A comparison of sampling methods for the detection of the invasive alga *Didymosphenia geminata* in New Zealand rivers

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Prepared for

Biosecurity New Zealand

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Reviewed by: *Approved for release by:*

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Executive Summary

- Regular surveillance for the non-indigenous, invasive diatom *Didymosphenia geminata* in New Zealand is part of Biosecurity New Zealand's incursion response to this organism. The sampling method initially used comprised collection of a pooled sample of algae growing on 25 stones at each river site surveyed. This method has been shown to be capable of detecting relatively low densities of *D. geminata*. However no work has been done to investigate whether alternative methods are more effective.

- The work described in this report has been undertaken at the request of Biosecurity New Zealand. Its main objective was to trial different sampling methods for *D. geminata* in order to identify those that minimise the probability of false negatives. Additional aims were: to quantify the sampling effort required to detect *D. geminata* when it is rare at a site (including a determination of "rare"); to calculate and compare the resources required for each sampling method; to qualitatively assess the relationship between visible *D. geminata* growth and cell densities in the water and benthos. Outcomes will be used to develop a standardised protocol for detecting *D. geminata* for surveillance in unaffected catchments, for monitoring affected catchments (e.g., by local authorities) and for other studies that require the ability to detect *D. geminata*.

- Our strategy was to undertake the sampling comparisons on rivers where *D. geminata* was known to be present, but which also included downstream reaches free of the alga. We sampled six sites on each of two rivers in which *D. geminata* had been recently reported. *D. geminata* was confirmed in the Ahuriri River 47–48 days before our initial survey, and in the Aparima River 11–12 days before the survey. The main survey was undertaken on 5-8 April 2006. Additional follow-up surveys were undertaken in both rivers.

- In the main survey, working in an upstream direction we selected sites that were visually free of *D. geminata*, through to sites where growth was clearly visible. At each site, we carried out:
    - visual assessments of macroscopic cover of *D. geminata*
        - rapid
        - detailed;
    - Sampling for later microscopic analysis
        - a 25-stone pooled sample of benthic algae (currently-used survey method)
        - detailed benthic sampling (multiple individual rocks, up to 40 per site)
        - filtration of material from the water column using a drift net deployed for a range of times, up to 10 minutes
        - deployment of four types of artificial substrate.

- During follow-up sampling to collect the artificial substrates, we also undertook further drift sampling and collection of 25-stone pooled samples.

- Overall results from all surveys indicated that detailed benthic sampling and filtration of material from the water column (drift sampling) both allowed us to detect *D. geminata* at sites farther downstream than the method currently in use for surveillance of rivers. Detailed visual assessments were no more effective at detecting *D. geminata* growths than an initial quick visual examination.

- Using the 25-stone pooled sample method, we picked up *D. geminata* at sites up to 10 km downstream from sites where colonies were visible. In contrast, both detailed benthic samples and drift samples gave positive results for *D. geminata* at all sites sampled in the initial survey, with the lowest densities found at the most downstream site in the Aparima, which was approximately 31 km downstream of the affected reaches.

- Higher intensity detailed benthic sampling (i.e., 25 and 40 rocks) was slightly more effective at picking up *D. geminata* than the low intensity sampling (10 rocks). High intensity drift sampling (10 minute deployment) was also slightly more effective at including *D. geminata* cells than lower intensity deployments.

- Patterns of visible *D. geminata* relative to cell densities in the benthos and water column differed in the two rivers. The more extensive downstream spread of cells in the water column of the Ahuriri River would result from the longer time since establishment of *D. geminata* in that River. Unexpectedly, cell densities were higher in the water column at the most severely affected site in the Aparima, even though percentage cover was much lower than that in the Ahuriri.

- We found no indication that the artificial substrates were any more effective than benthic or drift sampling at picking up *D. geminata* cells. Two types of substrate (textured plastic and unglazed ceramic (clay) tiles) provided a better surface for colonization by all algae, including *D. geminata*, than glass or acrylic slides. This pattern was consistent between rivers. Flooding prevented completion of the planned schedule of three retrieval dates for the substrates.

- In follow-up sampling on the Aparima River, we found *D. geminata* cells in drift samples collected from three additional sites up to 70 km downstream from the affected area. We discuss the possibility that some of these positive samples may have been due to cross contamination from the drift net. The net design has been altered to minimize such contamination.

- From the detailed benthic sampling, we determined that a reasonable estimate of rarity of *D. geminata* at a site is an average of 0.1 cells per sampling unit. In this case, a “sampling unit” is

a scanning effort of 10 mins applied to each individual sample collected. This estimate of rarity applied to the second sparsest density found in the surveys (Aparima River, site 5). Use of a standard statistical formula showed that to achieve 0.95 chance of detecting the species at a site where it is present at 0.1 cells per sampling unit, at least 30 sampling units must be collected. This is equivalent to 5 hours of scanning effort per site.

- The low efficiency of the 25-stone pooled sample method for detecting *D. geminata* cells is most likely to be due to the relatively lower effort put into microscope analysis of samples, compared to that for the detailed benthic samples. Possibilities for increasing the efficiency of sample scanning are discussed, viz. acid cleaning of samples, flow cytometry combined with automatic shape recognition, and genomic detection.

- Drift samples tended to be finer and more uniform in consistency than benthic samples. The smaller volume of material collected (compared to benthic samples) means that they may be better suited to both microscope and genomic detection methods, although in some cases drift samples may be bulkier than benthic samples.

- A comparison of the costs and effectiveness associated with each of the sampling methods shows that filtering samples from the water column is clearly the most efficient method (of those tested) for detecting *D. geminata* at low densities. We recommend that a 5 to 10-minute deployment become the standard methodology in future surveillance for *D. geminata*. For the next upcoming survey, both the 25-stone pooled sampling and the net methods should be used to confirm the performance of both.

- A supplementary objective of this work was to collect samples for a parallel study at the University of Waikato, viz. development of a genomic detection method for *D. geminata*. We subdivided 125 samples for this purpose. All were preserved in 70% ethanol within ~6 days of collection, and held at ~1° prior to dispatch.

1. Introduction

The invasive diatom *Didymosphenia geminata* (didymo) is a microscopic, single-celled alga that lives attached to stones and other substrates in rivers. Although individual cells are generally less than 150 µm long, this organism can form large, smothering mats through the production of masses of polysaccharide stalks exuded by each cell. These stalks also form the attachment to the substrate.

D. geminata was first identified in New Zealand in October 2004, in the Mararoa and lower Waiau Rivers, Southland. Within days of its confirmation as a new, unwanted organism in New Zealand, organised surveillance for *D. geminata* was initiated by Biosecurity New Zealand (BNZ), with visual surveys of rivers on Southland and Otago. This was followed within weeks by a more thorough survey of Southland Rivers, which involved sample collection and subsequent microscope examination of samples to determine whether *D. geminata* cells were present. No *D. geminata* was found in any of the samples examined (Kilroy 2004). Subsequent ongoing passive surveillance (OPS) by Fish and Game Councils, Regional/District Councils, Department of Conservation and Crown Research Institutes, who had been provided *D. geminata* identification sheets in December 2004, resulted in the submission of multiple suspect samples from throughout New Zealand. These proved negative until late September 2005, when the alga was confirmed in two South Island catchments. BNZ has subsequently undertaken regular surveillance, to track the spread of the organism to other rivers, and also to reconfirm on a quarterly basis its absence from further vulnerable catchments, particularly in the North Island. At the time of writing this report, the species was known to be present in six South Island river catchments (Waiau (Southland), Oreti, Aparima, Clutha, Waitaki and Buller)[1].

As part of the initial incursion response, a *D. geminata* query service was incorporated into BNZ's Exotic Pest and Disease Emergency hotline (0800 80 99 66). The number was advertised on handouts and public notices concerning *D. geminata*, as a source of further information (along with the BNZ website), and as the place to report finds of suspected *D. geminata*. Messages could also be emailed to BNZ via a link on their website. In all cases, suspected *D. geminata* samples are confirmed, or otherwise, by examination under the microscope. Currently, microscopic examination is essential for confirmation of identity. Experienced observers can be reasonably confident of making a correct identification from the macroscopic appearance of *D. geminata* colonies. However, there is potential for error because other diatoms (e.g., the native stalked diatom *Gomphoneis minuta* var. *cassieae*) form mats that may closely resemble *D. geminata*. To the inexperienced eye, a range of algal growths resemble *D.*

[1] *D. geminata* was found in a seventh catchment (Mataura, Southland) in August 2006.

geminata, hence the large number of negative suspected *D. geminata* samples received via OPS (see Duncan 2006).

The aim of regular surveillance is to detect *D. geminata* even if it is rare. To maximise the chances of achieving this, three main questions need to be considered.

1. **Are we sampling the most appropriate sites?** Is the environment suitable for *D. geminata* to colonise, grow, and possibly bloom? Is the site frequented by the major vectors of *D. geminata* (assumed to be humans)?

2. **Are we collecting samples from the site in the most efficient and appropriate way?** Are we targeting the most likely microhabitat in the site where the species will become evident first? Are we collecting enough material? If *D. geminata* is present, but rare, does our sampling methodology provide the best chance of including at least some cells?

3. **How confident can we be that *D. geminata* will be detected in the collected samples, if it is present?**

In the ongoing surveys, the first question has been addressed by: 1) referring to a stratification of suitability for colonisation and growth of *D. geminata* applied to river reaches throughout New Zealand (Kilroy et al. 2005b); and 2) consideration of recreational use, etc. of the sites (Duncan et al. 2005). For the third question, microscopic examination is the only option for checking samples at present, but work is underway to develop a genomic method.

The work described in this report specifically addresses the second question.

At the time of the first delimiting survey in Southland rivers (Kilroy 2004), *D. geminata* was confirmed in only one river system and was visible over the whole river length. Therefore it was not possible to test the effectiveness of different sampling methods. The appearance of *D. geminata* in further catchments since September 2005 presented an opportunity to do this. BNZ requested that NIWA submit a proposal for work to improve sampling efficiency, specifically to identify methodology that would increase confidence that *D. geminata* is being detected in a river, if it is present. The work recognises that field sampling techniques may be the weakest link in our ability to detect *D. geminata* at a river site, if it is present. If sampling techniques cannot reliably capture *D. geminata*, when it is present, then even the most sensitive laboratory method will not improve detection.

At the time of this study (first half of 2006), the survey method comprises benthic sampling, in which 25 rocks are retrieved from the river bottom at each sampling site, and algal material scrubbed from these rocks is pooled into a single container (Duncan et al. 2005). Replicate subsamples from the pooled sample are then examined under a microscope for presence or absence of *D. geminata* cells. Sampling to date has confirmed that these methods are capable of detecting *D. geminata* in samples from sites where the species was not visible. In all such cases, *D. geminata* was visible upstream (Duncan et al. 2005, Duncan 2006). However, in no case was it possible to estimate the probability of finding *D. geminata* associated with a specified sampling effort, given that visible colonies were present upstream. The corollary to this was that it was also not possible to state a level of confidence that failure to detect *D. geminata* in other samples represented absence of the organism at upstream sites. The sampling methods currently used in the ongoing surveillance allow us to say *only* that *D. geminata* was or was not detected in a given sample, and that macroscopic growths of *D. geminata* were or were not observed at a site.

To detect rare species, a general rule is that increasing the number of samples improves the chances of detection, with samples either randomly located, or stratified according to prior knowledge of the environmental preferences of the species concerned. Therefore, in this study, as well as comparing sampling techniques, we compared sampling intensities for their ability to pick up *D. geminata* at low densities.

We also attempted to identify a spatial relationship between the occurrence of visible colonies of *D. geminata*, and the density of microscopic *D. geminata* in samples collected using the various techniques at different intensities. The purpose of this was to illustrate the extent to which different sampling strategies are capable of detecting *D. geminata* at sites where there is a range of probabilities of it being present.

In a broader context, this study addresses two common issues in ecology: the design of surveys to detect rare species (Green & Young 1993; McArdle 1990), and the related issue of the determination of species occupancy in the landscape (Mackenzie & Royle 2005). Both these problems need to be linked to the practicalities of sampling and sample processing because the most sensitive and accurate method for detecting a species may also be the most time-consuming and costly. Assessment of sample analysis in the laboratory was not a specified aim of this study, though we do include a commentary on this aspect of detection.

To summarise, the main objective of the work was to investigate different sampling methodology that might increase the efficiency of monitoring the presence of *D. geminata* in rivers thereby minimising the probability of false negatives. In other words, when *D. geminata* is suspected to be present at a site, but is not visible, which

sampling method is most efficient at detecting it (i.e., picks it up at the highest concentrations)? Additional objectives were: 1) to quantify the sampling effort required to detect *D. geminata* when it is rare at a site (including a determination of "rare"); 2) to compare the resources required for each sampling method; 3) to assess issues related to laboratory processing of samples; 4) to collect samples for a parallel study at the University of Waikato, viz. development of a genomic detection method for *D. geminata*.

2. Potential methods for sampling *D. geminata* in rivers: a review

2.1. Benthic sampling

Since *D. geminata* is an attached benthic (bottom-living) alga inhabiting running waters, the most obvious sampling method is to collect periphyton from river substrata. This involves scraping or brushing algal growth from rocks and other submerged material, or collecting shallow surface-cores from streambeds covered with fine sediment. Methods are described in Biggs & Kilroy (2000). Samples may be collected quantitatively (from a known surface area) or qualitatively. General observations on periphyton community composition indicate that common benthic algal taxa tend to be broadly dispersed in river reaches that are homogeneous with respect to light and water velocity (e.g., Passy 2001). Thus small sub-samples would be expected to yield communities representative of the whole reach, at least for the common taxa. On the other hand, for a rare alga that is more sparsely distributed, more subsamples would be required to ensure that this particular alga is represented in the sample. Therefore, the more sub-samples collected over an area, the higher the likelihood of detecting any given rare taxon.

The benthic sampling protocol used in the ongoing surveillance program comprises collection of benthic samples pooled from 25 rocks at each site. Here, we compare the ability of this method to incorporate any *D. geminata* present with that of sampling multiple individual stones at three levels of intensity (i.e., increasing numbers of samples).

2.2. Visual assessments

Visual assessment methods range from broad-scale to detailed. For example, a broad-scale method is used as part of NIWA's National River Water Quality Monitoring Network data collection protocol (Quinn and Meleason 2002): sampling teams are asked to estimate the % cover of green algae and brown algal mats >2 mm thick, along 10 transects at their sampling sites. A more detailed method was developed for the

Stream Health Monitoring and Assessment Kit (SHMAK, a stream monitoring protocol for non-scientists – see Biggs et al. 2002), and this has been adapted for use in more rigorous monitoring programmes (method RAM-2 in Biggs & Kilroy 2000). For RAM-2, the %cover of different types of algae is assessed on at least 20 individual rocks collected along transects at a sampling site, with algae types separated on the basis of colour and thickness. Other types of visual assessments employed in ecology include the use of quadrats, and random point-centred estimates of cover. Visual assessment of periphyton cover depend very much on the categories selected for assessment (colour, texture, thickness, particular growth forms, etc.) and all the methods can be adapted to focus on any particular algal growth type.

2.3. Artificial substrates

Artificial substrates are a specialised method of benthic sampling and have long been employed in the study of periphyton for both monitoring and experimental purposes (see references in Biggs & Kilroy 2000). In both cases their main advantage over the use of natural substrates is that use of a standardised surface provides a very efficient way of testing the effects of other environmental differences among sites. Some common types currently in use are described in Biggs & Kilroy (2000). These include:

- plexiglass or glass slides mounted vertically or horizontally;
- unglazed ceramic (clay) tiles;
- simulated cobbles made of moulded plastic with a tomentose surface.

Observations to date on *D. geminata* suggest that the species grows most prolifically on stable rock substrates (though other substrates such as submerged plants and wood can also be colonised), and grows preferentially in waters with velocities up to 0.5 m/s, particularly at the upper end of that range (Kilroy et al. 2005a). The artificial substrate designs mentioned above can all be anchored securely (e.g., using concrete blocks), and therefore could all potentially be located in favourable habitat for *D. geminata*.

2.4. Water (drift) sampling

New infestations of *D. geminata* that are picked up before the bloom stage often comprise small colonies distributed sparsely over a wide area. These colonies expand and coalesce to form the complete cover seen in more advanced infestations. Multiple small colonies may result from the simultaneous release of many cells from a single (or small number) of sources, or from a larger mat or bloom of *D. geminata* upstream.

The time interval between cell release and the appearance of new colonies is currently unknown, but any spread of cells downstream must mean that at some stage there are cells in the water column (drift), but not in the benthos (or they are so rare in the benthos that only extremely intensive sampling will detect their presence). Drift sampling is undertaken using plankton nets or filters with mesh size appropriate for retention of *D. geminata* cells. Nets that can be tethered and left for a set time are preferable, to allow for filtration of suitably large volumes of water.

3. Methods

3.1. Study areas

Our strategy was to undertake the sampling comparisons on rivers where *D. geminata* was known to be present but which also included downstream reaches visually free of the alga. We therefore worked in two rivers where *D. geminata* had been reported recently: the Ahuriri River, Canterbury, and the Aparima River, Southland. Site locations were determined by accessibility and the requirement for sites to span unaffected and affected areas. Surveys were undertaken as follows:

Ahuriri River	Main survey	5 – 6 April
	First follow-up	17 – 18 April
	Second follow-up	12 May
Aparima River	Main survey	7 – 8 April
	First follow-up	25 April
	Second follow-up	17 May
	Third follow-up	25 May

3.1.1. Ahuriri River

D. geminata was first identified from the Ahuriri on 17 February 2006, 47–48 days before our main survey. Visual surveys of the river had been undertaken by the Department of Conservation and Environment Canterbury (ECan) prior to our survey. On 27 March, Dean Nelson, Dept of Conservation, Twizel, reported that growth had developed to a "reasonably thick" cover at the site where it was first found, and had extended downstream in scattered colonies. On 5 April, colonies were found as far downstream as the SH8 bridge. We sampled six sites, as follows (Figure 1a):

1.	Glenburn Station, approx. 1.2 km upstream of the lake outlet	unaffected
2.	Glenburn Station, 3 km upstream (at the foot of Chain Hills)	unaffected

3.	Just upstream of SH8 bridge	sparse
4.	Fishing access opposite Killermont Station	moderate
5.	Downstream of gauging station off Birchwood Road	thick
6.	Fishing access upstream of Gorge Bridge	sparse

3.1.2. Aparima River

D. geminata was confirmed in the Aparima on 28 March 2006, 11–12 days before our survey. At that stage, Stuart Sutherland (Southland Fish & Game) reported that the upstream limit of growth was Dunrobin Bridge, and colonies were visible to at least 13 km downstream. The main survey was undertaken at six sites (sites 4 to 9). A further three (sites 1, 2 and 3) were added in the first follow-up survey in order to extend the sampling as far downstream as possible. Sampling sites (Figure 1b) were:

1.	Thornbury, downstream of road bridge	unaffected
2.	Otautau, upstream of road bridge	unaffected
3.	Wrey's Bush, upstream of road bridge	unaffected
4.	Fishing access about 600 m north of Newton Greer Road	unaffected
5.	Fishing access opposite Sinclair Road	unaffected
6.	Fishing access opposite Gowan Hill Road	unaffected
7.	Fishing access 1 km upstream of Etal Stream	unaffected
8.	Mossburn – Wrey's Bush road, downstream of road bridge	sparse
9.	Dunrobin, downstream of road bridge	moderate

3.2. Field methods: main survey

In each river, the sampling team worked from downstream to upstream in order to minimize cross-contamination (i.e., introducing *D. geminata* cells into samples collected at unaffected sites). Sampling areas were river reaches up to 50 m long, containing both run and riffle areas. At each site, we first carried out a visual inspection similar to that carried out during the regular surveillance (Duncan et al. 2005). If no colonies of *D. geminata* were noticed, the site was confirmed as "visually unaffected" and the full set of sampling was undertaken as follows.

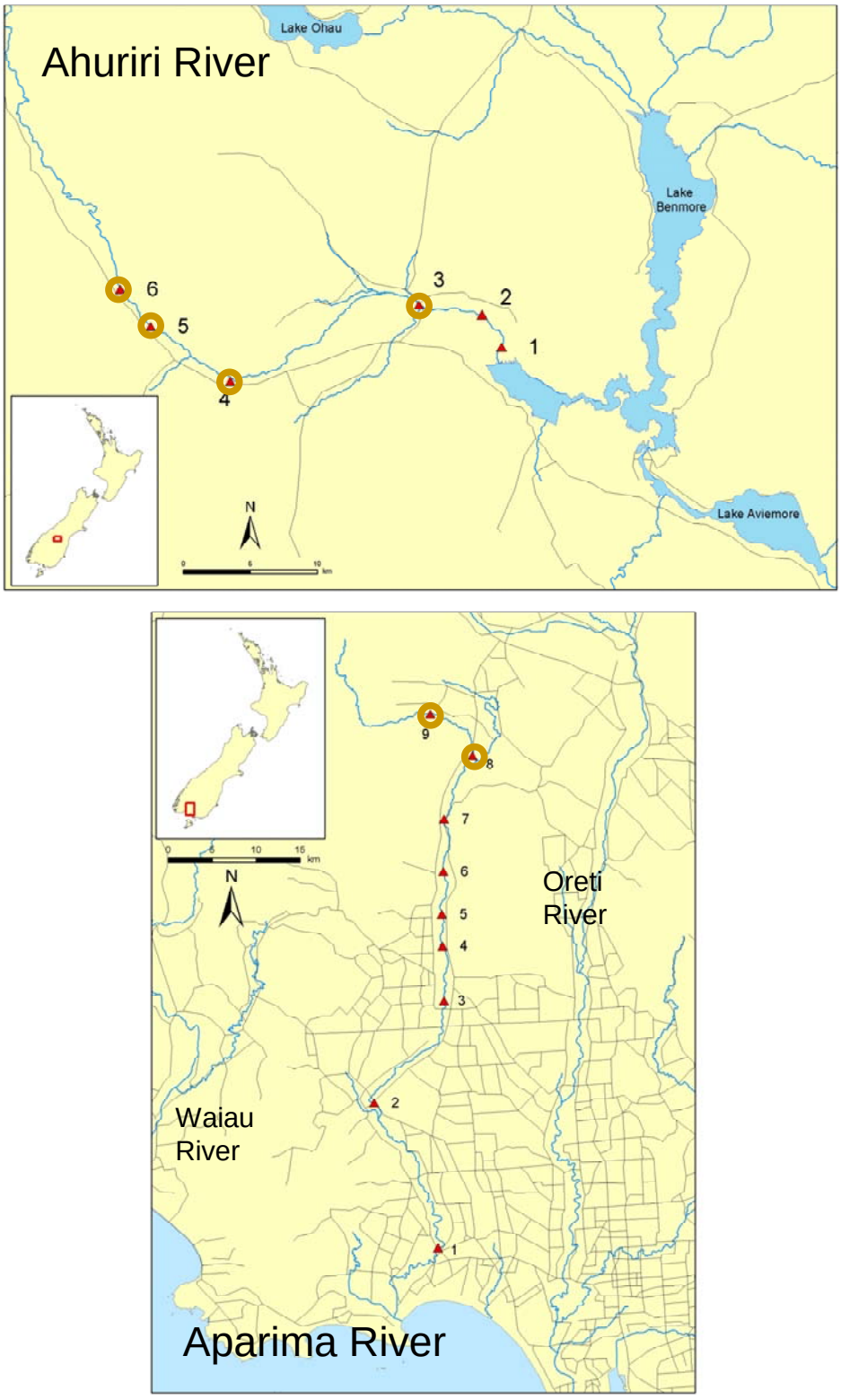

Figure 1: Locations of sampling sites (red triangles) on the Ahuriri River (top) and Aparima River (bottom). Brown circles indicate sites where *D. geminata* colonies were visible at the time of the main survey (4 – 7 April 2006). Roads are shown in black.

3.2.1. Visually unaffected sites

First, we marked the locations of 13 transects, approximately 4 m apart (Figure 2). Transect lengths were determined by the limit of depths and water velocities that could be sampled safely towards the centre of the channel (up to 0.8 m deep and/or up to 1 m/s water velocity).

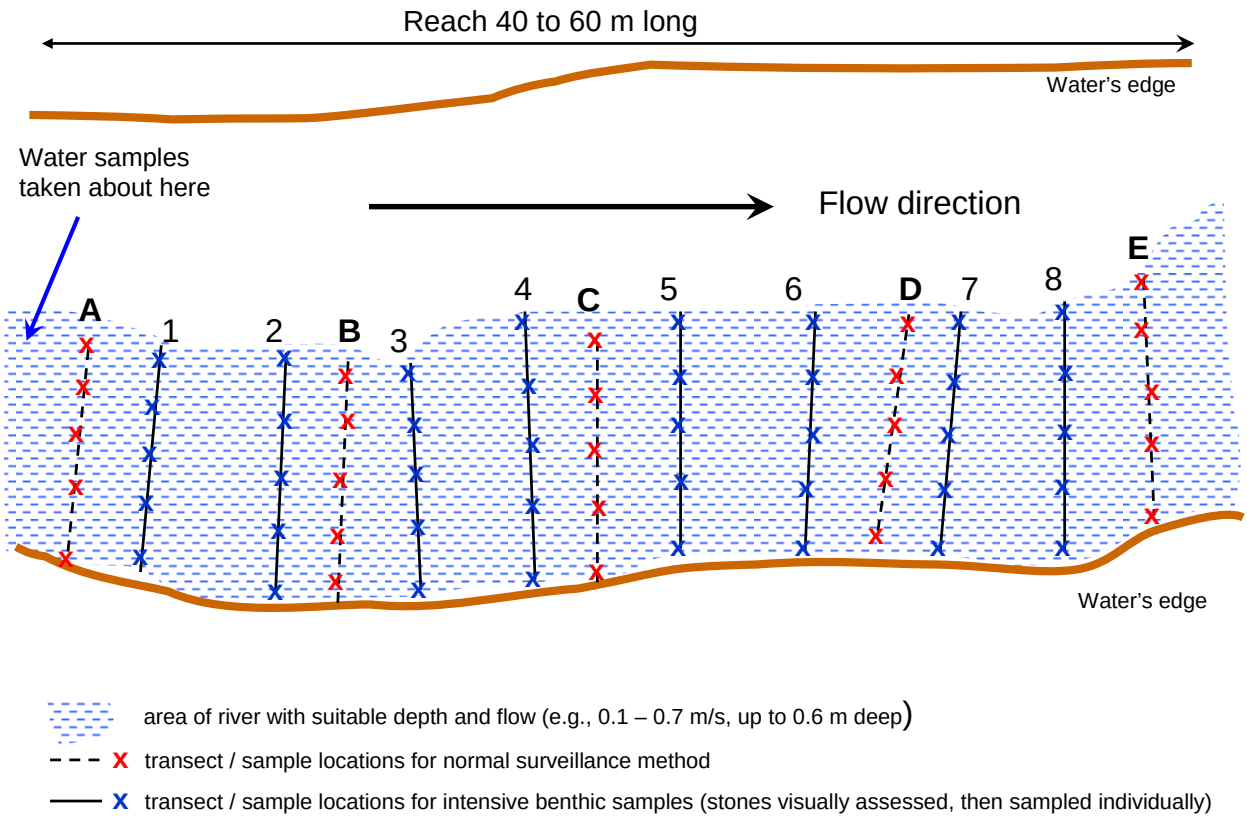

Figure 2: Sampling plan, showing designation of transects at sampling sites. Transects were located 3 – 6 m apart, with regular sampling transects interspersed with those for the detailed sampling. Water samples were collected from just upstream of the transects to prevent disturbed benthic material entering the net.

(a) Pooled sample (regular surveillance)

We collected five rocks along each transect A, B, C, D and E (Figure 2), with rock selection randomised by pre-determining the position on the transect, then picking up the first rock of manageable size encountered at that position. Using a clean nailbrush, algae were scrubbed from the surfaces of all 25 rocks and combined into a single container.

(b) Detailed benthic sampling and visual assessments

Using the same strategy as for the pooled sample, we collected five rocks along each of transects 1 to 8 (total of 40 rocks). For each rock::

- water depth and velocity at 0.6 depth were recorded at the sampling location of each rock;[2]
- algal cover was visually assessed by estimating the percentage cover of the rock surface by categories of algae based on colour and thickness. Categories are listed on the sample field sheet (Appendix 1);
- the x, y and z dimensions of the rock were recorded using a metal ruler (to the nearest 0.5 cm);
- using a clean nailbrush, all algal material was brushed off the rock and collected into an individual container (one per rock).

(c) Drift samples

We collected drift samples using a plankton net (Figure 3) deployed by attaching it to a reinforced metal rod driven into the river bed so that the net opening was positioned in the upper part of the water column (Figure 4). The net was deployed at the upstream end of each sampling site for successive periods of 10 minutes, 1 minute, and ~10 seconds. We sampled in fast flowing water (within limits of practicality and safety), on the principle that *D. geminata* cells are most likely to be held in suspension in increasing numbers as flow velocity increases. Water velocity at the net opening was measured or estimated[1].

The different deployment times represented different sampling intensities, with the water volume filtered (litres) calculated as:

area of net opening (m^2) x water velocity (m/s) x deployment time(s) x 1000.

Depending on water velocity, a 10-minute deployment represented a filtered volume of between 12,000 and 20,000 litres. The 10-second samples represented 200–350 litres. In addition, a 10-litre sample collected in a bucket was poured through the net,

[2] Unfortunately, due to instrument failure, water velocity measurements were taken only at sites 1, 2 and 3 in the Ahuriri River. At subsequent sites, notes were taken on relative flow velocities along the sampling area.

and a further 10-litre sample was poured through a small hand-held strainer (40 µm mesh).

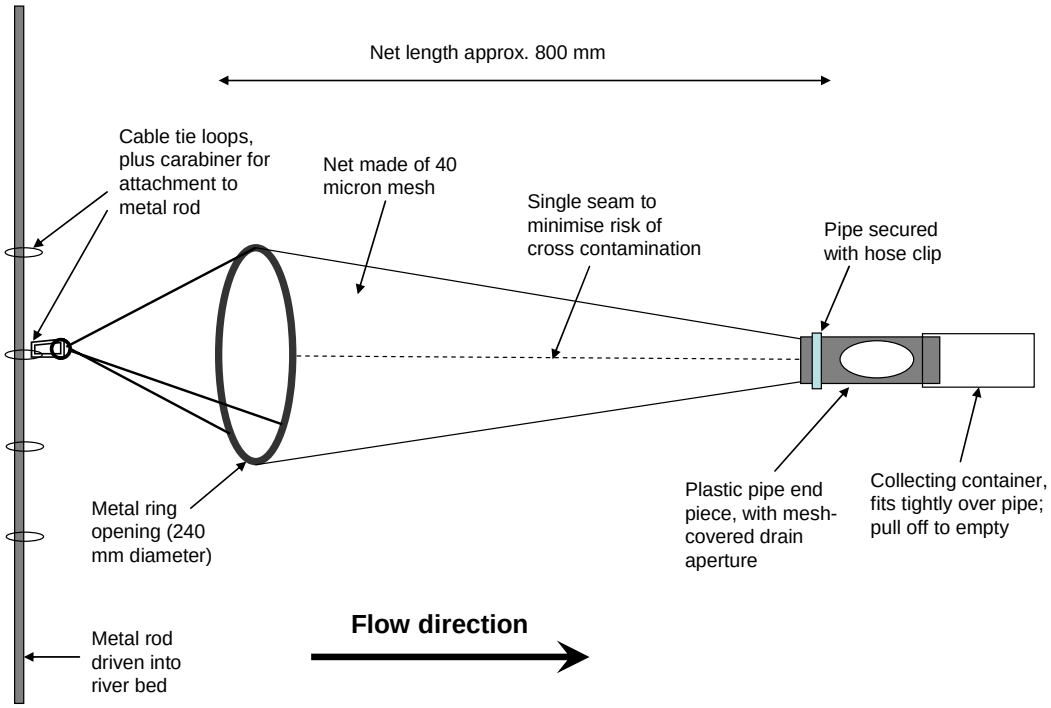

Figure 3: Schematic of drift net construction and deployment method

Figure 4: Drift net deployed in the Aparima River

(d) Artificial substrates

We deployed four different types of artificial substrate. To ensure that all were located in comparable hydraulic conditions, they were mounted adjacent to each other on concrete pavers, which also provided a stable anchor under normal river flow conditions (Figure 5). At each site we deployed three or six pavers, each carrying two of the four substrate types. We planned to collect substrates from one or two complete pavers on three subsequent occasions, approximately 10, 20 and 30 days following deployment. The purpose of multiple collections over time was to confirm whether *D. geminata* would colonise any of the substrates preferentially as the species spread downstream. Multiple collections were expected to provide a stronger indication of any preference (or not) than a one-off collection.

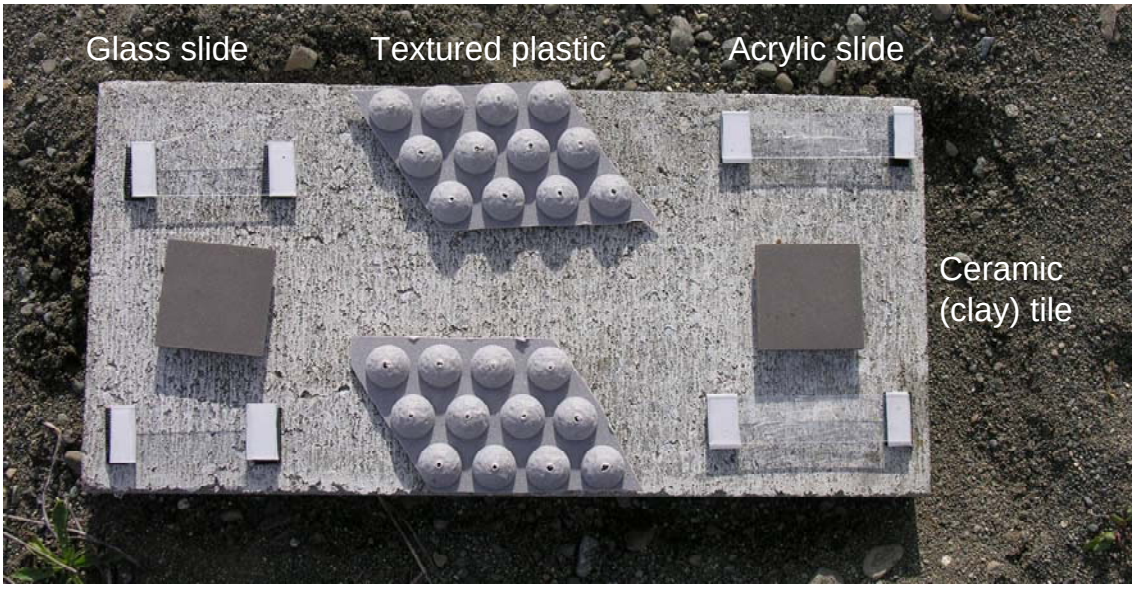

Figure 5: Artificial substrates. Two of each of four different substrate types were attached to 400 x 200 x 40 mm concrete paving blocks, using industrial Velcro. Up to six blocks were deployed at each sampling site.

3.2.2. Visually affected sites

Where the initial visual inspection gave a positive result (*D. geminata* colonies visible), the benthic sampling was reduced to a visual assessment and individual samples from 10 rocks only. This allowed us to estimate the overall % cover and *D. geminata* cell density on the substrate at these sites. Drift sampling and substrate deployment were carried out as at the unaffected sites. No 25-stone pooled sample was collected.

In all cases, samples were stored chilled until microscope examination.

3.2.3. Follow-up surveys

Follow-up surveys were undertaken in each river to retrieve the artificial substrates. On the first retrieval occasion, we randomly removed substrates from different pavers, rather than collecting the eight substrates from single pavers.

(a) Ahuriri River

Substrates were collected on 18 April, 12 days after deployment. Drift samples were collected from each site (10 min, 1 min , 10 sec and 10 L net samples). The remaining substrates were retrieved on 19 May. Some had been damaged in high flows.

(b) Aparima River

Substrates were retrieved on 25 April, 18 days after deployment. Both drift samples (as above), and 25-stone pooled samples were collected from the visually unaffected sites, and drift samples from sites 5 and 6, where *D. geminata* was visible. Because the initial survey yielded positive drift samples from the most downstream site, on 25 April we also collected drift samples and 25-stone pooled samples from three additional sites (Figure 1), in order to try to establish just how far downstream *D. geminata* cells were detectable.

On 25-26 April, the Aparima was affected by severe flooding. On 17 May, staff returned to the sites and found that all of the remaining substrates had been lost or damaged. A further set of drift and benthic samples was collected from the three most downstream sites in the Aparima River on 25 May.

3.3. Microscope analyses

In the laboratory, all samples from the initial surveys were allowed to settle (at least overnight) and excess water poured off. The remaining volume was measured, shaken to obtain a homogeneous suspension of algae, then measured aliquots of the suspensions were examined under an inverted microscope at a magnification of 125 x. Entire aliquots were scanned and numbers of *D. geminata* cells counted, distinguishing live cells (with intact or nearly intact chloroplasts) from dead cells (empty cells, or cells containing only fragments of chloroplasts).

For the drift samples, if low intensity samples contained more than 10 *D. geminata* cells in one subsample, we assumed that higher intensity samples would also contain many cells, and no further samples were processed for that site.

Collected substrates were processed as follows:

- Algal growth on textured substrates and tiles was brushed off using a clean toothbrush (a new brush for each site) and collected into individual new containers. Microscopic examination was as for the benthic/drift samples.

- Both glass and plastic slides were examined directly under microscope.

For consistency, a standard effort was applied to sample examination, of 10 mins scanning per sample. Except for the slides, this was equivalent to examination of 3 to 5 subsamples from each sample. The number of subsamples varied because denser samples (containing more algae) took longer to scan than sparse samples.

3.4. Data analysis

Field and laboratory data enabled calculation of the following:

1. For affected sites, mean percentage visible cover of *D. geminata* colonies;

2. At all sites, total densities of live and dead benthic *D. geminata* cells per unit area of river bed, using the surface area of stones sampled calculated from:

   $$\text{Surface area} = 1.59 + 0.811\,(xy + xz + yz)$$ (from Biggs & Kilroy 2001)

   Densities applicable to three different sampling intensities were calculated by using samples collected from pre-determined transects (Figure 2), viz. low intensity – transects 1 and 5 (10 samples), medium intensity – transects 1, 2, 3, 5 and 7 (25 samples), high intensity – transects 1 to 8 (40 samples);

3. Densities of live and dead *D. geminata* cells in the water column, per unit volume, determined for different sampling intensities represented by different deployment times;

4. Mean densities of *D. geminata* per sampling unit (defined as a 10 minute scanning effort applied to algal material collected from one rock or drift sample).

5. Densities of live and dead *D. geminata* cells per substrate unit for each type of substrate.

The data collected from a limited number of sites in two rivers only allow visual representation of the data and not a complete statistical analysis. For each river, we plotted cell densities vs. site for benthic, drift and substrate samples at each intensity tested. We qualitatively compared patterns in each river using simple plots of mean cell densities and % cover.

One aim of the study was to determine statistically the number of samples required to detect *D. geminata* at a specified, low, density in the benthos. The basis for this calculation was the collection and analysis of multiple samples at each site, from which a range of actual densities of *D. geminata* could be calculated. This would allow a definition of a cell density that realistically represents "rare" at a site. To calculate the number of samples required to detect the species at this specified density, we used the formula derived by Green & Young (1993),

$$n = -(1/m) \log \beta$$

where β is the probability of failing to collect a species from n samples, when it is actually present at the site with some mean density m.

In other words, n is the number of samples required to detect *D. geminata* (with a probability $1 - \beta$) if it is present with a mean density of at least m. If the probability of detection, $1 - \beta$, is taken to be 0.95, the formula is simply:

$$n = 3 / m \qquad \text{Equation 1}$$

The main assumption associated with this formula is that the distribution of the rare species in a river can be described by the Poisson distribution[3], which itself requires the following assumptions:

- Samples are independent of one another.
- The spatial distribution of the species is random (not clumped).

[3] The binomial distribution is the general rule with which we can determine the probability of getting a given result from a specified number of samples (trials), assuming random distribution and independence of samples. The Poisson distribution is a special case of the binomial, for when the probabilities are very small (e.g., when we are dealing with rare species). As mean values increase (i.e., species are no longer rare), the normal distribution (bell-shaped curve) starts to apply.

In this study we determined realistic ranges of values for *m* from our field collections, prior to calculating *n*. We also consider the validity of the above assumptions.

3.5. Collection of samples for genomic study

A major study being undertaken in parallel with the present project is development of a genomic detection method for *D. geminata* (Waikato University). A supplementary objective of the present work was to contribute samples to the Waikato University study. Subsamples of the material filtered from the water column were transferred to 10 ml centrifuge tubes and topped up with 98% ethanol to a final concentration of approximately 70%. In general the subsamples were the same volume as those examined in our 10-minute standard scan, so that detection rates from the same samples would be comparable. To eliminate cross contamination, sample transfer was carried out using a new disposable pipetter for each sample, and tubes were sealed immediately after addition of ethanol.

4. Survey results

4.1. Overall comparison

In Table 1, we present a summary of the results in terms of presence/absence of *D. geminata* (live and dead cells not distinguished) at all sites in both rivers, using each sampling method at the range of intensities specified above. These results are based only on the main sampling run and the first collection of substrates. Further details on the results for each sampling method, and for the additional sampling undertaken are given in the following sections, in which summary results are presented graphically. Detailed results are tabulated in Appendix 2.

4.2. Visual assessments

During the detailed visual assessments at the six sites initially assessed as being unaffected (see section 3.1), small growths on 6 rocks (all in the Ahuriri) were queried as possible *D. geminata* colonies. Microscope examination confirmed that none were positive. Cover at the six affected sites ranged from 0.2% to 46% (Appendix 2, Table A1). The lowest cover was recorded at Ahuriri site 3. Our aim in this case was to quantify the cover at a site where colonies were barely visible, and possibly only able to be picked up by an experienced observer.

Table 1: Summary comparison showing detection (presence – red cells, absence – yellow cells) of *D. geminata* at 6 sites in the Ahuriri and Aparima Rivers using different sampling methods. Purple cells indicate no data (samples either not collected or not analysed). Refer to text for the reasons why some site / sampling method combinations were not included. Refer to relevant sections (by method) for more detailed results.

Ahuriri River	Site:	**1**	**2**	**3**	**4**	**5**	**6**
Distance from top site (km):		**38**	**35**	**30.5**	**13**	**4**	**0**
Method	**Intensity**						
Visual							
25-stone pooled							
Benthic	High						
	Medium						
	Low						
Drift	High						
	Medium						
	Low						
	Very low						
	Extra low						
Substrates (after 12 days)	Textured						
	Clay tiles						
	Glass slides						
	Acrylic						

Aparima River	Site:	**4**	**5**	**6**	**7**	**8**	**9**
Approx. distance from top site		**35**	**31**	**25**	**18**	**8**	**0**
Method	**Intensity**						
Visual							
25-stone pooled							
Benthic	High						
	Medium						
	Low						
Drift	High						
	Medium						
	Low						
	Very low						
	Extra low						
Substrates (after 18 days)	Textured						
	Clay tiles						
	Glass slides						
	Acrylic						
25-stone pooled (after 18 days)							

4.3. Benthic sampling: 25-stone pooled sample

In the initial survey, 25-stone pooled samples from the six visually unaffected sites yielded two positive samples, site 2 in the Ahuriri, and site 4 in the Aparima. These sites were, respectively, approx. 5 and 10 km downstream of locations where *D. geminata* was visible. In the follow-up surveys in the Aparima (25 April), of the seven 25-stone pooled samples collected, just one was positive (site 4, Table 1). A single dead (empty) *D. geminata* cell was found in one subsample from this site. Surprisingly, the samples from sites 5, 6 and 7 were negative, even though they were closer to the area where *D. geminata* growth was visible.

4.4. Benthic sampling: multiple samples

In both rivers every intensive benthic sampling (40 rocks) yielded at least one sample containing *D. geminata* (Table 1). We found the minimum density possible (one *D. geminata* cell observed in one subsample from a single rock) at site 4, Aparima River. High-intensity samplings did not consistently yield higher overall densities than medium and low-intensity samples (Appendix 2, Table A1). However, in the Aparima River, the low intensity sampling at the most downstream site did not include the single positive sample found at that site. In both rivers, sites where *D. geminata* was visible were sampled only at the low intensity rate in order to estimate cell density, for comparative purposes. In the Ahuriri, but not in the Aparima, there was a pattern of increasing proportions of live cells in an upstream direction (Figure 6).

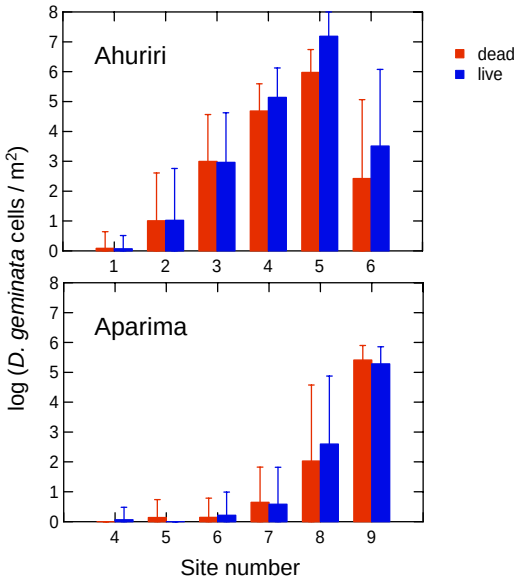

Figure 6: Calculated mean densities (by stone) of *D. geminata* at 6 sites each in the Ahuriri and Aparima rivers. Densities are derived from all stones sampled (i.e., 40 stones in the downstream sites), and are presented on a log scale for clarity (i.e., the numbers on the vertical scale are power values of 10 – e.g., 3 is 10^3 = 1000).

4.5. Drift samples

Cells were found in the drift samples at every site in both rivers in the initial survey (See Appendix 2, Tables A2 and A3 for details). Densities decreased, as expected, as distance from areas with visible growth increased (Figure 7). In both rivers there was a pattern of dead (empty) cells being picked up in drift samples in higher densities and/or farther downstream than live cells, except at Ahuriri, site 5, and Aparima, site 6, where *D. geminata* was present in quantity (Figure 8). Again, there was no clear tendency for higher intensity samples to yield higher densities of *D. geminata* (i.e., to be more efficient at including cells in the sample). However, in the Aparima River in particular, the small filter samples (xlow intensity, Tables 1, A3) were the least efficient at picking up *D. geminata* cells.

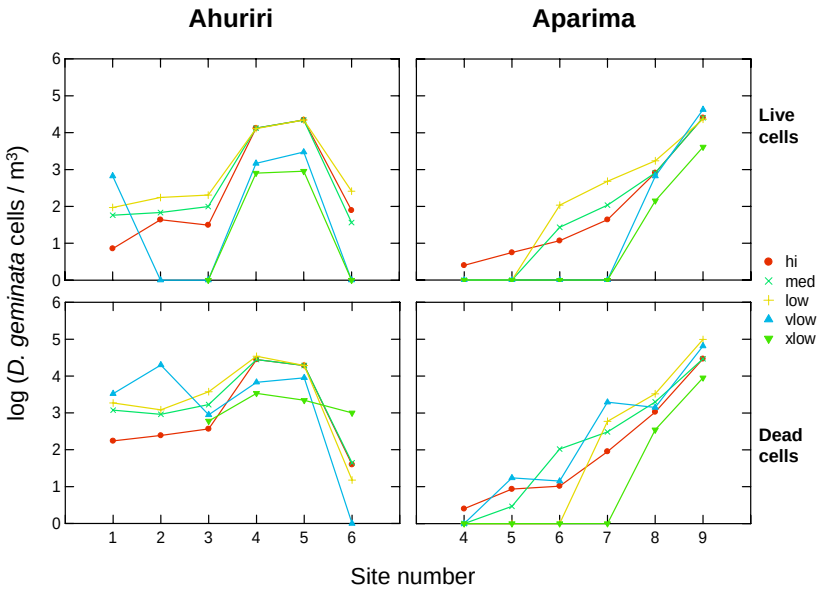

Figure 7: Calculated densities of *D. geminata* filtered from the water column at 6 sites each in the Ahuriri and Aparima rivers, main sampling occasion. Densities were derived for each sampling intensity used at the site (see key at right), and are presented on a log scale in cells/m^3, for clarity. Refer to Appendix 2, Table A1 for detailed results.

In both rivers, densities of cells in the water column increased markedly over time, as might be expected. In the first follow-up surveys in both rivers, drift samples from all sites yielded *D. geminata* cells. In the Aparima River, this included three additional downstream sites (sites 1, 2 and 3), which were sampled in order to try to establish a downstream limit of infestation. Further drift samples were collected from these three sites on 25 May because of the possibility that the earlier samples may have been positive for *D. geminata* as a result of cross-contamination from the sampling equipment. On 25 May, the most downstream site (1) yielded no positive samples, but *D. geminata* cells were found in some samples from sites 2 and 3 (Figure 9, Table A3).

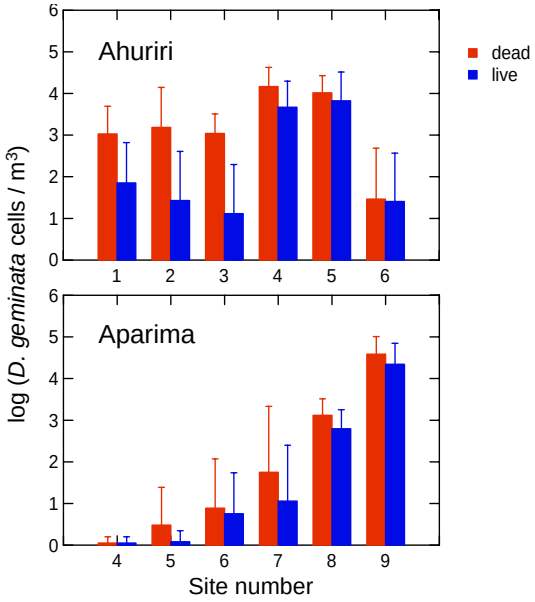

Figure 8: Mean densities (with standard deviations) of *D. geminata* filtered from the water column, main sampling occasion, Ahuriri and Aparima Rivers. Note higher densities of dead cells versus live cells. Means are calculated from all sampling intensities. Densities are presented on a log scale (in cells/m^3) for clarity.

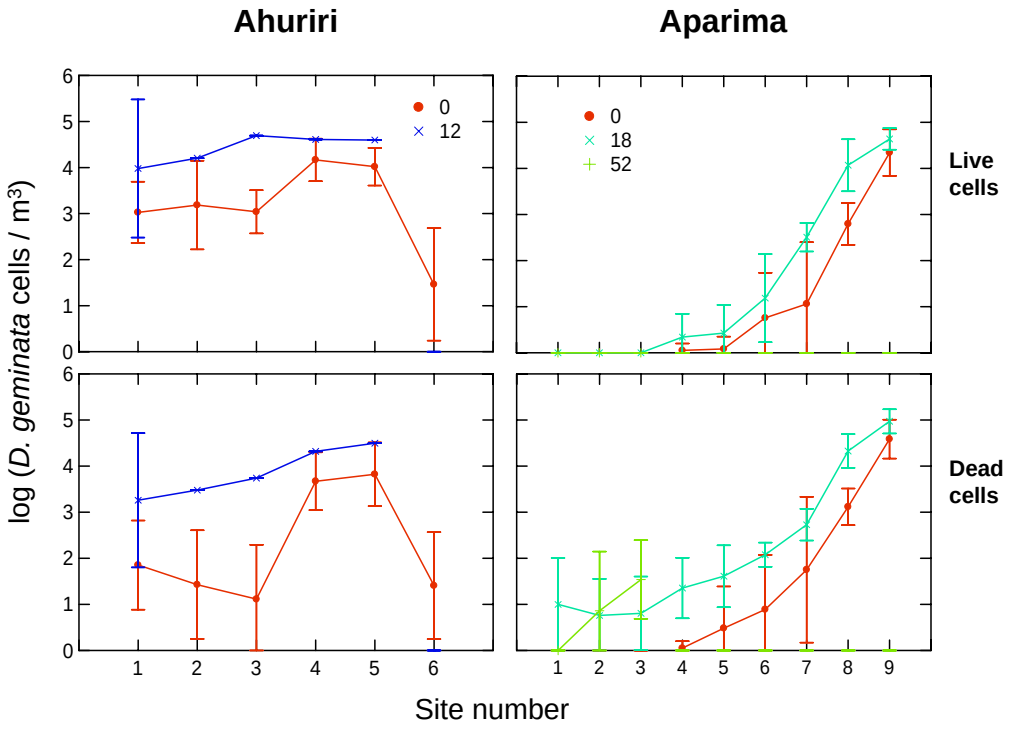

Figure 9: Mean densities (with standard deviations) of *D. geminata* filtered from the water column at 6 sites and two sampling occasions in the Ahuriri River, and at 9 sites over three sampling occasions in the Aparima River. Means are calculated from all sampling intensities. Densities are presented on a log scale (in cells/m^3) for clarity. Times (see keys in the two upper plots) are in days since the main sampling.

The tendency for drift samples to contain higher densities of dead cells than live cells was confirmed for the follow-up sampling in the Aparima (Figure 9).

4.6. Artificial substrates

A full set of artificial substrates was retrieved from the Ahuriri River 12 days following deployment, and from the Aparima River after 18 days, with the exception of those deployed at site 6 in the Ahuriri, which were inexplicably lost. Some individual substrates had been lost, but on the whole, the deployment method worked well under normal flows. As indicated (section 3.2.3), the planned three collection trips could not be completed because of subsequent flooding/high flows, in which most of the remaining pavers in the Aparima were lost completely, and most of those in the Ahuriri damaged.

Algal growth was evident on all remaining substrates, with the textured plastic showing the most obvious accrual (Figure 10). Both the glass and acrylic slides colonised on the underside as well as the top (attachment to the concrete pavers with Velcro left a gap of about 5 mm). In all cases, the substrates colonised with the algal species that were abundant on natural substrates at the site, as would be expected.

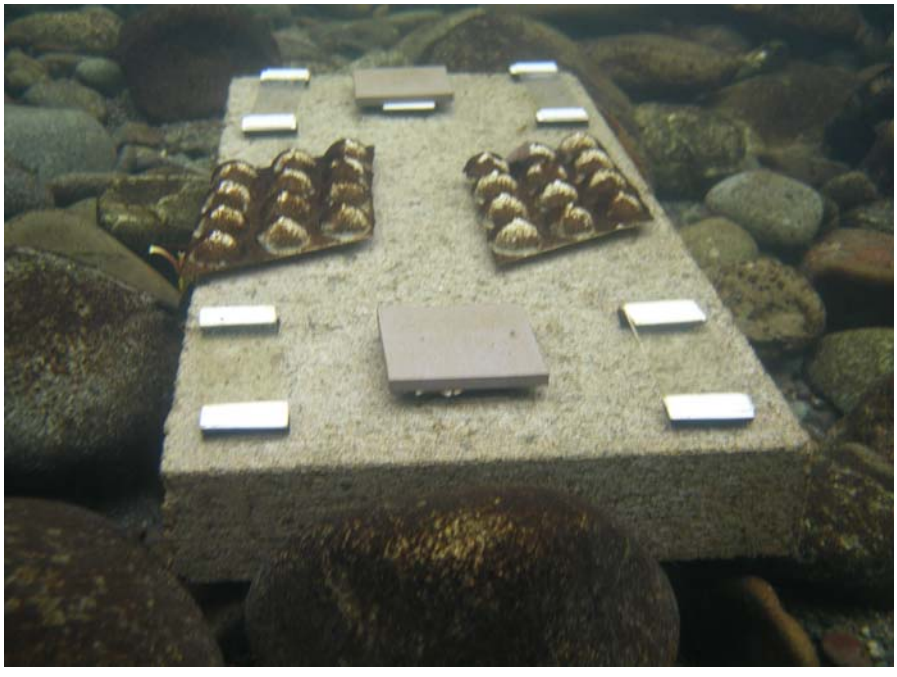

Figure 10: Artificial substrates in the Aparima River, following 18 days incubation under normal flows. Note the subtle greenish film on the glass slides (bottom of picture) and clay tiles, and compare with the prolific growth on the textured plastic.

The summary results (Table 1) indicate variations in colonisation by *D. geminata* between the two rivers. In particular, glass and acrylic slides generally gave positive results (*D. geminata* present) in the Ahuriri, but not in the Aparima, despite a longer incubation time in the latter river (18 days as opposed to 12 days). However, the densities of cells on these slides were very low (Figure 11).

While the textured plastic and ceramic tile substrates were most rapidly colonised by *D. geminata* in both rivers, neither colonised ahead of live cells being captured from the water column (compare Figures 9 and 11).

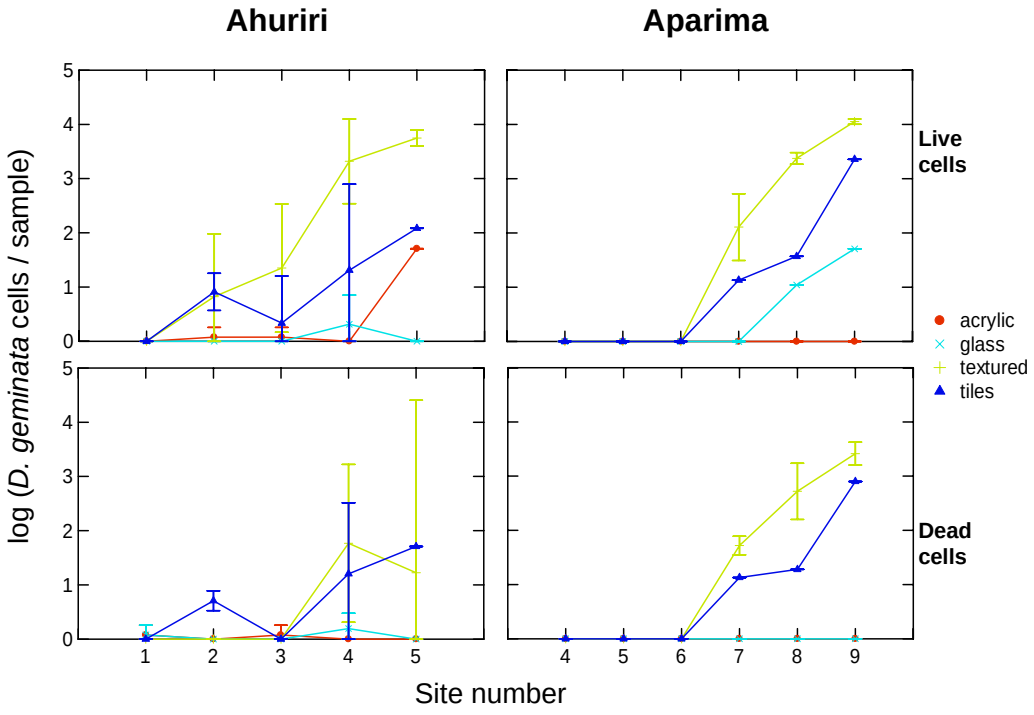

Figure 11: Mean numbers of cells per sample (with standard deviations) of *D. geminata* counted from four types of artificial substrate at 6 sites in the Ahuriri and Aparima Rivers. Densities are presented on a log scale for clarity. Note that the substrates in the Ahuriri were left in the river for 12 days and those in the Aparima for 18 days.

4.7. Samples for genomic study

A total of 125 samples was subsampled, preserved in ~70% ethanol and shipped to Dr. Brendan Hicks, University of Waikato. These included 13 benthic samples (25-stone pooled method), 22 substrate samples, with the balance being filtered material from the water column. In all cases, samples were held at ~1ºC while awaiting analysis and were processed within no more than 6 days. We expect algae to remain in good condition (i.e., viable) under these conditions.

4.8. Spatial relationships between visual cover and cell densities in the water and benthos

Patterns of visible *D. geminata* relative to cell densities in the benthos and water column differed in the two rivers (Figure 12). We did not sample upstream of the affected reaches in the Aparima, hence no peak appears in the latter plots. The main difference was the maintenance of maximum cell densities in the water column in the Ahuriri River well beyond the area of maximum % cover and benthic cell density. In the Aparima River, on the other hand, all three measures showed a consistent pattern. Note the different scales on the y-axes in Figure 12. A notable feature is that, in spite of very much higher percentage cover and benthic cell densities in the Ahuriri River, densities in the water column were markedly lower than those in the Aparima.

5. Benthic sampling to maximise the probability of detecting *D. geminata*

To calculate the number of samples required to detect a given low density of *D. geminata*, we first had to decide on an appropriate density to represent "rare". Second, we needed to determine the appropriate sampling units in which to express that density. In the present study, we were able to calculate cell density per unit area of river bed by measuring the dimensions of all rocks sampled. Using this method, the lowest mean density found in the present surveys was ~9 cells/m^2 (site 4, Aparima River). However, a more logical sampling unit for this calculation is simply one rock. Green and Young (1993) arbitrarily defined "rare" as <0.1 individuals per sampling unit (i.e., one individual detected in every 10 sampling units), with sampling units generally interpreted as quadrats. If we consider one rock as our unit (quadrat), in our case the lowest density detected (at Aparima River, site 4) was 1 cell per 40 sampling units (rocks), or 0.025 individuals per sampling unit. Since this is the minimum density possible in our intensive sampling, Green and Young's arbitrary definition of "rare" as < 0.1 individuals per sampling unit appears to be appropriate in our case. This density applies at site 5 in the Aparima River (Appendix 1, Table A3), where we calculated 26 cells/m^2 (Table 2).

Because we consistently examined only a proportion of the whole sample, for this calculation our sampling unit is taken to mean the sample proportion actually examined. Rather than standardising the proportion, we applied a standard effort of about 10 mins scanning per sample, equivalent to 3 to 5 subsamples.

Applying equation 1 (page 14), the number of sampling units required for a 0.95 chance of detecting of *D. geminata* in our samples, if it is present with a density of 0.1

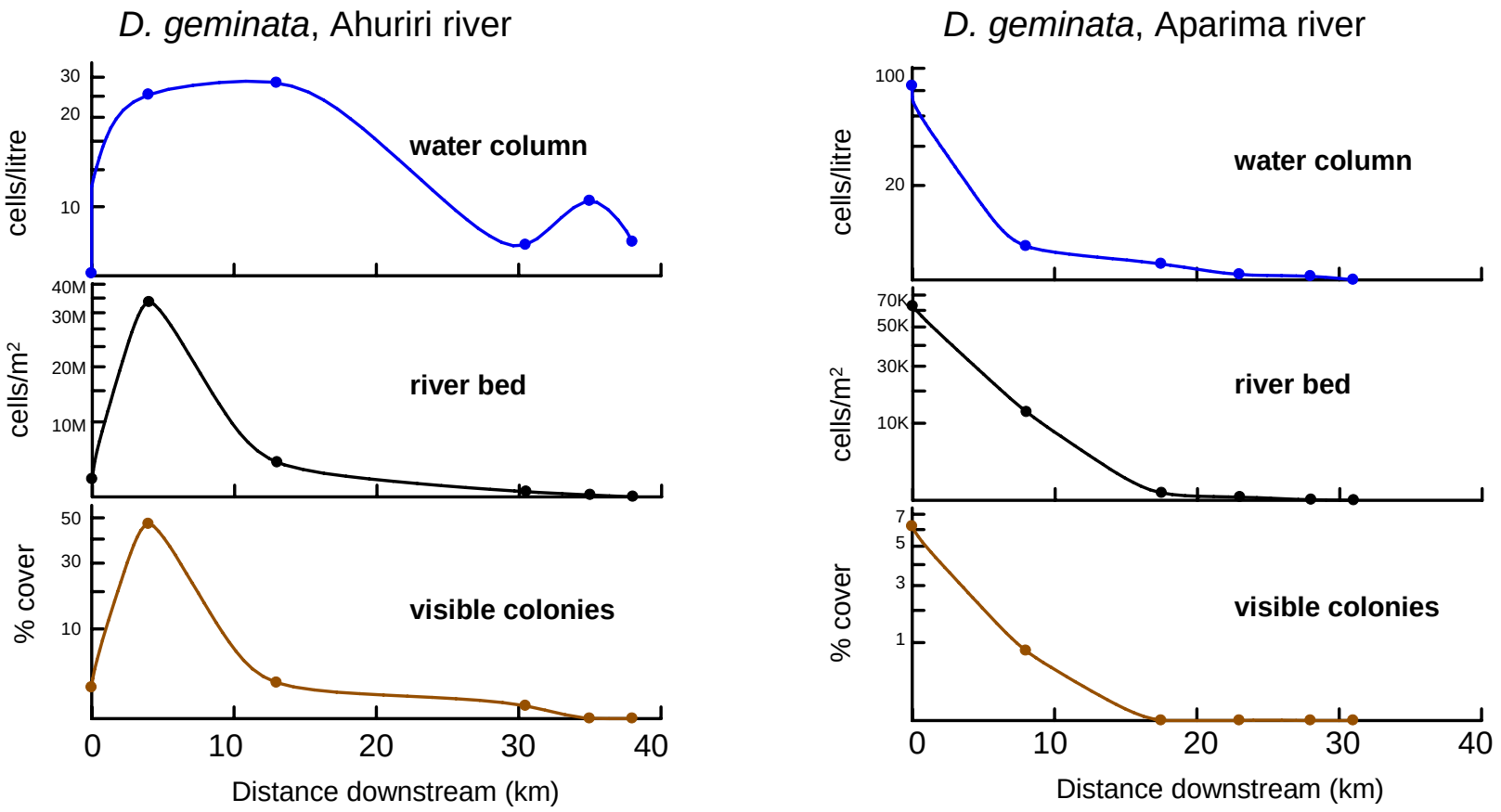

Figure 12: Mean percentage cover of *D. geminata* recorded in the Ahuriri and Aparima rivers (brown plots) showing visual relationship with mean total cell densities (live + dead cells) measured in the benthos (black plots) and water column (blue plots). Note that the data ranges on the vertical axes are different for the two rivers.

or more, is:

$$3 / 0.1 = 30$$

For a 0.95 chance of again detecting the density that we actually observed (0.025 individuals per sampling unit), assuming that this was the true density at that site, we would need to collect 3 / 0.025 = 120 samples.

As indicated above, this calculation depends on the assumption that the distribution of *D. geminata* cells in the rivers and at the sites follows a Poisson distribution. In a Poisson distribution, the mean and variance are approximately equal. This is true for site 1 in the Ahuriri, and sites 4, 5 and possibly site 6 in the Aparima (Table 2).

Table 2: Summary statistics calculated from benthic sampling at six sites each in the Ahuriri and Aparima Rivers. All benthic samples are included. Cell densities are per sampling unit (number of cells counted in a standard 10-minute scanning effort per sample). Sites in bold are those conforming to a Poisson distribution of cells, consistent with rarity of the organism (see Green and Young 1993).

River	Site	No. samples	Cells per sampling unit			
			Maximum	Minimum	Mean	Variance
Ahuriri	**1**	**40**	**1**	**0**	**0.05**	**0.05**
	2	40	56	0	4.7	135
	3	40	125	0	25	926
	4	10	262	5	104	8480
	5	5	206	105	156	1822
	6	5	114	0	28	2325
Aparima	**4**	**40**	**1**	**0**	**0.03**	**0.03**
	5	**40**	**2**	**0**	**0.08**	**0.12**
	6	**40**	**8**	**0**	**0.38**	**2.5**
	7	40	86	0	3.6	203
	8	10	180	0	43	4653
	9	6	476	119	271	24100

The conditions required for the Poisson distribution are independence of samples and a spatially random distribution of cells at the site. We consider that both these conditions are valid at sites where *D. geminata* is shown to be rare, which can be interpreted as sites where the cells have not yet, or have only just, established. In this case, the only *D. geminata* present will be cells that have settled out from the water

column and adhered to the substrate. Only when colonies start to grow and expand will distribution of individual stones be non-independent (i.e., the density on a particular rock can be influenced by that on a neighbouring rock).

With regard to random distribution, it is possible that cell settlement is a function of water velocity, for example, most cells might settle in areas of slower moving water. We tested this using data from the 3 sites in the Ahuriri River from which water velocity data were available. If cell settlement is preferentially in a particular water velocity, then this should be evident from a plot of water velocity vs. cell density. As Figure 13 shows, no such relationship is seen at any site. The slope of the linear regression line for site 2 suggests a negative relationship between cell density and water velocity, however, the relationship is non-significant ($R^2 = 0.043$, $P = 0.209$).

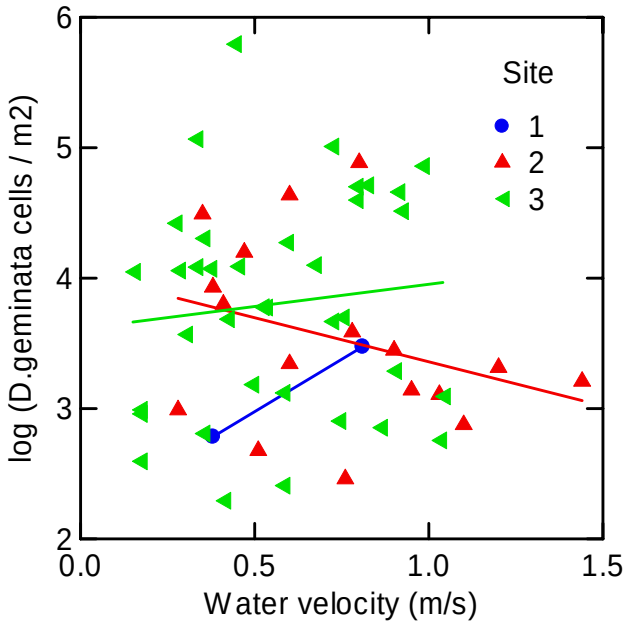

Figure 13: Benthic *D. geminata* cell densities (normalised to cells per unit area) plotted against water velocity measured at the sample collection point for sites 1, 2 and 3 in the Ahuriri River. Lines are the best fit linear regression. Density data are log-transformed data to conform to a normal distribution.

6. Comparison of sampling effort and resources

In Table 3 we compare the resources required and effort expended for each sampling method. We take into account time for sample collection and processing in the field, laboratory time for sample preparation and examination, and the cost of required equipment that is not common to all the methods. Drift sampling at the lower

Table 3: Comparison of costs, resources and effectiveness of the sampling methods tested. The overall rank is the total score (sum of ranked costs and effectiveness), re-ranked.

		Field			Lab					
			Special equipment *							
Method		Time (person - time per sample)	Details	Estimated cost ($)	Time / sample	**Total time + extra cost (per sample) ($) #**	**Ranked cost**	**Ranked effective ness###**	**Total scores**	**Overall rank**
Quick visual assessment		5 mins	nil	n/a	nil	8.33	1	10	11	**5**
Detailed visual		20 mins	nil	n/a	nil	33.33	2	10	12	**7**
25-stone pooled		30 mins	Brush, container	10	15 min	85.00	7	9	16	**9**
Detailed benthic	30 stones	90 mins	Brush, container	20	10 h	1170.00##	13	1	14	**8**
Drift	high (10 min)	35 min **	Net, container	10***	15 min	93.33	8	1	9	**4**
	medium (1 min)	25 min	Net, container	10	15 min	76.67	4	3	7	**1**
	low (10 sec)	25 min	Net, container	10	15 min	76.67	4	3	7	**1**
	very low (10 L)	25 min	Net, container	10	15 min	76.67	4	3	7	**1**
	x low (10 L filter)	25 min	Filter, container	25	15 min	60.83	3	8	11	**5**
Artificial substrates	Textured plastic	45 min deploy., 20 min collection	Substrate, adhesive & anchor	40	25 min	156.67	11	6	17	**10**

Method		Field			Lab	Total time + extra cost (per sample) ($) #	Ranked cost	Ranked effectiveness###	Total scores	Overall rank
			Special equipment *							
		Time (person - time per sample)	Details	Estimated cost ($)	Time / sample					
	Clay tiles	45 min deploy., 20 min collection	Substrate, adhesive & anchor	40	25 min	156.67	12	6	18	**12**
	Glass slides	45 min deploy., 20 min collection	Substrate, adhesive & anchor	40	15 min	140.00	9	8	17	**10**
	Acrylic slides	45 min deploy., 20 min collection	Substrate, adhesive & anchor	40	15 min	140.00	10	13	23	**13**

Notes:

*Equipment for each method includes only materials that are not common to all the methods. For example, this does not include decontamination equipment. No travel time is included as this is common to all methods, except for one extra site visit for substrate deployment. We assume that multiple substrates are deployed at one time, and extra time required is estimated to be 30 minutes per sample.

**Time allowed for drift sampling includes 10 minutes for decontamination of nets between successive sites.

***Plankton nets for drift sampling cost approximately $200.00 each. Assuming that each net can be used at least 20 times, this is costed at $10.00 per sample.

#Time is costed at a flat rate of $100 per hour.

##Since the additional cost for the 30-stone detailed sample is an order of magnitude greater than all other costs, the rank here should probably be weighted (doubled) to reflect this.

###Effectiveness is detection of *D. geminata*, with higher effectiveness applying to methods detecting cells farther downstream.

intensities ranked highest overall, followed by the highest intensity drift sampling. However, given the very small difference in cost between the different intensities, drift sampling at a higher intensity is preferable.

7. Discussion

The summary results (Table 1) indicate that both the detailed benthic sampling and drift sampling were capable of picking up *D. geminata* farther downstream than either visual inspections or the 25-stone benthic samples currently used in Biosecurity New Zealand's surveillance programmes. We found at least one *D. geminata* cell in the detailed benthic samples from every site in both rivers. In the Ahuriri River, *D. geminata* was detected in detailed benthic and drift samples 7.5 km downstream from where colonies were just visible (only 0.2% cover). We picked up cells using the current surveillance method only 3 km downstream from these colonies. In the Aparima, we picked up cells in both drift and detailed benthic samples at sites up to 23 km downstream of a site with 0.8% visible cover. At the most downstream site, only a single cell was found in the 40 benthic samples examined, and two cells (one live, one dead) in the 10-minute drift sample. In contrast, the current method gave a positive result 9.5 km downstream, but negative results thereafter.

Like the detailed benthic sampling, drift samples gave positive results at every site in the initial survey. However, the resources and sampling effort required are considerably less (Table 3). The highest intensity drift samples gave the best results in the initial surveys, but follow-up surveys in the Aparima river indicated that this was not necessarily the case (Appendix 1, Table A3). Drift sampling presents the most promising alternative to the present sampling protocol for increasing the probability of detecting *D. geminata* cells in an infected river at very low densities. Resources required for the drift sampling and the method currently used are similar.

Nets required for drift sampling are relatively expensive at approximately $200.00 each. We trialled a small filtering apparatus as an alternative. However, this proved quite fiddly to use and the results were not as reliable as those obtained using the net, because only small volumes of water could be passed through it. This apparatus would have the same contamination issues as the nets (see below), unless one filter was made for each site, in which case the costs are likely to be equivalent.

Detailed visual assessments provided no better information than casual inspections, though a very experienced observer might be expected to detect visible colonies earlier. By the time colonies become barely visible (Ahuriri River, site 3, 0.2 % cover), we calculated mean densities of approximately 25,000 cells per square metre

of cobble-covered river bed (live and dead cells combined, Appendix 1, Table A1), or an average of 25 cells (live and dead combined) per sampling unit examined (Table 2). At this site, cells were found on 80% of the stones sampled. Thus, as might be expected, by the time colonies appear, given a supply of propagules from upstream, the entire site may be infested.

In the current survey, we found no indication that artificial substrates were any more effective than benthic or drift sampling at picking up *D. geminata* cells. Textured plastic substrates and unglazed ceramic (clay) tiles provided the best surfaces for colonization by all algae, including *D. geminata*. We found single cells of *D. geminata* on both glass and acrylic slides at the two downstream sites in the Ahuriri. However, this was probably more to do with the even distribution of cells in the water of the whole river at the time of substrate collection (Figure 8), rather than any preference for settlement on these substrates over anywhere else.

7.1. Laboratory processing of samples

Although this study focused on an evaluation of sample collection methods, the procedure for examining samples in the laboratory following collection is clearly a crucial part of the process and deserves some mention. In terms of sampling effort in the field, the current method (25-stone pooled sample) is the same as our medium-intensity benthic sampling, with the difference that in the latter method, we collected samples from each stone individually, instead of examining subsamples from a pooled sample. We found more *D. geminata* cells in the detailed benthic method simply because the proportions of algal material on the stones collected and examined under the microscope were higher. The proportion of material examined in the detailed benthic samples ranged from 4% (prolific algal growth in the Ahuriri) to 20% (much thinner algae in the downstream sites of the Aparima). With a pooled sample collected from a site supporting thick algal growth, we would examine, at the most, 1% of the material collected, which itself would often comprise only a proportion of the material present on the rocks.

In the current method the pooled sample from 25 rocks is similar in terms of intensity (number of samples collected) to the estimate of 30 samples needed to detect *D. geminata* (0.95 confidence) at a density of 0.1 individual cells per sampling unit (rocks). However, the calculation is based on a standard scanning effort of 10 mins per sample, i.e., 5 hours scanning effort for every site to obtain a 0.95 chance of picking up at least one *D. geminata* cell, if it is present at that density. A similar effort applied to the pooled samples would likely improve the rate of detection, but 5 hours scanning effort per site is clearly a substantial and unrealistic requirement.

There are potential alternatives to microscope scanning of the raw samples, which would allow us to examine a much larger proportion of the sample in a shorter time. These include:

1. Treatment of samples with strong oxidising agents (e.g. acids). This is a standard technique for preparation of diatom samples for permanent slide mounts (Biggs and Kilroy 2000, page 106). Strong acids remove all organic material in samples, thereby considerably reducing the volume. The silica cell walls of diatoms are resistant to this treatment and remain in the sample. Theoretically, a 10-minute scanning effort applied to the cleaned sample would then cover a much larger proportion of the original sample. *D. geminata* cells may also stand out more clearly in a sample once clumps of other algae are removed. Drawbacks to this method are:

    - the extra processing takes time and uses consumables (acid and other chemicals);
    - if samples contain a lot of silt, acid cleaning may not reduce the volume significantly.

2. Passage though an automatic counter (flow cytometer) with shape recognition capability. Flow cytometry is routinely used to process marine plankton samples (Hall and Cumming 2003) and a major project in Europe has developed a system for automatic identification of diatoms on the basis of shape (du Buf and Bayer 2002). Drawbacks to the method are:

    - samples containing very thick and clumped algae would still need to be pre-processed (e.g., passing through sieves, homogenisation);
    - setting up a shape recognition algorithm to distinguish *D. geminata* from other cells may require significant resources (as yet unknown);
    - specialised equipment would be required.

3. Detection of cells in a sample using genomic methods. Work is currently in progress at the University of Waikato to develop a genomic detection method for *D. geminata*. As in microscope examination, only small subsamples can be examined at one time. However, if *D. geminata* containing DNA is present in a subsample, then detection is assured and does not depend on the vigilance or thoroughness of the operator.

As for benthic sampling, the ability to detect *D. geminata* cells in a drift sample must also be a function of the effort expended in the laboratory. Because net sampling concentrates small amounts of solid material from a very large volume of water, it is not necessary to sift through prolific benthic growth searching for individual *D. geminata* cells that happened to settle out at the sampling site. Proportions of the samples examined during our standard ~10 minute scan ranged from 1.5% to 5% for 10-minute samples, up to 100% for most 10 litre samples. The net samples contained high proportions of fine, amorphous organic matter and fragments of invertebrates and were generally easier to view than benthic samples. Most samples included only a few large-sized particles. The algae observed in these samples were generally not representative of the benthic algae but included more typical planktonic taxa. For example, in the Aparima River, most drift samples contained a large species of the diatom *Surirella*, and many contained *Pinnularia* species. Very few benthic samples contained these taxa.

The fine consistency of most of the net samples means that they would be more suitable for flow cytometry and shape recognition processing than the benthic samples. The smaller volume of material collected (compared to benthic samples) means that they are also better suited to both microscope and genomic detection methods. However, note that there may be occasions when drift samples are much more bulky than benthic samples, for example, when the water column contains high densities of suspended sediment or other debris, and benthic growth is very low, due to season or a recent flood.

7.2. Distribution of *D. geminata* through the rivers

The broad patterns of distribution of *D. geminata* shown in Figure 12 highlight the difference between rivers in the distribution of cells in the water column from upstream to downstream, i.e., a less marked downstream decline in cell concentrations in the water column at the time of the initial survey in the Ahuriri River than in the Aparima (Figure 8). The downstream decline was clear in the Aparima in both the initial and follow-up surveys. The difference is not surprising given the length of time that *D. geminata* since had been first reported in the two systems, viz. 47–48 days in the Ahuriri, versus 11–12 days in the Aparima. In addition cover at most severely affected site in the Ahuriri River was almost 50%, whereas in the Aparima it was less than 10%.

Despite higher cover in the Ahuriri, mean cell densities in the water column at the affected sites were considerably lower than those in the Aparima. There is no obvious explanation for this. One possibility (conjecture only) is that in the Aparima, the colonies were in some biological state that caused them to release cells. Cover at the

two sites of maximum cover in the two rivers was quite different. In the Ahuriri, the cover comprised thick mats typical of well-established *D. geminata*. In the *Aparima*, cover comprised many very small, dark-coloured colonies that could be identified visually as *D. geminata* only with close scrutiny. In an earlier study in the Mararoa and lower Waiau Rivers, we found approximately 250 live cells/litre (with variation from 56 to 560 cells/litre) (Kilroy et al. 2005a). This is considerably higher than any densities found in the present study, but probably not surprising given the massive growths of *D. geminata* in the Mararoa/lower Waiau system.

A pattern common to both rivers was the tendency for samples from the water column to contain more dead (empty) cells than live cells (chloroplasts evident), especially at downstream sites. This was particularly noticed in the two following sampling occasions in the Aparima River, when *only* dead (empty) cells were found in samples from sites 1, 2 and 3. While some may have been due to contamination (see section 7.3), those found on the day 52 sampling (see Figure 8) could not have been, as all sampling equipment used was new.

We might expect more dead cells than live cells to be transported in the water column simply because if cells become unhealthy for some reason (physical damage, parasites) they tend to fall off. During microscope studies it has been noted that when dead (empty) cells are present within the mat, they are very often detached (personal observations). However, at some stage, live cells must also detach, as this is how the species moves downstream. When and how this happens, and whether any environmental cues are involved, are unknown. No studies have been located that track the spread of given diatom species downstream in a river system. This is not surprising as the presence of a new diatom species in a river system is a very unusual situation that would normally go unnoticed. It is only the fact that *D. geminata* is an invasive species that makes it of interest.

One issue that arises from the pattern of mostly dead cells occurring downstream is that these cells may not yield DNA that could be detected by a genomic method. With live cells, a genomic method is likely to provide a more sensitive and objective means of detecting *D. geminata* cells than the microscope method. A comparison of the results from both methods using the 125 samples supplied to the University of Waikato will confirm whether sufficient genomic material was present.

7.3. Cross-contamination

Unfortunately our initial surveys did not include any sites free of *D. geminata* cells in the benthic samples. Therefore we had no measure of the maximum distance

downstream from visible colonies over which *D. geminata* cells are detectable in the water column. However, the fact that cells were found only in the high intensity (10 minute) drift sample at site 4 in the Aparima River, but were found in lower intensity samples further upstream, suggests that site 4 may have been close to the limit. As a check on this, during the follow-up visit to retrieve the artificial substrates, we also undertook a complete drift sampling run at all sites, including three new sites farther downstream. As expected, cell densities in the water column had increased in the 18 days since the initial survey, but we were surprised to obtain positive results in most samples from all three new sites. The most downstream site was around 70 km from our most downstream affected site.

This raised the possibility that some of the positive samples were due to contamination, since we were using the same net that had been used in previous surveys. Flooding meant that it was not possible to run a check on this until 4 weeks later. On 25 May, sites 1, 2 and 3 were re-sampled (drift and 25-stone pooled benthic sample) using completely new net and associated equipment. Negative samples from site 1 suggested that the previous positive result may have been due to contamination. The shape of the 18-day plot in Figure 9 is consistent with this, and also suggests contamination in the 18-day samples from site 2. The fact that only dead cells were detected at these three sites on day 18 is also consistent with contamination (the net had been cleaned with bleach prior to use at each site). However, we detected only dead cells in the 52-day samples, therefore this was probably a part of the natural pattern of drift of *D. geminata* cells downriver also. It is possible that the very large flood in the Aparima River on 26 April may have flushed out *D. geminata* growth to such an extent that by the time of the 25 May survey, the drift of cells downstream had not been restored to the levels of a month earlier. During the search for the artificial substrates following the flood, no *D. geminata* colonies could be seen at site 8, and the stones at site 9 appeared to be clean.

Therefore the question of whether contamination of samples occurred during the 25 April collections was not entirely resolved. Subsequent improvements to the design showed in Figure 3 have minimised this possibility. For example, our “prototype” single-seamed net was sewn with the stitching on the outside to provide a smooth, easy-to-clean, inner surface. However, the closed seam (intended to provide strength) trapped fine material. Therefore the seam style was changed to open, allowing material to pass through freely. Further modifications will be documented in future surveillance reports.

8. Conclusions and recommendations

From our surveys in the Ahuriri and Aparima Rivers, we conclude that filtering samples from the water column offers a more sensitive and cost-effective method for surveillance of *D. geminata* in rivers than the current sampling protocol (samples from a collection of 25 stones pooled into a single container). Detailed benthic sampling proved to be as sensitive as the drift sampling, however the effort required to both collect and process the samples is very much greater. To obtain a 0.95 chance of detecting *D. geminata* at a site where it is present, but rare (0.1 cells per sampling unit), at least 30 samples must be collected and processed. The effort required using microscopic methods would be 5 hrs (i.e., 30 samples at a standard 10-minute scanning effort). Benthic sampling showed that *D. geminata* was rare at four of the sites surveyed. At three of these these sites cells were detected in at least three of the drift samples, with only the standard 10-minute scanning effort applied. At the most downstream site in the Aparima River, we found *D. geminata* only in the most intensive drift sample. Artificial substrates were less efficient at picking up *D. geminata* cells than drift or detailed benthic sampling.

We recommend that a 10-minute deployment of the plankton net used in this study becomes the standard methodology for future surveillance for *D. geminata* in rivers. For the next survey planned, both the 25-stone pooled sampling and the net methods should be used to compare their performances. Cross contamination with the nets is a slight issue. The net design has been modified to minimise this. In addition, sampling teams must be rigorous in decontaminating the nets with 2% bleach solution, or similar, between sites to prevent the transfer of live cells (as is currently done for all other equipment, including footwear). In addition, to prevent contamination of subsequent samples with dead cells from an earlier positive sample, the nets should be rinsed and brushed thoroughly prior to each new deployment using the river water from the site about to be sampled. Full instructions on the use of the nets will be provided.

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Appendix 1: Main survey field data collection forms

Didymo sampling methods study, …… River

Site: ……..… Date: …… Time: …….

Sampling team: ……

Weather conditions: ……...

General observations on river conditions: …….

GPS upstream E: …….. N: …….

GPS downstream E: …….. N: …….

Water conductivity …….. Water temperature ……

Suggested order of sampling

1. Regular surveillance visual inspection, didymo present YES / NO

if **no** didymo, collect a pooled sample from 25 rocks on 5 transects sample no. ……..

2. Visual assessment and benthic sampling (use the same rocks) *see sampling forms and diagrams*

unaffected sites	8 transects visual, 8 transects benthic samples
affected sites	2 - 4 transects visual, 2 transects benthic samples

3. Water samples, all sites.

Deploy nets in areas with velocity approx 0.5 m/s, within main sampling area, while sampling is underway (*see diagram*). Transfer samples to Elkays. Tick off when done.

10 L (pour through net), duplicate samples	☐
~100 L, 10 secs, duplicate samples	☐
~1000 L, 1 min, duplicate samples	☐
~10,000 L, 10 mins, single sample (duplicate if very little material)	☐

4. Deploy artificial substrates, all sites

At each site put out 6 concrete tiles 180 x 380, each carrying 2 each of:

- Plexiglass slides
- Glass slides
- 50 x 50 mm unglazed ceramic tiles
- Textured plastic substrate, 70 x 100 mm (12 bobbles)

i.e., we need 108 pieces of each, plus a few spares.

Ahuriri River: visual assessments and benthic sampling. Transects 1 to 4 (total of 8). **Site: …… Date: ……..**

Periphyton (on exposed surfaces)		**Transect 1**					**Transect 2**					**Transect 3**					**Transect 4**				
Stone no.		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Sample label																					
Water depth (m)																					
Water velocity at 0.6 depth (m/s)																					
Stone dimensions	x																				
	y																				
	z																				
Bare rock (not slippery, no periphyton)																					
Thin mat/film:	bright green																				
(under 0.5 mm thick)	light brown / yellow																				
	black / dark brown																				
Medium mat:	green																				
(0.5 – 3 mm thick)	light brown																				
	black / dark brown																				
Thick mat:	green / light brown																				
(over 3 mm thick)	black / dark brown																				
Filaments, short:	green																				
(under 2 cm long)	brown / reddish																				
Filaments, long:	green																				
(over 2 cm long)	brown / reddish																				
Visible didymo?																					

Appendix 2: Results of visual, benthic and water column sampling for *Didymosphenia geminata* on the Ahuriri and Aparima Rivers

Table A1: Visual surveys and benthic sampling for *D. geminata* on the Ahuriri and Aparima Rivers (5–8 April 2006). Means are calculated from total cells estimated at a site divided by the total estimated area sampled. L = live cells; D = dead cells. - = no data collected.

Ahuriri River			**Site:**	**1**	**2**	**3**	**4**	**5**	**6**
	Distance from top site (km):			**38**	**35**	**30.5**	**13**	**4**	**0**
Method	Intensity	**Units**	**Cell type**			***D. geminata***			
Visual		% cover		0	0	0.2	1.6	47	1.2
Benthic	pooled	cells/ sample	L	0	86	1980	-	-	-
			D	0	0	1440	-	-	-
Benthic	40 rocks	mean cells/m^2	L	22	2900	15,000	-	-	-
			D	56	1100	10,500	-	-	-
	25 rocks		L	83	0	15,500	-	-	-
			D	0	900	14,500	-	-	-
	10 rocks		L	83	0	15,500	870,000	32,000,000	250,000
			D	0	900	14,500	200,000	1,700,000	21,000
	40 rocks	%rocks with Dg	L	2.5	25	80	100	100	80
			D	2.5	30	82.5	100	100	60

Aparima River			**Site:**	**4**	**5**	**6**	**7**	**8**	**9**
	Distance from top site (km):			**35**	**31**	**25**	**18**	**8**	**0**
Method	Intensity	**Units**	**Cell type**			***D. geminata***			
Visual		% cover		0	0	0	0	0.8	10.3
Benthic	pooled	cells/ sample	L	0	0	0	10	-	-
			D	0	0	0	10	-	-
Benthic	40 rocks	mean cells/m^2	L	9	0	111	690	-	-
			D	0	26	130	380	-	-
	25 rocks		L	15	0	84	280	-	-
			D	0	34	11	100	-	-
	10 rocks		L	0	0	210	570	63,000	280,000
			D	0	98	30	175	68,000	350,000
	40 rocks	%rocks with Dg	L	2.5	0	7.5	20	50	100
			D	0	5	5	25	70	100

Table A2: Summary results of drift sampling on two occasions in the Ahuriri River. Densities are in cells per litre of filtered water. L = live cells; D = dead cells. - = no data (no sample analysed)

			Site	1	2	3	4	5	6
			Distance from top site (km):	38	35	30.5	13	4	0
Sampling date	**Intensity**	Deployment time, or vol.	**Cell type**	**Cells / litre of water**					
5-6 April	high	10 min	L	0.01	0.04	0.03	-	-	-
			D	0.17	0.24	0.37	-	-	-
	medium	1 min	L	0.06	0.07	0.12	13.22	-	0.04
			D	1.19	0.92	1.68	28.02	-	0.43
	low	10 sec	L	0.09	0.17	0.20	12.90	22.10	0.31
			D	1.85	1.21	3.77	34.83	19.30	0.11
	vlow	10 L (net)	L	0.67	0.00	0.00	1.65	3.50	-
			D	3.33	20.00	1.46	6.85	9.00	-
	xlow	10 L (filter)	L	-	-	0.00	0.80	0.90	0.00
			D	-	-	0.60	3.40	2.20	0.00
18 April	high	10 min	L	-	-	-	-	-	-
			D	-	-	-	-	-	-
	medium	1 min	L	0.212	-	-	-	-	-
			D	0.689	-	-	-	-	-
	low	10 sec	L	0.940	-	-	-	-	-
			D	9.7	-	-	-	-	-
	vlow	10 L (net)	L	30	3	5.5	30	31.5	-
			D	129	16	50	41	40	-

Table A3: Summary results of drift sampling on three occasions in the Aparima River. Densities are in cells per litre of filtered water. L = live cells; D = dead cells. - = no data (no sample analysed)

Aparima River			Site:	1	2	3	4	5	6	7	8	9
		Distance from top site (km):		78	58	42	35	31	25	18	8	0
Sampling date	**Intensity**	**Deployment time, or vol.**	**Cell type**	**Cells / litre of water**								
7-8 April	high	10 min	L	-	-	-	0.001	0.005	0.01	0.043	0.81	-
			D	-	-	-	0.001	0.008	0.01	0.089	1.06	-
	medium	1 min	L	-	-	-	0	0	0.03	0.107	0.84	26
			D	-	-	-	0	0.004	0.10	0.305	2.00	29
	low	10 sec	L	-	-	-	0	0	0.11	0.482	1.82	23
			D	-	-	-	0	0	0	0.593	3.37	98
	vlow	10 L	L	-	-	-	0	0	0	0	0.75	62
			D	-	-	-	0	0.015	0.200	1.960	1.45	68
	xlow	10 L (filter)	L	-	-	-	0	0	0	0	0.15	4
			D	-	-	-	0	0	0	0	0.350	9.000
25 April	high	10 min	L	0	0	0	0.002	0.004	0.03	0.24	-	-
			D	0.003	0.003	0.006	0.004	0.012	0.08	0.24	-	-
	medium	1 min	L	0	0	0	0.006	0.009	0.05	0.29	-	-
			D	0.06	0.005	0.005	0.013	0.037	0.10	0.42	-	-
	low	10 sec	L	0	0	0	0	0	0.03	0.76	20.1	35
			D	0.04	0.04	0.042	0.035	0.022	0.25	0.73	30.2	73
	vlow	10 L (net)	L	0	0	0	0	0	0	0.20	2.30	86
			D	0	0	0	0.1	0.25	0.01	0.20	7.40	200
25 May	high	10 min	L	0	0	0	-	-	-	-	-	-
			D	0	0	0.006	-	-	-	-	-	-
	medium	1 min	L	0	0	0	-	-	-	-	-	-
			D	0	0.016	0.009	-	-	-	-	-	-
	low	10 sec	L	0	0	0	-	-	-	-	-	-
			D	0	0	0.134	-	-	-	-	-	-
	vlow	10 L (net)	L	0	0	0	-	-	-	-	-	-
			D	0	0.162	0.145	-	-	-	-	-	-