
***Didymosphenia geminata* experimental
control trials: Stage Two, Phase Two
(testing the effectiveness of Gemex™,
a chelated copper formulation)**

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Executive Summary

NIWA was contracted by MAF Biosecurity New Zealand (MAF BNZ) to conduct a programme of experimental trials on potential *Didymosphenia geminata* (didymo) control agents. MAF BNZ's primary objective was to determine if a cost-effective, timely, operationally feasible treatment could be developed that would cause 100% mortality of *D. geminata* in a river environment with minimal and acceptable effects on non-target species. Our approach to meet this objective was to carry out a structured three-stage investigation involving product screening (Stage 1), product testing (Stage 2 Phases 1 and 2) and river trials (Stage 3). At each stage of the programme, potential control agents were assessed in terms of: a) effectiveness against *D. geminata*; b) potential impacts on non-target biota (including humans); c) the feasibility of success; d) risks that could affect success; e) the duration of effective *D. geminata* control; and f) the cost to implement. The requirement for this thorough, systematic approach to minimise risk was balanced against the urgent need for operationally effective control tools to eliminate or suppress *D. geminata* (i.e., in addition to strategies to prevent spread, such as disinfection of equipment and personnel), and meant these studies had to be completed as rapidly as possible in a challenging experimental time frame. Based on knowledge gained during Stage 1 and Stage 2 Phase 1 product testing (Jellyman et al. 2006a), three of the initial ten chemicals (Gemex™, Organic Interceptor™, and Hydrothol®-191) were selected for further assessment in both laboratory-based fish toxicity testing and in static cobble screening trials on *D. geminata*. This report details the results of Stage 2 Phase 2, including non-target toxicity testing of four biocides on juvenile rainbow trout, rapid screening of biocides in static cobble trials and intensive trials of Gemex™ (a chelated Cu formulation) in flow-through artificial channels.

Effects of biocides on non-target species: effect of 1 h static laboratory exposure to biocides on juvenile rainbow trout survival for 4 d post-treatment

The laboratory testing results for Gemex™ showed 100% survival four days post-treatment of juvenile rainbow trout exposed to concentrations of up to 14 mg Cu/L (as chelated Cu) for 1 h (hardness, 36 mg CaCO₃/L). Four day survival was slightly decreased to 93% after 1 h exposure to 27 mg Cu/L. These data indicate that Gemex™ concentrations as high as 27 mg Cu/L (as chelated Cu) could be trialled for efficacy against *D. geminata* in artificial channels; preferably concentrations < 22 mg Cu/L (1 h LC₀₅) should be used to minimise potential effects on fish and increase the safety margins of using this product.

Toxicity of Hydrothol®-191 to juvenile rainbow trout increased rapidly at nominal concentrations ≥ 3 mg a.e./L, with 100% mortality after exposure to 4 mg a.e./L. A 1 hour exposure of rainbow trout to Organic Interceptor™ at ≥ 74 mg pine oil/L is likely to cause > 50% trout mortality. The laboratory tests on Degaclean®150 showed that 1 h exposures to ≥ 2 mg PAA/L will negatively affect fish. The results of the fish tests were used to design the cobble trials to rapidly screen the efficacy of biocides on *D. geminata*.

Rapid screening of the effect of biocides on *D. geminata* cell viability in static cobble trials

Both single and multiple exposures of *D. geminata* to ≤ 16 mg a.e./L Hydrothol®-191 were relatively ineffective at decreasing cell viability, therefore Hydrothol®-191 was not tested further.

Data from these and previous trials showed that a 1 h exposure to Organic Interceptor™ at a rate necessary to cause 95% mortality to *D. geminata* (i.e., approximately 100 mg pine oil/L) would cause severe mortality to resident fish species, therefore it was not considered for further assessment as a *D. geminata* control compound.

In these static screening trials, single and multiple exposures of *D. geminata* to Gemex™ unexpectedly had similar effects on cell viability once nominal Gemex™ concentrations were > 8 mg Cu/L. Multiple exposures are likely to be more effective in a flow-through environment. In addition, the short-term monitoring in the static cobble trials did not discriminate well between the efficacy of different treatments once *D. geminata* viability was $< 10\%$. In contrast, multiple exposures to Hydrothol®-191 or Organic Interceptor™ were clearly more effective than single exposures. Combinations of Gemex™ with either Hydrothol®-191 or Organic Interceptor™ were less effective than Gemex™ alone. Gemex™ at 6 mg Cu/L was more effective than 33 mg Cu/L K-Tea™ (another chelated Cu formulation). These results, in combination with the laboratory testing of juvenile trout, invertebrates and non-target alga resulted in the decision to intensively test Gemex™ in artificial channel trials at Monowai Experimental Facility.

Intensive trials of Gemex™ in artificial flow-through channels at Monowai Experimental Facility

The intensive Gemex™ trials at Monowai Experimental Facility on ~15 mm thick mats of *D. geminata* yielded promising results for the suppression or elimination of the alga. High *D. geminata* mortality rates (98%) for 5-10 weeks post-treatment were achieved with either single applications of 18 mg Cu/L Gemex™ (nominal concentration, 12 mg Cu/L measured) in medium to fast velocity water or multiple applications of 9 mg Cu/L Gemex™ (nominal concentration, 7 mg Cu/L measured) in medium velocity water. One hundred per cent mortality (required for elimination) was not achieved in these trials, but it is likely that these treatments would be more effective against thinner mats (< 5 mm), or biofilms of *D. geminata*. In which case, if the alga were treated with Gemex™ in a natural waterway when it was in the early stages of infestation, prior to the formation of mats, it might possibly be eliminated. However, the relationship between the effectiveness of Gemex™ and mat thickness was not tested in these trials. The low Cu concentrations measured in the channels could have been caused by several factors including rapid uptake of Cu by the *D. geminata* mats.

Gemex™ was less effective in slow velocity water (0.1 m/s) than medium to fast velocity (0.3-0.6 m/s), although the effect of water velocity was confounded by the fact that in slow velocity exposures, the *D. geminata* mats were exposed to substantially lower net amounts (i.e., total masses integrated over the exposure period) of chelated copper. The strategy used for attempting to eliminate *D.*

geminata in a natural waterway should take this into account; for instance, in slow-velocity waters, repeat dosing will probably be required. Post-treatment monitoring of *D. geminata* viability should be accompanied by measurements of water velocity.

The artificial channel trials showed that the mortality of *D. geminata* increased with increasing doses of Gemex™, increased water velocity and multiple applications of biocide. An application rate of 20 mg Cu/L is recommended for a field trial of Gemex™ in order to maximise effectiveness against *D. geminata* while minimising effects on non-target species.

The artificial channel trials showed 100% survival of the native fish *Galaxias* sp. after single 1 h exposures to Gemex™ of 18 mg Cu/L (as chelated Cu) (hardness, 12 mg CaCO₃/L). However, the data do suggest that fish might be negatively affected by three exposures to 5-9 mg Cu/L Gemex™ 24 h apart. Therefore, field trials of Gemex™ should take a precautionary approach by avoiding multiple applications 24 h apart, and further laboratory testing should examine the effect of multiple exposures and different time intervals on juvenile rainbow trout. The assessment of stream invertebrate survival was completed at an indicative level only, but clearly showed that stream invertebrates are far more resistant to Gemex™ exposure than the pond invertebrate *Daphnia magna* tested in earlier laboratory exposures (Jellyman et al. 2006a).

The data from the artificial channel trials suggest that it is likely that multiple treatments will be necessary to eliminate or suppress *D. geminata* in natural waterways, especially in slow velocity water. Treatments will probably best be repeated after approximately 5-10 weeks, or when regrowth is first detected. Ideally, follow-up applications could occur at lower doses (e.g., less than 10 mg Cu/L) than the initial treatment because Gemex™ will probably be more effective on degraded mats, non-visible colonies, or biofilms of *D. geminata* only detectable by microscopic assessment of delimitation samples. The benefit of repeated applications of Gemex™ will have to be weighed against the potential impact of increased doses of chelated Cu in the receiving environment. Any Gemex™ field trial would need to be accompanied by extensive monitoring and assessment of the fate of the chelated copper in Gemex™. Such monitoring should focus on: spatial distribution and density of surviving *D. geminata*; spatial distribution and concentration of chelated copper in waterway sediments *versus* time elapsed since last treatment; and the response of non-target species to Gemex™ treatment.

1. Introduction

Didymosphenia geminata Schmidt (didymo) is an invasive freshwater diatom that inhabits 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. Since its discovery this single-celled alga has been causing massive and problematic growths in a number of South Island waterways. The distribution of the alga has now significantly expanded, with the diatom recorded in at least 53 South Island rivers (including tributaries in the same catchment) as of September 2007. 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 primarily of extracellular polymers organised into stalks. The capability to secrete large quantities of highly organized extracellular polymer arrays differentiates *D. geminata* from other related benthic diatoms. Studies commissioned by MAF Biosecurity New Zealand (MAF BNZ) estimated that the uncontrolled spread of this species could have significant negative environmental, economic and cultural effects on New Zealand's freshwater rivers (Campbell 2005, Kilroy 2004, NZIER 2006). The NZIER eight-year economic impact assessment of *D. geminata* estimates potential present value impacts to be \$158 million (NZIER 2006).

The potential impacts of *D. geminata* on environmental and economic values have necessitated research on control or eradication measures. To date, there have been no published examples of attempts to control blooms of *D. geminata* (Kilroy 2004, Gee and Wells 2006). NIWA was contracted by MAF BNZ to conduct a programme of experimental trials on potential *D. geminata* chemical control agents. Our approach was to carry out a structured three-stage investigation involving product screening (Stage 1), product testing (Stage 2 Phases 1 and 2) and river trials (Stage 3) (Figure 1). The research programme progressed from controlled stream environments, with additional non-target toxicity testing in the laboratory, to final control agent validation in natural waterways (Figure 1). At each stage of the programme, potential control agents were assessed against the following criteria set by MAF BNZ as information requirements for developing control methods:

- **effectiveness** as a *D. geminata* biocide;
- **impacts** on non-target biota, including humans;
- **feasibility** of success in affected waterways;
- **risks** which could affect success;
- **time** or the duration of effective control;
- **cost** to implement treatment(s).

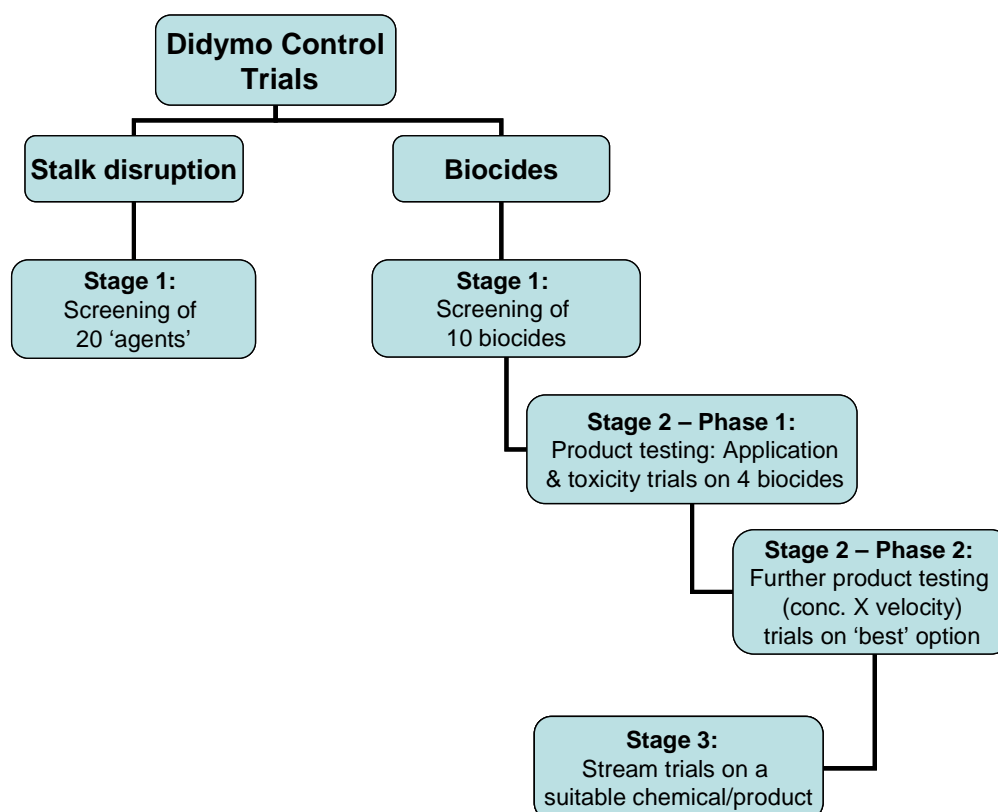


Figure 1: The organisation of the three-stage control study investigation.

The requirement for this thorough, systematic approach to minimise risk was balanced against the urgent need for operationally effective control tools to suppress or eliminate *D. geminata* (i.e., in addition to strategies to prevent spread, such as disinfection of equipment and personnel), and meant these studies had to be completed as rapidly as possible in a challenging experimental time frame. The ideal product would have high toxicity to *D. geminata* and minimal toxicity to non-target species. The assessment of the effectiveness, impacts, feasibility, risk, duration and cost of *D. geminata* control options began in a preceding report (Jellyman et al. 2006a), which included the results of biocide screening (Stage 1, complete early 2006) and product testing in stream-side channels and non-target toxicity testing (Stage 2 Phase 1, complete mid 2006). Based on knowledge gained during Stage 1 and Stage 2 Phase 1 product testing (Jellyman et al. 2006a), three of the initial ten chemicals (Gemex™, Organic Interceptor™, and Hydrothol®-191) were selected for further assessment in Stage 2 Phase 2.

This Stage 2 Phase 2 report documents: a) laboratory-based toxicity testing of biocides on non-target fish species b) further rapid product screening of biocides in static cobble trials on *D. geminata* and, c) the intensive Stage 2 Phase 2 trials of a chelated copper formulation called Gemex™ in artificial channel trials where we examined the effects of concentration, water velocity, and repeated treatments on efficacy against *D. geminata* and toxicity to non-target species (complete late 2006). Gemex™ was selected for these intensive trials because of its effectiveness against *D. geminata*, its lack of toxicity to fish and mild to moderate effects on non-target algae and invertebrates. The results of the Stage 2 Phase 2 testing were used to plan the first field trial of Gemex™ in a small stream to further measure ecosystem effects and the duration of impact on *D. geminata* (Stage 3) in a natural waterway (Clearwater et al. 2007). As the project moved into Stage 3, a thorough risk assessment of potential environmental effects was performed on Gemex™ including obtaining a resource consent from the regional council (Environment Southland) and a permit from the Environmental Risk Management Authority (ERMA) to use Gemex™ in containment.

‘Control’ of an unwanted species is an overarching term that includes different degrees of unwanted species reduction. The following definitions of biosecurity terminology were supplied by MAF BNZ and include internationally accepted terminology (IPPC 2007). ‘Control’ is the suppression, containment or eradication of a pest population. In the case of *D. geminata*, ‘suppression’ is the application of phytosanitary measures in an infested area to *reduce* the pest population (e.g., decreased live cell density or decreased biomass). ‘Containment’ is the application of phytosanitary measures in and around an infested area to *prevent the spread* of a pest. ‘Eradication’ is the 100% successful result of the application of phytosanitary measures to *eliminate* a pest from an area. MAF BNZ considers eradication as the removal of every individual and propagule of a species from New Zealand so that only re-introduction from beyond New Zealand’s borders would enable the re-emergence of the species. Achievement of eradication should be demonstrated by time-limited surveillance with a defined level of sensitivity. Whereas the term eradication is used by MAF BNZ to signify *national* eradication, the term ‘elimination’ is generally used to signify *local* eradication, such as removal of *D. geminata* from a catchment or from the North Island. For the purposes of this report elimination has been defined as a result of all samples negative for live *D. geminata* as measured by microscopic analysis of drift net and benthic samples² collected in at least two (preferably more) intensive delimitation surveys six months post-treatment, focused on the sites that were previously positive for *D. geminata*.

²At the time this research was completed, the methodology for detection of *D. geminata* by analysis for genetic material was not fully developed. Note that genetic analysis will detect both live and dead *D. geminata*, therefore any attempt to define elimination by absence of genetic material will have to allow for this.

2. Chemicals selected for further testing

2.1 Selection of chemicals

Based on knowledge gained during Stage 1 and Stage 2 Phase 1 product testing (Jellyman et al. 2006a), three of the initial ten chemicals (Gemex™, Organic Interceptor™, and Hydrothol®-191) were selected for further assessment in both laboratory-based fish toxicity testing and in static cobble screening trials on *D. geminata*. In order to progress our understanding of other potential control products, Degaclean®150 (a peracetic acid and peroxide-based product) was tested in fish toxicity trials, and K-Tea™ (a chelated copper formulation) and sodium hypochlorite were also tested on *D. geminata* in cobble screening trials. Both nominal and measured chemical concentrations can be reported, and they provide different types of information. Nominal concentrations are calculated, generally by preparation of solutions from a measured stock of chemical, while measured concentrations are those provided by some type of analysis of the experimental solution. Nominal and measured concentrations will sometimes differ substantially due to processes that remove or transform the analyte of interest in the experimental solution (e.g., metal ions binding to organic matter).

2.2 Gemex™ (chelated copper)

The chelated copper (Cu) formulation (nominal 20.36 g Cu/L, measured stock concentrations reported in each section, range 18.4-18.6 g Cu/L³) used in this research includes Cu as copper sulphate⁴ (CuSO₄·5H₂O, CAS Number 7758-99-8), and other non-toxic ingredients, including chelating compounds. To distinguish this chelated copper solution (formulated to be effective against algae) from other chelated copper products, this formulation has been named Gemex™. The active ingredient, copper, is toxic to aquatic organisms and has been used in many formulations as an algaecide and antifouling agent. In general, there have been very few adverse effects reported as a result of the large-scale and long-term use of copper in antifouling paints (both marine and freshwater). However, some coastal authorities such as those in California, are moving away from the use of copper-based marine paints because of concerns about its accumulation in coastal sediments (Johnson and Gonzalez 2004). Copper is an established algaecide and has been used extensively for this purpose since the

³ Gemex™ stock was delivered to NIWA Hamilton or the Monowai Experimental Facility immediately prior to the experiments labelled as nominal concentration 20.36 g Cu/L. The actual Cu concentration in different batches of the stock was measured after the experiments were completed (18.4-18.6 g Cu/L).

⁴ Copper sulphate anhydrous = CAS Number 7758-98-7; Gemex™ can also be prepared as a 3% solution.

1960s in the U.S.A. It is commonly used in chelated copper formulations in swimming pools, the aquarium trade and irrigation/hydro-power canals to control algae or fungi without harming desirable species such as fish or corals.

Copper sulphate has been approved by the U.S. Environmental Protection Agency for use on a repetitive basis in lentic systems such as ponds and lakes. Copper-based algaecides are often recommended because they are cheap, efficient, easily obtained and at the correct dose rates, are specific to algae and fungi. Copper has the disadvantage of being a cumulative element, and therefore it must be used sparingly, and with regard to its potential to accumulate in sediments. Copper is an essential element for most living organisms, therefore its *dietary* uptake and excretion is well-regulated and it does not biomagnify in food webs, although it can bioaccumulate with chronic exposure to very high dietary concentrations. *Dissolved* copper bioavailability and toxicity is much higher than dietary copper bioavailability, and dissolved copper toxicity is significantly affected by water quality, particularly the parameters of pH, alkalinity, hardness and dissolved organic matter (DOM). In general, copper toxicity will be higher in low hardness, low pH waters typical of South Island rivers. Copper toxicity is reduced however, in hard waters, for example in areas characterized by limestone formations. High concentrations of humic and fulvic acids (DOM) leaching from vegetation will bind copper and decrease its bioavailability and toxicity. *D. geminata* is known to be sensitive to copper, with no conspicuous growth in a Norwegian river system when Cu exceeded 8 µg Cu/L in a continuous exposure as a result of contamination from abandoned copper and zinc mines, and die-back when levels exceeded 0.015 mg Cu/L (15 µg Cu/L) (Lindstrom and Rorslett 1991). However, in this case the copper was probably not chelated, so it would have been toxic to most other aquatic organisms as well as *D. geminata*. In addition, it was not the only contaminant in the mine leachate; zinc concentrations were also slightly elevated and pH was slightly decreased. Although this raises the possibility of suppressing *D. geminata* by chronic dosing with low doses of dissolved copper, chronic exposure to dissolved copper will also probably be toxic to other aquatic organisms, thereby reducing the specificity of the control treatment. Specificity against *D. geminata* is one of the main advantages of using a pulse dose of high concentrations of Gemex™ compared to the other control agents that were tested.

The formulation of copper used in these trials (Gemex™) results in the chelation of copper in compounds that remain in solution over a wide range of pH and temperature. 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 such as copper carbonate. In contrast, in a solution of copper sulphate most

of the copper would be present as the free 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 dissociated 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. 2007).

Gemex™ was included in laboratory-based fish toxicity testing trials to systematically test the findings of the Stage 2 Phase 1 artificial channel trials that showed 100% survival of rainbow trout (*Oncorhynchus mykiss*) and common bullies (*Gobiomorphus cotidianus*) after 1 h exposure to 4 mg Cu/L (as chelated Cu) (Jellyman et al. 2006a). The cobble screening trials (Section 4 in this report) were used to establish the potential effectiveness of a wide range of Gemex™ doses against *D. geminata*, and the effect of multiple doses of Gemex™. In combination, the results of the cobble screening trials and the laboratory-based toxicity testing were used to design the intensive trials of Gemex™ in the Stage 2 Phase 2 artificial channel trials (Section 5 in this report).

2.3 Organic Interceptor™

Organic Interceptor™ is a herbicide used in the control of annual weeds, grasses and the brown-off of perennial species. Organic Interceptor™ is a contact weed killer made from pine oil and sold by Certified Organics Ltd⁵. 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 in New Zealand by the Agricultural Compounds and Veterinary Medicines Group (ACVM) and the Environmental Risk Management Authority (ERMA). The biocides in the formulation are not described or identified any further (e.g., with CAS Numbers⁶) because 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. can 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.

⁵ Supplied by H. Frith Ph. 09 525 3432

⁶ CAS Numbers (Chemical Abstracts Registry) are used as identifiers of chemicals and can be found in the Merck Index (1989).

Organic Interceptor™'s mode of toxicity against terrestrial plants is thought to be via desiccation.

Data from Stage 1 Phase 1 testing indicated that while 68–271 mg pine oil/L Organic Interceptor™ was effective against *D. geminata*, it was also toxic to rainbow trout (Jellyman et al. 2006a). Static cobble screening trials (Section 4 of this report) allowed us to determine if 100% *D. geminata* mortality could ever be achieved, even using very high exposure concentrations (i.e., 500 mg pine oil/L). These trials also allowed us to rapidly assess the likely effectiveness against *D. geminata* of multiple exposures to lower concentrations of Organic Interceptor™ that fish might potentially survive. These results were then compared to the fish toxicity testing results. Ultimately we were able to use these data to exclude Organic Interceptor™ from further testing.

2.4 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, though the same formulation labelled as DES-I-CATE II is used as a harvest aid for potatoes⁷. Endothall is a dicarboxylic acid, and is a selective contact herbicide that acts as a desiccant and defoliant. The dipotassium salt of endothall is used in the aquatic herbicide Aquathol® K (a liquid formulation) and Aquathol® Super K (a granular formulation); these formulations were recently registered by ERMA for use in New Zealand.

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 (mg acid equivalents/L, or mg a.e./L). Hydrothol®191 contains 53% active ingredient (dimethylalkylamine salt of endothall⁸), 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 on its use than Aquathol® K due to its greater toxicity to aquatic organisms, especially fish (at approximately 5 mg a.e./L). Hydrothol®191 is usually applied to lakes or channels with relatively low flows resulting in extended contact times. Different

⁷ Supplied by Elliot Chemicals Ltd., Brian Smith, 09 237 0431.

⁸The 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 Ecotox 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).

application rates might be appropriate where short contact times (between one and four hours) will occur in the high flow environments where *D. geminata* infestations are located.

Data from Stage 1 Phase 1 testing indicated that ≤ 2 mg a.e./L Hydrothol[®]191 was not sufficiently effective against *D. geminata*, so static cobble screening trials (Section 4 of this report) were used to determine if high concentrations (4-16 mg a.e./L) would cause 100% mortality of *D. geminata* regardless of non-target effects. In addition, we examined the effect on *D. geminata* of multiple exposures to lower concentrations, that fish might potentially survive (2-8 mg a.e./L). These results were then compared to the fish toxicity testing results. Ultimately we were able to use these data to exclude Hydrothol[®]191 from further testing.

2.5 Degaclean[®] 150

Degaclean[®] 150 is a biocide formulated for use in cooling water systems to prevent the settlement and build-up of biological slimes and fouling organisms, while minimising corrosion. Degaclean[®] 150 is an equilibrium solution of peracetic acid (PAA) (10% - 20%), acetic acid (25% - 90%), and hydrogen peroxide (> 10% - < 20%) and is recommended for use as a pulse dose exposure for approximately 15 min at 40 mg PAA/L to prevent biofouling. Degaclean[®]150 was included for testing because it is a strong oxidiser that might penetrate *D. geminata* mats more effectively than other products and it has the advantage of producing no (or minimal) toxic degradation products. It was also thought that fish might be sufficiently resistant to short (1 hour) exposures to Degaclean[®]150 to allow effective treatment of *D. geminata*. Degaclean[®]150 was supplied for use at NIWA Hamilton in fish toxicity testing by Degussa Peroxide Ltd, New Zealand, and was quantified during experimentation using a Reflectoquant[™] kit specialised for peracetic acid measurements.

2.6 K-Tea[™] (chelated copper)

K-Tea[™] is a chelated copper formulation registered for use as an algacide in the United States of America. K-Tea[™] was included in the product screening for direct comparison to Gemex[™], another chelated copper formulation, and because K-Tea[™] is a registered product. Product registration is a lengthy process, requiring extensive research, therefore having much of this data already available for assessment is an enormous advantage. K-Tea[™] (nominal 80 g Cu/L or 8% Cu, measured 98.6 g Cu/L) is derived from copper hydroxide and copper-triethanolamine complex. Triethanolamine is often abbreviated TEA. The pH of K-Tea[™] is slightly alkaline (8.4).

K-Tea™ is potentially not as stable in low water temperatures (< 15-17°C) as Gemex™, tending either to precipitate or to speciate toward increased concentrations of cupric ions (Cu^{2+}) rather than chelated copper compounds (source *Pers Comm 4 September 2006* Brett Ruth, RCI Australia) (no other data or references have been found on this subject). The effectiveness of K-Tea™ is significantly decreased by increasing water hardness (> 50 mg/L as CaCO_3) as are most Cu compounds (K-Tea™ MSDS 2006, SePRO Corporation, USA).

2.7 Sodium hypochlorite

Sodium hypochlorite (NaOCl) is an industry standard for sterilisation and is used as a bleach and disinfectant, and for the control of biofilms (which include bacteria and algae) in swimming pools and cooling towers. Sodium hypochlorite was used in this product-screening phase as it is readily available in 4% solution as household bleach and is a strong oxidant that might penetrate the *D. geminata* mats more readily than other types of chemicals.

Whilst it is not being reconsidered (it was tested in the Stage 1 trials) as a biocide for potential widespread use, knowing what concentration of NaOCl is effective against *D. geminata* may be useful in some situations where treated water would not be directly discharged into waterways; there is also the potential to neutralise NaOCl after it has been released. In addition NaOCl was included in the cobble screening trials as a positive control to establish whether total cell mortality could be achieved in the static cobble trials (as opposed to flow-through exposures in the channel trials). The product used in these trials was Pool Master Liquid Pool Chlor (Pool-Quip Ltd) (125 g/L NaOCl) (nominal).

3. Laboratory toxicity testing of fish

3.1 Introduction

Previous toxicity testing in Stage 2 Phase 1 of the Control Study was conducted on the green alga *Pseudokirchneriella subcapitata*⁹ 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 (Gemex™, Hydrothol®-191, Organic Interceptor™, and EDTA) (Jellyman et al. 2006a). Due to lack of availability of juvenile fish in June 2006, it was not possible to assess the impact of these biocides on fish in a controlled laboratory environment. As a result, recommendations in Jellyman et al. (2006a) regarding the impacts on fish species were based on information gathered from research papers, toxicity testing databases and the inclusion of fish in the artificial stream channel trials at the Monowai Experimental Facility. Jellyman et al. (2006a) made the recommendation that research be discontinued on EDTA and proposed that future trials on fish should only test the biocides: chelated copper (Gemex™), Hydrothol®191 and Organic Interceptor™. In addition to these three biocides, the Degaclean® 150 was also tested for its toxicity to fish.

Testing the effect of a short-term exposure (1 hour) to the biocide required modification of the standard toxicity testing protocols (from 48, 72 or 96 hour exposures) and suitable controls (i.e., 1 h exposure of fish to control water, followed by rinsing and transfer) were included in all the tests to take into account the effects of these modifications. A one hour exposure was used because data from Stage 2 Phase 1 trials had led to the observation that a 1 h dose of a biocide to a natural waterway was the one most likely to be effective against *D. geminata* while minimising effects on non-target species, and still being practical to undertake, given the quantities of biocide likely to be required. Juvenile rainbow trout were used for these trials because they are widely accepted as a sensitive indicator species for fresh water environments. For this reason, rainbow trout are used in standard toxicity test protocols world-wide to assess the potential effect of toxicants and effluents on aquatic ecosystems. Rainbow trout are also present in many of New Zealand's rivers, including those currently affected by *D. geminata*.

⁹ Formerly known as *Selenastrum capricornutum*.

3.2 Methods

3.2.1 Experimental animals

Juvenile rainbow trout (~36 d old, 0.9 ± 0.4 g) were obtained from the Eastern Fish and Game fish hatchery at Ngongotaha, transported to the toxicity testing laboratory (NIWA Hamilton) and held for 6 days prior to testing in 90L filtered, recirculating, dechlorinated Hamilton City tapwater (hardness 36 mg CaCO_3/L), with aeration in a constant temperature chamber (14°C), on a 16:8 hour light:dark cycle. Fish were fed two to three times per day, at a rate of 3-5% body weight/day.

3.2.2 Fish test methods

Fish were exposed to at least four concentrations of each chemical or a control solution for 1 h (Table 1). Each replicate contained five fish, and there were three replicates for each treatment. For each replicate the fishes were in an aerated 4 L container (Appendices 1 and 2). The exception was that fish were exposed in only one replicate of the two highest concentrations tested of Gemex™ (55 and 109 mg Cu/L). Fish were observed periodically throughout the exposure to assess their behavioural response to the chemicals. The solutions were vigorously aerated to simulate exposure in a high flow environment. After 1 h the fish were transferred in a soft net for a brief rinse in 20 L clean water, then placed in 4 L holding tanks with gentle aeration for follow-up observation of survival for 4 d post-treatment (see summary Appendix 3).

Presumed mortalities were noted at 1 h, but moribund/dead fish were left for 24 h to determine whether recovery would occur in fresh aerated water. Vigorous aeration was added to the holding tanks of one treatment for 24 h (Hydrothol, 3 mg a.e./L) to assist recovery of moribund fish, and better simulate a high flow environment. The pH, DO (mg O_2/L) and temperature was measured in the exposure solutions and daily in the holding tanks. All concentrations are nominal except for Degaclean® 150 and three of the Gemex™ solutions (nominal 8, 16 and 32 mg Cu/L).

Samples of the testing solutions were acidified (1% HNO_3 , trace metal grade) and measured for Cu and lead (Pb) concentrations by ICPMS (Hill Laboratories, Hamilton). Lead concentrations were measured because Pb is a potential trace contaminant of the Gemex™ concentrate, and was found to be below the detection limit (< 0.2 mg Pb/L) in the Gemex™ stock, and in the measured exposure solutions (detection limit < 0.002 mg Pb/L). Dissolved calcium (Ca) and magnesium (Mg) in dechlorinated Hamilton City Tapwater were 10.4 and 2.6 mg/L respectively (0.45 μm filtered, ICPMS, method APHA 3125 B 20th ed 1998) and water hardness was 36 mg CaCO_3/L , (calculated from dissolved Ca and Mg, method APHA 2340 B 20th ed 1998; also confirmed by titration against 0.01 M EDTA method APHA 2340C).

Results were analysed using the ToxCalc™ program (Tidepool Scientific Software) as recommended by the U.S. Environmental Protection Agency (Tidepool 1994).

Table 1: The 1 h treatment solutions that rainbow trout were exposed to during laboratory toxicity testing at NIWA Hamilton, including pH of the 1 h exposure solution (not the holding tank pH). Degaclean® 150 concentrations and a selection of the Gemex™ concentrations were measured.

Chemical	Units	Nominal Concentration	Measured Concentration	Replicates	pH
Control	--	--		5	7.3
Gemex™	mg Cu/L	4 ^A	-	3	8.0
		8	6.6	3	7.0
		16	13.7	3	5.3
		32	27.3	3	4.2
		64	(54.1) ^D	1	3.8
		128	(108.1) ^D	1	3.5
Hydrothol®-191	mg a.e./L	1	-	3	7.0
		2 ^B	-	3	8.0
		3	-	3	7.2
		4	-	3	8.1
Organic Interceptor™	mg pine oil/L	34	-	3	7.9
		46	-	3	8.1
		68 ^C	-	3	6.9
		90	-	3	7.0
Degaclean® 150	mg PAA/L	0.3	0.3	3	7.8
		1	1.3	3	7.8
		2	2.4	3	7.5
		3	3.8	3	7.9

^AThis was the highest concentration of Gemex™ (Rate 3) tested in the Stage 1 Phase 1 artificial channel trials of *D. geminata* (Jellyman et al. 2006a), with 100% survival of both rainbow trout and common bullies.

^BThis was the highest concentration of Hydrothol®191 (Rate 3) tested in the Stage 1 Phase 1 artificial channel trials of *D. geminata*, with 100% survival of both rainbow trout and common bullies.

^CThis was the lowest concentration of Organic Interceptor™ (Rate 1) tested in the Stage 1 Phase 1 artificial channel trials of *D. geminata*, with 0% survival of rainbow trout.

^DConcentrations calculated from average % difference between nominal and measured in lower concentrations.

3.3 Results -fish toxicity testing

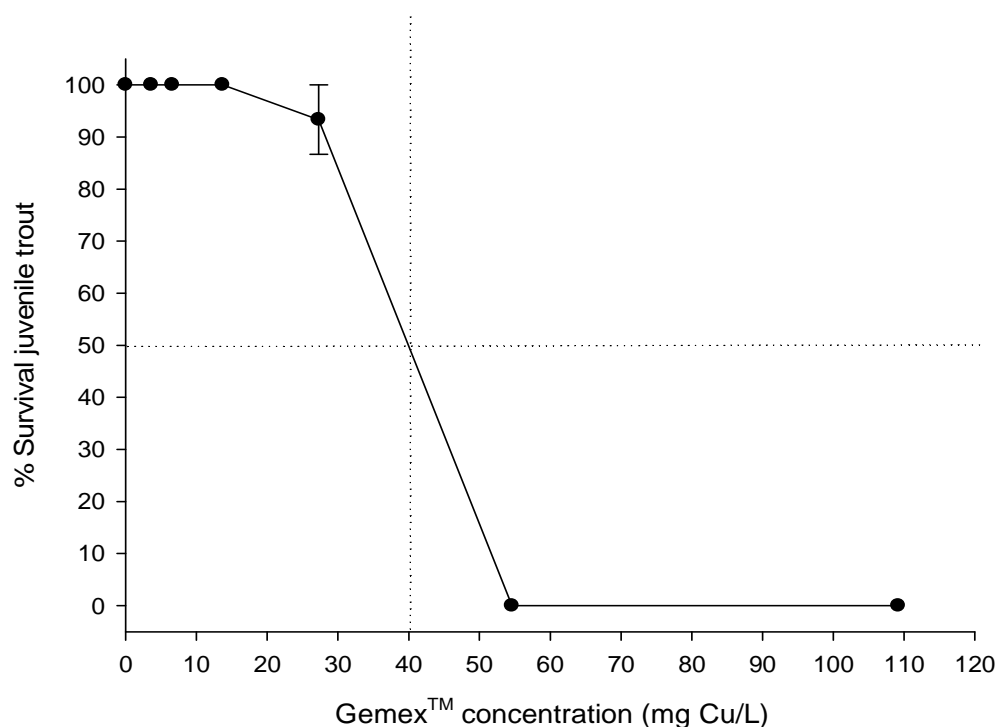


Figure 2: Percentage survival of juvenile rainbow trout 4 days after 1 h exposure to different concentrations of Gemex™ (measured). Dashed lines give an estimate of the LC₅₀ for each exposure but could be over- or underestimated if the response is non-linear.

3.3.1 Gemex™

Four days after a 1 h exposure there was 100% survival of juvenile rainbow trout exposed to Gemex™ at up to 14 mg Cu/L (as chelated Cu) (Figure 2, Table 2). During the exposure, fish behaved normally in 4 mg Cu/L, but were possibly slightly hyperactive in 7-27 mg Cu/L Gemex™. One fish died by 24 h after 1 h exposure to 27 mg Cu/L; no more fish died subsequently, so average survival was 93% 4 d later. In the one replicate tested at 54 mg Cu/L, all 5 fish initially survived the 1 h exposure and transfer to clean water (although they were dark in colouration and hyperactive), but 3 h later four fish were dead and one was moribund. All five fish were dead after approximately 30 min exposure to 108 mg Cu/L (as chelated Cu) Gemex™ in the one replicate tested. The low pH (3.5-4.8) of the solutions 27-108 mg Cu/L is outside the normal range encountered by rainbow trout and will have contributed to the toxicity of the solutions. The statistically-derived no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) for a 1 h exposure to Gemex™ were 27 and 54 mg Cu/L (as chelated Cu) respectively (Table 2). The LC₅₀ for a 1 h exposure to Gemex™ was 37±3 mg Cu/L (as chelated Cu), when survival was monitored for 4 d (96 hours) post-exposure.

Table 2: Toxicity parameters statistically-derived for 1 h exposures of juvenile rainbow trout to various chemicals (nominal concentrations except for Degaclean[®] 150 and Gemex[™]). Survival was assessed 4 days (96 h) after the 1 h exposures (s.d. = standard deviation for the statistically derived LC₅₀).

Chemical	Units	LC ₅₀ ^A	s.d.	NOEC ^B	LOEC ^C
Gemex [™]	mg Cu/L	36.9	3.2	27.3	54.6
Organic Interceptor [™]	mg pine oil/L	73.5	1.4	46.0	68.0
Hydrothol [®] -191	mg a.e./L	3.4	0.1	2.0	3.0
Degaclean [®] 150	mg PAA/L	2.9	0.1	1.3	2.4

^ALC₅₀: Statistically-derived concentration lethal to 50% of the test organisms after 1 h exposure.

^BNOEC: The statistically-derived concentration having No Observed Effect on the test organisms relative to the controls.

^CLOEC: The statistically-derived concentration having the Lowest Observed Effect on the test organisms relative to the controls.

3.3.2 Hydrothol®-191

There was 100% survival of fish exposed to 1-2 mg a.e./L Hydrothol®-191 for 1 h then transferred to freshwater, and 87% survival of fish exposed to 3 mg a.e./L (Figure 3). Fish behaved normally in 1 mg a.e./L solutions but in 2 and 3 mg a.e./L all fish ceased active swimming and fish in all replicates showed a loss of equilibrium (LOE) (effects were more severe in 3 mg a.e./L), recovering after transfer to fresh water. Survival was 0% after 1 h exposure to 4 mg a.e./L; all fish showed LOE approximately 30 min after exposure commenced, and upon transfer to freshwater were not actively ventilating their gills. Fish did not recover despite vigorous aeration of the holding tank water to promote gill ventilation (and simulate recovery in a river environment). The statistically-derived NOEC, LOEC and LC_{50} for the 1 h exposures were 2.0, 3.0 and 3.4 ± 0.1 mg a.e./L respectively. Survival was monitored for 4 days post-exposure (Table 2).

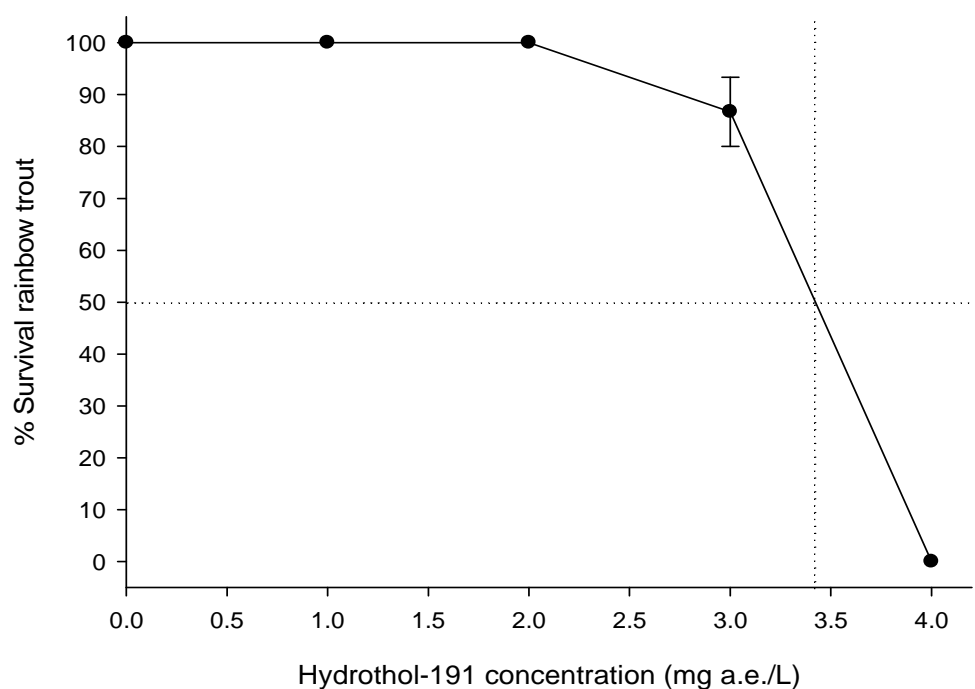


Figure 3: Percentage survival of juvenile rainbow trout 4 days after 1 h exposure to different concentrations of Hydrothol®-191 (nominal). Dashed lines give an estimate of the LC_{50} for each exposure but could be over- or underestimated if the response is non-linear.

3.3.3 Organic Interceptor™

Fish exposed to 34 mg pine oil/L Organic Interceptor™ behaved relatively normally, although some fish decreased their swimming activity and were darker in coloration than normal. Recovery was immediate in freshwater and survival was 100% 4 days later (Figure 4). Within 10 min of exposure to 46 mg pine oil/L Organic Interceptor™, 90% of all fish showed LOE but continued active gill ventilation and occasional swimming activity. Upon transfer to fresh water all fish recovered immediately although they ‘coughed’ occasionally and swam with slightly flared opercula (4 d survival was 100%). Similar responses were seen in 1 h exposure to 68 mg pine oil/L Organic Interceptor™ although gill ventilation movements were minimal by the end of the exposure, and not all fish recovered; survival was 67% 4 d later. After just 2-3 min exposure to 90 mg pine oil/L Organic Interceptor™ swimming activity had decreased, and after 1 h all fish had LOE and no ventilatory movements. Although the fish appeared dead, they were transferred to fresh water but no recovery occurred. The statistically-derived NOEC, LOEC and LC₅₀ for the 1 h exposures to Organic Interceptor™ were 46, 68 and 74± 1 mg pine oil/L respectively (Table 2). Survival was monitored for 4 days post-exposure.

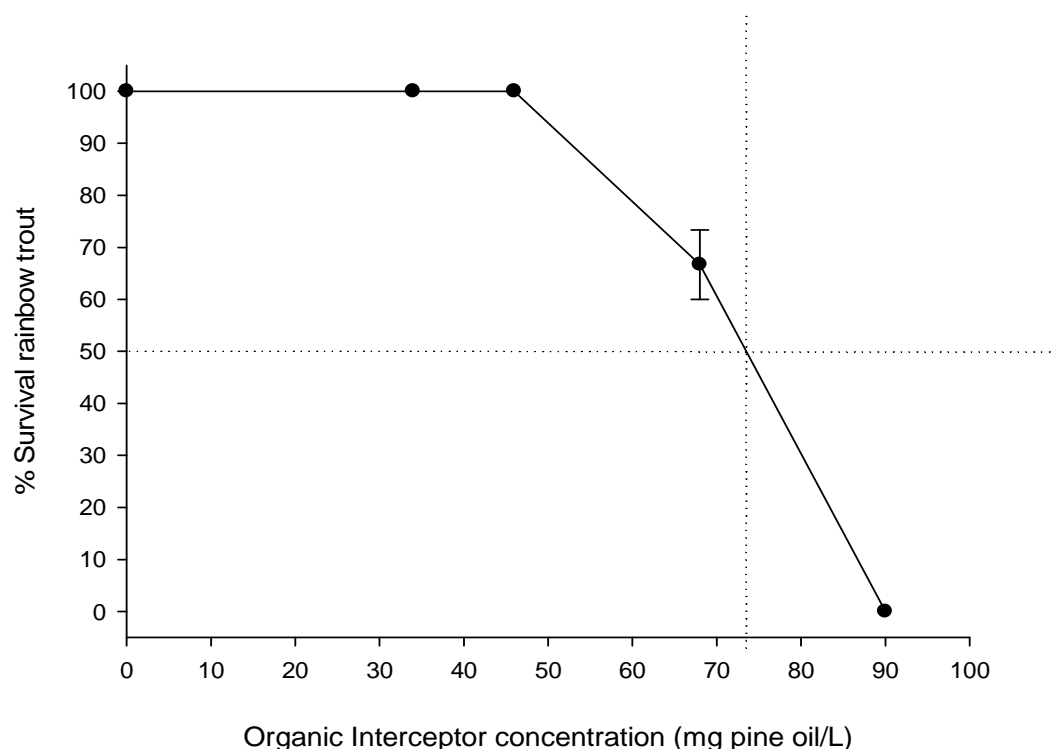


Figure 4: Percentage survival of juvenile rainbow trout 4 days after 1 h exposure to different concentrations of Organic Interceptor™ (nominal). Dashed lines give an estimate of the LC₅₀ for each exposure but could be over- or underestimated if the response is non-linear.

3.3.4 Degaclean® 150

Fish exposed to 0.3-1 mg PAA/L Degaclean® 150 appeared normal after 1 h exposure and survival was 100% 4 d later (Figure 5). Similarly fish appeared relatively normal during 1 h exposure to 2 mg PAA/L Degaclean® 150, although by the end of the exposure the rate of gill ventilation movements increased and one fish had LOE. Approximately 2 h after transfer to fresh water some of the fish were moribund and were dead 24 h post-exposure; 4 d post-exposure no more fish had died and survival was 80%. After approximately 50 min exposure to 3 mg PAA/L all fish appeared normal apart from slightly flared opercula, however 10 min later when the fish were about to be transferred 2-3 fish in each tank had LOE and no ventilatory movements (the deterioration was very rapid); all fish further deteriorated after transfer to fresh water and almost all fish were dead approximately 2 h later. All of the fish exposed to 3 mg PAA/L Degaclean® 150 for 1 h were dead 24 h post-exposure. The statistically-derived NOEC, LOEC and LC₅₀ for the 1 h exposures to Degaclean® 150 were 1.3, 2.4 and 2.9 ± 0.1 mg PAA/L respectively (Table 2). Survival was monitored for 4 days post-exposure.

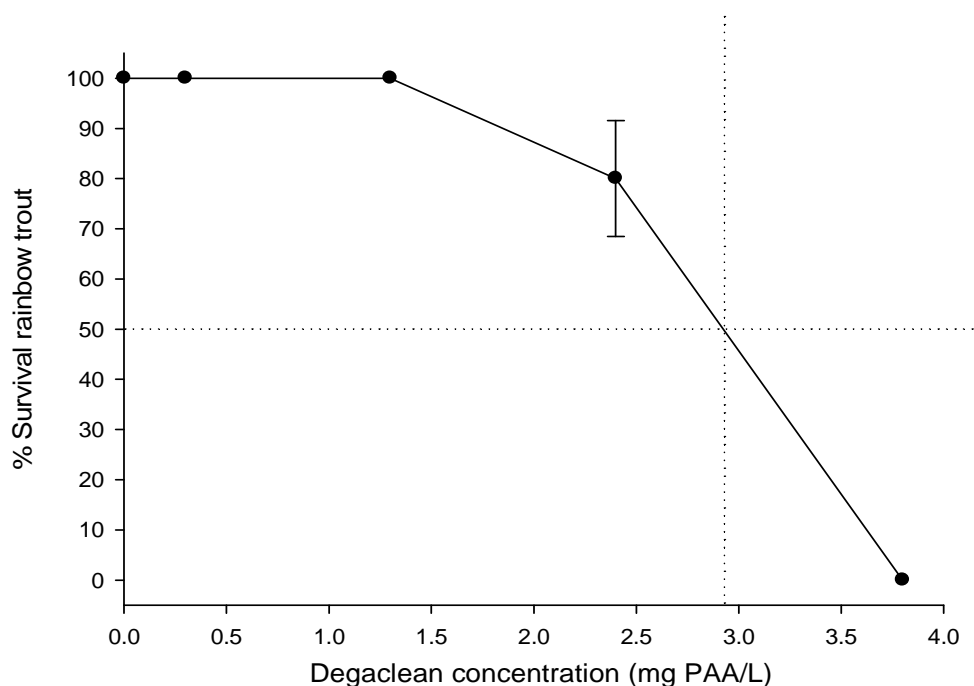


Figure 5: Percentage survival of juvenile rainbow trout 4 days after 1 h exposure to different concentrations of Degaclean (measured). Dashed lines give an estimate of the LC₅₀ for each exposure but could be over- or underestimated if the response is non-linear.

3.3.5 Reference toxicant results

The reference toxicity results for juvenile trout exposed to zinc sulfate indicated that the batch of fish used for these experiments were within the acceptable sensitivity range of ± 2 s.d. of the ongoing average for our laboratory. The 96 h LC₅₀ was 0.12 ± 0.10 mg Zn/L and the NOEC and LOEC were < 0.1 and 0.1 mg Zn/L respectively.

3.4 Discussion of fish toxicity testing results

The laboratory testing results for Gemex™ support the indicative findings of the artificial channel trials, which showed 100% survival of common bullies and rainbow trout up to 4 mg Cu/L. However, only eight or nine individuals of each species were tested in these exposures (Jellyman et al. 2006a). The results of the current study show 100% survival of juvenile rainbow trout exposed to up to 14 mg Cu/L (as chelated Cu) for 1 h (in hardness 36 mg CaCO₃/L). Survival was slightly decreased to 93% after 1 h exposure to 27 mg Cu/L. Fish were likely to have been negatively affected by a combination of the increasing chelated copper concentration and the decreasing pH. No attempt was made to isolate the effect of pH from Cu toxicity at this stage of the investigation because in the context of an application of the biocide to a natural waterway, this is a side-effect that would be difficult to mitigate. If the decision was made to use Gemex™ at concentrations around 27 mg Cu/L where fish survival was $< 100\%$, it might be worthwhile investigating the separate impact of low pH and Cu on fish survival. It is likely that the maximum concentration of Gemex™ to be used on *D. geminata* will be 20 mg Cu/L (as chelated Cu), and the dose-response data indicate that this concentration will have $< 5\%$ effect on trout survival (Figure 2, LC₀₅ ~22 mg Cu/L). The toxicity of the Gemex™ formulation to juvenile rainbow trout increases rapidly above 27 mg Cu/L (as chelated Cu). Longer exposures (> 1 h) will also increase trout mortality. Water quality will also affect the toxicity of Gemex™ to fish; in this experiment water hardness and alkalinity was low to moderate (~ 36 mg/L as CaCO₃) and pH was approximately 7.0. The water source was dechlorinated Hamilton City tapwater, therefore the organic matter content was relatively low. The toxicity of Gemex™ is likely to increase in acidic, soft waters, and waters with low concentrations of organic matter because all of these factors influence copper speciation and its bioavailability and toxicity to the fish gill.

In summary these laboratory testing data indicate that Gemex™ concentrations as high as 27 mg Cu/L (as chelated Cu) could be trialled for efficacy against *D. geminata* in artificial channels, although preferably concentrations < 22 mg Cu/L (~LC₀₅) should be used to minimise potential effects on fish and increase the safety margins of using this product.

Toxicity of Hydrothol®-191 to juvenile rainbow trout increased rapidly at concentrations ≥ 3 mg a.e./L. Fish swimming was rapidly affected by 3 mg a.e./L but most individuals recovered quickly in fresh water; 4 mg a.e./L was 100% lethal. These data support the artificial channel results in which there was 100% survival of both common bullies and juvenile rainbow trout exposed to 2 mg a.e./L for 1 h (Jellyman et al. 2006a). Hydrothol®-191 at 2 mg a.e./L was not particularly effective against *D. geminata*, decreasing cell viability by only 60%. Unless increasing Hydrothol®-191 concentrations to 3 mg a.e./L rapidly increases its efficacy against *D. geminata* to $> 95\%$ mortality, it is likely that this product would have unacceptable negative effects on local fish populations. Another option is to explore the effect on *D. geminata* of multiple exposures to Hydrothol®-191 at ≤ 2 mg a.e./L.

Juvenile rainbow trout are relatively susceptible to Organic Interceptor™ at concentrations effective against *D. geminata*. The laboratory data showed that a 1 hour exposure to ≥ 74 mg pine oil/L is likely to cause $> 50\%$ mortality. In the artificial channel trials at Monowai, no juvenile rainbow trout survived 1 h exposure to 68, 135, and 271 mg pine oil/L but common bullies had 66%, 100% and 33% survival respectively. However, only eight individuals of each species were tested in these indicative trials (Jellyman et al. 2006a). Organic Interceptor™ is relatively effective against *D. geminata*; 68, 135 and 271 mg pine oil/L decrease cell viability by approximately 85, 95 and 98% respectively (Jellyman et al. 2006a) but the higher concentrations will cause 100% fish mortalities. The laboratory fish data suggest that it is only worth investigating the effect on *D. geminata* of multiple exposures to Organic Interceptor™ at 30-60 mg pine oil/L to determine if the product could be used in a manner that might avoid harming fish.

The laboratory tests on Degaclean®150 showed that 1 h exposures to ≥ 2 mg PAA/L will negatively affect fish. Degaclean®150 probably physically damages the gill membranes of fish by first oxidising the protective mucus layer on the gill and then damaging the gill cells directly. This mode of action might explain the sudden irreversible deterioration in fish condition that occurred after approximately 50 min exposure to Degaclean®150 at 3 mg PAA/L. These data indicate that Degaclean®150 would have to be effective on *D. geminata* in 1 h exposures of ≤ 1 mg PAA/L in order to avoid negative effects on fish¹⁰.

¹⁰This product has not yet been tested further at Monowai Experimental Facility because it would require a modification to the resource consent that allows the operation of the facility (it was not included in the original list of products to be tested). Permission may be sought in the future.

4. Static screening trials of biocides

4.1 Introduction

After completion of the Stage 1 Phase 1 artificial channel trials of Gemex™, Hydrothol®-191, Organic Interceptor™ and EDTA, it was clear that EDTA did not warrant further testing as a potential control compound due to its lack of efficacy against *D. geminata* viability (Jellyman et al. 2006a). In preparation for the next round of intensive artificial channel trials, static screening trials of biocide effectiveness against *D. geminata* mats on cobbles were used to rapidly gather information about the likely effect of relatively high concentrations of Gemex™, Organic Interceptor™ and Hydrothol®-191. The trials also examined the effect of multiple exposures to low concentrations of each product, because this strategy could be used to reduce effects on non-target species, especially fish. As a starting point, multiple 1 h exposures spaced 24 h apart were selected as a practical method of repeatedly exposing *D. geminata*, and allowing time for non-target species such as fish to recover physiologically between treatments. River dosing equipment could probably be safely left *in situ* for 24-72 h if necessary. Another chelated copper formulation, K-Tea™, was included in the cobble testing for comparison against Gemex™. Sodium hypochlorite was tested at a wider range of concentrations than previously tested in Stage 1 to determine the threshold for high *D. geminata* mortality rates after 1 h exposures, and as a positive control to determine if > 98% cell mortality could be achieved in static cobble trials (see Section 2 for detailed information about each chemical). Lastly two combinations of chemicals were tested to determine if there would be a synergistic increase in toxicity to *D. geminata*.

The cobble trials are efficient and rapid to complete because sourcing of natural *D. geminata* mats on moveable cobbles avoids the time required to prepare and colonise artificial substrates (approximately 6-8 weeks, subject to flood events). Assessment of data from the Stage 1 Phase 1 trials also indicated that cell viability counts completed 24 h after the 1 h chemical exposure gave a good indicator of product effectiveness over the ensuing 28 d, as indicated by all three metrics (cell viability counts, chlorophyll a content (mg/m²) and ash free dry mass (g/m²)) (Jellyman et al. 2006a). On the other hand, a single 24 h viability assessment will not discriminate well between treatments with viability less than 10% as we have observed that viability will sometimes continue to decrease for approximately a week post-treatment. Cobble trials have the disadvantages that the *D. geminata* mats are exposed in a non-flowing (static) environment that will not mimic a flowing waterway as well as artificial channel exposures, and the naturally-sourced mats are more heterogenous than those grown simultaneously on the identical artificial substrates purpose-built for the Monowai Experimental Facility. In addition the mats on the cobbles often partially

detach several days after exposure and they only offer a small surface area for follow-up monitoring of cell viability that limits their usefulness for longer-term experiments. Keeping these limitations in mind, the cobble trials were an excellent method to rapidly gather information that was then used in combination with information from Stage 1 Phase 1 studies and the laboratory toxicity testing of chemical effects on non-target algae, invertebrates and fish to guide the design of the intensive channel trials (Stage 2 Phase 2). Ultimately we were seeking treatments that will cause >95% mortality of *D. geminata* cells in a flow-through exposure, with minimal effects on non-target species. The ideal result of course, would be 100% mortality of the *D. geminata* cells in a concentration range that will not negatively affect other species.

4.2 Methods

4.2.1 Cobble sourcing for *D. geminata* mats

Cobble screening trials on *D. geminata* were conducted between 21 and 25 August 2006 at the Monowai Experimental Facility (MEF) located at the Monowai Power Station, Southland, at the confluence of the Monowai and Waiau Rivers (Figure 6, and refer to Section 5.2.1 for more detailed information). Cobble sized substrates of approximately 5 to 10 cm diameter were selected from a ~90 m² area of the Waiau River bed. Substrates were visually assessed (using a divers mask) to select 180 rocks with maximum surface coating and thickness of *D. geminata* (Kilroy et al. 2006a). Selected cobbles had well-established mats (~ 2 cm thick), and were placed in the channels at MEF for 5 d (17 August 2006) to equilibrate to the facility conditions before chemical trials began. Initial viability of the *D. geminata* mats was established by a sub-sample of ten cobbles collected on the first day of the chemical exposures (22 August 2007).

4.2.2 Exposure solutions

On the days of the *D. geminata* exposures (22-24 August 2006), chemical dilutions in 49 L Monowai River water were prepared in 54 L fish bins (~ 70 x 40 x 30 cm) double-lined with plastic bags. Chemicals were mixed during dilution, and by aeration throughout the *D. geminata* exposure. Test chemicals were GemexTM, Hydrothol[®]-191, Organic InterceptorTM, K-TeaTM, sodium hypochlorite, or mixtures of these products (Table 3). When chemical mixtures were prepared, GemexTM was added to the 49 L and mixed prior to addition of the second chemical; no precipitation was observed.

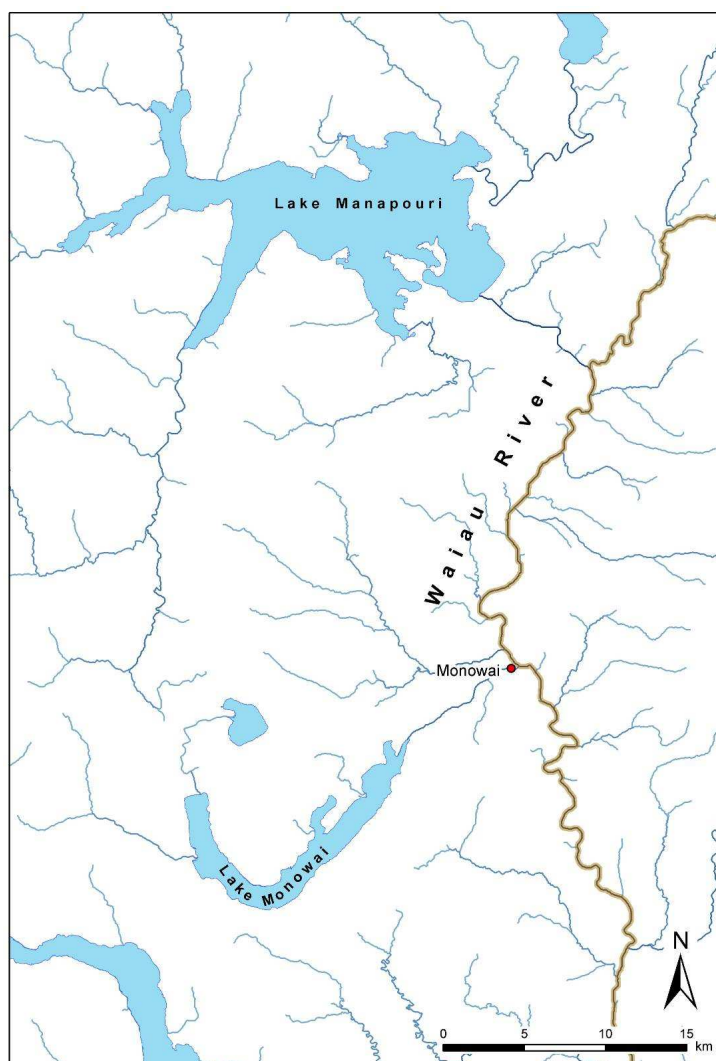


Figure 6: Map showing the location of the Monowai Experimental Facility. The thicker brown line indicates the stretches of river most heavily infested by *D. geminata*.

To commence each exposure, three replicate cobbles were transferred from the artificial channels into the exposure solutions in each fish bin. A control treatment was included with each set of trials. In the single exposures the cobbles were exposed to the chemical for 1 hour (a static exposure) then transferred back to the artificial channels. A series of multiple exposures were also performed so that the 1 h exposures were repeated daily over 3 d, for a total of three 1 h exposures. The effect of chemical treatment on didymo cell viability was measured by the neutral red staining method approximately 20 h after the chemical exposure (Kilroy 2005, Kilroy et al. 2006b) (see Section 4.2.3).

Temperature (°C) and dissolved oxygen (DO)(mg O₂/L) were measured during each chemical treatment and varied between 7.3-9.8°C and 11.3-14.9 mg O₂/L respectively over the 3 days of exposures. The pH was more variable but was pH 5-6 for most

exposures including controls and mixtures, pH 4 for Gemex™ concentrations ≥ 9 mg Cu/L, pH 7-8 for all KTEA solutions, pH 7 for 500 mg pine oil/L Organic Interceptor™, and pH 7 for 135 mg Cl/L NaOCl sodium hypochlorite. Lead concentrations in the Gemex™ stock were below detection (<0.0002 g Pb/L), and in the KTEA™ stock were 0.033 g Pb/L.

Table 3: Nominal chemical treatments (mg/L) tested in the Stage 2 Phase 2 cobble screening trials 22-25 August 2006. When mixtures were tested, concentrations are listed in the same order as the chemicals. The nominal Gemex™ and KTEA stock concentrations were 20 and 80 g Cu/L, measured stock concentrations were 18 and 99 g Cu/L respectively.

Chemical	Units	Single/Multiple exposure	Nominal Concentration	Nominal Concentration based on measured stock [Cu]
Control		Single	-	-
		Multiple	-	-
Gemex™	mg Cu/L (chelated)	Single	6, 12, 24	5, 11, 22
		Multiple	3, 9, 27	3, 8, 24
Hydrothol®-191	mg a.e./L	Single	4, 8, 16	-
		Multiple	2, 4, 8	-
Organic Interceptor™	mg pine oil/L	Single	192, 384, 500	-
		Multiple	32, 64, 102	-
Gemex™ / Hydrothol®-191	mg Cu/L /mg a.e./L	Single	4/4, 4/8	-
Gemex™ /Organic Interceptor™	mg Cu/L /mg pine oil/L	Single	2/32, 4/64, 8/64	-
KTEA	mg Cu/L	Single	3, 9, 27	4, 11, 33
Sodium hypochlorite	mg Cl/L	Single	5, 15, 45, 135	-

4.2.3 Assessment of *D. geminata* viability

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, Kilroy et al. 2006b). For each sample, five to six pieces of a *D. geminata* mat were placed into a 30 mL sample container already containing neutral red stain. Mat pieces were kept intact whilst in the stain, so the 'outer' surface (surface containing the cells) of the mat could be identified during slide preparation. This practice decreased stalk material to be examined on the slides and increased the likelihood of encountering *D. geminata* cells during microscope analysis. At least 100 *D. geminata* cells were enumerated for detection of those that might be live. Samples were analysed within one hour of staining, with no detectable decline in cell viability over this period. Kilroy et al. (2006b, see Appendices 1 and 2 in that document) has completed a thorough analysis of this technique including the validity of counting 100 cells (versus more/less cells) as an indicator of overall mat viability.

4.3 Results and discussion

4.3.1 Viability of the control treatments

The viability of the *D. geminata* mats in the control treatments was $89 \pm 4\%$ and $90 \pm 2\%$ for the single and multiple exposure controls respectively.

4.3.2 Multiple *versus* single exposures of *D. geminata*, and high concentrations of chemicals

Gemex™

Comparing the relationship between single and multiple exposures of Gemex™ in the cobble trials indicated (unexpectedly) that a single 1 h static exposure resulted in a lower percentage of viable cells than three static 1 h Gemex™ applications spaced 24 h apart (Figure 7).

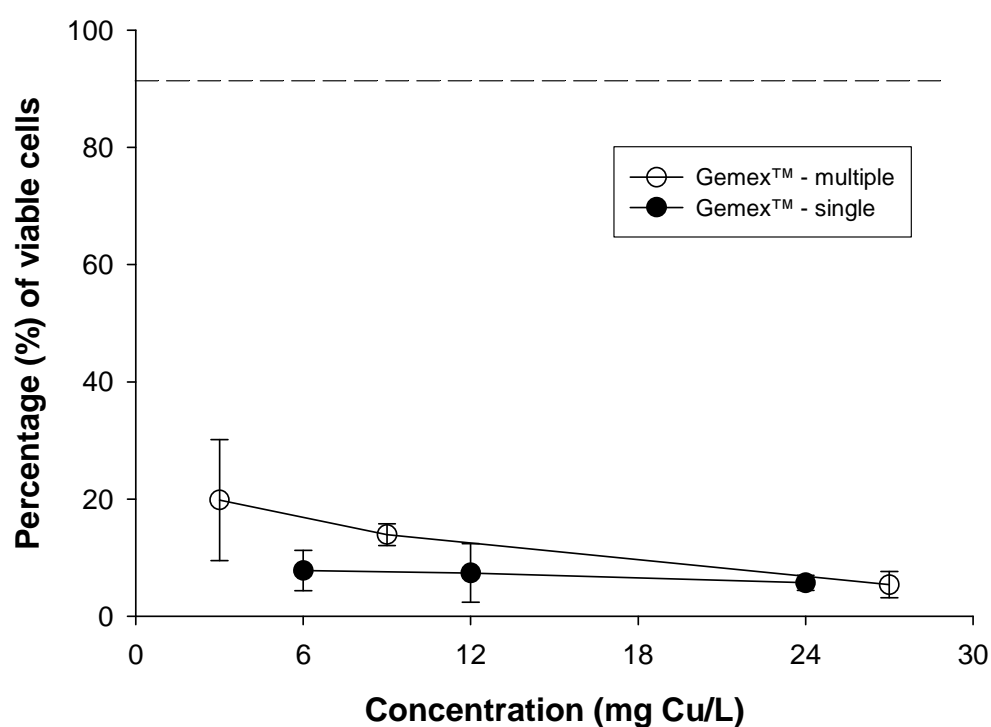


Figure 7: Comparison of average (\pm s.e.) *D. geminata* cell viability ~20 h after single (filled circles) or multiple (open circles) 1 h static Gemex™ exposures of mats on cobbles. Dashed line indicates the average of the single and multiple exposure controls. Chelated Cu concentrations are nominal.

A single 1 h exposure to 6, 12 and 24 mg Cu/L resulted in 8 ± 3 , 7 ± 5 , and $6\pm1\%$ viable cells respectively, whereas three 1 h exposures to 3, 9 and 27 mg Cu/L resulted in 20 ± 10 , 14 ± 2 and $5\pm2\%$ viable cells respectively. It is important to note that once the Gemex™ concentration was >12 mg Cu/L, the variability of the results decreased noticeably, suggesting that these concentrations were significantly more effective than lower concentrations. In addition the result that most defies the expected trend is the low effectiveness of the multiple exposure to 9 mg Cu/L; although these results were less variable than a single exposure to 6 or 12 mg Cu/L. Note that the viability measures were completed on two different sets of cobbles, so the single exposure results are not from the same mats as the multiple exposures. The effect of the two treatments (multiple versus single exposures) was not statistically significantly different once chelated Cu concentrations were > 8 mg Cu/L.

In future work a flow-through exposure would probably ensure better penetration and effectiveness of the Gemex™, particularly in multiple exposures. Another difference is that the actual mass of Gemex™ used in a one hour flow-through exposure would be far greater than that used in a static exposure, and would probably greatly increase the efficacy of the treatment. In addition a single viability assessment in these static trials was probably not sufficient to discriminate between treatments once the *D. geminata* viability was $<10\%$. Longer-term monitoring might have achieved this, because we have observed that after some treatments cell viability continued to decline >24 h post-treatment. However, longer-term monitoring was not conducted for these trials which were used for screening and rangefinder assessment only. Lastly observation of the actual condition of the *D. geminata* cells in these treatments 20 h post-treatment strongly suggests that these higher concentrations were more effective than concentrations <10 mg Cu/L.

Within the single exposures, increasing Cu concentrations above 6 mg Cu/L did not increase the effectiveness of the exposures, except to decrease the variability of the results. Decreased variability is significant because we have observed that in general, as treatment efficacy increases, variability remains very low, along with low viability. These data also showed that greater than 95% cell mortality could probably be achieved using 15-20 mg Cu/L (as chelated Cu) Gemex™ in flow-through exposures; concentrations that would probably be non-lethal to resident fish. A flow-through exposure is likely to significantly increase the effectiveness of Gemex™ treatments suggesting that $> 95\%$ mortality is an attainable goal.

Hydrothol®

In contrast to the Gemex™ data from cobble trials, multiple applications of Hydrothol®-191 resulted in lower percentage viable cells than single exposures. When directly contrasting single and multiple applications of Hydrothol®-191 at 4 and 8 mg a.e./L, cell viability was at least 20% lower in multiple exposures than for single exposures (Figure 8).

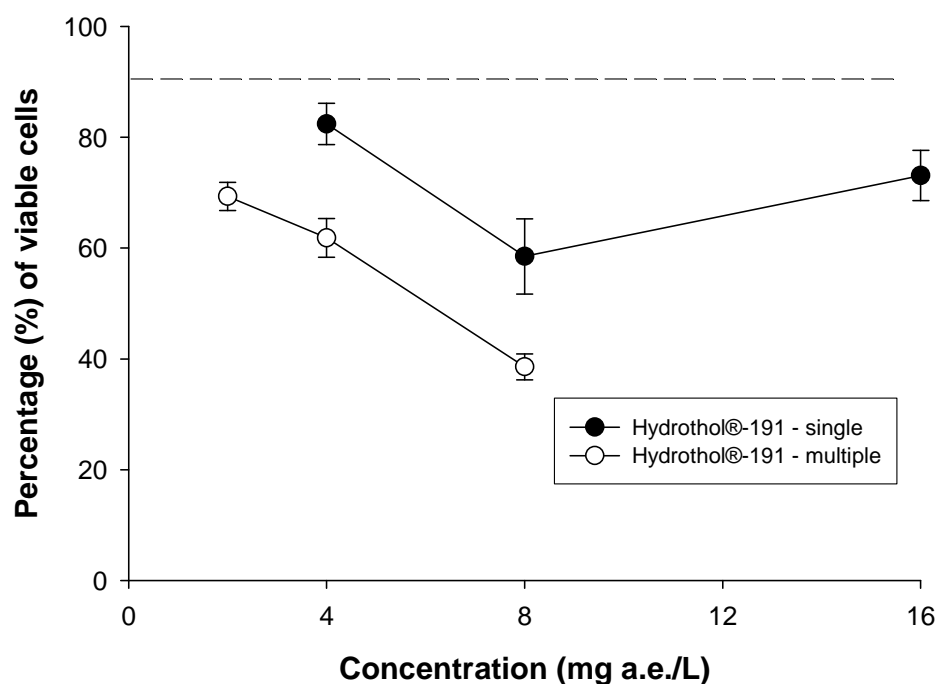


Figure 8: Comparison of average (\pm s.e.) *D. geminata* cell viability ~20 h after single (filled circles) or multiple (open circles) 1 h static Hydrothol®-191 exposures of mats on cobbles (nominal concentrations). Dashed line indicates the average of the single and multiple exposure controls.

The poor dose-response pattern of these data demonstrated that even relatively high concentrations of Hydrothol®-191 (> 5 mg a.e./L) were relatively ineffective at decreasing *D. geminata* cell viability. The maximum treatment rates recommended against aquatic macrophytes in the U.S.A. are 5 mg a.e./L only for areas where fish are not present, or where fish mortalities would be an acceptable side effect (WSDE 2001).

Organic Interceptor™

Single 1 h static exposures of *D. geminata* mats to 192-500 mg pine oil/L Organic Interceptor™ produced highly variable cell viability (Figure 9). The cobble trials did not replicate the results from the artificial channel trials (Stage 2 Phase 1), where exposure to 271 mg pine oil/L caused ~98% mortality. It is thought that although the Organic Interceptor™ solutions were vigorously mixed both before and throughout the cobble exposures, the mat penetration by the chemical in the static exposures is much less than occurred in the flowing water exposure provided by the artificial channel trials. There may have been some loss of chemical through adsorption to the container walls. This may have resulted in the highly variable viability after a single exposure to high concentrations of Organic Interceptor™. Three 1 h applications of 32-102 mg pine oil/L Organic Interceptor™ also did not cause significant mortality of *D. geminata* cells (Figure 9). Given that the laboratory testing of rainbow trout showed that exposure to > 60 mg pine oil/L would adversely affect fish, these data collectively indicate that, despite the limitations of the static cobble trials, Organic Interceptor™ cannot be used effectively against *D. geminata* without unacceptable negative effects on non-target species.

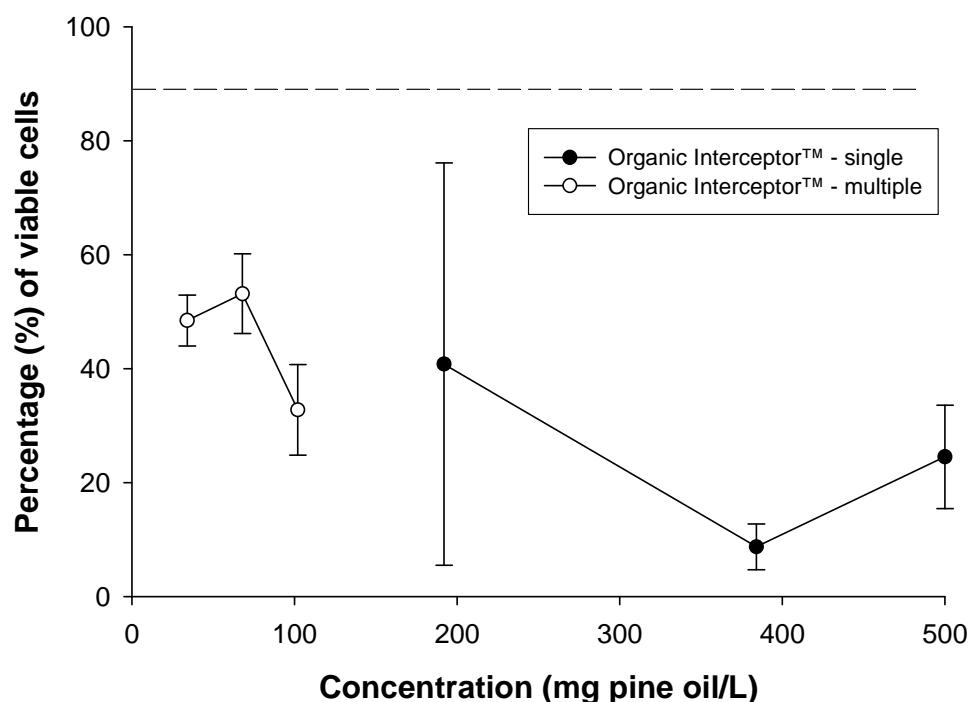


Figure 9: Comparison of average (\pm s.e.) *D. geminata* cell viability ~20 h after single (open circles) or multiple (filled circles) 1 h static Organic Interceptor™ exposures of mats on cobbles (nominal concentrations). Dashed line indicates the average of the single and multiple exposure controls.

Sodium hypochlorite (NaOCl)

In contrast to the Organic Interceptor™ data, a typical dose–response relationship was observed when *D. geminata* mats were exposed to NaOCl in static 1 h exposures (Figure 10). There was a significant relationship between decreasing cell viability and increasing concentrations of NaOCl when the data was fitted with an exponential decay curve ($y = y_0 + ae^{-bx}$) (ANOVA: $F_{2,3} = 1718.89$, $R^2 = 0.99$, $P = 0.02$). Cell viability was $0.7\% \pm 0.6$ at 135 mg Cl/L. Sodium hypochlorite is an oxidising agent and, in high enough concentrations, probably physically disrupts the *D. geminata* mats ensuring good penetration of the chemical and almost complete cell mortality.

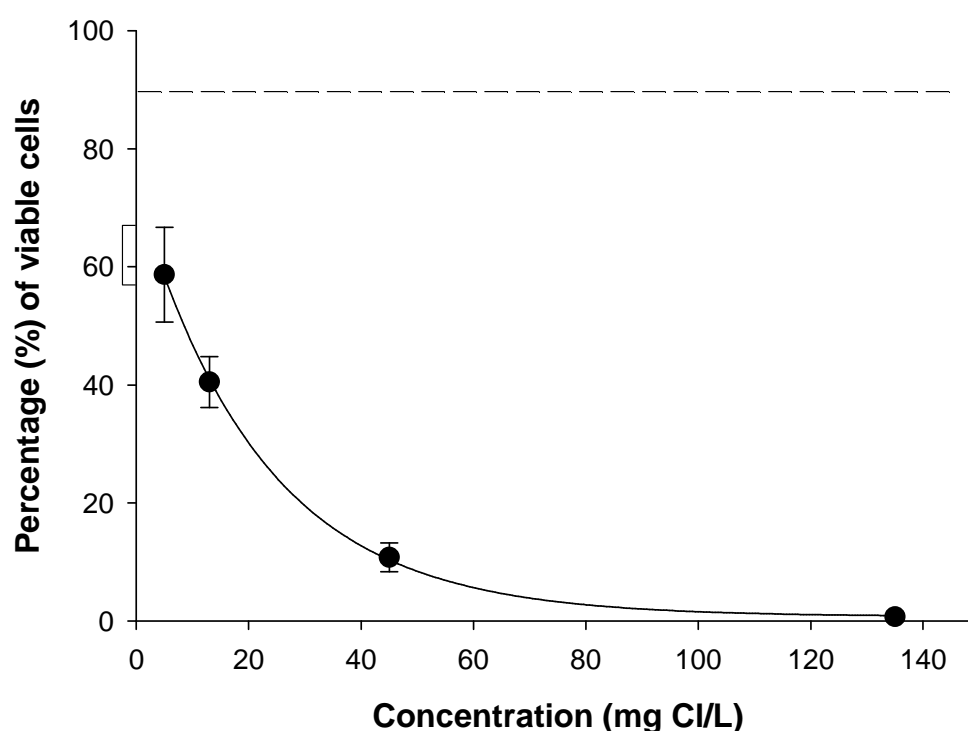


Figure 10: Average (\pm s.e.) *D. geminata* cell viability ~20 h after a 1 h single static exposure to sodium hypochlorite (nominal concentrations) of mats on cobbles. Data fitted with exponential decay curve. Dashed line indicates the average of the single exposure control.

4.3.3 Comparison of Gemex™ and K-Tea™ effectiveness

This comparison of two different chelated copper formulations clearly shows that over a range of potential copper concentrations Gemex™ is a more effective compound at reducing the survival of *D. geminata* cells (Figure 11). Cell viability was still at 14% ± 4.6 at 33 mg Cu/L (as chelated Cu) for K-Tea™, compared with 6% ± 1.3 at 24 mg Cu/L (as chelated Cu) for Gemex™.

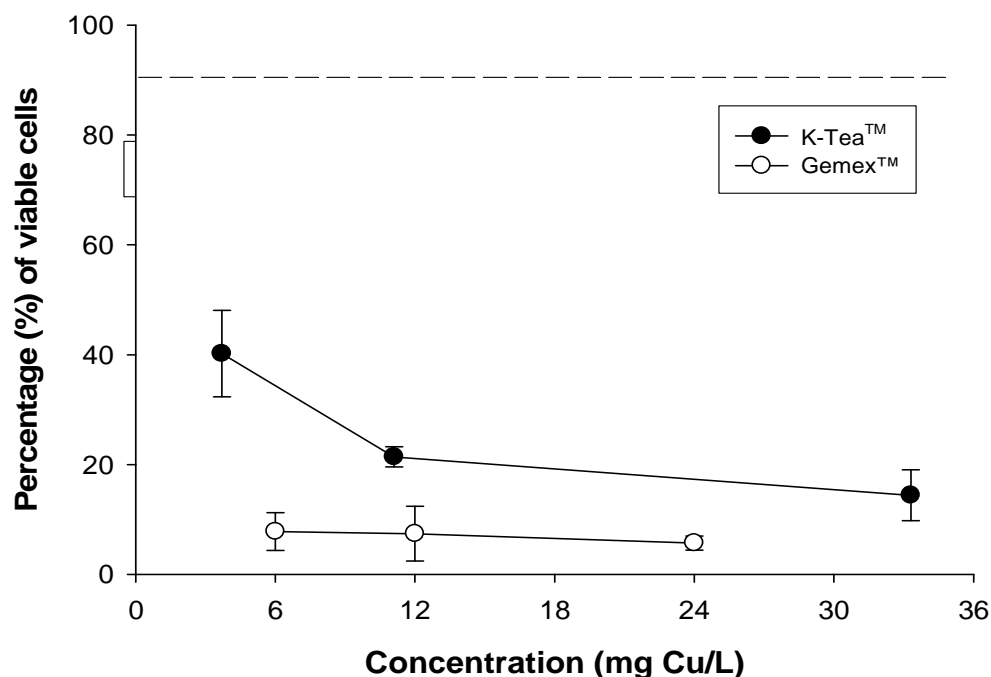


Figure 11: Comparison of average (± s.e.) *D. geminata* cell viability ~20 h after single 1 h static exposures to K-Tea™ (filled circles) or Gemex™ (open circles) of mats on cobbles (nominal concentrations). Dashed line indicates the average of the single exposure control.

4.3.4 Effect of chemical combinations on *D. geminata* cell viability

Gemex™ in combination with Hydrothol®-191 (at both 4 and 8 mg a.e./L) was ineffective in reducing cell viability of *D. geminata* below that achieved with exposure to Gemex™ alone at similar concentrations. Cell viability did not decrease below 50% with either of these combinations (Figure 12). Results were more variable for the combination of Gemex™ and Organic Interceptor™, however, the product combination was far less effective than Gemex™ alone. It is likely that both Hydrothol®-191 and Organic Interceptor™ decrease the toxicity of Gemex™ to *D. geminata* by decreasing the bioavailability of the chelated Cu to the algal cells.

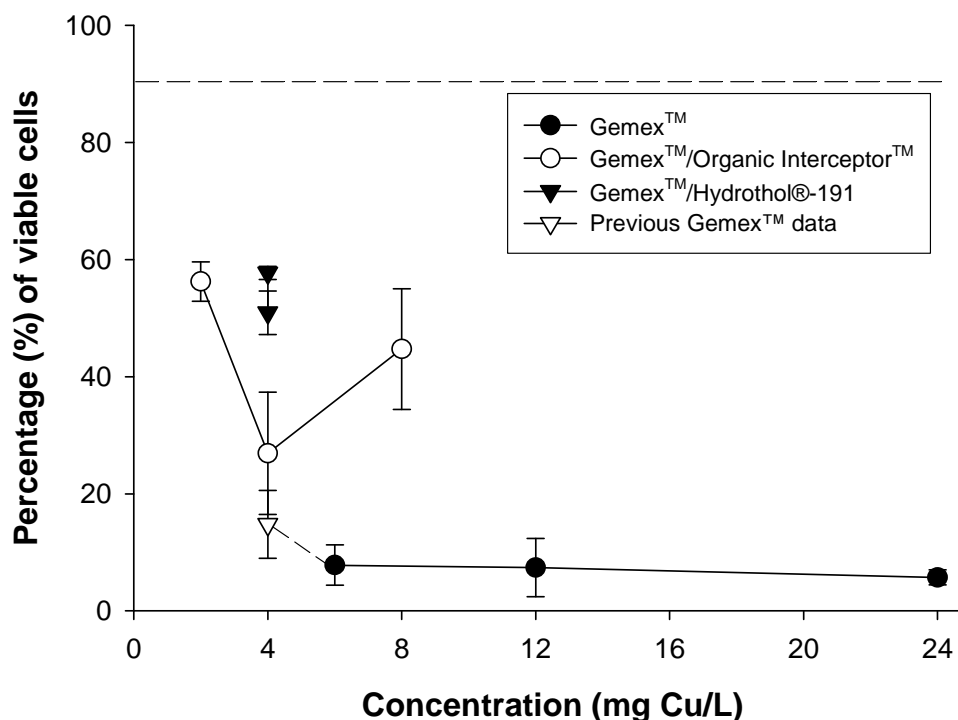


Figure 12: Comparison of average (\pm s.e.) *D. geminata* cell viability ~24 h after single 1 h static exposures to Gemex™/Hydrothol®-191 (closed triangles) and Gemex™/Organic Interceptor™ (open circles) contrasted against Gemex™ alone (closed circles, open triangle). For the concentrations of Hydrothol®-191 and Organic Interceptor™ used in this comparison see Table 3.

4.4 Conclusions, integration of laboratory toxicity tests and screening trials and recommendations for Stage 2 Phase 2 channel trials

One of the limitations of conducting the cobble trials in a static (non-flowing) environment is that, compared to a flowing water exposure, the static exposure probably results in lower penetration of the thick fibrous mats by chemicals (with the exception of high concentrations of sodium hypochlorite). It is likely that cell mortality on the outer surface of the *D. geminata* mats (where most cells are located) would be significant but that without getting thorough transfer of the biocides into the sub-surface layer of the mat, > 95% cell mortality is unlikely. As a result of these limitations to the cobble trials, we believe it was difficult to discriminate between treatments that could cause > 90% *D. geminata* cell mortality (or < 10% cell viability). It is likely that this is the main reason for any discrepancies between these cobble trial data and the more thorough analysis from Stage 2 Phase 1 in artificial channels using flowing water exposures. Nonetheless the rapid cobble screening was a time-efficient method to meet the aims of this trial and to quickly contrast a range of chemicals, concentrations, combinations and application methods.

4.4.1 Gemex™ and K-Tea™

When Gemex™ was combined with other biocides, it was apparent that not only were there no synergistic effects arising from the combinations, but the effectiveness of Gemex™ against *D. geminata* was actually decreased. Gemex™ was also compared to another chelated copper formulation, K-Tea™. Results showed that Gemex™ was more effective in reducing *D. geminata* cell viability than K-Tea™.

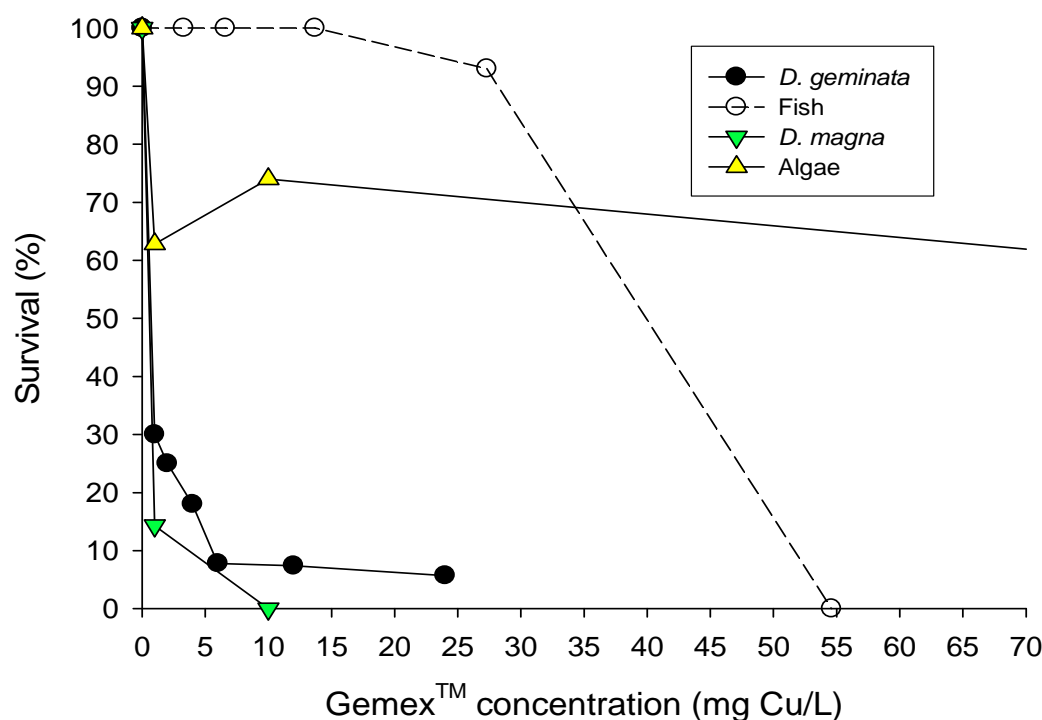


Figure 13: A summary of the percentage survival of all species tested to date after exposure to a range of nominal and measured (for higher concentrations tested on fish) Gemex™ concentrations. All responses shown are based on an exposure time of 1 h. The fish data are a combination of laboratory and field data for rainbow trout and bullies up to 4 mg Cu/L, then are laboratory data only for juvenile rainbow trout. The *D. geminata* data ≥ 6 mg Cu/L are static exposures only from the screening trials. The algal and *D. magna* data were static laboratory exposures.

Laboratory toxicity testing of Gemex™ has confirmed the indicative toxicity results from the biocide product testing trials (Stage 2 Phase 1) (Jellyman et al. 2006a). Further to the 100% survival of fish species at 4 mg Cu/L in artificial channel trials, laboratory toxicity testing showed that a 1 h application of Gemex™ had minimal effect on juvenile fish survival up to ~27 mg Cu/L (measured) (where 93% of fish tested were alive) (Figure 13). Fish are the non-target species of most concern as they are an important ecological component in most of the *D. geminata* affected waterways

and they will take the longest (perhaps years) to re-establish if killed in significant numbers by a *D. geminata* treatment. Non-target green alga growth was decreased only by 30-50% by 1 h exposures up to 100 mg Cu/L (nominal), suggesting that Gemex™ would be most effective against *D. geminata*, and as desired, non-target native algae would recover more rapidly. A 1 h exposure to a nominal concentration of 10 mg Cu/L Gemex™ was 100% lethal to the pond invertebrate *D. magna*, suggesting invertebrate losses could be significant in a treated waterway. On the other hand native mayfly species have been shown to be relatively tolerant to some dissolved metal exposures such as zinc, therefore direct testing of river invertebrate species is recommended. Lastly wide fluctuations in invertebrate density are typical of river environments due to flood events and seasonal changes in water quality, therefore invertebrate populations in a treated waterway would be expected to recover relatively quickly (within weeks) due to processes such as downstream migration and repopulation by resistant life stages. At this stage, Gemex™ appears to be the best product for further assessment because of its effectiveness against *D. geminata* and the scope to test higher concentrations of the biocide without harming fish species as well as moderate impacts on non-target algae.

4.4.2 Hydrothol®-191

Results from the cobble trials of Hydrothol®-191 (viability data for > 3 mg a.e./L) showed a marked difference in *D. geminata* cell survival when compared with viability results from Stage 2: Phase 1 channel trial data (Figure 14). For both single and multiple exposures of Hydrothol®-191, cell viability has not been recorded below 35%.

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. At concentrations above 2.0 mg a.e./L, fish species were negatively affected, with 100% mortality of juvenile rainbow trout observed at 4.0 mg a.e./L. The impact of Hydrothol®-191 on resident fish species coupled with its poor efficacy for *D. geminata* means that it will not be considered for further assessment as a *D. geminata* control compound.

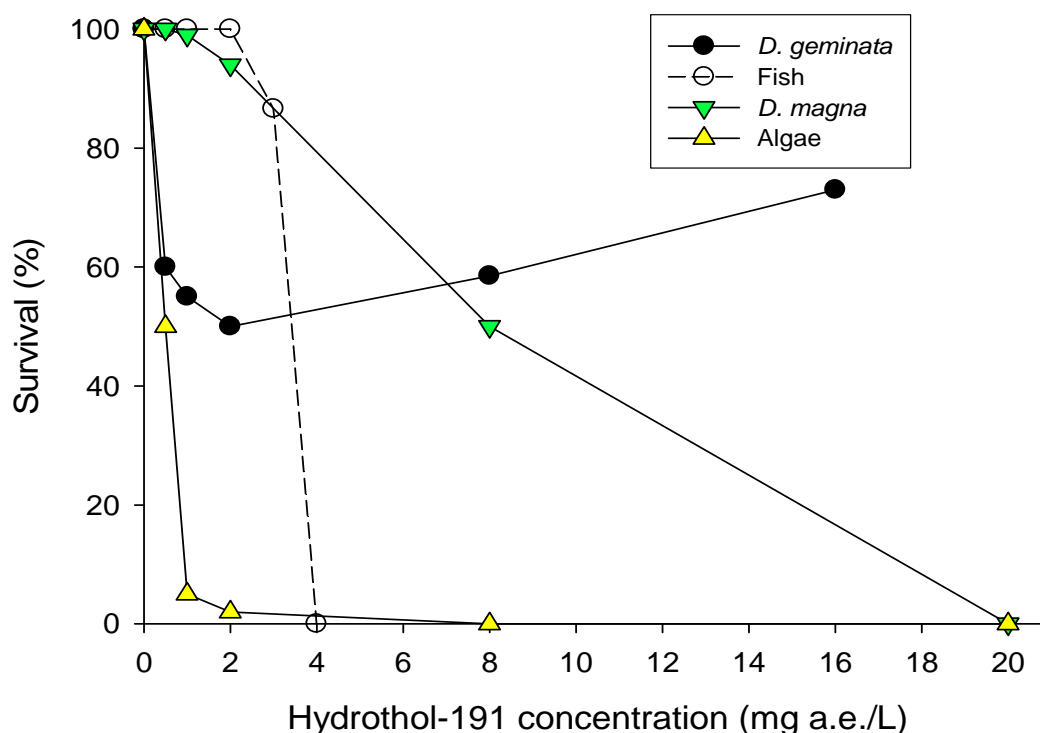


Figure 14: The combined responses of all species tested to date to a range of Hydrothol®-191 concentrations. All responses shown are based on an exposure time of 1 h.

4.4.3 Organic Interceptor™

The results for single static exposures of Organic Interceptor™ in the cobble trials were highly variable and contrasted those of previous trials (Stage 2 Phase1) as discussed earlier (Section 4.3.2), probably because the chemical did not penetrate the mats as effectively as in the flow-through exposures. For the purposes of discussion the cobble trial results for Organic Interceptor™ have been excluded from Figure 15.

The toxicity data indicate that a 1 h exposure of Organic Interceptor™ at a rate necessary to cause moderate mortality to *D. geminata* (i.e., ~100 mg pine oil/L¹¹) would cause severe mortality to resident fish species (Figure 15). Neither the highest multiple application rate of Organic Interceptor™ (102 mg pine oil/L) or Organic Interceptor™ in combination with Gemex™ were effective in causing considerable (> 90%) mortality to *D. geminata* in the cobble trials. Although local invertebrates and non-target algal species might not be significantly negatively affected by the

¹¹ Organic Interceptor™ is expressed in mg pine oil/ L. However, the compounds that comprise the biocide are trade marked and there is no certainty about the identity of the active ingredient that affects *D. geminata*.

application of Organic Interceptor™, its severe mortality to fish species means that it will not be considered for further assessment as a *D. geminata* control compound.

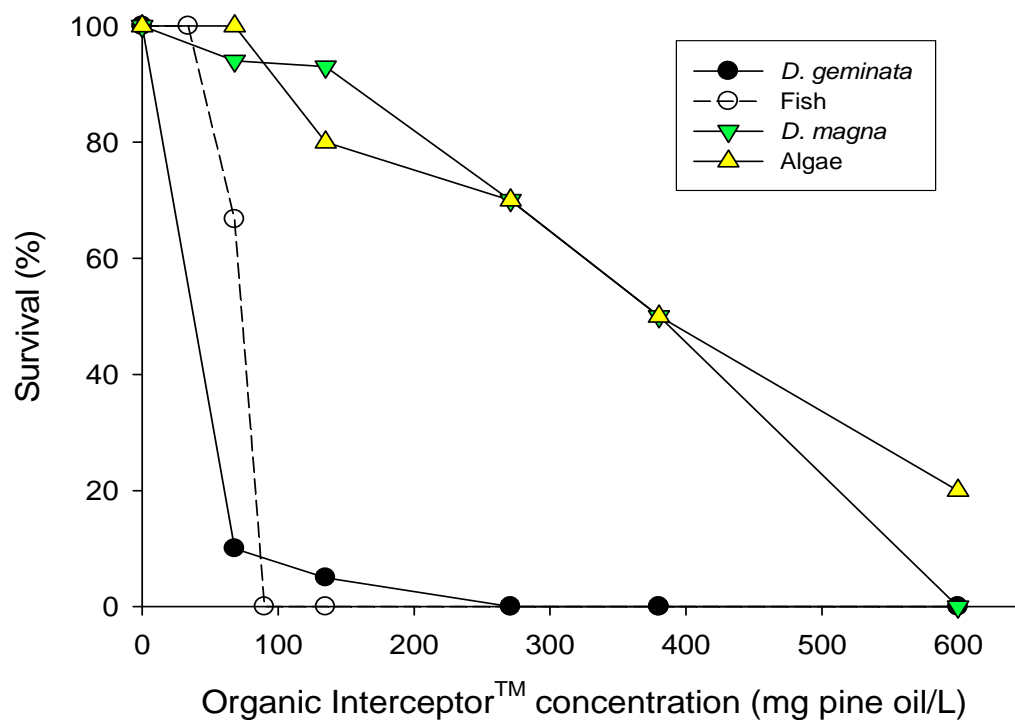


Figure 15: The combined responses (percentage survival) of all species (tested to date) to a range of Organic Interceptor™ concentrations. All responses shown are based on an exposure time of 1 h. Data on *D. geminata* mortality from the cobble trials have not been included in this graph for reasons outlined above.

5. Gemex™ refinement trials

5.1 Introduction

As summarised in Section 4.4, the data from the laboratory toxicity testing on fish and the last round of cobble trials supported the findings of Jellyman et al. (2006a) that Gemex™ is the most promising control agent for *D. geminata*. Gemex™ and Organic Interceptor™ were both effective against *D. geminata*, however it is clear that Organic Interceptor™ would be toxic to fish when applied at doses sufficient to control *D. geminata*. The cobble screening trials showed that Gemex™ could be applied in single doses (6-24 mg Cu/L, as chelated Cu) sufficient to cause high rates of *D. geminata* mortality (92-94%) even in a static environment. The next round of artificial channel trials were designed to explore the effect of three factors on Gemex™ toxicity to *D. geminata*:

1. Concentration: we tested a range of concentrations between 5-20 mg Cu/L, expecting that it was most likely that Gemex™ would be applied at 10-15 mg Cu/L.
2. Water velocity: in natural waterways *D. geminata* is found in waters flowing at an unusually wide range of velocities and is distinctive because it thrives at higher velocities (~1 m/s)(Kilroy et al. 2006a). Aquatic herbicides are not usually applied to flowing waters therefore we had no information on how changes in water velocity would influence the effectiveness of Gemex™. We thought it most likely that higher velocity waters would increase the penetration of Gemex™ into the *D. geminata* mats, but it was also feasible that longer contact times in slow velocity water would increase the toxicity of Gemex™.
3. Multiple applications: the cobble trials indicated that unlike Hydrothol®-191 and Organic Interceptor™, Gemex™ effectiveness was not increased by multiple applications. However, these were static trials and probably were not able to differentiate well between treatments that decreased *D. geminata* viability below 10%. It was important to test multiple exposures in a flow-through application, particularly as the cobble trials suggested that a dose of as little as 5 mg Cu/L could cause 92% mortality of *D. geminata*. It seemed feasible that one to two follow-up doses of only 5 mg Cu/L to a dying and degraded *D. geminata* mat might be more effective as a control method than a single higher dose of 10-15 mg Cu/L to an intact mat.

Although these trials were designed primarily to explore the effects of Gemex™ on *D. geminata*, we also took the opportunity to examine the effects on fish and invertebrates, albeit at an indicative level.

5.2 Methods

5.2.1 Study site, the Monowai Experimental Facility

The trial was carried out at the Monowai Experimental Facility (MEF). This is a purpose-built experimental streamside trough facility located on land owned by Pioneer Generation Ltd. at the Monowai Power Station, Southland (N 5478740, E 2091405). The hydro-electric power station at Monowai is supplied by a 1.2 km pipeline which is sourced from the upper Monowai River (a regulated waterway that drains Lake Monowai, see Figure 6). The experimental facility has the capacity to extract ~150 L/s from the penstocks. The water used at the facility has very low nutrient levels (see Appendix 4), but is considered suitable for growth of *D. geminata*, because *D. geminata* proliferates throughout the lower reaches of the Monowai River. The water that supplied the facility contained no *D. geminata* cells (last checked at 9 August 2006) as the water is diverted to the power station above the point where *D. geminata* has colonised the lower Monowai River. The facility consists of two 3000 L header tanks which supply 48 channels (10 × 10 × 150 cm) arranged in groups of six, radially around the two tanks (Figure 16). Each channel can be supplied with a maximum flow of approximately 3 L/s. The design of the system allows the water flow to each channel to be adjusted throughout its entire range without affecting the flow to other channels.

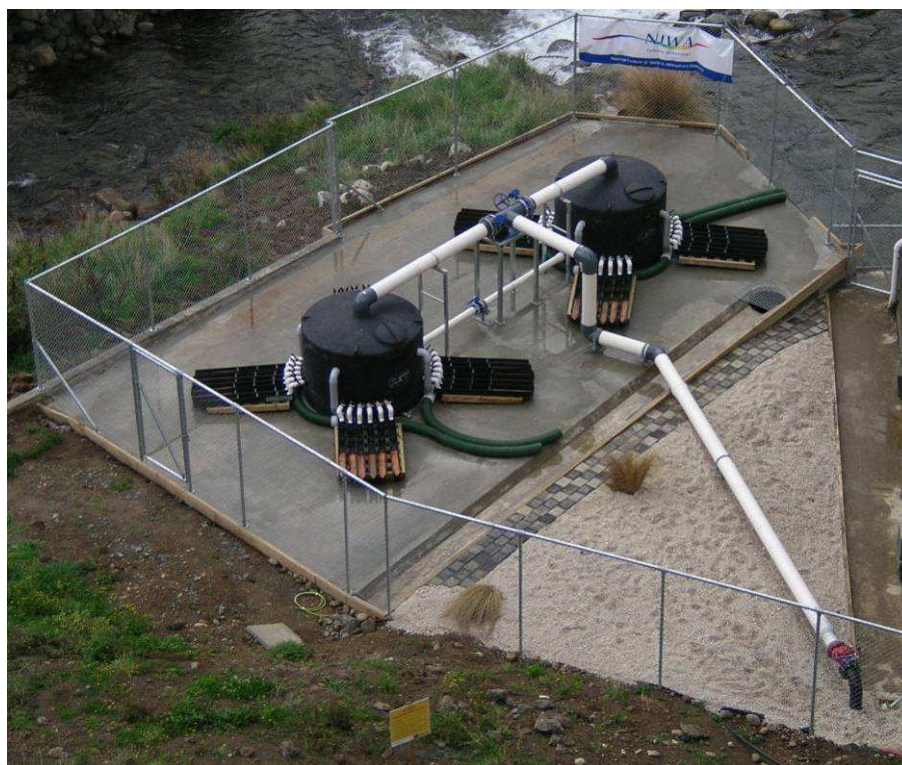


Figure 16: The Monowai Experimental Facility (MEF) used for Stage 2 field trials.

5.2.2 Colonisation of *D. geminata* substrates for the experimental facility

Six weeks prior to commencing the chemical exposures (9 August 2006), purpose-built substrates were placed in two rivers to allow colonisation with *D. geminata*. The substrates (9 cm x 50 cm) were constructed from a clear acrylic plastic base (55 cm x 9.5 cm x 0.6 cm), and a contoured plastic mould that simulates a cobbled river substrate. Artificial substrates provide a uniform sampling surface, and allow for easier and more reproducible sampling because they reduce heterogeneity in microhabitats (Biggs & Kilroy 2000). Forty-three substrates were bolted to concrete blocks and placed in the Waiau River ~150 m upstream of the confluence with the Monowai River (and the MEF) in ~0.75 m deep rapidly flowing water, oriented parallel to the current (longitudinally) (Appendix 1). Approximately 40 substrates were also placed in the Clutha River (north of Lake Dunstan N5583840, E2217980) as a back-up option in case changes in water quality associated with flood events (e.g., high turbidity, scouring) prevented colonisation of substrates in the Waiau River. Regular monitoring of the substrates and removal of objects fouling the bolts (e.g., grass, algae) allowed uniform growth of the *D. geminata* mats. A week before commencing the chemical exposures (19 September 2006), 43 colonised substrates were retrieved in high-flow conditions from the Waiau River, stacked with spacers in a large plastic tub without water, carried downstream to the MEF, screwed onto

untreated timber sections (55 cm x 9.5 cm x 5 cm) in the experimental channels and supplied with flowing water (~ 1 L/s) within 1-2 hours of being removed from the river (longer air exposure causes death of the algae, and filled containers causes water movement that detaches the algal mats). 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*. The substrates retrieved from the Waiau River were colonised with ~15 mm thick *D. geminata* mats that covered ~95% of the surface area. The *D. geminata* mats continued to grow in the MEF prior to chemical treatment¹².

5.2.3 Experimental design

As described in Section 5.1, the experiment primarily tested the effect of Gemex™ dose (5-20 mg Cu/L, as chelated Cu), water velocity, and multiple applications (three 1 h doses 24 h apart) (Table 4). Previous research had established that a 1 h contact time was more effective than shorter exposures (0.6 or 6 min) against *D. geminata*, and exposures much longer than 1 h were considered impractical for the logistics of dosing a river. Although not the main aim of the experiment, both fish and invertebrates were included in the exposures at an indicative level. The various treatments were assigned randomly to the channels to account for any effects of channel position. In order to test the effect of water velocity on chemical effectiveness (rather than the effect of water velocity on subsequent *D. geminata* growth), the water velocity was adjusted only for the day of the chemical exposure, and the following day was returned to an average flow velocity maintained for all of the channels (~0.4 m/s).

¹² The Clutha River substrates were a back-up option to the Waiau River and were monitored approximately weekly; good growth on the Clutha substrates was achieved by 12 September 2006, but high volume flows of turbid water removed much of the algal mats a week later. In the end, these substrates were not required for the trials.

Table 4: Gemex™ application frequency, concentration (mg Cu/L, as chelated Cu), water velocity, flow rate, volume of 2% Gemex™, and mass of Cu added to channels, and tested in the Gemex™ refinement trials in 1 h exposures in artificial channel trials. The 2% Gemex (20.36 g Cu/L nominal, 18.6 g Cu/L measured) was the stock solution provided by the manufacturer. Multiple 1 h applications were conducted 24 h apart on 3 consecutive days. (# reps = number of replicate channels).

Treatment	Applications	Nominal Gemex™ Treatment Concentration based on nominal stock [Cu] (mg Cu/L)	Nominal Gemex™ Treatment Concentration based on measured stock [Cu] (mg Cu/L)	Velocity	Velocity (m/s)	Flow rate (L/s)	Volume 2% Gemex™ added per replicate (L)	Mass (g Cu)	# reps
Control-slow	Single	0	0.0	Slow	0.1	0.25	0	0.0	2
Control-medium	Single	0	0.0	Medium	0.3	0.60	0	0.0	2
Control-fast	Single	0	0.0	Fast	0.6	2.00	0	0.0	2
Cu 5-slow	Single	5	4.5	Slow	0.1	0.25	0.22	4.1	3
Cu 5-medium	Single	5	4.6	Medium	0.3	0.60	0.53	9.9	3
Cu 5-fast	Single	5	4.6	Fast	0.6	2.00	1.77	32.9	3
Cu 10-slow	Single	10	9.1	Slow	0.1	0.25	0.44	8.2	3
Cu 10-medium	Single	10	9.1	Medium	0.3	0.60	1.06	19.7	3
Cu 10-fast	Single	10	9.1	Fast	0.6	2.00	3.54	65.8	3
Cu 15-medium	Single	15	13.7	Medium	0.3	0.60	1.59	29.6	3
Cu 20-slow	Single	20	18.2	Slow	0.1	0.25	0.88	16.4	3
Cu 20-medium	Single	20	18.3	Medium	0.3	0.60	2.12	39.4	3
Cu 20-fast	Single	20	18.3	Fast	0.6	2.00	7.07	131.5	3
Cu 5x3-medium	Multiple	5	4.6	Medium	0.3	0.60	0.53 ^A	29.6	3
Cu 10x3-medium	Multiple	10	9.1	Medium	0.3	0.60	1.06 ^A	59.1	3
TOTAL									42

^AEach of these volumes were added a total of 3 times to the channels.

5.2.4 Calibration of water velocities

We assessed the toxicity of the selected biocide to *D. geminata* at three water velocities. The water velocity was adjusted only for the day of the chemical exposure, and the following day was returned to an average water velocity maintained for all of the channels (~0.4 m/s). We altered both channel slope and water flows to manipulate water velocities as much as possible between the extremes available in the MEF system. Ultimately each channel was calibrated to flow at water velocities of either 0.1, 0.3 or 0.6 m/s (termed slow, medium, and fast respectively), which in the MEF channels corresponded to flow volumes of 0.25, 0.60 or 2.00 L/s. These velocities correspond to a typical range of those in natural waterways where *D. geminata* is known to thrive.

5.2.5 Preparation of chemicals and delivery of doses

Gemex™ exposures commenced on 27 September 2006, with all treatments prepared from a 2% Gemex™ stock (20.36 g Cu/L nominal, measured 18.6 ± 0.3 g Cu/L) for a range of concentrations (Table 4). The amount of biocide used in each channel was calculated according to the relevant flow volumes (0.25-2.00 L/s). The required concentration of 2% Gemex™ (calculated from nominal concentrations¹³) was added to a 20 L solar shower, and brought up to a total volume of 20 L. The shower was then suspended from a wooden rack (c. two metres high) above the channels (see illustrations, Appendix 1) immediately prior to the trial. Each solar shower was fitted with a dispensing hose and tap calibrated to dispense 20 L of Gemex™ solution over a one hour period (c. 320 – 350 mL/min). Water samples were taken from each channel during the Gemex™ application. The taps were positioned in the centre of the flow to allow thorough mixing and even dispersal of the biocides in the channel. Mixing was checked visually prior to the trials using food colouring. Biocides and application rates were randomised across all channels, to account for any differences in channel position. Lead concentrations measured in the Gemex™ stock (i.e., the nominal 20.36 g/L concentrate) were 0.0013 g Pb/L¹⁴.

5.2.6 Monitoring of water quality

Water quality parameters (temperature, pH and dissolved oxygen) were monitored during the application of the biocide using probes (YSI 95, YSI 60) placed

¹³ Gemex™ stock was delivered to the Monowai Experimental Facility immediately prior to the experiments labelled as nominal concentration 20.36 g Cu/L. The actual Cu concentration in the stock was measured after the experiments were completed (18.6 g Cu/L).

¹⁴ Note that once the stock is diluted approximately 1000-fold to make a 20 mg Cu/L solution for treating *D. geminata*, the Pb concentrations will also be diluted to ~0.0013 mg Pb/L.

approximately 10 cm downstream from the top of the substrates. Measurements were taken approximately 30 min after commencing the biocide application, along with collection of 15 mL water samples. Water samples were acidified (1% HNO₃, trace metal grade) and measured for Cu and lead (Pb) concentrations by ICPMS (Hill Laboratories, Hamilton). Lead concentrations were measured because Pb is a potential trace contaminant of the Gemex™ concentrate. Lead concentrations were less than the detection limit (<0.002 mg/L) in 65% of the samples, were 0.002-0.011 mg/L in 33% of the samples, and in one sample Pb was 0.035 mg/L. The ANZECC (2000) guideline (95% level of protection) for a chronic (long-term) discharge is 0.0034 mg/L. A one hour exposure to Pb concentrations ≤ 0.035 mg/L will have negligible effect on aquatic organisms (ANZECC 2000, Rogers et al. 2003, USEPA ECOTOX 2006, Meyer et al. 2007). Dissolved Ca and Mg at MEF was 3.19 and 1.05 mg/L respectively (0.45µm filtered, ICPMS, method APHA 3125 B 20th ed 1998) and water hardness was 12 mg CaCO₃/L, (calculated from dissolved Ca and Mg, method APHA 2340 B 20th ed 1998; also confirmed by titration against 0.01 M EDTA method APHA 2340C).

5.2.7 Inclusion of native fish in artificial channel exposures

From 20-26 September 2006 prior to the start of the experiment, fish were sourced for inclusion in the artificial channel exposures. Heavy rain and flooding in the Waiau River catchment prevented fish species used in the previous channel trials being located in sufficient numbers [juvenile rainbow trout (*Oncorhynchus mykiss*) and common bully (*Gobiomorphus cotidianus*)]. The only fish species that could be sourced in sufficient numbers were adults of a non-migratory galaxiid, southern flathead galaxias (*Galaxias* sp. S – uncertain species status¹⁵). Galaxiids were caught via electrofishing from a tributary of McKerchar Creek (5482485 N, 2097360 E) and transported back to the MEF. Fish used during the trial were 60 – 100 mm total length. Fish were contained in flow-through four-litre cages c. 20 cm x 20 cm x 25 cm, held in fish bins receiving the water flow from the end of the channels. Each cage had at least two sides of stainless steel mesh (mesh size 2 mm) or had 30 holes drilled into opposing sides (hole diameter 7 mm). Each fish cage contained 1-2 cobbles for refuge and to weigh the cages down. Fish cages were covered in shade cloth, or purpose built lids to stop fish escaping and prevent bird predation. Fish were fed on stream invertebrates prior to the commencement of the trial, then on frozen bloodworms (standard aquarium variety) during the trial. Fish were fed every second day from days 5 – 14 at a minimum rate of approximately 2 g/day.

¹⁵ Galaxias species with uncertain status have been assigned letters to distinguish them from one another –hence species “S”.

One fish cage containing three galaxiids was installed in each of the 42 channels used for the trial. Fish status was checked on at least four occasions: immediately after the application of the biocide (i.e., 1 hour), 1 d, 7 d and 14 d. If a dead fish was found during a status check, it was removed from the cage and frozen for later examination. A small proportion (7%) of the fish escaped during the first hour, and over the ensuing 14 day period 25% of fish escaped (the plastic containers continually deform under the pressure of the relatively high water flows, weakening the glued/heat-welded attachments between mesh and plastic or creating gaps in the mesh lids, despite continual maintenance). Fish that escaped were assumed to be alive (as they were not found inside the cage like dead fish) but nonetheless were excluded from statistical analyses to provide a conservative interpretation of survival data. The fish survival component of the trial was ended after 14 days. It was thought that after this time it would be difficult to distinguish fish mortality resulting from Gemex™ from the impacts of fish captivity in the experimental facility. Native galaxiids tend to feed at a high rate on a on diverse diet of invertebrate species; it was beyond our logistical capabilities to provide adequately diverse diets and cage maintenance past a 14 day period.

5.2.8 Inclusion of stream invertebrates in artificial channel exposures

Stream invertebrates were collected from a tributary of McKerchar Creek approximately 7 km from the MEF by gently scrubbing cobbles in a Surber sampler placed mid-stream. Approximately 10-15 invertebrates were added to 30 cages (1 mm mesh), with 1-2 cobbles added as substrate and weights to stabilise the cages (Appendix 1, Figure A1-4). The invertebrates were placed alongside the caged fish in the fish bins at the end of the artificial channels. The 30 cages of invertebrates were randomly assigned to the various treatments.

5.2.9 Assessment of *D. geminata* viability and cell density

The viability of *D. geminata* on all artificial substrates was determined prior to biocide application (Day 0). After biocide application, all substrates were sampled at day one, one week, two weeks, five weeks and 10 weeks post-treatment. Multiple exposures were sampled two and three days after treatment commenced (i.e., after two and three applications)¹⁶. *D. geminata* mats were progressively sampled upstream towards the tank end of the mat, to avoid re-sampling the same area.

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

¹⁶ Samples were taken on days 1, 8, 14-15, 33-34, 69-71 post-treatment.

2005, Kilroy et al. 2006b). For each sample, five to six pieces of a *D. geminata* mat were placed into a 30 mL sample container containing neutral red stain. Mat pieces were kept intact whilst in the stain, so the outer surface (surface containing the cells) of the mat could be identified during slide preparation. This practice decreased stalk material to be examined on the slides and increased the likelihood of encountering *D. geminata* cells during microscope analysis. At least 100 *D. geminata* cells were enumerated for detection of those that might be live. Samples were analysed within one hour of staining, with no detectable decline in cell viability over this period. Kilroy et al. (2006b, see Appendices 1 and 2 in that document) has completed a thorough analysis of this technique including the validity of counting 100 cells (versus more/less cells) as an indicator of overall mat viability.

At five and 10 weeks, samples were also collected for measurement of biomass and cell density in the remaining *D. geminata* mats. Water flow was briefly halted to each channel and a 30 mm diameter circle on the artificial substrate was cleared of all biological material using forceps and surgical scissors; samples were frozen for later analysis. The sample was homogenized in a known volume of water and 1 mL sub-samples were pipetted into a counting chamber on an inverted microscope. Subsamples were then scanned at approximately 100x magnification and *D. geminata* cells were counted in either 100 fields of view or 200 cells per sample. Absolute numbers of cells per square millimetre were calculated from the known areas of the counting cell and the numbers of fields of view. The remaining homogenized sample was quantified, dried at 105°C for 24 h, weighed, ashed at 400°C for 4 h and reweighed for measurement of ash free dry mass (biomass) according to standard methods (Biggs and Kilroy 2000).

Mat samples were also collected at five and 10 weeks for examination of species diversity and measurement of Cu concentration in the mat material. Four to five mat samples were cut from different locations in each channel and samples were frozen for later analysis. Mat sub-samples were scanned for estimates of species diversity.

5.2.10 Recirculation experiment

An additional experiment was conducted on 1 November 2006 to measure the rate of uptake of Gemex™ by a *D. geminata* mat, and the change in concentration of Gemex™ (dissolved Cu) in the exposure solution. The experiment was conducted on a single moderately thick (~20 mm) mat in one of the stream channels (unused for Stage 2 Phase 2 refinement trial) at the MEF. As the exposure was not replicated this gives indicative information only. The mat had been grown on an artificial substrate in the Waiau River at the same time as those used in the main Gemex™ experiment.

Although the mat on the substrate was not uniform enough for inclusion in the main trial (27 September 2006), the *D. geminata* had continued to grow in the MEF and was uniformly ~20 mm thick on 1 November 2006. A pump recirculated 50 L of a 18 mg Cu/L (nominal, as chelated Cu, calculated from the measured stock [Cu] of 18.6 g Cu/L) solution of Gemex™ over the mat for three hours at a rate of 1.2 L/s. Water samples were taken at 0, 5, 15, 30, 45, 60, 90, 120 and 180 minutes to measure changes in dissolved Cu and Pb over time. Mat samples were taken at the same time intervals to measure metal uptake. Changes in dissolved Cu concentrations were analysed using linear regression analysis and ANOVA. Dissolved Pb concentrations were measured only because Pb is a potential trace contaminant of the Gemex™ concentrate.

5.2.11 Data analysis for field trials

Statistical analyses were conducted using the SYSTAT® statistical software package (Version Number 11).

To assess *D. geminata* mortality the proportions of viable cells were initially 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). As a result, data could not be pooled for a comparison between cell viability at the start and end of the trial.

To better elucidate important relationships in the data, a statistical analysis was conducted in an attempt to exclude time zero cell viability measurements from further analyses, because if a statistically significant difference was found between pre- and post-treatment data, then post-treatment data could be analysed independently. A one-way analysis of variance (ANOVA) was conducted to test for differences in viability between pre-treatment (time 0) and post-treatment (time > 0) *D. geminata* mats. Control channels were excluded from this analysis, and the data pooled for all channels at time 0 (n=36) and time > 0 (n=186). A significant difference was indeed found between pre- and post-treatment data, so only data with time > 0 was kept in the GLM for further analyses. The new GLM compared cell viabilities among the 15 treatments (control, plus a range of Gemex™ treatments applied at three velocities) at time > 0.

Using the factors found to be significant in the GLM, thorough analyses assessing differences in water velocity, Gemex™ concentration and number of exposures were conducted. All analyses were assessed over time (measured on a continuous geometric

scale), using two-way ANOVA. 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 was detected, Tukey's HSD test was used to identify significant pairwise comparisons.

5.3 Results

5.3.1 Water quality analysis

Measured copper concentrations in the artificial channels were variable and although overall the concentrations were significantly different ($F_{4,38} = 36.89$, $P < 0.0001$) (Figure 17), a Tukey's analysis shows that not all treatments were statistically significantly different from one another (Table 5). The 5 and 10 mg Cu/L treatments were not significantly different (NSD), 10 mg Cu/L and 15 mg Cu/L treatments were NSD, and 15 mg Cu/L and 20 mg Cu/L treatments were NSD; all other treatments were significantly different from one another. In addition dissolved Cu in the artificial channels during the 1 h Gemex™ doses was on average only 72% of nominal Cu concentrations calculated from the measured stock Cu concentration (18.6 g Cu/L) ($R^2 = 0.82$, $F=170.0$, $P < 0.0001$) ($y=0.67x + 0.21$) (Figure 18).

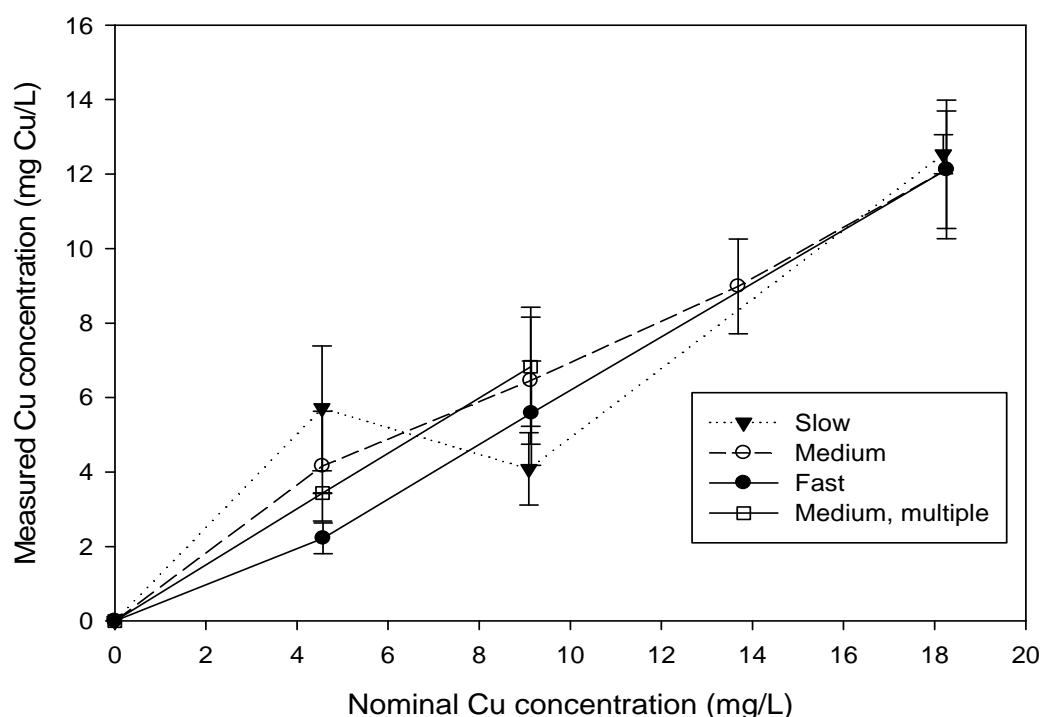


Figure 17: Nominal dissolved Cu concentrations during the 1 h Gemex™ doses (calculated from the measured stock of 18.6 g Cu/L) compared to Cu concentrations measured in the channels.

Table 5: Tukey's analysis of measured chelated Cu concentrations in the artificial channels. *** indicates the treatments were significantly different at $\alpha = 0.05$, and NSD =not significantly different.

Conc (mg/L)	0	5	10	15	20
0	-	***	***	***	***
5		-	NSD	***	***
10			-	NSD	***
15				-	NSD
20					-

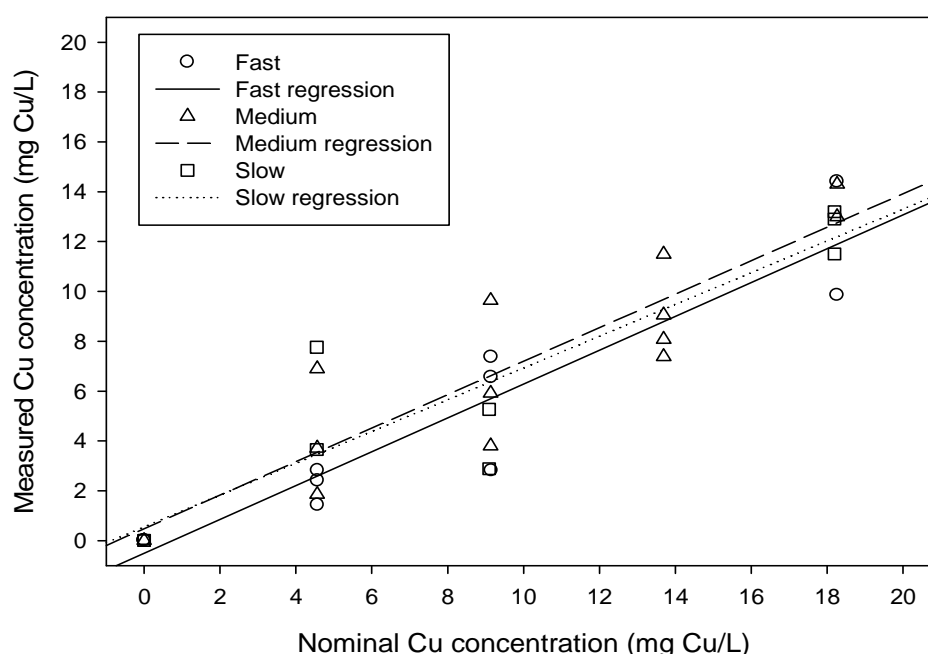


Figure 18: Nominal Cu concentration (calculated from the measured stock 18.6 g Cu/L) during the 1 h Gemex™ doses compared to measured copper concentration in the artificial channels (fast velocity = circles, solid line; medium velocity = triangles, dashed line; slow velocity = squares, dotted line).

A number of factors are likely to have contributed to these results. The flow rate for the addition of Gemex™ solutions to the channels was difficult to control (average time for addition 60 ± 2 min, range 41-90 min) and would have resulted in variations in concentration. However, the variation in Cu concentrations was not systematically related to the rate of addition. Precision pumps will be used in future experiments. Variations in the flow rates through the channels may have contributed to differences

in concentrations particularly in the slow velocity treatments where the volumes of Gemex™ added were lowest. Most importantly though, the water samples were taken approximately 10 cm downstream from the top of the mats and a subsequent experiment has shown that *D. geminata* mats rapidly absorb the chelated Cu (Section 5.3.2) and that even when a solution is recirculated over the mat dissolved Cu concentrations were variable over time. Only two water samples each were taken for the measured Cu concentrations in the slow velocity treatments at 5 and 10 mg Cu/L and the average values were strongly affected by a high concentration measured in one of the 5 mg Cu/L treatments (7.8 mg Cu/L).

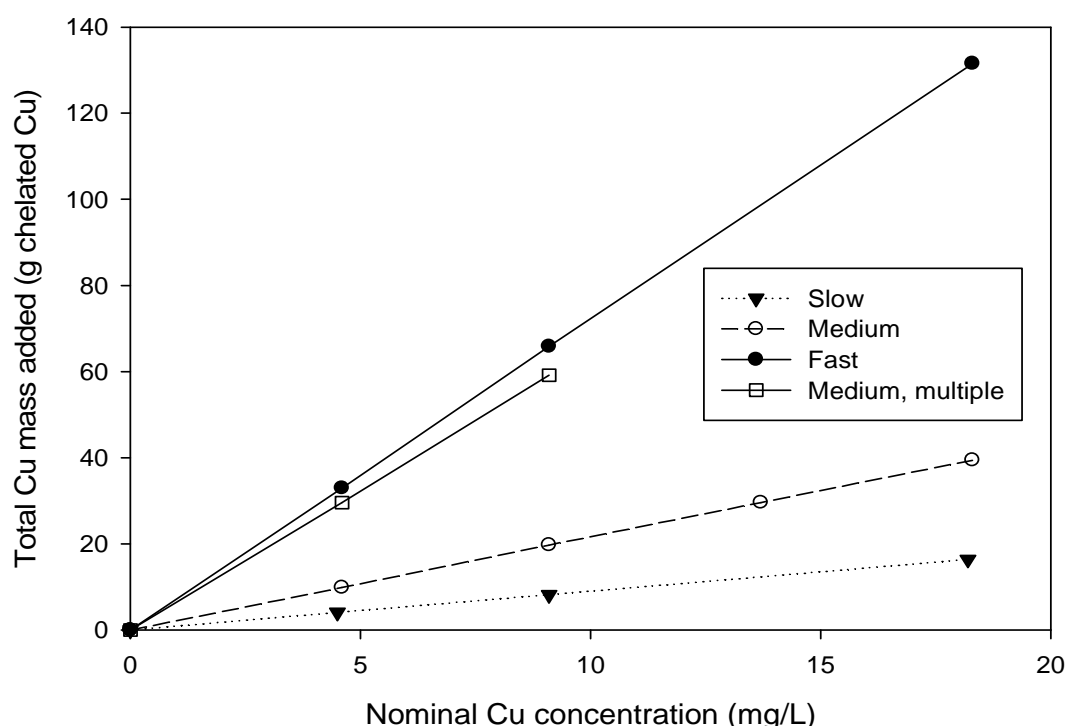


Figure 19: Total mass of Cu added to each channel during the 1 h Gemex™ doses compared to nominal Cu concentrations (slow velocity = triangle, medium velocity = open circles, fast velocity = filled circles, medium velocity multiple dose = squares). The mass of Cu for the multiple treatments is the sum of the three 1 h treatments, 24 h apart.

Even though the measured dissolved Cu concentrations in each channel were variable, the actual mass of Cu delivered to each channel would have more consistently matched the nominal treatment, as all Gemex™ solutions were made up according to the target calibration values and added to each channel in their entirety (Figure 19, Table 4). For example, if delivery rates were slow, a lower concentration would have

been delivered for a longer time in order to empty the delivery bag¹⁷. The different treatments received markedly different amounts of Cu (i.e., mass) depending on the water velocity, therefore combining the treatments by measured Cu concentrations would not accurately reflect the Cu exposure of the *D. geminata*. For these reasons the nominal treatment targets rather than the measured dissolved Cu concentrations will be initially used to compare the viability results in relation to the hydraulic treatments, although the measured values and mass will be taken into account in the final assessment. Lead concentrations were less than the detection limit (0.002 mg Pb/L) in the majority of the samples, the remainder were 0.002-0.011 mg Pb/L with one outlier at 0.035 mg Pb/L. In the untreated channels, lead concentrations ranged from less than the detection limit up to 0.006 mg Pb/L. A one hour exposure to 0.035 mg Pb/L would have minimal effect on juvenile rainbow trout (ANZECC 2000, Rogers et al. 2003).

5.3.2 Recirculation experiment

There was a large initial decrease in the concentration of GemexTM in the first five minutes of recirculation of the 18.2 mg Cu/L GemexTM solution (nominal) over the *D. geminata* mat, but after this sharp decline the concentration remained relatively stable over time (Figure 20). When fitted with a power curve ($y = ax^b$), the relationship between the concentration of GemexTM in the water column and time is statistically significant (ANOVA: $F_{1,8} = 7.11$, $R^2 = 0.43$, $P = 0.03$). The t_0 concentration of the solution was 17.1 mg Cu/L which is 6% lower than the nominal initial concentration of 18.2 mg Cu/L GemexTM; this difference could have been caused by rapid sorption of the chelated Cu by the hardware in the experimental system (e.g., pump and fish bin surfaces). Once the solution was pumped over the *D. geminata* mats, it decreased rapidly to approximately 70% of the initial 17.1 mg/L suggesting that the mat absorbed 30% of the chelated Cu and was then saturated. We calculate that if the mat in the artificial channel was approximately 0.045 m² (9 cm x 50 cm) and 30% of the chelated Cu in the solution was absorbed (17 mg/L x 50 L solution = 850 mg chelated Cu total; 850 mg x 30% = 255 mg) then the 2 cm thick mat absorbed approximately 5.7 g chelated Cu/m². On the other hand, copper concentrations steadily increased in the *D. geminata* mat for two hours after the exposure started, rather than saturating after five minutes. The copper concentrations reached a maximum of 19000 mg/kg dry weight. These data suggest the algal mats have a very high capacity to absorb copper. Using the ash free dry mass of untreated mats (280 g/m²) in the Stage 2 Phase 1 trials at MEF, the 19000 mg Cu/kg dry weight equates to approximately 5.3 g chelated Cu/m², which is in good agreement with the calculated Cu removal from the water.

¹⁷ Note that slow delivery rates did not explain all of the low measured Cu concentrations as there was no consistent relationship between length of time to deliver the GemexTM and measured Cu concentration.

This exposure was not replicated so the results are indicative only. Lead concentrations in the recirculation solution ranged between less than the detection limit (0.002 mg Pb/L) to 0.004 mg Pb/L.

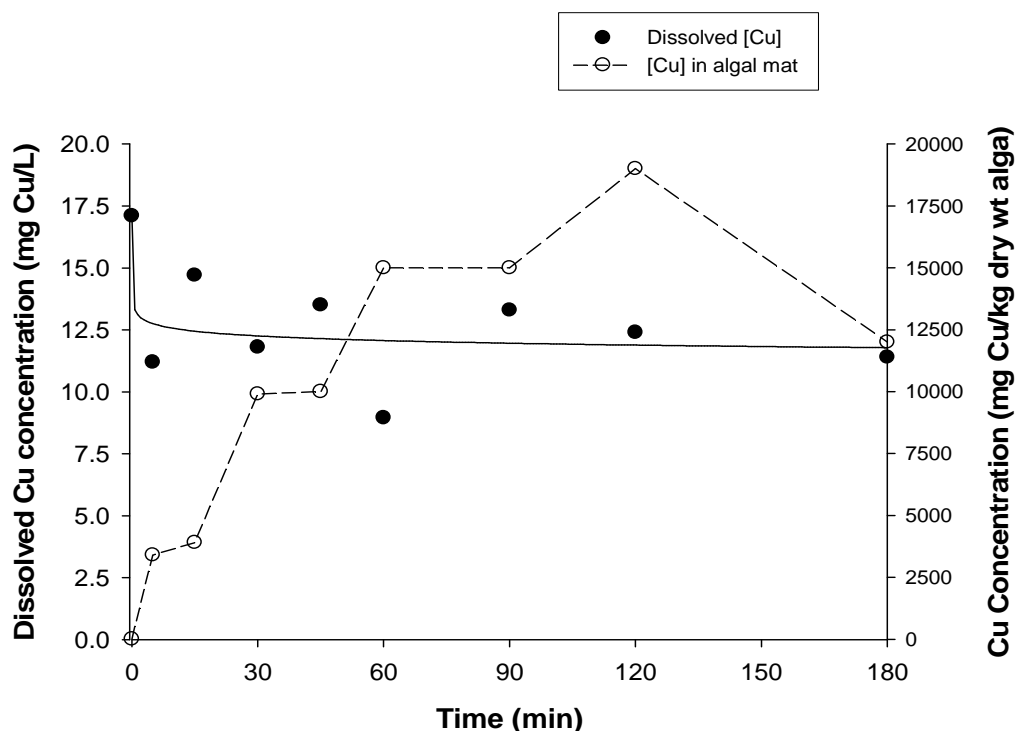


Figure 20: The change in Gemex™ concentration (dissolved Cu concentration, filled circles), and the change in copper concentration in the *D. geminata* mat (mg Cu/kg dry weight alga, open circles) following recirculation of 18.2 mg Cu/L (nominal, calculated from measured stock of 18.6 g Cu/L) over a *D. geminata* mat for three hours.

5.3.3 *D. geminata* viability

As expected, it was evident that the pre-treatment cell viability (time 0) was significantly different from the post-treatment cell viability (ANOVA: $F_{1,221} = 4369.6$, $P < 0.001$). So, to detect differences between treatments, the pre-treatment data was excluded from further analyses. Unless otherwise stated the following analyses apply to post-treatment data only.

The effect of time on cell viability was significant (GLM: $F_{1,184} = 34.8$, $P < 0.001$), and the interaction between time, concentration and velocity was also highly significant (GLM: $F_{6,184} = 5.88$, $P < 0.001$). As a result, the data could not be pooled for analysis as in Jellyman et al. (2006a).

Cell viability decreased with increasing Gemex™ concentrations (GLM: $F_{6,184} = 1998.8$, $P < 0.001$) and the effect of application rate differed among velocity treatments (GLM: $F_{6,184} = 20.8$, $P < 0.001$). The three water velocities also differed significantly in their effect on cell viability (GLM: $F_{2,184} = 36.1$, $P < 0.001$). These differences were further explored using two-way ANOVA (see below).

The average viability for the control material for slow, medium and fast water velocities was $95.1\% \pm 0.55$, $94.9\% \pm 1.11$ and $95.1\% \pm 0.81$, respectively (Figure 21). The consistent cell viability through time meant that there was no significant differences between velocity treatments (ANOVA: $F_{2,35} = 0.010$, $P > 0.05$). In contrast to the gradual linear decline in cell viability recorded in the Stage 2 Phase 1 trial (Jellyman et al. 2006a), there was no observed decline in the viability of control substrates for this 70-day trial (Figure 21).

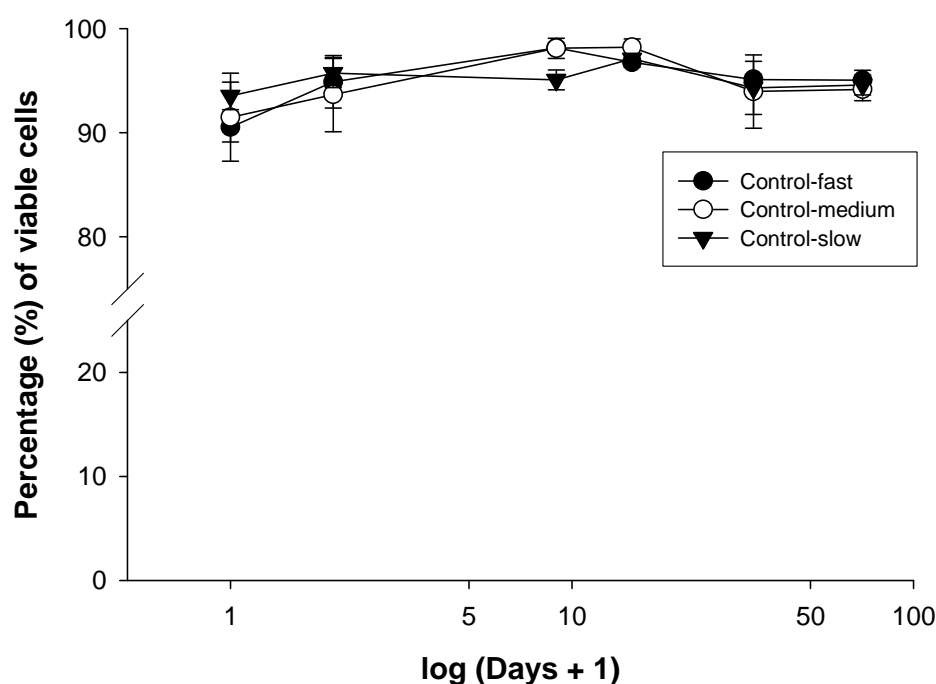


Figure 21: A comparison of the mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for the control substrates exposed to three different water velocities (slow (triangles), medium (open circles), fast (filled circles)) in artificial channels ($n = 3$) for 1 h during the Gemex™ treatment period.

Effect of 5 mg Cu/L Gemex™ in different water velocities

For substrates exposed to a 5 mg Cu/L dose (nominal) of Gemex™ there was a large initial decrease in cell viability for all water velocities (Figure 22). For the medium and fast velocity treatments cell viability was variable but generally declined and was at its lowest after monitoring on Day 33. Nonetheless there were no statistically significant differences in cell viability as a result of the different velocity treatments at 5 mg Cu/L (ANOVA: $F_{2,20} = 0.18$, $P = 0.83$), and no significant interaction of velocity and time on cell viability at 5 mg Cu/L (ANOVA: $F_{8,20} = 2.40$, $P = 0.054$). Dissolved Cu concentrations measured in these treatments were 6, 4 and 2 mg Cu/L for the slow medium and fast treatments respectively. A Cu mass of 4, 9 and 33 g Cu would have been added to each channel for the slow, medium and fast treatments respectively.

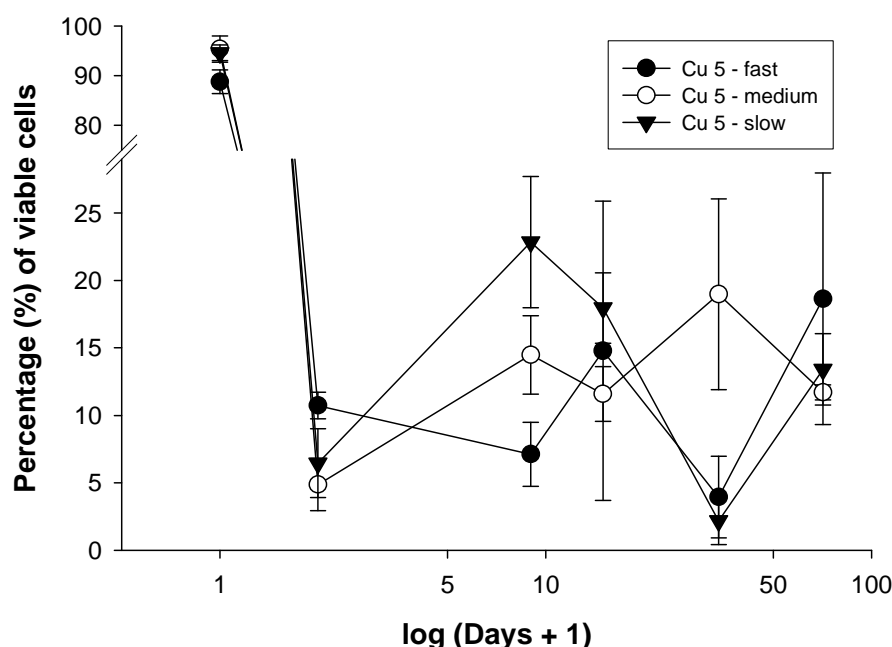


Figure 22: A comparison of the mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for the substrates exposed to 5 mg/L (nominal) of Gemex™ for 1 h in three different water velocities (slow = triangles, medium = open circles, fast = filled circles) in artificial channels. Note change in y-axis break from previous graph (n = 3).

Effect of 10 mg Cu/L Gemex™ in different water velocities

After a large initial decrease, *D. geminata* dosed with Gemex™ at a rate of 10 mg Cu/L (nominal) showed a gradual decline in cell viability (Figure 23). The cell viabilities were closely associated for the medium and fast water velocities, until a period of potential regrowth was seen in the final sampling period for the medium velocity treatment. There was a significant difference in cell viability as a result of the different velocity treatments at 10 mg Cu/L (ANOVA: $F_{2,20} = 4.12$, $P = 0.03$). A post hoc Tukey's test showed that only cell viabilities for the fast and slow velocities were significantly different from each other. There was no statistically significant interaction of velocity and time on cell viability at 10 mg Cu/L (ANOVA: $F_{8,20} = 0.47$, $P = 0.86$). Dissolved Cu concentrations measured in these treatments were 4, 7 and 6 mg Cu/L for the slow, medium and fast treatments respectively. A Cu mass of 8, 20 and 66 g Cu would have been added to each channel for the slow, medium and fast treatments respectively. The lower dissolved Cu concentrations measured in the slow velocity treatment might have contributed to the higher cell viability in this treatment.

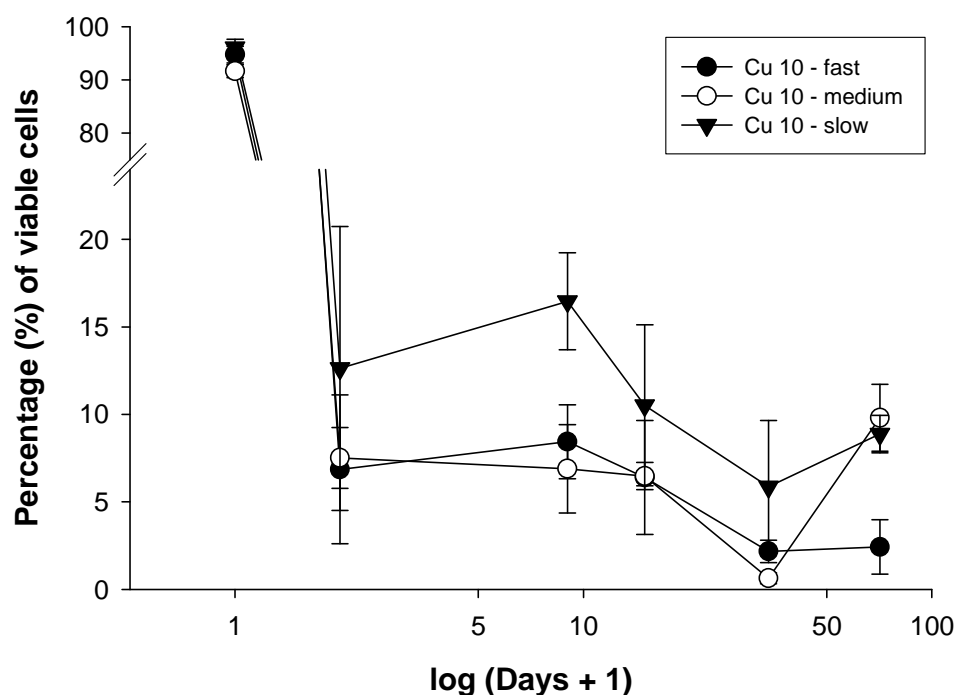


Figure 23: A comparison of the mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for the substrates exposed to 10 mg/L (nominal) of Gemex™ in three different water velocities (slow = triangles, medium = open circles, fast = filled circles) for 1 h in artificial channels ($n = 3$).

Effect of 15 mg Cu/L Gemex™ in medium velocity water

The effect of applying a 15 mg Cu/L dose of Gemex™ for 1 h was only tested at medium water velocity as insufficient channels were available to test more combinations of treatments. Cell viability initially decreased to only 2% but then increased slightly to 3 – 6% for one to five weeks (Figure 24). The final measurement of 12% cell viability at 10 weeks (70 d) indicated that regrowth had begun to occur, although there was no statistically significant effect of time on cell viability for the 15 mg Cu/L treatment (ANOVA: $F_{4,10} = 2.02$, $P = 0.17$) probably due in part to the variable viability at five weeks. Dissolved Cu concentrations measured in these channels were 9 mg Cu/L and a mass of approximately 30 g Cu would have been added to each one.

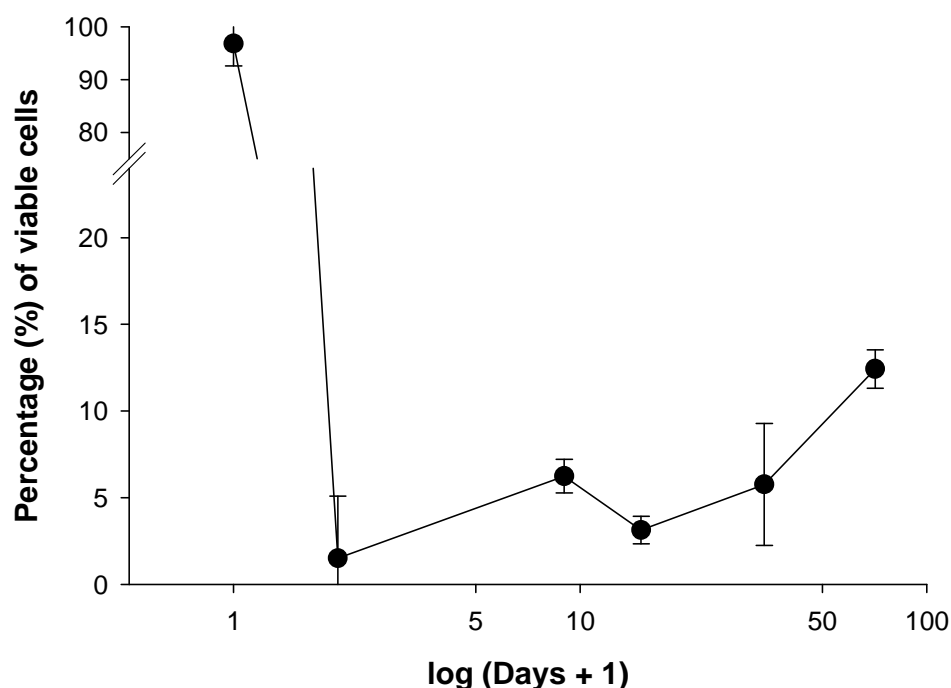


Figure 24: The mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for the substrates exposed to 15 mg/L (nominal) of Gemex™ for 1 h at a medium water velocity in artificial channels ($n = 3$).

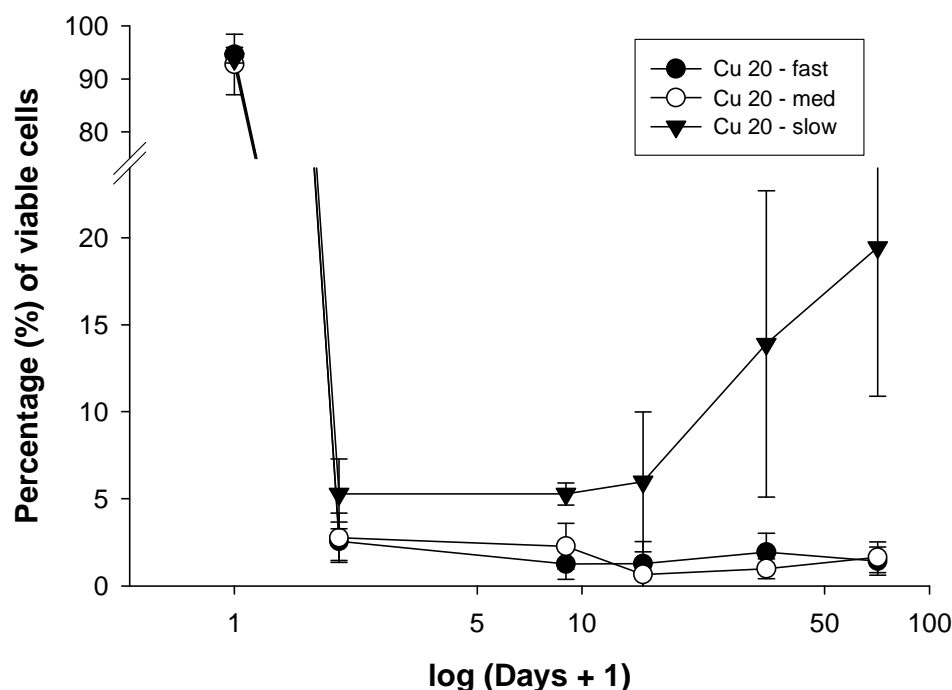


Figure 25: A comparison of the mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for the substrates exposed to 20 mg/L (nominal) of Gemex™ in three different water velocities (n = 3).

Effect of 20 mg Cu/L Gemex™ in different water velocities

Cell viability of *D. geminata* mats treated with Gemex™ at 20 mg Cu/L (as chelated Cu) (nominal) was very low and stable through time in both the medium and fast water velocities, initially decreasing to 3% 1 d post-treatment, then remaining at 1-2% for the following 10 weeks (Figure 25). In contrast, the mats in the slow velocity treatment initially decreased to 5% viability but 5 weeks post-treatment cell viability increased to 14% and was 19% by 10 weeks post-treatment suggesting significant regrowth had occurred (viability remained < 10% in one replicate and increased in the other two). Dissolved Cu concentrations measured in these three treatments were 12.1-12.5 \pm 1.9 mg Cu/L (Figure 17), but Cu mass added to each channel would have been approximately 16, 39 and 132 g Cu for the slow, medium and fast treatments respectively. There was a statistically significant difference in cell viability as a result of the different velocity treatments at 20 mg Cu/L (ANOVA: $F_{2,20} = 9.08$, $P = 0.002$). A post hoc Tukey's test statistically confirmed observations that both the fast and medium velocity treatments were significantly different from the slow velocity treatment. There was no statistically significant interaction between water velocity and time in the 20 mg Cu/L treatments (ANOVA: $F_{8,20} = 1.17$, $P = 0.36$).

Effect of different concentrations of Gemex™ in slow velocity water

When the effects of 1 h exposure to different Gemex™ concentrations in slow velocity water are compared, cell viability was $\leq 23\%$ but highly variable over time for all concentrations (Figure 26). There were no statistically significant differences in cell viability as a result of the different Gemex™ concentrations in the slow velocity treatments (ANOVA: $F_{2,20} = 0.57$, $P = 0.57$), but there was a statistically significant interaction between concentration and time on cell viability in the slow velocity treatments (ANOVA: $F_{8,20} = 3.06$, $P = 0.02$). Dissolved Cu concentrations measured in these treatments were 6, 4 and 13 mg Cu/L in the nominal 5, 10 and 20 mg Cu/L treatments respectively (Figure 17). Cu mass added to each channel would have been approximately 4, 8 and 16 g Cu for the 5, 10 and 20 mg Cu/L treatments respectively.

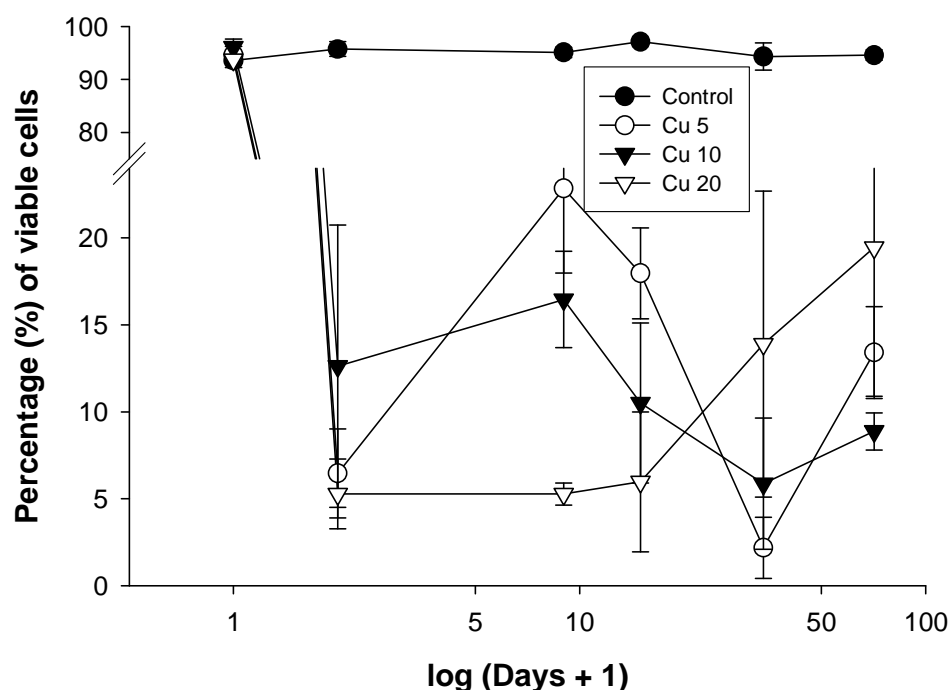


Figure 26: A comparison of the mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for the substrates exposed to three different concentrations of Gemex™ for 1 h in slow velocity (0.1 m/s) water in artificial channels (controls = filled circles, 5 mg/L = open circles, 10 mg/L = filled triangles, 20 mg/L = open triangles) (n=3).

Effect of different concentrations of Gemex™ in medium velocity water

When 1 h treatments of different concentrations of Gemex™ in medium velocity waters are compared, cell viability was consistently highest for the 5 mg Cu/L treatment (5-20%) while the 20 mg Cu/L treatment (1-3%) had the lowest viability throughout the 70 d following the treatment (Figure 27). Cell viability of *D. geminata* mats in both the 10 and 15 mg Cu/L treatments appeared to have increased to 10-12% after 70 d. These differences between treatment concentrations were statistically significant within the medium velocity treatments (ANOVA: $F_{3,30} = 16.46$, $P < 0.001$). A post hoc Tukey's test indicated that 10, 15 and 20 mg Cu/L were all significantly different from the 5 mg Cu/L treatment. The 20 mg Cu/L treatment was also statistically significantly different from the 10 mg Cu/L treatment. There was also a statistically significant interaction between time and concentration in the medium velocity treatments (ANOVA: $F_{12,30} = 2.44$, $P < 0.02$). Dissolved Cu concentrations measured in these treatments were 4, 7, 9 and 12 mg Cu/L in the nominal 5, 10, 15 and 20 mg Cu/L treatments respectively (Figure 17). Cu mass added to each channel would have been approximately 10, 20, 30 and 39 g Cu for the 5, 10, 15 and 20 mg Cu/L treatments respectively.

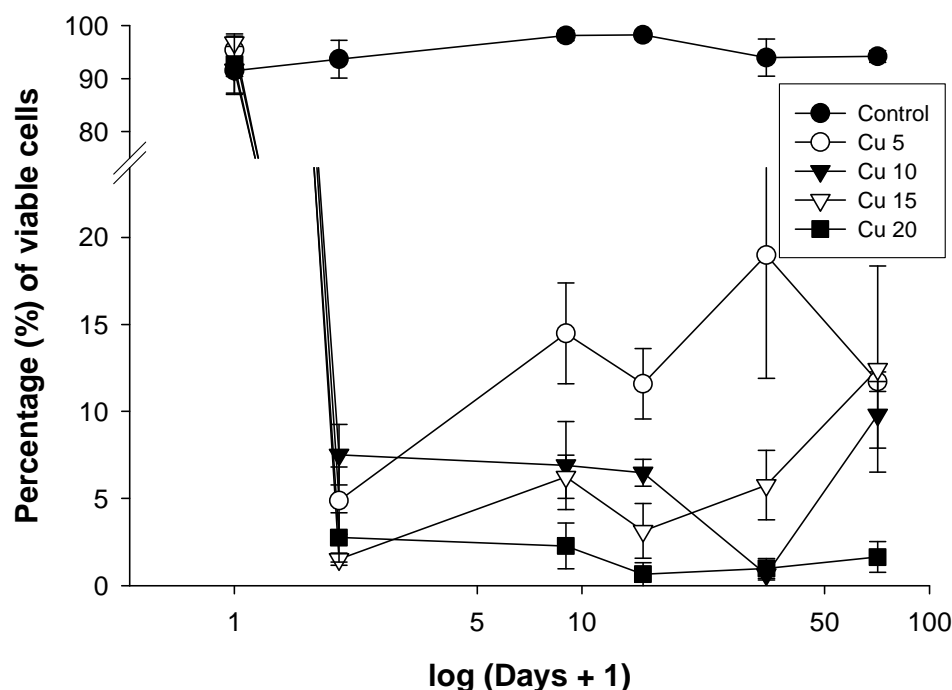


Figure 27: A comparison of the mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for the substrates exposed to four different (nominal) concentrations of Gemex™ for 1 h in medium velocity (0.3 m/s) water in artificial channels (controls = filled circles, 5 mg/L = open circles, 10 mg/L = filled triangles, 15 mg/L = open triangles, 20 mg/L = squares)($n = 3$).

Effect of different concentrations of Gemex™ in high velocity water

A comparison of *D. geminata* mats treated with 20 mg/L Gemex™ for 1 h shows that cell viability was consistently very low 70 d post-treatment in high velocity water while in contrast, cell viability in mats treated in slow velocity water was highly variable through time and significant regrowth had probably occurred by 14 d post-treatment in at least one replicate of this treatment (Figure 25). The differences in cell viability as a result of exposure to different Gemex™ concentrations in fast velocity water were statistically significant (ANOVA: $F_{2,20} = 5.68$, $P = 0.01$). A post hoc Tukey's test showed that only cell viabilities for the 5 mg Cu/L and 20 mg Cu/L treatments were statistically significantly different from each other. There was no significant interaction between concentration and time on cell viability in the fast velocity treatments (ANOVA: $F_{8,20} = 0.78$, $P = 0.63$). Dissolved Cu concentrations measured in these treatments were 2, 6 and 12 mg Cu/L in the nominal 5, 10 and 20 mg Cu/L fast velocity treatments respectively (Figure 17). Cu mass added to each channel would have been approximately 33, 66 and 132 g Cu for the 5, 10 and 20 mg Cu/L fast velocity treatments respectively.

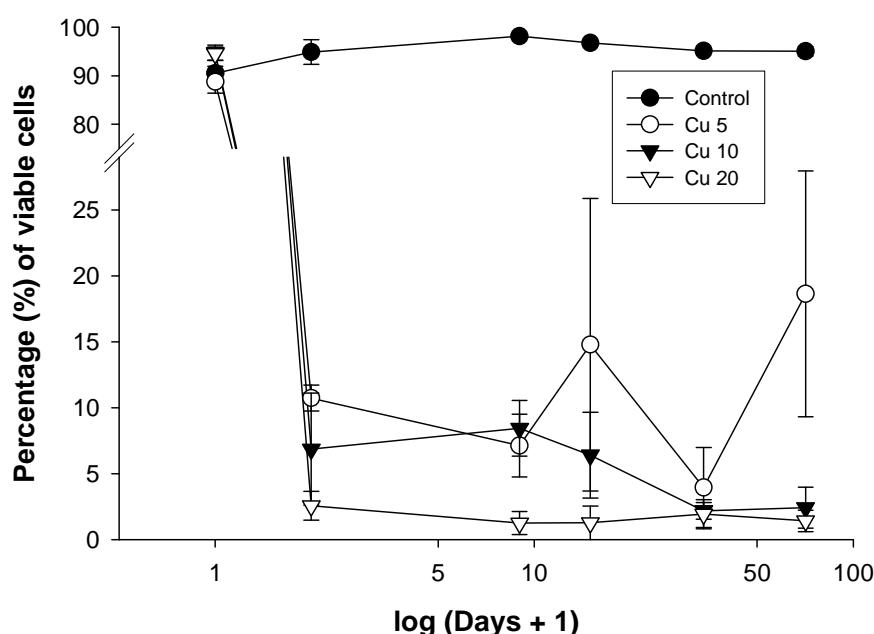


Figure 28: A comparison of the mean (± 1 S.E.) percentage of viable *D. geminata* cells as a function of time (days) for substrates exposed to three different (nominal) concentrations of Gemex™ for 1 h in fast velocity (0.6 m/s) water in artificial channels (controls = filled circles, 5 mg/L = open circles, 10 mg/L = filled triangles, 20 mg/L = open triangles) (n = 3).

Single versus multiple doses of 5 mg Cu/L Gemex™

A comparison of cell viability for 70 d after a single versus multiple 1 h applications of Gemex™ at 5 mg/L shows that although cell viabilities on days 1-3 post-treatment were similar between the two treatments (4-9%), they were consistently lower (2-7%) in the multiple treatment 1-5 weeks post-treatment (7-33 d) (Figure 29). Ten weeks (70 d) post-treatment however, it appears that significant regrowth occurred in the multiple treatment resulting in cell viabilities of 16%, similar to those in the single treatment (12%). On average the two treatments (single versus multiple applications of 5 mg Cu/L) were statistically significantly different (ANOVA: $F_{1,10} = 8.23$, $P = 0.02$), and there was no statistically significant interaction between application frequency and time (ANOVA: $F_{4,10} = 3.33$, $P = 0.06$). Dissolved Cu concentrations measured in these two treatments were 4 and 3 mg Cu/L for the single and multiple applications respectively. The Cu mass added to each channel would have been approximately 10 and 30 g Cu for the single and multiple applications respectively.

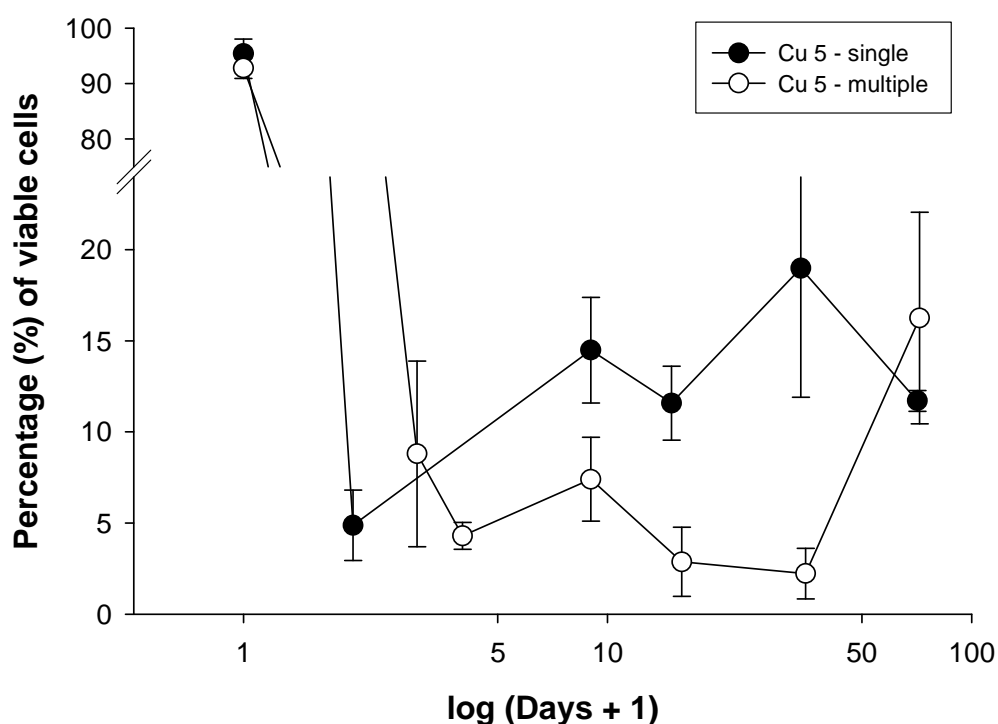


Figure 29: A comparison of cell viability in *D. geminata* mats at different times after a single (closed circles) or multiple (open circles) 1 h applications of 5 mg/L Gemex™ in medium velocity water (0.3 m/s) in artificial channels (n=3).

Single versus multiple doses of 10 mg Cu/L Gemex™

A comparison of cell viability for 70 d after a single, versus multiple 1 h applications of Gemex™ at 10 mg/L shows that on average the cell viabilities were lower in the multiple treatment (0.6-3.7%) two days to 5 weeks post-treatment compared to 0.6-7.5% in the single treatment (Figure 30). The low cell viability in the mats 5 weeks (33 d) after a single treatment was anomalous, even compared with other treatments (e.g., single treatments with 5, 15, or 20 mg Cu/L). Within the multiple application treatment, cell viability decreased slightly from 3.7% after two applications to 2.3% after the third application. The differences in cell viability between the single and multiple applications were statistically significant (ANOVA: $F_{1,10} = 10.05$, $P = 0.01$), and there was no significant interaction between application frequency and time post-treatment (ANOVA: $F_{4,10} = 0.62$, $P = 0.66$). Cell viability did however, on average change statistically significantly over time (ANOVA: $F_{4,10} = 9.67$, $P = 0.001$), regardless of Gemex™ application frequency, probably reflecting the trend toward slightly higher cell viability 10 weeks (70 d) post-treatment (4.5-9.8%). Dissolved Cu concentrations measured in each treatment were 6.5 and 6.8 mg Cu/L in the single and multiple treatments respectively. The Cu mass added to each channel would have been approximately 20 and 59 g Cu for the single and multiple treatments respectively.

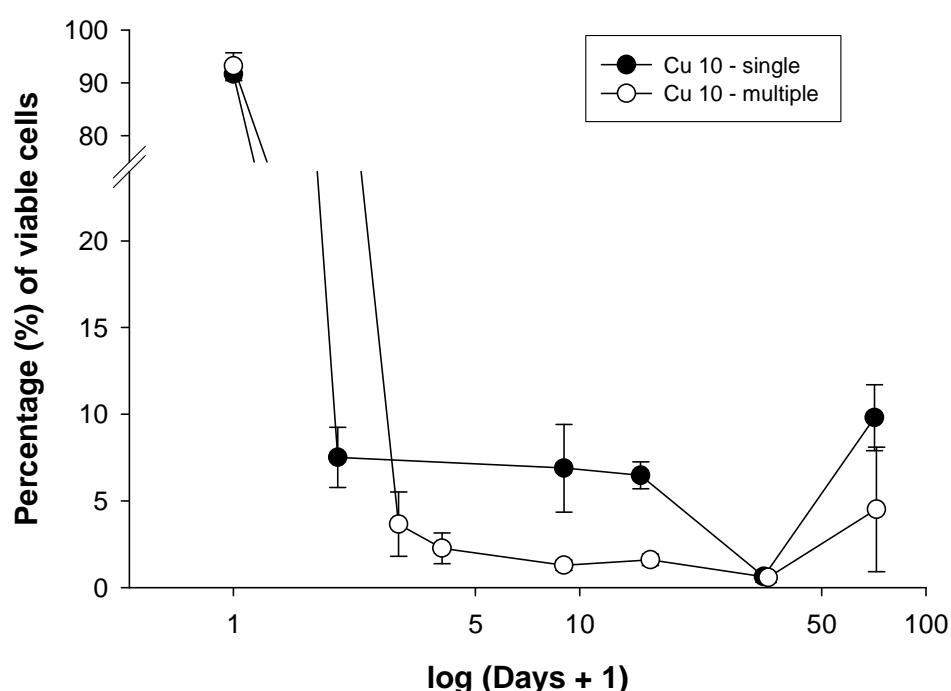


Figure 30: Comparison of cell viability in *D. geminata* mats at different times after a single (closed circles) or multiple (open circles) 1 h applications of 10 mg/L Gemex™ in medium velocity water (0.6 m/s) in artificial channels (n = 3).

A comparison of multiple doses of 5 mg Cu/L Gemex™ with a single dose of 15 mg Cu/L Gemex™

It is interesting to see the similarity in the post-treatment cell viability of *D. geminata* mats treated with the same quantity of Gemex™ either as three 1 h doses of 5 mg/L Gemex™ or one dose for 1 h of 15 mg/L Gemex™ (Figure 31). Cell viability decreased progressively from 8.8% after the second 5 mg/L Gemex™ application to 4.3% after the third application. Cell viability decreased to 1.5% after a single exposure to 15 mg/L Gemex™, but thereafter cell viability was 2-7% in both treatments for 5 weeks post-treatment. The two treatments were not significantly different (ANOVA: $F_{1,10} = 0.12$, $P = 0.74$), and there was no significant interaction between the treatments and time post-treatment (ANOVA: $F_{4,10} = 0.32$, $P = 0.86$). There were however, statistically significant changes in cell viability over time post-treatment (ANOVA: $F_{4,10} = 10.18$, $P = 0.001$), probably reflecting the increase in cell viability 10 weeks post-treatment to 12-16%. Dissolved Cu concentrations measured in these treatments were 3 and 9 mg Cu/L for the nominal 5 and 15 mg Cu/L treatments respectively, whereas the Cu mass added to each channel would have been 30 g Cu for both treatments.

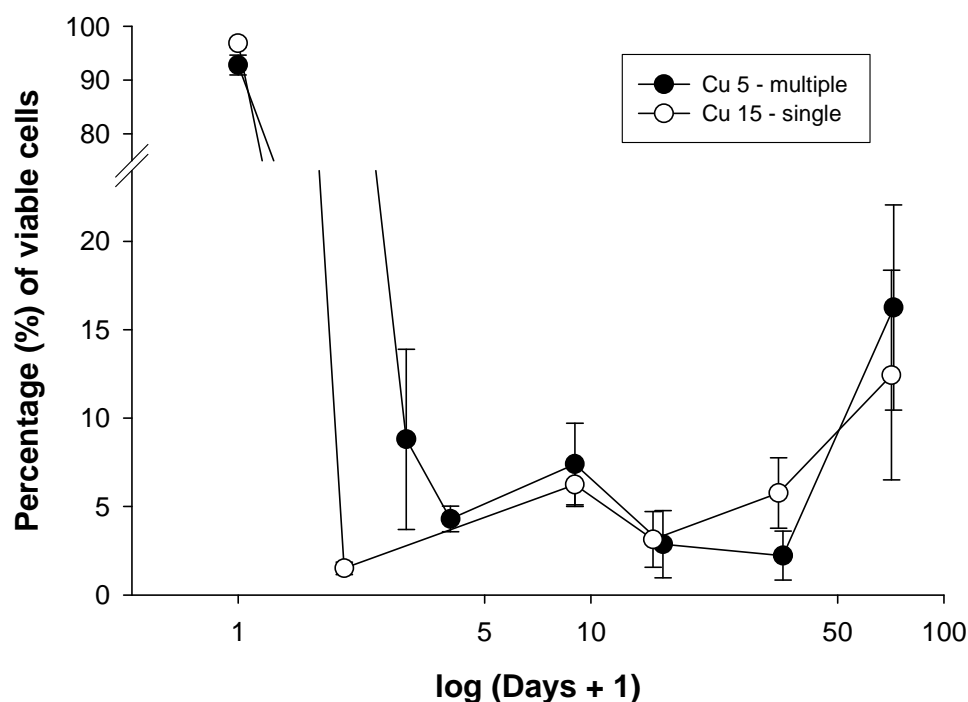


Figure 31: Comparison of the same quantity of Gemex™ being applied as either multiple 1 h applications (5 mg/L x 3 applications) (filled circles) or a single application 1 h (15 mg/L x 1 application) (open circles) in medium velocity water (0.3 m/s) in artificial channels (n = 3).

Effect of multiple doses of 5 mg Cu/L versus 10 mg Cu/L Gemex™

Increasing the concentration of the multiple doses from 5 mg/L Gemex™ to 10 mg/L resulted in a significantly lower cell viability (ANOVA: $F_{1,12} = 12.20$, $P = 0.004$) for ten weeks post-treatment (Figure 32). Cell viabilities in the *D. geminata* mats were on average 2-16% after multiple treatments with 5 mg Cu/L Gemex™ versus 0.6-4.5% after multiple treatments with 10 mg Cu/L. There was no statistically significant interaction between the concentration of the multiple application (5 or 10 mg Cu/L) and time (ANOVA: $F_{5,12} = 1.50$, $P = 0.26$), and there was no statistically significant change in cell viability over time post-treatment (ANOVA: $F_{5,12} = 2.28$, $P = 0.11$). Dissolved Cu concentrations measured in these treatments were 3 and 7 mg Cu/L for the nominal 5 and 10 mg Cu/L treatments respectively. The Cu mass added to each channel would have been 30 and 59 g Cu for the 5 and 10 mg Cu/L treatments respectively.

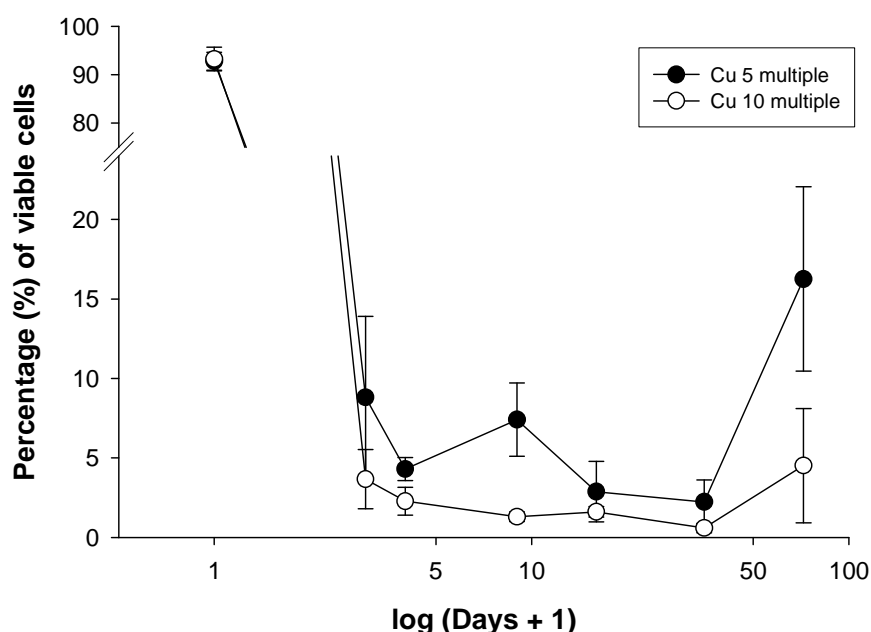


Figure 32: Comparison of cell viability in *D. geminata* mats at different times after multiple 1 h applications of either 5 mg/L (closed circles) or 10 mg/L (open circles) Gemex™ (nominal) in medium velocity water (0.3 m/s) in artificial channels (n = 3).

The relationship between cell viability and mass of chelated Cu

Percentage cell viability at either five or 10 weeks post-treatment also decreased with increasing net amounts of chelated copper added to each channel, as well as with increasing concentration (Figure 33). This comparison takes into account that more chelated Cu was added to each channel in the fast velocity treatments than in the slow velocity treatments (Table 4).

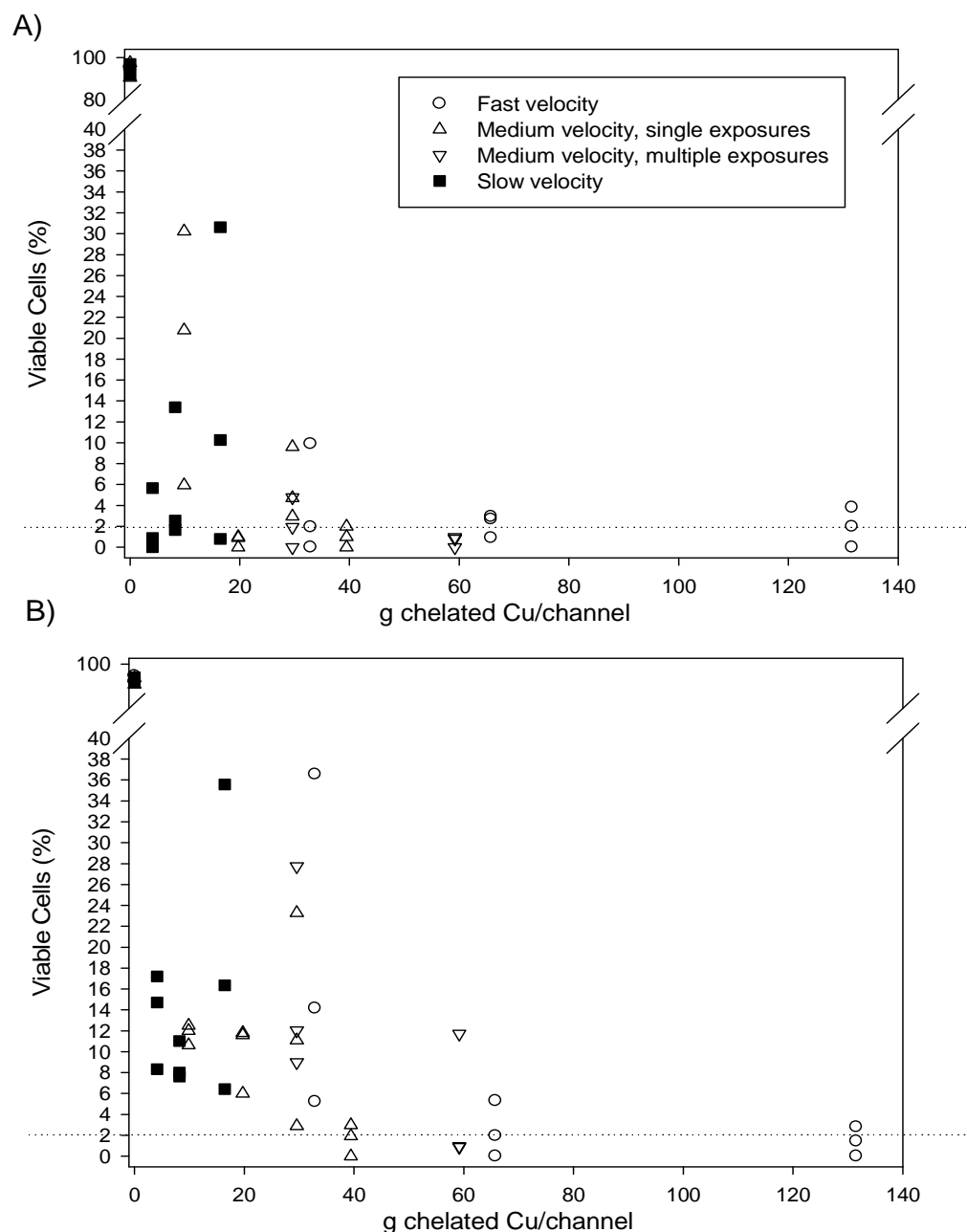


Figure 33: Changes in the percentage viable cells at either a) 5 weeks (33 d) post-treatment or b) 10 weeks (70 d) post-treatment with the mass of chelated Cu added to each channel. The area of *D. geminata* mat in each channel was 0.045 m², therefore a dose of 40 g/channel is equivalent to 0.9 kg/m². A threshold of 40 g/channel (or 0.9 kg/m²) appears to relate to low viability 5 weeks post-treatment.

At five weeks post-treatment, percentage cell viability was around the 2% threshold in treatments with > 40 g chelated Cu/channel, whereas by 10 weeks post-treatment cell viability was > 2% in most treatments.

This relationship shows that we were unable to test the effect of adding > 20 g chelated Cu/channel in slow velocity water because we could only manipulate channel slope and flow volumes in order to change water velocity. In other words there was a limit to how much copper we could add to the slow velocity treatments within the physical constraints of the experimental facility. The area of mat in each channel was 0.045 m^2 therefore a dose of 40 g/channel was equivalent to 0.9 kg/m^2 (note change in units).

5.3.4 *D. geminata* cell density

Cell density was highly correlated with the proportion of viable cells (ANOVA: $F_{1,22} = 69.60$, $R^2 = 0.77$, $P < 0.0001$) (Figure 34). As the proportion of viable cells increases, so does the density of cells per mm^2 . This indicates that there was about a 3 – 5 fold decrease in cell density in the treated substrates, which has important implications for cell viability analysis. Overall this relationship means that when cell viability and cell density are low (e.g., in Gemex™ treated mats that are dead and dying), expressing cell viability as a percentage results in an overestimate of relative live cell abundance compared to healthy mats with much higher densities of cells. For example 2% cell viability in mats with only 200 cells/ mm^2 is equivalent to 4 live cells/ mm^2 , whereas 95% cell viability in mats with 800 cells/ mm^2 is equivalent to 760 live cells/ mm^2 . Ideally when a detailed understanding of treatment effects is required, cell viability should be expressed as a function of cell density.

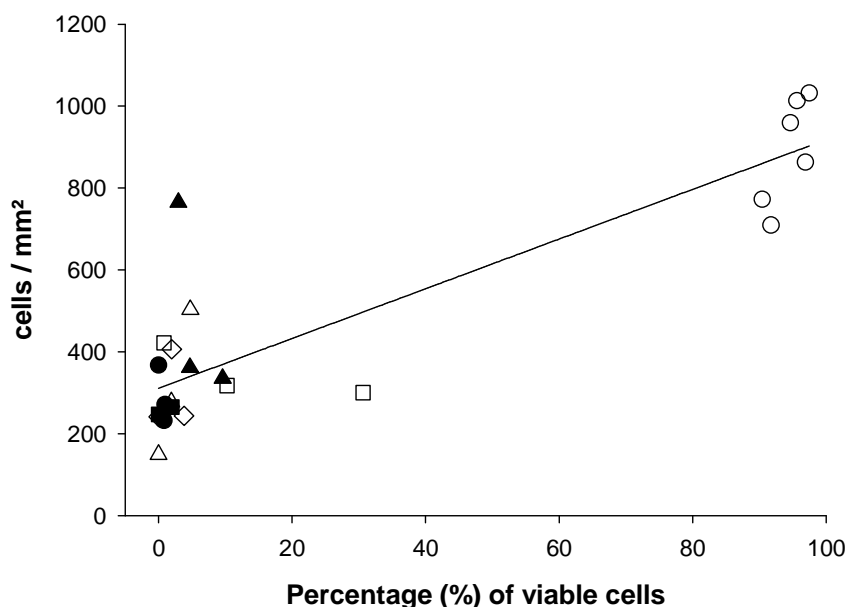


Figure 34: A comparison of *D. geminata* cell densities against the proportion of viable cells for different treatments 34 days (5 weeks) post-treatment. Control = open circles; 5 mg/L multiple = open triangles; 15 mg/L single = closed triangles, 10 mg/L multiple = closed circles; 20 mg/L single, slow velocity = open squares; 20 mg/L single, medium velocity = closed squares; 20 mg/L single, fast velocity = open diamonds.

There was a significant relationship between cell density¹⁸ 5 weeks post-treatment and the cumulative nominal dose of Gemex™ when the data was fitted with an exponential decay curve ($y = y_0 + ae^{-bx}$) (ANOVA: $F_{2,16} = 24.52$, $R^2 = 0.78$, $P < 0.0001$) (Figure 35). The negative exponential relationship shows that as the quantity of Gemex™ increases, the density of cells per mm² declines. There is no significant difference when cell densities are compared at 20 and 30 (10 x 3) mg Cu/L (ANOVA: $F_{1,4} = 0.43$, $P > 0.05$), indicating there appears to be little effect on cell density of adding increasing quantities of Gemex™ above ~20 mg Cu/L.

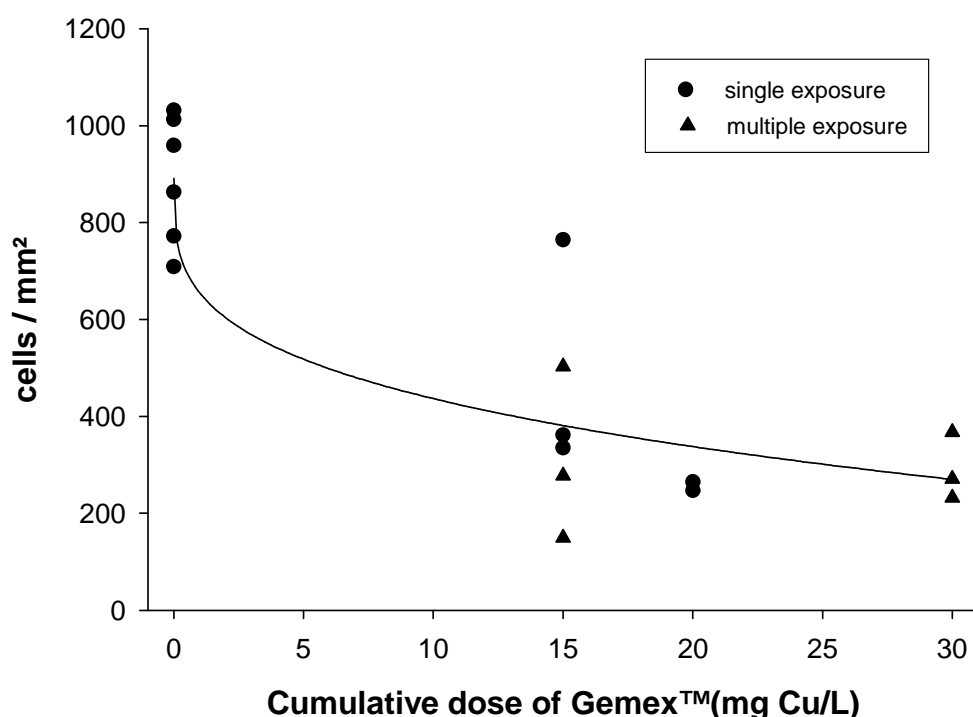


Figure 35: A comparison of *D. geminata* cell densities 5 weeks post-treatment plotted against the cumulative nominal dose of Gemex™ (i.e., adding multiple doses). Comparisons are for substrates exposed in medium velocity water to either a single dose (circles) of 15 mg/L or 20 mg/L Gemex, or multiple doses (triangles) of 5 mg/L or 10 mg/L Gemex™ (corresponding to 15 or 30 mg/L cumulative dose respectively). Control data was pooled because there was no significant difference between velocity treatments.

¹⁸ Note that cell density is a measure of the total number of cells in a sample and gives no information about the viability of the cells in that sample.

5.3.5 Effects of Gemex™ treatments on fish survival

For each treatment combination of concentration, velocity and number of applications (single or multiple) of Gemex™, fish mortality rates were assessed using nine adult non-migratory galaxiids per treatment (three fish per replicate cage, and three replicates for each treatment). A proportion (24%) of these fish escaped during the 14-day trial (Table 6), and the following discussion does not include escaped fish (although these are thought to have survived the treatments – see methods). Fish mortality was observed in three of the fifteen treatments, and these occurred one week after treatment. Two fish died in one replicate of the multiple 5 mg/L Gemex™ treatment (medium velocity), one fish died in the multiple 10 mg/L Gemex™ treatment (medium velocity) and one fish died in the single 20 mg/L Gemex™ high velocity treatment.

Table 6: Average fish (adult non-migratory galaxiid species, *Galaxias* sp. S) survival monitored over a two week period following a range of treatments of Gemex™. Note small changes in survival (%) between weeks one and two are a result of fish escapes, not further mortality, n = number of fish.

Treatment	Velocity	1 hour		1 week		2 weeks	
		n	% survival	n	% survival	n	% survival
Control	Fast	3	100	3	100	1	100
Control	Med	6	100	3	100	2	100
Control	Slow	6	100	6	100	6	100
Cu 10	Fast	6	100	6	100	5	100
Cu 10	Med	9	100	8	100	8	100
Cu 10	Slow	7	100	6	100	3	100
Cu 15	Med	9	100	7	100	6	100
Cu 20	Fast	9	100	6	83	4	75
Cu 20	Med	9	100	9	100	9	100
Cu 20	Slow	8	100	6	100	6	100
Cu 5	Fast	9	100	9	100	6	100
Cu 5	Med	9	100	9	100	9	100
Cu 5	Slow	9	100	9	100	7	100
Cu 10 x 3	Med	6	100	9	89	8	88
Cu 5 x 3	Med	9	100	9	78	9	78

5.3.6 Effects of Gemex™ treatments on stream invertebrate survival

The effects of the Gemex™ treatments on stream invertebrates were only assessed at an indicative level. In general the mortalities that were observed mostly occurred

immediately post-treatment (1 h) and were primarily individuals of the mayfly *Coloburiscus* sp. and lesser numbers of the mayfly *Deleatidium* sp. (Table 7). Stoneflies appeared more resistant to the Gemex™ treatments with very few mortalities observed, although fewer individuals were included in the treatments. There appeared to be a trend toward slightly lower survival in the higher Gemex™ dose treatments. Nonetheless there was no statistically significant effect of water velocity (ANOVA: $F_{3,31} = 1.94$, $P = 0.14$) or Gemex™ concentration (ANOVA: $F_{3,20} = 0.29$, $P = 0.82$) on the number of invertebrates surviving one hour post-treatment. In addition, there was no statistically significant effect of water velocity or concentration on the survival of any of the four taxa one hour post-treatment. These statistics should be interpreted with caution as not all treatments were replicated, and different numbers of individuals were assessed in the replicates.

Table 7: The percentage of invertebrates alive after one hour for a given Gemex™ treatment. Not all treatments had three replicates, so the number of replicates per treatment is indicated (# reps). The percentage of each invertebrate species alive following a 1 h treatment of Gemex™ is also shown. Numbers in parentheses indicate the number of replicates available for that species for a particular treatment.

Treatment	Velocity	# Reps	Total % Survival (1h)	<i>Deleatidium</i> sp.	<i>Coloburiscus</i> sp.	<i>Stenoperla</i> sp.	<i>Zelandobius</i> sp.
Control	Fast	2	100	100 ⁽²⁾	100 ⁽²⁾	-	-
Control	Med	1	100	-	-	-	-
Control	Slow	1	100	100 ⁽¹⁾	100 ⁽¹⁾	100 ⁽¹⁾	-
Cu 5	Slow	2	100	100 ⁽²⁾	100 ⁽²⁾	-	-
Cu 5	Med	2	100	100 ⁽²⁾	100 ⁽²⁾	-	100 ⁽¹⁾
Cu 5	Fast	2	83	100 ⁽²⁾	0 ⁽¹⁾	-	100 ⁽²⁾
Cu 10	Slow	2	94	86 ⁽¹⁾	-	-	100 ⁽¹⁾
Cu 10	Med	2	86	100 ⁽²⁾	70 ⁽²⁾	-	75 ⁽²⁾
Cu 10	Fast	2	94	90 ⁽²⁾	100 ⁽²⁾	100 ⁽¹⁾	100 ⁽¹⁾
Cu 15	Med	1	100	100 ⁽¹⁾	100 ⁽¹⁾	-	100 ⁽¹⁾
Cu 20	Slow	2	78	100 ⁽²⁾	50 ⁽¹⁾	-	100 ⁽¹⁾
Cu 20	Med	3	100	100 ⁽³⁾	100 ⁽³⁾	-	100 ⁽¹⁾
Cu 20	Fast	3	85	83 ⁽³⁾	85 ⁽³⁾	100 ⁽¹⁾	100 ⁽²⁾
Cu 5 x 3	Med	3	100	100 ⁽²⁾	100 ⁽³⁾	100 ⁽¹⁾	100 ⁽²⁾
Cu 10 x 3	Med	3	100	100 ⁽³⁾	100 ⁽³⁾	-	100 ⁽²⁾

6. Discussion and recommendations for field trials

6.1 Treatment efficacy

The intensive Gemex™ trials at MEF yielded promising results for the control of *D. geminata*. For an algaecide/biocide to effectively suppress¹⁹ a species in the field, at least 95-98% mortality should be achievable in a controlled environment. However, if elimination is to be achieved, 100% mortality should be reliably attained in a controlled environment. In these trials of well-established 10-20 mm thick mats, high *D. geminata* mortality rates (98%) were achieved with either single applications of 20 mg Cu/L (nominal) Gemex™ in medium to fast velocity water or multiple applications of 10 mg Cu/L (nominal) Gemex™ in medium velocity water (Figure 36). A single application of 20 mg Cu/L Gemex™ resulted in < 2% cell viability for longer than the multiple applications of 10 mg Cu/L Gemex™, so for this criterion the highest application rate is recommended for a field trial, based on the results of the artificial channel trials. The artificial channel trials showed clearly that the mortality of *D. geminata* increased with increasing Gemex™ dose, increased water velocity and multiple applications of biocide. Each of these factors had a threshold above which the long-term efficacy of Gemex™ increased markedly. Gemex™ doses ≥ 10 mg Cu/L (the higher, the better) appeared to be required for cell viability to remain low for ~5-10 weeks without obvious regrowth. Treatment in medium to fast water velocities (≥ 0.3 m/s) resulted in much lower cell viability, and for longer, than treatment in slow velocity water. Multiple treatments with 10 mg Cu/L Gemex™ resulted in lower *D. geminata* viability for longer (~70 d) than multiple treatments with 5 mg Cu/L. Obviously the effects of increasing Gemex™ concentrations on non-target species will provide an upper limit to an acceptable application rate in a natural environment (Section 6.6).

Together these results suggest that Gemex™ treatments of well-established mats might achieve suppression of *D. geminata* but are unlikely to eliminate the alga from a natural waterway. On the other hand, the possibility still remains that if *D. geminata* was treated with Gemex™ in a natural waterway when it was in the early stages of infestation, prior to the formation of mats, the alga might possibly be eliminated. Gemex™ penetration and efficacy is likely to be much higher when the alga is only present in the biofilm, or in small colonies ≤ 5 mm thick. However, the relationship between Gemex™ effectiveness and mat thickness was not tested in this study so it remains an assumption. Treatment efficacy would also be higher if the treatment was

¹⁹ See introduction for definition of the terms, “control”, “suppression” and “elimination” of *D. geminata*.

repeated; elimination from a natural waterway would require ongoing surveillance for the alga followed up by Gemex™ treatments.

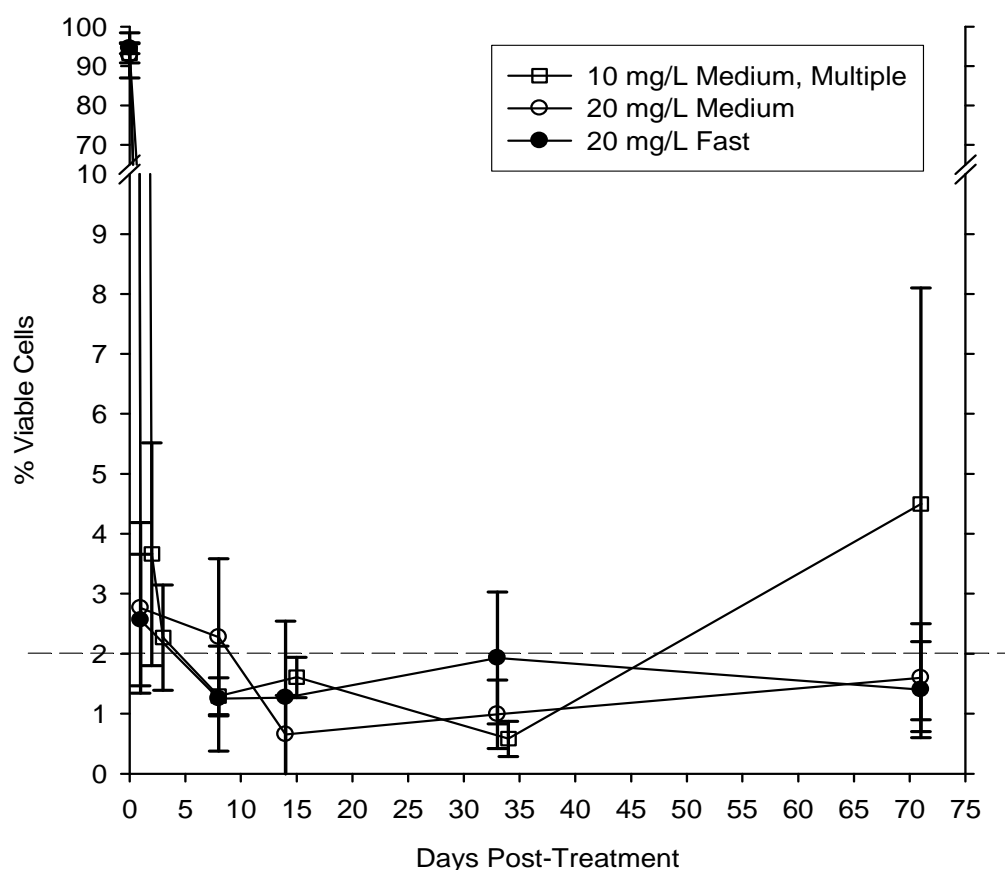


Figure 36: Comparison of cell viability in *D. geminata* mats treated with either multiple 1 h applications of 10 mg Cu/L Gemex™ in medium velocity water (open squares) or a single 1 h application of 20 mg Cu/L Gemex™ in medium (open circles) or fast velocity water (closed circles) in artificial channels. Dashed line indicates 2% viable cells.

6.2 Nominal versus measured chelated Cu concentrations

The dissolved Cu concentrations measured in the nominal 20 mg Cu/L treatments were only 12 mg Cu/L. It has been suggested that perhaps the measured concentrations should be used to describe the treatments, in which case the target concentration for a field trial might be lower than 20 mg Cu/L. However, as discussed earlier the low measured Cu concentrations were not only a result of lower than expected Cu concentrations in the stock solution (18.6 g Cu/L, rather than 20.4 g

Cu/L) but also potentially: a) losses of Cu to the hardware in the system (e.g., delivery bags and channel walls); b) rapid absorption of Cu by the algal mats or; c) variation in the Gemex™ dosing rates to the channels. If the latter two factors strongly influenced the measurements, then the nominal concentrations describe the treatments better than the measured concentrations. In addition, despite any differences in nominal and measured dissolved Cu concentrations, the total mass of Cu delivered to each channel would have been relatively consistent with the nominal concentration (see Section 5.3.1). There was a strong relationship between the total mass of Cu delivered to each channel and efficacy of Gemex™ against *D. geminata*, with an apparent threshold at 40 g/channel or 0.9 kg/m² corresponding to the most effective treatments (10 mg Cu/L fast velocity – 66 g Cu/channel; 20 mg Cu/L medium velocity – 39 g Cu/channel; 20 mg Cu/L fast velocity – 132 g Cu/channel; 10 mg Cu/L medium velocity multiple exposures – 59 g Cu/channel)(Figure 33, Table 4). It is likely that in these artificial channels, the shallow water depth and high turbulence promoted Cu absorption by the *D. geminata* mats, whereas in a natural waterway absorbance would eventually be limited by water depth. In a natural waterway, water velocity and depth will be highly variable and many factors will contribute to decrease the efficacy of Gemex™. For example, high concentrations of organic matter (e.g., silty organic sediment) will readily absorb the chelated Cu in Gemex™. Adsorption of Gemex™ to organic matter in a natural waterway is expected to significantly limit the downstream distance that it will be effective. Taking these factors into consideration, we recommend that the first field trial of Gemex™ uses the highest feasible concentration of product, after taking into account the likely effects on non-target species. It is recommended therefore, that for the field trial the target Cu concentration is 20 mg Cu/L (as chelated Cu).

6.3 The relationship between water velocity and net Gemex™ copper exposure within channels versus copper concentration

The water velocity in the artificial channels and the water flow were related by the factors:

$$\text{Flow (m}^3\text{/s)} = \text{velocity (m/s)} \times \text{width (m)} \times \text{depth (m)}$$

We were unable to manipulate channel width, and we could only alter water depth by changing the slope of the channel. Most of the increase in water velocity from 0.1 to 0.6 m/s was therefore achieved by increasing flows through the system. Thus, the observation that Gemex™ efficacy increased in higher velocity water is confounded by the fact that in order to attain the same Gemex™ concentration in high velocity water, the net amount of chelated Cu applied to the system was increased markedly because the total volumes of water passing over the *D. geminata* mats were much

higher. The relationship between the net amount of chelated Cu and cell viability is relatively strong (Figure 33) and the changes in cell viability after three treatments with 5 mg Cu/L Gemex™ in medium velocity water were almost identical to those after a single treatment with 15 mg Cu/L Gemex™ in medium velocity water. Unfortunately at slow velocities we were only able to test the effect of adding 5-18 g chelated Cu/channel (0.1-0.4 kg/m²), whereas five weeks post-treatment, the channels that had received ≥ 40 g chelated Cu/channel (0.9 kg/m²) had cell viability reduced to around 2% (Figure 33).

These data strongly suggest that in a natural waterway Gemex™ will be much less effective in areas of low velocity water such as shallow river margins. The strategy used for attempting to eradicate *D. geminata* in a natural waterway should be manipulated to take this into account. For example, if it is the net amount of chelated Cu delivered per unit area that determines the post-treatment cell viability, then the dosing regime (capped at a maximum concentration of 20 mg/L, for 1 hour) could potentially be targeted to achieve > 0.9 kg chelated Cu/m² in the slow velocity margins, by repeat doses spaced 2-5 weeks apart (dosing strategies and the accompanying decision-making process is discussed in more detail below). In any case, post-treatment monitoring of *D. geminata* viability should be accompanied by measurements of water velocity for each mat sample, as significant differences in water velocity would be expected between the margins of a waterway and the centre of the flow. On the other hand, as the 1 h dose of Gemex™ moves downstream through a natural waterway, the processes of dispersion, dilution, and adsorption will result in the pulse of Gemex™ spreading out and decreasing in concentration as it moves downstream. As a result locations further downstream will receive doses which are progressively longer with lower concentrations of Gemex™. This effect will be more marked in areas of slow velocity, deep water. The longer exposures in slow velocity water may mitigate against the reduced effectiveness observed in the slow velocity trials in the artificial channels, at least partly by causing the slow velocity areas to be exposed to a similar net mass of Cu as the faster velocity areas.

The data also indicate conversely that Gemex™ effectiveness is increased with increasing water velocity. We were unable to test velocities > 0.6 m/s due to the physical constraints of the testing facility at MEF but *D. geminata* is found at velocities of 1 m/s (Kilroy et al. 2006a). We predict that Gemex™ efficacy will be highest in high velocity areas of a natural waterway; measurements of water velocity along with post-treatment *D. geminata* viability in a field trial will be the best way to test this prediction.

6.4 Cell viability and cell density

Observation on the gross appearance of the *D. geminata* colonized substrates strongly suggest that mat thickness, biomass and cell density decreased markedly by 2.5 and 5 weeks post-treatment, especially in the channels treated with 10-20 mg Cu/L Gemex™ in medium to high water velocities (*Clearwater and Jellyman pers. obs.*). The same changes were observed in all other Gemex™ treatments relative to the controls but were not as obvious. In contrast mat thickness, biomass and cell density has increased noticeably over time in the untreated controls. These observations are reflected in the measured three to five fold decrease in cell density with decreased cell viability in the Gemex™ treated substrates at 5 weeks post-treatment. The decrease in cell density has important implications for the interpretation of changes in cell viability as Gemex™ treated *D. geminata* mats degrade, because in treatments with low cell density, percentage viability overestimates the proportion of live cells in the treated *D. geminata* mats per unit area by 3 – 5 times compared to the control treatments, hence Gemex™ is even more effective than the cell viability results indicate. Cell density will decrease in the treated mats as cells die and degrade, and are not replaced by growing material; also we have observed microscopically (during viability counts) that as *D. geminata* cells die many of them become detached from their stalks and could then be flushed from the mats.

An alternative explanation for the large difference in cell density between treated and untreated mats is that cells are being released in response to a chemical stress (i.e., the application of Gemex™). Cell detachment in response to different treatments was observed in the *D. geminata* survival studies (Kilroy et al. 2006b). Cazaubon (1988) recorded large increases in cell densities of diatoms in the water column in response to physical disturbances, but whether Gemex™ will disrupt cell physiology and cause *D. geminata* cells to drift is unknown. Cell drift in response to Gemex™ application could be monitored in future experimental trials at the MEF or in field trials.

6.5 Fate of chelated Cu

Dissolved copper concentrations measured in the channels were approximately 28% lower than the nominal copper concentrations (calculated from the Cu concentrations measured in the stock (18.6 g Cu/L)) applied to each mat. The recirculation experiment demonstrated that within five minutes of application, sorption processes by the *D. geminata* mats can decrease chelated Cu concentrations by approximately 30%, but these processes were probably less significant in the treatment channels as the water samples were taken from the top end of the *D. geminata* mats within seconds of the solution entering the channel. Alternatively the chelated Cu may have been

sorbed to the distribution equipment such as the solar showers and hoses, or the container walls, or the distribution of the Gemex™ concentrate to the channels may have occurred at a slower rate than calculated, or water flows were higher than measured. It is likely that a combination of these factors affected dissolved Cu concentrations.

The recirculation experiment data suggest that the *D. geminata* mats rapidly became saturated with chelated Cu. Presumably if the area of the mat, or perhaps the mass of alga was increased, more of the chelated Cu would have been absorbed. We can calculate that if the mat in the artificial channel was approximately 0.045 m² (9 cm x 50 cm) and 30% of the chelated Cu in the solution was absorbed (17 mg/L x 50 L solution = 850 mg chelated Cu total; 850 mg x 30% = 255 mg), then the 2 cm thick mat absorbed approximately 5.7 g chelated Cu/m². In order to obtain 20 mg/L Gemex™ in a 0.5 m³/s stream (e.g., Princhester Creek) for 1 h, we would apply 1768 L of 20.36 g/L Gemex™, or 36.0 kg chelated Cu in total. Assuming the waterway was 2 m wide and completely covered with *D. geminata* mats, this would be sufficient chelated Cu to be absorbed 3 km downstream, as long as metal absorption did not decrease significantly with decreasing dissolved Cu concentrations. We plan to treat when *D. geminata* mats are much less dense than 100% cover, therefore we calculate that there should be sufficient chelated copper to be absorbed at 5.7 g/m² for at least 6 km downstream from the treatment point.

The accumulation of chelated Cu in the dead and dying mats may retard the regrowth of the *D. geminata* colony, or the rate of regrowth may simply be a function of the number of viable cells remaining post-treatment. The former explanation would fit with the trend occasionally observed in the highest dosed mats for cell viability to continue to decline for several weeks post-treatment.

6.6 Effects on non-target species

The results from the artificial channel trials support data collected so far that show 100% survival of fish to single 1 h exposures of Gemex™ concentrations < 20 mg Cu/L (as chelated Cu). The four fish mortalities in the Gemex™ refinement trials occurred between 1 day and 1 week after the Gemex™ treatment, and were not correlated with Gemex™ concentrations or volumes (e.g., two mortalities occurred in the 5 mg/L multiple treatment [net Gemex™ volume 1.6L] whereas only one occurred in the single 20 mg Cu/L treatment in fast velocity water [net Gemex™ volume 7.1L]). It is possible that the diet in captivity was inadequate and caused some of the mortalities, as galaxiids tend to have higher feeding rates than salmonids and eat diverse diets. On the other hand, the fact that three of the mortalities occurred in the

multiple exposures suggests that 24 h between repeat applications of Gemex™ may be insufficient for resident fish to recover from the stress of the chemical exposures. We recommend that further laboratory testing examine the effect of multiple exposures on juvenile rainbow trout and that monitoring of post-treatment survival continue past the standard 4 d to at least 7 d. In the meantime, field trials of Gemex™ should avoid multiple applications 24 h apart. The rainbow trout toxicity tests could be followed up by focussed laboratory testing of rarer non-migratory galaxiid fishes to test for interspecies differences in sensitivity, because galaxiid species are often found in ‘threatened’ populations, existing only in isolated areas such as the upper Taieri River catchment.

The assessment of stream invertebrate survival was completed at an indicative level only, but clearly showed that stream invertebrates are far more resistant to Gemex™ exposure than the pond invertebrate *D. magna* tested in earlier laboratory tests (Jellyman et al. 2006a). Gemex™ treatment appeared to decrease invertebrate survival by 20-30%. Mayfly larvae may be more susceptible to Gemex™ than other genera. We recommend laboratory testing on mayfly larvae to determine mortality rates under more precisely controlled conditions than were possible at MEF.

6.7 Effect of water quality on Gemex™ toxicity

Water hardness has a significant influence on Cu toxicity to aquatic biota. Increasing hardness protects against the toxicity of Cu ions (i.e., Cu²⁺) to aquatic biota. However, the Cu in Gemex™ is present in a chelated form, therefore hardness will probably have less effect on the toxicity of Gemex™ to aquatic biota than its influence on the toxicity of ionic Cu.

Hardness was 36 and 11 mg/L as CaCO₃ in the fish toxicity trials in Hamilton, and at Monowai Experimental Facility respectively. Both of these hardness concentrations are relatively low; moderate hardness is considered to be ~ 60 mg/L as CaCO₃. It is possible that the lower hardness at Monowai will result in slightly higher toxicity of Gemex™ to aquatic biota.

6.8 Potential for *D. geminata* regrowth and treatment strategy in field trials

Another sampling of the *D. geminata* mats at MEF was scheduled for approximately 15 weeks post-treatment to show whether cell viability would start to increase in the mats treated with 20 mg Cu/L Gemex™ in medium to fast velocity water. Unfortunately this monitoring could not be completed because a large flood event

mobilized large quantities of coarse sediment that scoured out the *D. geminata* mats on the substrates in the artificial channels. In fact, timing the application of Gemex™ at a time when frequent high flow events are expected is one possible strategy we have proposed to maximise the efficacy of this tool. However, the usefulness of a high flow event for physically removing alive or dead *D. geminata* material would have to be weighed up against the logistical challenges of a high flow event and the higher priority to treat an infestation as soon as possible to improve the chances of successful elimination.

Another consideration for effective treatment strategies is to take steps to minimise the spread of *D. geminata* within a waterway, particularly if it has been discovered in the very early stages of infestation. Once we understand what stimuli cause *D. geminata* cells to detach from their stalks we might decide to, for example, avoid disturbing early colonies unduly to minimise the spread of viable cells downstream. Other less subtle factors should be taken into consideration, such as preventing the movement of stock through an infested waterway, and restricting human access for recreation.

Despite the inability to sample 15 weeks post-treatment (due to a flood destroying the mats) the data from the artificial channel trials suggest that it is likely that multiple treatments will be necessary to eliminate or suppress *D. geminata* in natural waterways, especially in slow velocity water. Treatments will probably best be repeated after approximately 5-10 weeks (i.e., when regrowth was first observed in the artificial channels) or when monitoring shows regrowth is starting in the natural waterway, rather than on the 24 h schedule trialled in the artificial channels. Ideally follow-up applications could occur at lower doses (e.g., < 10 mg Cu/L) than the initial treatment because Gemex™ will probably be more effective on degraded mats, non-visible colonies, or biofilms of *D. geminata* only detectable by microscopic/genetic assessment. The benefit of repeated applications of Gemex™ will have to be weighed against the potential impact of increased doses of chelated Cu in the receiving environment. Any Gemex™ field trial would be accompanied by extensive monitoring and assessment of the fate of the chelated copper in Gemex™ in order to collect precise information on its spatial distribution, concentration in sediments and biota, and effects on *D. geminata* and non-target biota. Treatment strategies will be highly site-specific, and will also depend on seasonal factors such as ambient water temperatures and flow regimes.

The following lines of information would inform our selection of treatment strategy (follow-up treatments) in field trials:

1. spatial distribution and density of surviving *D. geminata*;

2. spatial distribution and concentration of chelated copper in waterway sediments *versus* time elapsed since last treatment; and
3. response of non-target species to Gemex™ treatment, particularly fish.

It is expected that the Gemex™ treatment will be most effective against *D. geminata* over the one to four km reach immediately down stream of the application point, based on the approximate calculation of there being sufficient chelated Cu for uptake of an effective dose by the mats for up to 6 km downstream and the expectations that:

- a) a Gemex™ treatment would have to be located a significant distance (e.g., 2 km) upstream of an area of 100% cover by *D. geminata* mats to ensure that the waterway was not infested upstream of the treatment point; and
- b) the effectiveness of the chemical would be reduced from the theoretical calculation of 6 km by the processes of adsorption to sediments and other non-algal substrates, as well as dispersion and dilution.

Follow-up applications could be positioned further downstream than the initial treatment site if delimitation data shows *D. geminata* has been successfully eliminated in upstream areas.

It is recognised that pest species are most effectively controlled through strategic use of multiple control tools that take into account the ecology and physiology of the target species. It has been observed that flood events significantly reduce the biomass of *D. geminata* in a waterway, and our artificial channel research has shown that 3 repeat applications completed 24 h apart achieve higher *D. geminata* mortality than one application. We will use our knowledge of all of these factors to strategically deploy Gemex™ to the greatest effect on *D. geminata*, for instance, immediately after high flow events, but at the lowest concentrations possible in order to mitigate any potential negative environmental effects of chelated copper. The proposed application methods, treatment site(s) and pre- and post-treatment monitoring programme have been described in detail in the Assessment of Environmental Effects document submitted in support of the application for a resource consent to conduct field trials of Gemex™ in Princhester Creek and/or Monowai River, Southland (Jellyman et al. 2006b) and in the Gemex™ Treatment Contingency Plan (Version 3, Clearwater and Jellyman 2007).

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9. Appendix 1: Pictures of experimental facilities



Figure A1: Juvenile rainbow trout were exposed to biocides for 1 h in 4L containers, then removed to holding tanks for observation for a further 4 days.



Figure A1-2: Juvenile rainbow trout used for laboratory toxicity testing.



Figure A1-3: Artificial substrates: (A) construction, (B) orientation in river and (C) growth on the substrates prior to the start of refinement trials.

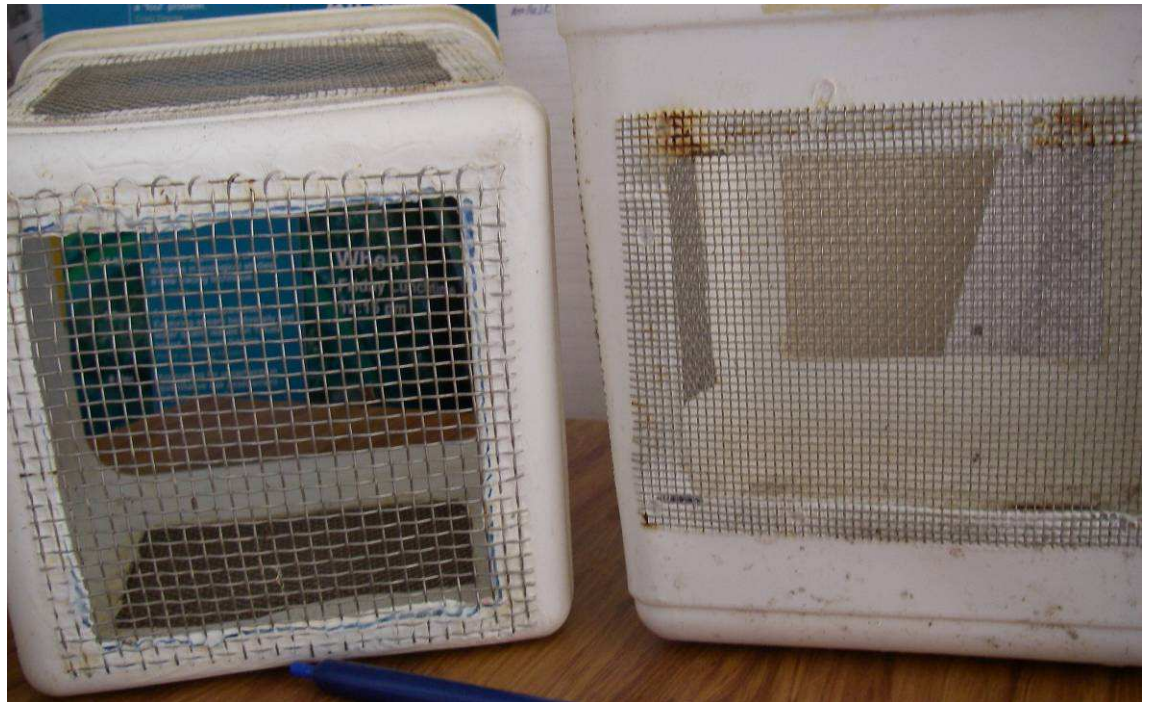


Figure A1-4: Fish cages (4L) used in Gemex™ refinement trials in artificial channels. Similar cages with 1 mm mesh were used for invertebrates.



Figure A1-5: Experimental setup for the application of Gemex™ to the channels.

10. Appendix 2: Summary of fish toxicity testing conditions

Summary of test conditions used in the juvenile rainbow trout (*Oncorhynchus mykiss*) bioassay.

Project Name: Didymo Trials	Project No:MAF07203
Test Protocol:	NIWA SOP 28.1 (NIWA, 2004) modified for short-term exposures of 1 h as detailed in methods section above.
Test Materials:	Organic Interceptor™, Hydrothol®191, Gemex™ (chelated copper formulation), or Degaclean® 150
Test Organisms:	<i>Oncorhynchus mykiss</i> (juveniles ~36d old 0.9 ± 0.4 g)
Source:	Ngongotaha Fish Hatchery (Eastern Fish and Game)
Organisms/Container:	5
Test Concentrations:	Control, and test solutions as listed in Table 1
Replicates:	5 for controls, 3 for test solutions
Reference Toxicant:	Zinc sulphate
Test Duration:	1 h exposure to test solution, survival monitored for 96 hours
Dilution Water:	Dechlorinated Hamilton City Tap Water (hardness ~36 mg/L as CaCO ₃)
Test Chambers:	4 L plastic containers, lined with plastic bags
Lighting:	16: 8h light: dark
Temperature:	$15 \pm 1^\circ\text{C}$
Aeration:	Gentle aeration
Chemical Data:	Temperature, pH, dissolved oxygen
Effect Measured:	Mortality (behavioural changes noted)
Test Acceptability:	Mean control mortality no greater than 10%, reference toxicant 96 h LC ₅₀ < ± 2 s.d. long term average.
Test Compliance:	Achieved

11. Appendix 3: ToxCalc™ results for rainbow trout toxicity testing

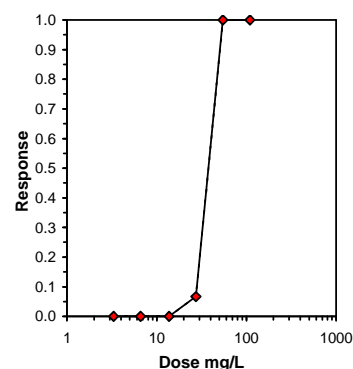
Gemex™ exposure for 1 h, rinsed and survival monitored for 96 h.

Acute Fish Test-96 h survival					
Start Date:	7/08/2006	Test ID:	2425/LO3	Sample ID:	BNZ-Biosecurity New Zealand
End Date:	11/08/2006	Lab ID:	SC-Sue Clearwater	Sample Type:	GEMEX-Chelated Copper Formulation
Sample Date:	7/08/2006	Protocol:	EC2000-Environment Canada	Test Species:	OM-Oncorhynchus mykiss
Comments:	Fish exposed for only 1 h, survival monitored for 96 h (mg Cu./L)				
Conc-mg/L	1	2	3	4	5
Control	1.0000	1.0000	1.0000	1.0000	1.0000
3.3	1.0000	1.0000	1.0000		
6.59	1.0000	1.0000	1.0000		
13.7	1.0000	1.0000	1.0000		
27.3	1.0000	0.8000	1.0000		
54.6	0.0000				
109.2	0.0000				

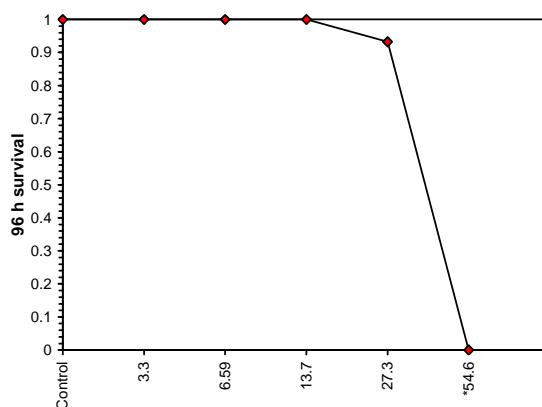
Conc-mg/L	Mean	N-Mean	Resp	Not Resp	Total	N	Fisher's Exact P	1-Tailed Critical	Number Resp	Total Number
Control	1.0000	1.0000	0	25	25	5			0	25
3.3	1.0000	1.0000	0	15	15	3	1.0000	0.0500	0	15
6.59	1.0000	1.0000	0	15	15	3	1.0000	0.0500	0	15
13.7	1.0000	1.0000	0	15	15	3	1.0000	0.0500	0	15
27.3	0.9333	0.9333	1	14	15	3	0.3750	0.0500	1	15
*54.6	0.0000	0.0000	5	0	5	1	0.0000	0.0500	5	5
*109.2	0.0000	0.0000	5	0	5	1	0.0000	0.0500	5	5

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Fisher's Exact Test	27.3	54.6	38.608	

Trimmed Spearman-Kärber			
Trim Level	EC50	95% CL	
0.0%	36.869	33.728	40.303
5.0%	37.608	33.138	42.682
10.0%	37.664	35.782	39.645
20.0%	37.664	35.782	39.645
Auto-0.0%	36.869	33.728	40.303



Dose-Response Plot



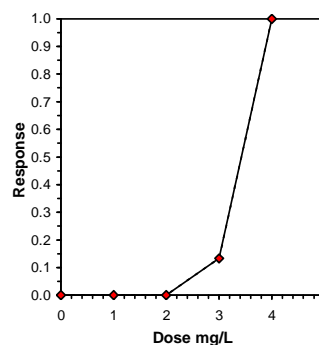
Hydrothol -191 Fish exposed for 1 h then rinsed, survival monitored for 96 h.

Acute Fish Test-96 h survival					
Start Date:	7/08/2006	Test ID:	2425/LO2	Sample ID:	BNZ-Biosecurity New Zealand
End Date:	11/08/2006	Lab ID:	SC-Sue Clearwater	Sample Type:	HYD-Hydrothol 191 Algaecide
Sample Date:	7/08/2006	Protocol:	EC2000-Environment Canada	Test Species:	OM-Oncorhynchus mykiss
Comments:	Fish exposed for only 1 h, survival monitored for 96 h (mg a.e./L) aci				
Conc-mg/L	1	2	3	4	5
Control	1.0000	1.0000	1.0000	1.0000	1.0000
1	1.0000	1.0000	1.0000		
2	1.0000	1.0000	1.0000		
3	0.8000	0.8000	1.0000		
4	0.0000	0.0000	0.0000		

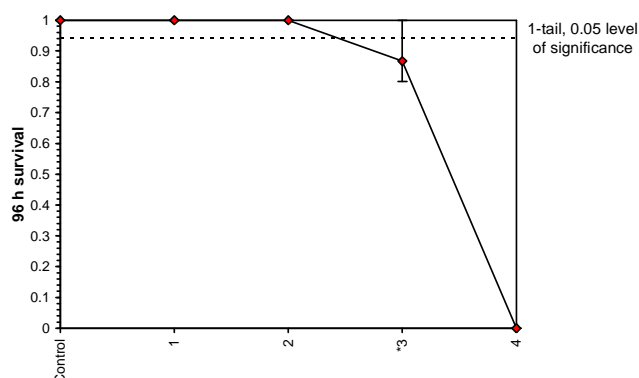
Conc-mg/L	Transform: Arcsin Square Root						N	1-Tailed			Isotonic	
	Mean	N-Mean	Mean	Min	Max	CV%		t-Stat	Critical	MSD	Mean	N-Mean
Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	5				1.0000	1.0000
1	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	3	0.000	2.466	0.1107	1.0000	1.0000
2	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	3	0.000	2.466	0.1107	1.0000	1.0000
*3	0.8667	0.8667	1.1865	1.1071	1.3453	11.587	3	3.536	2.466	0.1107	0.8667	0.8667
4	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	3				0.0000	0.0000

Auxiliary Tests				Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)				0.60617	0.825	1.71729	6.5
Equality of variance cannot be confirmed							
Hypothesis Test (1-tail, 0.05)				NOEC	LOEC	ChV	TU
Bonferroni t Test				2	3	2.44949	0.05886
							0.06196
							0.0198
							0.00378
							0.01978
							3, 10

Linear Interpolation (200 Resamples)					
Point	mg/L	SD	95% CL(Exp)	Skew	
IC05	2.3750	0.2134	2.1125	3.7925	1.2354
IC10	2.7500	0.1978	2.2250	3.4850	0.2310
IC15	3.0192	0.1374	2.4538	3.2938	-0.7742
IC20	3.0769	0.0557	2.9154	3.3354	0.1726
IC25	3.1346	0.0522	2.9832	3.3769	0.1726
IC40	3.3077	0.0418	3.1865	3.5015	0.1726
IC50	3.4231	0.0348	3.3221	3.5846	0.1726



Dose-Response Plot



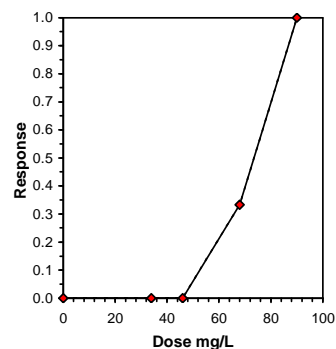
Organic Interceptor. Fish exposed for 1 h and survival monitored for 96 h.

Acute Fish Test-96 h survival					
Start Date:	7/08/2006	Test ID:	2425/LO1	Sample ID:	BNZ-Biosecurity New Zealand
End Date:	11/08/2006	Lab ID:	SC-Sue Clearwater	Sample Type:	OI-Organic Interceptor
Sample Date:	7/08/2006	Protocol:	EC2000-Environment Canada	Test Species:	OM-Oncorhynchus mykiss
Comments:	Fish exposed for only 1 h, survival monitored for 96 h mg pine oil/L				
Conc-mg/L	1	2	3	4	5
Control	1.0000	1.0000	1.0000	1.0000	1.0000
34	1.0000	1.0000	1.0000		
46	1.0000	1.0000	1.0000		
68	0.8000	0.6000	0.6000		
90	0.0000	0.0000	0.0000		

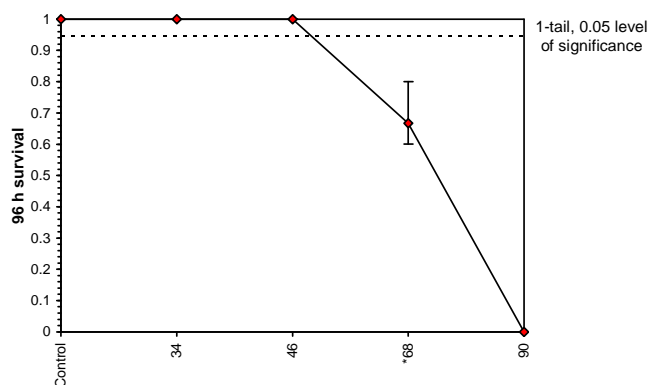
Transform: Arcsin Square Root								1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	5				1.0000	1.0000
34	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	3	0.000	2.466	0.1028	1.0000	1.0000
46	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	3	0.000	2.466	0.1028	1.0000	1.0000
*68	0.6667	0.6667	0.9598	0.8861	1.1071	13.299	3	9.248	2.466	0.1028	0.6667	0.6667
90	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	3				0.0000	0.0000

Auxiliary Tests				Statistic		Critical	Skew	Kurt					
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)				0.60617		0.825	1.71729	6.5					
Equality of variance cannot be confirmed													
Hypothesis Test (1-tail, 0.05)				NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test				46	68	55.9285		0.05397	0.05681	0.11677	0.00326	1.2E-05	3, 10

Linear Interpolation (200 Resamples)					
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	49.300	0.700	48.145	53.920	1.2516
IC10	52.600	1.399	50.290	61.840	1.2516
IC15	55.900	2.099	52.435	69.760	1.2516
IC20	59.200	2.798	54.580	77.680	1.2516
IC25	62.500	2.959	56.725	76.937	0.5780
IC40	70.200	1.702	65.580	77.130	0.1561
IC50	73.500	1.418	69.650	79.275	0.1561



Dose-Response Plot



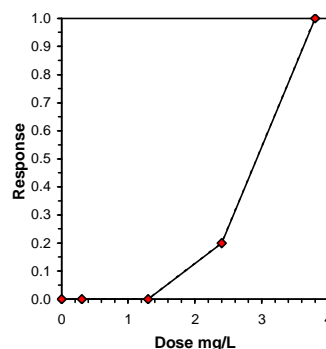
Degaclean® 150 exposure for 1 h, rinsed and survival monitored for 96 h.

Acute Fish Test-96 h Surviv					
Start Date:	8/08/2006	Test ID:	2425/LO5	Sample ID:	BNZ-Biosecurity New Zealand
End Date:	12/08/2006	Lab ID:	SC-Sue Clearwater	Sample Type:	PAA-Peracetic acid
Sample Date:	8/08/2006	Protocol:	EC2000-Environment Canada; Test Species:	OM-Oncorhynchus mykiss	
Comments:	Fish exposed to Degaclean for 1 h, %S at 96 h (mg PAA/L) peracetic acid				
Conc-mg/L	1	2	3	4	5
Control	1.0000	1.0000	1.0000	1.0000	1.0000
0.3	1.0000	1.0000	1.0000		
1.3	1.0000	1.0000	1.0000		
2.4	0.8000	1.0000	0.6000		
3.8	0.0000	0.0000	0.0000		

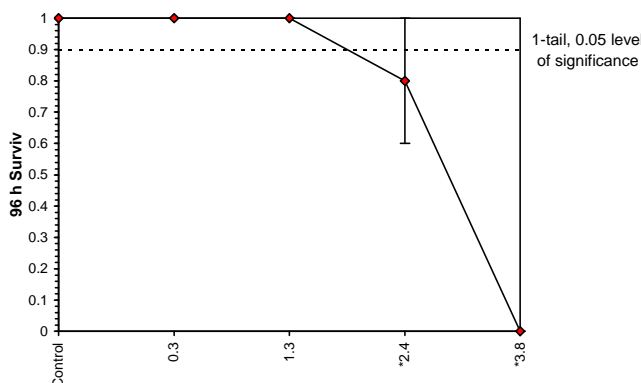
Conc-mg/L	Mean	N-Mean	Transform: Arcsin Square Root				N	t-Stat	1-Tailed		Isotonic	
			Mean	Min	Max	CV%			Critical	MSD	Mean	N-Mean
Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	5				1.0000	1.0000
0.3	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	3	0.000	2.560	0.1753	1.0000	1.0000
1.3	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	3	0.000	2.560	0.1753	1.0000	1.0000
*2.4	0.8000	0.8000	1.1128	0.8861	1.3453	20.637	3	3.395	2.560	0.1753	0.8000	0.8000
*3.8	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	3	16.354	2.560	0.1753	0.0000	0.0000

Auxiliary Tests					Statistic		Critical	Skew	Kurt					
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)					0.50148		0.851	0.11901	8					
Equality of variance cannot be confirmed														
Hypothesis Test (1-tail, 0.05)					NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test					1.3	2.4	1.76635		0.10222	0.1076	0.73893	0.00879	1.1E-08	4, 12

Linear Interpolation (200 Resamples)					
Point	mg/L	SD	95% CL(Exp)	Skew	
IC05	1.5750	0.2653	1.2863	3.4545	1.7197
IC10	1.8500	0.2858	1.2725	3.2990	0.7691
IC15	2.1250	0.2678	1.2588	3.1435	0.0851
IC20	2.4000	0.2278	1.2450	2.9880	-0.5645
IC25	2.4875	0.1869	1.4375	3.0387	-0.7175
IC40	2.7500	0.1273	2.0150	3.1910	-0.5511
IC50	2.9250	0.1061	2.3125	3.2925	-0.5511



Dose-Response Plot



12. Appendix 4: Water chemistry data

Table A4-1: Average water chemistry measurement for the water at MEF.

Parameter	Units	Data
Temperature	°C	12.1
Dissolved Oxygen	% Sat	100.3
Dissolved Oxygen	ppm	10.6
Dissolved Calcium	ppm	3.2
Dissolved Magnesium	ppm	1.1
Hardness (as mg CaCO ₃ /L)	ppm	12
Instantaneous Discharge	m ³ /sec	13.95
Visual Clarity	m	6.12
Turbidity	NTU	0.46
Hydrogen ion concentration	pH	7.35
Electrical conductivity	µS/cm @ 25°C	39.0
Ammonia (NH ₄)	ppb	5
Nitrate (NO 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

Table A4-2: Average water chemistry measurements taken during the Gemex™ trials.

Treatment	Velocity	Reps	DO	pH	°C
Control	Fast	2	13.45	6.96	10.30
Control	Med	2	11.26	6.21	9.90
Control	Slow	2	11.37	7.04	10.25
Cu 10	Fast	3	12.38	4.09	9.37
Cu 10	Med	3	12.95	4.07	10.00
Cu 10	Slow	3	11.45	4.64	10.10
Cu 15	Med	3	12.48	3.71	9.33
Cu 20	Fast	3	13.37	3.62	9.90
Cu 20	Med	3	14.12	3.67	9.47
Cu 20	Slow	3	12.34	3.71	9.57
Cu 5	Fast	3	12.89	4.66	10.20
Cu 5	Med	3	11.56	5.46	10.13
Cu 5	Slow	3	11.27	5.64	9.63
Cu 10 x 3	Med	3	14.18	3.69	9.83
Cu 5 x 3	Med	3	12.70	4.42	9.83