Studies on the survivability of the invasive diatom *Didymosphenia geminata* under a range of environmental and chemical conditions

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Cathy Kilroy
Amy Lagerstedt
Andrew Davey
Karen Robinson

Prepared for

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National Institute of Water & Atmospheric Research Ltd
10 Kyle Street, Riccarton, Christchurch
P O Box 8602, Christchurch, New Zealand
Phone +64-3-348 8987, Fax +64-3-348 5548
www.niwa.co.nz

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Reviewed by: *Approved for release by:*

Sue Clearwater Brian Sorrell

Executive Summary

- Since it was first identified in New Zealand in October 2004, the invasive freshwater diatom *Didymosphenia geminata* (a single-celled alga) has been recorded in at least seven catchments in the South Island. To date, the species has not been recorded in the North Island.

- In an effort to contain the spread of *D. geminata*, within two months of the discovery of the alga in New Zealand, preliminary conservative decontamination methods based on the microbial biosafety literature were identified and promoted by Biosecurity New Zealand for decontaminating risk goods that may have come into contact with the alga. Two months later, in February 2005, the decontamination methods were amended and re-issued based on initial results from experimental studies. In November 2005, an extensive "Check Clean Dry" behaviour change campaign was launched to further increase public awareness of the decontamination methods.

- The study reported here is part of Biosecurity New Zealand's ongoing effort to provide validated information to enable freshwater users to reduce the spread of *D. geminata*. After previously determining how to quickly kill the alga with decontamination treatments, the next priority was to determine how long *D. geminata* cells might survive if removed from a river and left untreated.

- As part of an evaluation of the risk associated with transport of *D. geminata* by a range of potential vectors, this laboratory-based study assessed the survivability of *D. geminata* under a range of temperature, light and moisture conditions. Once the optimum temperature–light–moisture regime was estimated, trials to determine the survivability of the alga under different water quality conditions (salinity, pH, municipal water, dilutions of detergent/cleaning agents) were conducted under these optimum survival conditions to develop worst-case scenarios for risk of survival outside waterways. The information has been used to augment the current decontamination recommendations because the "Check, Clean, Dry" campaign has necessitated cleaning on a much larger scale than when the original decontamination measures were initially validated, and more options are required.

- The work was further extended to include an assessment of one specific item that has been identified as a prime candidate for transfer of *D. geminata* between rivers and possibly into New Zealand: felt-soled wading boots.

- In all trials, cell viability in *D. geminata* was assessed using the neutral red staining technique. Live cells take up this stain in cytoplasmic vacuoles (appearing as deep red spots under the microscope). Dead cells do not take up the stain. For every sample tested, we counted at least

100 cells, distinguished stained cells (assumed viable) from unstained or empty cells (assumed dead), and then calculated the proportion of stained cells. A review and evaluation of the neutral red staining technique is included as an Appendix. The risk of false negatives (counting cells as non-viable when in fact they are viable) is present, but can be avoided. We conclude that the method is appropriate and cost-effective in the present context.

- The primary temperature–light–moisture experiment was undertaken in a controlled temperature unit with temperature set at 28, 20, 12 and 5 °C. Different covers over *D. geminata* samples provided light treatments ranging from full fluorescent lighting to complete darkness. Wet treatments were kept immersed in river water (topped up as necessary). In damp treatments, *D. geminata* pieces were drained, then allowed to dry out as the experiment progressed. Survival in extreme temperatures (40 °C water and <0 °C) was assessed in separate experiments.

- Survival of *D. geminata* was strongly dependent on light, temperature and moisture. The rate of decline in cell viability was faster at higher temperatures. In all cases, cells held in the dark died faster than those receiving some light, and survival of cells in damp colonies declined faster than in wet colonies.

- At 28 °C, no viable cells remained in any treatment after three days. Survival at lower temperatures was much longer than anticipated, and for practical reasons all three trials had to be terminated before 100% mortality was achieved. Models fitted to the data for each treatment combination enabled predictions to be made of the time required to decline to 5% survival. These times range from just over 1 day (damp colonies at 28 °C in the dark) to over 8 months (wet colonies at 5 °C in low light). Lower rates of survival in damp colonies were due to desiccation, which occurred faster at higher temperatures.

- The extreme temperatures tested were rapidly lethal to *D. geminata*. Mat fragments soaked in 40 °C water required between five and 20 minutes exposure for 100% cell mortality. To translate these experimental results into operational recommendations, we allowed for temperature differentials which can occur at the interface of a cold item soaked in hot water. We recommend that the temperature for hot tap water used to decontaminate gear be no less than 45 °C, with soaking times of at least 20 minutes for non-absorbent items or of at least 20 minutes plus whatever additional time is required for thorough saturation of absorbent items. Domestic water heating cylinders often deliver water to outlet taps at between 45 and 55 °C, but this may vary, so those wishing to use hot water treatment to decontaminate gear should check the temperature at the beginning and end of the treatment to ensure that it remains at no less than 45 °C. Previous recommendations for using very hot water for rapid treatment (at least 60 °C for at least one minute) still apply, where practical, for temperature resilient items and where temperature compliance can be assured.

- Freezing caused 100% mortality and there was no difference in response between freezing at -2 °C and -15 °C, although the time taken for cells to freeze solid occurs faster in lower temperatures.

- For practical reasons, the temperature trials in the primary experiment had to be run consecutively and the unexpected duration of the trials meant that optimum conditions for all subsequent trials had to be estimated. We determined that a temperature of 9 °C in high light would be near optimal. Survival rates for the first 30 days of the 5 and 12 °C trial (wet treatments, all light levels except dark) were similar, hence this estimate is considered reasonable. All remaining trials on the effects of water quality conditions were therefore undertaken at 9 °C in high light.

- In tests on a range of seawater dilutions, full-strength seawater (3.1% w/v salt) was lethal to all *D. geminata* cells in the samples tested after 4 hours. Half-strength seawater killed most cells within one day but residual, possibly viable, cells persisted for at least 30 days. Greater dilutions were even less effective, with 10% seawater yielding better survivability than river water. For effective decontamination, immersion in full-strength sea water for at least 4 hours is recommended. Immersion in estuarine water of 50% seawater or less cannot be recommended as a reliable decontamination method. Immersion times required for 100% mortality in seawater between half and full strength are likely to be too long to be practical.

- A suite of pH levels was made up in river water by adding lime (for high pH) and hydrochloric acid (for low pH). As expected, *D. geminata* did not withstand exposure to very low or very high pH (pH 1 and pH 11), but survived well in the range between pH 4 to 9.5, which covers the entire pH range of natural freshwaters in New Zealand. Extrapolating the result that survival occurs at pH 4 suggests that cells may withstand pH levels in bird guts from time to time, however the accompanying conditions in bird guts (high temperatures and darkness) are extremely unlikely to support live *D. geminata* cells passing through. Transport of *D. geminata* by birds is considered more likely to be on feathers or feet than in the gut, although the relative risk of such transport between catchments is estimated to be small compared to transport by humans for the following reasons: the natural tendency of birds to groom and clean their feathers, the natural tendency of feathers to shed water and debris and the estimated rapid desiccation of cells during flight under dry weather conditions.

- Under optimal survival conditions, 14 products were tested for their effectiveness in killing *D. geminata* cells over a range of dilutions and contact times. Products included "generic" constituents of household cleaners, products recommended for general decontamination purposes at biosecurity checkpoints at New Zealand's border, products currently recommended for decontamination of *D. geminata*, cleaners marketed as environmentally friendly, and commonly available detergents (dishwashing liquids with no special environmental claims). The aim was to identify concentrations required for 100% mortality of

D. geminata at a range of contact times from 1 minute to 1000 minutes (16 h 40 mins) and thus provide options for longer soaking times at reduced concentrations of products, especially for high volume/commercial users who wish to soak gear overnight at less expense to themselves, their business and the environment.

- Three constituents of common cleaning products [borax, sodium percarbonate and sodium dodecyl sulphate (SDS, an anionic surfactant)] were less effective than commercial detergents containing mixed ingredients that increase product effectiveness. For example, 5% nappy cleaner (main active ingredient sodium percarbonate) killed all *D. geminata* cells within one minute, but the equivalent concentration of the pure active ingredient did not. Borax was ineffective at the concentrations tested and SDS required high concentrations for complete mortality.

- Concentrations of sodium metabisulphite recommended for use at the border were not fully effective for *D. geminata*. A disinfectant currently in use at the border (Virkon®) had been found in a previous test to be effective in one minute at the recommended concentration.

- The current recommendation for household bleach as a *D. geminata* decontaminant was re-confirmed (i.e., a 2% solution for 1 minute). Longer contact times with lower concentrations were not fully effective, indicating that the low concentrations of chlorine in swimming pool water or treated tap water should not be recommended for treatment of contaminated items.

- A swimming pool cleaner (active ingredients, quaternary ammonium compounds – quats, which are cationic surfactants) required high concentrations for complete mortality of *D. geminata*, even at long exposure times. Five hundred times the stated recommended dose for killing algae was needed to kill all *D. geminata* cells in one minute. Quat compounds are known to be toxic against micro-organisms, but specific information about concentrations in quat-based products is needed before their effectiveness can be assessed.

- Ethanol was tested because this is used as a preservative for *D. geminata* genomic material. The recommended concentration of 70% was completely effective within 10 minutes. Lower concentrations were also effective over longer times. We conclude that use of ethanol for *D. geminata* preservation poses minimal risk of spreading live cells.

- Cleaning products marketed with environmental claims were less effective for killing *D. geminata* than commonly available detergents (dishwashing liquids). For example, 2% solutions of the former were needed for 100% mortality within 1000 minutes, whereas 0.1 or 0.5% solutions of the latter were effective. Very high concentrations (>50%) of the environmentally friendly products were needed for a one minute kill, and one product was not fully effective at 100%. The original finding that exposure to 5% regular dishwashing liquid for one minute was 100% lethal to *D. geminata* was confirmed. The difference between the

two groups of products may simply reflect lower concentrations of active ingredients in the "non-toxic" group, rather than different, less toxic ingredients.

- To assist Biosecurity New Zealand in providing practical recommendations for decontaminating risk goods against *D. geminata*, a summary table was provided ranking all effective products and methods based on their relative effectiveness, and the following additional factors: availability, cost, toxicity/irritation to humans, corrosiveness, possible effect on other organisms, and biodegradability. It is recognised that not all methods will be practical in all situations and users must exercise judgement. Our recommendation is to select the highest ranked methods that are practical for the situation. Regardless of rank, all products and methods recommended in the table are effective provided that the specified contact times and concentrations (if applicable) are used. This means that all of the potentially contaminated material (interior as well as exterior) must be in direct contact with the decontamination agent for the full required contact time. Such contact may not be easily achieved for absorbent materials such as felt soles, foam, etc., and therefore soaking for prolonged times will be necessary. Because *D. geminata* mortality increases with temperature, the effectiveness of all the chemical treatments is likely to increase with temperature.

- The question of longevity of decontamination baths was considered, i.e., how often should decontaminants be replenished? It is concluded that a precautionary approach is desirable, since the active ingredients are "used up" with use, and also biodegrade over time. Household bleach solutions should be changed daily, and more often with heavy use. Other products recommended for decontamination will generally be biodegradable, a process that starts as soon as they are in solution and in contact with particulate organic matter. Therefore solutions other than bleach should be renewed at least every other day, and preferably daily.

- Trials on the efficacy of wading boots as vectors for *D. geminata* showed that in the short term (four to five hours), felt soles, leather boot tops and neoprene waders *all* present a high risk of transferring cells if they are not decontaminated. However rubber gumboots present a much lower risk. In the longer term (36 hours), felt soles harbour live *D. geminata* cells much more successfully than the other materials tested.

- Trials to compare the efficacy of decontamination methods on felt soles indicated that solutions containing surfactants (nappy cleaner, dishwashing liquid) soaked into felt soles faster than those containing bleach, though neither type of solution appeared to soak to their maximum potential depth within one minute. Spraying the felt soles was much less effective than soaking and is not recommended for any porous or absorbent material.

- It was not feasible to experimentally test if the depth to which decontamination solutions penetrate felt soles would be sufficient to kill every *D. geminata* cell which could potentially be forced deep within the soles from the weight of footsteps in affected rivers. However, the

finding that decontamination solutions at ambient temperatures do not passively soak quickly into felt soles coupled with the finding that felt soles remain damp for long periods and harbour relatively large amounts of live *D. geminata* cells indicates the need for precaution. Combining the power of heat with the power of detergent is highly recommended for decontaminating felt soles. The entire felt sole needs to be completely immersed for 30 minutes in hot tap water at no less than 45 °C (uncomfortably hot to touch) containing 5% dishwashing liquid or nappy cleaner. If hot water alone is used, careful attention is needed to ensure the temperature of the water is maintained at no less than 45 °C for 40 minutes to ensure the interior of the felt reaches a sufficient temperature. Alternatively, freezing felt-soled waders until solid is also effective.

- Relying on ambient drying as a stand-alone treatment for decontaminating highly absorbent risk goods such as felt-soled waders is not recommended in situations where use between waterways is frequent (daily, weekly or even monthly). Dessication of *D. geminata* mat fragments to a water content of less than 83% has previously been shown to be 100% lethal, however cells kept damp and cool (< 20 °C) can remain viable for months. Felt soles that were dried at ambient conditions for 36 hours and were barely wet to the touch still harboured viable cells. Drying should only be relied upon as a decontamination treatment if great care is taken to actively and completely dry the felt (such as by using a heat source where temperatures around the felt are assured of reaching 30 °C). Once the felt appears dry, complete dryness must be confirmed by a tactile inspection of the felt pile to the base of the fibres. Once completely dry, items must remain dry for at least 48 hours before use in another waterway. These findings for felt soles can be extrapolated to other absorbent materials.

1. Introduction

Ever since the invasive diatom *Didymosphenia geminata* was first discovered in the lower Waiau and Mararoa Rivers, Southland, New Zealand, in October 2004, considerable effort, led and funded by Biosecurity New Zealand, has been directed towards understanding its autecology (the study of a single organism) and effects (environmental, economic and social), and investigating possible control options. *D. geminata* forms thick, smothering mats over river substrates, and can attain biomass that far exceeds current guidelines on biomass levels for the maintenance of recreational and biodiversity values in New Zealand Rivers (e.g., Kilroy et al. 2005a). A serious problem with *D. geminata* is its ability to grow prolifically in low-nutrient rivers in which noticeable algal growth would rarely have occurred. Effects have been estimated to be significant (Campbell 2005, Branson 2005). In parallel with the research, surveillance has been undertaken to define the distribution of the organism and attempts have been made to prevent or slow its spread to new catchments.

In the early stages of the incursion, a priority was to contain the organism within its original catchment. This required development of methods for decontaminating materials and equipment that may have come into contact with *D. geminata* colonies or cells. This work was completed within four months of the first report of *D. geminata*, with preliminary guidelines available within two months (Kilroy 2005). The results indicated that *D. geminata* was relatively easy to kill. Further, the fact that we had difficulty maintaining live colonies in the laboratory for long enough to complete the experiments suggested that *D. geminata*'s survivability out of its natural environment was probably low. Although some very preliminary tests showed that viable cells could persist for at least nine days in the laboratory, given a suitable temperature (see Appendix 1 of Kilroy 2005), overseas information suggested that no culture of *D. geminata* had ever progressed past "a few feeble divisions" (D. Czarnecki, Loras Culture Collection, pers. comm.[1]).

Concurrently with the research programme, discussions were underway on the related problem of the potential survival time of *D. geminata* if cells/colonies are removed from a river and remain untreated. In other words, what is the risk associated with *not* cleaning everything that might have picked up live *D. geminata*? How long might cells survive in/on risk goods such that they could re-establish and grow if introduced into another river? These questions assume that the major vectors for spreading *D. geminata* are humans. While this is likely to be an accurate assumption, other potential

[1] David Czarnecki, who was probably one of the world's leading authorities on the culture of diatoms, sadly died in May 2006.

vectors clearly exist. The most obvious of these are birds and animals (on fur/hide, feathers, feet and through the gut), including domestic livestock.

In late September 2005, *D. geminata* was identified in several other rivers, including the Buller River, approximately 600 km from the closest affected reach in the Mararoa catchment. The locations of the new findings – in widely separated rivers popular for fishing and other recreational activities – strongly suggested human-mediated dispersal and may indicate that *D. geminata* reached the other rivers much earlier, and possibly as a completely separate incursion in to New Zealand. Human-mediated dispersal was supported by the results of a nationwide survey in October 2005, which failed to identify incursions into any new catchments (Duncan et al. 2005), which would have been expected if *D. geminata* was undergoing random spread by means of wind, birds, etc. Importantly, no outbreaks of *D. geminata* were found in the North Island, and none have been detected in subsequent surveys to date.

Following the discovery of *D. geminata* in new rivers and the subsequent nationwide survey (Duncan et al. 2005), in December 2005 Biosecurity New Zealand requested studies be conducted to determine survivability of *D. geminata* in a range of environmental conditions that would encompass a wide range of scenarios for its transport from affected rivers. The objective was to provide information for a detailed assessment of the risk of transport of *D. geminata* by various means and under various conditions, within New Zealand. The work was also expected to contribute to our understanding of how live *D. geminata* reached New Zealand initially, and to allow assessment of the risk of further incursions from overseas.

An additional objective was to provide information that could be used to refine the current decontamination recommendations. One outcome of the identification of *D. geminata* in more rivers and catchments in September/October 2005 was that Biosecurity New Zealand reviewed its controlled area policy for the organism. Instead of declaring controlled areas around each area or catchment known to be affected, a decision was taken to expand the controlled area to encompass the entire South Island. The emphasis broadened to include specific efforts to prevent its spread to the North Island, Stewart Island and other outlying islands. To this end, a countrywide public awareness/behaviour change campaign was launched, which was also aimed at continuing to slow the spread within the South Island. A crucial part of the campaign was the message that people needed to decontaminate equipment, clothing, etc. when moving between *any* rivers, regardless of whether *D. geminata* was known to be present or not. This has been implemented in Biosecurity New Zealand's "Check, Clean, Dry" campaign. An effect of this directive was that decontamination escalated from the small amount associated with a relatively remote Southland catchment, to treatment of equipment on a massive scale. It soon became clear that the

recommendations resulting from the initial decontamination studies were inadequate to cover all situations. Problems reported included: high cost of treating large items repeatedly; concern about the environmental effects of disposal of large quantities of detergents and other chemicals; concern about damage to equipment through the repeated use of bleach, salt, etc. The proposal for work on survival of *D. geminata* was therefore extended to include trials on a range of alternative decontamination products, chemicals and protocols.

In this report, we document the results of both the above aspects of survival of *D. geminata*, that is, survival potential in the absence of decontamination, and survival after treatment with a range of potential decontamination agents. The work was further extended to include an assessment of one item identified as a prime candidate for transfer of *D. geminata* between rivers: felt-soled wading boots, now almost universally used by recreational fishermen. The report is structured into four parts:

- In Part 1, we describe experiments investigating the survivability of *D. geminata* under different light, temperature and moisture regimes.

- In Part 2, we examine the survival responses of *D. geminata* under gradients of water conductivity (salinity) and water pH.

- In Part 3, we present trials on the survival of *D. geminata* following treatment by fourteen chemicals and products over a range of concentrations and exposure times.

- Part 4 covers tests to determine the efficacy of felt-soled wading boots as a vector of *D. geminata*, and to assess potential decontamination methods.

Each part is formatted as a stand-alone report, and includes a discussion on the implications of the results. Recommendations are made where appropriate. The main results and recommendations are summarised in a concluding section.

In all four studies, we determined cell viability by means of the neutral red staining technique, using the method developed for the initial decontamination study (Kilroy 2005). This method is also being used in ongoing trials to identify potential chemical control agents (Jellyman et al. 2006a, b). Because the results of both the present trials and the control trials depend on the accuracy and reliability of distinguishing live cells from dead cells, we provide a review of the technique (Appendix 1).

2. Part 1. Trials on the survivability of *D. geminata* under different light, temperature and moisture regimes

2.1. Introduction

There are numerous scenarios under which cells or colonies of the invasive alga *D. geminata* might be transferred out of an affected river. Examples of human-assisted transfer are: trapped in clothing, footwear, hair or under fingernails; caught in fishing gear or the wheels and underside of vehicles; trapped inside kayaks and jetboat engines; mixed with a catch of whitebait, which is then frozen.

If decontamination procedures are followed, then the risk of live cells being transferred to another river will be substantially reduced, if not eliminated. But what are the risks if no chemical decontamination measures are taken? For example, if wet boots are left in a garage (e.g., temperature less than 12 °C), and are still partly damp when next used a week later, could any *D. geminata* cells trapped on the underside still be viable? Transfer by means other than human-assisted may also be an issue, for example, how long would cells survive trapped in bird's feathers, or in the hoof of a deer or cow? Could cells survive in the dark, warm, acidic conditions inside a bird or animal gut?

To provide information for assessing the likelihood of transfer posed by scenarios such as these, we determined survival times for *D. geminata* under four temperature regimes, and three or four light levels. The experimental design also included treatments in which *D. geminata* was maintained immersed in river water versus drained fragments that were allowed to dry naturally over time. In short-term tests, we investigated survivability in extreme temperatures.

2.2. Methods

The long-term trials were undertaken in the NIWA Christchurch laboratories (MAF-approved controlled environment), using a Contherm Phytotron Climate Simulator equipped with 12 36W fluorescent tubes (Figure 1). Lights were set on a 16 h : 8 h light : dark regime. At each temperature, light and moisture treatments (see section 2.2.1) were run simultaneously. Shorter term tests on extreme temperatures were conducted separately.

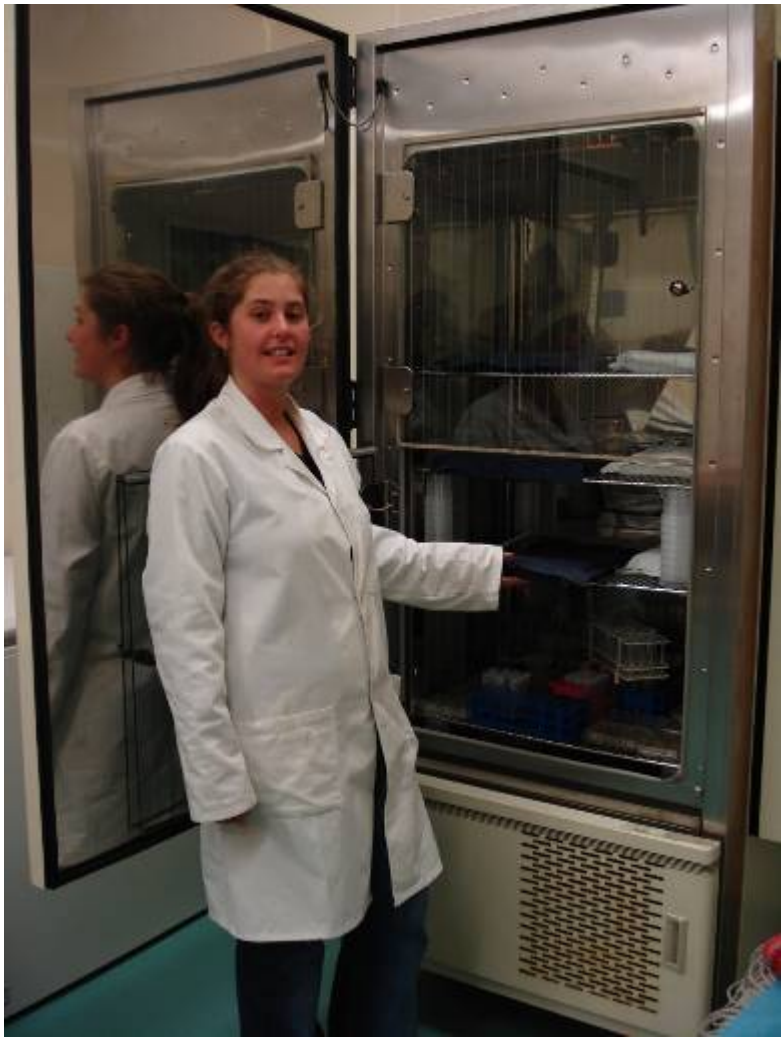

Figure 1: The Contherm climate simulator unit, with lights off.

2.2.1. Long-term experiments

D. geminata experimental set-up

Healthy *D. geminata* colonies *in situ* on stones were collected from the Waitaki River and transported to the laboratory in 2-litre containers with river water, on ice within a secondary container (a large chilly bin). The time between collection and arrival at the laboratory was typically about 4 hours. On arrival at the laboratory, colonies were removed from rocks and cut into small portions (approximately 10 x 10 x 5 mm), ensuring each portion contained an area of healthy brown cells. These portions were transferred to their respective treatments, with replication (n = 3), with replicates always sourced from different rocks.

Each treatment replicate comprised 12 colony portions placed individually into 35 mm diameter clear, covered acrylic Petri dishes. The 12 portions allowed testing on 12 separate occasions over time. Sets of 12 Petri dishes were then placed in larger trays, with lids, which ensured that the replicate pieces from single rocks were held in more or less similar conditions.

Temperature treatments

We ran trials under four different temperature regimes, with the climate simulator set at 28, 20, 12 and 5 ºC. These temperature settings maintained temperatures within approximately 5 degrees of the target temperature, which were logged at 30 min intervals using a Hobo tidbit temperature logger.

Light treatments

We initially set up three light treatments to simulate high, medium and dark conditions. Because there was little difference in response to our high and medium light treatments in the 20 ºC trial, we added a low light treatment for the 12 ºC and 5 ºC trials. Light treatments were achieved by placing different types of covers over the larger trays holding groups of Petri dishes. Light levels within the trays were measured in the climate simulator (lights on) using a LiCor light meter. Treatments were:

- High light[2]: transparent covered trays with no additional cover, measured light 65 $\mu mol^{-2}\ s^{-1}$;
- Medium light: transparent covered trays with an extra single layer of white cotton fabric, measured light 24 $\mu mol\ m^{-2}\ s^{-1}$;
- Low light: transparent covered trays with an extra single layer of navy cotton fabric, measured light 4 $\mu mol\ m^{-2}\ s^{-1}$;
- Dark: solid black covered trays with all apertures between lid and base covered with black tape, measured light 0.

[2] Compared with natural light, the levels tested appear very low: full sunlight is approx. 1500 $\mu mol\ m^{-2}\ s^{-1}$ compared with our maximum of 65 $\mu mol\ m^{-2}\ s^{-1}$. However, the levels used are fairly typical for laboratory experiments. They account for attenuation of light by the water column in rivers, and the variation of natural light throughout the day as opposed to constant delivery for 16 h in the experiments.

Moisture treatments

Each of the trays held 24 Petri dishes, 12 each in two moisture treatments:

- wet – immersed in river water, and topped up as necessary throughout the experiments;
- damp – colony fragment with water drained off, allowed to dry naturally as the experiment progressed.

Time intervals

At the start of all trials we tested a colony portion from each rock used to obtain an estimate of cell viability (as % viable cells) at time = 0. The 12 colony pieces in each treatment replicate were removed (in random order) for viability testing at pre-determined intervals. Initially intervals were set at three hours following placement in the treatment, 6 hours, 12 hours, 24 hours, 48 hours, 4 days, 6 days, then every 2 days thereafter. In the 20 ºC trial, it was clear that viability was declining minimally over the first two days. Therefore in subsequent trials, the first readings were undertaken one or two days following placement and the shorter time intervals were omitted. Higher survival rates than expected meant that we also extended the sampling interval to 7-10 days or more towards the ends of the trials.

Live-dead cell determinations

All live–dead cell determinations were carried out using a neutral red staining technique (Kilroy 2005). This technique is discussed in detail in Appendix 1. The procedure followed in the present trials was as follows. A stock solution of neutral red dye was made up by dissolving 200 mg of the dye powder in 200 ml of distilled water (a 1% w/v solution). This was further diluted to 5% for use with *D. geminata*. Colony fragments were transferred from their Petri dishes to vials containing about 20 ml of the diluted neutral red stain solution. We also transferred as much as possible of any visible cell debris deposited on the bottom of the Petri dishes. The colony was roughly homogenized in the stain solution (using scissors or vigorous shaking) to ensure that the stain was thoroughly mixed and in contact with all cells in the sample. The vials were then left for 15 – 20 minutes to allow the stain to penetrate. After completion of the staining time, the vials were shaken and subsamples of the suspension of stalks and cells pipetted onto labelled glass slides. The fragments were teased apart using dissecting needles to eliminate clumps before dropping a glass coverslip into place.

Prepared slides were scanned under a Leica DMLB microscope at magnifications of x100 to x400. If stained cells were observed, counts were made of stained and unstained cells up to a total of at least 100 cells and used to calculate the percentage of stained (live) cells in the subsample. Counts were made on transects covering different parts of the subsample, to ensure that the cell count was representative of the entire slide. Previous trials had shown that using this method, a count of 100 cells was sufficient to obtain a consistent estimate of the percentage of stained cells (see Appendix 2). Towards the end of the 20 °C trial, we started counting empty frustules as a third category. Initially, few empty cells were observed in the samples and these were not enumerated on the grounds that empty frustules had likely been dead for some time (longer than the duration of the test) and therefore were not contributing to the mortality occurring in the samples as a result of the light/temperature treatment. However, increasing numbers of empty cells were noted as the trial proceeded. It was clear that these empty cells should be included as unstained cells. Subsequently all counts included stained, unstained and empty cells. We consider that their omission in the earlier counts would have made minimal difference to the result as very few empty cells were observed in the early stages of the trials.

Statistical analysis

The proportion of viable cells in each sample was analysed using a Generalised Linear Model (GLM) with a binomial error distribution and a logit link function. Change in cell viability over time (hours) was modelled as a function of temperature (5, 12, 20 and 28 °C), light (0, 24, 65 µmol m^{-2} s^{-1}) and moisture (wet, damp). All terms in the model were evaluated for significance using F-ratio tests to correct for over-dispersion and $\alpha = 0.05$ was used for significance testing.

For an analysis of this type, ideally all treatments should be undertaken simultaneously, all material should be collected at the same time and place, all sampling should be undertaken at the same time intervals, and all light and temperature treatments should be equivalent. Practical constraints (including availability of space in temperature-controlled units, and availability of microscopes and analysts) meant that we had to run the trials consecutively rather than simultaneously (i.e., complete one temperature before moving on to the next one)[3]. As a result, material had to be sourced separately for each temperature. To minimise differences, the same collection site was used for all trials, and, for a valid statistical comparison, we must assume that there was no difference in population vigour or

[3] An exception was that another unit was used for the 5 °C trial because of the unexpected need to re-run the trial at 12 °C (see section 2.3.1). Light conditions in the alternative unit were tested and found very similar to those in the Contherm Climate Simulator.

response to temperature changes over the period of collection (March to August 2006). No information exists (to our knowledge) to support or refute this assumption. Because the same controlled temperature unit was used for all the replicates, light conditions were the same in all experiments (but see footnote 3). Note that the low light treatment was not included in the formal analysis because it was run only for the two lowest temperatures. However, a commentary on these results is included.

2.2.2. Experiments at extreme temperatures

To complete the range of temperatures tested we also included sub-zero and high temperature treatments. In the freezing trial, portions of *D. geminata* colonies were transferred to Petri dishes, as described above, with replicates originating from different rocks. No water was added. In the high temperature trial, colonies were held in polyethylene centrifuge tubes. The subsequent experimental procedure was the same as for the longer term trials. Treatments were:

1. freezing to -15 °C (a standard large chest freezer), 2 h exposure of damp colony fragments before thawing out and testing for viability;

2. freezing to -2 °C (frozen food compartment within a small refrigerator), 4 h exposure of damp colony fragments before thawing out and testing for viability;

3. maintenance at 40 °C (immersion into pre-heated river water in a water bath maintained at 40 °C), 5 minute and 20 minute exposures.

All trials were carried out in the dark, with controls maintained at 12 °C. In an additional test to verify the initial results of the freezing treatments, we repeated the treatments, using new material. The frozen material was then immersed in river water and transferred to the climate simulator at 12 °C, with controls. After four days, viability tests were carried out on three replicate pieces from each freezing treatment, and on three replicate controls.

2.3. Results

2.3.1. Temperature regimes

Logged temperatures for the 20 ºC trial are shown in Figure 2. The diurnal fluctuation is to be expected because of the heat produced by the banks of fluorescent lights in the light phase of the cycle. For example, in the 20 ºC trial, temperatures were always a few degrees above the target temperature when the lights were on, and remained at or

just below the target temperature with the lights off. This produced a mean temperature over the entire trial of 21.8 ºC.

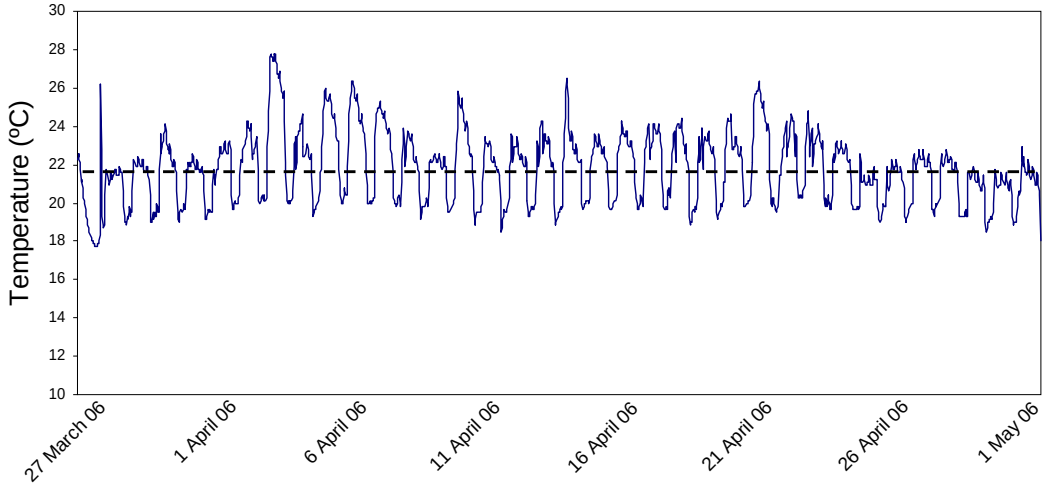

Figure 2: Temperatures logged at 30 min intervals during the 20 ºC trial with the climate simulator set at 20 ºC. Actual mean temperature over the incubation period is shown by the dotted line (21.8 ºC, standard deviation 1.85 ºC).

At the end of the 12 ºC trial, a download of the temperature data revealed large fluctuations at the beginning of the trial, which were not obvious during the course of the trial. Because these fluctuations were suspected to have seriously compromised the results, the trial was re-run simultaneously with the 5 ºC trial. The latter was run in an alternative, secure, controlled temperature room, using exactly the same light treatments as used in the Contherm unit. The light source was similar (fluorescent tubes) and light measurements confirmed that the light levels reaching the *D. geminata* colony pieces differed from those in the Contherm by no more than 10%.

In the following discussion, we refer to the trials using the intended temperature values (28, 20, 12 and 5), even though the recorded means varied from these values by up to 3 ºC. Results from the first 12 ºC trial are not considered further, and from this point on the 12 ºC trial refers to the re-run.

2.3.2. Survivability under different light, temperature and moisture regimes

As expected, the proportion of viable *D. geminata* cells in all treatments declined over time ($P < 0.00001$), but the rate of decline was contingent upon the environmental conditions. Cell viability declined faster in the dark than under medium or high light conditions ($P < 0.003$). The rate of decline in cell viability was faster at higher

temperatures (P < 0.00001) and the effect of temperature on survival was more pronounced under damp conditions than under wet conditions (P < 0.00001). In all cases survival of cells in damp colonies declined faster than in wet colonies. Complete results of the statistical analysis are shown in Appendix 3.

Models fitted to the data for each treatment combination (Figure 3) enabled predictions to be made of the time required to achieve a given survival level, and these are shown in Table 1 for 5% survival. (Since 100% mortality is a probabilistic concept in this context, time to reach this point will always be infinity.) These times varied from just over one day (28 °C, dark, wet conditions) to more than 250 days (5 °C, low light, wet conditions).

Table 1: Predicted time (days) to 5% cell viability under different environmental conditions.

		Temperature (°C)			
Moisture	**Light level**	**5**	**12**	**20**	**28**
Wet	**High**	231 *	93*	59 *	1.4
	Medium	182 *	74 *	51 *	1.5
	Low	252 *	79 *	nd	nd
	Dark	80 *	45	25	1.2
Damp	**High**	77 *	42	13	1.6
	Medium	86 *	60 *	14	1.7
	Low	112 *	51	nd	nd
	Dark	57	34	10	1.3

* mean cell viability <5% not achieved in laboratory trials.
nd = no data

Low light treatments were tested only at 5 and 12 °C, therefore these could not be included in the main analysis, but models were fitted to these data for comparison (Figure 4), and times to 5% survival predicted (Table 1). A separate analysis on data for the two lower temperatures showed no significant difference in the rate of *D. geminata* decline between high, medium and low light levels. Complete results are in Appendix 3.

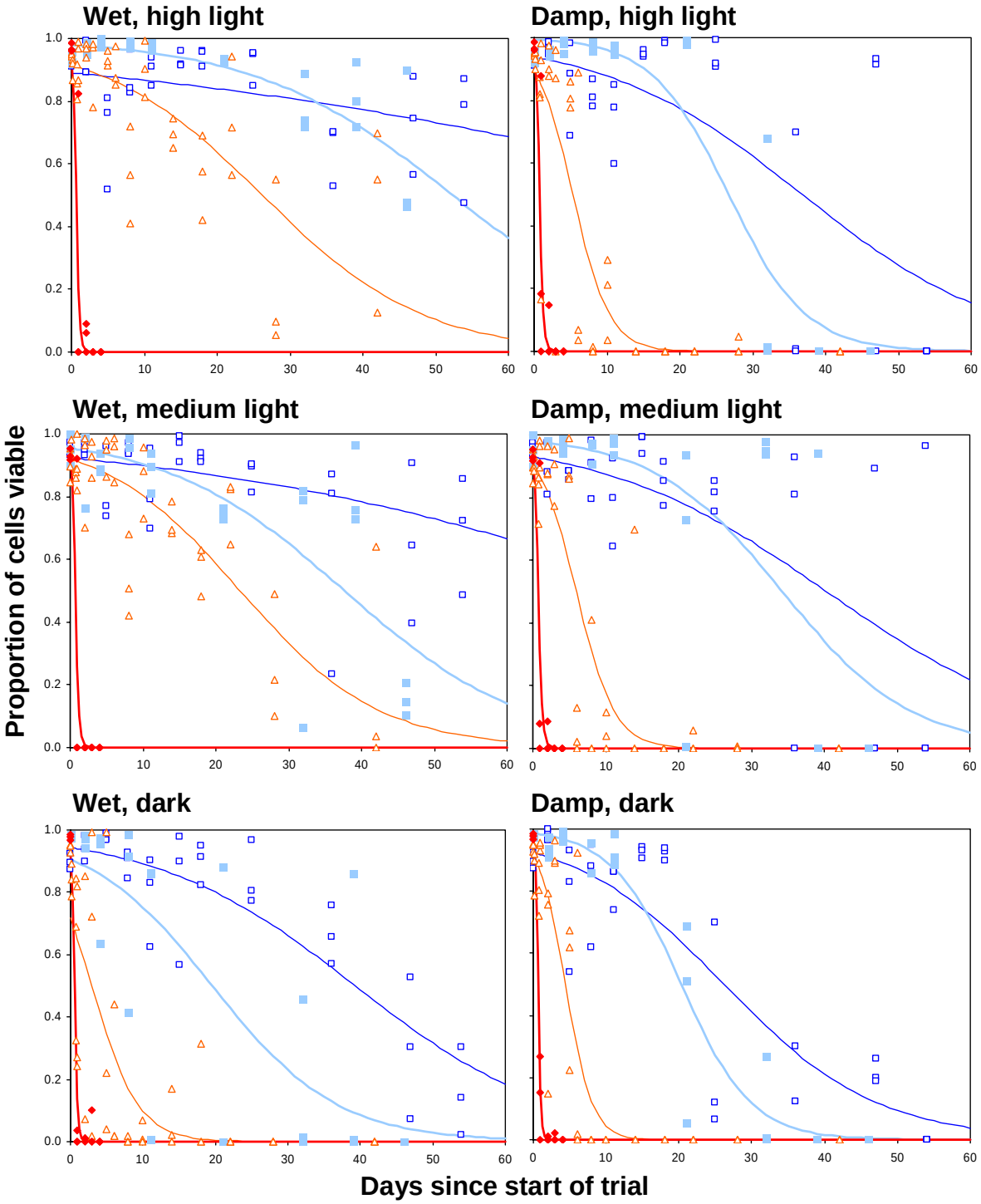

Figure 3: Survivability of *D. geminata* [as proportions of viable (stained) cells] over time in four different temperature regimes, at three light levels. Data points for three replicates are shown. Fitted lines are models determined from the data.
◆ 28; ▽ 20; ◆ 12; □ 5 °C.

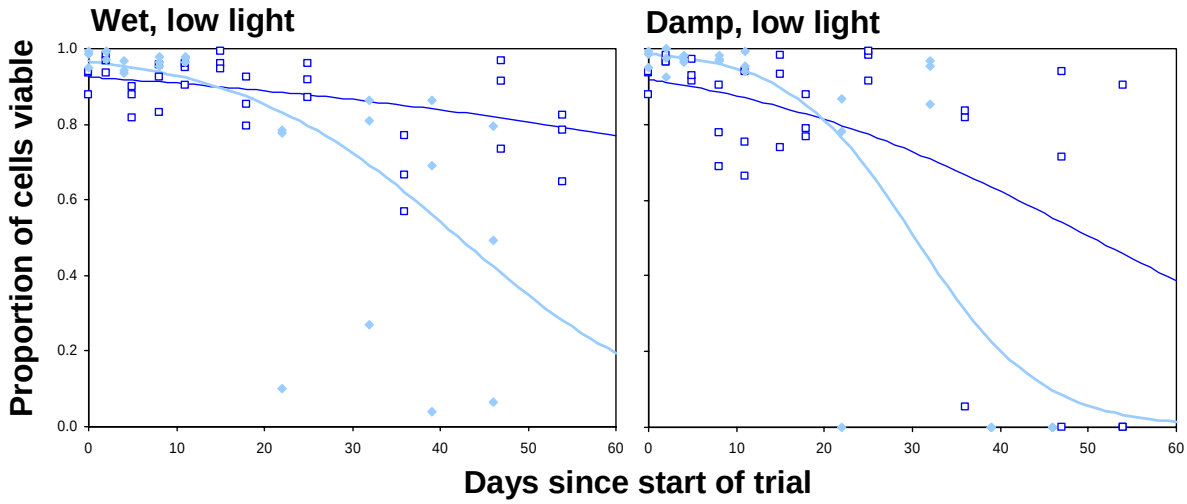

Figure 4: Survivability of *D. geminata* [as proportions of viable (stained) cells] over time in for the low light level treatment included at 12 and 5 °C only. Compare with Figure 3. ◆ 12; □ 5 °C.

Results for the hot water and freezing trials are shown in Table 2. Our tests showed that *D. geminata* does not survive freezing, at either just below freezing point (-2 ºC) or substantially below (-15 ºC). The cells did not revive when returned to room temperature. After 20 minutes at 40 ºC, no stained cells were observed, but after 5 minutes 47% of cells were still viable.

Table 2: Summary results of freezing and high temperature trials, with controls

Trial	Treatment temperature (ºC)	Exposure time (from room temperature)	% stained cells (mean of three replicates with standard deviation)
Freezing	**–15**	**2 hours**	**0**
	control	0	94 ± 2.1
	–2	**4 hours**	**0**
	control	0	86 ± 2.0
Freezing, then returned to 12 ºC for 5 days	**–15**	**2 hours**	**0**
	–2	**4 hours**	**0**
	control	0	87 ± 9.7
High temperature	40	5 minutes	47 ± 4.5
	40	**20 minutes**	**0**
	control	0	86 ± 2.0

2.4. Discussion

2.4.1. Survivability under different light, temperature and moisture regimes

Except in the 28 ºC trial, *D. geminata* appeared to be capable of survival for much longer than anticipated. Our intention at the start of the experiments was to run all trials until no live cells could be found. This was possible only in the trial at 28 ºC, in some of the 20 °C trials (all damp treatments and the dark, wet treatment), and in the damp light treatment at 12 °C. Table 1 indicates that potential survival could be as long as 250 days (more than 8 months), given low temperatures and a little light. This result was completely unexpected as previous work suggested that survival of *D. geminata* out of the river was limited (Kilroy 2005).

Many cells that took up the neutral red stain after up to 54 days appeared to be normal, i.e., staining with the same appearance as cells analysed within 12 hours of removal from the river (Figure 5).

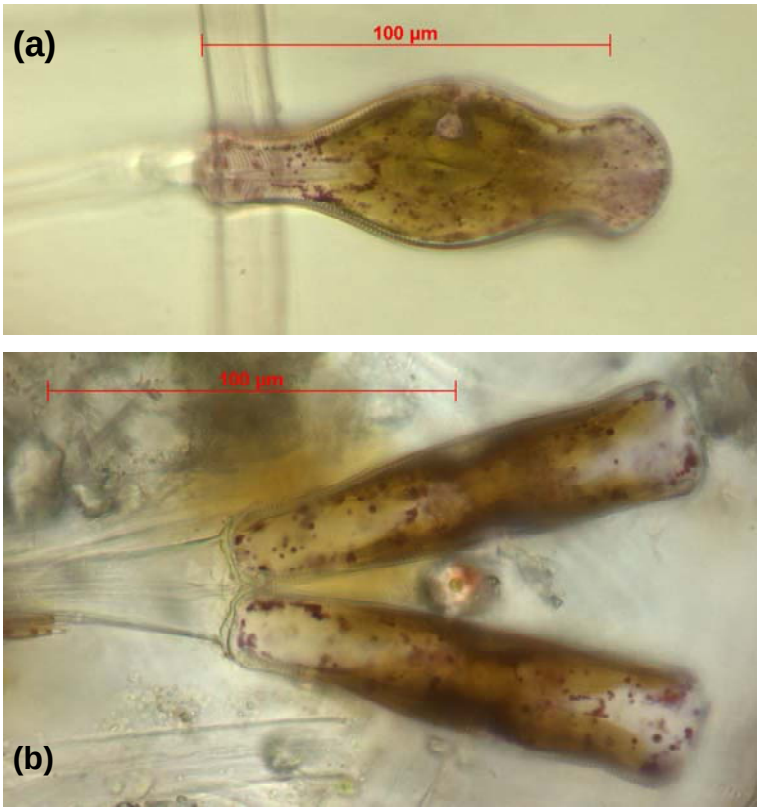

Figure 5: (a) A stained (live) *D. geminata* cell photographed within 12 hours of removal from the river. (b) Cells from the light, wet treatment, 12 ºC (initial) trial, photographed on 1 July, 52 days after the start of the experiment. Note that (a) shows the top view of the cell – the characteristic coke-bottle shape of *D. geminata*; in (b) the cells are viewed from the side.

At 28 ºC survivability was limited under all light treatments and there was little difference between wet and damp treatments. After 3 days the damp colonies were desiccated and dead, but all cells in the wet treatment were also dead, suggesting that high temperature was the main cause of mortality. In contrast, in the 20 ºC trial, wet colonies under high and medium light still contained up to 50% viable cells after 37 days.

Lack of significant differences in survival between high, medium and low light treatments at 12 and 5 °C indicates that at these temperatures even minimal light levels permit survival. This is consistent with freshwater diatoms in general, which are well known for their ability to function under and adapt to very low light conditions (Hill 1996). Although light is essential for photosynthesis, many diatoms inhabit environments with extremely low, or even complete lack of light. For example, diatoms survive the complete darkness of polar winters (Peters and Thomas 1996), or in deep sediment layers (Wasmund 1987). In the absence of photosynthesis, diatoms may survive by entering a dormant state (e.g., Gibson et al. 2003) or by obtaining nutrients (carbon) from organic sources (heterotrophy) (Tuchman et al. 2006). Experiments on marine diatom species have shown that Antarctic species maintained in the dark at low temperatures (< 1 ºC) resumed photosynthesis after up to 10 months in complete darkness (Peters and Thomas 1996). In contrast, related temperate species did not survive past ~50 days in darkness at temperatures of 8 to 15 ºC (Peters 1996). Peters and Thomas (1996) described a morphological response to darkness in cells that subsequently resumed photosynthesis as “clumping of the protoplasm”. Such a transition to a “resting cell” stage has been regularly recorded in diatom cells, with the cell contents described as “dense, dark cytoplasmic matter and rounded plastids” (McQuoid and Hobson 1996). It should be noted that the resting stage is not the same as a cyst or spore, as there is no resistant cell covering. The resting stage is a means to prolong viability when, for example, resources (e.g., light) are scarce, but resting cells are no more resistant to heat or desiccation than normal vegetative cells (McQuoid and Hobson 1996).

At 20, 12 and 5 °C, *D. geminata* survival in the dark was significantly lower than in the high, medium and low light treatments. The chloroplasts in most cells that failed to stain (and therefore were interpreted as dead) appeared to be degenerated (Figure 6a). However, some cells with condensed, rounded chloroplasts were observed, especially after prolonged exposure to the dark, wet treatments at 5 and 12 °C (Figure 6b). When subsamples from these colonies were reintroduced to light, we found that percentage cell viability increased, suggesting that these cells with condensed chloroplasts were resting cells.

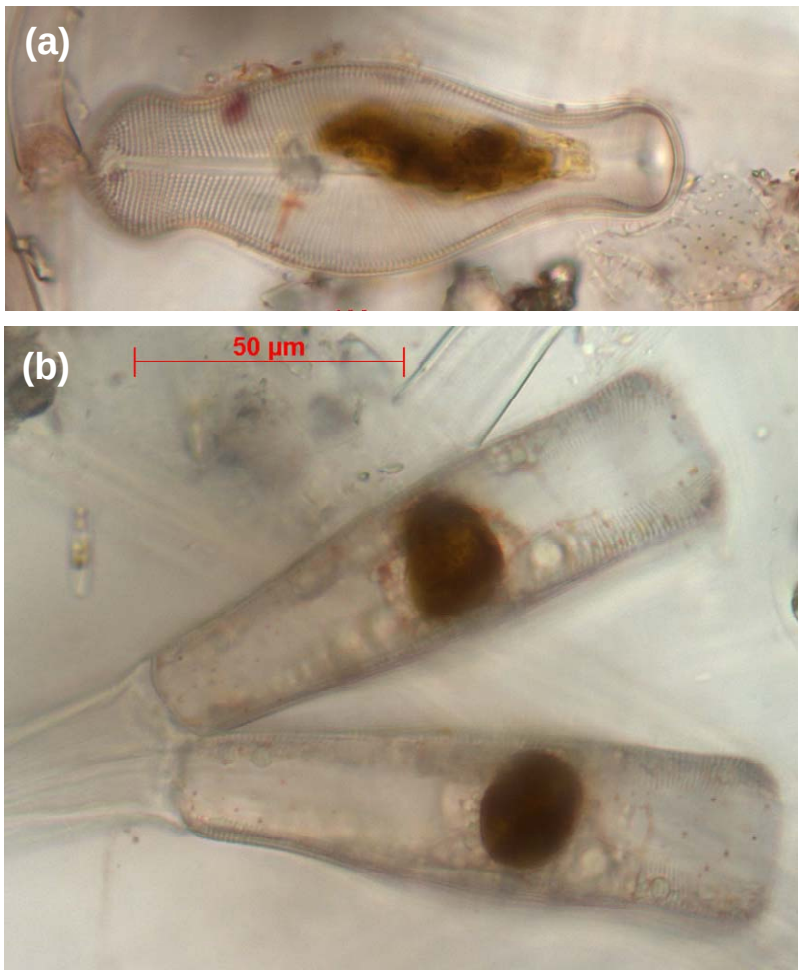

Figure 6: (a) Typical appearance of cell with a degenerated chloroplast from a dark treatment (wet samples), following staining. This example was photographed after 38 days in the dark at 12 ºC. (b) Cells with the condensed, rounded chloroplasts typical of resting cells. Note the slight staining within the cells.

There have been few reports of resting cells in freshwater diatoms, and virtually all are from centric or araphid diatoms (diatoms lacking the characteristic central slit [“raphe” – see footnote 4, p. 17] seen in *D. geminata*). No examples were located from freshwater stalk-forming species (McQuoid and Hobson 1996), though Jewson et al. (2006) described resting cell formation in a marine stalk-forming diatom, as a response to burial in sand, which was the specific habitat of the species. Our observations on *D. geminata* were therefore unusual. They indicate that the survival times estimated for dark, wet conditions at 5 and 12 °C (Figure 3, Table 1) are probably underestimates.

No resting cells were observed at 20 ºC. The diffuse, irregular appearance of the cell contents in unstained cells at this temperature suggested that *D. geminata* in the dark was not utilising heterotrophic food sources, and was not forming resting cells.

Overall, our results reflect an anticipated result, that survival in wet conditions is temperature-dependent at all light levels, with low temperatures favouring longer survival. Low temperatures slow down the metabolic rate, and thus prolonged survival at lower temperatures is to be expected because the cells use up any available resources (e.g., dissolved oxygen, nutrients) more slowly. It is difficult to compare these results with those for other diatoms because most studies consider cell growth rather than cell survival. The predictable overall pattern of increasing viability with decreasing temperature suggests that running the experiments consecutively rather than simultaneously did not have a major effect on the outcome as a result of possible seasonal differences in cell health.

We noted that many cells detached from their stalks during the course of the trials. Our observations suggested that cell detachment was more prevalent at lower temperatures, and in the dark treatments. Further trials would be needed to confirm this. Checks of detached cells showed that many were motile, and therefore definitely alive.[4] Cell detachment may be a response to stressful environmental conditions, which facilitates dispersal to more favourable conditions. Cohn et al. (2003) found differential rates of detachment in four diatom species as a function of temperature, though all species tested tended to lose adhesion as temperature increased. The processes leading to detachment/loss of adhesion are unknown. Indeed, there appears to be far more literature on substrate adhesion in diatoms than on cell detachment from the substrate or stalks (Cooksey and Wigglesworth-Cooksey 1995, Wetherbee et al. 1998, Higgins et al 2003, but see Cohn et al. 2003). With respect to *D. geminata*, cell detachment in response to changes in environmental and chemical conditions may possibly be important in determining the effectiveness of potential control measures.

Over all light treatments, the decline in percentage viability of damp colonies reflected the time taken for colonies to desiccate in the different temperature regimes. This explains the great variability in percentage survival seen at the longer exposure times: desiccated replicates tended to have no live cells, whereas replicates still holding moisture could have high proportions. In previous trials it was established that *D. geminata* requires a moisture content >83% for survival (Kilroy 2005), at which moisture content mats barely appear damp. We did not measure moisture content in the present trials, but did note stained (live) cells in some colony pieces visibly dry on the outside, but moist in the interior. The chloroplasts in desiccated cells migrated to the cell walls and shrank (Figure 7), and eventually took on a bleached appearance.

[4] Motility is seen in most diatoms that, like *D. geminata*, possess a raphe system. This is a pair of longitudinal slits in the silica cell wall, through which mucilage is extruded to effect movement across a surface (Hoagland et al. 1993).

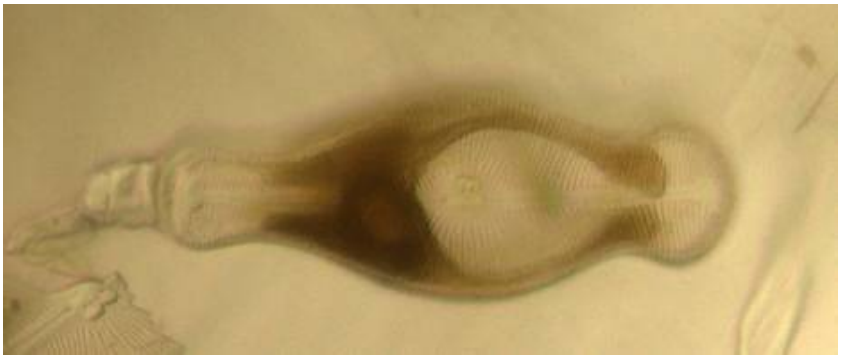

Figure 7: Typical appearance of a cell from a damp, low light treatment, following staining. This example was photographed after 23 days in the 12 ºC (re-run) temperature regime.

Transfer of these results to various scenarios of transport of *D. geminata* can be undertaken on a case-by-case basis, by answering the following questions:

- How long will the cells be out of a suitable river?
- How much water are they immersed in? (i.e., are they likely to dry out?)
- What temperature regime are they likely to be exposed to?
- Are they in darkness or will they receive some light?

Other environmental information such as a change in pH or salinity may also be relevant (see Section 3).

2.4.2. Hot water treatment

Exposure to 40 °C was lethal to *D. geminata* after just 20 minutes, with the effect starting to appear after only five minutes, at which time there was 47% survival in the treated colonies compared to over 80% in the controls (Table 2). Given that *D. geminata* is a cool-temperate diatom species (from its broad distribution – see Kilroy et al. 2005b), this is consistent with published data for other algal species. For example, in an experimental study on planktonic algae from the UK, 35 ºC was found to be lethal to all species (including diatoms) except for one representative of the cyanobacteria (Butterwick et al. 2005). Two tropical diatoms were shown to be unaffected by exposure to 42 °C for up to 45 minutes, however, after four days this temperature was lethal (Rajadurai et al. 2005).

Current decontamination recommendations include treatment in 60 °C water for one minute (http://www.biosecurity.govt.nz/files/pests-diseases/plants/didymo/didymo-cleaning-methods.pdf). This treatment was not specifically tested in the previous

trials, except to validate the neutral red staining technique (cells were killed by immersion in 60 °C water, and these dead cells did not take up the stain). Since heat treatment to 50 °C or more has long been used to kill algae cells experimentally (e.g., Crippen and Perrier 1974, Fisher and Harrison 1996), it is reasonable to accept that the method is robust. Extreme heat deactivates cells by breaking down essential enzymes and other cell constituents, though some diatom species are more thermo-tolerant than others (Rousch et al. 2003). In the present trials we have demonstrated that immersion at 40 ºC kills *D. geminata*, but requires between 5 and 20 minutes exposure for 100% mortality. Thus there is scope for a recommendation for treating *D. geminata* contaminated items with water direct from a domestic or workplace hot water supply.

The recommended temperature for water storage (in water heating cylinders) is 60 ºC, but the recommended water temperature at hand basins and showers is 45 ºC, and should not be greater than 55 ºC (EECA 2003). A hot water system operating under these recommendations will have water hot enough to treat items, *provided that* the temperature can be maintained for long enough. Despite the EECA recommendations, many hot water systems still deliver direct to the taps at >60 ºC (e.g., this is the case at NIWA, Christchurch). In these cases, hot water treatment will be much more likely to be effective. Because of the variability in the temperature of hot water delivered at the taps, it is advisable for anyone wishing to use hot water treatment to check the temperature of their supply. It should also be noted that the rate of cooling of water depends on a range of factors, apart from initial water temperature. These include: ambient (room) temperature, water volume, water surface area, thermal properties of the container in which it is held, temperature and volume of the items being treated.

To minimise cooling during hot water treatment, we recommend that all steps are taken to maintain an initial water temperature of at least 45 ºC (uncomfortable to touch) for at least 20 minutes (longer for absorbent items). For example, a possible protocol might be to place the items to be decontaminated in a pre-warmed chilly bin (rinse with hot water before adding the items), then add enough hot water (direct from the hot tap, maximum temperature) to immerse them completely, cover and leave for at least 20 minutes. The water temperature should be checked at the beginning and end of the treatment to ensure that it remains at no less than 45 ºC. If not, then the hot tap water will need to be supplemented with water heated separately. See Section 5.4 for a further discussion on results of heat treatment for felt-soled waders.

2.4.3. Freezing treatment

We found that no cells took up neutral red stain following freezing and thawing. The temperature once freezing had occurred had no effect, thus freezing in the frozen food compartment of a small refrigerator had exactly the same consequences for the cells as

freezing in a large chest freezer at -15 ºC. In light of the anomalous staining response to depressed pH (see Section 3), one question raised was: would frozen, thawed cells "come back to life" if returned to favourable conditions? Given that the chloroplasts of frozen cells appeared to be physically affected by the treatment (as were cells following prolonged dark treatment, Figure 6), this scenario seemed highly unlikely and proved not to be the case.

The existence of freshwater diatom communities in polar environments indicates that survival after freezing is possible for some diatom species. However, these communities are either terrestrial (soil-living) taxa, or are associated with cyanobacterial mats and appear to survive through a freeze-drying process. In freeze-drying, ice crystals gradually sublime from within the cells, which retains the cell structure (McKnight et al. 1999). Other algae have been shown to withstand cycles of freezing and thawing (Hawes 1990), but in general, freezing and thawing is harmful to algal cells. While cryopreservation is commonly used for long-term storage of algal cultures, cryoprotective agents are generally essential (Brand and Diller 2004). That a large, temperate diatom such as *D. geminata* should be highly vulnerable to the freezing – thawing process was therefore predictable.

For decontamination purposes, freezing may provide a useful option for treating certain items where application of chemicals is not desirable or not feasible. It will be important to allow enough time for the entire item to cool to below the freezing point.

3. Part 2. Trials over gradients of water conductivity (salinity) and pH

3.1. Introduction

While temperature and light are major variables influencing growth rates and viability of freshwater algae (DeNicola 1996, Hill 1996), water chemistry factors are well known as drivers of diatom community composition. Many diatom taxa have narrow tolerance ranges for both pH and salinity. Indeed, the differing pH tolerances of diatoms provided early evidence of the effects of acid rain (from industrialisation) on lakes in Northern Europe through comparisons of current diatom community composition with that of historical communities whose silica frustules remained preserved in sediments (Batterbee et al. 1999). Similarly, historical reconstructions of sea-level changes and tsunamis are provided through the signatures left in sediments by diatoms with differing tolerances for seawater (Denys and De Wolf 1999, Chagué-Goff et al. 2002). While several diatom *genera* have representative species in both freshwater and marine environments (Mann 1999), individual *species* appear to be restricted to a particular salinity range.

Ecological information currently available for *D. geminata* indicates that this species is exclusively freshwater, and inhabits alkaline (pH>7) rather than acidic waters (Kilroy *et al.* 2005b). The implication is that for both salinity and pH there will be thresholds beyond which survival is not possible. In the following trials we therefore manipulated both these variables to try to identify those thresholds.

In terms of the risk of transfer of *D. geminata* to other rivers, specific questions relevant to survivability in varying pH or salinity might be:

- Can a kayak (or other equipment) that has been used in a *D. geminata*-affected river be decontaminated by washing it out in an estuary? What immersion time would be required to be sure that all live cells were killed?
- Can jet boats be decontaminated by pumping seawater through the jet system?
- Might *D. geminata* cells survive passage through the acidic conditions in bird guts? pH in bird guts can be as low as 1 or as high as 4 (Secor and Diamond 1998, Thouzeau et al. 2004) and tends to be in the range 2 -3 (Withers 1992).
- How long would colonies survive if transferred from an infested river to the slightly acidic conditions of fresh rain water or water affected by humic acids (peat)?
- Liming of rivers has been suggested as a possible means to control *D. geminata* growth. From what we already know about *D. geminata*, there is no reason to expect that this would be successful. Liming is generally undertaken to raise water pH, but *D. geminata* apparently thrives in alkaline environments, and indeed may generate those conditions on a diurnal basis through photosynthetic activity (Davies-Colley and Wilcock 2004, Kilroy et al. 2005a). But what is the upper pH limit for survival of *D. geminata*? Does it differ from the generally accepted limit for most algae (pH 10 – lethal to most, pH >11 – lethal to almost everything (Clearwater and Hickey 2003)?

3.2. Methods

3.2.1. Seawater trial

Solutions of 1%, 10% and 50% seawater were prepared using seawater from Lyttelton Harbour, and filtered Waitaki River water. The series was completed with 100% seawater and Waitaki River water (control treatment, 0% seawater). All solutions were held at approximately 9 °C, in a 16 h : 8 h light:dark environment. Small pieces of *D. geminata* colonies were placed in each of the solutions in covered Petri dishes (as in the temperature trials) in groups, which were sampled progressively over time. We

sampled three replicates at each time, with each replicate originating from a different rock. Colony pieces were sampled at 1 hour, 4 hours, 24 hours, 4, 9, 14, 18, 25 and 33 days. In an additional trial, survivability in 100% sea water was tested (relative to controls held in river water) after 1, 10, and 30 minutes, then at 30 minute intervals up to 4 hours. In all cases, viability tests were conducted as described in Section 2.2.1.

3.2.2. pH trial

A series of solutions of different pH were made up by titration of molar HCl into river water, or addition of lime (CaO) of known concentrations, as follows:

- pH 1 (equivalent to extreme conditions within animal or bird guts),
- pH 4 (equivalent to mild conditions within animal or bird guts; also approximates the lowest pH naturally encountered in stream water),
- pH 8 (ambient river water, control treatment),
- pH 9.5 (hard water, lime added at 100 mg/l – equivalent to ~ 70 ppm Ca),
- pH 11 (very alkaline, lime added at 400 mg/l – equivalent to ~290 ppm Ca),
- pH 12 (extremely alkaline, lime added at 1000 mg/l).

We followed the same procedure as used in the seawater trials in that sets of three replicates at each pH level were prepared, and viability was tested after set times. All treatments were held at approximately 9 °C, in a 16 h : 8 h light : dark environment. Viability tests were conducted as described in Section 2.2.1.

3.3. Results

3.3.1. Seawater trial

In the 33-day trial, only 100% seawater achieved 100% mortality, and that occurred relatively rapidly (4 hours) (Figure 8, Appendix 4). In 50% seawater a few cells still took up stain after 30 days (<1%). The control and 1% seawater treatments declined at a similar rate. Surprisingly, the colonies held in 10% seawater showed enhanced survival at the end of the trial. An additional set of tests to determine a more precise survival time for *D. geminata* in 100% seawater showed that at 9 °C, occasional stained cells (<0.2%) were observed in colonies immersed for up to 3.5 hours (Figure 9).

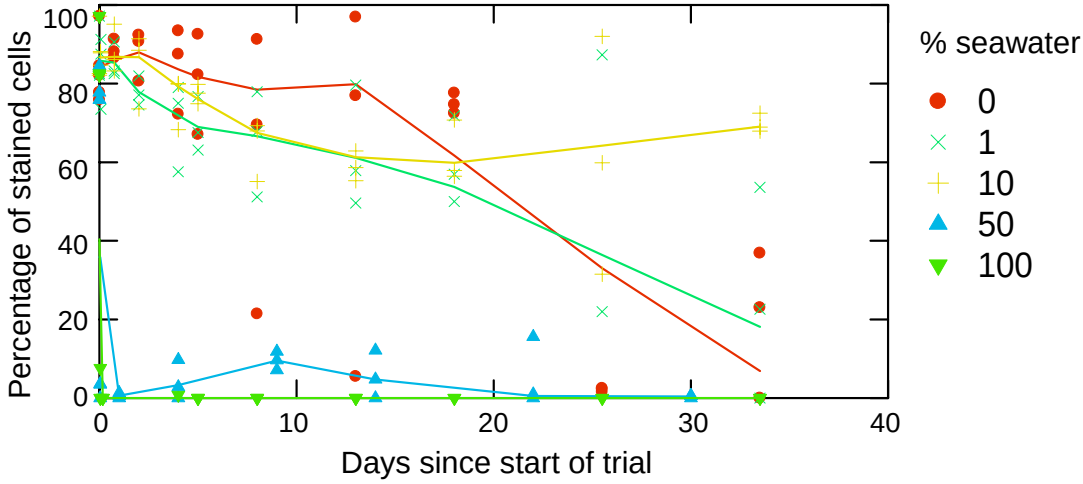

Figure 8: Percentages of stained (live) *D. geminata* cells counted from subsamples of colonies incubated at ~9 °C, in different concentrations of seawater. The control treatment was river water (0% seawater, red dots). Lines are fitted through the data points using the LOWESS smoothing function in SYSTAT v. 10.[5]

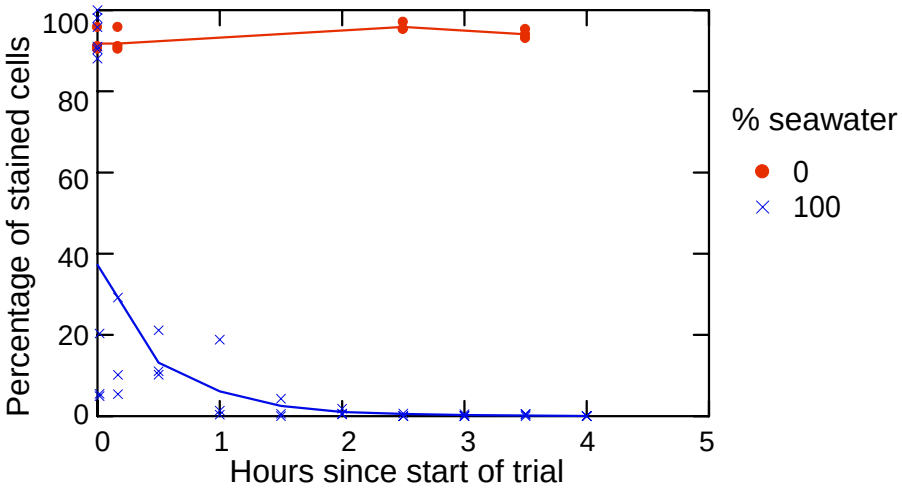

Figure 9: Percentages of stained (live) *D. geminata* cells counted from subsamples of colonies incubated at ~9 °C, in 100% seawater (blue crosses) versus river water (red dots) for up to 4 hours. Lines are fitted as in Figure 8.

3.3.2. pH trial

D. geminata declined rapidly in both very high and very low pH environments. Complete mortality at pH 1 was almost immediate, but took slightly longer at pH 12

[5]"LOWESS produces a fitted line by running along the x values and finding predicted values from a weighted average of nearby y values. The surface is allowed to flex locally to better fit the data, with the tension set at 0.5 (half the points are included in the running window)." (From SYSTAT v. 10).

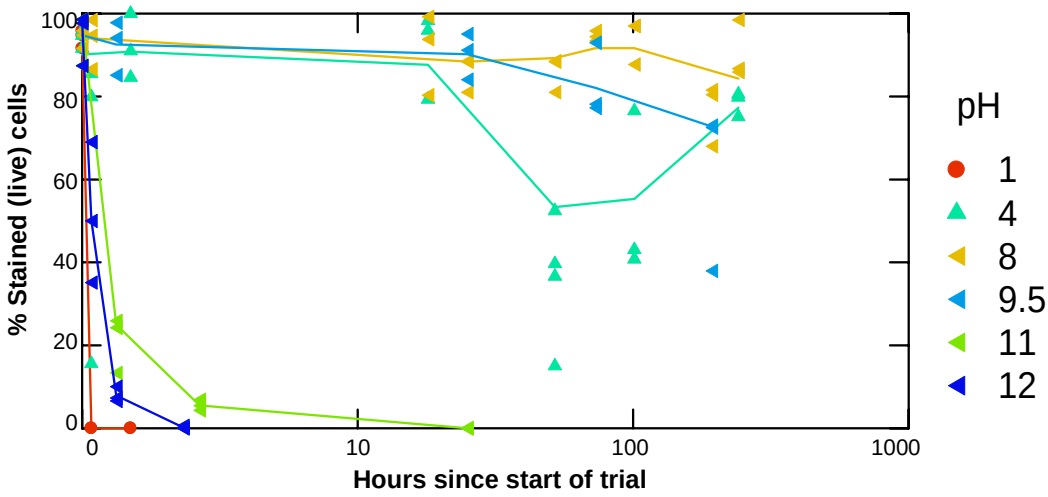

Figure 10: Percentages of stained (live) *D. geminata* cells counted from subsamples of colonies incubated at ~9 °C, in river water adjusted to different pH levels. pH = 8 is plain river water (control). Lines are fitted through the data points as in Figure 8.

and 11 (Figure 10, Appendix 4). For up to about 4 days, survival in the relatively high pH of 9.5 differed little from that in the control (pH 8 – Waitaki River water). Results from the last set of samples suggested that survivability was declining faster in pH 9.5, however, only a longer trial could confirm this.

The results for pH 4 were anomalous. At the first sampling time (5 mins), one of the samples contained a very low proportion of stained (live) cells (blue-green upright triangles, Figure 10). However, on the two subsequent occasions, all replicates returned high percentages; these were followed by further low counts. In all cases, we noted that the cells appeared normal, but did not take up the stain (Figure 10). As a check for viability and stain uptake, we incubated fresh subsamples in the pH 4 river water for 48 hours, then returned these colonies to plain river water. A set of controls was retained in river water for the same duration. We found no difference in percentage viable cells (as indicated by uptake of neutral red stain) between the pH 4 - treated colonies and the controls. Stained cells also had normal-looking chloroplasts. It was therefore concluded that the *D. geminata* was not adversely affected by exposure to pH 4 for 10 days (compared to the control) (Figure 10), but for some reason this pH environment interfered with uptake of neutral red in healthy cells (see Appendix 1 for further discussion).

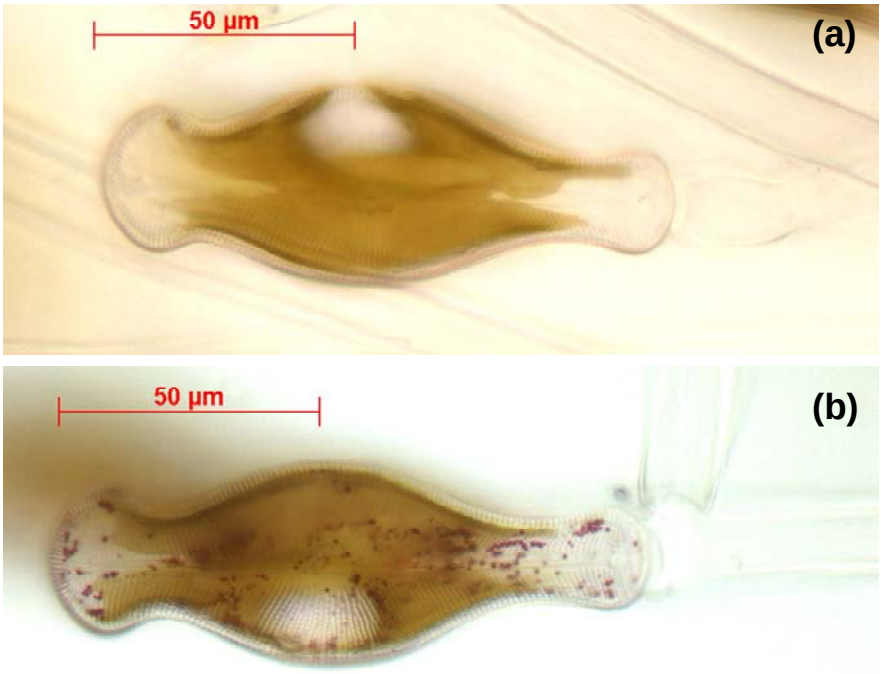

Figure 11: (a) *D. geminata* cell exposed to pH 4 for 2 days, after staining. The chloroplast appears to be intact and healthy. (b) A cell returned to river water after 2 days exposed to pH 4. In the river water, high proportions of cells took up neutral red stain normally, indicating viability.

3.4. Discussion

3.4.1. Seawater trial

The apparent ability of some *D. geminata* cells to survive for up to 3 or 4 hours in full strength sea water, and much longer in diluted sea water was surprising. Given this species' original distribution in oligotrophic, montane or boreal streams, our expectation was that it would not tolerate highly elevated electrolyte concentrations. The sea water used had conductivity of 49.7 mS/cm, and salinity of 3.2% (both measured at 18.8 °C, standardised to 25 °C). This is more dilute than the salinities reported from the open seas around New Zealand (e.g., 3.43 to 3.54 %, Sutton & Wiles 2001), and is equivalent to a sodium chloride concentration of approximately 3.1 % (calculated using published data on the composition of seawater, Turekian 1976). In contrast, the conductivity of water from the Waitaki River is approximately 60 µS/cm.

In the earlier decontamination trials (Kilroy 2005), a 2% salt solution (v/v) was shown to kill all *D. geminata* cells after 10 min exposure. This is equivalent to approximately 4% w/v, and is consistent with the present results which indicate that seawater with 3.1% salt (w/v) is lethal to *D. geminata* after no more than 4 hours. During the counts,

we noted that in some cells the stain was taken up in larger paler vacuoles, which, as discussed in Appendix 1, may indicate that they were dying. However, other cells stained normally (Figure 12).

Half-strength seawater killed most cells within one day, but stained cells were observed in samples until the trial ended at 30 days. At this stage the cells were not staining normally and may not have been viable (see Appendix 1). However, it was clear that progression to complete mortality was slow. Improved survival of cells in 10% seawater (compared with the control) may be a result of increased nutrient availability, or an isotonic environment compared to the cell interior. There is no obvious explanation as to why this did not also occur in the 1% seawater, which declined at the same rate as the control.

Our results suggest that the recommended immersion time in full strength seawater to ensure a complete kill of *D. geminata* should be no less than 4 hours.

The results in diluted seawater suggest that immersion of boats and equipment in estuarine water may only partially kill any *D. geminata* present. Accordingly, immersion in estuarine water of 50% seawater or less cannot be recommended as a reliable decontamination method. The probable times to 100% mortality in dilutions greater than 50% could be estimated, but given that full strength seawater requires 4 hours, these times are likely to be too long to be practical. Another issue is that salinity in estuaries varies with the tidal cycle, and also varies spatially.

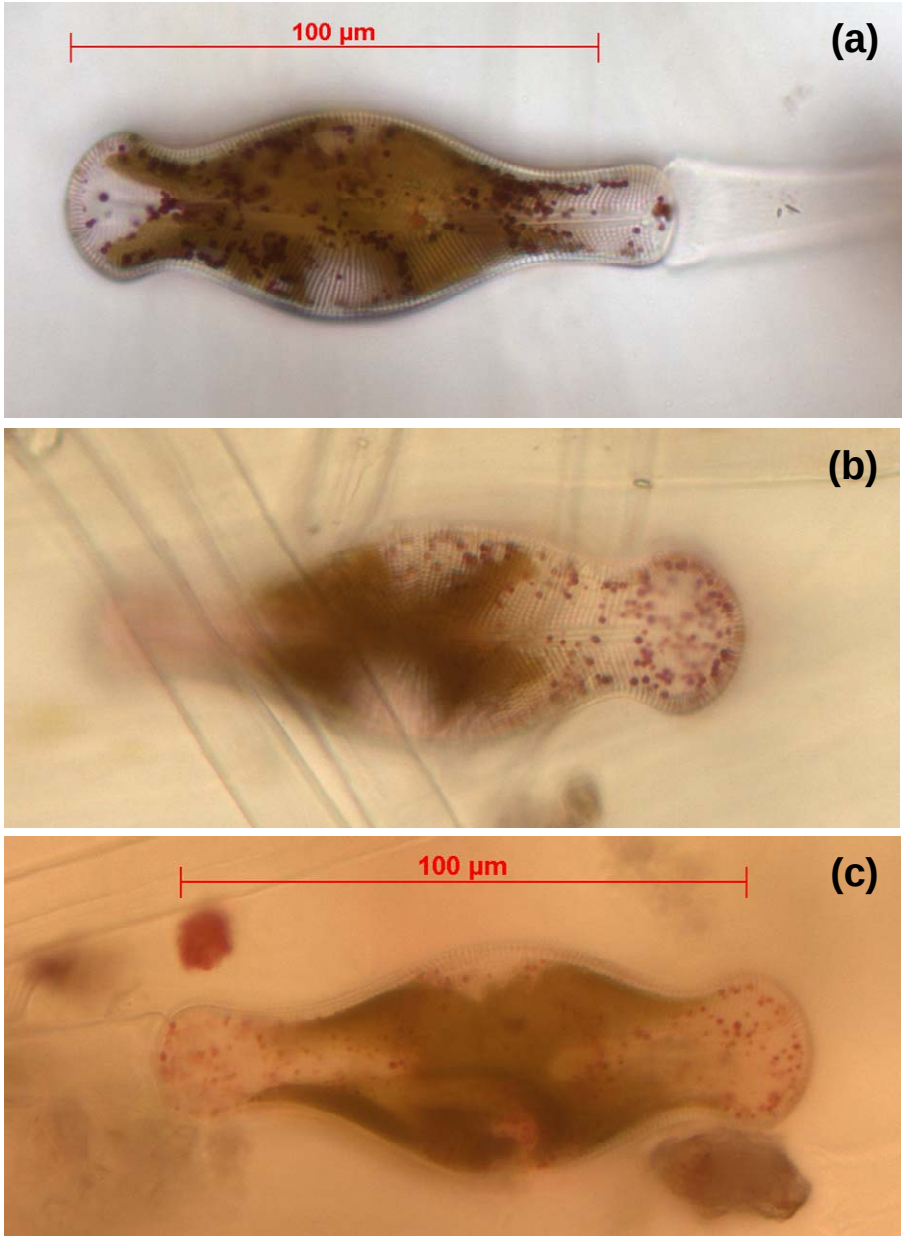

Figure 12: Appearance of cells interpreted as live following immersion in seawater solutions. (a) Live cell following 2 days exposure to 10% seawater. (b,c) Live cells after 1 hour's exposure to 100% seawater. Note the shrunken chloroplast in (b) and paler stain in (c).

3.4.2. pH trial

The demise of *D. geminata* in extremely high and extremely low pH environments was expected. For the high (alkaline) pH range, this is consistent with the Clearwater and Hickey (2003) assessment of pH>11 being lethal to everything, and pH 10 being lethal to most things. However, good survival for 4 days in pH 9.5 and almost

certainly good survival at pH 4 indicates that *D. geminata* has a wide pH tolerance, at least in the short term. Given that the prolific growth of this species generates periodic high pH values in its natural environment (e.g., pH up to 9.3, Kilroy et al. 2005a), short-term tolerance is not surprising. Additionally, records on *D. geminata* distribution in the UK suggest that its pH tolerance extends to at least pH 9, though there are no records of occurrence in waters with pH < 6.4 (Martyn Kelly, UK, pers. comm.). Good survival for at least 10 days in pH 4 is more surprising for a species that is generally found in alkaline waters. This result does not necessarily imply that *D. geminata* could colonise natural waters with such a low pH, but it does suggest a wider tolerance than assumed to date. The pH range of New Zealand's major rivers is 6.0 – 9.7 (median 7.7) (NIWA data, National River Water Quality Monitoring Network 1989 - 2003), thus on the basis of pH, *D. geminata* could survive short-term exposure in all of these.

So, could *D. geminata* survive passage through the acid conditions of bird guts? Typically the gastric pH of vertebrates is 2 – 3 (Withers 1992), but may fluctuate up to pH 5 in some birds (Thouzeau et al. 2004) and in extreme cases may fall to < 1 (Secor & Diamond 1998). Clearly, *D. geminata* does not survive at pH 1. However, our results indicated that pH 4 would allow survival. Given potential fast passage of food through bird guts, especially smaller birds (Karasov 1999), there may be potential to survive the pH environment in some cases. However, it should be remembered that low pH is not the only characteristic of bird and animal guts that creates possible adverse conditions for *D. geminata*. As we have seen from our light – temperature – moisture trials (Section 2), a combination of high temperature and darkness can cause rapid mortality. Since birds and large animals that might ingest *D. geminata* are warm-blooded, this combination of factors – and also enzyme activity – may have more effect than exposure to low pH.

The body temperatures of birds tend to be several degrees higher than that of humans and other mammals (Withers 1992). Bird metabolic rates are correspondingly higher, therefore they need to consume large volumes of food and pass it through their guts rapidly. Retention times can be less than 1 hour (Karasov 1999), but for large birds may be much longer. Ducks and other relatively large waterfowl are the most likely birds in New Zealand to ingest live *D. geminata* direct from rivers. In a study on dispersal of a bryozoan by ducks, Charalambidou *et al.* (2003) found that most propagules passed through the gut within 4 hours. Nevertheless, it would have to be a very large clump of *D. geminata* that could undergo, say, a 1-hour passage through a duck gut without attaining the bird's body temperature (39 – 44 °C, McNab 2003) for at least 20 minutes. On this basis, it seems that survival of *D. geminata* cells after passage through animal or bird guts is extremely unlikely, unless this diatom is able to form some kind of encysted, resistant form. No evidence exists for this, to our knowledge. Any transfer via birds and animals seems more likely to be on the exterior,

on feet, feathers or fur. An assessment of this risk may be made by referring to the results of the temperature – light – moisture trials (section 2 of this report).

4. Part 3. Trials on chemicals and products that could potentially be used as decontamination agents

4.1. Introduction

The main criteria for the final selection of decontamination compounds recommended in the earlier decontamination study (Kilroy 2005), apart from proven ability to kill *D. geminata* cells, were:

- Products were widely and easily available to the public (e.g., products commonly sold in supermarkets).
- Products were inexpensive.
- A variety of compounds needed to be recommended, because some products would be more suitable than others when used in different situations (e.g., diluted bleach is cheap and effective, but can damage some materials and fabrics).

Since Biosecurity New Zealand's "Check, Clean, Dry" campaign has greatly broadened the scale of decontamination activities, it has become clear that the original recommendations, while still valid, are not flexible enough to provide for all situations. For example, the currently recommended products all produce fast results (100% kill in less than one minute). To achieve this, high product concentrations must be used. When treating large items, the cost of the cleaning products can be significant. Additionally, disposal of large quantities of used solution is a potential environmental problem, particularly where there is no piped drainage system. Factors that need to be considered when recommending decontamination agents therefore should include the following:

1. Availability – Is the product widely sold or does it have to be ordered from a specialist supplier?
2. Expense – Can the product be used at a low concentration to make it more economical? Is it relatively cheap in a concentrated form?

3. Effect on humans – Is the product non-toxic to humans and pets? Is it an irritant? Is protective clothing required?

4. Effect on metals, fabrics, plastics, etc. – Does the product adversely affect any materials? It is acknowledged that some reagents may be unsuitable or especially suitable for particular types of gear.

5. Effect on other organisms – Will the products harm plants, invertebrates, etc, if disposed of into the environment?

6. Biodegradability – Does the product break down when released into the environment, without any adverse long-term legacy?

A further issue is that, while decontamination recommendations can be made for *D. geminata*, this is not the only undesirable organism that has invaded freshwaters in New Zealand and not the only organism for which decontamination methods have been researched (e.g., Matheson et al. 2004). Thus, ideally, any recommendations for preventing the spread of *D. geminata* should also be effective on other freshwater weeds and pests. In the trials described below, we focus on effectiveness of agents on *D. geminata*. Steps taken in the selection of products for testing are discussed first, followed by a description of the trials, and results for individual products. In the discussion, we evaluate each product tested against the criteria listed above.

Note that throughout this section, we draw on information obtained from discussions with Dr Scott Belanger (Procter & Gamble, USA).

4.2. Choice of products tested

The initial brief for these trials was to identify three or four generic constituents of household cleaning products, then test these individually for their effectiveness in killing *D. geminata*. In principle it would then be possible to transfer the test results to a wide range of proprietary products that contained these generic constituents (or closely related chemicals). The reason for the approach is that there are hundreds of cleaning products on the market, all of which may well have some capability in terms of decontamination of items. There is therefore no basis for recommending one proprietary product over another, unless all can be tested (which is clearly not possible). Testing common ingredients appears to be a logical way to overcome this. However, two major problems meant that this approach was not practical.

1. Cleaning products, including detergents, contain a very wide range of ingredients. Some families of compounds may be common to many products

(e.g., anionic surfactants, cationic surfactants), but there is much variability within these families in terms of toxicity and biodegradability. Other ingredients (e.g., chlorine oxidisers, other oxidising agents, alcohol-based compounds) are used in various forms in different products. The huge variety of ingredients used means that few are truly generic.

2. Many commercially available cleaning products do not list ingredients on the packaging and for commercial reasons most manufacturers do not make these freely available. In New Zealand, there is no legal requirement for constituents to be listed (as there is for foods). Therefore, even if identification of generic components were straightforward, consumers would be unable to relate test results from these back to individual products.

Consequently, advice was sought from experts as to appropriate products to test. Rather than try to identify which products were commonly used in many commercially available cleaners, the focus was more on selecting products that were acceptable according to the six selection factors. General information about the products tested follows.

In Appendix 5, we present MSDS data for some of the products (where available).

4.2.1. Borax (sodium tetraborate, $Na_2B_4O_7 \cdot 10H_2O$)

The following information is lifted directly from en.wikipedia.org/wiki/Borax (sourced September 2006):

"Borax, also called sodium borate or sodium tetraborate, is an important boron compound. It is a soft white many-sided crystal that dissolves easily in water. If left exposed to dry air, it slowly loses its water of hydration and becomes the white chalky mineral tincalconite ($Na_2B_4O_7 \cdot 5H_2O$). Commercially sold borax is usually partially dehydrated.

"Borax is widely used in detergents, water softeners, soaps, disinfectants, and pesticides. Its use in detergents is due to its ability to bind to and solvate dirt particles in addition to producing peroxides which have a bleaching effect. One of its most widely advertised uses was as a hand-cleaner for industrial workers. It is used in making enamel glazes, glass and strengthening pottery and ceramics. It is also easily converted to boric acid or borate, which have many applications. It is also used to make buffer solutions that are used in chemical analysis. …Large amounts [of borax] are ... used in production of sodium perborate monohydrate for use in detergents. "When used in a mixture, borax can be used to kill carpenter ants and fleas. Despite its

use as an insecticide and reputation as a toxin, the LD_{50} toxicity of borax is about the same as that of table salt (both are around 3,000 mg/kg body mass)."

Boron is well known to be toxic to plants, especially on soils of arid and semi-arid regions (Nable et al. 1997). In New Zealand, borax is recommended by many agencies as an economic and environmentally friendly alternative to commercially available cleaning products and insecticides for crawling insects. For example, brochures produced by Auckland Regional Council, Marlborough District Council and the University of Canterbury all mention the use of borax. (For example, see, www.marlborough.govt.nz/content/docs/environmental/Being_Cleaner_and_Greener_Around_the_Home.pdf and www.arc.govt.nz/arc/library/w30650_2.pdf).

Borax is available from pharmacies.

4.2.2. Sodium percarbonate (oxygen-based oxidiser)

Sodium percarbonate ($2Na_2CO_3 \cdot 3H_20$) is the stated active ingredient in nappy (diaper) cleaner. As a potential decontamination agent, it has the advantage of producing almost no environmental legacy. The following information has been taken directly from en.wikipedia.org/wiki/Sodium_percarbonate (sourced September 2006):

"When dissolved into water, it releases H_2O_2 [hydrogen peroxide, a bleaching agent] and soda ash (sodium carbonate). … The pH of the resulting solution is typically alkaline, which activates the H_2O_2 for bleaching. The dry powder contains about 30% w/w H_2O_2.

"Sodium percarbonate is an ingredient in a number of home and laundry cleaning products. It is the primary ingredient in OxiClean products and a component of Cillit Bang crystals. It offers many of the same functional benefits as liquid hydrogen peroxide. It dissolves into water rapidly to release oxygen and provides powerful cleaning, bleaching, stain removal and deodorizing capabilities. ….

"According to http://www.chem-world.com/sodium_percarbonate, compared with chlorine bleaching chemicals that leave contaminating residues in the environment, sodium percarbonate is an environmentally friendly chemical which decomposes into oxygen, water and natural soda ash when subjected to moisture. Sodium percarbonate is increasingly being used as the substitute for sodium perborate in detergent formulations due to its lower dissolving temperature in water.

"Detergent or bleach compositions formulated with sodium percarbonate have a strong stain removal capability. It is very effective as a laundry presoak for heavily stained articles. It is color safe. It brightens colors and prevents fabric from becoming yellowed or darkened. Sodium percarbonate is effective as a disinfectant on both bacteria and viruses."

We were unable to find any supplier of sodium percarbonate in small quantities. It appears to be sold only as a bulk commodity (25 kg, minimum quantity), but it is relatively inexpensive ($120.00 + gst per 25 kg).

4.2.3. Nappy cleaner

Nappy cleaner (Napisan®, powder formulation) was included in the original decontamination trials at a limited range of concentrations. The range was expanded in the present trials to determine the efficacy of longer exposure to lower concentrations and to confirm the concentration required for a one min kill. In both trials, we used the granular formulation ("Everyday soaker"), in which the active ingredient is stated to be 25.7% sodium percarbonate. Other ingredients in Napisan (complete nappy treatment) are: anionic surfactant (alkyl benzene sulphonate), sodium carbonate, sodium silicate [information from Clorox Ltd.].

4.2.4. Sodium dodecyl sulphate (SDS)

SDS ($C_{12}H_{25}OSO_3Na$) is relatively safe, biodegradable anionic surfactant that is "used in household products such as toothpastes, shampoos, shaving foams and bubble baths for its thickening effect and its ability to create a lather. The molecule has a tail of 12 carbon atoms, attached to a sulfate group, giving the molecule the amphiphilic properties required of a detergent" (en.wikipedia.org/wiki/Sodium_dodecyl_sulfate). It is also known as sodium lauryl sulphate (see MSDS data in Appendix 5 for more synonyms). SDS has laboratory applications, in polyacrylamide gel electrophoresis.

In general, anionic surfactants are toxic to fish and less so to primary producers, therefore they are not expected to be the critical component in a product used as a decontamination agent for an alga. Anionic surfactants are the major ingredient in most detergents and the major class in use is the linear alkylbenzene sulphonates (LAS). While LAS are regarded as biodegradable (and certainly more so than their precursors), their degradation can be slow and complex and new alternatives are gradually being introduced (Scott and Jones 2000). SDS, on the other hand, biodegrades rapidly.

Like all detergent surfactants (including soaps), SDS can cause irritation to the skin and eyes. SDS is commonly used in research laboratories as the standard skin irritant with which other substances are compared. SDS is regarded an extremely low-risk compound in terms of long-term effects such as carcinogenesis.

SDS must be purchased from a specialist chemical supplier.

4.2.5. Sodium hypochlorite (chlorine-based oxidiser)

Sodium hypochlorite (NaClO) is the active ingredient in household bleach, usually a 35 – 40 g/litre solution of sodium hypochlorite (or 3.5 – 4%). Household bleach was tested in the earlier trials and a 2% solution with 1 min contact time recommended as a decontamination procedure. In the present trials, we tested lower concentrations with longer contact times, using a product with a stated sodium hypochlorite concentration of 35 g/L (3.5%) sodium hypochlorite (equivalent to 0.8% Cl, or 8000 ppm Cl).

Sodium hypochlorite is the basis of water chlorination of drinking water supplies (concentration approx. 0.2 ppm Cl – NZ Ministry of Health) and swimming pools (concentration approx. 1 – 3 ppm Cl). Thus, its toxic effect on microorganisms is well known. The effects are broad-spectrum. Further problems include the corrosive effect of sodium hypochlorite on fabrics and other materials, and potential hazard associated with its use (see Appendix 5). It is, however, cheap and readily available.

4.2.6. Quaternary ammonium compounds

Quaternary ammonium compounds (quats) include cationic surfactants, characterised by a core nitrogen atom with alkyl and other functional groups covalently bonded to the nitrogen. The salts of these molecules are used as disinfectants, surfactants, fabric softeners, and antistatic agents. While they are less effective surfactants than the anionics, they are more effective disinfectants, being less toxic to fish, but better algaecides. As algaecides, they are available in many formulations, with benzalkonium chloride being the best known. Benzalkonium chloride is a mixture of alkylbenzyl dimethylammonium chlorides of various alkyl chain lengths. Alkyl derivatives with more than 14 carbons are very absorbent, bind to sediments, and eventually biodegrade. Compounds with <14 carbons biodegrade quickly and completely. Benzalkonium chloride is considered safe for human use in eyewashes, hand and face washes, mouthwashes, spermicidal creams, etc.

In the current trials, we tested 303 Clearall, marketed as an algaecide/bactericide for swimming pools (a proprietary mixture). Information on the container states: “303

Clearall contains a complex cationic copolymer". The product supplier has supplied further details, viz. that 303 Clearall contains 60% ai by weight and the content is 1,2-Ethanediamine, polymer with (chloromethyl) oxirane and N-methylmethanamine. This implies a mixture of many quats, which may be impossible to define exactly (information via John Clayton, NIWA).

4.2.7. Sodium metabisulphite

Sodium metabisulphite ($Na_2S_2O_5$) was included in the trials specifically because it has been recommended to Biosecurity New Zealand as one of the reagents suitable for generic use for disinfection of gear at the border. When dissolved in water, it releases the gas SO_2, which is the toxic ingredient. Uses of this chemical are as an equipment sanitizer in home-brewing, and as a knock-out for chlorine residual in the water treatment industry. Because SO_2 causes breathing difficulties in some people, it has fallen from use as an equipment sanitizer in recent times (en.wkipedia. org/wiki/Sodium_metabisulfite). The chemical is available from homebrew and winemaking specialist shops in New Zealand.

4.2.8. Ethanol

Ethanol (C_2H_5OH) is unlikely to be used as a decontaminant by the public, but since ethanol is used as a preservative for molecular material, and *D. geminata* may therefore be transported in ethanol solution, both regionally and internationally, it is important to define thresholds of mortality to ensure zero risk.

4.2.9. Cleaning products marketed with environmental claims

Some cleaning products are marketed as being non-toxic, biodegradable, natural, ecologically sensitive, etc. Several are on the market and are based on different active ingredients. We chose three: B.E.E. ™ multi-surface cleaner (blend of non-ionic and anionic surfactants, etc., claimed to be "New Zealand's most environmental cleaning products"); citrus-based cleaner (no ingredients stated); Simple Green® (mixture of citric acids, acetic acids, sulfonic acids, alcohols). B.E.E.™ products and Simple Green® are available in supermarkets. Citrus Based Cleaner is available by mail order, but several citrus-based products are sold in stores throughout New Zealand.

4.2.10. Commonly available detergents

The following widely used dishwashing liquids were tested: Down-to-Earth®, Palmolive® and Sunlight®. None is strongly marketed as being environmentally outstanding, though Down-to-Earth® advertises a recyclable bottle. We used the basic variety of each (i.e., with no added fragrance or anti-bacterial properties). Down-to-Earth® was included in the original decontamination trials (Kilroy 2005), but was only tested at one concentration. In the present trials, we tested a range of concentrations and exposure times. Down-to-Earth® lists anionic surfactant, pH stabilisers, and antimicrobial agent as ingredients. No ingredients appear on the containers of the other two products.

4.2.11. Virkon®

Virkon® S is a broad spectrum virucidal disinfectant, which is currently used at the border as a decontamination agent for imported equipment. It is also widely used by other organisations (e.g., St John ambulances) as a cleaner/disinfectant. The listed ingredients are: dipotassium peroxodisulphate (or dipotassium peroxomonosulphate), sodium chloride, sulphamic acid, malic acid, sodium hexametaphosphate (a buffer), sodium dodecyl benzene sulphonate (a detergent), amaranth color (an indicator). Note that the detergent here is an LAS (see section 4.2.4 above). Virkon® is sold as a powder which mixes with water to produce a pink solution that is stable for about a week. When the pink colour fades, the solution needs to be replaced. We have tested Virkon® at the recommended concentration and a single contact time in a previous trial. Tests on Virkon were not repeated in the present study, but we discuss the result along with those of the current trial.

4.3. Methods

Each product was tested on *D. geminata* sourced from the Waitaki River no more than 4 days previously, and held at approximately 9 °C in the climate simulator, in a 16 h : 8 h light : dark regime under the “light” treatment described in Section 2. Concurrent trials at 12 and 5 °C showed that after 20 days, light-treated colonies had not declined in % viability compared to viability tested at time 0. Survival was higher than at 20 and 28 °C, and lower light levels. Therefore we considered that 9 °C (wet colonies, high light) was an appropriate estimate of optimal conditions.

In all cases we tested survival of *D. geminata* over a wide range of concentrations of the product range (usually spanning 2 – 3 orders of magnitude). Exposure times were standardised to 1, 10, 100 and 1000 minutes, with the longest time equivalent to prolonged overnight exposure (16 hours 40 minutes). Concentrations tested were

based on manufacturers' recommendations (for products designed for controlling algae) or on published toxicity data (for basic ingredients tested). In the following results, choices of concentrations are explained for each product and results are presented graphically. Note that in all graphs, the red circles are the control treatment (plain river water).

4.4. Results

Results for each of the products tested are summarised below in graphical form. Numerical data are included as Appendix 6.

4.4.1. Borax

We selected test concentrations based on published boron toxicity information (Dyer 2001). Each unit of boron is equivalent to 8.8 units of borax. We started at a very low level (0.5 mg B/l) reported to cause minimal mortality in a very sensitive organism (the rainbow trout *Oncorhynchus mykiss*), and tested up to 1000 mg B/l, 10 times the dose reported to cause 100% mortality in rainbow trout (from Dyer 2001). No appreciable mortality of *D. geminata* occurred at any concentration (Figure 13a)

4.4.2. Sodium percarbonate

In the previous decontamination trials, 5% nappy cleaner (25.7% sodium percarbonate) killed 100% *D. geminata* in one minute. We therefore started the trial using a 1% sodium percarbonate, which is equivalent to the active ingredient in just under 4% nappy cleaner. Both higher and lower concentrations were tested. We found that total mortality occurred only after 1000 minutes in 0.1, 0.5 and 1% solutions, and after 100 minutes in a 2% solution (Figure 13b). At this stage, in all cells the chloroplast retained its colour, but lost definition at the edges (compare Figure 14 with Figure 11a). A 5% solution did not quite kill all cells after 10 minutes. At this concentration, it was difficult to get all the solid granules into solution at 9 °C.

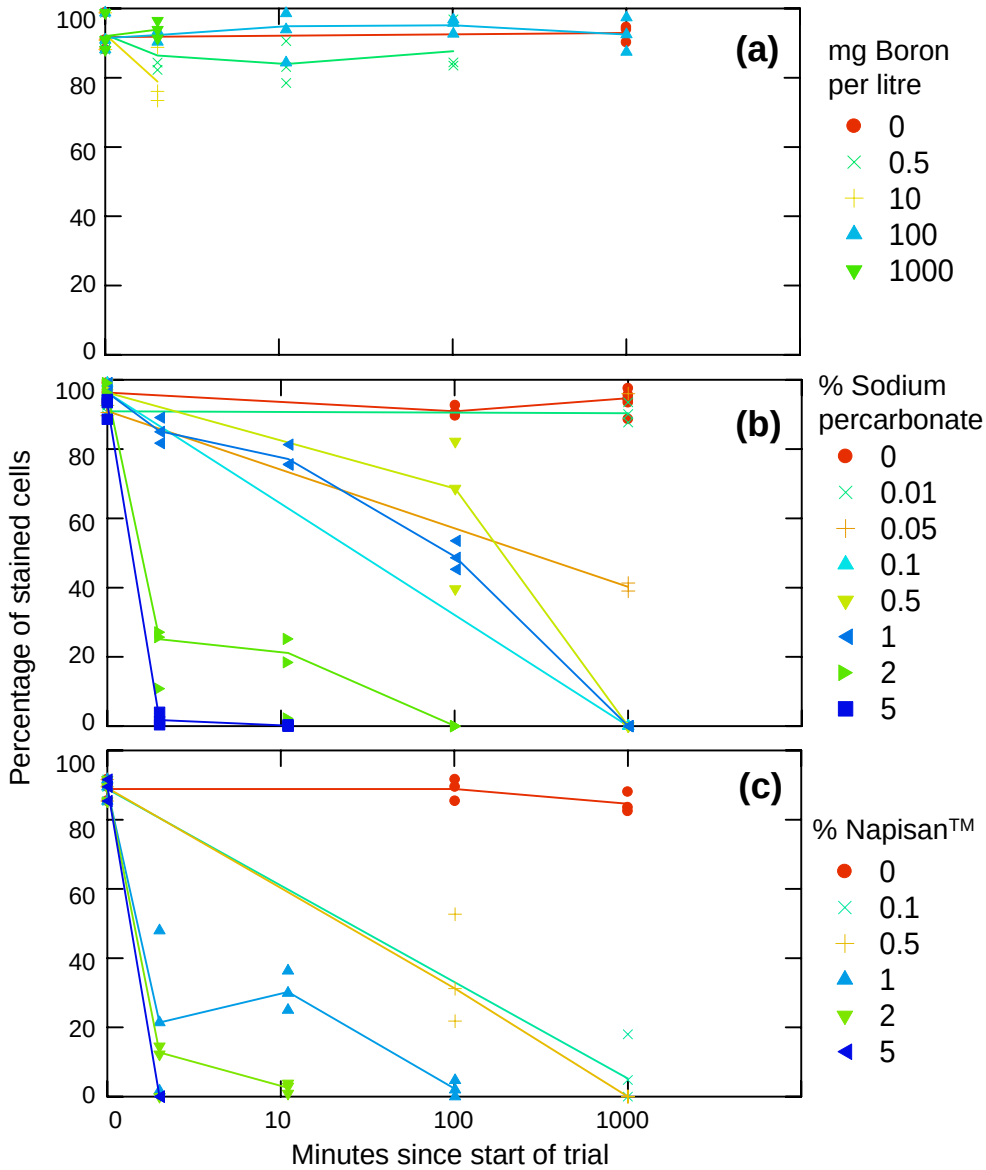

Figure 13: Percentages of stained *D. geminata* cells counted following exposure of colonies to a range of concentrations of (a) borax, (b) sodium percarbonate, and (c) Napisan, for up to 1000 minutes. Three replicates were examined for each time-concentration combination.

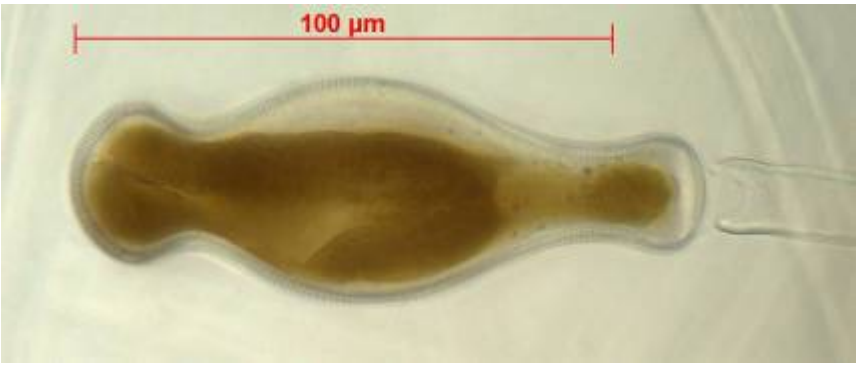

Figure 14: Typical appearance of a *D. geminata* cell, following staining with neutral red, after immersion in 1% sodium percarbonate for 1000 minutes at 9 °C.

4.4.3. Nappy cleaner

As in the earlier trials (Kilroy 2005), no stained cells were found after exposure of colonies to 5% nappy cleaner (Napisan) for one minute. None of the lower concentrations tested achieved 100% mortality in less than 10 minutes (Figure 13c), however all cells were dead following 1000 minutes in a 0.5% solution. Stained cells generally looked normal and those not staining (assumed dead) were similar to cells treated in sodium percarbonate (Figure 14).

4.4.4. Sodium dodecyl sulphate

Belanger et al. (1996) reported "no observable effects concentrations" (NOEC) for two anionic surfactants of 553 and 608 µg/l (approx. 0.00005%). In the absence of more information, we started with concentration 1000 times this level (0.05%), expecting to obtain a response. This concentration did not quite kill all cells after 1000 minutes, but a 0.1% and 0.5% solutions did (Figure 15a). Only a 1% solution achieved complete mortality after one minute. At this concentration, it was quite difficult to get the surfactant into solution, as the substance is very bulky (specific gravity 0.4). Unstained cells following treatment typically had chloroplasts with a "curly" appearance (Figure 16).

4.4.5. Household bleach (sodium hypochlorite)

The product tested had a stated sodium hypochlorite concentration of 35 g/L (3.5%). Our trial included 1% household bleach (which had been shown to be effective after one min in the earlier trials) and two lower concentrations of 0.5% and 0.1%. Therefore sodium hypochlorite levels were 0.035, 0.07 and 0.35%. The 1% household

bleach solution was almost effective, with a very few stained cells seen in just one of the three replicates after 1 min exposure. Neither of the lower concentrations was 100% effective, even after 1000 minutes (Figure 15b).

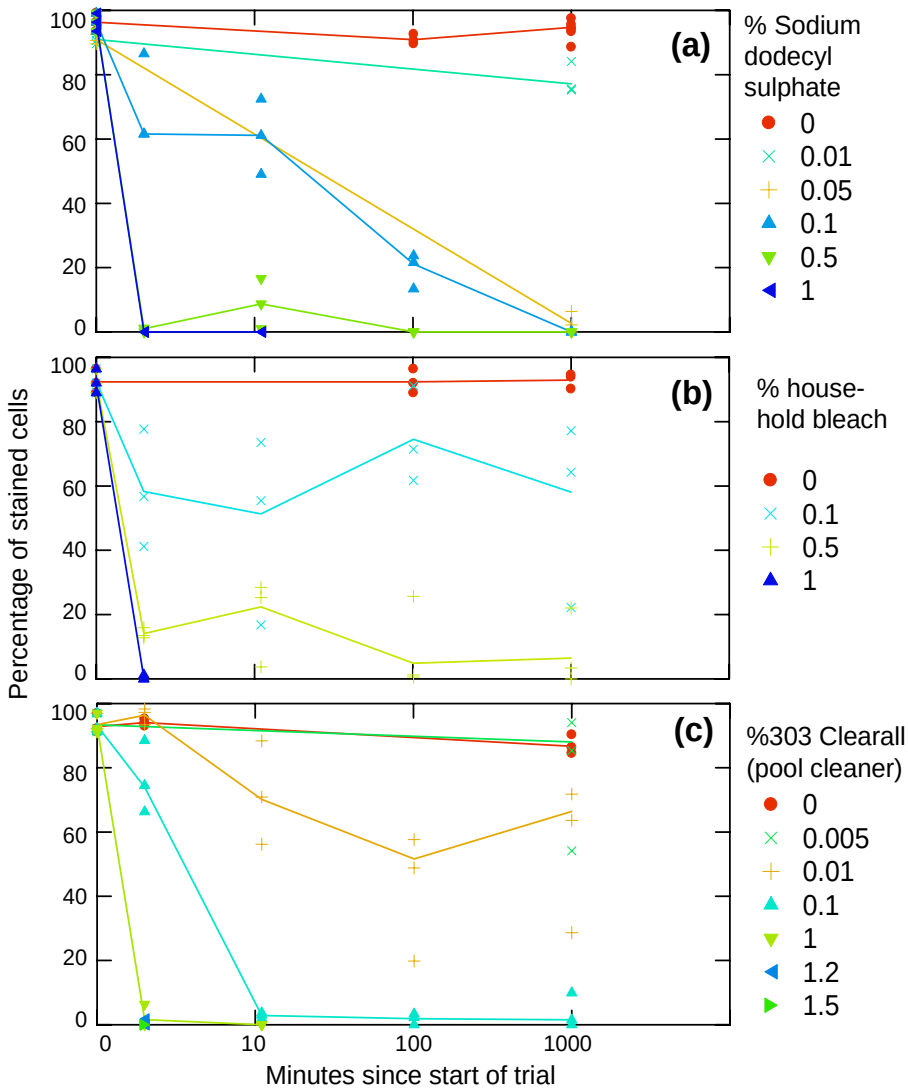

Figure 15: Percentages of stained *D. geminata* cells counted following exposure of colonies to a range of concentrations of (a) SDS, (b) household bleach, and (c) Clearall, for up to 1000 minutes. Three replicates were examined for each time-concentration combination.

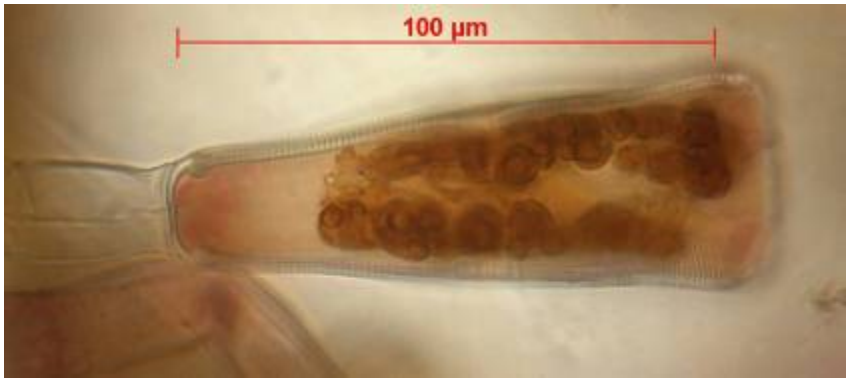

Figure 16: Typical appearance of a cell, following staining with neutral red, after immersion in 0.1% sodium dodecyl sulphate for 1000 minutes at 9 °C.

4.4.6. 303 Clearall (quat mixture)

The manufacturer's recommendation for treatment of "algae-infested swimming pool water" is a dose of 30 ml Clearall per 1000 litres, a concentration of 0.003%. Given that this was suggested as a long-term treatment, our concentrations started slightly higher (0.005%), at which dose there was no discernable difference from the control. The 0.1% concentration was not fully effective after 1000 minutes, and a relatively high dose of 1.5% was needed before we could detect no stained cells after one minute (Figure 15c).

4.4.7. Sodium metabisulphite

Paul Champion, NIWA, provide the following advice: "Sodium metabisulphite is used to sterilise wetsuits, usually adding a teaspoon (~5 g) into a large plastic bag with around a litre of water. The suit is then sealed inside the bag with the liquid. The sterilisation agent is SO_2 gas, released when sodium metabisulphite is dissolved in water, with 1000 ppm produced from 1.5 g sodium metabisulphite in a 20L container. See (http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/V5030E/V5030E0b.htm)". From this we calculated that 5 g in 1 litre of water is equivalent to approx 70 000 ppm SO_2. Our concentration range therefore started at slightly lower than this. Even the very high concentration tested (200 000 ppm SO_2, or ~15 g sodium metabisulphite/ litre) did not achieve complete mortality of *D. geminata* in 10 minutes (Figure 17a). At half that strength, 100 minutes was required.

4.4.8. Ethanol

We tested 70% strength (the recommended concentration for preservation of samples for DNA extraction), and lower concentrations. A few stained cells were present after

a one minute exposure to 70%, but none after 10 minutes (Figure 17b). Progressively lower concentrations took longer to kill all cells, with 20% requiring 1000 minutes.

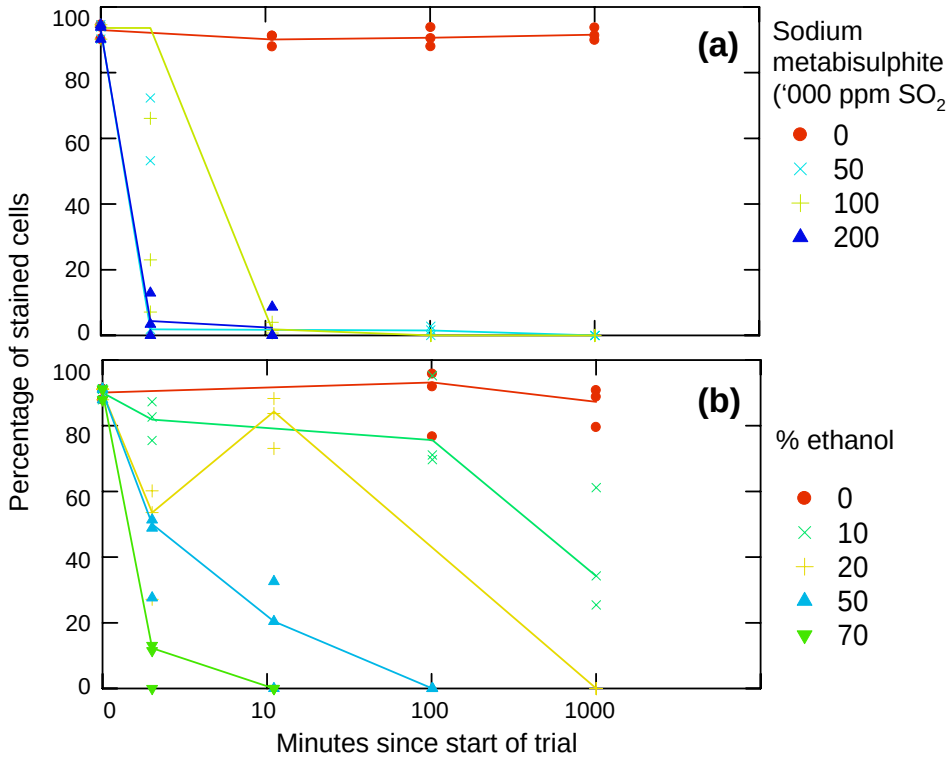

Figure 17: Percentages of stained *D. geminata* cells counted following exposure of colonies to a range of concentrations of (a) sodium metabisulphite (b) ethanol for up to 1000 minutes. Three replicates were examined for each time-concentration combination.

4.4.9. Cleaning products marketed with environmental claims

Selected concentrations for the three cleaners carrying "environmental" claims were centred around the 5% currently recommended for detergent. In all three products, a 5% solution was not fully effective after 10 minutes exposure, and 100 minutes was sufficient time only for Citrus Based Cleaner. No stained *D. geminata* cells were found after 1000 minutes in all three products at 2% concentration. However, 50% Simple Green® was needed to achieve complete mortality after just one minute, and 100% Citrus Based Cleaner. No concentration of the B.E.E.™ product was completely effective after only a minute (Figure 18).

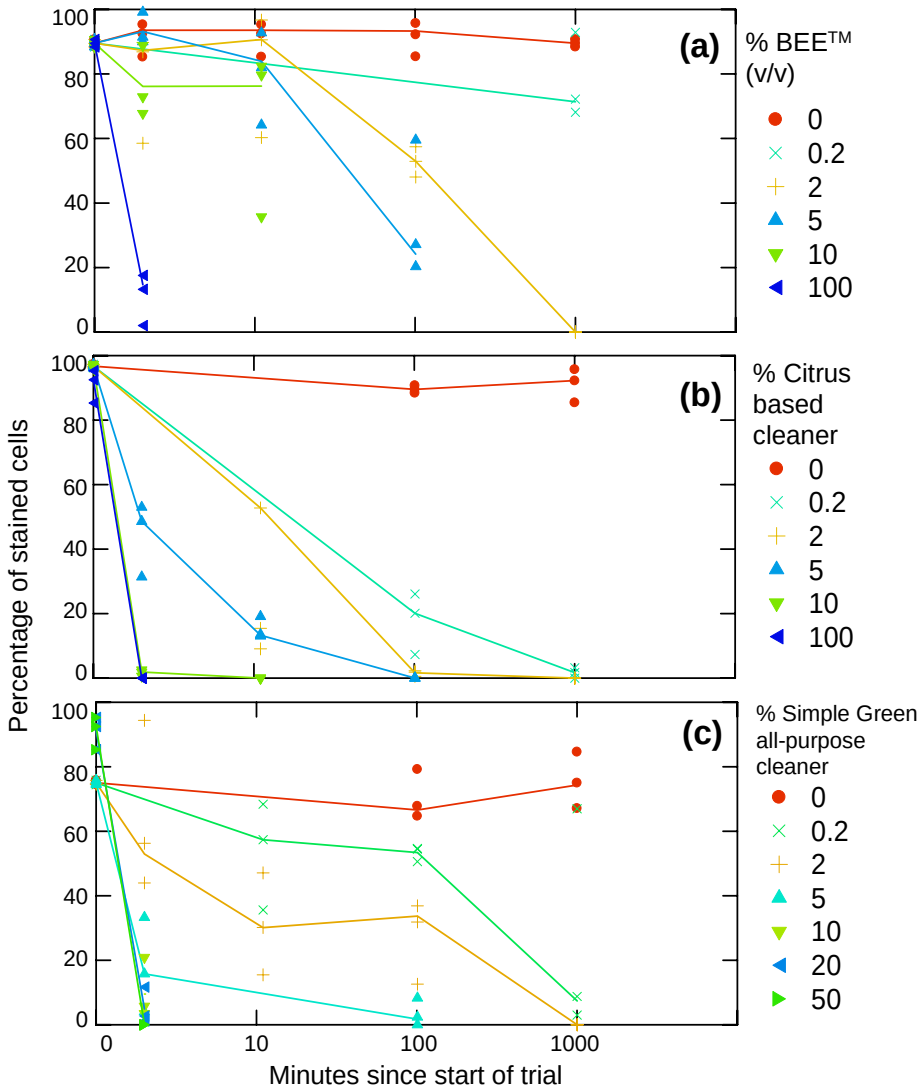

Figure 18: Percentages of stained *D. geminata* cells counted following exposure of colonies to a range of concentrations of three "environmentally friendly" products: (a) B.E.E. all purpose cleaner, (b) Citrus Based Cleaner, and (c) Simple Green. Three replicates were examined for each time-concentration combination.

4.4.10. Commonly available detergents

The three dishwashing liquids tested showed a similar response in terms of percentages of stained *D. geminata* cells following exposure. In all cases, a 5% solution was required for a 100% kill. Down-to-Earth® and Sunlight® at 0.1% were also completely effective after 1000 minutes. A very few stained cells were seen in the equivalent treatment with Palmolive® (Figure 19).

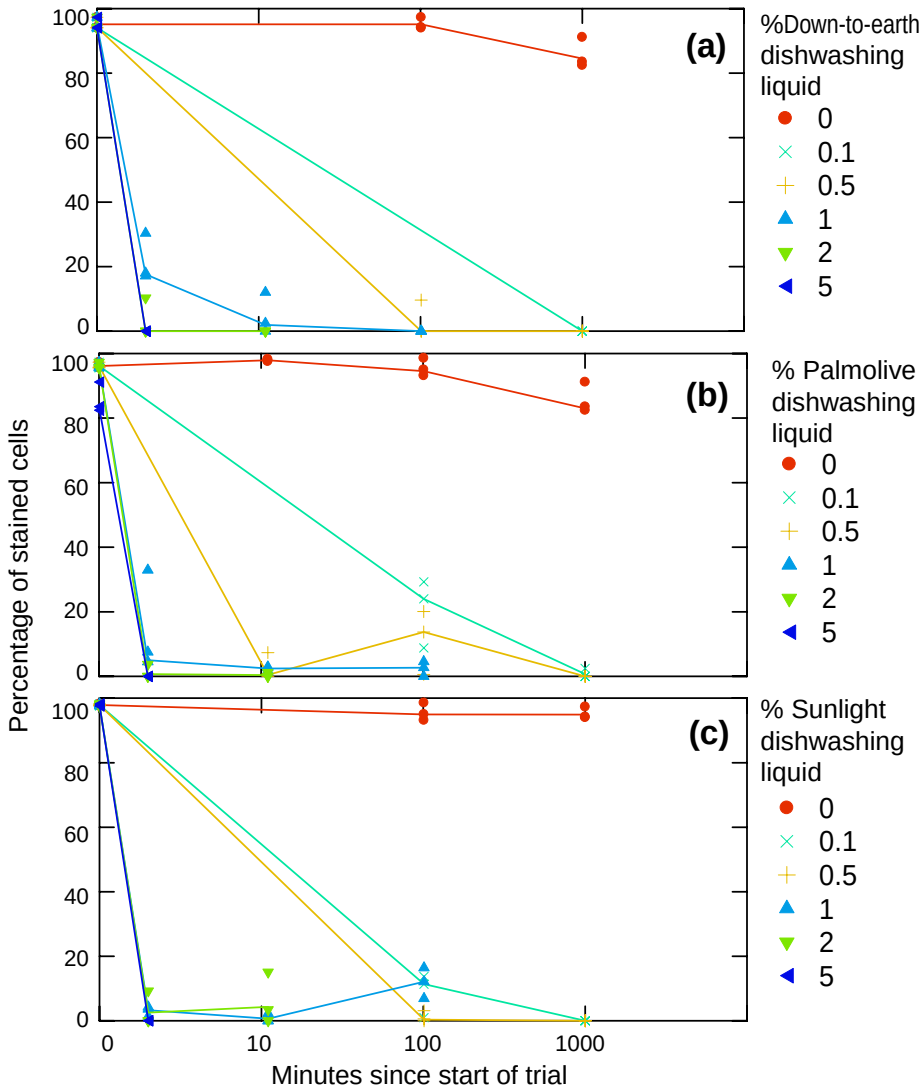

Figure 19: Percentages of stained *D. geminata* cells counted following exposure of colonies to a range of concentrations of three common dishwashing liquids: (a) Down-to-Earth®, (b) Palmolive®, and (c) Sunlight®, for up to 1000 minutes. Three replicates were examined for each time-concentration combination.

4.4.11. Virkon®

A 1% solution of Virkon® S was made up as directed on the packaging and three replicate *D. geminata* colony pieces were immersed in the solution for 30 seconds and 1 minute. No stained cells were found following these treatments. Parallel controls in river water showed 70% survival.

4.5. Discussion

4.5.1. General evaluation of products tested

Trials using basic cleaning ingredients rather than manufactured products indicated that this may not be the most effective approach. It was surprising, for example, that borax used at a very high concentration apparently had no effect whatsoever. One reason for this may be that the toxicity of some reagents is dependent on other environmental factors. For example, low temperatures can moderate the effects of some toxins (e.g. chlorine (Rajagopal et al. 2002), endothall and diquat (Netherland et al. 2000)). In the present study, we established that *D. geminata* has improved survivability at lower temperatures, therefore all the trials on decontamination reagents were purposely carried out at a relatively low temperature (and in a light environment), in order to determine the maximum survival times. In other words, we allowed for the worst-case scenario.

Water pH or alkalinity can also affect the effectiveness of toxins. In his analysis of boron toxicity, Dyer (2001) listed % mortalities for all organisms at two contrasting alkalinity levels. In that analysis, for boron derived from borax, a lower alkalinity appeared to be associated with higher mortalities in most cases. Since the Waitaki River has even lower alkalinity than that stated by Dyer (2001) (NIWA data), we may well have been recording the worst-case reactions to borax (i.e., *D. geminata* has a very high tolerance for this chemical, at least up to the times and concentrations tested). Water pH is also critical for obtaining maximal effectiveness of chlorine as a disinfectant agent. Thus monitoring of chlorine in swimming pool water is accompanied by pH checks. Differing pH levels alter the nature of the chemical reaction. If pH is too high (>8), the Cl combines with oxygen to form a less toxic molecule, and if pH is too low (< ~7), free chlorine is produced, which is a severe irritant.

The contrast between *D. geminata*'s responses to sodium percarbonate and nappy cleaner highlight that testing the isolated active ingredient in a product may not be indicative of the product's usefulness as a decontamination agent. There are at least two reasons for this. First, as for borax, sodium percarbonate may also have a pH requirement for maximum toxicity, which was not met in our trials. Second, although sodium percarbonate is stated to be the active ingredient in Napisan, other constituents (which make up 75% of the product) almost certainly contribute to the product's effectiveness against *D. geminata*.

The trial on the anionic surfactant, sodium dodecyl sulphate (SDS), confirmed that relatively high concentrations need to be used before it will effectively decontaminate

against *D. geminata*. As mentioned, these surfactants are already known to be more toxic to higher animals than to algae. SDS is also a bulky chemical and a severe irritant to human/mammalian skin, therefore it has limited usefulness in this context, except to demonstrate that it is not likely to be the anionic surfactant *per se* in any commercial detergent that is the active ingredient causing *D. geminata* mortality.

In the present more detailed trials, we had hoped to identify some lower effective concentrations of decontamination reagents currently recommended by Biosecurity New Zealand. Our results suggest that the recommendation for household bleach (sodium hypochlorite) should not change (2% bleach for 1 minute) since a very few cells still took up the stain after a 1-minute exposure to 1% bleach. The stain was light, but otherwise not abnormal, suggesting probable viability (see Appendix 1). The earlier trials (Kilroy 2005) had shown 1% bleach to be 100% effective. The difference may have been due to the lower temperature of the present trials. Unlike the other chemicals tested, longer exposure to lower concentrations did not lead to increasing mortality rates. This is presumably because the chlorine in the solution was used up in the first minute of exposure. The lowest concentration tested was 0.1 % household bleach, which is equivalent to ~8 ppm chlorine. At this concentration 60 – 80% of cells remained viable after 1000 minutes. Since typical swimming pool and drinking water chlorine concentrations are 1 – 3 ppm and 0.2 ppm respectively (see Section 4.2.5), this suggests that soaking in chlorine-treated swimming pool or drinking water will not be effective for decontamination purposes.

The quat-based pool cleaner ("303 Clearall") required surprisingly high concentrations for killing *D. geminata*, given that these cationic surfactants are generally highly toxic to microorganisms (e.g., Nalecz-Jawecki et al. 2003). Clearall is a clear, sticky, viscous liquid that was quite difficult to handle. Information supplied by the retailer states: "303 Clearall is effective at all pH values and compatible with all other swimming pool chemicals. In fact it works synergistically with chlorine, and together they are each more effective at killing bacteria and algae than separately." Instructions on the container also recommend its joint use with a chlorine product to control "black spot" algae. The instructions also imply that a long contact period is necessary, but no times are specified. Complete mortality of *D. geminata* within one minute required a concentration of 1.5%, which is 500 times the strongest dose recommended by the manufacturer. This equates to 0.9% a.i. (active ingredient, using the distributor's stated concentration of 60%). We did not identify precisely the concentration needed for a complete kill within 1000 minutes, but it lies between 1% and 0.1%, or <0.6% > 0.06% a.i. (Figure 15c).

A separate test of a product containing 3% benzalkonium chloride (see Section 4.2.6) (NIWA report DDT06501, also see http://www.unclejacks.co.nz/4.htm) showed that a

4% solution (i.e., 0.12% benzalkonium chloride) achieved 100% mortality of *D. geminata* cells in one minute. A diluted solution of 0.0015% benzalkonium chloride was effective after 1000 minutes.

It is difficult to reconcile the difference in response between the two products with no information about the active ingredient concentrations in 303 Clearall. The high concentration of Clearall needed to kill all cells rapidly suggested that the concentration of active ingredient was low. However 303 Clearall's stated %a.i. of 60% is high.

Sodium metabisulphite produced a disappointing result: even high concentrations did not affect all cells within 10 minutes. Further, a concentration slightly lower than that that was recommended for use at the border by MAF Quarantine (70 000 ppm SO_2 – see Section 4.2.5) had not killed all cells after 100 minutes. However, we noted that the cell contents of the few remaining stained cells at that stage did not look "normal" (i.e., diffuse and misshapen chloroplasts). We assume that there can be a stage between a healthy cell and a dead cell when parts of the cytoplasm may be damaged by the decontamination agent, but some cell vacuoles (where the stain is taken up) still remain intact (see Appendix 1). Whether such cells are still viable is unknown, but the prudent approach would be to assume that they are viable.

Sodium metabisulphite was presumably recommended for use at the border because its application is simple, it does not require immersion of large items in large volumes of liquid (because the active substance is a gas), does not damage fabrics such as neoprene, and does not require rinsing following treatment. For *D. geminata*, our results suggest that, unless very long contact times are possible, sodium metabisulphite may not be the most satisfactory method of decontaminating items that may contain/carry live *D. geminata*.

During the trial on sodium metabisulphite, we did not notice any unduly unpleasant SO_2 fumes, even using our high concentrations, but only a very slight odour. However, some people may be more sensitive than others to the fumes and this potential is clearly highlighted in the MSDS data (Appendix 5).

As expected, 70% ethanol killed all cells within 10 minutes. The few residual stained cells after the one minute exposure were unexpected, but, as noted above for sodium metabisulphite, they did not look healthy, despite taking up the stain. Both 50% and 20% ethanol were effective (within 100 and 1000 minutes, respectively). It is concluded that if ethanol is used as a preservative for *D. geminata* samples, then the recommended concentration of 70% is adequate to also kill all cells rapidly. There is plenty of margin for error because concentrations down to 20% also achieve a 100%

kill if left for 1000 minutes. This time would normally be achieved because samples are generally stored before use.

The three products marketed as "environmental" (non-toxic, biodegradable, natural, etc.) were not particularly effective at killing *D. geminata*. Even the 2% solutions necessary for a 100% kill within 1000 minutes were concentrated compared with other products. A visual examination of the results suggests that Citrus Based Cleaner was the most effective, followed by Simple Green, then B.E.E. surface cleaner. Given that all three products are strongly marketed as being environmentally safe, this result is not particularly surprising. We conclude that use of such products for decontamination defeats their purpose (of being environmentally friendly) because they have to be used in such high concentrations to be effective. This also makes them very expensive: none of these products was cheap in its concentrated form (Table 3). No ingredient information was on the packaging of any of these products. Biosecurity New Zealand already holds ingredient information and MSDS data on Simple Green.

In contrast, the three "ordinary" dishwashing liquids tested required much lower concentrations. The 5% needed in all three cases for complete mortality within one minute is fairly concentrated compared to what might be used to wash dishes. However, a 50 x dilution (i.e., 0.1%) of Down-to-Earth or Sunlight caused 100% mortality of *D. geminata* cells within 1000 minutes. Because 0.1% Palmolive was not completely effective after 1000 minutes, the recommended concentration for prolonged overnight (16 h 40 minutes) soaking of contaminated gear using dishwashing liquids in general would need to be 0.5%. We have no specific information on ingredients but assume that these relatively high quality dishwashing liquids are manufactured using safe and approved ingredients.

It was suggested initially that the lowest common denominator in commercial detergents (dishwashing liquids) should be tested, i.e., the cheapest. The problem with this approach is that often the low cost of a product results from the use of outdated ingredients; in particular they may contain nonylphenol and its ethoxylates (NP/NPE). NPEs have been widely used as surfactants in detergents, but are now known to break down to form the endocrine disruptor NP, which may have long-term harmful effects on wildlife and possibly humans (S. Belanger, pers. comm.). Moves towards restricting the use of NPEs are being debated in some countries, but not in others (see www.aperc.org/docs/qa110502.htm).

Refer to Section 6 for a summary of effective concentrations and exposure times for all products tested, along with information relating to the six factors to be considered when recommending decontamination agents (Section 4.1). The summary also

includes other methods tested (e.g., heat), as well as summary information on products tested independently of this study (see Section 4.5.2, below).

4.5.2. Other products considered

In addition to the products and chemicals tested, we also considered others.

Iodine. Iodine is an excellent anti-microbial agent, but is expensive. Repeated human exposure to iodine can lead to chronic poisoning and birth defects. Occasional exposure to iodine during a hospital stay is not a health risk, but it cannot be recommended for cleaning gear on a regular basis. It is also persistent in the environment.

EDTA (ethylenediaminetetraacetic acid). This is a chelating agent (chelation: reversible binding to a cation (metal ion)) commonly added to detergents to reduce water hardness by binding Ca and Mg ions. It is not directly toxic to algae, though it can affect algal growth by sequestering essential nutrients. EDTA is persistent in the environment, therefore regular use as a decontamination method is not desirable.

Acetic acid (vinegar). Acetic acid is a common ingredient of many household cleaners, such as glass cleaners, car cleaners, householder detergents and cleaners, ironing aids. Its function is normally to neutralise bases. Vinegar (5% to 18% acetic acid) is often recommended as an ecologically friendly household cleaner (for example, see http://www.arc.govt.nz/arc/library/w30650_2.pdf. Its pH is around 3. However, any biocidal effect of acetic acid (or vinegar) results from its low pH. Given that water with pH 4 has little effect on *D. geminata* (see Section 3), the probable effectiveness of vinegar would be marginal, particularly if used on a wet item that could dilute it further.

Information on ability to kill *D. geminata* is also available for several other products. For example, fire control additives have been tested to determine whether the concentrations used in fire fighting are sufficient to mitigate the risk of spreading D. geminata from river to river via fire-fighting equipment (Kilroy 2006). Two commercially available "didymo" decontaminants have also been tested independently (see: http://www.unclejacks.co.nz/4.htm, and NIWA reports for project DDT06501 (in letter format)). In all cases, the tests were undertaken using the same methodology as described for the present study, including maintenance of low temperatures.

4.5.3. How long does it take for decontamination solutions to lose their effectiveness?

Over the summer of 2005 / 06, in some catchments affected by *D. geminata*, public decontamination units were set up at convenient locations, such as petrol stations and motels. A question that arose from this practice was: for how long can a large container of solution be used before it stops being effective? In other words, how often should the decontaminant be replenished? There is no straightforward answer to this because the time depends on many factors, the main ones being:

- the type of decontaminant;
- the number of times it is used, and the surface area of non-absorbent items and the volume of absorbent items cleaned;
- the amount of dirt, especially organic material, that enters the solution;
- the volume and surface area of the solution;
- possibly temperature and exposure to sunlight;
- initial product concentration will also affect the longevity of cleaning effectiveness, though it is assumed that products will be used at the recommended concentrations.

The number of variables involved and the difficulty of monitoring factors such as number of uses and amount of organic material in the solution means that it would be difficult to generalise from any trials to test this. For example, we could set up a typical solution in the laboratory, and "clean" a set number of items over a given time, with regular testing to see if the solution still kills *D. geminata* cells. But how realistic would it be to relate this to a similar solution set up at a different temperature, outdoors, and used for cleaning a wide range of different items? Comments follow in relation to common products.

1. The chlorine in a household bleach solution is volatile: it gradually gases off and the solution loses its antimicrobial properties. The process of chlorine depletion is accelerated by photodegradation (especially if it is exposed to the sun), and by the chlorine demand of the water. Chlorine demand is caused by suspended or dissolved organic material in the water, and by various inorganic ions that are oxidised by the chlorine. Any items dipped into the solution for decontamination also cause chlorine demand. This means that a household bleach solution needs to be changed frequently if it is to remain effective. Depending on usage, this might be once or twice a day, but

the household bleach solution should certainly be renewed at least every two days and more frequently with heavy usage or heavily soiled items. Test kits to determine the chlorine concentration of swimming pools may be useful in determining chlorine strength. It is beyond the scope of this report to review these.

2. Decontamination solutions based on quats should remain active for considerably longer than household bleach solutions because the action of quats is partially catalytic (i.e., the active ingredient damages cells without altering its chemical structure). These compounds do not have a "demand" in the same way that chlorine does, so the solution can be re-used many times. However, over time, microbial action will change the molecules and render them less toxic. This is simply biodegradation taking place. Presumably the rate at which biodegradation occurs depends on environmental conditions (light, temperature). The half life of certain quats can be a matter of hours in wastewater (Scott and Jones 2000). Given that we expect these compounds to last longer than the chlorine in bleach solutions, but that biodegradation will set in quickly in the presence of oxygen, the turnover time should probably be not much longer than for household bleach (e.g., quats solutions should be renewed every two to three days).

3. The sodium percarbonate in nappy cleaner releases hydrogen peroxide when dissolved in water. Hydrogen peroxide gradually loses its strength over time as it decomposes into water and oxygen. The anionic surfactants in nappy cleaner also biodegrade, in the presence of oxygen with widely varying half lives, which can be as fast as one day (Scott and Jones 2000). Nappy cleaner solutions should, in that case, also be renewed on a one to two day basis, and more frequently with heavy usage or heavily soiled items.

Overall, it would seem preferable to take a precautionary approach with regard to decontamination baths. Household bleach solutions should be changed daily, and more often with heavy use. Other products recommended for decontamination will generally be biodegradable, a process that starts as soon as they are in solution and in contact with particulate organic matter. Therefore solutions other than bleach should be renewed at least every other day, and preferably daily.

5. Part 4. Felt-soled wading boots as vectors of *D. geminata*, and determination of effective decontamination methods

5.1. Introduction

Within hours of the initial identification of *D. geminata* from the lower Waiau River, at least one NIWA staff member suggested that the most likely vector – assuming the species had arrived from overseas – was felt-soled wading boots (R.M. McDowall, NIWA, pers. comm.). The suggestion was also made independently at that time from a number of other sources (e.g., Maurice Rodway, Southland Fish & Game, pers. comm.). Bothwell et al. (2006) presented circumstantial evidence linking the rise in popularity of felt-soled wading boots with the start of the expansion of *D. geminata*'s global range in the early 1980s. These boots are now universally popular with anglers. They come in various forms, but the common feature is a thick (~10 mm) outer sole made of dense, felt-like material, which is non-slip in most river conditions. Millions of entwined fibres in the sole make ideal traps for small algal cells. Because of the density and thickness of the felt, the soles, once wet, may take weeks to dry. In a favourable temperature and light environment, the prolonged presence of moisture could enable algal cells to survive for a considerable time. As we have shown in Part 1 of this report, *D. geminata* could also survive for several weeks at low temperatures (< 12 C), given a little light.

The only direct evidence we have to date on the effectiveness of felt-soled wading boots as vectors of algae was an informal trial at NIWA in January 2005. A staff member noticed that the felt soles of his wading boots were still damp after three weeks storage in an open room. Using a stiff toothbrush, we thoroughly brushed material from the soles into a tray, followed by rinsing with tap water. Subsamples of the resulting slurry of silt were examined under a microscope. We found apparently healthy, growing, green unicells (Chlorophyta). No diatoms were seen, but the presence of live green cells strongly suggested potential for live diatoms to be transported by the soles.

The present project had two aims:

1. To compare the efficacy of the felt used in wading boots as a vector of live *D. geminata* with that of other materials commonly worn by recreational river users.

2. To assess the effectiveness of recommended decontamination methods for killing *D. geminata* cells trapped in felt soles.

5.2. Methods

5.2.1. Materials comparison

We obtained second-hand felt-soled wading boots for the trials. The upper parts of the boots were stitched leather with a fabric tongue section. These were compared with more traditional waders with gumboot-style (rubber) boots and neoprene leggings. Thus, for the first part of the trial, we compared four materials: felt soles, gumboot soles and sides, leather boot-style uppers and neoprene. The felt soles used were new soles, which were attached to the boots with waterproof cement. Three or four replicates of each material were included in the trial.

All the materials were exposed to *D. geminata* in a heavily infested reach on the Waitaki River, at Duntroon. Wading boots were worn for approx. one minute of continuous walking around on *D. geminata* colonies. The neoprene was laid on the substrate for 30 seconds and was walked over. Our aim was to contaminate all items thoroughly. All items were then placed in separate plastic bags and transported direct to the laboratories in Christchurch, which took four to five hours. On arrival at the lab, we immediately simulated a typical scenario of rinsing the boots/waders, then leaving them to dry out, except that all items were also individually scrubbed using a hard-bristled toothbrush for four to five minutes per item in an attempt to retrieve algal cell contaminants. To minimize the amount of water used, we rinsed with a fine-spray wash bottle. All washings were collected into labelled containers and allowed to settle overnight in a 12 °C environment. The washed items were transferred to a room with a temperature starting at 5 °C and rising to approx. 15 °C, where they were hung out (waders), or laid on their sides (wading boots). After 36 hours drying, we thoroughly repeated the cell retrieval process, as before, using a new set of clean brushes for each item. Again, the washings were collected into containers.

After 12 hours of settling, surplus water was poured off each sample, and the volume of the residual concentrated sample was noted. This was shaken up to obtain a homogenous solution, and then 1.5 ml aliquots were pipetted into the well of an inverted microscope. We examined each aliquot at a magnification of x125. All *D. geminata* cells were counted from at least 10 fields of view, distinguishing healthy cells (with intact chloroplasts) from dead or empty cells. From the counts, we calculated the numbers of *D. geminata* cells retrieved from each item. Counts from the first retrieval allowed us to compare densities of live cells (per item) that could potentially be transferred. Total counts (live plus dead cells) from the second retrieval indicated the effectiveness of the first retrieval, and counts of live cells indicated the potential for survival of cells within the material.

5.2.2. Decontamination methods

We originally planned to undertake direct trials to compare the efficacy of different reagents for killing *D. geminata* on contaminated felt soles. Sections of sole were to be dosed with *D. geminata* in the laboratory, by vigorous rubbing with freshly collected colonies. Our trials were unsuccessful because it turned out to be very difficult to get enough live cells into the soles using this method. Considerable sampling and microscope effort was needed to find a very small number of cells. As an alternative, we conducted a series of observations using dyed reagents to determine how well they penetrated the felt. The felt soles used were white (Simms Fishing Products, Replacement Felt), so any bright coloration was clearly visible.

Soles were cut into pieces approximately 70 x 50 mm. One side was covered with waterproof sticky tape to simulate the upper surface of the sole stuck to the bottom of the boot. Pieces were first soaked in river water, then some immersed in a dyed solution of 5% nappy cleaner and others in 2% household bleach. After exactly one minute, the pieces were lifted out, turned over and a cut was made cross-wise, so that the interior of the sole could be seen and the level of the dye noted. Since bleach immediately bleached any dyes, the level of penetration was checked by micro-pipetting tiny drops of dye onto the material at various levels. Bleaching of the drops was used to indicate how far the solution had penetrated. Soaking times of 20 minutes were also checked.

Using the same technique and nappy cleaner only, we compared penetration after spraying and soaking.

The potential effectiveness of heat treatment of wading boots was tested by tracking the temperature of hot water following immersion of wet wading boots. The hot temperature trials (Section 2.4.2) showed that 20 minutes exposure to 40 °C will kill *D. geminata* cells. We tested two water temperatures on wading boots: very hot tap water (>55 °C) and hot tap water at approximately the recommended kill temperature (45 °C, see section 2.4.2). Water at each temperature was held in a chilly bin (insulated cooler box) and in a non-insulated plastic bin with a cover. In each case, we used 15 litres of hot water per pair of boots, which was enough to fully cover size 10 wading boots. Prior to the trials, the boots were soaked in cold water in a cool room to simulate a worst-case scenario of treatment immediately after use in cold river water. The cold boots were directly transferred to the hot water after draining for two minutes. Their starting temperature was 4.6 °C. In each case, we measured the starting temperature of the water in the bins just before addition of the boots. After addition, the water was mixed thoroughly around the boots, and the temperature checked at intervals of 1-10 minutes for 40 minutes. Ambient (room) temperature was 17 °C.

5.3. Results

5.3.1. Materials comparison

Numbers of cells counted from each material type are summarised in Table 3. The first cell retrieval yielded high numbers of both live cells and live + dead/empty cells for the felt soles, leather boot tops and neoprene, but very few cells from the gumboots (smooth rubber). The second attempt yielded an average of three times more live cells from the felt soles than the leather boot tops and almost 12 times more than the neoprene, whereas the gumboots yielded no cells (Figure 20). Similar percentages of live and total cells were retrieved from the felt soles and the leather boot tops during the second attempt, but much lower percentages were retrieved from the neoprene.

Table 3. Numbers of *D. geminata* cells per item retrieved from four types of material after exposure in a severely affected river (means of 3 or 4 replicates, with standard deviations in parentheses). The percentages were calculated from the numbers of cells counted in two retrieval attempts combined.

	First Cell Retrieval (after ~ 5 h)		Second Cell Retrieval (after 36 h)			
	Live cells	**All cells**	**Live cells**	**% of live remaining***	**All cells**	**%of total remaining***
Felt sole	11 000 (10 400)	19 500 (7900)	290 (50)	4.1 (2.4)	1880 (820)	9.8 (6.5)
Rubber boot	3.9 (5.5)	23 (33)	0	0	0	0
Leather boot top	14 200 (19 000)	20 200 (20 000)	93 (50)	3.1 (3.4)	1020 (510)	9.7 (8.6)
Neoprene	15 050 (4000)	47 600 (22 300)	26 (19)	0.2 (0.1)	2700 (1930)	5.2 (3.1)

*Percentages calculated as the number of cells counted in the second retrieval as a percentage of the total number of cells from both retrieval attempts.

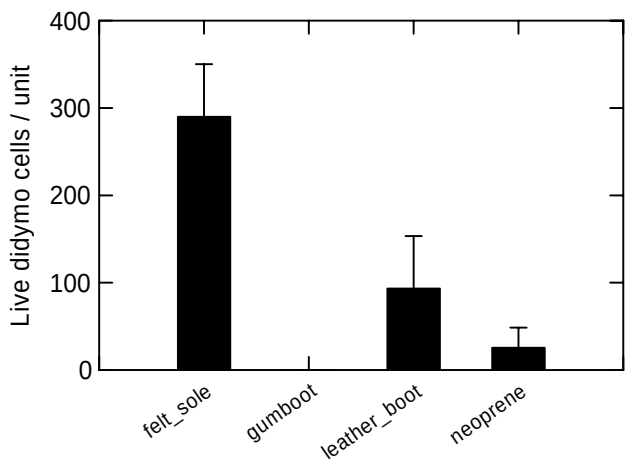

Figure 20. Comparison of live *D. geminata* cells retrieved from four types of material after 36 hours at 5 - 15 °C. n = 4, error bars are standard deviations.

5.3.2. Decontamination methods

The following observations were made:

- After one minute, blue-dyed nappy cleaner had soaked ~6 mm into the ~10 mm thick felt soles, or approximately two thirds of the sole thickness.

- After 20 minutes, the dyed nappy cleaner had penetrated slightly deeper into the sole, but still had not reached the waterproof tape.

- After 20 minutes, the household bleach solution appeared to have soaked in to less than one third of the thickness of the felt (as indicated by dye spots added after cutting the sole open), although there is uncertainty about the accuracy of this method of determination.

- Dyed nappy cleaner sprayed onto the sole surface until the colour was uniform (2–3 seconds) penetrated to approx. 2 mm, or one fifth of the sole thickness.

- In the hot water trials, water in the chilly bin held its temperature well. With starting temperatures of 55.5 °C and 48.5 °C, the temperatures had fallen by 5.5 and 4.5 °C respectively 20 minutes after cold, wet boots were added to 15 litres of hot water (after mixing). After a further 20 minutes, with the chilly bin lid shut, the temperature fell a further 2 °C in both cases, so that the water temperature was still greater than 40 °C after 40 minutes. In the covered non-insulated container (single boot, 7.5 litres of water, same starting temperatures as above), the temperature decreased after 20 minutes to 48 and 38.5 °C, respectively.

5.4. Discussion

The materials comparison showed that, as suspected, felt soles harboured live *D. geminata* cells more successfully than other materials tested. In the short term, just as many live cells were retrieved from the boot tops and neoprene as from the felt soles. However, after 36 hours in a variable temperature regime, we were able to retrieve many more cells from the felt soles. Highest total cell numbers were counted from the neoprene in both washes, simply because the surface area washed was greater, but far fewer (and a much smaller proportion) of live cells survived the 36 hours drying time.

From this, we conclude that in the short term (the four to five hours taken to transport the boots and waders to the laboratory), boot tops, felt soles and waders *all* present a

high risk of transferring *D. geminata* cells if they are not decontaminated. The risk from gumboot waders is far less, but it should be remembered that our contamination time was extremely brief (one minute). Old worn gumboots that may have cuts and irregularities on the soles could potentially pick up many more cells; therefore these boots should be decontaminated using one of the recommended methods, not just washed with water.

After 36 hours, the felt soles barely felt damp, but we were still able to rinse live cells from them that had escaped the first scrubbing. Note that parts of the leather boot tops were still obviously damp, so it was not surprising to find live cells still present on them. Given that the soles can feel dry, yet still be damp inside, it is considered that these pose the greater risk.

Our tests on decontamination agents were preliminary. However it was clear that nappy cleaner soaked in much better than household bleach solution, presumably because of the surfactants it contains.

Soaking was three times more effective at penetrating the dense felt than spraying. Spraying of felt soles cannot be recommended as a decontamination method because a short spray (which is what would generally be applied) barely penetrated past the surface. Recommended contact times and concentrations of any cleaning method will only apply to situations where all of the potentially contaminated material (interior as well as exterior) is in direct contact with the decontamination agent for the full required contact time. Such contact may not be easily achieved for absorbent materials such as felt soles, foam, etc., and therefore soaking for prolonged times will be necessary.

We were unable to test the assumption that *D. geminata* cells could penetrate through the entire depth of the sole. However, if waders are worn in an affected river for long periods, every footstep that comes into contact with algae will force cells up through the felt fibres. Similarly, water flowing past and to a small extent through the soles could wash out cells trapped inside. Therefore, it seems reasonable to assume that the entire sole could become impregnated with cells. If this is the case, then quick and complete decontamination is difficult because our simple dye tests showed that decontaminant solutions do not quickly soak into a wet sole.

Previous decontamination trials in 2005 showed that when *D. geminata* cells and mats dried to a water content of less than 83%, 100% mortality occurred (Kilroy 2005). Complete drying would successfully decontaminate felt-soled waders, but relying upon ambient conditions to achieve this is not recommended in situations where use between waterways is frequent (daily, weekly or even monthly) because not only is it

difficult to determine when the sole is sufficiently dry, boots left standing on their soles, as we have seen, can remain damp for several weeks (see Section 5.1). Drying should only be relied upon as a decontamination treatment if great care is taken to actively and completely dry the felt (such as by using a heat source where temperatures around the felt are assured of reaching 30 °C). Once the felt appears dry, complete dryness must be confirmed by a tactile inspection of the felt pile to the base of the fibres. Once completely dry, the felt sole must remain dry for at least 48 hours before use in another waterway.

Compared to drying, a relatively quick method to ensure the demise of all *D. geminata* cells which may be trapped inside a felt sole is application of a lethal temperature - heating or freezing. For heating to be effective, the deepest interior of the boot would need to be held at or above at least 40 °C for at least 20 minutes (see Table 2). As a precaution, a longer immersion time (40 minutes) at a higher temperature (not less than 45 °C is needed to ensure that all parts of the boot attain at least 40 degrees for at least 20 minutes. As suggested in Section 2.4.2, this could involve placing the boots in a chilly bin with hot water. Our trials showed that heat treatment could easily be achieved using hot tap water (over 48 °C) in a chilly bin, even if the boots were cold (less than 5 °C) and wet when immersed. In many cases, hot water direct from the tap would be hot enough, but any water less than 45 °C (i.e., the lower threshold for the current tap water temperature recommendation – see Section 2.4.2) may not be adequate. Ideally the temperature of the water should be measured at the start and finish of the treatment, to ensure that it is no less than 45 °C for the duration of the treatment. As a rough guide, water at 55 °C and over is too hot for most people to tolerate; immersing a hand into water hotter than 45 °C feels uncomfortably hot. If you can comfortably hold your bare hand in the hot water at the start of the treatment, then the water is probably not hot enough.

If a chilly bin is not available, then a covered plastic container (e.g., a washing up bowl) could be used. In either case, we recommend thorough mixing of the hot water around and in the boots initially and after 10 minutes, with full immersion of the boots for 40 minutes, to allow time for the heat to penetrate. An additional measure would be to soak using a hot solution of 5% nappy cleaner or detergent, in which case the treatment time could be reduced to 30 minutes.

Freezing is straightforward: boots could be placed in a freezer overnight. The entire boot needs to be frozen solid for this to be effective.

Finally, felt-soled waders are effective at transferring live cells because they are thick, dense, and absorbent. This means that cells can easily become trapped within the fibres, which are so closely packed that the material retains moisture for much longer

than smooth, non-absorbent materials. High density also makes the material resistant to soaking with decontaminants. Other materials have similar properties, therefore these findings for felt-soled waders can be extrapolated to cover *any* absorbent material. The thicker and denser the material, the better it will be at holding moisture (and live cells), the slower it will be to dry out and the longer it will take to soak completely with decontaminant solutions.

6. Conclusions

The studies described in this report aimed to provide information to assist Biosecurity New Zealand in decision-making related to preventing or delaying the further spread of the invasive alga *Didymosphenia geminata*.

(1) *Evaluation of the risk of further spread of* D. geminata *associated with a wide range of activities*.
The results of the temperature – light – moisture trials indicated that *D. geminata* is capable of survival outside its natural (river) environment for much longer than previously thought. At low temperatures (~5 °C), we found 70 – 80% survival in samples held immersed in river water with some light after one month, and some cells still appeared to be viable after almost two months. Viability decreased as temperature increased, until at 28 °C, most cells died within a day, regardless of light availability. At the lower temperatures, viability declined faster in complete darkness than in light. The amount of light did not appear to be critical. Models constructed from the data allowed predictions of time for a population to reach 5% viability under different environmental conditions. The longest predicted time was over eight months (5 °C, wet, low light). Even at 20 °C, survival could be up to two months (wet, high light).

Survival of damp colonies depended on time taken for colonies to desiccate. Previous decontamination trials in 2005 showed that when *D. geminata* cells and mats dried to a water content of less than 83%, 100% mortality occurred (Kilroy 2005). With the benefit of over two years of observations on the spread of *D. geminata* in New Zealand since it was first discovered, coupled with the experimental evidence on the lethality of cell desiccation, it can be concluded that natural cell desiccation has likely been responsible for preventing substantial spread throughout New Zealand. While *complete* drying of any risk item would result in sufficient cell desiccation to prevent spread, there are difficulties in providing generic drying recommendations because of the variable time it takes for various items to reach *complete* dryness – inside and out, and the effect of relative humidity on drying times. Errors in judgement and perception on what constitutes “dry” can lead to accidental spread. As an example, an individual may consider their gear to be completely dry, and in fact it may feel dry to many, but to others with heightened senses, the slight smell of odour-causing bacteria,

mould and mildew will indicate otherwise. *D. geminata* does not have a distinctive smell or any other indirect indicator that suggests its microscopic presence. Consequently, a precautionary approach should be taken for drying. The current recommendation that any item be completely dry for 48 hours before use in another waterway remains prudent, considering the risk. Freshwater users who move frequently between rivers are at highest risk of spreading *D. geminata* and must not rely solely on ambient drying as a decontamination method.

The other environmental conditions tested – water salinity and pH – indicated that *D. geminata* may survive for days or weeks in estuarine waters (less than 50% seawater), and in waters with pH between ~4 and 9.5, which covers the pH range of most New Zealand freshwaters. These results may be applied to a wide range of transport scenarios for risk estimation; e.g., survival of passage through bird guts is considered unlikely, due as much to high temperatures and lack of light as to low pH.

(2) *Review of recommendations for decontamination procedures and products*

A summary of all product trials in which 100% mortality of *D. geminata* was achieved is presented in Table 4. For completeness, drying, hot water, freezing and seawater results are also included. The main variable reported is time taken to achieve 100% mortality of *D. geminata*, as assessed using the neutral red staining technique. To assist Biosecurity New Zealand in providing practical recommendations for decontaminating against *D. geminata*, all the effective products and methods were ranked based on their relative effectiveness, and the following additional factors: availability, cost, toxicity/irritation to humans, corrosiveness, possible effect on other organisms, and biodegradability. It is recognised that not all methods will be practical in all situations and users must exercise judgement. Our recommendation is to select the highest ranked methods that are practical for the situation. Regardless of rank, all products and methods recommended in the table are effective provided that the specified contact times and concentrations (if applicable) are used. This means that **all** of the potentially contaminated material (interior as well as exterior) must be in **direct contact** with the decontamination agent for the full required contact time. Such contact may not be easily achieved for absorbent materials such as felt soles, foam, etc., and therefore soaking for prolonged times will be necessary.

It should be noted that the results reported in parts 1 and 2 of these trials (see sections 2 and 3 – temperature–light–moisture trials and pH / conductivity trials) apply only to survival of *D. geminata* in still water. They should not be extrapolated to running water environments, which have very different physical and chemical conditions. In still water however, it is clear that *D. geminata* mortality increases with temperature. Therefore chemical decontamination will be more effective than the results of these studies suggest if treatment is carried out at warmer temperatures. The detrimental

effect of higher temperatures on *D. geminata* survival should, if possible, be incorporated into decontamination procedures.

The trials on felt-soled wading boots showed the felt soles do appear to present a greater risk of transfer of *D. geminata* than the other types of materials tested. However, in the short term (a few hours), boot tops, felt soles and neoprene waders *all* present a high risk of transfer if they are not decontaminated. Decontamination tests on felt soles (using wet boots) indicated that (a) soaking in a product containing surfactants (e.g., nappy cleaner, dishwashing liquid) was more effective than bleach, (b) soaking was superior to spraying, and (c) complete penetration of the soaking solution into the entire thickness of the sole was uncertain. Heat treatment may be more effective, and it was demonstrated hot tap water (≥45 °C – uncomfortably hot to touch) in an insulated container can remain hot enough for 40 minutes to ensure that all parts of the boot attain the minimum killing temperature, even if cold, wet boots are immersed. Adding nappy cleaner or dishwashing liquid provides additional decontaminating power. An alternative treatment is freezing.

Complete drying would decontaminate felt-soled waders; however reliance solely on ambient drying is not recommended where use between waterways is frequent (daily, weekly or even monthly) because not only is it difficult to determine when the sole is sufficiently dry, but boots left standing on their soles, as we have seen, can remain damp for several weeks (see Section 5.1). Drying should only be relied upon as a decontamination treatment if great care is taken to actively and completely dry the felt (such as by using a heat source where temperatures around the felt are assured of reaching 30 °C). Once the felt appears dry, complete dryness must be confirmed by tactile inspection of the felt pile to the base of the fibres. Once completely dry, the felt sole must remain dry for at least 48 hours before use in another waterway.

Any absorbent material will present a similar elevated risk as felt soles. We recommend highlighting this risk in future decontamination instructions for the public.

Table 4. Comparison of the effectiveness of methods and products tested on *D. geminata* and their rank according to operational suitability for compliance with Biosecurity New Zealand's Check Clean Dry public awareness campaign to reduce the spread of the alga.[1]

Method or product, with units[1]	Typical price and package size	Level or concentration	1. Time to 100% mortality (minutes, unless stated)	2. Price per litre of solution for 100% mortality[2]	3. Availability[2]	4. Irritant to skin / throat / etc.[3]	5. Corrosive to metals, rubber, etc.[3]	6. Toxicity to other organisms[3]	7. Biodegrad ability[3]	Rank[4]
Drying[5]	N/A	< 83% moisture	Varies; ≥ 48 h	N/A	N/A	N/A	N/A	N/A	N/A	**3**
Heat (hot water)	N/A	45 °C 60 °C	20 1	N/A	N/A	medium medium	N/A	N/A	N/A	**1** **2**
Freezing	N/A	-2 to -15 °C	Need to freeze solid	N/A	N/A	N/A	N/A	N/A	N/A	**1**
Seawater	N/A-	50 (~1.6% w/v NaCl) 100 (~3.1% w/v NaCl)	> 30 days 4 h	N/A	N/A	N/A	medium	N/A	N/A	**3**
Salt (NaCl) (% v/v)	$2.00 / kg	2 (~4% w/v NaCl) 5 (~10% w/v NaCl)	10 1	8 ¢ 20 ¢	supermarket	low	medium	N/A	N/A	**7** **8**
pH (hydrochloric acid) pH (lime @ 1000 mg / l) pH (lime @ 400 mg / l)	-	1 11.9 10.8	<5 80 24 h	-	-	-	-	-	-	**-**
Sodium percarbonate	$120 / 25 kg	0.1 2	1000 100	0.5 ¢ 24 ¢	specialist	medium	high	low	N/A	**6** **13**
Napisan (% v/v)	$6.68 / kg	0.5 5	1000 1	3.4 ¢ 33.5 ¢	supermarket	low	medium	medium	high	**7** **11**
Sodium dodecyl sulphate (% w/v)	$81.00 / 500 g	0.1 0.5 1	1000 100 1	16.2 ¢ $1.62	specialist	medium	low	low	high	**9** **14** **12**
Household bleach (% v/v c/ ≥ 35 g/L NaHCl)	$~2 / litre	0.5 1 2	> 1000 (not effective) 10 1	- 2 ¢ 4 ¢	supermarket	- low? medium	high	high	N/A	**-** **6** **6**
303 Clearall (quat mixture) (% v/v)	$83.31 / 5 litres	1 1.5	10 1	16.7 ¢ 25 ¢	specialist	medium	medium	medium	medium	**10** **12**
Sodium metabisulphite ('000 ppm SO_2)	?	50 100	1000 100	-	specialist	? ?	medium	medium	N/A	**-**
Ethanol (% v/v)[6]	?	20 50 70	1000 100 10	-	specialist[6]	-	high	low	high	**-**
Citrus based cleaner (% v/v)[7]	$80.50 / litre[7]	2 5 10 100	1000 100 10 1	$1.61 $4.25 $8.50[7]	mail order	low	low	low?	high?	**18** **16** **15** **15**

BEE all purpose cleaner (% v/v)	$6.00 / 500 ml	2	1000	24 ¢	supermarket	low	low	medium	high?	**12**
Simple Green (% v/v)	$12.68 / litre	2 50	1000 1	25 ¢ $6.34	supermarket	low	medium	medium	medium?	**14** **14**
Down-to-Earth dishwashing liquid (% v/v)	3.49 / litre	0.1 1 2 5	1000 100 10 1	0.4 ¢ 17.5 ¢	supermarket	low	low	medium	medium?	**4** **5** **6** **6**
Palmolive dishwashing liquid (% v/v)	$3.81 / 900 ml	0.5 2 5	1000 10 1	2.1 ¢ 8.4 ¢ 21 ¢	supermarket	low	low	medium	high	**5** **6** **7**
Sunlight dishwashing liquid (% v/v)	$3.21 / 900 ml	0.1 5	1000 1	0.4 ¢ 17.8 ¢	supermarket	low	low	medium	high	**4** **6**
Virkon[8] (% w/v)	$7.00 / 50 g	1	1	$1.40	specialist	high?	medium	medium	low?	**14**
Uncle Jack's (3% benzalkonium chloride) (% v/v)[8]	$10 / litre	0.1 4 (soak) 100 (spray)[9]	1000 1 1	1 ¢ 40 ¢ $10.00	specialist	medium	medium	medium	high	**7** **13** **17**
Snot-off (% v/v)[8]	$25.00 / 500 ml	0.1	1	5 ¢	specialist	medium	low	medium	low	**7**
Firetrol (fire retardant) (% v/v)[8]	N/A	6	2	-	-	-	-	-	-	**-**
Hydroblender soap (% w/v)[8]	N/A	0.03	< 36 h	-	-	-	-	-	-	**-**
Fire suppressant foam (% v/v)[8]	N/A	0.3	< 12 h	-	-	-	-	-	-	**-**

[1] All products were tested in the temperatures range 5 – 9 °C. Summary results from methods and products tested in previous trials are included for comparison (see section 4.5.2 for more details).
[2] Assessments of price and availability are based on the experimenter's findings at one place (Christchurch) and time (mid 2006), and therefore may vary for other locations and times.
[3] Relative qualitative assessments of irritation, corrosiveness, non-target toxicity and biodegradability are based on Material Safety Data Sheets (see Appendix 4) and comprehensive knowledge by review experts of the scientific literature in algal toxicology and industrial detergent chemistry.
[4] Ranking system**:** rank scores were applied to columns 1 to 7, with lowest scores applied to favourable properties, e.g. fastest time to mortality, lowest relative price, easiest availability, etc. The final rank is taken from the sum of the scores for products that have complete information in the columns numbered 1 to 7. Where the criterion was inapplicable to a method (signified by N/A), the lowest score was applied. A question mark after a relative assessment implies a "best guess". Ranking criteria are discussed in Section 4.1. A dash (-) means inappropriate for ranking.
[5] Effective drying times will vary due to the properties of the risk good (density, porosity, 3-D structure) and ambient conditions (temperature, light, humidity). A precautionary recommendation is that items must be completely dry for at least 48 hours before they can be safely used in another waterway; e.g., if an item takes five days to dry, seven days must elapse before the item can be safely used.
[6]A special license is required to purchase pure ethanol.
[7] Much lower prices apply when purchased in bulk.
[8] Tested by NIWA in previous independent trials.
[9] Spraying is NOT recommended for decontaminating risk goods which are porous or absorbent.
NOTES: A. The rankings are based on the criteria listed in Section 4.1 and it is recognised that not all methods will be practical in all situations. Our recommendation is to look for methods that are practical for the situation, with the best ranking. Regardless of rank, all methods are effective provided that the specified contact times and concentrations (if applicable) are used.
B. Contact times and concentrations apply to situations where **all** of the potentially contaminated material (interior as well as exterior) is in **direct contact** with the decontamination agent for the contact time. Such contact may not be easily achieved for absorbent materials (felt soles, foam) and therefore soaking for prolonged times will be necessary. Refer to Sections 5 and 6 for further discussion.

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Appendix 1. The neutral red staining technique for distinguishing viable from nonviable cells: a review and evaluation

Introduction

An early challenge in our work on the invasive diatom (alga) *Didymosphenia geminata* in New Zealand was identification of a method for distinguishing live cells from dead cells. Such a method was essential for evaluation of decontamination reagents, and for assays in experiments to assess the effectiveness of potential control products. The neutral red staining technique (Crippen and Perrier 1974) was trialled as a possible method in the early stages of the research programme, following advice from Dr Max Bothwell (Environment Canada and Biosecurity New Zealand Didymo Technical Advisory Group Member) (Bothwell et al. 1989). The method proved to be simple to apply, did not require specialised equipment (other than a good microscope), yielded immediate results, and appeared to work well with *D. geminata*. Trials showed that live *D. geminata* cells took up the stain within 10-15 minutes of contact with a diluted solution. The stain was clearly visible under the microscope, even at low magnifications of 200 x or even less, and appeared as deep crimson-purple spots or granules scattered throughout the cell (see photographs,Section 2.4.1, and Figure A1.1). Often the granules appeared to be oscillating (presumably Brownian motion – random motion of tiny particles suspended in a fluid). Heat-killed controls did not take up the stain at all. The technique has therefore been used in all subsequent trials of reagents for decontamination and control purposes (Kilroy 2005, Jellyman et al. 2006 a,b), and also in the survivability experiments described in the main part of this report. Observations on the condition of the cell contents (chloroplasts) have helped to corroborate the results of neutral red assays.

During extensive use of the technique for a range of trials with *D. geminata*, we have been satisfied that the technique has produced unambiguous results on most occasions. However, we have observed some inconsistencies in the appearance of stained cells that have made interpretation more difficult. This review aims to provide further information on neutral red staining including its mode of action, applications and methods of usage. The intention is to obtain some understanding of the reasons for and the implications of our ambiguous results. Information has been collated from literature searches on neutral red, and on mechanisms and pathways to cell death in algae and other organisms. An evaluation is provided of the effectiveness of the neutral red technique for assessing cell viability in *D. geminata*.

Methods for assessing cell viability: a quick overview

Assessment of changes in algal cell or population viability is required in many ecological and toxicological studies. In ecological studies, live diatoms are often

determined by a visual assessment (under the microscope): live cells are taken to be those containing intact or near-intact chloroplasts, while dead cells are those comprising only the outer silica cell wall (termed the frustule), or with very reduced cell contents (e.g., Brussard et al 1998). When whole algal communities are being investigated, methods may include determination of changes in dissolved oxygen (DO, a measure of active photosynthesis), measures of chlorophyll *a*, and direct measures of photosynthetic activity using pulse amplitude modulation (PAM). Methods used in toxicology are similar. A standard approach for assessing effects of toxicants is to measure changes in growth rates using direct cell counts using flow cytometry (Hall and Cumming 2003), sometimes combined with direct fluorescence measurements (Aidar et al. 1996). Alternative indirect spectrophotometric methods may be used (Ma et al. 2002). For tests on whole communities, DO and chlorophyll *a* may be measured, as well as counts of intact cells (Belanger et al. 1996).

Staining techniques provide an additional means of distinguishing live cells from dead cells. Vital stains comprise a group of reagents that are taken up by living cells only and are non-toxic, at least in the short term (Crippen and Perrier 1974). For example, the fluorescent stain FDA (fluorescein diacetate) is commonly used in microscopic counts of a range of types of algae and bacteria (Li et al 1996) and in more automated methods using spectrophotometry (Amano et al 2003). FDA is taken up by live cells and converted to the fluorescent compound fluorescein, which can be viewed under a microscope equipped with the appropriate optics. Other stains are taken up by dead cells but not by live cells (e.g., Evans Blue, Gallagher 1984). For greater precision in distinguishing cells that are still viable from those that are truly dead, combined techniques have been devised, e.g., combining enzyme and staining assays (Agustí and Sanchéz 2002).

What is neutral red?

Neutral red (also called toluylene red) is 3-amino-7-dimethylamino-2-methylphenazine hydrochloride, with the chemical formula $C_{15}H_{17}N_4Cl$. It is a weak cationic dye. The raw chemical is a black metallic-looking powder that is very soluble in water and ethanol, producing a deep crimson solution. Between pH 6.8 and 8 the red colour changes to yellow, hence neutral red is a pH indicator. Neutral red is also a vital stain. It is taken up by living cells without immediate adverse effects on cell function.

Applications and mode of action

Applications of neutral red staining range from veterinary to medical to ecological. For example, in the veterinary field, the stain has been used to assess the effectiveness of antifungal agents (Fukuda et al. 1996). In medicine it has been identified as a

potential vital stain in eye surgery (Jackson et al. 2005), and used in evaluation of the effects of laser therapy (Hawkins and Abrahamse 2005). In bacteriology it has been used to confirm the presence of certain bacterial strains (Jonsson 1989). Applications in ecology include estimation of patterns of viability over time in marine phytoplankton (Gallagher 1984). Neutral red is the basis for a cytotoxicity assay (Babich and Borenfreund 1992), in which it is applied as a supravital stain in human toxicology tests (e.g., Babich et al. 2005). Supravital staining is a procedure in which living tissue cells isolated from the body are placed in a nontoxic dye solution so that their vital processes may be studied. Some applications directly utilise the pH indicator property of neutral red, e.g., in investigations of membrane transport in the alga *Chara corallina* (Berecki et al. 2001). The stain has also been used to track the condition of animal cells in ecotoxicity tests (Zurita et al. 2005), and in cytological studies on algae (Becker and Hickisch 2005).

The following description of the mode of action of neutral red is taken from www.ib.amwaw.edu.pl/invittox/prot/64.htm. "Neutral red (NR) is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells." This description refers specifically to a cytotoxicity assay used for animal cells (Babich and Borenfreund 1992).

Lysosomes are organelles within cells, with the functions of food and waste digestion. They are enclosed within a membrane and contain enzymes in acidic conditions. Some biology textbook descriptions place lysosomes only in animal cells (e.g., see: Cell biology article in Wikipedia, McGraw Hill Science and Technology Encylopedia); plant cells (including diatoms) logically have a lower requirement for such organelles as they obtain food autotrophically (building up molecules through photosynthesis). However, other descriptions place lysosomes in all eukaryotic cells (e.g., see "lysosome." Encyclopædia Britannica. 2006. Encyclopædia Britannica Premium Service. www.britannica.com/eb/article-9049557). Note that eukaryotic cells are cells that contain discrete membrane-bound organelles, and are distinguished from prokaryotic cell that have no organelles. The latter include bacteria and cyanobacteria, with all other plant and animal cells eukaryotic.

The definition of lysosomes from www.biology-online.org is more broadly based: "a class of morphologically heterogeneous cytoplasmic particles in animal and plant tissues characterised by their content of hydrolytic enzymes and the structure-linked latency of these enzymes. The intracellular functions of lysosomes depend on their

lytic potential *[i.e., their ability to cause cell death by lysis - rupture of cell membranes and loss of cytoplasm]*. The single unit membrane of the lysosome acts as a barrier between the enzymes enclosed in the lysosome and the external substrate. The activity of the enzymes contained in lysosomes is limited or nil unless the vesicle in which they are enclosed is ruptured. Such rupture is supposed to be under metabolic (hormonal) control."

Whatever they are called, membrane-enclosed organelles with acidic contents are known to occur in diatoms (Vrieling et al. 1999). These include microbodies, lysosomal active vesicles and silica deposition vesicles (Vrieling and Lee 2002). A classical description of the cellular structure of the diatom *Nitzschia palea* indentified circular vesicles and "special" vesicles (Drum 1963), which may be interpreted as lysosomal-like bodies. Membrane-bound acid-filled organelles in diatoms would be expected to take up and precipitate neutral red in the same way as described for animal cells by Babich and Borenfreund (1992). Ehara et al. (1996) studied the uptake of neutral red into vacuoles of the large green alga *Micrasterias pinnatifida* (a desmid), which, as we observed in *D. geminata*, accumulated the stain as dark red precipitated granules in the vacuoles. These authors propose explanations for the transport of neutral red into cell vacuoles and its retention there under different conditions, which are largely consistent with our observations and are discussed below.

Anomalous results in *D. geminata* experiments

As mentioned, in most of our trials neutral red staining produced unambiguous results. Cells either stained with many small dark granules throughout the cell, or they did not take up the stain. However, there were some exceptions. Our observations fell into one of the following nine categories. In subsequent discussion, these categories will be referred to by the letters **A** to **I**. Some examples are shown in Figuure A1.1, labelled according to these letters.

The three expected results were:

A. Cells that took up the stain generally had more or less normal chloroplasts, though often there was slight shrinkage. Such shrinkage was also reported by Crippen and Perrier (1974).

B. In cells that did not take up neutral red, if chloroplasts were present they appeared to be damaged. As indicated in the main report, chloroplast appearance was often characteristic for the treatment being applied. We observed changes in colour, loss of definition of the margins, gross changes in shape, shrinkage, and rupture of the chloroplasts.

C. Most populations contained some empty frustules. These had no cell contents and did not stain. Very few were observed in the short term trials. In the longer term trials, increasing numbers of empty frustules were counted as the experiment proceeded, especially after about 30 days.

Anomalous results were:

D. Cells took up the stain as dark granules (to a greater or lesser extent) but the chloroplasts were clearly affected by the treatment. In these cases, stained granules were present, but the chloroplasts were severely contracted and misshapen, and often displaced to the side of the cell. For example, this was the case in a few cells after short-term (1 minute) exposure to 70% ethanol, after longer-term (24 h or more) exposure to 50% or 100% sea water, and after exposure for variable times to some of the detergents tested. These cells were counted as stained (viable).

E. Cells did not take up the stain, but the chloroplasts appeared to be normal, with well defined edges and the typical shape seen in healthy, untreated cells. This was the case in cells exposed to pH 4 for up to 2 days. These cells were counted as unstained (non-viable).

F. Cells took up the stain, but the granules were paler and sometimes larger than those seen in typical healthy controls, with an "oily" rather than a granular appearance. This was most often seen in the dark treatments, in low concentrations of some of the detergents, and occasionally after exposure to high pH. Chloroplasts often contracted. These cells were counted as stained (viable).

G. Cells took up the stain, but instead of dark granules visible through out the cell, the granules were concentrated in particular areas, or in streaks, sometimes as if clustering at the edge of a large vacuole. Brownian motion was generally absent. Chloroplasts were usually somewhat contracted. This was seen from time to time in both the long-term experimental treatments, and in the treatments involving chemicals. These cells were counted as stained (viable).

H. Cells took up the stain as a solid orange-red colour filling the entire cell, and not as granules. This response was seen only in cells in the 5 and 12 °C dark treatments, after about 30 days. These cells were counted as unstained (non-viable).

I. In a very few cases, cells contained stained granules, but few or no other cell contents.

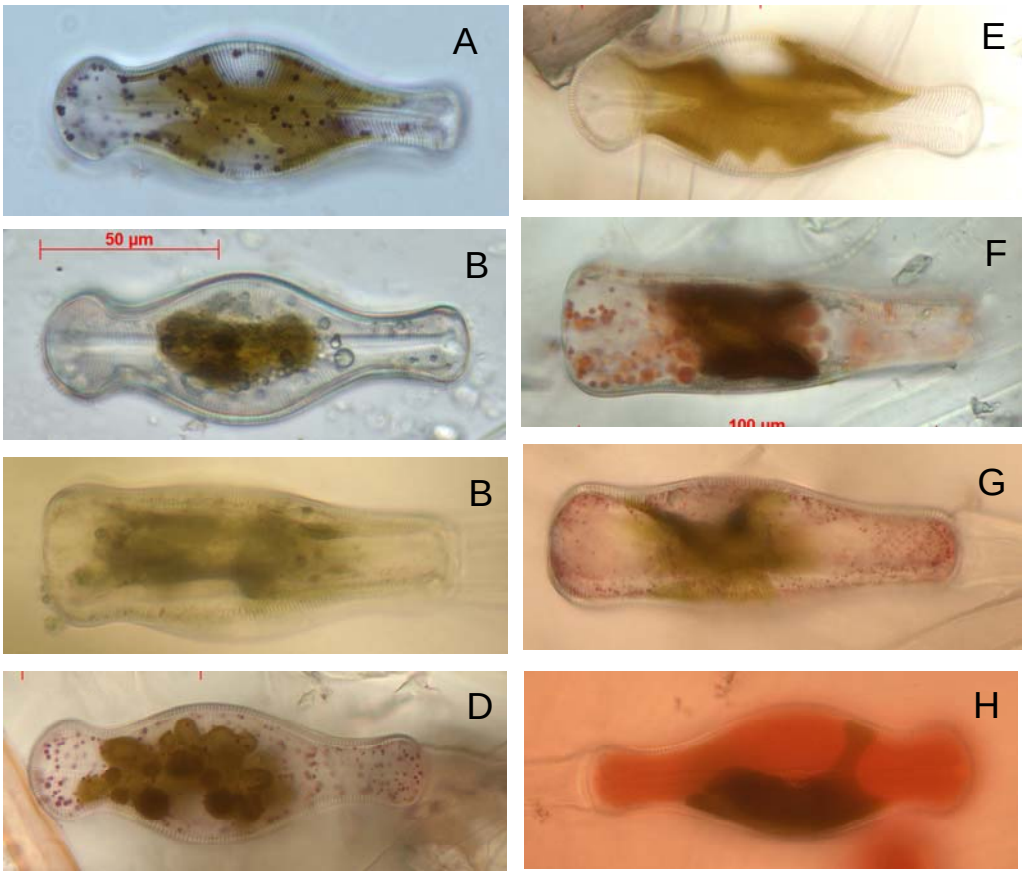

Figure A1.1. Examples of the categories of staining observed during trials with *D. geminata*. See text for explanation of each letter. Note that cell A was motile and therefore definitely alive. This was the typical appearance of live cells.

Cell death, chemical changes and the uptake of neutral red

In this section we attempt to reconcile the appearances of cells in our experiments (categories **B** to **I** above) with the processes taking place within the cells. (Category **A** is explained in the foregoing discussion about uptake of neutral red by live cells.)

It is well established that cell death in multicellular organisms takes place via a number of mechanisms. A major division is between accidental cell death (necrosis – death from damage) and programmed cell death (PCD – self-destruction of cells by the organism, as a normal part of the life cycle or in response to infection, nutrition or light stress, etc) (Bidle and Falkowski 2004). Several mechanisms of PCD are recognised, which differ between plants and animals (van Doorn and Woltering 2005). The existence of PCD in unicellular organisms has been increasingly recognised in the last decade (Kirchman 1999), although transfer of the concept of PCD to unicellular organisms presents some semantic difficulties (Franklin et al. 2006). However, for this

review we are merely interested in what happens within the cell when cells die. There are at least three possible pathways. These are discussed below, with comments on the neutral red stain responses in our experiments on *D. geminata*, and reference to the nine categories listed above.

1. Marine diatoms under stress (e.g., nutrient or oxygen stress) have been shown to undergo lysis, or rupture of cell membranes and leakage of the cytoplasm from the cell (Brussard et al. 1997). The appearance of increasing numbers of empty cells over time in the long-term trials suggests that cell lysis under stress has taken place. Cells retaining some cell contents at this stage, but not taking up the stain generally had reduced chloroplasts, suggesting that lysis was underway (category **B**). Increasing numbers of empty frustules appearing in the later stages of the long term experiments indicates increasing completion of cell lysis (category **C**).

2. In non-lysosomal PCD in plants, the cell contents gradually shrink, but the lysosomes and other organelles remain intact until a late stage of the process (van Doorn and Woltering 2005). This process has not been demonstrated in diatoms, but may be what we are seeing in category **D**. At what point the process is irreversible is unknown, and all cells with granular stains were counted as live cells. Category **I** cells may also be explained by this process: these were counted as dead, despite some staining, since they clearly could not be viable with no chloroplast.

3. In autophagy, the lysosomes rupture and internal cell membranes are rearranged until the entire cell is filled with a membrane-enclosed vacuole (van Doorn and Woltering 2005). It is possible that this corresponds to what we saw in category **H**. These cells were deemed to be dead (unstained) because their mode of stain uptake was atypical and in all cases the chloroplasts were severely reduced.

Thus different pathways to cell death might explain our categories **B**, **C**, **D**, **H** and **I**. Categories **E**, **F** and **G** may be explained by environmental or physiological factors altering the uptake of neutral red.

Ehara et al (1996) observed differences neutral red uptake in *Micrasterias pinnatifida*, which depended on the pH of the external medium. The stain precipitated and formed dark red fibrils (granules) within the cell vacuoles if the cells were cultured in pH 8 but not in pH 5. They explained this by the ionization state of neutral red at different pH levels. In low pH (acid) conditions it is strongly ionized (i.e. it carries positive charges and can therefore bind to other ions), but in alkaline conditions (pH > 7) is non-ionized. Non-ionized molecules pass through membranes much more efficiently

than ionized molecules. However as soon as the non-ionized neutral red molecules pass through vacuole (lysosome) membranes into the acidic conditions inside, they becomes ionized and cannot pass back through the membrane. Hence the stain accumulates in the vacuoles (Ehara et al. 1996). The failure of *D. geminata* cells held in a pH 4 solution to take up neutral red (category **E**) therefore precisely corresponds to these earlier observations. Neutral red uptake in our pH 4 treatments presumably resumed because photosynthesis in *D. geminata* gradually raised the pH of the treatment solution (Davies-Colley and Wilcock 2004).

Category **F**, pale stain as opposed to deep red granules, may also stem from the interference of some chemical treatments with the passage of neutral red through organelle membranes, and possibly with associated internal pH elevation that reduces the red colouration of the stain (Ehara et al. 1996, Berecki et al. 2001).

Finally, the most likely explanation for category **G** is over-long exposure to the stain. While we aimed to standardise staining time to 15-20 minutes, the necessity for examining slides immediately meant that there was some variability. There was also a variable delay before prepared slides could be examined. Although neutral red is a vital stain, it is eventually lethal to cells (Crippen and Perrier 1974). Vdovenko (2000) studied morphological changes in the parasite *Blastocystis hominis* following neutral red staining and concluded that development of larger vacuoles in cells was due to degenerative changes as the cells died.

A further consideration is whether diatom cells will form resting cells or spores in response to experimental treatments (see section 2.4.1 in the main report). Gallagher (1984) noted that cells of the marine diatom *Skeletonema costatum* in culture could often be in a resting state, under which they did not take up neutral red. This was established by the parallel use of another stain, Evans Blue, which was taken up by dead cells in which the membrane is porous, but not by viable cells with an intact membrane. In this case therefore, the neutral red assay was not distinguishing live cells from dead cells, but active cells from inactive cells (which could be either dead or in a resting state). This is a very important distinction but is only relevant for *D. geminata* if this species forms resting cells. As discussed (section 2.4.1, see Figure 6b) we found evidence that *D. geminata* may form resting cells in some circumstances (e.g., in prolonged darkness at low temperatures). Previous studies have also found that diatom resting cells formed during light deprivation revert to their vegetative state within 3-4 days of exposure to light and a temperature suitable for growth (Sicko-Goad et al. 1989, Peters and Thomas 1996).

Evaluation of neutral red assay

It is concluded from the above discussion that the mode of action of neutral red can be explained with reference to the physiology of cells and their organelles, and to the changes that that take place when cells die. The anomalous result in the trials described in the main part of this report can also be explained reasonably well. The important question is: how confident can we be that our counts of percentage viability in *D. geminata* populations based on stained (live) vs. unstained (dead) cells actually reflect the real viability of that population?

For each individual cell counted there are four possible outcomes:

- the cell has taken up the stain and is alive;
- it has taken up the stain and is dead;
- the cell has not taken up the stain but is alive;
- it has not taken up the stain and is dead.

The third scenario is of most concern since this could lead to a false negative (or Type 1 error in statistical terms): we count the cell as dead when in fact it is alive. The four scenarios are summarised in Table A1.1, linked to the nine categories of cells observed in our trials.

From Table A1.1 it is clear that use of the neutral red staining technique does present some risk of both false positives and false negatives. However, it should be stressed that these occurrences are outnumbered by orders of magnitude by unambiguous counts. If ambiguous results are documented during the counting process (as has been done throughout these trials) then they can be considered separately and possibly explained. As we have seen, noting anomalous results in the pH 4 trials has enabled us to find a precise explanation for the result, and to confirm that this was indeed a false negative, as was suspected. Any future such results can be interpreted in the same way if low pH in the medium is confirmed. *D. geminata*'s apparent ability to form resting cells is also a potential source of false negatives. However, the appearance of such cells is very characteristic (Figure 6b). As long as analysts are aware of this potential then cells can be identified as probable resting cells and counted as viable.

Table A1.1 Matrix of possible outcomes of microscopic examination of cells stained with neutral red, with notes on the observations made during viability counts of *D. geminata*.

	Cells live	**Cells dead**
Cells stained **(all declared live, except categories H and I)**	**Category A** *Expected result* Cells stain throughout with multiple dark granules, often showing Brownian motion. Chloroplasts normal (well defined margins and typical shape) or slightly contracted. **Category F** Pale staining may indicate the progression of normal physiological processes. **Category G** If the stain is left for too long, then granules may cluster and become streaky.	**Category D** *Possible false positive* Cells take up the stain in granules, more or less as normal, but chloroplasts abnormal. Cells probably in the process of dying, but staining technique does not provide an absolute threshold line. **Category F** *Possible false positive* Very pale staining could indicate the effect of a chemical on membrane transport, which may eventually be lethal. **Category H** Solid red colour throughout cell was interpreted as dead because chloroplast was severely reduced. **Category I** Interpreted as dead, despite presence of stained granules, because no chloroplast present.
Cells unstained **(all declared dead)**	**Category E** *Possible false negative* Cells unstained but otherwise normal, due to the effect of low external pH blocking neutral red transport into vacuoles. **Category B** *Possible false negative* Condensed cell contents: possible "resting" cells.	**Category B** *Expected result* No stain, damaged or reduced chloroplasts. **Category C** *Expected result* Empty frustules.

False positives are possible in other commonly used staining techniques because of difficulty in establishing a threshold that divides viable cells from nonviable cells (Agustí and Sanchéz 2002). There is a continuum from a fully alive cell to a completely dead cell, which requires more complex methodology to define. For example, Agustí and Sanchéz (2002) used a multiple step method to achieve unambiguous differentiation between live and dead cells. The method was as follows:

1. Addition of an enzyme (DNAase) to quantitative phytoplankton samples and incubation for 15 minutes at 37 °C, to break down the DNA in any dead or damaged cells;

2. Addition of Trypsin, for hydrolysis of phospholipids, with a further incubation for 30 minutes at 37 °C;

3. Addition of a Trypsin inhibitor to halt the Trypsin digestion.

4. Undigested cells left after this digestion process were considered to represent live cells (with intact membranes). These could be enumerated quantitatively under the microscope, and percentage viable cells calculated from counts undertaken on undigested control samples.

Complications were that digestion rates were very sensitive to enzyme concentration, and to temperature. Also phytoplankton samples needed to be quantitative to ensure that all replicates were comparable.

The above is presented simply as an example of a method that could have been used instead of the neutral red method in an attempt to avoid false positives or negatives – i.e., judging cells to be alive when in fact they are dead, or vice versa. The description highlights the complexity of such methods compared with a simple vital staining technique that allowed many tests to be undertaken in a short time-frame. Agusti and Sanchéz (2002) pointed out that the digestion method avoids misleading counts resulting from physiological interactions with the stain (such as we have found with pH and neutral red), as well as unambiguously separating live cells from dead cells. Nevertheless, they found almost perfect agreement between their digestion method and use of the commonly used stain fluorescein diacetate (FDA).

A further alternative to reduce the possibility of false positives and negatives would have been to undertake parallel estimates of *D. geminata* viability using a second staining technique. We tried one option, Evans Blue, which could be termed a mortal stain rather than a vital stain because it is taken up through the compromised membranes of dead or dying cells, but is not taken up by live cells (Gallagher 1984) [Evans Blue is almost identical to and works in the same way as Trypan Blue, which appears to have wide medical research applications.] Gallagher (1984) was able to quantify numbers of resting cells in planktonic diatom populations by using both neutral red and Evans Blue on parallel samples. Unfortunately, in our trials *D. geminata* failed to stain satisfactorily with Evans Blue. Stained and unstained cells were not distinguishable under the microscope. It has been noted in many studies that all species do not take up all stains in the same way (e.g., Crippen and Perrier 1974, Agusti and Sanchéz 2002).

From a risk assessment point of view, false positives are not a major problem. They represent an overestimation of the numbers of live cells in a sample, and therefore the attachment of more risk to a treatment than is really warranted. The results of the

chemical trials where false positives are suspected indicate that the risk, if the positives were correct, is small. For example, the very small numbers of stained cells found after short exposure to 70% ethanol were probably not viable. After a slightly longer exposure time, 100% mortality becomes definite (see Figure 13b).

Summary and conclusions

- The vital stain neutral red has long had applications in many branches of biology and new applications continue to be described. Its usefulness rests on the ease with which it is taken up by living cells (resulting in a red stain), and inability of dead cells to take up the stain.

- Use of neutral red for distinguishing between live and dead cells of *D. geminata* in trials on the survivability of this organism is consistent with other applications of the technique.

- A review of the mode of action of neutral red, and pathways to cell death has helped to explain anomalous observations during *D. geminata* survivability trials. These anomalies include both false negatives (cells not taking up the stain when they are in fact alive) and false positives (cells taking up the stain when they are most likely dead).

- In order to supply accurate information for risk assessments, false negatives must be avoided. False negatives as a result of low pH in the medium surrounding *D. geminata* colonies are easily recognisable because the cell chloroplasts remain in a healthy condition, and the pH of the medium can be checked. False negatives resulting from the formation of resting cells are also relatively easy to recognise from the characteristic dense rounded chloroplast.

- False positives are less serious in terms of risk assessments. The worst-case scenario is that they suggest a risk when there is none. In most cases the suggested risk would be small.

- Other staining techniques as well a neutral red have issues with uncertainty over separating fully alive from completely dead cells on the continuum between alive and dead. More precise methods are available but are complex, and also have different complications.

- We conclude that the neutral red staining technique has provided a simple, cost-effective, and largely accurate method for distinguishing live and dead *D. geminata* cells. The information in this review may assist in improved interpretation of future assessments using the technique.

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Appendix 2: Tests to determine optimum numbers of cells to count for reliable estimation of percentages of stained cells in subsamples

A series of trials was conducted to determine the optimum cell count required to give a reliable estimate of the proportions of stained and unstained cells in a subsample. Counts were made on a series of slides, following the method described in Section 2. Numbers of stained and unstained cells counted in successive fields of view (or transects – depending on the density of cells in the sample) were recorded separately. The cumulative counts after each field or transect were used to calculate a series of percentages of stained cells. We then plotted the total number of cells counted versus the percentages of stained cells calculated for each total. A stable percentage indicates that sufficient cells have been counted. As the total number of cells counted increases, differences in the proportions of stained:unstained cells in single fields or transects have a smaller influence on the calculated percentage. If stained and unstained cells are distributed relatively evenly thoughout the sample, then the percentage will stabilise at a relatively low total count. If stained and unstained cells are very patchy (i.e., parts of the slide are heavily biased towards either type of cell), then a larger count will be needed before the percentage stabilises.

Results for eight samples are shown in Figure A1.1. In all eight cases, the percentage of stained cells at least started to stabilise before the cell count was terminated. We had previously assumed that a count of 100 cells would be sufficient for a reliable estimate. From the graph, note that in four of the five samples in which >100 cells were counted, the calculated percentage of stained cells did not vary by more than five percentage points after the count exceeded 100. In sample six, the percentage fell by only six points following a 105 cell count, and by 143 cells, the gap had narrowed to 5.5 points. For three samples, we counted less than 100 cells. In all three cases, the percentage of stained cells stabilised over the last three or more fields counted.

We conclude from these trials that 100 cells appears to be an adequate cell count in most cases, and extra effort in substantially increasing the required count would not be warranted for the probable increase in accuracy obtained. It was important to ensure that the areas counted cover the entire slide. In most cases, final counts exceeded 100, particularly where cells were abundant in the sample. Some slides in our trials had very sparse cells. In these cases the entire slide was scanned and all cells counted. Thus, accuracy did not depend on the number of cells counted. Levelling off of the calculated percentages in samples 3, 5 and 7 (Figure A2.1) shows that in these cases, even lower counts would have been adequate.

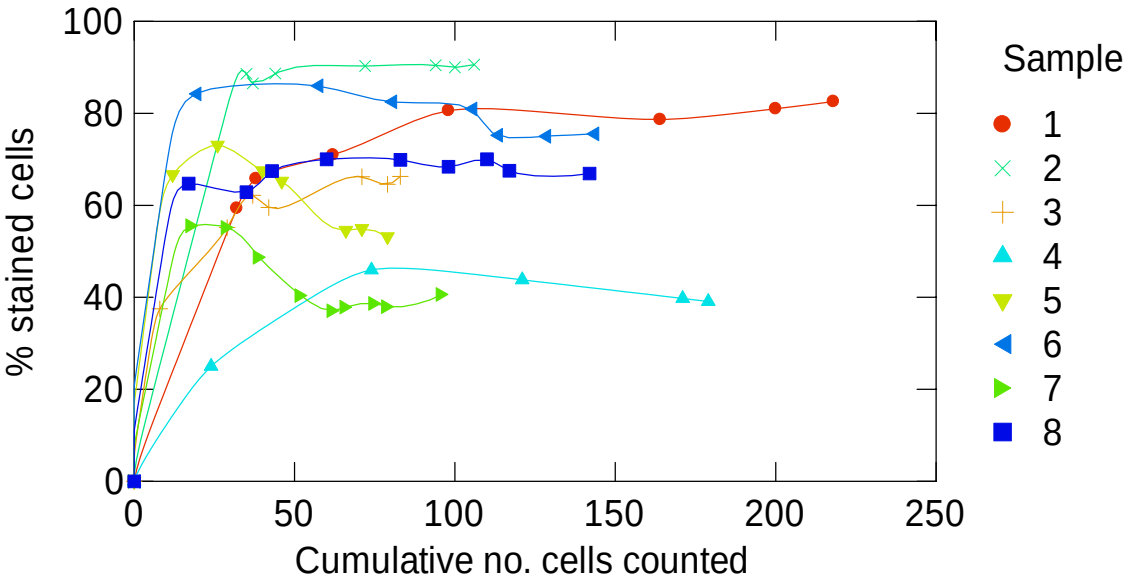

Figure A2.1: Percentages of stained cells (versus unstained) calculated progressively over cell counts of up to 200, plotted against the number of cells counted. The choice of 100 as the optimum count appears to be justified.

Appendix 3: Temperature – light – moisture trials: results of statistical analyses

Table A3. 1 Analysis of deviance table showing the effect of temperature (5, 12, 20 and 28 °C), light (dark, medium, light) and moisture (damp, wet) on the proportion of viable *D. geminata* cells.

Term	df	Deviance	MS	F	P
temp	3	24172.66	8057.55	470.22	<0.00001
light	2	3605.03	1802.52	105.19	<0.00001
temp:light	6	1174.95	195.83	11.43	<0.00001
trays(temp:light)	24	411.26	17.14	-	-
moisture	1	1792.29	1792.29	35.10	<0.00001
temp:moisture	3	1346.34	448.78	8.79	0.00041
light:moisture	2	720.62	360.31	7.06	0.00389
temp:light:moisture	6	605.05	100.84	1.97	0.10937
trays:moisture	24	1225.66	51.07		
time	1	26838.98	26838.98	351.16	<0.00001
temp:time	3	11842.63	3947.54	51.65	<0.00001
light:time	2	1167.03	583.52	7.63	0.00272
temp:light:time	6	419.32	69.89	0.91	0.50161
trays:time	24	1834.32	76.43	-	-
moisture:time	1	1996.27	1996.27	99.43	<0.00001
temp:moisture:time	3	1291.46	430.49	21.44	<0.00001
light:moisture:time	2	87.40	43.70	2.18	0.13530
temp:light:moisture:time	6	87.75	14.63	0.73	0.63122
trays:moisture:time	24	481.84	20.08	-	-
stones(trays:moisture:time)	576	13875.18	24.09	-	-
total	719	76652.97	-	-	-

Table A3.2. Analysis of deviance table showing the effect of temperature (5 and 12 °C), light (low, medium, light) and moisture (damp, wet) on the proportion of viable *D. geminata* cells.

Term	df	Deviance	MS	F	P
temp	1	289.13	289.13	2.61	0.13215
light	2	33.29	16.65	0.15	0.86161
temp:light	2	92.14	46.07	0.42	0.73885
trays(temp:light)	12	1329.22	110.77		
moisture	1	1016.69	1016.69	29.28	0.03249
temp:moisture	1	0.55	0.55	0.02	0.90192
light:moisture	2	116.13	58.07	1.67	0.21725
temp:light:moisture	2	213.78	106.89	3.08	0.37379
trays:moisture	12	416.63	34.72		
time	1	10218.95	10218.95	226.65	0.00438
temp:time	1	1592.23	1592.23	35.31	0.00007
light:time	2	36.53	18.27	0.41	0.67317
temp:light:time	2	37.07	18.54	0.41	0.74080
trays:time	12	541.04	45.09		
moisture:time	1	966.76	966.76	28.62	0.03321
temp:moisture:time	1	59.40	59.40	1.76	0.20953
light:moisture:time	2	86.54	43.27	1.28	0.30330
temp:light:moisture:time	2	53.74	26.87	0.80	0.45241
trays:moisture:time	12	405.40	33.78		
stones(trays:moisture:time)	288	8100.73	28.13		
total	359	24606.78	68.54		

Appendix 4: Numerical data: seawater and pH trials

The following tables show numerical values for the results presented in Figures 8 to 10. Percentage live *D. geminata* values are the means of three replicates in each case. Standard deviations (s.d.) are included.

Seawater trial

Seawater strength (%)	Exposure time (hours)	Exposure time (days)	% live cells	s.d.
1	2	0.08	84	9.4
	18	0.75	85	4.5
	46	2	78	3.8
	90	4	71	11.4
	114	5	69	6.9
	186	8	66	13.5
	306	13	62	15.5
	426	18	60	11.2
	615	25.5	36	45.4
	807	33.5	25	26.9
10	1.5	0.06	87	1.9
	18	0.75	88	6.2
	46	2	84	9.6
	90	4	76	6.7
	114	5	77	2.5
	186	8	64	7.8
	306	13	59	3.8
	426	18	62	7.9
	615	25.5	61	30.3
	807	33.5	70	2.4
50	1	0.04	2	2.0
	25	1	1	0.7
	97	4	4	5.0
	217	9	10	2.4
	337	14	6	6.1
	529	22	6	8.7
	721	30	0	0.3
100	1.1	0.045	3	4.4
	4	0.17	0	0.0
	90	4	0	0.0
	114	5	0	0.0
	186	8	0	0.0
	306	13	0	0.0
	426	18	0	0.0
	615	25.5	0	0.0
	807	33.5	0	0.0

Short-term trial to compare full-strength seawater with river water

Seawater strength (%)	Exposure time (hours)	% live cells	s.d.
River water	0	94	4.7
	2.5	96	2.3
	3.5	94	0.3
100	0.02	10	1.0
	0.17	15	1.1
	0.5	14	8.8
	1	7	12.6
	1.5	1.6	6.1
	2	1.0	10.4
	2.5	0.2	0.7
	3	0.2	0.4
	3.5	0.3	0.3
	4	0.0	0.0

pH trial

Reagent	pH	Exposure time (hours)	Exposure time (days)	% live cells	s.d.
HCl	1	5 min	0.0035	0	0.0
	1	30 min	0.02	0	0.0
	4	5 min	0.0035	60	38.9
	4	30 min	0.02	92	7.7
	4	17	0.7	91	10.4
	4	51	2	36	15.5
	4	100	4	53	20.0
	4	240	10	79	2.9
control (river)	8	0	0	94	4.2
	8	5 min	0.0035	93	6.1
	8	17	0.7	91	9.7
	8	24	1	86	4.3
	8	51	2	86	4.3
	8	72	3	95	1.2
	8	100	4	94	5.4
	8	192	8	77	7.5
	8	240	10	90	7.0
lime	9.5	20 min	0.014	92	6.5
	9.5	24	1	90	5.6
	9.5	72	3	83	8.8
	9.5	192	8	61	20.1
	10.8	20 min	0.014	21	6.8
	10.8	100 min	0.06	6	1.3
	10.8	24	1	0	0.0
	12	5 min	0.0035	51	17.0
	12	20 min	0.014	8	1.8
	12	100 min	0.06	0	0.3
	12	24	1	0	0.0

Appendix 5: MSDS's for tested decontamination products

Sample material safety data sheets (MSDS) are reproduced below for some of the potential decontamination products tested. Not all were available. A Material Safety Data Sheet contains information for handling or working with a particular substance. They include information such as toxicity, flammability, health issues and handling procedures.

Note that the following MSDS data are for general information only, and have been abbreviated in places. It would be advisable to source the MSDS from the retailer or manufacturer when actually dealing with these chemicals.

1. Safety (MSDS) data for borax decahydrate

Common synonyms
Borax decahydrate, sodium tetraborate decahydrate, sodium borate decahydrate
Formula : $Na_2B_4O_7$ 10 H_2O
Physical properties

Form: white crystals
Stability: Stable
Melting point: 75 C
Boiling point: 320 C
Specific gravity: 1.73
Water solubility: moderate

Principal hazards

- Borax is harmful if swallowed.
- Borax dust is very irritating if breathed in.
- There is some evidence that prolonged or repeated exposure to borax may cause reproductive defects.

Safe handling: Wear safety glasses.
Emergency

Eye contact: Immediately flush the eye with water. If irritation persists, call for medical help.
Skin contact: Wash off with soap and water.
If swallowed: Call for medical help if the amount swallowed is significant.

Disposal

Small amounts may be flushed down the sink unless local rules prohibit this. Larger amounts should be stored for disposal as solid waste.

Protective equipment

Safety glasses.

2. Material Safety Data Sheet for sodium percarbonate

Product Name: Sodium Percarbonate
Chemical Name: Sodium Carbonate Peroxyhydrate
Synonyms: PCS, Sodium Percarbonate, Sodium Carbonate Peroxide

Components	Formula	CAS No.	Percent
Sodium Percarbonate	$2Na_2CO_3 \cdot 3H_2O_2$	15630-89-4	>88
Sodium Carbonate	Na_2CO_3	497-19-8	12 approx

1. Hazards Identification

Emergency overview:

- Toxicity effects principally related to its irritating properties.
- Does not present any significant hazard for the environment.
- Supports combustion of other substances (oxidizing product)

Potential health effects:

General: Irritating to mucous membrane, eyes and skin.

Inhalation: Slight nose and throat irritation. At high concentrations, cough. In case of repeated or prolonged exposure: risk of sore throat, nose bleeds,chronic bronchitis.

Eye contact: Severe eye irritation, watering and redness, can cause burns to the eye. Risk of serious or permanent eye lesions

Skin contact: In case of repeated contact: risk of dermatitis.

Ingestion: Severe irritation of the mouth, throat, esophagus and stomach. Bloating of stomach, belching. Nausea, vomiting and diarrhea.

2. First-Aid Measures

Inhalation: Remove the subject from dusty environment.

Consult with a physician in case of respiratory symptoms.

Eye contact: Flush eyes with running water for 15 minutes, while keeping the eyelids wide open. Consult with an ophthalmologist in all cases.

Skin contact: Remove contaminated shoes, socks and clothing; wash the affected skin with running water. Clean clothing. Consult a physician in case of persistent pain or redness.

Ingestion: Consult with a physician in all cases. If the subject is completely conscious, rinse mouth and administer fresh water. Don't induce vomiting. If the subject is unconscious, loosen collar and tight clothing, lay the victim on his/her left side, give nothing by mouth. Keep warm with blanket. Don't induce vomiting.

3. Fire-Fighting Measures

Flash Point: Not applicable

Flammability: Non-flammable

Auto-ignition temperature: Not applicable

Danger of explosion: Non-explosive

Common extinguishing methods: Water

Specific hazards: Oxidizing substance. Oxygen released on exothermic decomposition may support combustion. Pressure burst may occur due to decomposition in confined spaces or containers.

4. Accidental Release Measures

Precautions:

Observe the protection measures given under sections 3 and 7.

Avoid materials and products which are incompatible with the product (see section 7)

Avoid direct contact of the product with water.

Cleanup methods:

Collect the product with suitable means, shovel or sweep, avoiding dust formation

All receiving equipment should be clean, dry, vented, labeled and made of material that is compatible with the product.

Don't return spilled or contaminated material to inventory.

Clean the area with large quantities of water.

For disposal methods, refer to section 13.

4.Handling and Storage

Handling:

Clean and dry process piping and equipment before using this product.

Never return unused product to original storage container.

Keep away from incompatible products.

Containers and equipment used to handle the product should be used exclusively for that product.

Avoid any contact with water or humidity.

For more information, consult the supplier.

Storage:

In a dry area.

Protect from direct sunlight.

Keep away from heat sources

Keep away from reactive products (see section 7).

Store in vented containers.

Store at temperatures less than 40 (104)

5. Exposure Controls/Personal Protection

Skin protection: For brief contact, few precautions other than clean body-covering clothing should be needed. When prolonged or frequently repeated contact could occur, use protective, full body clothing, such as PVC or rubber, impervious to this material.

Respiratory protection: For many conditions, no respiratory protection may be needed; however, in dusty or unknown atmospheres or when exposures exceed limit values, use a NIOSH approved dust respirator.

Other precautions: Safety shower and eyewash stations.

6. Physical and Chemical Properties

Appearance: White granular solid.

Odor: None

Bulk Density: 0.80-1.0g/cm3

Solubility: 140g/L @ 24°C, pH 3% solution: 10.0-11.0

Decomposition Temperature: Self-accelerating decomposition with oxygen release starting from 50°C.

7. Stability and Reactivity

Stability: Stable, under certain conditions (see below).

Conditions to avoid: ·Heat/Sources of heat. Moisture.

Materials to avoid: Water. Acids. Bases. Salts of heavy metals. Reducing agents. Organic materials. Flammable substances.

Other information: Decomposition releases steam/heat.

8. Toxicological Information

Acute toxicity:

Oral route –LD50, rat(combined sexes),1034 mg/kg.

Dermal route-LDLo, rabbit, >2000mg/kg.

Inhalation,LC0, 1 hour, rat, >4580mg/m3.

Irritation:

Eyes, severe damage, rabbit.

Skin, slightly irritating, rabbit.

Sensitization:

No sensitization was noted when administered as a 75% w/v mixture during induction and as a 25% w/v mixture at challenge.

Comments: Toxic effect linked with irritant properties.

9. Ecological Information

Acute ecotoxicity:

Fish, pimephales promelas, LC50, 70.7mg/L

Fish, Pimephales promelas, NOEC, 96 hours, 1mg/L.

Crustaceans,Daphnia pulex, EC50, 4.9mg/L.

Mobility

Air: Not applicable.

Water: Considerable solubility and mobility.

Soil/sediments, percolation: Non-significant adsorption.

Abiotic degradation

Air: Not applicable

Water: Significant hydrolysis.

Degradation products: sodium carbonate, carbon dioxide, bicarbonate, carbonate, hydrogen peroxide.

Soil: Hydrolysis.

Potential for bioaccumulation: Non-bioaccumuable.

Comments: Toxic for aquatic organisms. Nevertheless, hazard for the aquatic environment is limited. Not bioaccumuable. Abiotic degradation. Low toxicity of degradation products.

10. Disposal

Dispose of in an approved waste facility operated by an authorized contractor in compliance with federal, state and local regulations. The empty and clean containers are to be recycled or disposed of in conformity with local regulations.

11. Transport Information

D.O.T. Proper Shipping Name:
Oxidizing solid, n.o.s,(sodium carbonate peroxyhydrate)
UN Number: 1479
Hazard Class: 5.1
Label(s): 5.1(Oxidizer)
Packing Group: II

3. Material Safety Data for Sodium Dodecyl Sulphate

1. Product Identification

Synonyms: Sodium lauryl sulfate; Duponol; Dodecyl sodium sulfate; Sulfuric acid, monodecyl ester, sodium salt
CAS No.: 151-21-3
Molecular Weight: 288.38
Chemical Formula: $C_{12}H_{25}OSO_3Na$

Ingredient	CAS No	Percent	Hazardous
Sodium Dodecyl Sulfate	151-21-3	90 - 100%	Yes

3. Hazards Identification

Emergency Overview

WARNING! HARMFUL IF SWALLOWED OR INHALED. CAUSES IRRITATION TO SKIN, EYES AND RESPIRATORY TRACT. MAY CAUSE ALLERGIC SKIN OR RESPIRATORY REACTION. FLAMMABLE SOLID.

SAF-T-DATA(tm) Ratings (Provided here for your convenience)
Health Rating: 1 - Slight
Flammability Rating: 3 - Severe (Flammable)
Reactivity Rating: 1 - Slight
Contact Rating: 3 - Severe (Life)
Lab Protective Equip: GOGGLES & SHIELD; LAB COAT & APRON; VENT HOOD; PROPER GLOVES
Storage Color Code: Red (Flammable)

Potential Health Effects

Inhalation: Causes irritation to the respiratory tract. Symptoms may include coughing, shortness of breath. May cause allergic reaction in sensitive individuals.
Ingestion: Large doses may cause gastrointestinal distress, nausea and diarrhea.
Skin Contact: Mildly irritating to skin, causes dryness and a rash on continued exposure. May cause allergic skin reactions.
Eye Contact: Causes irritation, redness, and pain.
Chronic Exposure: Chronic exposure may cause skin effects.
Aggravation of Pre-existing Conditions: Persons with pre-existing skin disorders or impaired respiratory function may be more susceptible to the effects of the substance.

4. First Aid Measures

Inhalation: Remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion: Give large amounts of water to drink. Never give anything by mouth to an unconscious person. Get medical attention.
Skin Contact: Immediately flush skin with plenty of soap and water. Remove contaminated clothing and shoes. Get medical attention. Wash clothing before reuse. Thoroughly clean shoes before reuse.
Eye Contact: Immediately flush eyes with plenty of water for at least 15 minutes, lifting lower and upper eyelids occasionally. Get medical attention immediately.

5. Fire Fighting Measures

Fire: Flammable Solid! Classified as a flammable solid according to DOT test methods.
Explosion: Fine dust dispersed in air in sufficient concentrations, and in the presence of an ignition source is a potential dust explosion hazard.
Fire Extinguishing Media: Water spray, dry chemical, alcohol foam, or carbon dioxide.
Special Information: In the event of a fire, wear full protective clothing and NIOSH-approved self-contained breathing apparatus with full facepiece operated in the pressure demand or other positive pressure mode.

6. Accidental Release Measures

Ventilate area of leak or spill. Wear appropriate personal protective equipment as specified in Section 8. Spills: Pick up and place in a suitable container for reclamation or disposal, using a method that does not generate dust.

7. Handling and Storage

Keep in a tightly closed container, stored in a cool, dry, ventilated area. Protect against physical damage. Containers of this material may be hazardous when empty since they retain product residues (dust, solids); observe all warnings and precautions listed for the product.

8. Exposure Controls/Personal Protection

Airborne Exposure Limits: None established.
Ventilation System: A system of local and/or general exhaust is recommended to keep employee exposures as low as possible. Local exhaust ventilation is generally preferred because it can control the emissions of the contaminant at its source, preventing dispersion of it into the general work area. Please refer to the ACGIH document, Industrial Ventilation, A Manual of Recommended Practices, most recent edition, for details.
Personal Respirators (NIOSH Approved): For conditions of use where exposure to the dust or mist is apparent, a half-face dust/mist respirator may be worn. For emergencies or instances where the exposure levels are not known, use a full-face positive-pressure, air-supplied respirator. WARNING: Air-purifying respirators do not protect workers in oxygen-deficient atmospheres.
Skin Protection: Wear impervious protective clothing, including boots, gloves, lab coat, apron or coveralls, as appropriate, to prevent skin contact.
Eye Protection: Use chemical safety goggles. Maintain eye wash fountain and quick-drench facilities in work area.

9. Physical and Chemical Properties

Appearance: Fine, white or slightly yellow powder.
Odor: Slight fatty odor.
Solubility: 10g/100g water.
Specific Gravity: 0.4 @ 15C/4C
pH: No information found.
% Volatiles by volume @ 21C (70F): 0
Boiling Point: No information found.
Melting Point: No information found.

Vapor Density (Air=1): No information found.
Vapor Pressure (mm Hg): No information found.
Evaporation Rate (BuAc=1): No information found.

10. Stability and Reactivity

Stability: Stable under ordinary conditions of use and storage.
Hazardous Decomposition Products: Oxides of carbon, sulfur and sodium oxide may form when heated to decomposition.
Hazardous Polymerization: Will not occur.
Incompatibilities: Strong oxidizers, acids.
Conditions to Avoid: Heat, flames, ignition sources and incompatibles.

11. Toxicological Information

Toxicological Data: Oral rat LD50: 1288 mg/kg; Inhalation rat LC50: > 3900 mg/kg; irritation data: skin human, standard Draize, 25 mg/24-hour, mild; eye rabbit, standard Draize, 250 ug, mild. Investigated as a mutagen, reproductive effector.
Reproductive Toxicity: Has caused mutagenic and teratogenic effects on laboratory animals.
Cancer Lists

NTP Carcinogen	Ingredient	Known	Anticipated	IARC Category
	Sodium Dodecyl Sulfate (151-21-3)	No	No	None

12. Ecological Information

Environmental Fate: No information found.
Environmental Toxicity:
96 Hr LC_{50} fathead minnow (fry): 10.2 mg/L;
96 Hr LC_{50} fathead minnow (juvenile):17 mg/L;
96 Hr LC_{50} fathead minnow (adult):22.5 mg/L;
96 Hr LC_{50} rainbow trout: 4.6 mg/L (Static).

13. Disposal Considerations

Whatever cannot be saved for recovery or recycling should be managed in an appropriate and approved waste disposal facility. Processing, use or contamination of this product may change the waste management options. State and local disposal regulations may differ from federal disposal regulations. Dispose of container and unused contents in accordance with federal, state and local requirements.

14. Transport Information

Domestic (Land, D.O.T.)
Proper Shipping Name: FLAMMABLE SOLIDS, ORGANIC, N.O.S. (SODIUM LAUREL SULFATE)
Hazard Class: 4.1
UN/NA: UN1325
Packing Group: III

15. Regulatory Information

Australian Hazchem Code: None allocated.
Poison Schedule: None allocated.
WHMIS:
This MSDS has been prepared according to the hazard criteria of the Controlled Products Regulations (CPR) and the MSDS contains all of the information required by the CPR.

16. Other Information

NFPA Ratings: Health: 1 Flammability: 2 Reactivity: 0
Label Hazard Warning:
WARNING! HARMFUL IF SWALLOWED OR INHALED. CAUSES IRRITATION TO SKIN, EYES AND RESPIRATORY TRACT. MAY

CAUSE ALLERGIC SKIN OR RESPIRATORY REACTION.
FLAMMABLE SOLID.
Label Precautions: Do not breathe dust.
Avoid contact with eyes, skin and clothing.
Wash thoroughly after handling.
Use only with adequate ventilation.
Keep container closed.
Keep away from heat, sparks and flame.
Label First Aid:
If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. In case of skin contact, immediately flush skin with plenty of soap and water. Remove contaminated clothing and shoes. Wash clothing before reuse. If swallowed, give large amounts of water to drink. Never give anything by mouth to an unconscious person. In all cases, get medical attention.
Product Use: Laboratory Reagent.

4. Safety (MSDS) data for sodium hypochlorite solution

Common synonyms

Clorox, liquid bleach

Formula: NaClO (in water)

Physical properties

Form: colourless liquid with a strong odour

Stability: Unstable: light and heat sensitive; readily decomposes on heating to around 40 C

Specific gravity: approximately 1.21

Principal hazards

- Sodium hypochlorite is corrosive and can cause burns to skin and eyes.
- The liquid is harmful if swallowed and in contact with the skin. The fumes are harmful if inhaled.
- If acid is added to sodium hypochlorite solution, chlorine gas (poisonous!) is released. Since bleach may be deliberately or inadvertently added to other cleaning agents in use - some of which are acidic - this is a common way in which people may be exposed to chlorine.
- The solution is unstable, gradually decomposing over time to release both dissolved and gaseous products. Consequently, pressure may build up in sealed containers, so solutions in storage should be allowed to vent the products of decomposition.
- Sodium hypochlorite is a fairly strong oxidizing agent, so reacts vigorously with many reducing agents. Products of the reaction with amines and ammonia may be both toxic and explosive.

Safe handling

Wear safety glasses. Work in a well ventilated area. Do not add the liquid to a known acid, or to any material which may possibly be acidic.

Emergency

Eye contact: Immediately flush the eye with water. If irritation persists, call for medical help.

Skin contact: Wash off with soap and water. If the skin is left red or inflamed, seek medical aid.

If swallowed: Call for medical help.

Disposal

Small amounts of liquid may be disposed of down the sink unless local rules prohibit this. When discarding down the sink, ensure that plenty of water is used to thoroughly flush away this material.

Protective equipment: Safety glasses

5. MSDS for 303 Clearall

(available in MS Publisher format)

6. Safety (MSDS) data for benzalkonium chloride

General

Synonyms: parasterol, alkyl benzyl dimethylammonium chloride, alkyl dimethyl benzylammonium chloride, benirol, cequartryl, drapolene, enuclene, germitol, gesminol, rodalon, ammonyx, zephiran chloride, various further trade names

Use: medical disinfectant

Molecular formula: (mixture)

CAS No: 8001-54-5

EC No:

Physical data

Appearance: white or light yellow/grey solid, or colourless aqueous solution

Melting point:

Boiling point:

Vapour density:

Vapour pressure:

Specific gravity: 0.98

Flash point: 250 C

Explosion limits:

Autoignition temperature:

Stability

Stable. Incompatible with strong oxidizing agents, moisture. Hygroscopic.

Toxicology

Corrosive, toxic - causes burns. Harmful by inhalation, ingestion and through skin contact. May cause reproductive defects. May act as a mutagen.

Toxicity data

ORL-RAT LD50 240 mg kg-1

ORL-WMN lowest published toxic dose 266 mg kg-1

IPN-RAT LD50 14.5 mg kg-1

Risk phrases

Harmful by inhalation

Harmful in contact with skin

Harmful if swallowed

Causes burns

Transport information

Hazard class 8 (miscellaneous dangerous substances).

Packing group III (lowest hazard)

Personal protection

Safety glasses, adequate ventilation, gloves.

Safety phrases

In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

After contact with skin, wash immediately with plenty of soap-suds.

Wear suitable protective clothing.

Wear suitable gloves.

Wear eye / face protection.

7. MSDS for sodium metabisulfite

1. Product Identification

Synonyms: Sodium pyrosulfite; pyrosulfurous acid, disodium salt

CAS No.: 7681-57-4 Sodium Metabisulfite; 7631-90-5 Sodium Bisulfite.

Molecular Weight: 190.11

Chemical Formula: Na2S2O5 (sodium metabisulfite) and NaHSO3 (sodium bisulfite)

2. Composition/Information on Ingredients

Ingredient	CAS No	Percent	Hazardous
Sodium Metabisulfite	7681-57-4	100%	Yes
Sodium Bisulfite	7631-90-5	< 0.001%	No

3. Hazards Identification

Emergency Overview

WARNING! HARMFUL IF SWALLOWED OR INHALED. CAUSES IRRITATION TO SKIN, EYES AND RESPIRATORY TRACT. MAY CAUSE ALLERGIC RESPIRATORY REACTION. REACTS WITH ACIDS AND WATER RELEASING TOXIC SULFUR DIOXIDE GAS.

Potential Health Effects

Inhalation: Causes irritation to the respiratory tract. Symptoms may include coughing, shortness of breath. May cause allergic reaction in sensitive individuals.

Ingestion: May cause gastric irritation by the liberation of sulfurous acid. An asthmatic reaction may occur after ingestion. Large doses may result in nausea, vomiting, diarrhea, abdominal pains, circulatory disturbance, and central nervous system depression. Estimated fatal dose is 10 gm.

Skin Contact: Causes irritation to skin. Symptoms include redness, itching, and pain.

Eye Contact: Causes irritation, redness, and pain. Contact may cause irreversible eye damage. Symptoms may include stinging, tearing, redness, swelling, corneal damage and blindness.

Chronic Exposure: No information found.

Aggravation of Pre-existing Conditions: Some individuals are said to be dangerously sensitive to minute amounts of sulfites in foods. Symptoms may include broncho constriction, shock, gastrointestinal disturbances, angio edema, flushing, and tingling sensations. Once allergy develops, future exposures can cause asthma attacks with shortness of breath, wheezing, and cough.

4. First Aid Measures

Inhalation: Remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion: Induce vomiting immediately as directed by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention.

Skin Contact: Wipe off excess material from skin then immediately flush skin with plenty of water for at least 15 minutes. Remove contaminated clothing

and shoes. Get medical attention. Wash clothing before reuse. Thoroughly clean shoes before reuse.

Eye Contact: Immediately flush eyes with plenty of water for at least 15 minutes, lifting lower and upper eyelids occasionally. Get medical attention immediately.

5. Fire Fighting Measures

Fire: Not considered to be a fire hazard.

Explosion: Not considered to be an explosion hazard.

Fire Extinguishing Media: Use any means suitable for extinguishing surrounding fire. Do not allow water runoff to enter sewers or waterways.

Special Information: In the event of a fire, wear full protective clothing and NIOSH-approved self-contained breathing apparatus with full facepiece operated in the pressure demand or other positive pressure mode.

6. Accidental Release Measures

Ventilate area of leak or spill. Wear appropriate personal protective equipment as specified in Section 8. Spills: Pick up and place in a suitable container for reclamation or disposal, using a method that does not generate dust. Cautiously spray residue with plenty of water, providing ventilation to clear sulfur dioxide fumes generated from water contact. US Regulations (CERCLA) require reporting spills and releases to soil, water and air in excess of reportable quantities. The toll free number for the US Coast Guard National Response Center is (800) 424-8802.

7. Handling and Storage

Keep in a tightly closed container. Protect from physical damage. Store in a cool, dry, ventilated area away from sources of heat, moisture and incompatibilities. Releases toxic sulfur dioxide gas when in contact with water, ice. Keep away from acids, water, ice, and oxidizing agents. Use only with appropriate protective equipment. Do not use in unventilated areas such as holds of fishing boats, walk in coolers or confined spaces. Containers of this material may be hazardous when empty since they retain product residues (dust, solids); observe all warnings and precautions listed for the product.

8. Exposure Controls/Personal Protection

Airborne Exposure Limits: -ACGIH Threshold Limit Value (TLV): 5mg/m3 (TWA) for sodium bisulfite and for sodium metabisulfite, A4 Not classifiable as a human carcinogen.

Ventilation System: A system of local and/or general exhaust is recommended to keep employee exposures below the Airborne Exposure Limits. Local exhaust ventilation is generally preferred because it can control the emissions of the contaminant at its source, preventing dispersion of it into the general work area. Please refer to the ACGIH document, Industrial Ventilation, A Manual of Recommended Practices, most recent edition, for details.

Personal Respirators (NIOSH Approved): If the exposure limit is exceeded, a half-face respirator with an acid gas cartridge may be worn for up to ten times the exposure limit or the maximum use concentration specified by the appropriate regulatory agency or respirator supplier, whichever is lowest. A

full-face piece respirator with an acid gas cartridge may be worn up to 50 times the exposure limit, or the maximum use concentration specified by the appropriate regulatory agency, or respirator supplier, whichever is lowest. For emergencies or instances where the exposure levels are not known, use a full-facepiece positive-pressure, air-supplied respirator.

WARNING: Air-purifying respirators do not protect workers in oxygen-deficient atmospheres.

Skin Protection: Wear impervious protective clothing, including boots, gloves, lab coat, apron or coveralls, as appropriate, to prevent skin contact. (neoprene, polyvinyl chloride).

Eye Protection: Use chemical safety goggles and/or full face shield where dusting or splashing of solutions is possible. Maintain eye wash fountain and quick-drench facilities in work area.

9. Physical and Chemical Properties

Appearance: White to yellow white crystalline granules.

Odor: Slight odor of sulfur dioxide.

Solubility: Very soluble in water, insoluble in alcohol.

Specific Gravity: 1.48

pH: Aqueous solution is acidic.

% Volatiles by volume @ 21C (70F): 0

Boiling Point: Not applicable.

Melting Point: 150C (302F)

Vapor Density (Air=1): No information found.

Vapor Pressure (mm Hg): No information found.

Evaporation Rate (BuAc=1): No information found.

10. Stability and Reactivity

Stability: Strength diminishes somewhat with age. Gradually decomposes in air to sulfate, generating sulfurous acid gas. Contact with moisture (water, wet ice, etc.), will release toxic sulfur dioxide gas.

Hazardous Decomposition Products: Oxides of sulfur and sodium may form when heated to decomposition.

Hazardous Polymerization: Will not occur.

Incompatibilities: Water, acids, alkalis, sodium nitrite, oxidizers, aluminum powder.

Conditions to Avoid: Moisture, heat, flames, ignition sources and incompatibles.

11. Toxicological Information

Sodium Metabisulfite [7681-57-4]: No LD50/LC50 information found relating to normal routes of occupational exposure. Investigated as a tumorigen, mutagen and reproductive effector. Sodium Bisulfite [7631-90-5]: Oral rat LD_{50}: 2000 mg/kg. Investigated as a tumorigen and mutagen.

--------\Cancer Lists\----------

	NTP Carcinogen---		
Ingredient	Known	Anticipated	IARC Cat.
Sodium Metabisulfite (7681-57-4)	No	No	3

Sodium Bisulfite (7631-90-5)	No	No	3

12. Ecological Information

Environmental Fate: No information found.

Environmental Toxicity: No information found.

13. Disposal Considerations

Whatever cannot be saved for recovery or recycling should be managed in an appropriate and approved waste disposal facility. Processing, use or contamination of this product may change the waste management options. State and local disposal regulations may differ from federal disposal regulations.

Dispose of container and unused contents in accordance with federal, state and local requirements.

14. Transport Information

Not regulated.

16. Other Information

NFPA Ratings: Health: 3 Flammability: 0 Reactivity: 1

Label Hazard Warning: WARNING! HARMFUL IF SWALLOWED OR INHALED. CAUSES IRRITATION TO SKIN, EYES AND RESPIRATORY TRACT. MAY CAUSE ALLERGIC RESPIRATORY REACTION. REACTS WITH ACIDS AND WATER RELEASING TOXIC SULFUR DIOXIDE GAS.

8. Safety (MSDS) data for ethyl alcohol

Common synonyms:

Ethanol, alcohol, grain alcohol, fermentation alcohol, fermentation ethanol

Formula: C_2H_5OH

Physical properties

Form: colourless fragrant liquid

Stability: Stable, but highly flammable

Melting point: -144 C

Boiling point: 78 C

Water solubility: miscible in all proportions

Specific gravity: 2.12

Explosion limits: 3.3 - 24.5%

Principal hazards

- Contact with the eyes can cause considerable irritation.
- "One-off" consumption of small amounts of ethanol is not likely to be harmful, but consumption of large amounts can be (and has been) fatal. Chronic (long-term) ingestion of ethanol may lead to damage to a variety of organs, such as the liver, and may increase the risk of cancer.

- Ethanol is very flammable, so constitutes a fire risk.

Safe handling

Wear safety glasses. Ensure that no sources of ignition, such as a gas flame, hot plate or hot air gun, are present in the working area. Check that ventilation is good; use a fume cupboard if possible.

Emergency

Eye contact: Flush the eye with plenty of water. If irritation persists call for medical help.

Skin contact: Wash off with water.

If swallowed: If the quantity swallowed is large, call for medical help

Disposal

Small amounts of ethanol can be flushed down a sink with a large quantity of water, unless local rules prohibit this. Do not forget that this material is very flammable, so precautions must be taken to ensure that flammable vapour does not build up in the sink or drains.

Protective equipment: Safety glasses

9. MSDS for Simple Green

http://industrial.simplegreen.com/ind_msds.php (Simple Green All-purpose cleaner – degreaser)

Appendix 6: Numerical data: product trials

The following table shows numerical values for the results presented in Figures 13, 15, 17, 18, and 19. Percentage live *D. geminata* values are the means of three replicates in each case. Standard deviations (s.d.) are included. The two control values given are means calculated from all the experiments, at time = 0, and at all other times (up to 1000 minutes); *n* = >42 in each case.

Product	Concentration	Exposure time (minutes)	% live cells	s.d.
Controls (river water)	-	0	92	5.5
	-	1 - 1000	90	6.4
Borax (mg Boron / litre)	0.5	1	87	5.8
	0.5	10	84	6.1
	0.5	100	88	7.4
	10	1	79	8.2
	100	1	92	1.7
	100	10	92	7.3
	100	100	95	2.2
	100	1000	92	5.0
	1000	1	94	2.5
Sodium percarbonate (%w/v)	0.01	1000	90	2.9
	0.05	1000	59	32.3
	0.1	1000	0	0.0
	0.5	100	63	21.8
	0.5	1000	0	0.0
	1	1	85	3.7
	1	10	77	3.3
	1	100	49	4.1
	1	1000	0	0.0
	2	1	21	9.0
	2	10	15	11.8
	2	100	0	0.0
	5	1	2	1.9
	5	10	0.1	0.2

Product	Concentration	Exposure time (minutes)	% live cells	s.d.
nappy cleaner (% w/v)	0.1	1000	8	9.3
	0.5	100	35	15.8
	0.5	1000	0	0.0
	1	1	24	23.2
	1	10	30	5.7
	1	100	2	2.4
	2	1	9	7.8
	2	10	3	1.5
	5	1	0	0.0
Sodium dodecyl sulphate (% w/v)	0.01	1000	78	5.0
	0.05	1000	3	3.2
	0.1	1	70	14.4
	0.1	10	61	11.7
	0.1	100	20	5.4
	0.1	1000	0	0.0
	0.5	1	1	0.7
	0.5	10	9	7.8
	0.5	100	0	0.0
	0.5	1000	0	0.0
	1	1	0	0.0
	1	10	0	0.0
Household bleach (3.5% sodium hypochlorite) (%)	0.1	1	59	18.3
	0.1	10	49	29.0
	0.1	100	75	15.0
	0.1	1000	55	28.7
	0.5	1	14	1.6
	0.5	10	19	13.5
	0.5	100	9	14.3
	0.5	1000	8	11.9
	1	1	0.4	0.7
303 Clearall (quat mixture) (%)	0.005	1000	78	21.0
	0.01	1	96	2.6
	0.01	10	72	16.1
	0.01	100	42	19.8
	0.01	1000	55	22.9
	0.1	1	76	11.2
	0.1	10	3	0.9
	0.1	100	2	1.7
	0.1	1000	4	5.3
	1	1	2	3.5
	1	10	0	0.0
	1.2	1	1	1.1
	1.5	1	0	0.0
Product	**Concentration**	**Exposure time (minutes)**	**% live cells**	**s.d.**

Sodium metabisulphite ('000 ppm SO_2)	50	1	42	36.4
	50	100	1	1.4
	50	1000	0	0.0
	100	1	32	30.5
	100	10	2	1.7
	100	100	0	0.0
	100	1000	0	0.0
	200	1	5	6.7
	200	10	3	5.0
Ethanol (%)	10	1	82	5.9
	10	100	79	14.4
	10	1000	40	18.6
	20	1	47	17.5
	20	10	82	7.8
	20	1000	0	0.0
	50	1	43	13.0
	50	10	18	16.5
	50	100	0	0.0
	70	1	8	7.2
	70	10	0	0.0
BEE all-purpose surface cleaner (%)	0.2	1000	78	13.3
	2	1	78	17.3
	2	10	83	19.5
	2	100	53	4.7
	2	1000	0	0.0
	5	1	94	4.9
	5	10	80	14.4
	5	100	36	21.0
	10	1	77	11.0
	10	10	66	26.2
	100	1	11	8.0
Citrus based cleaner (%)	0.2	100	18	9.6
	0.2	1000	2	1.6
	2	10	26	23.6
	2	100	2	0.9
	2	1000	0	0.0
	5	1	44	11.4
	5	10	15	3.4
	5	100	0	0.0
	10	1	2	1.0
	10	10	0	0.0
	100	1	0	0.0
Product	**Concentration**	**Exposure time (minutes)**	**% live cells**	**s.d.**
Simple Green (%)	0.2	10	54	16.7
	0.2	100	53	2.3

	0.2	1000	26	35.4
	2	1	65	26.3
	2	10	31	15.8
	2	100	27	12.8
	2	1000	0	0.0
	5	1	18	14.8
	5	100	4	4.3
	10	1	10	9.5
	20	1	5	5.7
	50	1	0	0.0
Down-toEarth dishwasing liquid (%)	0.1	1000	0	0.0
	0.5	100	3	5.6
	0.5	1000	0	0.0
	1	1	22	7.4
	1	10	5	6.4
	1	100	0	0.0
	2	1	3	6.0
	2	10	0	0.0
	5	1	0	0.0
Palmolive dishwashing liquid (%)	0.1	100	21	10.6
	0.1	1000	1	1.4
	0.5	10	3	4.0
	0.5	100	11	9.9
	0.5	1000	0	0.0
	1	1	15	15.6
	1	10	2	0.6
	1	100	2	2.3
	2	1	1	2.2
	2	10	0	0.7
	5	1	0	0.0
Sunlight dishwashing liquid (%)	0.1	100	9	6.8
	0.1	1000	0	0.0
	0.5	100	1	1.6
	0.5	1000	0	0.0
	1	1	3	1.5
	1	10	1	1.3
	1	100	12	4.8
	2	1	3	5.3
	2	10	6	7.9
	5	1	0	0.0