Protocol for the collection of samples for delimiting surveys for *Didymosphenia geminata* for microscopic and DNA analysis

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Protocol for the collection of samples for delimiting surveys for *Didymosphenia geminata* for microscopic and DNA analysis

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Appendix 1: Habitat and appearance of *Didymosphenia geminata*

Appendix 2: Sample data sheet for the macroscopic/microscopic presence of *Didymosphenia geminata*

Reviewed by: Approved for release by:

Donna Sutherland Ross Woods

Executive Summary

At the request of Ministry of Agriculture and Forestry Biosecurity New Zealand (MAFBNZ), a delimiting survey protocol has been developed for the collection of visual, benthic and drift net samples for both microscopic and DNA analysis of the invasive, exotic diatom (alga) *Didymosphenia geminata* (didymo).

The protocol lists the sampling equipment required as well as detailing the collection methods for four types of sample: visual, benthic, drift net and sub-samples of the drift net sample for DNA analysis. The visual sample is collected when an algae suspected of being didymo is visible at the sampling site. The benthic sample is designed to provide a systematic sample of the algal communities at the site by pooling the algal scrapings from five rocks from each of five transects sampling the range of stream habitats at the sampling site. The drift net allows collection of drift from upstream of the sampling site increasing the chances of detection of *D. geminata* if it is present in the river but very rare in the benthos at the site. Sampling for DNA analysis requires a sub-sample of the drift net sample to be taken and preserved in alcohol. Analysis of the DNA in samples is an extremely sensitive means of detecting the presence of *D. geminata*, and therefore also provides the best assurance that a negative result represents absence of the species.

Prevention of cross-contamination is critical to avoid false positives and the infection of new sites and steps are outlined to minimise this. The use of higher bleach concentrations ensures denaturation of DNA that could otherwise cause a false positive by DNA analysis.

Illustrations of *D.geminata* at various stages of macroscopic growth are provided to help determine whether *D. geminata* is present at the site. An example field survey form is also provided.

Preface

The non-indigenous, invasive diatom *Didymosphenia geminata* was first detected in a New Zealand river in October 2004. Although the initial sighting was in the lower Waiau River, Southland, the species was found to have already affected almost the entire length of the Waiau's tributary, the Mararoa River. The first delimiting survey to determine whether *D. geminata* was present in other rivers was undertaken in December 2004, in neighbouring Southland rivers. In that survey, visual inspections were made at each site, and then multiple benthic (stone scrapings) and drift net (invertebrate nets, 500 µm mesh) samples were collected. All samples were examined microscopically. No *D. geminata* was detected at any site (Kilroy 2004). For almost a year, the species was not reported from any other river, until late September 2005, when it was simultaneously found growing in the Buller, Hawea, Oreti and upper Waiau Rivers. A nationwide delimiting survey in October 2005 detected *D. geminata* in one further river, the Von. In that survey, the visual and benthic sampling protocols described in this report were used for the first time. Further national delimiting surveys were undertaken in February 2006, April 2006, August 2006, November 2006, February 2007 and May 2007.

In an effort to improve the reliability of the surveys, trials were undertaken in April – May 2006 to develop methods that would help minimise the risk of false negatives. It was found that use of a fine-meshed drift net enabled more efficient microscopic detection of *D. geminata* if it was growing upstream, compared to the benthic sampling method (Kilroy and Dale 2006). Drift net sampling was therefore included in all subsequent delimiting surveys. Because *D. geminata* cells may be found in benthic samples but not in drift net samples, both methods are included in the updated protocol.

In parallel with the delimiting surveys and trials, work has been undertaken on development of a DNA probe for detection of *D. geminata*. This method has turned out to be extremely sensitive. If a sample contains just a single *D. geminata* cell or fragment of DNA, the method will yield a positive result. Conversely, if the method gives a negative result, there is a high probability that *D. geminata* is truly absent from the sample. The extreme sensitivity of the method means that cross-contamination can easily yield false positives. At the time of writing, the DNA method was still in the development stage.

In this report we present detailed protocols for collection of benthic and drift net samples for microscopic analysis, and for taking sub-samples from drift net samples for subsequent use in validating the DNA detection technique. The protocols are those used in the May 2007 delimiting survey. The aim is to ensure that all subsequent surveys undertaken by didymo partners adhere to the same methodologies and decontamination practices. This should ensure that scientifically defensible samples are taken and the incidence of either false positive or false negative results is reduced.

Sampling team leader:

Please read through this entire document before starting your sampling programme to ensure that you are aware of and understand the sampling and disinfection protocols.

1. Briefing

1.1. Purpose

The purpose of the survey is to detect if *Didymosphenia geminata* (didymo) is present in selected high risk tributaries and rivers to provide information for short-term containment options and longer term management options.

1.2. Objective

To collect aqueous and ethanol-fixed algal samples using a combination of visual, drift net and benthic collection methods ensuring that procedures are followed to prevent cross-contamination of samples and sites.

1.3. Scope

Organisations may sample any rivers that are of interest or concern, but for the sampling effort to be most cost effective it would be prudent to include sites that have 'high use' and/or 'high value' and a high probability of didymo establishment and growth (see section on site selection). These include catchments, tributaries and rivers that provide suitable habitat and/or are used for various recreational and commercial activities. Rivers already known to be infected with didymo should not be included in the survey to reduce the possibility of cross contamination between samples and sites and to maximize the efficient use of resources.

At the start of the survey, each team should be issued with a list of site names, GPS locations and 1:50,000 scale topographical maps for each site to be sampled.

1.4. Site selection

Site selection should be determined by a site ranking system based on the NIWA Likely Environments Model (Kilroy et al. 2005), human activity at a site, likelihood of transfer of *D. geminata* to the site, ease of access to the site and the social/cultural value of that site. The MAFBNZ national delimiting survey reports contain details of the site selection process, including the ranking criteria. The most recent report (Duncan 2007) lists the top 240 New Zealand sites considered for those surveys. Survey reports are available on the didymo page of the MAFBNZ website.

1.5. Communications and confidentiality

Refer members of the public with suspected findings of *D. geminata* to the MAF Biosecurity New Zealand exotic pests and diseases hotline at 0800 80 99 66.

Refer members of the public requesting information on *D. geminata* to the MAF Biosecurity New Zealand home page http://www.biosecurity.govt.nz then click on "didymo".

All media enquiries should be referred to the Communications Manager of MAF Biosecurity New Zealand.

Confidentiality should be maintained at all times. Please do not disclose information about any aspect of the survey to others not participating in the survey.

1.6. Contacts

Questions regarding site selection, conduct of surveys and analysis of samples should be referred to NIWA, Christchurch, ph 03 348 8987, or the agency conducting the survey.

MAF Biosecurity New Zealand Communications:

Refer all media enquiries to the Communications Manager MAF Biosecurity New Zealand.

1.7. Feedback

Sampling and disinfection methods and ways of reducing the potential for cross-contamination between samples and sites can always be improved. If you can see any way of improving the equipment or procedures or find any problems with the equipment or procedures, please contact the Operations Manager, MAF Biosecurity New Zealand.

1.8. Any questions?

Any questions or issues should be directed to the Operations Manager, MAF Biosecurity New Zealand.

2. Sampling preparations

2.1. Sample types

In this survey you will collect FOUR types of samples in the following order.

1. **Visual sample for microscopic analysis.** Any suspicious-looking algal growth that resembles *D. geminata* will be collected first. Note that we do not expect visual samples from every site. See Appendix 1 to see what didymo looks like.

2. **Drift net sample in water for microscopic analysis.** This is a sample of material filtered from the water column through a drift net (10 minute deployment).

3. **Drift net sub-sample in alcohol for DNA analysis.** This must be a sub-sample of the above drift net water sample. Do not re-deploy the net. **Note that the DNA detection technique is extremely sensitive to the presence of very small quantities of didymo. This increases the possibility of false positive results from contamination of samples from other sites. Please read and follow the instructions in section 3.5 and 3.7.3 very carefully to reduce the possibility of false positive results.**

4. **Benthic sample in water for microscopic analysis:** Pooled sample of the scrapings from 5 stones from each of 5 transects (25 stones total) to form one benthic sample per site.

Refer to Section 3 for full sampling instructions, including decontamination procedures.

2.2. Sampling equipment

Specialised sampling equipment such as the drift nets or complete sampling kits are available from Instrument Systems, NIWA Christchurch ph 03 3437890. Common or heavy items should be sourced locally. These items are listed separately below.

2.2.1. Benthic and drift net sampling kit contents (supplied)

DRY ITEMS (to be kept dry throughout sampling)

- Wooden scrapers (disposable ice-cream sticks) *(dispose of after use at one site)*
- Disposable pipettes for rinsing or sampling from bedrock *(dispose of after use at one site)*
- Paper towels
- Containers for samples (e.g., 130 ml stackable "Elkays", plus lids). These are available from Biolab Limited, Christchurch, ph 0800 933 966, Stock number LBS 31130 – 130 ml container with separate caps.
- Zip lock plastic bags
- 40 micron mesh pieces about 10 cm square (one needed for each site sampled)
- Waterproof marker
- Pencil
- Chilly bins for transporting samples
- Sample data field sheets (see Appendix 2 for an example field sheet)
- 1:50,000 scale topo maps showing the location of each site
- Short disposable gloves (several pairs per site)
- Long cow examination gloves to wear under short gloves (have your partner make holes on uppermost back side of gloves and tie together with a string to keep gloves from rolling down and to keep water from slipping in during benthic rock sampling). They should be available from your local veterinarian
- Rubbish bags for used scrapers, pipettes, towels, gloves, etc.

WET ITEMS (store in a leak-proof bin or bag)

- Drift net, pre-net
- Something to attach the net to the tethering rod (e.g., cable ties, large S hook)
- Rubbish bag, or a plastic bin with a lid, for transport of the net, pre-net and cod-end, wet with disinfectant, from site to site.

2.2.2. Benthic and drift net sampling equipment (to be supplied locally)

*DRY ITEMS (*to be kept dry throughout sampling)

- GPS unit

WET ITEMS (store in a leak-proof bin)

- Large plastic bin with lid to hold wet items when not in use, during transport
- Container to collect scrapings, e.g. 2 litre ice-cream containers or similar
- A metal knife may also be useful – anything that can be wiped thoroughly clean
- 10 L buckets (at least two) – for collecting rocks, etc. during the benthic survey
- Reinforcing rod or warratah fence post for tethering the drift net
- Warratah driver or small sledge hammer
- Waders – Rubber or neoprene and with rubber soles (not felt soles) to reduce the risk of transferring didymo between sites.

2.2.3. DNA sampling kit contents (supplied)

DRY ITEMS (to be kept dry throughout sampling)

- 14-ml graduated screw-top tubes with cap (one for each site)
- Disposable pipettes (one for each screw top tube)
- Zip lock bags for samples – 1 small bag per DNA tube, 1 large bag for all DNA tubes together
- Parafilm strips for sealing the screw-top tubes
- Paper towels
- Rubber bands
- 70% ethanol sufficient for all DNA tubes.

2.2.4. Decontamination kit contents (to be supplied locally)

WET ITEMS (store in a leak-proof bin)

- Foot bath: large plastic bin for standing in to disinfect wader boots and other gear; also useful for storage of wet items when not in use, during transport
- Measuring jug (to measure the bleach): 5% = 500 ml bleach in 9.5 litres of water
- "Janola" household wipes with active ingredient benzalkonium chloride (for disinfecting hands and arms instead of using bleach)
- 5 L bottle of household bleach. While any bleach will do, our preference is to use "Janola" brand because it is widely available, is high strength, and contains both sodium hypochlorite and sodium hydroxide that both kill didymo. However, any bleach that contains at least 31.5 g/L of sodium hypochlorite is acceptable. Check that the "used by" date has not expired.
- 10 L bucket for making up 5% bleach solution
- Sprayer (optional) for spraying 5% bleach on waders and equipment
- Scrub brush (plastic handle with wide-spaced plastic bristles) to assist with applying bleach solution to warratah and other wetted items.

2.3. Sample labelling and pre-packaging

To minimize handling and the risk of cross-contamination, please label and pre-package sampling containers before going in the field. Labels must include:

- sampling team names (**NOT JUST INITIALS**)
- sample number (this is the unique site number that appears on the supplied sample form and map for each site)
- sampling method
- date.

The only labelling in the field will be for any visual samples, as these are not collected at every site.

Pre label for each site:

- 1 Elkay container for the net sample for microscope analysis (use permanent marker pen)
- 1 Elkay container for the benthic sample (use permanent marker pen)
- 1 screw-top sampling tube for DNA sample collection (use pencil, not permanent marker. The tubes will have ethanol in them, which, if it leaks, will dissolve permanent marker ink).

Pre-package equipment for the DNA sample collection as follows:

- pre-fill the labelled DNA screw top sampling tubes with ethanol up to the 12 ml mark
- ensure the top is screwed on firmly
- wrap each tube in a paper towel secured with a rubber band
- place each tube in an individual zip lock bag with pipette and strip of parafilm to use as a seal once the sub-sample has been added.

Also assemble:

- 2 pairs of disposable gloves
- 3 wooden scrapers
- 3 disposable pipetters
- a 10-cm square piece of 40 micron mesh.

Place the two labelled Elkays (plus a spare one in case a visual sample is collected), the DNA sample package, the gloves, scrapers, pipetters and mesh in a larger plastic bag so that you have a single pack of containers and disposable items for each site.

3. Field methods

3.1. Staff assignment

At the start of the day assign one person to “wet” duties (i.e. net deployment, stone gathering and disinfection) and the other person to “dry” duties (form filling, rock scraping and sub-sampling for DNA). Keeping these activities separate will reduce the

possibility of cross-contamination. The duties of each person are indicated in the following instructions (WET COLLECTOR and DRY COLLECTOR).

3.2. Sampling sites

A "site" is any river reach. Reach length is variable, but should encompass areas with stony substrate within reasonably easy reach of the access point. Up to 25 – 50 m length is suggested, depending on river conditions. We assume highest chance of infestation by didymo is at or immediately downstream of public access points. Sampling should be done down stream of the access point. If the site location suggested by the GPS map reference or site description is unsuitable, it is acceptable to use nearby access points, as long as there are no significant tributaries between the suggested site and the new site.

In each river, sample from upstream to downstream (to eliminate any chance of transferring didymo, if it is present, upstream in a system).

Stay safe at all times. When in the water, velocity (m/s) × depth (m) should be less than 1.

3.2.1. Order of sampling

Start by inspecting the site for didymo and collecting visual samples if appropriate, then start the drift net procedure, and then do the benthic sampling.

3.2.2. Site description and inspection

Dry collector

Once the sampling site has been decided upon, complete the site information on data sheet, as necessary. Site location and GPS coordinates are pre-designated. Check the GPS coordinates and if they differ from those on the form, record the new coordinates. If site is unsuitable for sampling, still fill in the form and return it with the other samples stating clearly why the site was not sampled, e.g. the site was in flood and it was not safe, it had a muddy bottom, etc.

Set out the bag of pre-labelled containers and disposable equipment for this site and a further zip-lock bag in which you will place the two or three samples in water collected from this site.

Wet collector

Set out the equipment that has been decontaminated from the previous site:

- net and associated parts
- buckets (for collecting rocks)
- containers (for collecting scrapings).

3.3. Visual sample

See section 3.7.1 for sampling hygiene instructions.

Wet collector

1. Inspect the whole site area for any algae colonies that look like didymo. Check submerged plants and wood, as well as stones. Refer to Appendix 1 for didymo appearance and habitat information.

2. Collect samples of any alga suspected of being didymo and place in a 130 ml Elkay container. Top up the container with river water until it is about 80% full and cap firmly.

3. Place any disposable equipment used (scraper, pipetter) in rubbish bag.

Dry collector

1. Label Elkay container with sampling team, site code, sample type (VISUAL), and date. Place in the zip-lock bag and put aside until the next samples in water are ready.

2. Record sample and site comments on the sample form (Appendix 2) for the site.

3.4. Drift net sample in water for microscopic analysis

See section 3.7.2 for sampling hygiene instructions.

Wet collector

1. Drive the tethering pole into the river bed at a suitable location (reasonably fast flowing water towards the centre of the channel – safety considerations will determine possible flow/depth combinations (velocity (m/s) × depth (m) < 1). The tethering pole should be located upstream of the stone-gathering transects, so that none of the bed-material disturbed is caught in the net when gathering the stones. Figure 1 shows a diagram of a drift net and how it should be deployed.

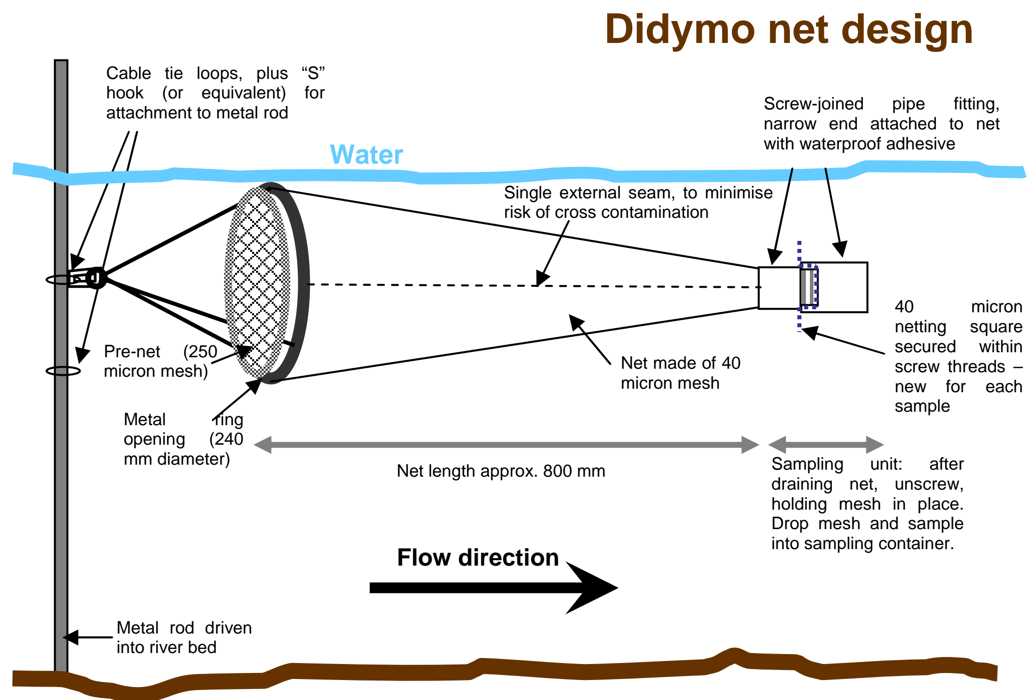

Figure 1: Diagram of net design and method of deployment

2. At the first site, use the new net and pre-net directly (no pre-cleaning required). If the net and pre-net have been used, deploy the net without the netting square for about 5 minutes to remove any disinfectant from the previous site before adding the netting square (see below). Also back-wash any debris from the pre-net.

3. Attach the sampling unit to the main part of the net by placing one of the netting squares over the end of the threaded part of the sampling unit, then screwing the two parts together so that the net is secured tightly and is taut.

4. Place the pre-net over the mouth of the main net. The tethering cords of the main net and the force of the water will hold it in place.

5. Attach the assembled net with pre-net and sampling unit to the pole using a cable tie or “S” hook, carabiner or similar. Depth should be so that the top of the net opening is just below the water surface. The objective of the high placement in the water column is to reduce the amount of sand and large bits of organic matter saltating along and above the river bed entering the net. Ensure that there are no bubbles in the net and if the water is flowing fast that the net is low enough to stop any air getting into the net.

6. Leave in the water for 10 minutes. (While the net is deployed, you can begin and often complete the collection of 25 stones for the benthic sample, provided you remain downstream of the net at all times. **DO NOT walk in front of the net while it is being deployed** as this could allow dead didymo cells to wash off waders, etc and into the net, and then be identified in the analysis).

7. After the 10-minute deployment, detach the net from the pole. Hold up vertically to allow water to drain out.

8. If any material has accumulated on the sides of the net, wash down by splashing on water from the outside.

9. Most of the sample should have accumulated in the sampling unit. If water is backing up into the main part of the net because of material blocking the end filter, drain off through the side of the net. Allow as much water as possible to drain off before loosening the screw (e.g., no more than a centimetre or two above the sampling unit).

10. As you unscrew the lower part of the unit, hold the netting square firmly, then release the mesh square with the sampled material into the pre-labelled Elkay sampling container. Make sure this is the pre-labelled sampling container for the NET sample. Wash out any material left on the net or sampling unit with a small amount of water (use the pipettes provided).

11. Once the sample has settled in the Elkay, a 2-ml sub-sample for DNA analysis is taken at this stage (see section 3.7).

12. After the DNA sub-sample has been taken, cap the sampling container securely and place in the zip-lock bag with the visual sample (if one was collected).

13. Place all disposable equipment used in rubbish bag.

Dry collector

Check that the container is labelled correctly for the site and labelled NET. Record sample and site comments on the sample data field sheet (Appendix 2) for the site. Note the drift net number. It is engraved on the plastic cod-end of the net.

3.5. Drift net sub-sample in alcohol for DNA analysis

NOTE: The DNA sample is a sub-sample of the drift net sample.

See section 3.7.3 for sampling hygiene instructions.

Dry collector

1. Check that the container is labelled correctly for the site

2. Put on the pair of disposable latex gloves.

3. After the drift net sample has been transferred to the Elkay, tip the container so that the filtrate collects at one side. Wait until the suspended material concentrates at the bottom of the container – this could take a few minutes. Use a new disposable pipette to suck up about 2 ml of this concentrated material, and squirt this into the pre-labelled, pre-ethanol-filled 14-ml screw-top tube.

4. Cap securely and wrap a strip of Parafilm round the cap junction. Slightly stretch the parafilm as it is wrapped around the sample tube and firm with the fingers to ensure a good seal.

5. Wrap sample tube in paper towels and secure with rubber band. Place in zip lock bag and place in chilly bin.

6. Put the used pipette into the rubbish bag.

7. Write on site sample data field sheet (Appendix 2) that a DNA sample has been collected. Note that you will need to make a photocopy of the sample form to go with the DNA sample (see section 3.8).

8. Don’t take off the gloves – you’ll need them on for the stone scraping.

3.6. Benthic sample in water for microscopic analysis

See section 3.7.1 for sampling hygiene instructions.

1. Determine the approximate locations of five transects from which you'll collect samples. Space every few metres (depending on reach length) but if the site contains different habitat (runs, riffles, pools – see definitions on sampling form), make sure that the transects include each habitat type, e.g., three transects in runs, two in riffles.

Wet collector

1. Depending on river size, transects will extend across to the opposite bank, or to water up to about 0.7 m deep (as long as it is flowing at a safe velocity). **STAY IN SAFE DEPTHS/VELOCITIES AT ALL TIMES.** Velocity (m/s) × depth (m) should be less than 1 for you to be safe.

2. Start inspecting the site at the **upstream** end. At each transect walk across the reach and collect rocks from in front of you at 5 more or less equally spaced points.

3. Pool the five rocks from the transect into a bucket. These can be pooled with rocks from other transects.

Dry collector

1. Keeping the disposable latex gloves on….

2. For each set of pooled stones, use a disposable wooden scraper to scrape a sub-sample of algae from each stone and transfer to an Elkay sampling container pre-labelled for BENTHIC samples. If algal growth is very thin, use a metal blade, rather than the wooden scrapers provided. Try to include all types of algae present (distinguishing algae by colour and texture). **NB. Please sample all rivers and scrape all stones even if there appears to be no algae present.** After scraping stones with little or no visible algae, wash the scraped area of the stone with the pipette filled with water from the bottom of the bucket, until the sample container is about 80% full.

3. One sampling container of algae is sufficient as long as it contains all types of algae present.

4. Place all disposable equipment in rubbish bag.

5. Record sample and site comments on the sample data field sheet (Appendix 2) for the site.

6. Put the sampling container in a zip lock plastic bag with any other samples in water from the site.

7. Place the zip lock bag with all (2 or 3) samples in water from the site into the chilly bin.

8. If samples have to be stored before dispatch (see section 3.8), keep them in a dark, cool place. DO NOT FREEZE.

3.7. Sampling hygiene and decontamination

It is very important that *all* these hygiene precautions are followed carefully, after collecting samples at each site, both to prevent spreading didymo from one catchment to another, and to minimize the possibility of contaminating samples with live or dead cells or DNA, which could lead to false positives.

Dry collector

1. With gloves still on, add household bleach to the foot bath bin, 500 ml per 9.5 litres of water.

Wet collector

1. Add river water to the bin to make up a 5% solution.

2. Clean all sampling items as detailed in the following sections, including all buckets, containers, waders, warratah and anything that has come in contact with the water.

3. Use scrubbing brush to scrub any surfaces, as necessary.

4. Wipe arms and hands with "Janola" wet wipes or similar with benzalkonium chloride as the active ingredient

5. Place all items that have been cleaned back into the now empty bath ready for transport to next site.

6. Refer to details below.

3.7.1. Procedure for benthic sampling hygiene

1. Keep used equipment and containers well away from unused/disinfected items and take measures to ensure water does not drip on them.

2. Use a new set of disposable equipment in each river or tributary and place items in rubbish bag directly after use.

3. Non-disposable items must be decontaminated using the recommended treatment soaking in 5% bleach for 15 minutes,

4. The person scraping benthic samples and sub-sampling the net sample will wear a pair of disposable gloves to help reduce the possibility of cross-contamination between sites.

5. Start inspection and sampling at the clean, upstream end of site, river or tributary and work downstream.

7. Remove all macro material from hands, boots/waders, drift net, pre-net, clothing, equipment, warratah, etc, before leaving each sampling and inspection site.

8. After completing sampling at each site, spray, soak and scrub with 5% bleach solution (500 ml to 9.5 litres) all potentially contaminated boots/waders, containers and equipment, etc for at least one minute. Tip out bleach solution well away from water.

9. Wipe arms and hands with “Janola” wet wipes or similar with benzalkonium chloride as the active ingredient

3.7.2. Procedure for drift net sampling hygiene

The following is the procedure for decontamination and cleaning of the net and pre-net. It is most important to strictly follow the procedure to reduce the possibility of false positives.

1. **Before** sample collection, rinse out the net, pre-net and separated sampling unit in the river to flush out any **visible drift material** caught in crevices, by attaching the cleaned net to the tethering pole without the sampling unit, and allowing water to flow through it for about 5 minutes. This should flush out any bleached cells still trapped in the netting or seam from the previous site. Decontamination will have killed the cells, but any remaining dead cells still are possible source of a false positive.

2. **After** sample collection, rinse out the net, pre-net and separated sampling unit in the river to flush out any **visible drift material** caught in crevices by attaching the cleaned net to the tethering pole without the sampling unit, and allow water to flow through it for about 5 minutes. This should flush out any cells still trapped in the netting or seams.

3. Transfer the net, pre-net and separated pieces to a container of at least **5% bleach and soak for about 15 minutes.** Make sure that all parts of the net and pre-net are fully immersed in the solution for the entire soaking time.

4. Spray the tethering pole with 5% bleach solution.

5. Place the wet (with disinfectant) drift net, pre-net and separated pieces into a plastic rubbish bag for transport to the next site.

6. Disinfection using the bleach solution should be carried out in a place where splashing and/or disposal of the used solution will not cause environmental damage.

7. At the end of each sampling day, visually check all potentially contaminated boots/waders, clothing and equipment to ensure they are cleaned, disinfected, dry and ready for the next inspection and sampling cycle.

8. At the end of the day, rinse the drift net and pre-net in didymo-free water to remove all traces of bleach and hang out to dry in the shade. This is to prolong the life of the net as they degrade and disintegrate if exposed to strong bleach for too long.

3.7.3. Procedures for DNA sampling hygiene

Disposable gloves must be worn and disposed of when sampling has finished at the site.

1. Put on disposable gloves.

2. Before handling anything else, remove all the things you need for DNA sampling from the vehicle while your hands are perfectly dry.

3. Remove the pre-labelled zip lock bag containing a pre-labelled (for that site), pre-ethanol-filled screw top sampling tube, parafilm strip, pipette, paper towel, and rubber band.

4. Carry out the sub-sampling and preservation procedure carefully so as to not spill any potentially infected water on anything.

5. Immediately dispose of the pipette into the rubbish bag.

6. Wipe outside of container with Janola wipes before placing sample tube in zip lock bag.

3.8. Sample return

If samples have to be stored before dispatch (e.g., over weekends), keep in a cool, dark place (fridge). DO NOT FREEZE.

Please photocopy all the sample collection sheets. One copy goes with the visual/ benthic/drift net samples to the microscopic analysis laboratory, the other with the ethanol samples to the DNA analysis laboratory.

All samples, including DNA samples, together with sample sheets, should be **couriered for overnight delivery** to the laboratory carrying out the analysis.

4. Acknowledgements

Thanks to Eric Edwards of the Department of Conservation to his ideas for reduction of cross-contamination and to Frances Velvin of MAF Biosecurity New Zealand for her insightful contributions to the protocol.

5. References

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Appendix 1: Habitat and appearance of *Didymosphenia geminata*

1 Good habitat for didymo is:

1. **Moderate velocity waters** (i.e., not in very still waters, not in very fast flowing waters more than ~1 m/s). Didymo may be present in backwaters of rivers (out of direct flow, but subject to constant water movement), so these also need to be inspected/sampled. Virtually all places where didymo has been sighted have been wadeable, though the alga may occur on the margins of faster-flowing, deeper areas. In the early stages of colonization, it is likely that didymo will not be in fast-flowing areas (> 1 m/s).

2. **Well-lit stream bed** (but this does not preclude areas that are in shade for part of the day).

3. **Stable substrates**: i.e. most likely to occur on substrate of cobble-size or larger (>150 mm diameter), but smaller rocks may be examined in areas of very stable flow.

In other words, **look for stable cobbles in open, wadeable areas with moderately flowing water**. Particularly look for rocks with small, tightly adhering light brownish bumps (see photos), but include rocks, plants and other substrates with all types of algae. After high flows didymo may still be found in protected patches on the downstream side of large rocks.

2. Appearance

Young colonies (Figure A1) form brown/pinkish-brown round bobbles on rocks and stones. Note the difference between didymo colonies in Figure 1 and the red algae colonies in Figure A2. Photographs of later stages of didymo growth are shown in Figures A3 and A4.

Figure A1: Small *D. geminata* (didymo) colonies

Figure A2: Colonies of a red alga that look a bit like *D. geminata.*

Figure A3: A later stage of the growth of *D. geminata*.

Figure A4: A more advanced stage of the growth of *D. geminata*.

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Appendix 2: Sample data sheet for the macroscopic/microscopic presence of *Didymosphenia geminata:*

Site Number………..

Sample site number:………………………… **River**: …………..……………………………………

Site location description:……………………………………… ……………………………………..

GPS: NZMG E:…………………… **NZMG N:**……………………….. OR. **Map ref:**…………...….

Date: ……………………………….... **Time**:……………….……………………………………...

Sampling team leader:……………………………..…………………………….……………….....

Sampling officer:…………………………….……………………………….………………………

Drift net ID Number:……………………………..……………………………

Email address for reporting of results:…………………………………………………………

Samples: label with site no., river name, site location, sample type, date, team leader.

Sample type	Sample no.	Habitat types run, riffle, pool)	Didymo visible? (yes/no)	% Didymo cover if visible	Notes (e.g. other algae present, suitability of site)
Visual sample					
Drift net sample					
Pooled sample from 25 stones					

Water clarity (tick box)

Clear ☐

Slightly turbid ☐

Very turbid ☐

DNA sample taken?

Yes ☐

No ☐

Include this form (duplicate if DNA sample is taken) with the samples and courier overnight to the laboratories:

Lab only:

Analysis facility: ……………………………………

Results	Positive	Negative	Comments on sample (didymo cell count and health)
Visual sample	☐	☐	
Drift net sample	☐	☐	
Pooled sample from 25 stones	☐	☐	

Analyst: Name:……………………………………….………..**Date:**……………………

Didymo Samples Database:

Data entry date: ……….. **Data entry operator:** ……………… **Organisation:** …………………