Tests to determine the effectiveness of methods for decontaminating materials that have been in contact with *Didymosphenia geminata*

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Biosecurity New Zealand

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Contents

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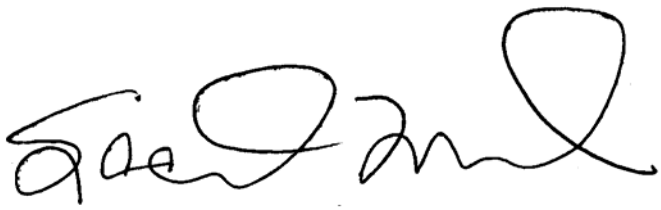


Scott Larned

Executive Summary

- NIWA was requested by Biosecurity New Zealand to test the effectiveness of a range of products as decontaminating agents for materials (clothing/equipment) that may be contaminated with the invasive diatom *Didymosphenia geminata*. Currently the species appears to be confined to the Mararoa and lower Waiau Rivers in Southland, and measures are in place to try to prevent its spread into other rivers.

- Trials were conducted to find a method for distinguishing live and dead diatom cells. A staining technique using neutral red dye was considered to be the best general method available. Neutral red dye is taken up by the cytoplasm of live cells, but not dead cells. Observations on the microscopic appearance of cells can provide supplementary evidence of the effectiveness of some treatments.

- In preliminary tests carried out on 22 December, the neutral red staining technique indicated that 5% solutions of nappy cleaner and two household antiseptic liquids (active ingredients chlorhexidine and chloroxylenol) rapidly killed cells of *Cymbella kappii* (a diatom related to *D. geminata*).

- The tests were repeated on 18-20 January 2005 using *D. geminata* mats collected on 16 January. Salt solutions, detergent, and bleach were also tested. The earlier results were confirmed for <1 min treatment with 5% nappy cleaner and chlorhexidine antiseptic. Treatment with 5% (v/v) salt solution for 1 min resulted in very few dyed cells, either outside or inside the mat. A longer treatment time (10+ min) was more effective, and a 1% solution less effective. Mats treated for <1 min with 5% detergent, chloroxylenol antiseptic and bleach also appeared to be effective, however the results were inconclusive because very low concentrations of dyed cells in control (untreated) mats indicated that the *D. geminata* mats were deteriorating rapidly in the laboratory.

- Further *D. geminata* colonies were collected on 3 February and transported to the laboratory the same day. To try to enhance their survival, colonies were left attached to rocks from the river bed, were immersed in at least 20 times their volume in river water, and were maintained at a cooler temperature. The tests were repeated on these colonies, this time with replication.

- These repeat tests confirmed that immersion in 5% solutions of nappy cleaner, chlorhexidine and chloroxylenol antiseptic liquids, and detergent rapidly (30 sec) killed *D. geminata* cells. In addition, a 1% solution of household bleach was found to be equally effective. Five percent salt solution was effective, but a 2% solution was slightly less reliable. All solutions were made up by volume.

- The tests with salt solution indicated that prolonged immersion in sea water (*at least* 1 hour) may be an effective method of decontaminating larger equipment such as the outer surfaces of boats and kayaks, but this remains to be tested.

- The effects of desiccation on *D. geminata* colonies were also investigated. After 15 h desiccation of a large mat in partially sunny conditions few live cells remained either outside or inside the mat. After 48 h the mats had dried completely and no live cells were found.

- In a more detailed desiccation test, no live cells were found in mats that had dried to a water content of less than 83%. The time taken to reach this point would vary according to initial water content, but in the warm (~20 °C), shaded conditions of the present trial, took up to three days.

- Heat treatment was not tested directly in these trials, however rapid heating to 60 °C is a standard laboratory method for killing algal cells.

- The rapid response of cells to a mild dishwashing detergent, along with the poor viability of *D. geminata* colonies in warm laboratory conditions (>22 °C) suggest that *thorough* shampooing and rinsing in warm to hot water, will be effective in killing any cells lodged in hair following swimming, particularly if the hair is dried before shampooing.

- Based on these results, recommendations for decontaminating materials that may have come into contact with *D. geminata* are:

    - Soak and scrub in a 5% (v/v) solution of either salt, nappy cleaner, household antiseptics (chlorhexidine or chloroxylenol based) or detergent for about 1 minute.

    - Or, soak and scrub in a 2% solution of household bleach for 1 min.

    - Or, immerse contaminated items in hot water for 2 min, to a final temperature of at least 60 °C.

    - After swimming in an affected waterway, allow hair to dry completely, then shampoo for *at least* 1 minute, and rinse thoroughly using warm to hot water.

    - For large items of equipment, desiccate completely by drying for an extended period of time (e.g., 48 hours) *after* all parts of the item (and therefore algal mats on or within it) appear to be dry.

1. Introduction

Didymosphenia geminata, a Northern Hemisphere invasive diatom (type of alga), has been declared by Biosecurity New Zealand to be an unwanted organism. The species is currently blooming in the Mararaoa and lower Waiau Rivers, Southland, along approximately 150 km of the main stem of the river. Surveys in neighbouring catchments have suggested strongly that the species is currently confined to the Mararoa/lower Waiau catchment. A priority for Biosecurity New Zealand is therefore to ensure that the species is not spread by humans into other catchments while future management options are being considered. Transfer via fishing equipment, boats, clothing, etc., seems the most likely means of spread of the species at this stage (although dispersal via birds and animals cannot be ruled out).

Completely banning access to the affected river was not considered to be a feasible option. Therefore, in addition to measures planned to discourage use of the river, methods were needed to decontaminate any equipment, clothing or parts of the body that might have come into contact with river water. Biosecurity New Zealand needed to be confident that any methods recommended were actually effective in killing *D. geminata* cells. NIWA was therefore asked to carry out simple tests using a range of commonly available solutions, such as bleach, nappy cleaner, antiseptics, disinfectant, detergent, saline solutions, and hot water. Biosecurity New Zealand also requested information about the viability of *D. geminata* during and following desiccation.

Since live colonies of *D. geminata* could not be delivered to the laboratory immediately, just prior to the Christmas holidays 2004, preliminary trials were undertaken using other algae. This report describes those preliminary trials as well as subsequent tests in January and February 2005 using live *D. geminata*.

2. Determining the viability of diatom cells

Before starting tests on potential decontamination products, an effective means of determining cell viability was required. Three methods were considered:

1. Microscope examination of samples for viewing intact chloroplasts and other cellular structures.

2. Measurement of chlorophyll *a* (if no chlorophyll *a* is present, the cell will be unable to photosynthesise, and therefore will not be viable).

3. Staining techniques (which also involves microscope examination).

Trials in November/December 2004 showed that the first two options were not universally useful.

Trial 1: Microscope examination. Samples of *D. geminata* immersed in a 10% salt solution showed no consistent microscopic features that distinguished them from untreated samples. One problem is that all samples contain some dead cells and we need to be able to distinguish these from live cells in any control (untreated) samples. Nevertheless, this technique may be useful with other reagents (bleach, cleaning products) in combination with other methods.

Trial 2: Measurement of chlorophyll a. Freshly collected periphyton (mainly the green alga *Spirogyra* sp. in a mixed algal community) was blended to make a homogeneous suspension. Known quantities were added to solutions of 5% chlorine bleach for 1 minute and 10% salt for 1, 10 and 30 minutes. Measured volumes of the suspension in the treatment solution were then filtered, and the filters rinsed with distilled water. The filters were then placed in 90% ethanol in centrifuge tubes, and boiled for 5 mins to extract the chlorophyll *a*. The concentration of the extracted chlorophyll *a* was read using spectrophotometric methods. For the full method, see Biggs and Kilroy (2000). The bleach treatment immediately destroyed the chlorophyll *a* pigment, confirming the bleach was effective in killing cells of this periphyton community. However, none of the salt treatments had any effect on chlorophyll *a*. Such a strong salt solution (about three times the strength of sea water) would be expected to have a detrimental effect on freshwater algal cells, but measuring chlorophyll *a* provided no indication of this.

The third option, a staining technique, was therefore investigated. Crippen and Perrier (1974) described the use of dyes for distinguishing live and dead marine plankton, including diatoms. They found that both Evans blue and neutral red dyes were effective on diatoms. The semipermeable membranes of living plant cells exclude Evans blue dye, which is taken up only by dead cells. Neutral red[1] stains parts of the cytoplasm (but not the chloroplasts) of living cells only. Since a supply of aqueous solution of neutral red was available, and Evans blue was not obtainable immediately, the former dye was trialled (see section 3.1) and found to give satisfactory results. Neutral red stain was therefore used for the tests described below. This method was backed up with assessments of chloroplast colour changes and cell appearance.

[1] The neutral red vital staining technique is designed to show the progression of pH in the food vacuoles of ciliates, and cytoplasmic parts of various protests. The dye is also used to distinguish between the white blood cell types, eosinophils, neutrophils and basophils, by staining the cell granules differently (Clark 1973).

3. Methods

The tests were conducted in three stages.

1. Initial tests were undertaken on 22 December 2004 using periphyton comprising mainly the diatom *Cymbella kappii* collected from the Selwyn River, Canterbury, earlier the same day. *C. kappii* is very common in clear running water and tends to bloom in stable, low-flow conditions. The genus is closely related to *Didymosphenia*. Many *Cymbella* species produce stalks and some, including *C. kappii*, exude copious mucilage. Cells of *C. kappii* are very much smaller than *D. geminata* (up to 40 μm long compared to >120 μm). These preliminary tests had to be done using *C. kappii* because indicative results were required urgently before 24 December, and it was not possible to obtain *D. geminata* samples in time.

2. The tests were repeated on 18-20 January 2005 using *D. geminata* collected from the Mararoa River on 16 January 2005. However, problems keeping the algal mat viable meant that a full set of tests could not be completed, and no formal replications were undertaken.

3. Further trials were carried out on 3-5 February 2005, this time using material collected on 3 February. Because of the difficulty in keeping the mats alive for the 18-20 Jan tests, extra steps were taken to maintain the mats in the laboratory (section 3.3.2).

3.1. Staining technique

On the first two testing runs (22 December and 18 January), the effectiveness of neutral red dye for distinguishing live cells from dead cells was established. Portions of algal mat were killed quickly by immersing them in hot water (>60 ˚C). Subsamples of the heat-killed algae and untreated algae (immersed in tap water at room temperature, ~18 ˚C) were then added to 20 ml of a 10% solution of the stock neutral red dye. After approximately 15 min in the dye, small samples were transferred to glass microscope slides, teased apart for ease of viewing, moistened with distilled water as necessary, then covered with glass coverslips. Slides were examined under a Leica DMLB light microscope at magnifications ranging from x100 to x400. Representative cells were photographed using a Zeiss Axiocam digital imaging system attached to the microscope.

The 15 min immersion time was chosen following trials to find the optimum time for dye treatment. In a 10% solution, cells took up the dye quickly (within minutes) but

the colour did not become more pronounced in cells if they were left for longer than about 15 mins.

This general method was applied in all subsequent tests of potential sanitising agents, with immersion of portions of algal mat in the selected treatment for the prescribed times, rather than heat treatment. Samples lifted from treatment solutions were rinsed in either tap water (22 December and 18-20 January trials) or river water (3-5 February trials) in order to remove as much reagent as possible before staining. No further tests were undertaken on the heat treatment used as the control.

3.2. Reagents tested

The potential sanitising agents tested were all household products that are readily available at low cost from supermarkets. Ideally, any cleaning agents suggested must be easy to obtain, and cheap.

Treatments tested were as follows (reagent, concentration, duration of treatment):

1. 22 Decmber 2004, tests on *C. kappii*

Nappy cleaner[2]	5%	~30 secs
Chlorhexidine antiseptic[3]	5%	~30 secs
Chloroxylenol antiseptic[4]	5%	~30 secs

2. 18-20 January 2005, tests on *D. geminata*

18 January		
Salt	1%	1 min, 10 min, 60 min
Salt	5%	1 min, 10 min, 60 min
Nappy cleaner	2%	1 min, 20 min
Nappy cleaner	5%	1 min
Chlorhexidine antiseptic	2%	1 min, 20 min
Chlorhexidine antiseptic	5%	1 min, 20 min

[2] Napisan, powder form
[3] Savlon. Active ingredients of undiluted product: 3 mg/ml chlorhexidine gluconate, 30 mg/ml Cetrimide, 35 mg/L isopropyl alcohol.
[4]Dettol. Active ingredient: chloroxylenol 48 mg/ml

19 January		
Chloroxylenol antiseptic	5%	1 min
Detergent[5]	5%	2 min
Household bleach	5%	1 min
18-20 January		
Desiccation		

3. 3-5 February 2005, replicated tests on *D. geminata* (treatments selected to confirm previous successful results, i.e., no stained cells seen following treatment)[6].

Nappy cleaner	5%	30 sec
Bleach	1%	30 sec
Salt	2%	1 min, 10 min
Salt	5%	1 min
Chlorhexidine antiseptic	5%	30 sec
Chloroxylenol antiseptic	5%	30 sec
Detergent	5%	30 sec
Desiccation		

All treatment dilutions were by volume (v/v, including the solid materials – salt and nappy cleaner).

3.3. Procedures

3.3.1. Tests on *C. kappii*

Treatment solutions were prepared in tap water (temperature 16 °C). Duplicate subsamples of the treated material were examined for each treatment. A control (untreated, dyed) sample was also examined.

[5] Down-to-Earth dishwashing liquid.
[6] The intention was to test progressively stronger solutions and longer immersion times for each agent if the most dilute solution and shortest immersion times showed any live cells in treated *D. geminata*.

3.3.2. Tests on *D. geminata*

18-20 January tests

Large sections of mature *D. geminata* mat were collected from the Mararoa River by Brian Goodger, Agriquality New Zealand, on 16 January. These were packed into plastic bags with sufficient river water to cover them, and shipped to Christchurch on ice. Mats were retained on ice until required for sampling. Prior to sampling, mat sections were transferred to open trays in the laboratory, and covered with tap water (temperature ~17 °C). Air was bubbled through the water periodically, to maintain some water movement. The water was changed as necessary to maintain a cool temperature.

Sections of mat approx. 10 x 8 x 1.5 cm were transferred to glass beakers containing the treatment solutions, and tap water (as controls). After immersion in the treatment solution or control tap water for the specified time, 2 or 3 fragments were cut from different parts of the upper side of the treated mat. Fragments were also cut from the inside (i.e., not in direct contact with the treatment fluid). The inside samples were to check whether the treatment fluid would penetrate to the inside of the mat. The fragments were transferred to the staining solution and gently pulled apart as necessary, to ensure that all cells would be in contact with the stain. After staining, small subsamples from each fragment were transferred to microscope slides, teased apart further, mounted under cover slips, as noted in section 3.1, then examined under the microscope (section 3.3.4).

3-5 February tests

For the trials on 3-5 February 2005, colonies of *D. geminata* were collected from the Mararoa River (by Brian Goodger) on 3 February and transported to Christchurch on ice the same day. All mats were left attached to river stones or plants, and placed in 2-litre screw-top transparent plastic jars, which were then topped up with river water to about 90% full. In the laboratory, the stones were sampled directly from the collecting containers, which were kept on ice between sampling. The changes to collecting procedure and laboratory maintenance were necessary because the large mats collected for the previous trials deteriorated very rapidly after arrival at the laboratory.

All reagents were diluted using river water (after filtering through a 60 μm screen), rather than tap water. Using river water ruled out any potential effects of a change in water chemistry on the treatments. The samples were kept on ice during the course of the tests, as a cooler temperature helped to maintain their viability. To ensure that the

effects of temperature were not included, reagents were chilled to approximately the same temperature as the sample water before the treatments began.

Four replicate mat pieces (approx 2 x 1 x 1 cm) were subjected to each treatment, with each replicate cut from a different colony (from at least two different containers). Matching control pieces were transferred directly to the dye solution. Separate containers were used to treat each piece. Subsamples from each portion were transferred onto glass slides (one slide per portion), mixed and teased apart, then covered with cover slips (two per slide) and examined (see section 3.3.4). Mixing ensured that cells from all through the mat were in the samples examined, including any live cells persisting on the inside.

3.3.3. Desiccation tests

Two desiccation trials were undertaken. The first test was to obtain an idea of the time taken for complete drying of large growths of *D. geminata* under natural conditions (e.g., exposure to sunshine for at least part of the day). The second test was aimed at determining the survival of viable cells in mats of different water contents, dried for different lengths of time.

On 18 January, a large mat, up to 3 cm thick, was removed from water and allowed to dry naturally in a tray that was tilted so that water drained out. The mat was exposed to sunlight for part of the day through a west-facing open window. Similar conditions might be expected if mats were drying in situ in the river-bed following a reduction in river levels. Samples were taken from different parts of the mat after 15 and 48 h (5 replicates on each occasion), stained, and examined under the microscope.

On 3 February, 32 pieces of *D. geminata* colony, each approx. 3 x 3 x 1 cm, were transferred to pre-weighed, numbered aluminium foil weighing dishes. Each piece was drained briefly after removal from the water. At the start of the trial, small fragments were removed from sample numbers 1 – 4, and stained for subsequent examination under the microscope for the presence of stained (i.e., live) cells. The first four samples (minus the removed fragments) were then weighed (including the weighing dish), transferred to a drying oven at 105°C for approx. 8 h, until all the water had been driven off, then re-weighed. This allowed calculation of the % water content of the initial samples. The remaining 28 pieces of mat were laid out on a tray and allowed to dry slowly over the next 3 days. At 8-14 h intervals, 3 or 4 pieces were removed, and the procedure for samples 1 – 4 repeated. The last samples were weighed and dried approximately 78 h after the start of the trial. Drying was undertaken relatively

slowly (not in direct sunlight) to ensure that cells from mats with a range of water contents were examined.

3.3.4. Microscope examination

Each slide mount was examined under the microscope by scanning the entire sample at x200, and re-examining individual cells at x400 if traces of dye were seen. If any live cells (i.e., with red-stained granules in the cytoplasm) were observed, an estimate of the proportion of live cells vs. dead cells in the sample was obtained by counting 100 cells and noting the number stained vs. unstained. All cells were counted within a narrow plane of focus in random fields along vertical transects.

During microscope examination, qualitative observations were also made about the condition of the cells, particularly the chloroplasts and stalks. Other features of the samples were noted, such as presence or absence of staining in other algae.

Representative microphotographs were taken using the digital imaging system (see section 3.1).

4. Results

4.1. Effectiveness of neutral red dye for distinguishing live from dead cells

Figure 1 shows representative cells of *C. kappii* and *D. geminata* after heat treatment, compared with control (untreated) cells. In both cases, no red-dyed cells were seen in the heat-treated samples and the chloroplasts changed colour from the typical golden-brown of diatoms to yellow-green. However, in the untreated samples, after staining many of the cells contained deep red granules and often streaks of red throughout the cell. In the *C. kappii* trial it was noted that stained cells were still motile, therefore clearly still alive.

It was noticed throughout the trials that the neutral red dye did not always affect the cells in exactly the same way. Often many small moving granules were visible in the clear part of the cell. Sometimes larger granules were present. Occasionally the cells had an overall pink tinge, or pink streaks. This is consistent with observations on the intestinal parasite *Blastocystis* by Vdovenko (2000), who showed that changes in the dyed appearance of the cells occurred progressively as length of exposure to the dye increased. The changes appeared to be associated with degeneration of the cell.

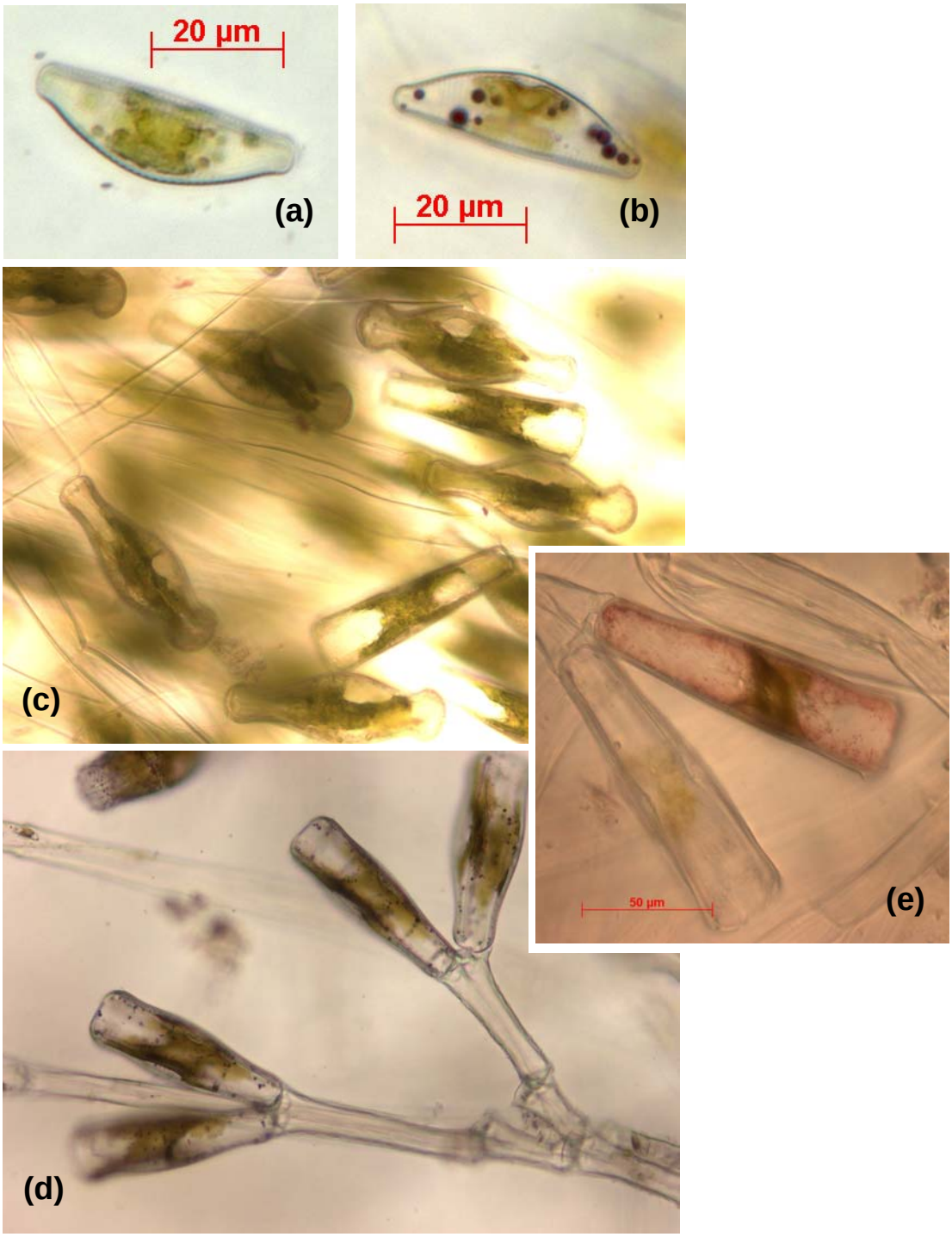


Figure 1: **Trial to test effectiveness of dye technique for distinguishing live and dead cells. (a) Heat treated *Cymbella kappii*, after staining; (b) untreated *C. kappii* after staining; (c) heat-treated *Didymosphenia geminata*, after staining; (d) untreated *D. geminata* (side views); (e) a pair of *D. geminata* cells after staining. The lower cell is not viable. Note the speckled red appearance of stained cells.**

4.2. Tests on *C. kappii*

All three treatments on *C. kappii* resulted in no stained cells and no visible motility in the treatment subsamples. On the other hand, a control sample contained many stained and motile cells. Examples are shown in Fig. 2.

4.3. Tests on *D. geminata,* 18-20 January

The results are summarised in Table 1. Examples of the visual effects of treatments, with some control (untreated) cells for comparison, are shown in Fig. 3. For an overall comparison, Fig. 4 shows examples of healthy, unstained cells.

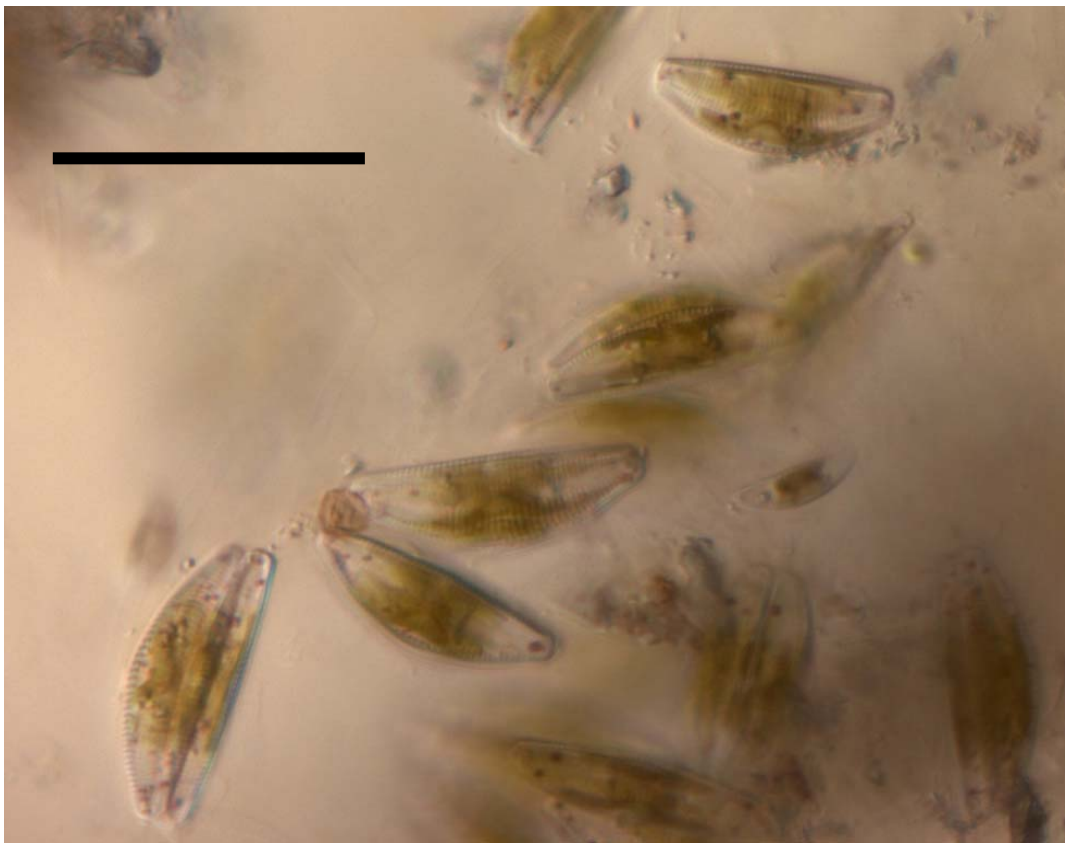
All treatments reduced the numbers of cells staining, compared to the controls. Cells on the outside of the mat were more severely affected than those on the inside. This was especially evident with the 1% salt solution. Deterioration of the controls meant that the effect of prolonging exposure to the decontamination agents could not be demonstrated clearly. In the 60 min treatment for both 1% and 5% salt solutions, no stained cells were found in samples from inside both treated and untreated (control) mats.

No stained cells were found in samples from the outside of mats immersed in 5% solutions of Nappy cleaner or Chlorhexidine antiseptic. However, the 5% Chlorhexidine antiseptic was less effective than Nappy cleaner at penetrating to the middle of the mat. Some cells from the inside of the mat were still taking up stain after 20 minutes in the former solution. Weaker (2.5%) solutions of Nappy cleaner and Chlorhexidine antiseptic were also less effective.

No stained *D. geminata* cells were found in subsamples after immersion of mats for approx. 1 min in 5% solutions of bleach, Chloroxylenol antiseptic or detergent. However, at this stage the mats were deteriorating, with only ~15% live cells present in the control sample (outer part of mat) (Table 1). Therefore the tests are inconclusive.

Because the mats deteriorated so quickly, it was not possible to undertake replicated tests using this material.

(a)



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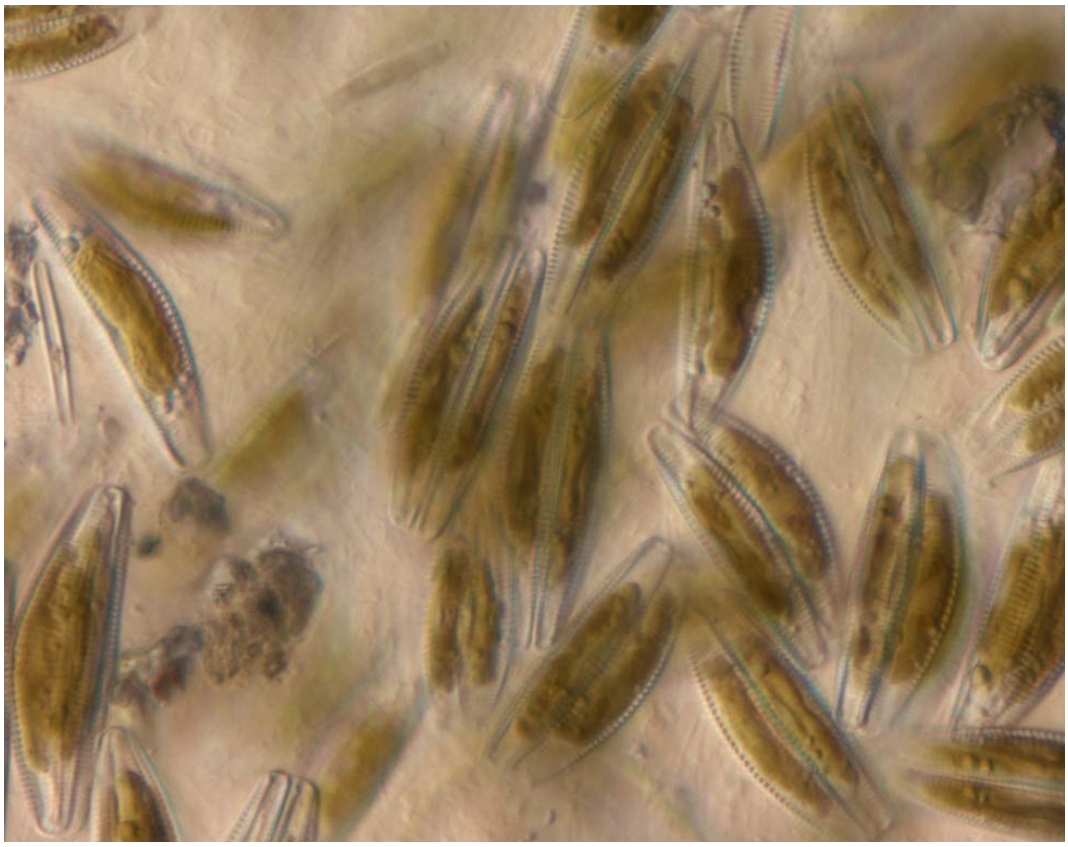

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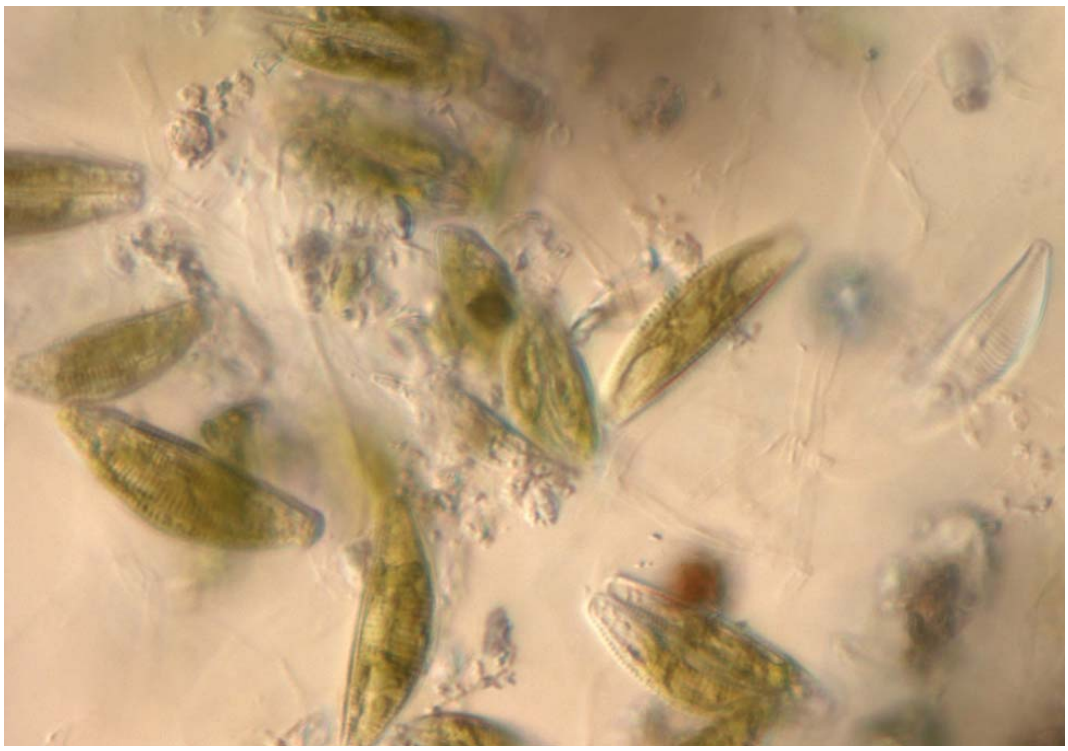

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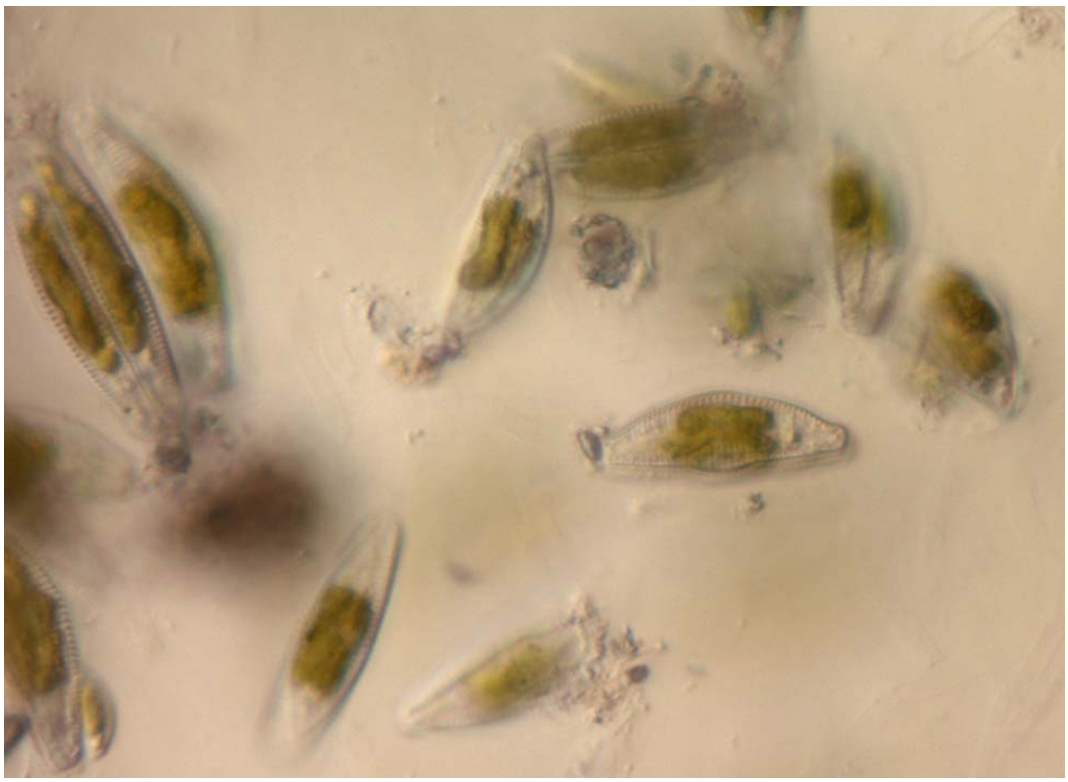

Figure 2: ***Cymbella kappii*****. (a) untreated cells, showing dye-stained cytoplasm; (b) treated with 5% Napisan; (c) treated with 5% Savlon; (d) treated with 5% Dettol. All treatments for ~ 30 sec. Scale bar in (a), 50 μm.**

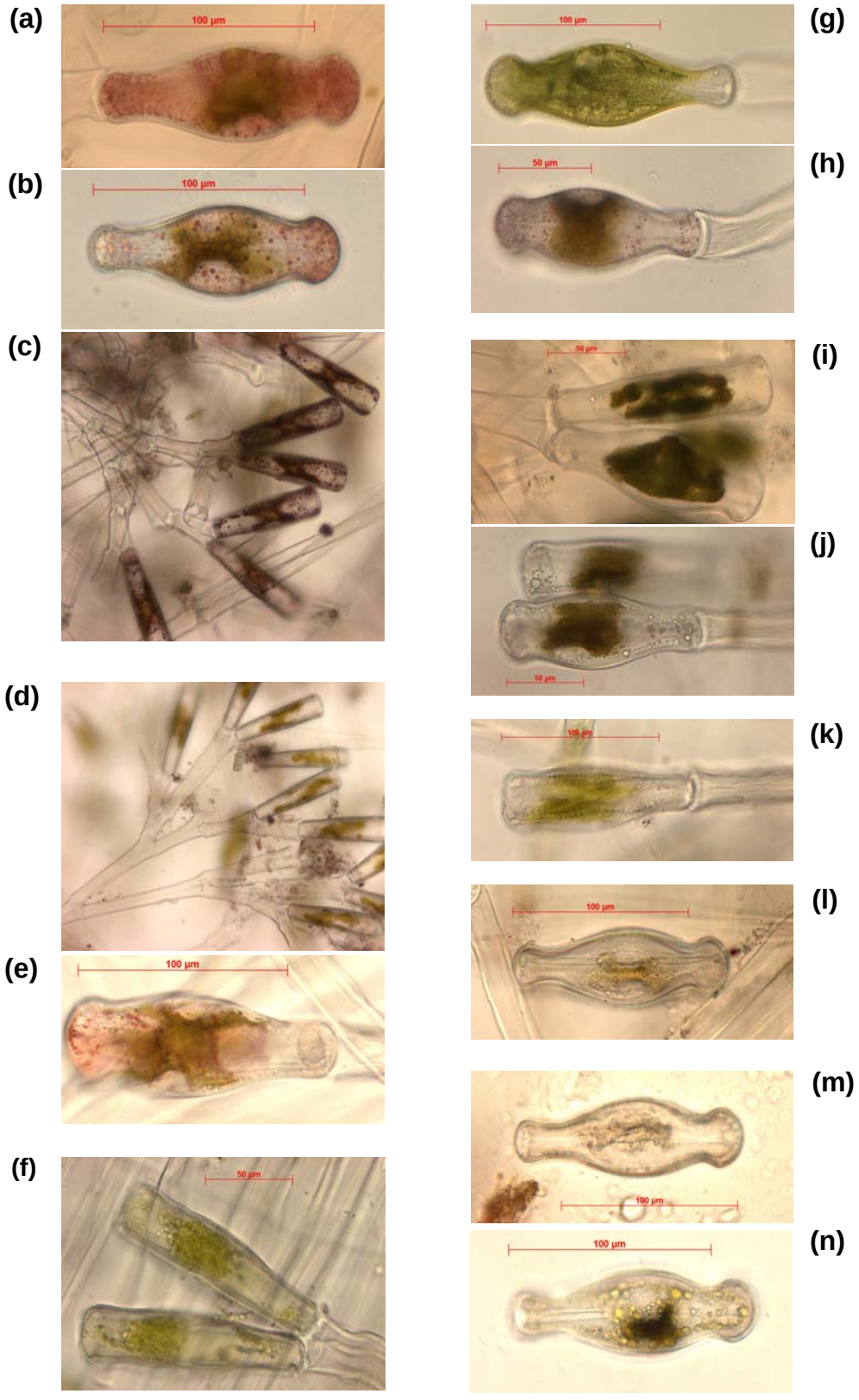

Figure 3: **Examples of cell appearance following different treatments, and after staining for 15 mins. (a), (b), (c) Control mats (no treatment); (d), (e) after salt treatment; (f) nappy cleaner; (g), (h) chlorhexidine antiseptic, outer and inner cell; (i), (j) chloroxylenol antiseptic, outer and inner cells; (k) detergent; (l) bleach; (m), (n) desiccation, outer and inner cells.**

Table 1: Summary of results of trials to determine effectiveness of decontaminating agents against *D. geminata,* 18-20 January 2005. Tests were undertaken on pieces of mat approx. 10 x 8 x 2 cm. Mat location refers to samples taken from the outer part of the mat, and samples cut from the inside of the mat. Cells that take up neutral red stain are assumed to be live; dead cells do not take up the stain.

Agent	Conc. (%, by vol.)	Treatment time (mins)	Mat location	% stained, treated	% stained, control	Observations on treated sample
Salt	1	1	outside	3	63	
		1	inside	14	43	
		10	outside	<1%	nd	Many cells with large and small air bubbles
		10	inside	12	nd	
		60	outside	<1%	8	Most *D. geminata* dead, but many small *Encyonema* stained
		60	inside	2	nd	
Salt	5	1	outside	1	61	
		1	inside	<1	50	Very slight stain in a very few cells in treatment solution
		10	outside	<1	66	
		10	inside	<1	11	
		60	outside	none seen	9	
		60	inside	none seen	none seen	
Nappy cleaner	2.5	~1	outside	21	50	Many other diatoms present in treated mat, none seen to be stained
		~1	inside	9	43	
		20	outside	none seen	nd	Greenish chloroplasts, diffuse-looking
		20	inside	none seen	nd	
Nappy cleaner	5	~1	outside	none seen	50	
		~1	inside	none seen	43	
Chlorhexidine antiseptic	2.5	~1	outside	<1	50	

Agent	Conc. (%, by vol.)	Treatment time (mins)	Mat location	% stained, treated	% stained, control	Observations on treated sample
		~1	inside	2	43	
		20	outside	none seen	nd	Chloroplasts in treated mat brownish-green, not diffuse
		20	inside	2	nd	
Chlorhexidine antiseptic	5	~1	outside	none seen	50	
		~1	inside	10	43	
		20	outside	none seen	nd	
		20	inside	8	nd	
Chloroxylenol antiseptic	5	~1	outside	none seen	15	Chloroplasts turned greenish and contracted, with bubbles in cells
		~1	inside	none seen	2	Green colour less pronounced, but many bubbles in cells"
Detergent	5	1	outside	none seen	15	Similar reaction as Chloroxylenol antiseptic
		1	inside	none seen	2	
Bleach	5	~30 sec	outside	none seen	15	Immediate colour change in mat. Chloroplasts bleached
		~30 sec	inside	none seen	2	" "
Desiccation		15 h	outside	6	50**	Most cells with contracted chloroplasts, and air bubbles
		15 h	inside	2	43	" "
		48 h	outside	none seen	50	Chloroplasts bleached, bubbles present, and most cells detached from stalks
		48 h	inside	none seen	43	Chloroplasts often darker coloured

nd = not done
**Desiccation controls all taken as mat condition at time of draining off water. This was also the control for the Nappy cleaner and Chlorhexidine antiseptic 1-min trials

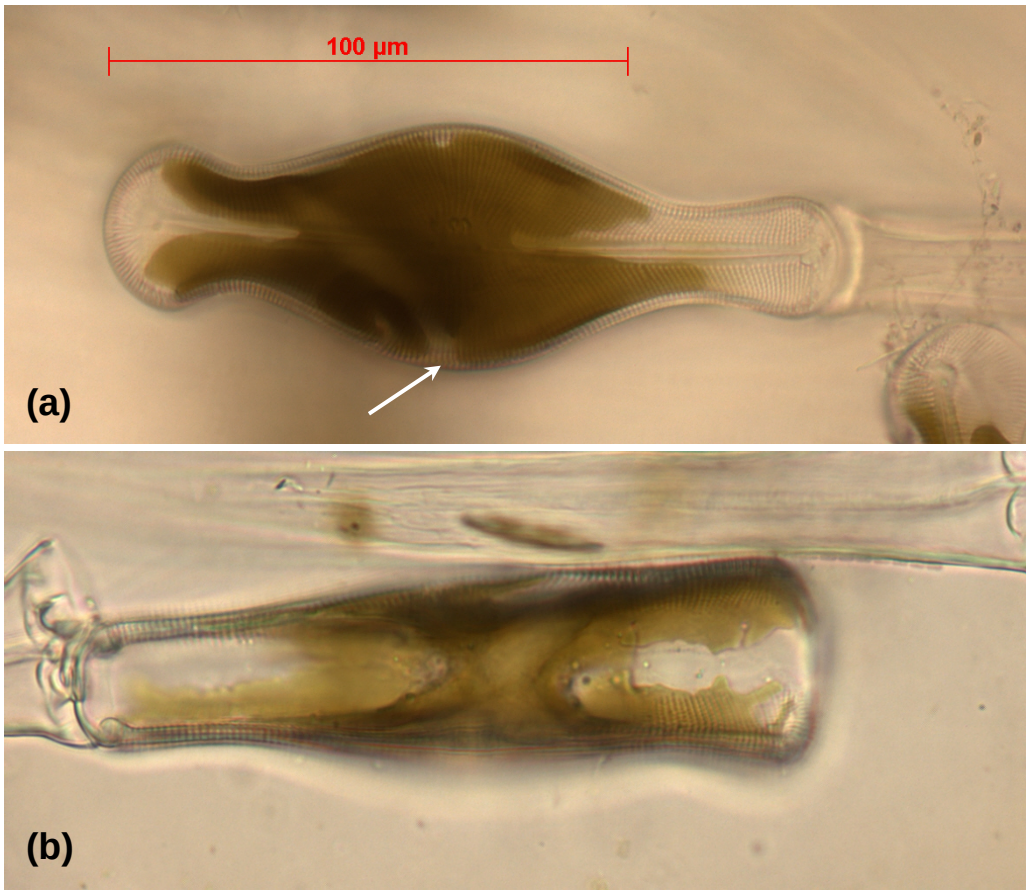

Figure 4. **Examples of healthy *Didymosphenia geminata* cells, unstained. (a) Top view; (b) side view. Note the golden-brown chloroplasts, which are characteristic of diatoms. The chloroplast is H-shaped, has well-defined margins, and in tope view typically shows an indentation or fold at the widest part of the cell, usually much more marked on one side than the other (arrowed in (a)).**

4.4. Tests on *D. geminata*, 3-5 February

The results are summarised in Table 2. The three "sessions" refer to sets of tests undertaken with no breaks (1 – 3 hours) with control samples tested at intervals.

With the exception of 2% salt for 1 minute, all the treatments yielded no stained cells after microscope examination of 4 replicate samples of treated, stained material. The 2%, 1-min salt treatment showed a tiny proportion of stained cells. A repeat of the test produced the same result. When the time of exposure to 2% salt was increased to 10 min, or the concentration increased to 5%, no stained cells were detected. A 1% solution of bleach was also effective after 30 s immersion, therefore it was not necessary to test stronger solutions.

Table 2: **Summary of results of trials to determine effectiveness of decontaminating agents against *D. geminata*, 3-4 February 2005. "Session" refers to a set of trials carried out with no interruptions. Tests were undertaken on pieces of healthy colony (or whole, young colonies) approx. 2 x 1 x 1 cm. Cells that take up neutral red stain are assumed to be live; dead cells do not take up the stain.**

Session	Treatment agent	Concentration (%, by vol.)	Treatment time	No. replicates	% cells stained	sd
1	control			13*	58	16.1
	Nappy cleaner	5	30 s	4	0	-
	Bleach	1	30 s	4	0	-
2	control			12	53	14.1
	Salt	2	1 min	4	0.25	0.50
	Salt	2	10 min	4	0	-
	Salt (repeat)	2	1 min	4	0.25	0.50
	Salt	5	1 min	4	0	-
3	control			4	46	7
	Chlorhexidine antiseptic	5	30 s	4	0	-
	Chloroxylenol antiseptic	5	30 s	4	0	-
	Detergent	5	30 s	4	0	-

* includes desiccation trial initial counts.

4.5. Desiccation tests

In the desiccation trial on 18-20 January, a small proportion of live cells was detected in the partly dried mat after 15 h. After 48 h, the mat was thoroughly dry and no live cells could be found (Table 1). The chloroplasts in all the cells were contracted, many contained bubbles, and many were bleached (Fig. 3m, n).

The results of the desiccation trial on 3-5 February are summarised in Fig. 5. Figure 5a shows that the percentage of live cells found in subsamples varied considerably at the start of the trial when the samples were still hydrated, but after about 24 hours an overall decline had begun. Water content reduced slowly from the maximum of over 95% (Fig. 5b). The later stages of the decline (<80% water content) were not recorded

in this trial. However, below moisture contents of approximately 83%, no live cells were detected in any samples (Fig 5c). Mats with water content of less than ~83% appeared to be only very slightly damp, and no water pooled on the mat surface when slight pressure was applied.

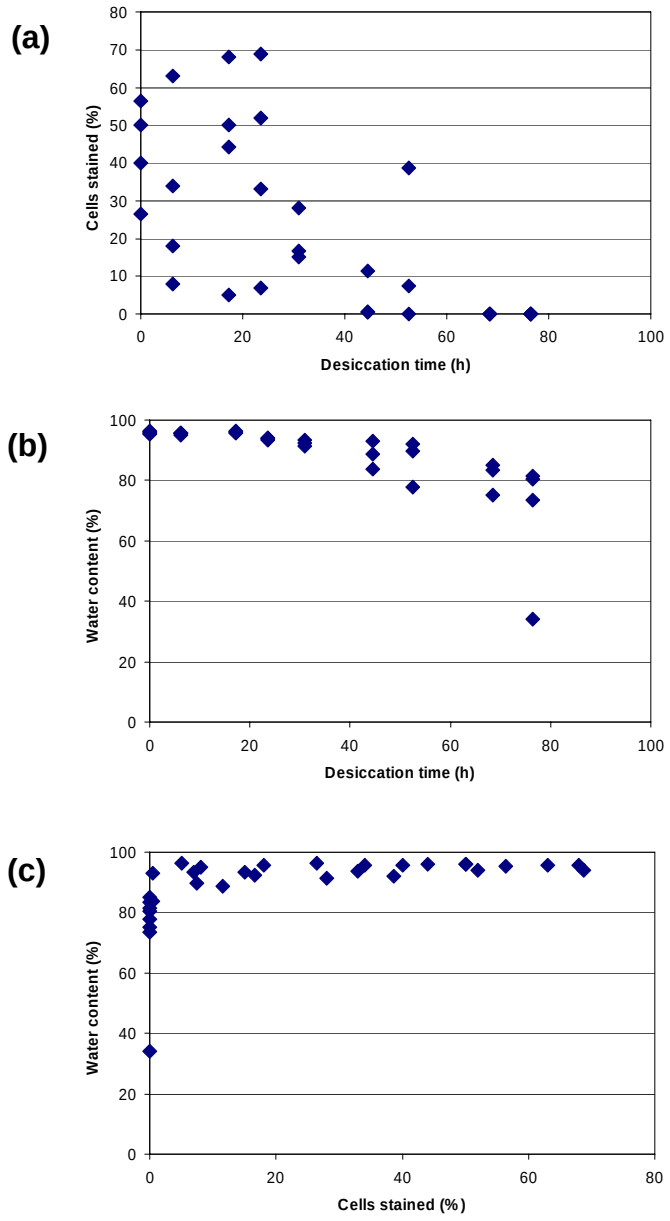

Figure 5. **Summary results of the desiccation trial on 3-5 February. (a) Percentage of stained cells found in subsamples plotted against desiccation time. (b) Water content of the mat plotted against desiccation time. (c) Water content plotted against the percentage of stained cells found, showing that below about 83% moisture, no stained cells were detected.**

4.6. Observations on cell condition following treatment

When applied to *Didymosphenia* colonies, some of the treatments caused rapid visible changes to the colonies and cells. These constitute further evidence that the cells were no longer viable. Figures 2 and 3 show examples of chloroplast structure in treated and healthy cells. Notes from the 18-20 January tests are included in Table 1, and Table 3 summarises observations on both the macroscopic appearance of colonies and the microscopic appearance of cells. In the desiccation treatments, it was very obvious that, as the mat dried, the cells gradually became detached from their stalks.

5. Discussion

The staining tests on *D. geminata* on 18-20 January confirmed the results of the earlier trials on *C. kappii*, that immersion the algal mats in 5% solutions of Nappy cleaner, Chlorhexidine antiseptic or Chloroxylenol antiseptic for less than 1 minute was effective in preventing any cells from throughout the mats tested from taking up the neutral red dye. From the initial trials on the heat-treated vs. control samples, we can infer that cells that did not take up the dye were dead.

Because the *D. geminata* mats used in the 18-20 January trial deteriorated very quickly, the results of the trials on Chloroxylenol antiseptic, as well as additional trials using household bleach and detergent, cannot be relied upon. In the subsequent tests carried out on 3-5 February, solutions that were not effective in the earlier tests (1% salt, 2.5% Nappy cleaner) were not re-tested. Repeat, replicated tests confirmed that 5% solutions of Nappy cleaner, Chlorhexidine and Chloroxylenol antiseptics, and also detergent, were equally effective desanitising agents. A 30 s immersion in all four solutions was sufficient. A solution of only 1% household bleach was also effective after 30 s. Thus there is a good choice of reagents that can be used for rapid and effective sanitisation of materials that may have come into contact with *D. geminata*. All the reagents used are easily available from supermarkets at low cost. Household bleach, in particular, can be hazardous; however, warnings are printed on all commercially available brands.

It is stressed that for effective sanitisation, the reagents should be used at the concentrations and times given, otherwise there is no guarantee of penetration to the inside of large pieces of mat. Although 5% nappy cleaner, antiseptics and detergent were effective in 30 s in the tests, a 1-min treatment is suggested to ensure that all cells are treated. A 2% bleach solution is suggested for the same reason. In addition the items being treated need to be *immersed* or *soaked* in the treatment agent. Clear instructions to first remove any visible fragments of algae from items to be treated will help to ensure that subsequent chemical treatment is completely effective.

Table 3. Summary of observations on *D. geminata* colony and cell colour and form following treatments

Treatment	Conc. (% by vol.)	Treatment duration	Macroscopic appearance	Microscopic appearance			Illustrated in :
				Chloroplast colour	Chloroplast form	Other	
No treatment, unstained			greyish white with golden brown sheen at surface	golden-brown	clearly defined edges, H-shaped, indentation (fold) visible to one side of the cell		Fig. 4
No treatment, stained				golden-brown	edges defined, H-shaped, but tendency to be shrunken away from cell walls		Fig. 3(a)
Salt	2	1 min	greenish tinge at surface of mat	golden-brown - greenish	irregular shape, undefined edges	bubbles present in cells	Fig. 3(d)
	5	1 min					
Nappy cleaner	5	30 s	greyish white with golden brown sheen at surface	greenish - golden-brown	diffuse edges, no well-defined shape	mat tends to float	Fig. 3(f)
Chlorhexidine antiseptic	5	30 s		definite green	diffuse edges		Fig. 3(g)
Chloroxylenol antiseptic	5	30 s		brown to brown-green	contracted; diffuse edges		Fig. 3(i),(j)
Bleach	1	30 s	light brown-green after 30 sec, (gradually changing to green and becoming very pale after 2-3 min)	pale green	contracted; irregular edges; granular appearance		Fig. 3(l)
Detergent	5	30 s		brown-green	diffuse edges; contracted	bubbles present in cells	Fig. 3(k)
Desiccation		24 h	greyish white, dull brown or greenish at surface	pale yellow to yellow-green to dark brown	mildly to severely contracted	cells become detached from stalks; bubbles present	Fig. 3(m),(n)

Salt solution also appeared to be a reasonably effective sanitisation agent, although only the 5% (v/v) solution worked rapidly (1 min). The 19 January tests indicated that a longer immersion even at 5% might be necessary to guarantee that all *D. geminata* cells are killed. Nevertheless, even though salt is apparently not quite as effective as the household products tested, it may be the only practical method for decontaminating boats where they can be moved from the river through to the sea. Full strength sea water contains approximately 3.5% (w/v) salt, which is equivalent to ~1.6% v/v (the density of salt is just over 2 g/ml). The replicated tests on *D. geminata* indicated that 10 minutes immersion in salt solution of at least 2% v/v strength would eliminate all live cells. It is suggested that *at least* 1 hour's immersion in sea water would be necessary to ensure that no cells remain viable, but this remains to be tested.

The tests on 19 January highlighted the great difficulty in maintaining *D. geminata* mats in a healthy condition in the laboratory. The mats were held in trays filled with cool water, with air bubbling through the water. Even though they looked and smelt "normal" after 3-4 days, the staining procedure indicated that the proportion of viable cells was declining rapidly. No literature has been located reporting the successful culture of *D. geminata*. Attempts by Sherbot & Bothwell (1993) failed.

Failure to maintain healthy colonies meant that the tests undertaken on 18-20 January could not be repeated. The experimental procedure involved completing each test before the next one could be started. It was not possible to preserve material on slide mounts and examine it later as Crippen & Perrier (1974) reported that preserving agents caused the dye to leach from diatom cells. Therefore dyed material had to be examined immediately after staining for the standard 15 mins, and this limited the time available for the treatments themselves. Nevertheless, the January tests provided useful screening tests indicating that a range of agents appeared to be effective in killing *D. geminata* cells.

In the February tests, the trials commenced within 6 h of removal of *D. geminata* colonies from the Mararoa River. This, along with the extra precautions taken to maintain the colonies (see section 3.3.2), and possibly the fact the colonies were still attached to rocks or plants, resulted in improved cell survival over the course of the tests. Nevertheless, the average proportion of live cells in the control samples declined as the tests proceeded (Table 2). Because of variability in the cell counts the differences were not statistically significant, but very probably indicated a trend. Some of the material supplied for the February tests is being maintained in a controlled temperature room at NIWA, Christchurch, to evaluate the longer-term viability of mats in water from different sources. The material has been examined 2 and 9 days after collection (see Appendix 1).

During the tests, a few informal observations were made on the viability of other organisms within the mat following treatment with decontaminating agents. Other live algae were observed only in the salt solutions, where cells of *Spirogyra* and *Zygnema* still took up the red stain after immersion in 2% salt for 1 min. Small chironomids were also seen moving. After a 30-sec treatment with bleach, nematodes were seen to be still moving. In all other treatments, no live algae or other organisms were noted.

Desiccation of a large mat of *D. geminata* in the January trial showed that cells within the mats are able to survive for at least 15 h out of the water, including a period of exposure to direct sunlight. In the more rigorous desiccation trial in February some cells survived for 52 h. However, survival depends very much on the amount of water retained in the mat. No live (stained) cells were detected in pieces of mat in which the moisture content had fallen below about 83%, regardless of how long it took to reach this level of water content (up to 3 days, Fig. 5). At around 83% moisture content, the mats still looked damp, but barely released water when slight finger pressure was applied to the surface. At less than 80% moisture, the mats were dry at the surface. The water content of the sample containing live cells after 52 h was 90%. At this moisture content, mats still looked wet and water pooled on the surface of the mat under very slight finger pressure.

The present drying trial was undertaken in relatively warm conditions (~20°C). Under cooler conditions, drying would be delayed and presumably some cells would remain viable for longer, providing the mats were holding sufficient water. This could be tested in the laboratory by running similar drying trials at different ambient temperatures. The present trials were undertaken in light and dark conditions. Desiccation in the dark (for example, inside a piece of equipment) could also take longer and may allow live cells to persist for longer.

If large items of equipment are used in the affected rivers, drying out may be the only practical means of decontamination, since immersion in chemicals may be difficult to achieve unless it is possible to shift boats and kayaks into sea water (see above). The results of the desiccation trials indicate that drying *must* be complete to be absolutely sure that no *D. geminata* cells survive.

The ability of live *D. geminata* cells to persist in fragments of wet mat for at least 52 h (in a day / night light cycle and under relatively warm conditions) explains how this diatom could have been initially transported into New Zealand from overseas.

One effective method of killing algae that was not directly tested in these trials is heat. Rapid heating to 60 °C was the recommended laboratory procedure for killing cells to validate the staining method used to distinguish live and dead cells (Crippen & Perrier

1974). More recent literature has cited heat as an effective method for killing diatoms. For example, Forbes & Hallegraef (2002) suggested that heating ballast water to just 35 °C was an effective strategy for preventing the transport of live marine diatoms in ballast tanks. The informal trials to determine the viability of *D. geminata* under different conditions (Appendix 1) strongly suggest that temperatures >22 °C are detrimental to *D. geminata* survival compared with a temperature of around 14 °C. Heat treatment can therefore be recommended as a further option for sanitising materials that have come into contact with *D. geminata*. Exposure to a temperature of 60 °C is recommended. The easiest way to achieve this is to immerse the item in hot water, ensuring that the temperature stays greater than 60 °C after introduction of the item.

A further agent that was not directly tested was shampoo. This is relevant because it may be necessary to sanitise hair after swimming in an affected river. The fact that the very mild dishwashing detergent tested was just as effective as antiseptic and disinfectant solutions suggests that shampoo is probably also effective. However, to ensure that live cells do not persist in wet hair, an extra precaution would be to allow hair (and any *D. geminata* cells trapped within it) to dry completely before shampooing. Then shampoo for at least 1 minute, following by thorough rinsing in warm to hot water.

The primary aim of these trials was to test procedures for killing *D. geminata* cells on clothing and equipment that had been in contact with the alga, rather than *D. geminata* in situ. Mat fragments/cells on clothing and equipment will usually be subject to drying, elevated temperatures and cell deterioration in between leaving the river and being decontaminated. Thus, even though the viability of cells in the mats declined in the laboratory, the same situation would apply to any *D. geminata* removed from the river on clothing or equipment. Accordingly carrying out tests on relatively large, intact mat pieces ensured that the probable worst-case scenario was covered (a large piece of mat lodged in equipment or clothing). The initial advice to river users – to first remove any obvious algae fragments from clothing and equipment – is an important step, before proceeding with any chemical or heat treatment.

If it is necessary to test agents for killing live *D. geminata* in situ, then trials will need to be undertaken in streamside channels alongside the affected rivers, probably the only way to maintain healthy colonies long enough to run the tests. The results of the present trials are indicative only of what might occur in a natural situation.

6. Conclusions

D. geminata proved to be relatively easy to kill rapidly, using simple treatments with materials cheaply and readily available in supermarkets: nappy cleaner, household disinfectants, detergent and bleach. Salt solutions (>2% v/v) killed *D. geminata* cells, but appeared to be less reliable than the cleaning agents. Desiccation of *D. geminata* mats was also effective. However, in order to be 100% confident of this method, the desiccation needs to be complete. Mats composed of >83% water may contain live cells, even though at this level of moisture the mats appear damp rather than wet. Mats may take several days to dry to less than 83% moisture, the time taken depending on ambient conditions.

Heat treatment to kill *D. geminata* was not tested directly in these trials, but heating to at least 60°C is an accepted laboratory technique for killing algal cells. Treatment of hair following swimming was also not tested directly, but a combination of drying, shampooing, and rinsing with warm to hot water should eliminate any live cells.

These results may be used to formulate recommendations to the public for the decontamination of any materials or equipment that may have come into contact with this unwanted organism.

7. Recommendations

Any clothing, equipment or other material that may have come into contact with *D. geminata* may be sanitised by soaking and scrubbing in 5% solutions of either nappy cleaner, household antiseptics (chlorhexidine or chloroxylenol based) or detergent for about 1 minute. Soaking is necessary to ensure complete immersion of all cells for at least 30 s; scrubbing is advisable in order to dislodge any pieces of the algal mat from crevices, and to break up larger fragments. The tests showed that these agents were effective after 30 s immersion. However, to allow for complete penetration to the inside of larger algae fragments, and for a margin of error in estimating time, a 1-min treatment is suggested. Immersion in 1% household bleach for 30 s was also effective. For the same reasons, a 1-min treatment with 2% solution is recommended.

Alternatively, immersion of contaminated items in hot water for 2 min, to a final temperature of at least 60 °C, is recommended.

The effectiveness of shampoos as decontaminating agents was not tested directly. It is therefore recommended that after swimming in an affected waterway, hair should be allowed to dry before shampooing for *at least* 1 minute, then rinsing thoroughly using warm to hot water.

Salt solution of at least 2% was also largely effective in killing *D. geminata* cells. The results were less conclusive than for the commercial cleaning products, therefore the latter are preferred decontamination methods. However immersion in sea water may be the only practical means to decontaminate some boats. *At least* 1 hour in full strength sea water is recommended, but further testing of this is desirable.

For effective treatment of equipment using desiccation, drying *must* be complete, since damp mats can still support some live cells. Drying for an extended period of time (e.g., 48 hours) *after* the mats appear to be dry is recommended. Exposure to direct sunlight assists in the drying process.

Advice to the public based on the results of the tests reported in this document are now available on Biosecurity New Zealand's website: www.biosecurity.govt.nz/pests-diseases/plants/didymo/index.htm.

8. Biosecurity precautions during the trials

The following summarises the precautions taken to prevent the spread of *D. geminata* during the course of the tests described in this report, in accordance with "Permission to multiply the unwanted organism *Didymosphenia geminata*" granted by Biosecurity New Zealand (Appendix 2).

- In accordance with the permission granted by Biosecurity New Zealand (see Appendix 2) all tests were undertaken in the rooms specified.

- Standard laboratory rules were followed at all times, as specified for: Physical Containment Level 2 in AS/NZS Standard 2243.3.2003, in accordance with MAF Standard 154.02.17: Transitional Facilities for Biological products, as specified in Appendix 3; and according to protocols set out n the NIWA, Christchurch Quarantine Manual (Sutherland 2004).

- Room A184 was locked during the time the trials were being undertaken, but personnel were not actively working on them.

- Prepared slides were transferred to the microscope room (room A170) in a sealed container and were returned to room A184 immediately after examination.

- All discarded *D. geminata* mats were held in room A184 in buckets of 10% bleach.

- At the conclusion of the tests, all *D. geminata* mats were wrapped in plastic and tinfoil, transferred to room A190, and autoclaved at 15 psi for at least 15 mins.

- Water used was filtered though a 300-μm sieve. Material caught in the sieve was autoclaved as above. The filtered water was made up to 10% bleach solution and left for at least 24 hours, then evaporated from a hard surface.

- All microscope slides, lab gloves, paper towels, disposable plasticware, etc. used during the tests were autoclaved, as above, before disposal.

- All other equipment and containers used were either soaked in 10% bleach and sun dried before washing, or autoclaved.

- Ongoing tests for viability of *D. geminata* colonies are being conducted in the controlled temperature room off room A190.

9. Acknowledgements

Murray Smith and Brian Goodger (Agriquality New Zealand) are thanked for organizing collection and collection (respectively) of the algal material used in the trials. Karen Robinson assisted with the trials, including microscope photography. Barry Biggs, Donna Sutherland, Scott Larned and Christina Vieglais are thanked for their constructive reviews of the experimental design and earlier versions of this report.

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Appendix 1

Preliminary trials to determine the viability of *Didymosphenia geminata* under laboratory conditions

Because the January trials indicated some difficulty in maintaining live *D. geminata* colonies in the laboratory, some of the colonies collected on 3 February were held in a range of laboratory conditions. Fragments were removed from colonies under each treatment after 2 and 9 days, and cells examined (4 counts per treatment) after staining as described for the trials of decontaminating agents. The results are summarised below. Briefly, cells did not survive in warm conditions (~22°C) in either tap water or river water, but all colonies held in a controlled temperature room at ~14.6°C, in a 16:8 h light:dark cycle contained some live cells after 9 days. In fact, in many of the treatments, the proportion of live cells appeared to have *increased* between 2 and 9 days. The techniques used did not allow for an absolute count of cells so there is no way of telling from these tests whether the live cells were those remaining after many other cells had become detached from their stalks, or whether the colonies were growing and dividing. Further subsampling may provide some clues. It was interesting that after 9 days a few live cells were noted without stalks and apparently showing motility, which is commonly seen in related types of diatoms.

Treatment			2 days		9 days	
			% stained cells		% stained cells	
Location	Water	Treatment	mean	s.d.	mean	s.d.
Laboratory	tap	Detached colony, high water volume	0.25	0.50	0	0
Laboratory	tap	Detached colony, low water volume	0.25	0.50	0	0
Laboratory	river	Detached colony, high water volume	0	0	0	0
Laboratory	river	Detached colony, low water volume	0	0	0	0
CT room	tap	Detached colony, high water volume	28	13	51	17
CT room	tap	Detached colony, low water volume	33	29	39	33
CT room	river	Detached colony, high water volume	24	23	62	21
CT room	river	Detached colony, low water volume	55	22	61	11
CT room	distilled	Detached colony, high water volume	19	14	75	15
CT room	deionized	Detached colony, high water volume	25	34	41	17
CT room	river	Colonies in situ on rocks, with air bubbler	13	9	6	10
CT room	river	Colonies in situ on rocks, no bubbler	46	7	34	11

Laboratory temperature: 17 – 23 °C

Controlled temperature room: 14.6 °C

Tap water: conductivity, 121.4 μS/cm, pH ~7.8

River water: conductivity, 62.1 μS/cm, pH ~7

Appendix 2

Permission to multiply unwanted organism *Didymosphenia geminata*

Pursuant to sections 53(2) and 53(3) of the Biosecurity Act 1993 ("the Act"), I hereby give permission to Dr. Barry Biggs, Regional Manager, National Institute of Water and Atmospheric Research Ltd (NIWA), Christchurch, New Zealand, or any person acting under his direction or control, to multiply or otherwise act in such a manner as is likely to encourage or cause the multiplication of *Didymosphenia geminata*, an organism listed in the register of unwanted organisms maintained under section 164C of the Act.

This permission is subject to the following conditions:

1. The multiplication of *Didymosphenia geminata* must take place in either of the following facilities, as appropriate, operated by NIWA at Christchurch:
   - the MAF approved transitional facility (Room A190);
   - the facility being prepared for approval as a containment facility under the Act (Room A184); or
   - the microscopy laboratory (Room A170).
2. This permission is limited to *Didymosphenia geminata* detected through general surveillance within New Zealand, or progeny derived as a result of these detections.
3. The organism may only be multiplied to enable NIWA to carry out its obligations under contract BFG/77/2003, purchase order N003275, with Biosecurity New Zealand, namely for the purposes of organism identification and studies to determine effective treatments for eradicating the organism.
4. All activities conducted with *Didymosphenia geminata* must follow proper quarantine procedures as specified in the Biosecurity New Zealand Standard 154-02-17: "Transitional Facilities for Biological Products", particularly section 4.4 on disposal which refers to AS/NZS Standard 2243.3.2002 (Safety in Laboratories), and section 4.10 on record keeping.
5. At the conclusion of the contract specified above, all *Didymosphenia geminata* held at NIWA must be destroyed by autoclaving or by another method that NIWA can demonstrate to my satisfaction will result in the complete destruction of *Didymosphenia geminata* organisms.
6. *Didymosphenia geminata* must be restricted to areas of the facilities at NIWA described above that can be secured to prevent entry of personnel not authorised to work with *Didymosphenia geminata.*

Signed at Wellington this day of 2005

Peter Thomson
Chief Technical Officer
Biosecurity New Zealand
Ministry of Agriculture and Forestry