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**Protocol for microscopic analysis of  
samples for the detection of  
*Didymosphenia geminata***

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**NIWA Client Report: CHC2007-071  
August 2007**

**NIWA Project: MAF07503**



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## **Protocol for microscopic analysis of samples for the detection of *Didymosphenia geminata***

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*Prepared for*

**MAF Biosecurity New Zealand**

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Reviewed by:

Approved for release by:

Handwritten signature of Donna Sutherland in black ink.Handwritten signature of Brian Sorrell in black ink.Handwritten signature of Karen Robinson in black ink, featuring a large, stylized 'K' and 'R'.

Donna Sutherland and Karen Robinson

Brian Sorrell



## Executive Summary

- At the request of Biosecurity New Zealand, a protocol has been developed for microscope scanning of algal samples aimed at detecting the invasive, exotic diatom (alga), *Didymosphenia geminata*, if it is present in a sample.
- The protocol requires use of an inverted microscope, and involves scanning subsamples of collected material for a standard length of time (10 minutes) at magnifications of up to at least 100 x.
- Prevention of cross-contamination is critical to avoid false positives, and steps are outlined to minimise this.
- Reporting procedures are included.
- Illustrations of *D. geminata* cells are provided to help determine the condition of cells observed, and to distinguish them from other diatoms.



## 1. Introduction

This document describes a protocol for microscope scanning samples of algae collected from rivers and streams, with the objective of detecting cells of the non-indigenous, invasive diatom *Didymosphenia geminata*, if they are present in the sample. The protocol has been requested by Biosecurity New Zealand to ensure that samples examined in different laboratories are treated in exactly the same way. The protocol also includes steps to minimise the chances of cross-contamination of samples, and decontamination procedures.

## 2. Objective and approach

The objective of microscopic scanning of samples is to detect *D. geminata* cells in sub-samples if they are present in the sample. If *D. geminata* is common in a sample, this is straightforward. If *D. geminata* is rare, then the chances of finding cell(s) increases with the proportion of the sample scanned, and with the accuracy of scanning.

The amount of algal material in samples varies considerably. Because of the large variation, standardising the proportion of a sample examined is impractical. For example, it is feasible to examine 100% of the material in very sparse samples, but for very dense samples examining even 1% would take hours of effort. Standardising the proportion of a sample examined is also somewhat artificial because the benthic area or water volume sampled also varies widely and cannot be easily measured in the field. Therefore standardising scanning effort is a logical means of treating samples in a comparable way. For realistic handling of large numbers of samples, scanning time is standardised to 10 – 15 minutes per sample. In most cases, this equates to scanning three subsamples (as defined below) of every sample. The time range allows for different working speeds of microscope operators.

Completely accurate scanning means that if there is one *D. geminata* cell in a subsample, it will be found. Very careful scanning of complete subsamples is therefore essential.

## 3. Samples

Two kinds of samples are collected: *benthic samples*, which are scrapings from rocks and other substrates on the river bed; and *drift samples*, which are obtained by filtering river water through 40 micron plankton netting. Both types of samples are treated in

exactly the same way for microscopic analysis although both types can be quite variable in the amount and type of material they contain. Both will have been collected into labelled containers for transport to the laboratory.

## 4. Equipment

- A good-quality inverted microscope with magnification up to at least 100 × (e.g., eyepiece 10 × and objective 10 ×). Higher magnifications are desirable (up to 400 ×) to allow closer examination of specimens.
- Cover glasses to fit the microscope sample chamber (well).
- Disposable pipetters.
- Tweezers (× 2, for handling cover glasses, for holding items in bleach solution).
- Squirt bottle with tap water.
- Tissues, paper towels.
- Decontamination kit (bleach solution, autoclave bags, disposal buckets).

## 5. Procedure

1. Before you start the analysis, make sure that there is enough clean bench space around the microscope to place samples, data sheets, etc.
2. Start with a **clean** microscope well. If in any doubt about the last use of the well, go through the following steps:
  - unscrew the well unit and discard the cover glass,
  - clean the well parts in bleach solution (at least 2%, for at least 1 minute),
  - wipe dry with a tissue,
  - rinse well with tap water and wipe dry with a new tissue,
  - reassemble the well unit with a new cover glass; handle the glass with a clean tweezers (used only for this purpose)

3. Samples containing a very high proportion of water to solid material should be allowed to settle for several hours (e.g., overnight) and excess water poured off (see section 6.2).
4. Before starting on a sample, check that the sample number on the datasheet and on the sample container correspond.
5. Shake up the sample to make a uniform suspension, then remove the lid from the sample container and place upper side down on the bench (so that no sample is transferred to the bench).
6. Using a new disposable pipetter for each sample, pipette out 1.5 to 2 mls into the clean microscope well unit. To keep the sample in suspension, stir with the pipetter. The idea is to get a representative subsample of the sample, so take it from the middle of the suspension. If the sample is very lumpy, you may need to withdraw algae fragments individually. If necessary, break up fragments in the container lid using the pipetter.
7. If the sample is very thin even after pouring off excess water (see 4.3), do not shake it up, but pipette solid material directly from the bottom of the container.
8. Use the same pipetter for all subsamples from the same sample. Leave it in the sample to ensure that it is not contaminated with material from other samples.
9. For ease of viewing, make sure the subsample is evenly distributed over the well.
10. If the subsample is very thick, use a smaller aliquot and dilute with a little tap water from the squirt bottle until you can see individual algae cells clearly.
11. Scan the subsample through the microscope at a magnification of approximately 100 x (the exact magnification will depend on the configuration of eyepiece and objectives on your microscope). At this magnification, *D. geminata* cells can be distinguished clearly. Use higher magnifications to confirm uncertain identifications. For example, in side view (see 3<sup>rd</sup> photo in Appendix) *D. geminata* and other diatoms may look very similar (wedge-shaped).
12. To the untrained eye, some species of native diatoms may look similar to *D. geminata*. Refer to the Appendix for comparative photographs of *D. geminata*

and common stream diatoms native to New Zealand. Definitive identification of *D. geminata* must be based on the characteristic size (30-50 µm width x 80-130 µm length) and the unique shape (like a curved bottle with bulbous ends).

13. Scan so that you view the entire subsample, working on vertical transects. (Vertical transects seem to be easier on the eyes than horizontal transects, which can induce nausea in some people.)
14. On completion of the first subsample scan, discard the material into a waste bucket then rinse out the microscope well thoroughly with tap water from the squirt bottle before pipetting in the next subsample. Repeat steps from 4.5 for the second and third subsamples (or more if the subsamples can be scanned rapidly). The aim is for an experienced microscopist to spend approximately 10 – 15 minutes on each sample (including changing subsamples).
15. If any *D. geminata* cells are seen in the subsample, note whether they are live (with intact golden-brown chloroplasts), unhealthy (discoloured or contracted chloroplasts) or dead (empty cells – no cell contents visible). Refer to Appendix for sample photographs of healthy and unhealthy *D. geminata*.
16. If **multiple live *D. geminata* cells** (e.g., three cells or more) are seen in the first subsample, this is a **positive** result. It is not usually necessary to examine further subsamples, though with less than say five cells, it is a good idea to reconfirm the result by checking one further subsample. In the event of a positive result, please notify Biosecurity New Zealand at [didymo@maf.govt.nz](mailto:didymo@maf.govt.nz) (refer to Section 7).
17. If **mostly dead/unhealthy *D. geminata* cells** (empty cells) are seen in the first subsample, this is a **suspect positive** result. Cells may be unhealthy because the sample was contaminated by bleach in the field, because the sample was previously desiccated, etc. Further subsamples should be examined to check for the presence of live cells (up to the 10 – 15 min. limit). In the event of a suspect positive result, please notify Biosecurity New Zealand at [didymo@maf.govt.nz](mailto:didymo@maf.govt.nz) (refer to Section 7).
18. If **only one or two *D. geminata* cells** (live or dead) are seen in the first subsample, examine further subsamples as usual to confirm the presence of didymo in the sample. If no more cells are seen, this is a **suspect positive** result because when only a few cells are observed, the source may be due to

cross-contamination.<sup>1</sup> Treat the sample with caution and report as suspect positive. Please notify Biosecurity New Zealand at [didymo@maf.govt.nz](mailto:didymo@maf.govt.nz) (refer to Section 7).

19. The sample is **negative** if **no didymo cells** are found in the three subsamples.
20. If you are keeping voucher samples (see 24), for very thin samples the subsamples may need to be transferred to the voucher container and not discarded.
21. After discarding the third subsample, unscrew the well unit, decontaminate and rinse thoroughly (see section 4.2). The cover glass may be re-used after cleaning, but **ONLY** if the previous sample contained no didymo cells. If the previous sample contained any didymo cells, discard the glass and insert a new one.
22. To ensure that the microscope unit is free of didymo, following any sample containing didymo cells, scan the cleaned unit under the microscope *before* adding the next sample, paying particular attention to the edges.
23. Complete the data sheet for the sample you have just examined. Include didymo cell count and health. Re-check that the data sheet and sample correspond.
24. If voucher samples are being kept, at this stage transfer subsamples from the sample container to the appropriate voucher containers.
25. Before starting the next sample, discard the disposable pipetter, replace the lid on the previous sample container and move to a “processed samples” location.
26. Processed samples are normally retained frozen until a directive is received from the client that they are no longer needed.

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<sup>1</sup> Cross-contamination of a sample with *D. geminata* can occur via laboratory practices and/or field sampling. The most likely field contamination scenario is when cells are trapped in the sampling net. These should always appear in any subsequent samples as dead/unhealthy cells because of exposure to bleach during cleaning between sites. However, since cells at a positive site could be dead/unhealthy for other reasons, it is not possible to distinguish such contamination from a positive sample. On the other hand, positive samples containing live cells will almost certainly be true positives. In these cases, repeat field sampling is recommended.

## 6. Preventing cross-contamination

Some precautions to prevent cross-contamination of samples are included in the procedure above and are summarised here:

- Place sample container lids on the bench top side down;
- Use a new disposable pipetter for each new sample;
- Avoid placing used pipettors directly onto the bench;
- Thoroughly clean the microscope well and cover glass between samples;
- After a positive sample use a new cover glass, and be particularly thorough about cleaning the well unit.
- Check (scan) the cleaned, empty well for didymo cells after any positive samples.
- If any sample is spilt on the bench, etc, wipe off immediately with a clean tissue or paper towel that has been dipped in bleach solution (at least 2%) or ethanol (90%);
- If any sample comes in contact with your hands, scrub them with warm soapy water, taking care to clean under fingernails, and rinse thoroughly.

## 7. Decontamination procedures

1. All used tissues, paper towels, pipettors, cover glasses, and any other material that might have come into contact with the samples should be disposed of into autoclave bags, which should be treated before disposal into the general refuse by either freezing overnight, or autoclaving at 15 psi for 20 minutes.
2. All discarded liquid sample material should be emptied into a bucket and treated with bleach (final concentration of at least 2%) before disposal into the sewerage system.
3. At the end of each day's work, the microscope well unit should be thoroughly cleaned (see section 4.2) and the bench wiped down with bleach solution or ethanol.

## 8. Reporting results

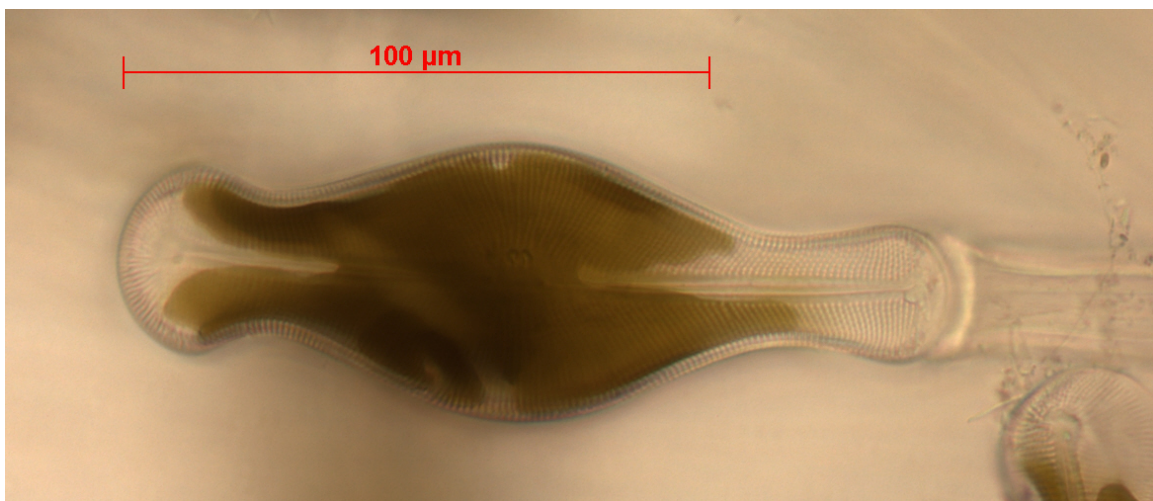
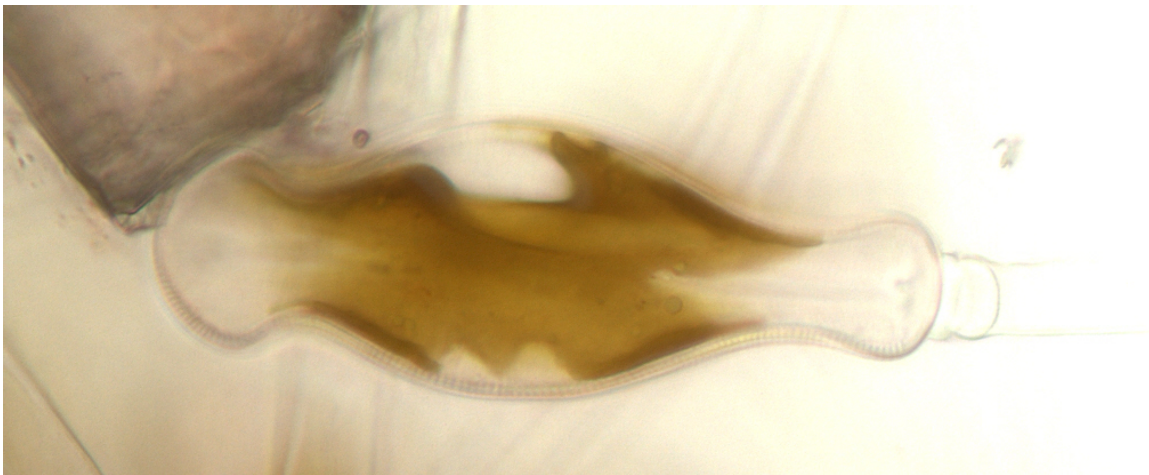
- 1 Please notify Biosecurity New Zealand at [didymo@maf.govt.nz](mailto:didymo@maf.govt.nz) of all positive and suspect positive results.
- 2 It is important to include in the report a description of the number and condition of cells found in a sample. This provides valuable information that can be used to decide the didymo status of the sampled site and whether further sampling is desirable. A presence/absence report usually does not provide sufficient information on which to make management decisions.
- 3 Sample analysis reporting data:
  - **Sample ID number / site number** (these numbers should correspond)
  - **River name**
  - **Location on river** (e.g., number of metres upstream of dam, confluence, etc.)
  - **GPS coordinates** (NZMG easting and northing)
  - **Date / time of sample collection**
  - **Sample type** (visual, benthic, drift)
  - **Sample status** (positive, suspect positive, negative)
  - **Number and condition of cells** (counts of cells less than 5, 5-10 cells, >10 cells; live: healthy, unhealthy, shrunken chloroplasts; dead: empty frustules)
  - **Name of sample collector**
  - **Client/owner**
  - **Method of analysis** (e.g., Biosecurity New Zealand Didymo Microscopic Analysis Protocol v. Mar 2007)



**APPENDIX. Appearance of *D. geminata*, and distinguishing live and unhealthy cells**

**Healthy cells**

Healthy, live cells have a roughly H-shaped golden-brown chloroplast with characteristic folds on the lateral margins, usually one larger than the other. Chloroplast edges are well-defined.



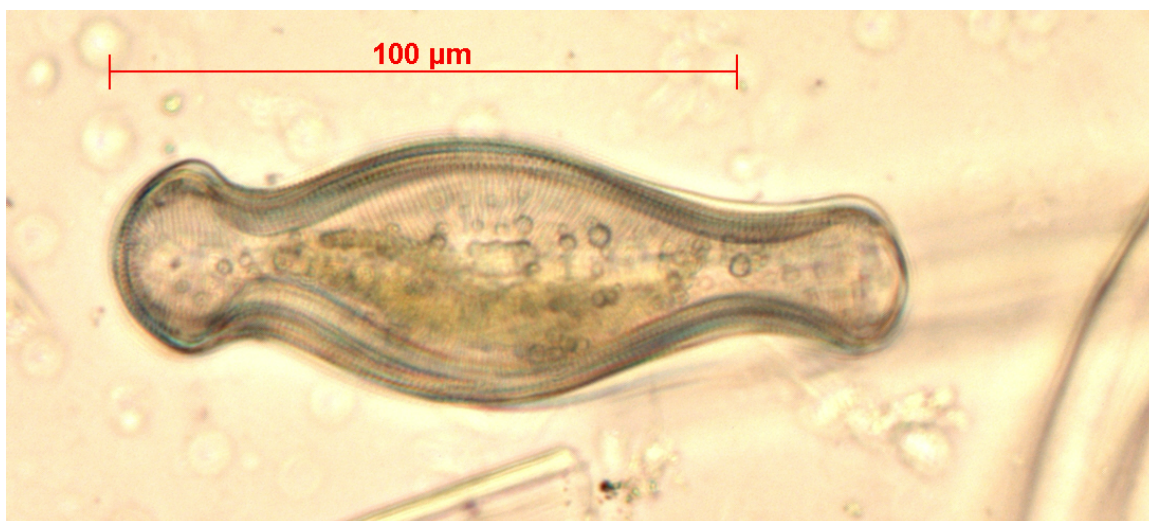
**Healthy didymo cells: large, well-defined chloroplasts**

### Unhealthy cells

After exposure to bleach, the chloroplast immediately starts to look degraded, losing its shape and well-defined edges, as well as its colour. The top photo below is of cells (side view) after 1 minute in 5% bleach, and the lower after 5 minutes.

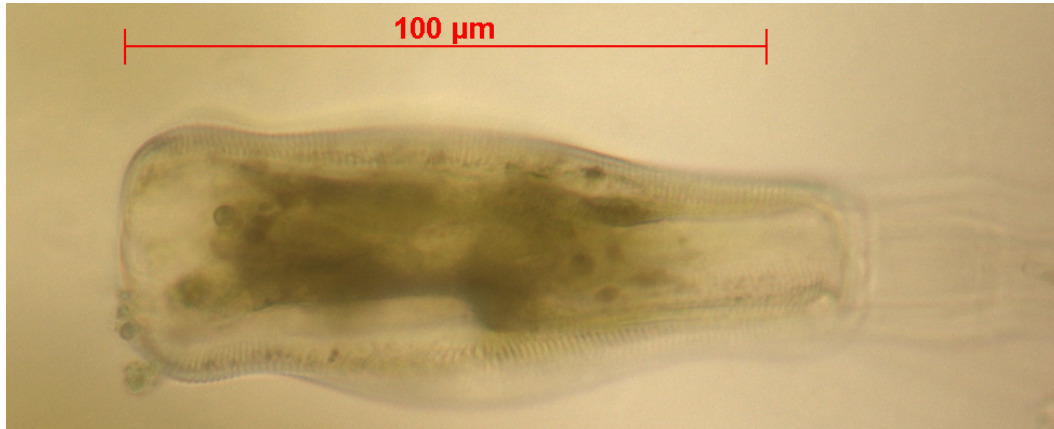


**Unhealthy didymo cells: 1 minute exposure to bleach**



**Unhealthy didymo cells: 5 minutes exposure to bleach**

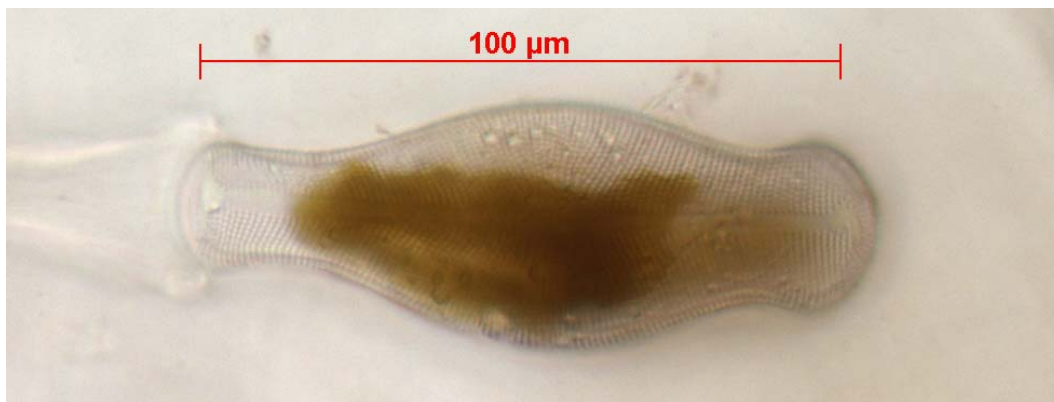
After 1 minute's exposure to 70% ethanol, the chloroplasts also degrade and change colour (below). In this case, the valves appear to be splitting apart.



**Unhealthy cells: exposed to ethanol**



**Unhealthy cells: exposed to warm temperature**



**Unhealthy cells: exposed to seawater**

**Comparison of didymo with common stream diatoms:** The diatoms shown are typical representatives of common NZ genera, shown to scale. Size ranges refer to the length (longest dimension) of the species shown and closely related species. Other species could have different sizes. Definitive identification of didymo must be based on the characteristic size (30-50  $\mu\text{m}$  width x 80 – 130  $\mu\text{m}$  length) *and* the unique shape (curved bottle with bulbous ends).

