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Rapid Screening of Multiple Compounds for Control of the Invasive Diatom *Didymosphenia geminata*

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ABSTRACT

Didymosphenia geminata is a freshwater diatom that inhabits streams and rivers worldwide. The discovery of this diatom in a New Zealand river in October 2004 was the first occurrence of mass growths of the alga in the southern hemisphere. Massive proliferations of the alga in New Zealand waterways led to urgent research to identify control or eradication options with potential to protect environmental and economic interests. The first stage of the control program involved rapid screening trials to test initial effectiveness of 10 biocides against *D. geminata*. Immediate efficacy was assessed by measuring *D. geminata* cell viability following biocide exposure for a range of contact times (up to 1 h), while longer-term effectiveness was determined after 15 d following biocide exposure for 1 h. Analysis of immediate biocidal effects showed that five biocides significantly reduced *D. geminata* cell viability below that of control treatments. From the longer-term assessment, a biocide effect was observed for four biocides (only two of which were identified from immediate-effects trials). A decision support matrix was used to evaluate the 10 biocides from which it was concluded that Gemex™ (a chelated copper compound), EDTA, Organic Interceptor™ (a pine oil formulation), and Hydrothol®191 be tested in further trials. A 1 h contact time is recommended because it was the most effective short-term exposure duration against *D. geminata* and is a realistic duration for potential river application trials. The utility of a rapid screening approach and its contribution to a larger *D. geminata* control research program are also discussed.

Key words: biocide, didymo, incursion, management.

INTRODUCTION

Didymosphenia geminata (Lyngbye) Schmidt (didymo) is a freshwater diatom that inhabits streams, rivers, and lakeshores in the boreal and alpine regions of the Northern Hemisphere (i.e., Europe, Asia, North America; Beltrami et al. 2008a). The discovery of this diatom in the Waiau River, New Zealand (October 2004) was the first occurrence of mass growths of the alga in the southern hemisphere (Whitton et al. 2009). A review of research on *D. geminata* shortly after its discovery indicated that the alga was capable of massive proliferations in natal waters, and that these blooms could have detrimental aesthetic effects as well as significant impacts on water chemistry and the aquatic flora and fauna (Sherbot and Bothwell 1993, Kawecka and Sanecki 2003, Beltrami et al. 2008b).

Since its discovery in the southern region of New Zealand, *D. geminata* has significantly expanded its distribution and is now recorded in many of the major South Island river systems (although it is not currently in the North Island). As found in other countries, the largest blooms of the alga are generally associated with oligotrophic conditions from lake outflows (Sherbot and Bothwell 1993, Jonsson et al. 2000). Under bloom conditions, *D. geminata* forms dense, degradation-resistant fibrous mats (composed largely of stalk material) that can often cover the entire stream bed at thicknesses up to 30 mm (C. Kilroy unpubl. data), even in relatively high water velocity (>1 m s^{-1}). These blooms have been found to alter water chemistry (e.g., pH, dissolved oxygen) and algal and macroinvertebrate communities in New Zealand waterways (Kilroy et al. 2005, 2009). While no effect of *D. geminata* on fish communities has yet been found in these waterways (Shearer et al. 2007), there are concerns about its impact on endangered native fish species and New Zealand's world-renowned trout fisheries.

Because New Zealand is very dependent on freshwater resources for power generation, tourism (e.g., trout fisheries, international image) and agriculture (e.g., water intakes, irrigation), the potential environmental and economic impacts of *D. geminata* were considered substantial (Campbell 2008). At the start of this research (January 2006), seven South Island catchments were infested with *D. geminata*, but GIS-based predictive modeling research suggested that >70% of river sections (stream order >3) throughout New Zealand were highly suitable for *D. geminata* blooms (Kilroy et al. 2008). The potential impacts and spread of *D. geminata* made it necessary to urgently investigate control or eradication measures. A literature review and responses solicited from diatom experts world-wide revealed no published or reliable anecdotal examples of attempts to contain, control, or eradicate blooms of *D. geminata* (Kilroy 2004). As part of the New Zealand government's incursion re-

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sponse to *D. geminata*, the National Institute of Water and Atmospheric Research Ltd. (NIWA) investigated a range of potential *D. geminata* control techniques. The potential distribution and effects of *D. geminata* were investigated simultaneously in separate projects contracted by MAF Biosecurity New Zealand (http://www.biosecurity.govt.nz/didymo).

Worldwide, little was known about the ecology of D. *geminata*. Moreover, a lack of fundamental knowledge about how to respond to an incursion of an invasive micro-organism in riverine environments created a further challenge. Three broad categories of control (biological, chemical, and mechanical) were considered, but only chemical control was considered feasible given the available time, regulatory requirements, and desired control level (significant suppression–eradication). Our approach was to implement a structured four-stage process for this investigation (Figure 1), progressing from controlled to natural stream environments, with additional nontarget species toxicity work performed in the laboratory. Compounds and processes to degrade stalk material were investigated in a separate, laboratory-based study (Jellyman et al. 2006). In this paper we describe the techniques and results of our rapid biocide-screening process (Stage 1) in static exposures, which enabled us to select a number of products for testing in more rigorous stream-side channel trials in conjunction with laboratory toxicity testing (Stage 2). Once a potential biocide was identified, the effect of variations in concentration and velocity were trialed and the application technique was refined (Stage 3) so that a full-scale stream trial to measure ecosystem effects and the duration of control on *D. geminata* in a natural waterway could be conducted (Stage 4). The following seven criteria were used at each stage of the study to evaluate the effectiveness of each biocide and assess whether a product was suitable for further testing: (1) toxicity to *D. geminata*, (2) potential to remove *D. geminata* stalk material, (3) contact time, (4) ease of application including risks to human health, (5) potential for damage of key nontarget species and ecosystem functioning, (6) cost of materials, and (7) restrictions or regulatory requirements from government agencies.

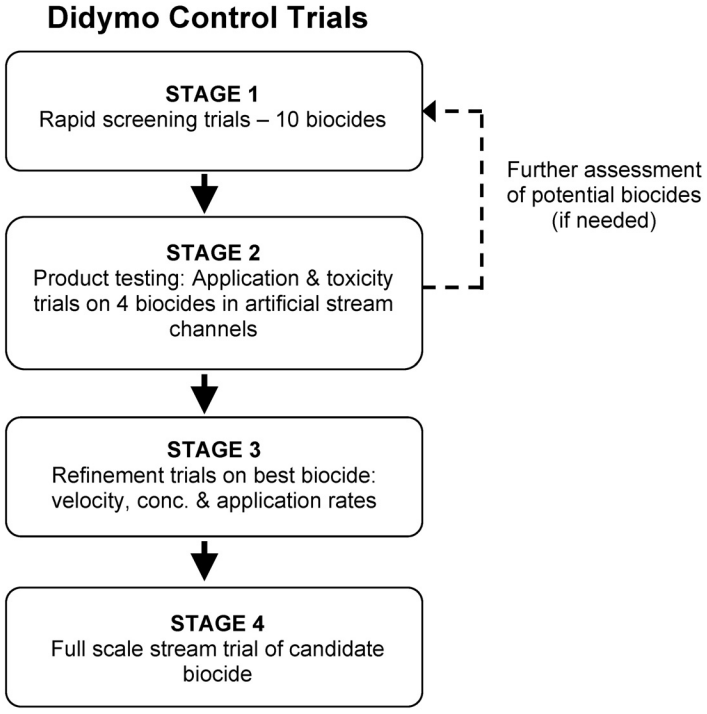

Figure 1. Organization of the 4-stage didymo control study investigation.

Problematic mass growths of *D. geminata* have been reported much more widely in recent years, even from countries with a long record of didymo occurrence (Whitton et al. 2009). Despite a global trend of increasing mass *D. geminata* blooms, this study was the first to investigate options for control of the alga. In this paper we report the efficacy of 10 biocides on *D. geminata*, using immediate and longer-term effects on a range of algal indices. We also analyze the effectiveness of our approach for identifying a control method, with a view to informing similar biosecurity challenges. The results of Stages 2 to 4 of this control study will be reported elsewhere (Clearwater et al. 2007, Jellyman et al. 2010, Clearwater et al. 2010).

MATERIALS AND METHODS

Study Site

Trials were carried out on the banks of the Monowai River, Southland, New Zealand (45°46'55"S, 167°35'28"E), and within the river itself (due to biosecurity risks it was not considered acceptable or practical to undertake trials in a laboratory). The Monowai River is a regulated waterway that drains Lake Monowai as part of the hydro-electric power scheme that discharges into the Waiau River. The majority of the Monowai River is diverted into a hydro-canal 6 km downstream of the lake, with a residual flow of 500 L s^{-1} maintained down the lower river. The regulated residual flow in the lower Monowai River provided a stable flow environment to conduct experimental trials, and the flat banks of the lower river provided a suitable area to perform rapid screening trials. The lower Monowai River had moderate riparian shading, stable cobble–boulder sized substrate and a relatively uniform channel with a low to moderate gradient. The experimental site was placed about 20 m above what had been determined as the upstream limit of *D. geminata* to prevent live *D. geminata* cells immigrating into the treatments (potentially confounding evaluation of treatment effectiveness). The water chemistry of the Monowai River was considered suitable for growth of *D. geminata* because the diatom proliferated throughout the lower reaches. The Waiau River was heavily infested with *D. geminata* and was therefore an excellent source of *D. geminata* mats growing on small cobbles and rocks for container trials.

Selected Biocides

Given the paucity of information regarding any chemical treatment of *D. geminata*, we based our biocide selection on knowledge of existing products used nationally and globally for controlling algae, aquatic weeds, and diatoms. Ten products (Table 1) were chosen to be evaluated for their efficacy against *D. geminata*. We selected test concentrations that corresponded to their highest recommended application rate

(if a commercially available product) or to phytotoxic concentrations in the US Environmental Protection Agency ECOTOX database (http://cfpub.epa.gov/ecotox/). Gemex™ was selected because chelated copper formulations are used worldwide as algaecides that are cost-effective, easily-manufactured, and reasonably specific to algae. The particular formulation of chelated copper used in the trials was selected because of its stability over a wide range of pH and temperatures, and we chose to identify it as Gemex™. *Didymosphenia geminata* was thought to be sensitive to copper, with no conspicuous growth in a polluted Norwegian river system when copper concentrations exceeded 8 µg L^{-1} (Lindstrom and Rorslett 1991), although copper was only one of many contaminants present in the river. Zinc sulphate was trialed because it is known to be effective in controlling algae and some native macroinvertebrate taxa (i.e., endemic mayflies) can tolerate up to 30 mg L^{-1} of Zn (Hickey 2000). Organic Interceptor™ is a pine oil-based formulation registered for use as a terrestrial herbicide in New Zealand to control annual weeds and grasses. Germanium dioxide was selected because it is known to inhibit or kill specific diatoms at concentrations around 1 to 10 mg L^{-1}, possibly by substituting for silicon (Markham and Hagmeier 1982), and is likely to present a low environmental risk because it is thought to have limited bioavailability. Simazine (6-chloro-N,N'-diethyl-1,3,5-Triazine-2,4-diamine), used as an algaecide and a preemergence herbicide, is toxic to some species of diatoms at concentrations <1 mg L^{-1} (http://cfpub.epa.gov/ecotox/); however, its use in natural water bodies has been prohibited in the United States since 1996 (USEPA 2006). The quaternary ammonium compound (QAC), 303 Clear-All, was selected because QAC are extensively used for control of bacteria, fungi, and algae and are known to suppress plant growth at concentrations of 3 to 5 mg L^{-1} (Walker and Evans 1978). Chlorine is an industry standard for sterilization, and in this research sodium hypochlorite (NaOCl) was used as a readily available form of chlorine. Although NaOCl was likely to be a nonspecific toxicant, we selected it for this trial because it could act as a positive control. Ethylenediaminetetraacetic acid (EDTA) is used to detach marine biofouling diatom cells from their pads or stalks, possibly by chelating cations (e.g., Ca^{2+}), thereby significantly altering the molecular composition and integrity of the extracellular mucilage. The marine diatom cells separate from their pads after a couple of hours (Chen and Stewart 2000), so EDTA was included in product screening for its potential to cause detachment of *D. geminata* mats from riverine substrates. Hydrothol®191 (a dimethylalkylamine salt of endothall) has been used to control aquatic vegetation and filamentous algae for more than 30 years. It is regarded as moderately toxic to a variety of aquatic biota but is particularly toxic to diatoms; at >1 mg L^{-1} it rapidly inhibits photosynthesis (Axler et al. 1994). A related formulation (Aquathol K) had also been recently registered in New Zealand for macrophyte control. Diquat (diquat dibromide) is the active ingredient in Reglone®, a herbicide used for macrophyte control in New Zealand lakes for 50 years. Reglone® contains 20% diquat dibromide and is a selective herbicide that controls many unwanted target weed species in fresh waters (e.g., *Elodea* sp., *Egeria* sp., *Lagarosiphon* sp., and hornwort). Diquat desiccates plant tissue and disrupts cell membranes, but the effect of diquat on algae is known to be dependent on water quality, especially turbidity (Hofstra et al. 2001).

Experimental Design

Because a selection of biocides with a variety of mechanisms of toxicity was tested, the response time in the didymo colonies was expected to vary markedly (Table 1). For example, some products were expected to cause significant and immediate cell mortality upon contact with the biocide and other products were expected to affect didymo colonies over a longer time period. To evaluate both the immediate and longer-term efficacy of the 10 selected biocides, a two-step screening design was used. Didymo-coated pebbles (<80 mm dia) were used to test immediate effects (step 1), and didymo-coated cobbles (<200 mm dia) were used to assess longer-term impacts by the biocides (step 2). Pebbles and cobbles with both a "healthy" surface coating (i.e., an even, well-attached, rich-brown color with minimal sediment) and uniform thickness of *D. geminata* were selected from an 80 m^2 area of the nearby Waiau River bed. All didymo-coated rocks (pebbles and cobbles) were exposed to 50 L of biocide diluted in Monowai River water to a range of nominal concentrations (Table 1). Exposures were static, but tanks were vigorously aerated over the course of the trial to simulate river turbulence and enhance transfer of the

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

Biocide	Test Concentration	Mode of action	Response time
Sodium hypochlorite	5 mg Cl L^{-1}	Photosynthetic inhibitor	Immediate
Gemex™	5 mg Cu L^{-1}	Photosynthetic inhibitor	Immediate
Diquat	5 mg Reglone® L^{-1}	Cell toxicant	Immediate
QAC	0.02 mL product L^{-1}	Membrane disruption	Immediate
Simazine	1 mg a.i. L^{-1}	Metabolic disruption	Immediate
Zinc sulphate	10 mg Zn L^{-1}	Cell toxicant	Immediate
Organic Interceptor™	1000 mg pine oil L^{-1}	Photosynthetic inhibitor	Immediate
Hydrothol®191	1 mg a.e. L^{-1}	Photosynthetic inhibitor	Longer term
EDTA	10 mg EDTA L^{-1}	Stalk/mat detachment from substrate	Longer term
Germanium dioxide	10 mg Ge L^{-1}	Cell toxicant	Longer term

biocide through the mats. Tanks were also shaded to maintain constant water temperatures, which were measured every 15 min (all exposure solutions were maintained at 15 ± 0.5 C).

The efficacy of each biocide was evaluated with three replicate exposures, and within each replicate, three pebbles (step 1) and one cobble (step 2) were exposed to the biocide. Two more pebbles were assigned to each replicate for controls and exposed to 50 L of Monowai River water and aerated turbulence without biocide. For each replicate in step 1, the three biocide-exposed pebbles were withdrawn after either 36, 360, or 3600 s (1 h) and placed in separate tanks of fresh, aerated river water. In step 2, the single cobble was removed after 3600 s. The pebbles (36, 360, and 3600 s) were destructively sampled for live/dead cell counts at the conclusion of the final contact time (3600 s; Kilroy 2005). The two control pebbles were sampled after 0 and 3600 s to determine any changes in cell viability as a result of the trial conditions.

After being treated for 3600 s, each cobble was placed into the Monowai River and left for 15 d. Cobbles were randomly placed on one of six transect lines to control for variation in water velocity. Three replicate cobbles for each biocide and six untreated control cobbles were placed on the transect lines (36 cobbles total). At the start and conclusion of the trial, water depth (cm) and velocity (m/s) were measured for each cobble (at "mat height", 5 cm above river bed) using a current meter (Marsh-McBirney Flo-Mate™ Model 2000 accuracy ± 2%) to determine the hydraulic stresses under which *D. geminata* mats were exposed prior to live/dead cell enumeration (Kilroy 2005).

Laboratory Procedures

Changes to the viability of *D. geminata* mats were determined microscopically using neutral red staining to distinguish live and dead didymo cells in fresh material (Kilroy 2005). The neutral red stain (25 mL of a 0.004% solution) distinguished recently dead cells still containing chloroplasts from those that were still physiologically active because live cells take up the stain into their organelles. For each sample, at least 100 *D. geminata* cells were enumerated to calculate percentage live cells; these counts included all cells viewed (i.e., those containing chloroplasts that did or did not take up stain, and empty cells). Samples were macerated prior to the introduction of the stain to allow uniform stain uptake throughout the mat. Stained samples were analyzed within 1 h of staining, with no detectable decline in cell viability over this period. For treated pebble samples (i.e., 36, 360, and 3600 s), the three replicates for the 3600 s contact time were analyzed first, and if >85% of cells were viable the remaining contact times (i.e., 36 and 360 s) were not examined.

Two 30 mm diameter circular samples of the *D. geminata* mats were cut and scraped from the cobble substrates after 15 d and frozen for later measurement of chlorophyll *a*, ash-free dry mass (AFDM) and total cell density (Biggs and Kilroy 2000). Each sample was homogenized using a hand blender and made up to a known volume. Duplicate subsamples of known volume were filtered through glass fiber filters. For AFDM, one of the duplicates (a preweighed filter plus filtered sample) was dried at 105 C for 24 h, reweighed, then ashed at 400 C for 4 h and weighed for a final time. Chlorophyll *a* was extracted from the second filter-plus-sample using boiling ethanol, and concentrations of chlorophyll *a* were read spectrophotometrically at 663 nm, including acidification to correct for phaeophytins. In each case, we calculated quantities per m^2 of stone surface, based on the area of the sampling circle (0.00141 m^2).

Live/dead cell enumeration using neutral red staining proved difficult for the cobble substrates in the longer-term trials (step 2) because of the high numbers of "empty" cells present within the *D. geminata* mats. Empty cells contained no chloroplasts, but the cause could not be determined (e.g., cell mortality due to initial biocide application or an unknown environmental variable affecting them after relocation). These empty cells were still usually attached to the stalk material. While live/dead cell enumeration was conducted on fresh samples 15 d post-treatment, most of these viability samples had <100 cells containing chloroplasts; therefore, live cell density was also measured using a larger volume of the frozen samples. The cell density analysis produced a quantitative estimate of the absolute number of "live" cells in a sample, those taken to be cells containing intact chloroplasts. This analysis assumes that any cells that were going to die after biocide exposure no longer contained chloroplasts 15 d post-exposure. The 1 mL subsamples were extracted from the homogenized solution made up for biomass estimation (see earlier description), and all *D. geminata* cells containing chloroplasts were counted until either 250 cells or up to 100 fields of view (100× magnification) were examined on an inverted microscope. Absolute numbers of live cells per sample were calculated from the known areas of the microscope well and the numbers of field of view examined.

Data Analysis

Immediate effects (step 1 - pebbles). To reduce the number of biocides to be analyzed, a two-stage analysis was adopted. The first stage identified biocides that significantly reduced cell viability at the longest contact time (3600 s) in comparison to untreated controls that were immersed in water for 3600 s (these controls were the 0 s contact time in the Generalized Linear Model [GLM]) using a one-tailed one-sample *t*-test for each biocide ($\alpha = 0.10$ to lower the probability of a Type II error). The second stage compared the performance, over a range of contact times (0 to 3600 s), of five biocides identified in Stage 1 as being capable of reducing cell viability. The proportion of viable cells was modeled using a GLM with a binomial error distribution and a log-link function. Preliminary analyses showed no significant variation among replicates for each biocide; therefore, this term was dropped from the model, leaving just two predictors: biocide (a fixed factor with 5 levels: chlorine, Gemex™, Organic Interceptor™, QAC and zinc sulphate) and contact time (a fixed factor with 4 levels: 0, 36, 360, and 3600 s). Contact time was measured on a continuous scale and $\log_{10}$-transformed. Because the log of 0 is undefined, we approximated this control with a value of 0.36, two $\log_{10}$ units below the

next lowest treatment of 36 s. The significance of model terms was evaluated using *F*-ratio tests, which account for over-dispersion in the data ($\alpha = 0.05$).

Longer-term effects (step 2 - cobbles). Ash free dry mass (AFDM), chlorophyll *a*, and the ratio of AFDM to chlorophyll *a* (autotrophic index [AI]) of *D. geminata* on rocks was analyzed using one-way analysis of variance (ANOVA; $\alpha = 0.05$). There were 11 levels of the treatment factor: 10 biocides and a control (those that had been immersed in water for 3600 s). The assumption of homogeneous variances was tested using an F_{max} test. Following a significant main effect, Dunnett's test was used to examine which biocides differed significantly from the control group. Habitat characteristics (water velocity and depth) were analyzed using regression analysis, against the response variables: chlorophyll *a*, AFDM, AI, percentage cell viability, and live cell density.

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

Decision Support Matrix

A decision support matrix was a subjective tool used to evaluate the attributes sought in an ideal *D. geminata* control compound. This was achieved by ranking each biocide against seven key criteria (Table 2). Overall, the management aim behind these criteria was to prevent a potential long-term decrease in abundance and diversity of resident aquatic species by controlling the invasive alga *D. geminata*. The rankings for most attributes ranged from 1 to 3; however, the more important attributes (e.g., effectiveness against *D. geminata*) were assigned higher ranks (Table 2).

Effectiveness ranked the potential of the biocide to impact *D. geminata* without consideration of nontarget effects. Cell mortality rates were used as indicators of short-term effectiveness, and the level of chlorophyll *a* relative to controls after 15 d was the longer-term measure used. Biocides were ranked from 0 to 5 depending on the effectiveness of these measures, with biocides effective for both measures receiving higher ranks (4 to 5) than those that were effective in just one category (ranks 0 to 3) (Table 2).

Stalk removal potential was the ability of a biocide to remove or degrade *D. geminata* stalk material and was assessed qualitatively during the experiment and from available ecotoxicity data and material safety data sheets. A major ecological impact of *D. geminata* is the establishment of dense mats of extracellular stalk material, which may also prevent the complete penetration of biocides (and 100% kill of *D. geminata* cells). The ability to disrupt or penetrate the mats would be a favorable attribute of a biocide. The stalk removal potential of a biocide was ranked as either being possible or not possible.

Contact time assessed how much time was required for the biocide to interact with the *D. geminata* mats and result in a high mortality rate, regardless of whether the mortality occurred immediately or after a delay. Shorter contact times were preferred because less biocide would be required to treat an affected waterway, which is particularly important because the alga blooms in fast-flowing waterways. Biocides were ranked 1 to 4 based on the time taken to cause 50% *D.*

TABLE 2. CRITERIA AND RANKINGS USED TO CONSTRUCT THE DECISION MATRIX.

	Rank					
	5	4	3	2	1	0
Effectiveness	>90% mortality & Chl *a* < Control	>80% mortality & Chl *a* < Control	>65% mortality Or Chl *a* < Control	>50% mortality Or Chl *a* < Control	>25% mortality Or Chl *a* < Control	<25% mortality & Chl *a* > Control
Stalk removal potential				Possible		Not Possible
Contact time		Very fast	Fast	Moderate	Slow	
Application ease			Readily mixes	Requires pre-mixing	Difficult to apply	
			Standard equipment	Standard equipment	Special equipment	
Non-target impacts		No impact	Low impact on algal/plant species only	Moderate impact on macroinvertebrates and fish	Severe impact on fish and other vertebrates	High rates of fish mortality
Cost			Cheap	Moderate	Expensive	
Restrictions			Available, Registered in NZ	Available, Unregistered in NZ	Supply problems, Unregistered	

geminata cell mortality (very fast = ≤36 s, fast = 37 to 360 s, moderate = 361 to 3600 s, and slow = >3600 s).

Application ease ranked what state the biocide was supplied in (solid or liquid), whether it would mix readily with water, and whether there were safety concerns (particularly human health) with handling the product. The ideal biocide would be a liquid that mixed readily with river water from a stock solution (without premixing) and could be applied in a manner that achieved consistent river coverage, with safety easily managed with minimal specialist equipment. The biocides were ranked 1 to 3 for application ease.

Nontarget impacts were ranked from available ecotoxicity data. The ideal biocide would kill only *D. geminata*; however, a more practical management goal was to find a biocide that did not cause significant fish mortality (because these populations take the longest time to recover). Biocides were ranked 0 to 4 based on their potential to impact nontarget species (for further details see Table 2).

Cost was estimated for each of the biocides based on a 1 h application to a 5 m^3/s river. The quantity of each biocide was estimated based on the amount needed to cause >90% *D. geminata* cell mortality. Because product prices and quantity needed were highly variable, the biocides were broadly categorized as cheap, moderate, or expensive.

Restrictions on the use of the biocides were also evaluated in the decision matrix. Biocides needed to be registered for use and readily available in New Zealand to receive the highest rank of three. Biocides that received lower ranks were either unregistered or were difficult to obtain in large quantities.

RESULTS

Immediate Effects of Biocides on *D. geminata*

The mean (± S.E.) proportion of viable cells on control rocks (those that had been immersed in water for 3600 s) was 0.95 ± 0.01. Only five biocides significantly reduced the proportion of viable cells when applied at the longest contact time (3600 s): chlorine, Gemex™, Organic Interceptor™, QAC, and zinc sulphate (Table 3). The effectiveness of all five biocides also increased with contact time (GLM, $F_{1,47}$ = 127.9, $p < 0.001$). There was a significant interaction between biocide and contact time (GLM, biocide * contact time interaction: $F_{4,47} = 5.0$, $p = 0.002$; Figure 2). Overall, Gemex™ was the most effective, particularly at short contact times; 76.2% of cells died after 36 s contact time and 94.3% died after 3600 s. Organic Interceptor™, QAC, and chlorine achieved ≥50% mortality only at the longest contact time. Zinc sulphate had only a minor effect on cell viability (Figure 2).

TABLE 3. ONE-TAILED, ONE-SAMPLE T-TESTS COMPARING THE MEAN PROPORTION OF CELLS ALIVE AFTER 3600 S CONTACT TIME FOR EACH BIOCIDE WITH THE AVERAGE PROPORTION OF VIABLE CELLS ON CONTROL ROCKS IMMERSED IN WATER FOR 3600 S (0.95). EXPOSURE AT MAXIMUM CONCENTRATION GIVEN IN TABLE 1. $N = 3$ REPLICATE SAMPLES FOR EACH BIOCIDE. SIGNIFICANT DIFFERENCES ARE INDICATED IN BOLD ($\alpha = 0.10$)

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

Longer-term Effects of Biocides on *D. geminata*

Habitat characteristics (water velocity and depth) had no significant effect ($p > 0.05$) on any of the following responses: percentage cell viability, live cell density, chlorophyll a, AFDM, or AI. There was a weakly significant effect of treatment on AFDM (ANOVA: $F_{11,23} = 2.7$, $p = 0.023$); AFDM was significantly higher in the diquat treatment than the control (Dunnett's test: $q = 2.3$, $p < 0.05$), but no other biocides had a significant effect on AFDM. We did not observe an increase for any treatment in the biomass of *D. geminata* over the course of the 15 d trial. There was also no effect of treatment on chlorophyll *a* (ANOVA: $F_{11,23} = 1.9$, $p = 0.097$), and only four products had lower mean chlorophyll *a* measurements than the control (EDTA, Gemex™, Hydrothol®191, and Organic Interceptor™; Figure 3A).

Of the four products that resulted in average live cell densities lower than that of the control (EDTA, chlorine, Organic Interceptor™, and Gemex™), only EDTA was significantly different from the control 15 d post-treatment (t-test: $t_7 = 3.6$, $p < 0.01$; Figure 3B). Hydrothol®191 contained some samples with very low cell densities but samples were highly variable.

Although average AI values were higher in *D. geminata* cobbles treated with either Gemex™, EDTA, or Organic Interceptor™, there was no statistically significant treatment ef-

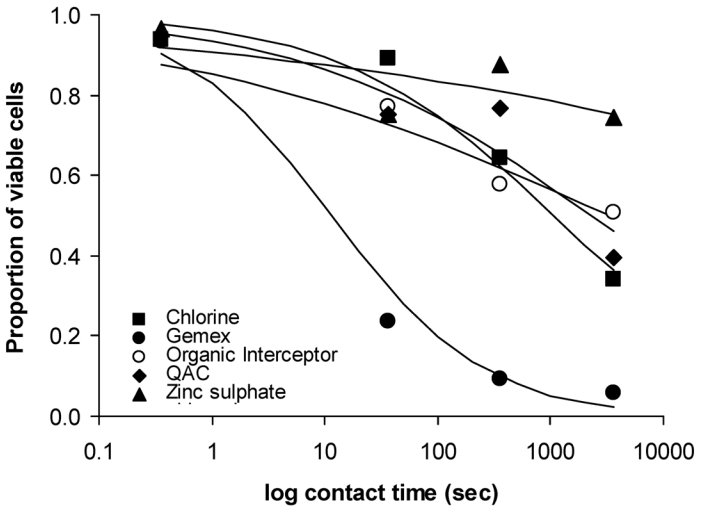

Figure 2. Relationship between cell viability and contact time for the five selected biocides found to be significantly different from the viability of the control substrates (n = 3). Lines indicate predictions from a Generalized Linear Model.

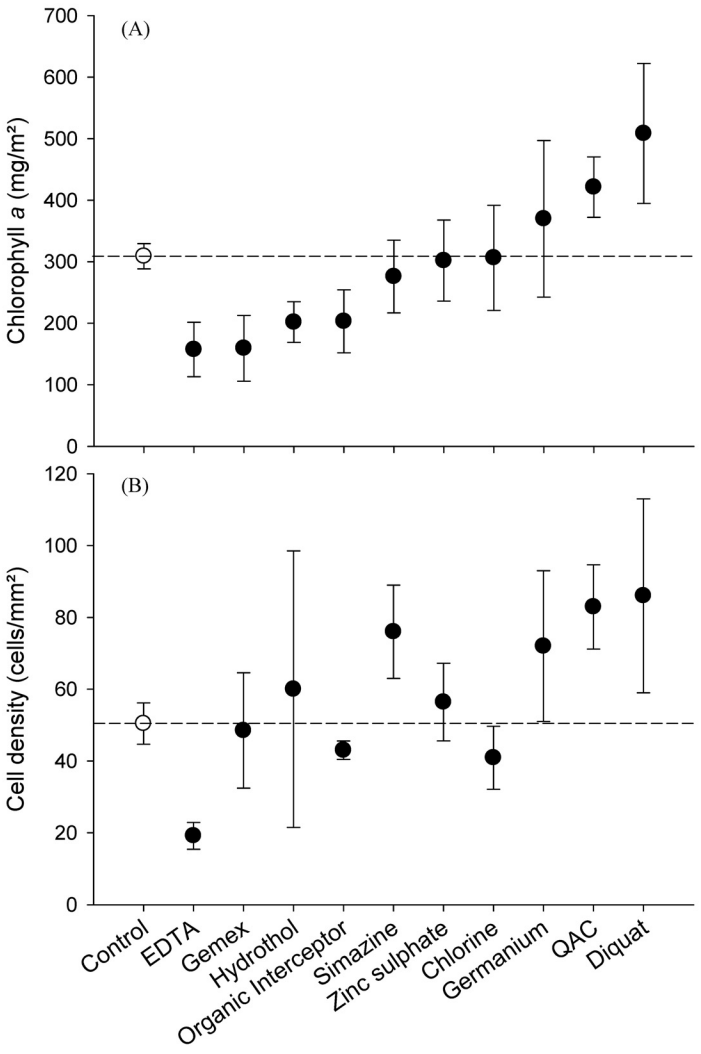

Figure 3. Mean (±1 S.E.) *D. geminata* measurements of (A) chlorophyll *a* and (B) cell density for cobbles 15 d after treatment with 10 biocides and a control (open circle). The dashed line represents the mean control value. n = 3 replicate rocks per biocide and 6 replicate rocks for the control treatment.

fect (ANOVA: $F_{11,23} = 0.9$, $p = 0.52$). However, higher AI values for these biocides indicated that at 15 d post-treatment, more dead *D. geminata* cells or stalk material per unit biomass were produced than was found in control samples.

The percentage of live cells from the control cobble substrates (measured by neutral red counts) after 15 d was highly variable. This high level of control substrate cell variability meant we could not distinguish any effect on longer-term cell viability caused by individual biocides from background cell mortality. However, the cell viability data were still useful for comparing the relationship between the percentage of live cells and the chlorophyll a measurement for a sample. The relationship between the percentage of live cells and chlorophyll a was variable ($R^2 = 0.05$, $p = 0.44$), indicating that the amount of live material in the samples was poorly correlated with the percentage of live didymo cells. In contrast, total live cell density was highly correlated with chlorophyll a ($R^2 = 0.67$, $p < 0.001$), signifying that *D. geminata* cells are the dominant live material in these algal communities.

DISCUSSION

The results of the screening trials indicated large differences in the immediate and longer-term efficacy of some products. A comparison of all analyses (both immediate and longer-term) consistently showed that germanium dioxide, diquat, and simazine were the least effective biocides. Immediate effects trials identified five biocides (Gemex™, chlorine, QAC, Organic Interceptor™, and zinc sulphate) that significantly reduced the percentage cell viability of D. geminata, but showed Gemex™ resulted in the highest cell mortality. The results from these immediate effects trials clearly demonstrated that these biocides would be the most effective against *D. geminata* after a 1 h exposure. However, because water/mat samples were not taken throughout the biocide exposure period, we could not be certain whether the uptake of the active ingredients was faster in some biocides than others. While some biocides may be more readily absorbed by *D. geminata* mats because of their chemical and solubility properties, such biocide traits would be likely to make it a more effective control tool against didymo, particularly in a fast-flowing river. Thus, this was seen as an acceptable bias during the initial rapid screening of these compounds, but it was a factor to consider during later biocide refinement testing.

As predicted, products identified as having a "longer-term" response time (e.g., EDTA; Table 1) only had a detectable effect on *D. geminata* 15-days post-exposure. In evaluating the efficacy of the biocides in longer-term trials, percentage cell viability data were not the main response variable used. The distribution of *D. geminata* cells across the surface of the mats became sparse and heterogeneous, with a large increase in empty cells and a large reduction in total cell density. While this outcome was not unexpected, we decided that percentage cell viability counted on 100 cells (for the longer-term data) was too variable for statistical analysis when large numbers of dead cells were present. Because water velocity and depth appeared to have had little systematic effect on live cell density during the 15 d post-treatment period, we instead concentrated on metrics that were more robust at the algal community level (i.e., chlorophyll a, AFDM, AI, and total cell density). Total cell density was the most useful for rigorous analysis because it provided a quantitative estimate of the absolute number of "live" cells in a sample without being prejudiced by the heterogeneous nature of the *D. geminata* mats over the longer trial period. In addition to the biocides identified in the immediate-effects trial, the cell density analysis showed that EDTA and Hydrothol®191 (although variable) could cause a large reduction in density of *D. geminata* cells. All other algal community metrics showed EDTA as a leading candidate, and for the most part Hydrothol®191.

None of the biocides at trial concentrations caused 100% mortality of *D. geminata* cells. The most effective biocide at causing cell mortality was 5 mg L^{-1} Gemex™, although it only killed 94% of the cells during the 1 h (3600 s) exposure. A 1

h biocide application to a river is feasible and realistic for a field trial, particularly for Gemex™. This time period should be adequate for instream mixing of biocides into most interstitial spaces within streams, and this exposure duration seems to kill most didymo cells. The change in cell mortality was <20% with increased biocide exposure time for the most effective immediate-impact biocide (Gemex™), so further increases in cell mortality are likely to come from increased concentrations rather than increased exposure times.

Other products were not tested in this trial for various reasons (e.g., malathion, which we believe is inappropriate for use with diatoms and would significantly affect nontarget species), but the rapid screening procedures we used for testing biocides was quick and replicable if future products are discovered that merit investigation. Regeneration of mats was not directly measured during the longer-term trial, and Stage 2 trials would assess this issue further. This trial did not test combinations of treatments, but testing of such combinations could produce synergistic effects that may be more effective for both short- and longer-term control of *D. geminata* than a single biocide. Long-term controls that alter water chemistry (e.g., sodium bisulphate addition to decrease pH) also have the potential to control *D. geminata* and could be considered for further investigation; however, it was not feasible to test such products using the rapid screening trial design.

Conducting several more short-term screening trials using a wider range of exposure concentrations and products would be useful; however, the urgency of the research and the remote location of the trial site (chosen because of its *D. geminata*-free water source and biosecurity concerns) meant that more intensive trials (Stage 2) commenced without further screening (although more static screening trials conducted at the end of Stage 2 provided useful information for Stage 3).

To select products for more intensive and controlled testing in Stage 2 (see Figure 1), we designed a decision support matrix to assess the 10 biocides used in the rapid screening trials in a transparent manner (Table 4). The decision support matrix detailed the characteristics we sought in an ideal *D. geminata* control compound and evaluated each biocide with respect to our best available knowledge of the seven criteria previously described. The rankings for each criterion are described in the methods, and for each criterion, the higher the ranking the more desirable the characteristic. We decided that four biocides would be selected for Stage 2 trials. Based on the total rank values from the decision support matrix analysis, these four biocides were Gemex™, EDTA, Organic Interceptor™, and Hydrothol®191 (Table 4). In Stage 2 trials, biocide concentrations would be set higher than those in this study because data indicated that higher concentrations and not longer exposure times would be the most practical approach for 100% *D. geminata* mortality in a lotic environment.

This research only conducted trials for the upper limit of recommended application concentrations. Screening concentrations greater than this range may have improved product efficacy. In retrospect we should have established a relatively high threshold for acceptable product efficacy (e.g., 99% cell mortality) under experimental conditions because the difficulty of exposing a micro-organism to a biocide in a natural waterway will generally result in lower efficacy compared to results in a laboratory setting.

To evaluate the various chemicals, we found a decision matrix that ranked various product characteristics was a useful tool, particularly for communicating our research to stakeholders and funding agencies. A decision matrix has the advantage of transparent product selection. In our study, the product that ranked highest in the rapid screening assessment (i.e., Gemex™) performed best through Stages 2 and 3 and was subsequently used in a full-river trial (Stage 4; Clearwater et al. 2010a). Therefore, we recommend both rapid screening assessments and decision matrices as useful tools to evaluate chemical products being tested for use on invasive aquatic microorganisms.

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TABLE 4. DECISION SUPPORT MATRIX FOR THE EVALUATION OF THE TEN BIOCIDES USED IN THE RAPID SCREENING TRIALS. BIOCIDES ARE RANKED FOR EACH CHARACTERISTIC AS DESCRIBED IN THE TEXT. A HIGHER TOTAL RANK INDICATES A MORE SUITABLE BIOCIDE FOR USE AGAINST *D. GEMINATA*.

Biocide	Effectiveness	Stalk removal potential	Contact time	Application ease	Non-target impacts	Cost	Restrictions	Total
Gemex™	5	0	4	3	2	3	2	19
EDTA	3	2	1	1	3	2	2	14
Organic Interceptor™	3	0	2	2	1	3	3	14
Hydrothol®191	3	0	1	3	2	2	3	14
Sodium hypochlorite	3	0	2	3	0	3	2	13
Diquat	0	0	1	3	2	3	3	12
Zinc sulphate	3	0	1	2	2	2	1	11
Simazine	3	0	1	3	1	2	1	11
QAC	2	0	2	2	1	3	1	11
Germanium dioxide	1	0	1	1	2	1	1	7

LITERATURE CITED

Axler, R., C. Owen, J. Ameel, E. Ruzycki and J. Henneck. 1994. Effects of Hydrothol®191 (amine salt of endothall) on algae in eutrophic ponds: Phytotoxicity and persistence. Lake Reserv. Manage. 9:53-61.

Beltrami, M. E., S. Blanco, F. Ciutti, C. Cappelletti, C. Monauni, S. Pozzi, F. Rimet and L. Ector. 2008a. Distribution and ecology of *Didymosphenia geminata* (Lyngbye) M. Schmidt (Bacillariophyta) in Trentino watercourses (Northern Italy). Cryptogamie Algol. 29:141-160.

Beltrami, M. E., C. Cappelletti, F. Ciutti, L. Hoffman, and L. Ector. 2008b. The diatom *Didymosphenia geminata*: distribution and mass occurrence in the Province of Trento (Northern Italy). Verh. Internat. Verein. Limnol. 30:593-597.

Biggs, B. J. F. and C. Kilroy. 2000. Stream Periphyton Monitoring Manual. National Institute of Water & Atmospheric Research Ltd. 222 pp.

Campbell, M. L. 2008. Organism impact assessment: risk analysis for post-incursion management. ICES J. Mar. Sci. 65:795-804.

Chen, X. and P. S. Stewart. 2000. Biofilm removal caused by chemical treatments. Water Res. 34:4229-4233.

Clearwater, S. J., P. G. Jellyman, B. J. F. Biggs, C. W. Hickey, N. Blair and J. S. Clayton. 2007. Didymosphenla geminata experimental control trials: Stage Two Phase Two (testing the effectiveness of Gemex™, a chelated copper formulation. National Institute of Water & Atmospheric Research Ltd. MAM2006-174. 89 pp.

Clearwater, S. J., P. G. Jellyman, B. J. F. Biggs, C. W. Hickey, N. Blair and J. S. Clayton. 2010. Pulse-dose application of chelated copper to a river for *Didymosphenia geminata* control: effects on macroinvertebrates and fish. Environ. Toxicol. Chem. *In press.*

Hickey, C. W. 2000. Ecotoxicology: laboratory and field approaches, pp. 313-343. *In*: K. J. Collier and M. J. Winterbourn (eds.). New Zealand stream invertebrates: ecology and implications for management. New Zealand Limnological Society, Christchurch.

Hofstra, D. E., J. S. Clayton and K. D. Getsinger. 2001. Evaluation of selected herbicides for the control of exotic submerged weeds in New Zealand: II. The effects of turbidity on diquat and endothall efficacy. J. Aquat. Plant Manage. 39:25-27.

Jellyman, P. G., S. J. Clearwater, B. J. F. Biggs, N. Blair, D. C. Bremner, J. S. Clayton, A. Davey, M. R. Gretz, C. Hickey and C. Kilroy 2006. Didymosphenia geminata experimental control trials: Stage One (screening of biocides and stalk disruption agents) and Stage Two Phase One (biocide testing). National Institute of Water & Atmospheric Research Ltd. CHC2006-128. 115 pp.

Jellyman, P. G., S. J. Clearwater, J. S. Clayton, C. Kilroy, N. Blair, C. W. Hickey and B. J. F. Biggs. 2010. Controlling the invasive alga Didymosphenia geminata: an ecotoxicity assessment of four potential biocides. Arch. Environ. Toxicol. Chem. *In press.*

Jonsson, G. S., I. R. Jonsson, M. Bjornsson and S. M. Einarsson. 2000. Using regionalisation in mapping the distribution of the diatom species Didymosphenia geminata (Lyngbye) M. Smith in Icelandic rivers. Verh. Internat. Verein. Limnol. 27:340-343.

Kawecka, B. and J. Sanecki. 2003. Didymosphenia geminata in running waters of southern Poland—symptoms of change in water quality? Hydrobiologia 495:193-201.

Kilroy, C. 2004. A new alien diatom, Didymosphenia geminata (Lyngbye) Schmidt: its biology, distribution, effects and potential risks for New Zealand fresh waters. National Institute of Water & Atmospheric Research Ltd. CHC2004-128. 34 pp.

Kilroy, C. 2005. Tests to determine the effectiveness of methods for decontaminating material contaminated with Didymosphenia geminata. National Institute of Water & Atmospheric Research Ltd. CHC2005-004. 30 pp.

Kilroy, C., B. J. F. Biggs, N. Blair, P. Lambert, B. Jarvie, K. L. Dey, K. Robinson and D. Smale. 2005. Ecological studies on Didymosphenia geminata. National Institute of Water & Atmospheric Research Ltd. CHC2005-123. 36 pp.

Kilroy, C., S. T. Larned and B. J. F. Biggs. 2009. The non-indigenous diatom Didymosphenia geminata alters benthic communities in New Zealand rivers. Freshw. Biol. 54:1990-2002.

Kilroy, C., T. H. Snelder, O. Floerl, C. C. Vieglais and K. L. Dey. 2008. A rapid technique for assessing the suitability of areas for invasive species applied to New Zealand's rivers. Divers. Distrib. 14:262-272.

Lindstrom, E. and B. Rorslett. 1991. The effects of heavy metal pollution on periphyton in a Norwegian soft-water river. Verh. Internat. Verein. Limnol. 24:2215-2219.

Markham, J. W. and E. Hagmeier. 1982. Observations on the effects of germanium dioxide on the growth of macro algae and diatoms. Phycologia 21:125-130.

Shearer, K. A., J. Hay and J. W. Hayes. 2007. Invertebrate drift and trout growth potential in didymo (Didymosphenia geminata) affected reaches of the Mararoa and Oreti Rivers: April and August 2006. Cawthron Institute. 73 pp.

Sherbot, D. M. J. and M. L. Bothwell. 1993. Didymosphenia geminata (Gomphonemaceae). A review of the ecology of *D. geminata* and the physicochemical characteristics of endemic catchments on Vancouver Island. National Hydrology Research Institute. 66 pp.

USEPA (US Environmental Protection Agency). 2006. Reregistration eligibility decision for simazine. United States Environmental Protection Agency. Prevention, Pesticides and Toxic Substances (7508P). EPA 738-R-06-008, April 2006. 267 pp.

Walker, J. R. L. and S. Evans. 1978. Effect of quaternary ammonium compounds on some aquatic plants. Mar. Pollut. Bull. 9:136-137.

Whitton, B. A., N. T. W. Ellwood and B. Kawecka. 2009. Biology of the freshwater diatom Didymosphenia: a review. Hydrobiologia 630:1-37.