qPCR detection versus microscopy observations for assessing presence–absence of *Didymosphenia geminata* in Quebec rivers (Canada)

Sandra Kim Tiam, Vincent Laderriere, Carole-Anne Gillis, Claude Fortin and Isabelle Lavoie

ABSTRACT

Microscopic versus qPCR (quantitative polymerase chain reaction) analyses were compared for the detection of *Didymosphenia geminata* in biofilms and water filtrates from seven Gaspésie rivers (Canada). For the qPCR approach, two DNA extraction kits (QIAamp DNA Micro Kit, Qiagen and PowerSoil DNA Isolation Kit, Mo Bio Laboratories) and two pairs of primers were considered. The pair of primers D602F/D753Rext did not amplify *D. geminata* DNA whereas the pair of primers D602F/D753R was specific for *D. geminata*. Presence-absence diagnosis based on qPCR and microscopic analyses were consistent: *D. geminata* was detected in six of the seven rivers, both in the biofilm and filtrate samples. However, technical replications were needed at certain sites to observe the presence of *D. geminata* cells by microscopy. This underscores the necessity of replicate analyses, which is cost-effective to achieve when using qPCR due to the capacity to process tens of samples in a single PCR run in the context of a large scale assessment.

Key words | biofilms, diatoms, *Didymosphenia geminata*, microscopy, qPCR

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INTRODUCTION

Didymosphenia geminata is a stalk-forming freshwater diatom ranging in size from 65 to 161 μm (Diatoms of the United States; available at https://westerndiatoms.colorado.edu). This alga is native to North America and northern Europe (Patrick & Reimer 1975). In recent years, *D. geminata* has become more prevalent in rivers and streams across large biogeographical scales (Spaulding & Elwell 2007). Moreover, *D. geminata* has been reported as an exotic species in the southern hemisphere (Kilroy & Unwin 2011; Sastre *et al.* 2013). In Quebec, paleolimnological work was carried out to assess the historical presence and abundance of *D. geminata* cells in the Matapedia River catchment. Results showed that cells, albeit rare, were present at least since the early 1970s but that there was a recent 200-fold increase in prevalence of cells within the last decade (Lavery *et al.* 2014). Under low nutrient and stable hydrological conditions, *D. geminata* can produce large benthic mats (Cullis *et al.* 2012). Thick mats have the potential to alter the aquatic food base and consequently have disruptive impacts to higher trophic levels (Larned *et al.* 2007; Gillis & Chalifour 2010; Anderson *et al.* 2014; James & Chipps 2016). It was not until 2006 that *D. geminata* mats were first reported in eastern Canada (Gillis & Chalifour 2010). Thick mats covering 100% of the riverbed spanning over 35 km of river were registered (Gillis & Chalifour 2010). Between 2007 and 2010, *D. geminata* mats were reported in multiple rivers across the Gaspésie Peninsula region (Gillis & Chalifour 2010) and concurrently

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observed throughout rivers in the northeastern United States (Khan-Bureau *et al.* 2014) raising concerns for managers and angler groups. The presence of *D. geminata* cells in a river does not always result in visual observations of dense proliferations on the river bed. In fact, surveys conducted in New Zealand by Kilroy & Unwin (2011) highlighted that cells may be microscopically identified in benthic samples at sites showing no evidence of *D. geminata*. Research efforts to understand potential drivers of *D. geminata* prevalence and severity in Gaspésie rivers are currently underway (Gillis in prep. [[to be updated]]). Robust and detailed surveys assessing presence–absence of cells in water and biofilm as well as the presence of mats are needed to test hypotheses to further identify water chemistry parameters that may control this species biogeography and severity. Limited regional scale monitoring efforts to detect *D. geminata* in rivers across the Gaspésie Peninsula were made by the Quebec government from 2006 to 2009 (MDDEP 2009). Detection surveys have not been made since and therefore currently limit our ability to understand factors limiting *D. geminata* presence and mat severity. A more precise and recent species distribution survey is therefore needed.

The aim of this study was to evaluate the efficiency of a molecular-based technique (real-time quantitative polymerase chain reaction: qPCR) for detection of *D. geminata* cells in Quebec rivers in comparison to traditional microscopy-based assessments.

METHODS

Study sites

This study was conducted in seven rivers of the northeastern Gaspésie Peninsula in Quebec, Canada (Figure 1): Cap-Chat, Madeleine, Grande-Vallée, Sainte-Anne, York, Dartmouth and Saint-Jean. Mean values of water chemistry parameters, sampled between 2012 and 2014, for five of the sampled rivers were obtained via the Quebec

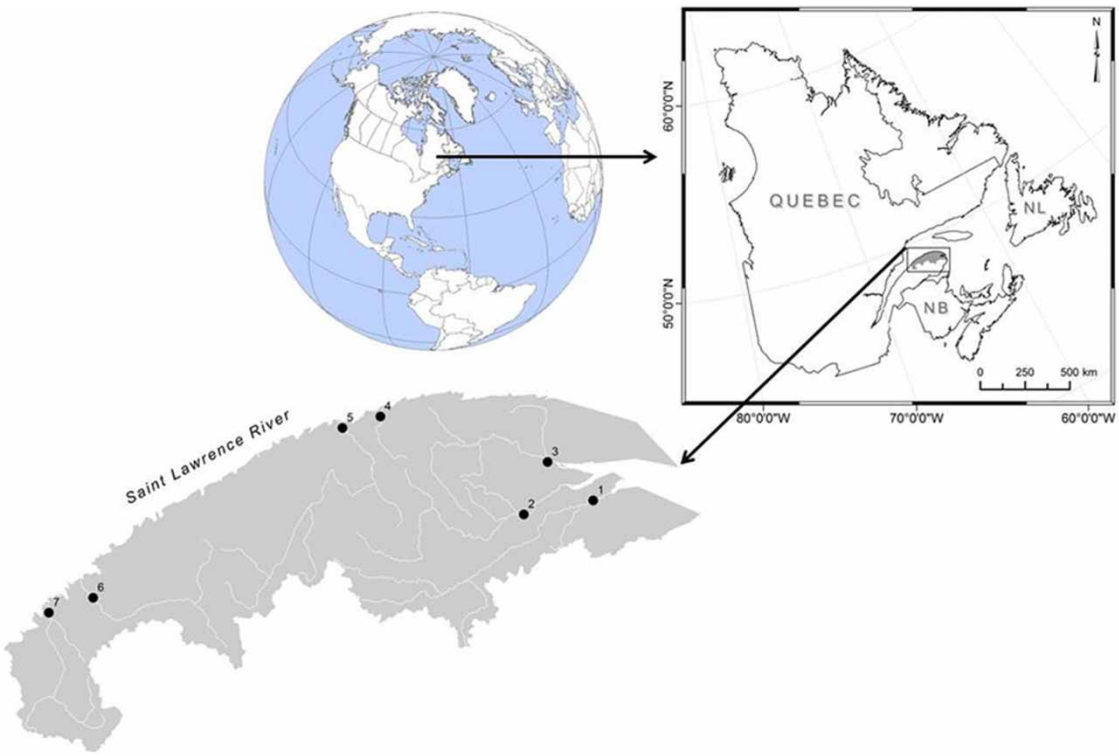

Figure 1 | Study area showing the seven sampled rivers in the Gaspésie region, Québec, Canada. 1: Saint-Jean, 2: York, 3: Dartmouth, 4: Grande-Vallée, 5: Madeleine, 6: Sainte-Anne, 7: Cap-Chat.

government water quality monitoring program (MDDELCC 2016) (Table 1). Benthic and drift samples were collected at seven sites between July 13th and 16th 2015 following the methods described later in the section entitled 'Biofilm and water column sampling'.

Biofilm and water column sampling

As suggested in Kilroy (2004), drift-net samples from the water column may provide an additional integrated assessment of the presence of cells in upstream portions of the watershed. For this reason, biofilm and water filtrate samples were collected to evaluate the ability of qPCR to detect *D. geminata* cells in Quebec rivers in comparison to traditional microscopy-based assessments within these two matrices. Biofilms sampled at each site were obtained by scraping eight rocks of similar size (composite sample; total surface scraped reaching about 64 cm^2 at each site) selected in fast and slow-flowing environments within the reach. The biofilm was collected using a toothbrush and by carefully rinsing each rock with 70% ethanol. Drifting *D. geminata* cells in the water column were sampled using a plankton net (Wisconsin net; mesh size = 40 μm) submerged for 10 minutes at a depth of about 50 cm from the bed of the river. Water velocity was estimated using a float method, based on the average time for a floating object to travel a known distance. The volume of water filtered during sampling was estimated by multiplying the water velocity estimate by the surface area of the net opening. The net was then rinsed multiple times with 70% ethanol to remove all collected drifting particles. All material potentially in contact with *D. geminata* cells was soaked in 5% hydrochloric solution between each site to kill live cells and to denature DNA as suggested in Cary *et al.* (2007).

Didymosphenia geminata detection by microscopy

Samples preserved in 70% ethanol were vigorously shaken and 1 mL aliquots were transferred into 2 mL tubes. An aliquot of 100 μL of each sub-sample was placed on a microscope slide (after homogenization using the pipette to avoid clumps being collected) and dried on a hot plate at low heat. Two replicate slides were mounted per

Table 1 | Sampling location, catchment description, water chemistry (±SD) and history of *D. geminata* presence

Site	River	Catchment size (km^2)	River length[a] (km)	Year of first cell detection[b]	Visual presence of didymo mats during field survey	Sampling site distance from the mouth of the river	NH_3 (mg/l)	DOC (mg/l)	Conductivity (μS/cm)	pH	Total P (mg/l)
1	Saint-Jean	1,109	118.2	2009	No	7.73	0.01 ± 0.00	1.0 ± 0.5	233 ± 27	8.1	0.002 ± 0.002
2	York	1,028	110.2	2008	Yes	21.58	0.01 ± 0.01	2.1 ± 1.2	218 ± 39	8.0	0.003 ± 0.002
3	Dartmouth	962	85.5	Undetected	Yes	6.23	n.a.	n.a.	n.a.	n.a.	n.a.
4	Grande-Vallée	172	25.1	Not surveyed	No	3.67	n.a.	n.a.	n.a.	n.a.	n.a.
5	Madeleine	1,232	126.3	2008	No	4.40	0.01 ± 0.00	2.0 ± 1.8	254 ± 75	8.1	0.003 ± 0.004
6	Sainte-Anne	822	67.1	2006	Yes	7.91	0.01 ± 0.01	1.9 ± 1.0	150 ± 34	8.0	0.003 ± 0.004
7	Cap-Chat	739	62.8	2008	No	3.55	0.01 ± 0.00	2.6 ± 1.4	156 ± 40	8.0	0.005 ± 0.007

n.a., not available.
aSource for watershed area and river length data: *Conseil de l'eau du Nord de la Gaspésie*.
bSource: MDDEP (2009) results from filtered river water analysed in microscopy from surveys carried out between 2006 and 2009.

sample. *D. geminata* cells were counted under a *Reichert-Jung Polyvar light microscope under* 100× magnification. The observation and identification of *D. geminata* was relatively simple as the cells were easily identifiable from other particles due to their large size and distinctive shape (Figure 2).

Didymosphenia geminata detection by qPCR

Selection of primers

Two pairs of primers (D602F/D753Rext and D602F/D753R) developed specifically for detection of the 18S ribosomal DNA sequence of *D. geminata* obtained from Cary *et al.* (2014) and Cary *et al.* (2006) respectively (Table 2), were tested.

D. geminata DNA extraction methods

Two commercial DNA extraction kits were considered for this study: QIAamp DNA Micro Kit (Qiagen) and PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc). The QIAamp DNA Micro Kit is suitable for samples with a small amount of genetic material and was chosen because cells of *D. geminata* were likely to represent a small fraction of the total biomass, especially in biofilms where mucilaginous stalks are abundant. The PowerSoil DNA Isolation Kit is especially designed to minimize inhibitors effects in soil such as humic acids, also present in aquatic environments. Samples preserved in 70% ethanol were vigorously shaken and 1 mL aliquots were transferred into 2 mL tubes and centrifuged for 4 min at 8,000 g at room temperature. The pellet was re-suspended in 1 mL milliQ water for DNA extraction.

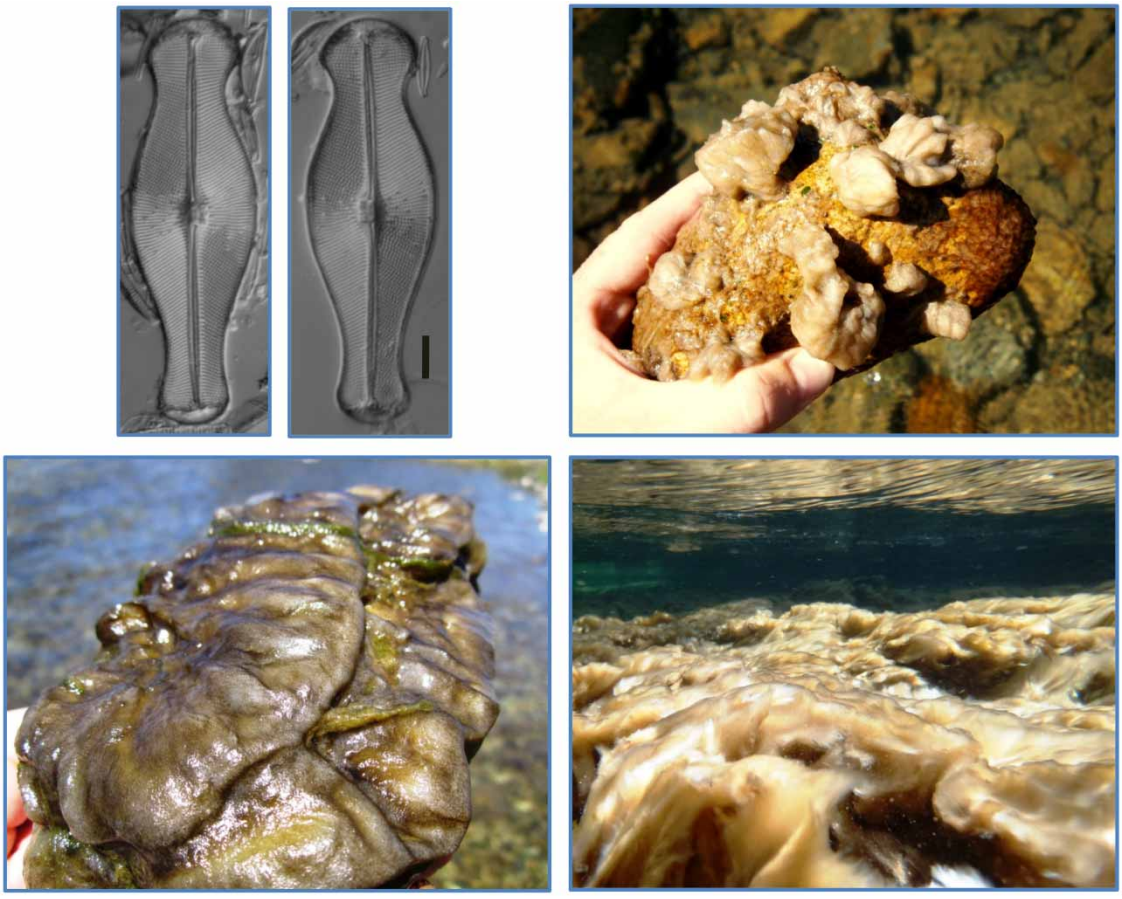

Figure 2 | *Didymosphenia geminate* cells (scale bar = 10 microns) and mat development stages in Gaspésie rivers.

Table 2 | Sequence of the primers tested in the study for the detection of *D. geminata* and lambda DNA as a positive control

Gene name	Organism	Associated primers	Primer (5′-3′)	Peak of fusion (°C)
18s	*Didymosphenia geminata*	D602F	GTT GGA TTT GTG ATG GAA TTT GAA[a]	80.5
18s	*Didymosphenia geminata*	D753Rext	GAC TTA CGT CGA TGA ATG TAT TAG CA[b]	
18s	*Didymosphenia geminata*	D753R	AAT ACA TTC ATC GAC GTA AGT C[b]	
	Bacteriophage lambda isolated from the heat-inducible lysogen *E. coli* (cI857 S7)	CALF	GCC AGG TCA TCT GAA ACA G[a]	90
	Bacteriophage lambda isolated from the heat-inducible lysogen *E. coli* (cI857 S7)	CALR	TCT GCG ATG CTG ATA CCG[b]	

Abbreviations: *18S* – ribosomal DNA 18S.
[a]Forward primer.
[b]Reverse primer.

QIAamp DNA micro kit

A 100 μL aliquot was transferred into clean tubes containing 100 μL of glass beads (<106 μm-diameter, Sigma), as well as 180 μL of ATL buffer and 1 μL of Reagent DX (Qiagen). Tubes were vortexed for 20 min and cell disruption was ensured by the use of a mechanical cell disruption device for 2 min (Disruptor Genie, Scientific Industries). Then, 20 μL of proteinase K (Qiagen) and 1 μL of lambda DNA [100 pg/μL] (New England BioLabs) were added to the tubes and mixed by vortexing for 15 s. Lambda DNA was used as an internal control. Tubes were incubated at 56 °C overnight and mixed by vortexing for 10 s each 10 min for the first hour of incubation. The end of the extraction was performed according to the manufacturer's instructions in the section 'Isolation of genomic DNA from tissue samples'. The DNA was stored at −20 °C until use for qPCR.

Powersoil DNA isolation kit

A 100 μL aliquot was transferred into PowerBead tubes (Mo Bio) containing 60 μL of solution C1 (Mo Bio), 20 μL of proteinase K (Qiagen), 1 μL of lambda DNA [100 pg/μL] (New England BioLabs) and glass beads (<106 μm-diameter, Qiagen). Samples were homogenized by vortexing for 20 min and cells were mechanically broken as described above. Tubes were incubated at 56 °C overnight and mixed by vortexing for 10 s each 10 min for the first hour of incubation. The end of the extraction was completed according to the manufacturer's instructions in the section 'Isolation of genomic DNA from tissue samples'. The DNA was stored at −20 °C until use for qPCR.

Real-time quantitative PCR

Real-time quantitative PCR reactions were performed in a C1000™ Thermal Cycler (BIO-RAD) using a CFX96 Touch™ Real-Time quantitative PCR Detection System (BIO-RAD) with one cycle at 95 °C for 2 min and 50 amplification cycles at 95 °C for 10 s, 55 °C for 15 s and 68 °C for 15 s. Reactions were conducted in multiplate low-profile 96-well unskirted PCR Plates (BIO-RAD). Each well contained 10 μL of SsoAdvanced universal SYBR Green Supermix (2X, BIO-RAD), 2 μL of DNA extract, *D. geminata* 18S ribosomal DNA primer pair at a final concentration of 300 nM for each primer for a final volume of 20 μL. For negative controls, DNA extract was replaced by diethylpyrocarbonate-treated water (DEPC-treated water). For positive controls, the wells contained 10 μL of SsoAdvanced universal SYBR Green Supermix (2X, BIO-RAD), 2 μL of lambda DNA [100 pg/μL] (New England BioLabs), the primers CALF and CALR at a final concentration of 500 nM for each primer and DEPC-treated water for a final volume of 20 μL. Each sample was analysed with technical duplicates. Specificity was determined for each reaction from the dissociation curve and the sequencing of the PCR product. This dissociation curve was obtained with the SYBR Green fluorescence level during gradual heating of the PCR products from 60 to 99 °C. These PCR products were sent for sequencing, and the sequences obtained were treated with a blast analysis.

DNA recovery calculation

For DNA recovery calculation, the samples were spiked with 100 femtograms of Lambda DNA as an internal standard at the beginning of the extraction procedure. For the PCR conditions described above, the addition of the internal standard gave a threshold cycle (Ct) of 21.5 (Tanguay personal communication) for a recovery of 100% and corresponds to 2,000 copies of the target gene. The recovery of each sample was calculated according to the following formula:

$$\text{Recovery} = \frac{2,000/2^{(\text{Ct,IS}-21.5)}}{2,000}$$

where Ct, IS is the Ct of the internal standard Lambda DNA.

A recovery of 1 corresponds to the maximal recovery and indicates that all Lambda DNA is recovered in the DNA extract and that PCR inhibitors are absent in the PCR reaction.

RESULTS AND DISCUSSION

Primers selection

Amplifications with high Ct values were recorded (Ct values >36) using the primer pair D602F/D753Rext (Cary *et al.* 2014), suggesting a non-specific amplification of *D. geminata*. Melting curves also presented multiple peaks in each sample. These results led us to the conclusion that the primer pair D602F/D753Rext do not amplify the 18S ribosomal DNA sequence of *D. geminata*. Amplifications with Ct values between 21.5 and 36.5 were recorded using the primer pair D602F/D753R (Cary *et al.* 2006), and all samples presented the same fusion temperature of 80.5 °C, characteristic of the 18S ribosomal DNA sequence of *D. geminata*. Final qPCR products were sent for sequencing to the *Plateforme de séquençage et de génotypage des génomes, Centre Hospitalier de l'Université Laval*. The obtained sequences were blasted on the NCBI database (available at http://blast.ncbi.nlm.nih.gov/), and the correspondence with the 18S ribosomal DNA sequence of *D. geminata* was confirmed for all samples. We concluded that the primer pair D602F/D753Rext published in Cary *et al.* (2014) was not specific to *D. geminata*. The results presented in the following sections were obtained with the pair of primers D602F/D753R (Cary *et al.* 2006).

DNA recovery efficiencies

Mean recovery efficiencies calculated with the lambda DNA as an internal standard are shown in Table 3. The results revealed low recovery using the Qiagen kit (11.5 ± 3.4%). For certain samples this kit even failed at recovering any lamdba DNA (Table 4). The Mo Bio extraction kit yielded a higher recovery efficiency (17.5 ± 3.1%) and null values were not encountered. The low performance of the Qiagen kit was in part due to the recovery values of zero in the water filtrate matrix for Saint-Jean, Cap-Chat, Grande-Vallée, and Sainte-Anne rivers (Table 4).

Recovery efficiencies are affected by two main causes. First, low recovery may result from low DNA yield which is particularly encountered in the case of complex matrices such as environmental samples. Low DNA concentrations have also been reported by Uyua *et al.* (2014) for biofilm extractions in a study evaluating several protocols, and DNA concentrations equal to zero were even obtained for one of the tested approaches. Decreasing recovery efficiencies with increasing matrix complexity was reported by Yáñez *et al.* (2005). The authors spiked different water matrices with *Legionella pneumophila* and obtained recovery efficiencies of 59.9% for distilled water, 42.0% for

Table 3 | Recovery calculated using lambda DNA as an internal standard for all samples, samples extracted with MoBio or Qiagen kit for biofilm or water filtrates, (mean ± SE, n = the number of samples)

Samples	Mean recovery (%)
All samples ($n = 28$)	14.5 ± 2.3
Biofilm ($n = 14$)	16.4 ± 3.3
Water ($n = 14$)	12.6 ± 3.3
MoBio ($n = 14$)	17.5 ± 3.1
MoBio, biofilm ($n = 7$)	21.3 ± 5.0
MoBio, water ($n = 7$)	13.7 ± 3.4
Qiagen ($n = 14$)	11.5 ± 3.4
Qiagen, biofilm ($n = 7$)	11.6 ± 3.9
Qiagen, water ($n = 7$)	11.4 ± 5.6

Table 4 | Recovery calculated using lambda DNA as an internal standard for samples from the seven Gaspésie rivers in the biofilm and water filtrates using MoBio and Qiagen

River	Matrix	Recovery (%) Qiagen	Recovery (%) MoBio
Saint-Jean	Biofilm	8.8	28.1
	Water	**0.0**	33.0
York	Biofilm	6.7	46.3
	Water	17.7	9.2
Cap-Chat	Biofilm	27.4	10.7
	Water	**0.0**	10.3
Dartmouth	Biofilm	20.7	17.8
	Water	31.2	16.4
Madeleine	Biofilm	0.7	15.9
	Water	31.0	6.3
Sainte-Anne	Biofilm	**0.0**	14.8
	Water	**0.0**	11.2
Grande-Vallée	Biofilm	16.7	15.2
	Water	**0.0**	9.7

potable water and 36.0% for cooling tower water. It seems reasonable to assume that recovery efficiency will be even lower in environmental samples, as observed in this study. Bletz *et al.* (2015) also pointed out that differential efficiency among DNA extraction methods influenced the detection of an amphibian pathogen (*Batrachochytrium dendrobatidis*). This result underscores the importance of taking into account recovery efficiencies, particularly to avoid false negatives. Secondly, recovery can be affected by the presence of PCR inhibitors, which represent an important issue because their occurrence may lead to false negatives or quantitative errors of several orders of magnitude (Bar *et al.* 2012; Jones *et al.* 2015). Inhibitors naturally found in the environment, such as humic acids, polysaccharides or metals, are designated as intrinsic inhibitors. Substances such as phenols, alcohols or salts introduced during sample preparation are known as extrinsic inhibitors. Our results suggest that the extraction procedure led to the recovery of low percentages of the environmental DNA or that the extraction protocol did not successfully remove PCR inhibitors (or PCR inhibitors were introduced during extraction procedures).

DNA recovery efficiencies were higher for samples from the biofilm matrix compared to the water filtrate matrix (21.3 ± 5.0 and 13.7 ± 3.7% mean respectively). This result was surprising since lower recovery rates were expected for biofilms than for water filtrates; benthic samples have been described as recalcitrant DNA sources in terms of both DNA yield and the potential persistence of PCR inhibitors (Uyua *et al.* 2014). DNA extraction was anticipated to be less effective for biofilms due to their dense tri-dimensional structure composed of a variety of organisms embedded in an exo-polysaccharide matrix compared with free-living organisms (or colonies) from the water column. This thick matrix can be strongly cohesive and particle size has been shown to influence the extraction where lower particle size offers a higher surface for the chemical to react during DNA extraction (Demeke & Jenkins 2010). High amounts of PCR inhibitors were also expected to be present in the biofilms samples due to the exo-polysaccharides component, particularly in the biofilms containing *D. geminata* and long polysaccharides stalks (Whitton *et al.* 2009; Bothwell *et al.* 2012). As mentioned earlier, polysaccharides may be potent enzymatic inhibitors (Pandey *et al.* 1996; Monteiro *et al.* 1997), and may adsorb significant amounts of metals (Kaplan *et al.* 1987), which are also known as PCR inhibitors affecting the enzymatic activity or damaging DNA.

D. geminata in Gaspésie rivers

Detection of *D. geminata* cells

The presence of *D. geminata* was detected by qPCR in six of the seven rivers monitored in July 2015 (Table 5), both in the biofilm and water filtrate samples (cells were not detected in the Grande-Vallée River). The results from the microscopy analyses were consistent with the molecular-based approach, both for biofilm and water filtrate samples; *D. geminata* cells were observed at all sites except in the Grande-Vallée River.

D. geminata concentrations in biofilm and water

In the biofilms samples, the highest cell densities were recorded in the York, Madeleine and Sainte-Anne rivers (1,200, 750 and 630 cells/cm^2, respectively) whereas the lowest cell densities were observed in Cap-Chat and Dartmouth rivers (20 and 30 cells/cm^2, respectively) (Figure 3).

Table 5 | *Didymosphenia geminata* detection results obtained by qPCR and microscopy analyses for biofilm and water filtrate samples for the seven rivers sampled in July 2015

		Biofilm		Water	
Site	**River**	**qPCR**	**Microscopy**	**qPCR**	**Microscopy**
1	Saint-Jean	Detected	Detected	Detected	Detected
2	York	Detected	Detected	Detected	Detected
3	Dartmouth	Detected	Detected	Detected	Detected
4	Grande-Vallée	Non detected	Non detected	Non detected	Non detected
5	Madeleine	Detected	Detected	Detected	Detected
6	Sainte-Anne	Detected	Detected	Detected	Detected
7	Cap-Chat	Detected	Detected	Detected	Detected

These results provide an interesting overview of the abundance of *D. geminata* cells in the biofilm of the seven rivers sampled. Moreover, *D. geminata* mats were observed in the York, Sainte-Anne and Dartmouth rivers. In the Madeleine River, high cell densities were recorded in the biofilm but mats were not present; on the contrary in Dartmouth River where cell densities in biofilms were low, mats were observed. This emphasises the fact that the presence of *D. geminata* cells in a river does not always result in visual observations of dense proliferations on the river bed (Kilroy & Unwin 2011). Further, it also reiterates the importance of distinguishing mat growth from cell division, where optimal nutrient conditions for cell division differ from optimal nutrient conditions for mat growth (Kilroy & Bothwell 2011; Cullis *et al.* 2012).

In water filtrates, the highest cell densities were recorded in the Cap-Chat and Sainte-Anne rivers (28,430 and 17,430 cells/m^3, respectively) and the lowest cell densities were observed in the Dartmouth and Saint-Jean rivers (100 and 120 cells/m^3, respectively). High densities of *D. geminata* cells in the water filtrates, as observed in the Cap-Chat and Sainte-Anne rivers, provide an integrated assessment of the presence of cells in upstream portions of the watershed, which could possibly indicate extensive mats or widespread growth upstream because mat coverage can be positively correlated with cell density (Ellwood & Whitton 2007). The high propagule pressure from the water column in Cap-Chat could result in mat formation further downstream if local conditions are suitable.

An interesting result to note is the fact that the presence of *D. geminata* could have been overlooked in the biofilm (Cap-Chat and Dartmouth rivers) and in the water filtrates (Dartmouth and St-Jean rivers) if only one technical replicate had been processed under the microscope (presence observed in only one of the two replicates) (Table 6). This emphasises the necessity of replicate analyses, which are cost-effective to

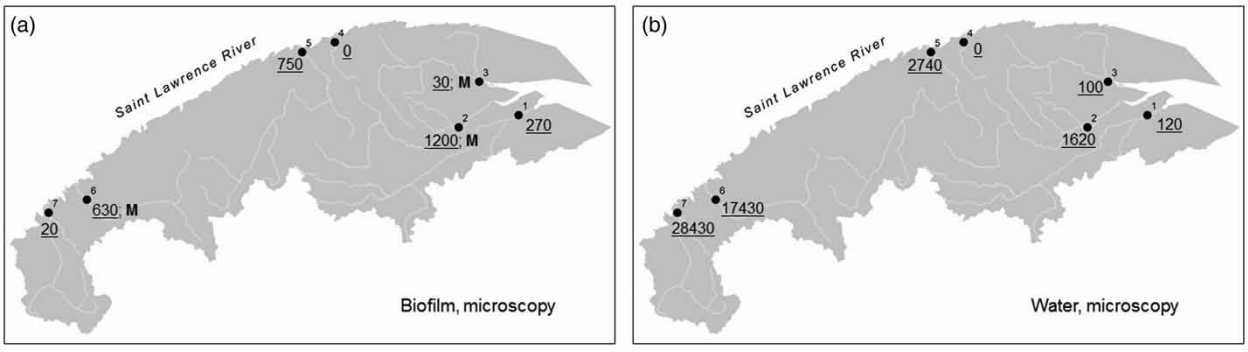

Figure 3 | *Didymosphenia geminata* cell density estimated by microscopy analyses on biofilms (a) and water filtrates (b) for the seven Gaspésie rivers sampled in July 2015. The underlined numbers denote the mean ($n = 2$) *D. geminata* density in biofilm sample in cells/cm^2 and in water samples in cells/m^3. **M**: presence of mats.

Table 6 | *Didymosphenia geminata* cells counted by microscopy in a 100 µL aliquot of biofilm or water for the seven Gaspésie rivers sampled in July 2015

		Number of *D. geminata* cells counted in a 100 µL biofilm aliquot		Number of *D. geminata* cells counted in a 100 µL water aliquot	
Site	River	Technical replicate #1	Technical replicate #2	Technical replicate #1	Technical replicate #2
1	Saint-Jean	8	9	0	3
2	York	30	47	10	25
3	Dartmouth	0	2	0	2
4	Grande-Vallée	0	0	0	0
5	Madeleine	23	25	18	28
6	Sainte-Anne	14	26	200	281
7	Cap-Chat	0	1	330	591

Samples were counted in duplicates. At each site, biofilms sample is a composite sample (total surface scraped reaching about 64 cm^2); biofilm was then transferred into a tube filled up to 50 mL with water from the site. Drifting *D. geminata* cells in the water column were sampled using a plankton net (Wisconsin net; mesh size = 40 µm) submerged for 10 minutes at a depth of about 50 cm from the bed of the river, cells were then transferred into a tube and filled up to 50 mL with water from the site.

achieve using qPCR in the context of a large scale assessments compared to microscopy-based assessments.

CONCLUSIONS

To enhance our knowledge of *D. geminata* distribution across broad geographical scales, it is necessary to gain a better understanding of regional drivers of cell presence and mat severity (of mat growth and extracellular stalk growth). While *D. geminata* mats are typically assessed visually, the absence of dense mat formation is not an indicator of this species absence. Cells can be present in the water column and in the biofilm without producing thick benthic mats. In the absence of these macroscopic accruals, presence–absence diagnosis is traditionally performed by microscopy-based assessments. At present, information is lacking for predicting occurrence, severity and persistence of nuisance mats towards facilitating management and mitigation measures (Cullis *et al.* 2012). By yielding precise distribution maps of *D. geminata* occurrence in rivers we can more effectively test hypotheses for identifying threshold values for presence–absence and mat development. Hence, future management of water resources could be strategically focused on vulnerable and suitable habitats where cells have not yet been found.

Two DNA extraction kits (QIAamp DNA Micro Kit; Qiagen and PowerSoil DNA Isolation Kit; Mo Bio Laboratories) and two pairs of primers were considered in this study. The pair of primers D602F/D753Rext (Cary *et al.* 2014) did not amplify *D. geminata* DNA whereas the D602F/D753R (Cary *et al.* 2006) was specific to *D. geminata*. The Mo Bio kit yielded better recovery efficiencies than the Qiagen kit. Additionally, recovery efficiency was null for certain samples extracted with the Qiagen kit. qPCR revealed the presence of *D. geminata* cells in six of the seven studied rivers sampled in July 2015. These results are consistent with the data obtained by microscopy analyses and thus confirm the ability of the qPCR based assay for the presence–absence diagnosis of *D. geminata* in environmental samples. The small volume of sample used for qPCR or microscopy analyses decreased the probability of detecting *D. geminata* cells. The main benefit of a molecular detection of *D. geminata* is the rapidity, sensitivity and low cost of the approach, allowing for the possibility of analysing multiple replicate samples for a site and therefore increasing the confidence in negative results.

Upgrading the method to be able to provide a quantitative estimate of the *D. geminata* cell density is an interesting avenue to consider. Before this can be accomplished, higher recovery efficiencies would have to be obtained. Evaluating and untangling the contribution of both extraction efficiency and PCR inhibition on DNA recovery is necessary. For example, the influence of PCR inhibition on recovery could be estimated by adding an internal standard after the extraction procedure step instead of just before. Also, analyses based on detecting amplification curve anomalies by comparing kinetics parameters of test and reference reactions (kinetics outlier detection) could be used to help distinguishing PCR inhibition from DNA recovery problems, as suggested in Jones *et al.* (2015). It could also be recommended to conduct more than one extraction per sample (technical replicate at the extraction step) in order to avoid false negatives due to extraction problems or sub-sampling effects. qPCR estimates of cell density would provide valuable information to more precisely characterize the degree of colonisation of the species in terms of propagule pressure.

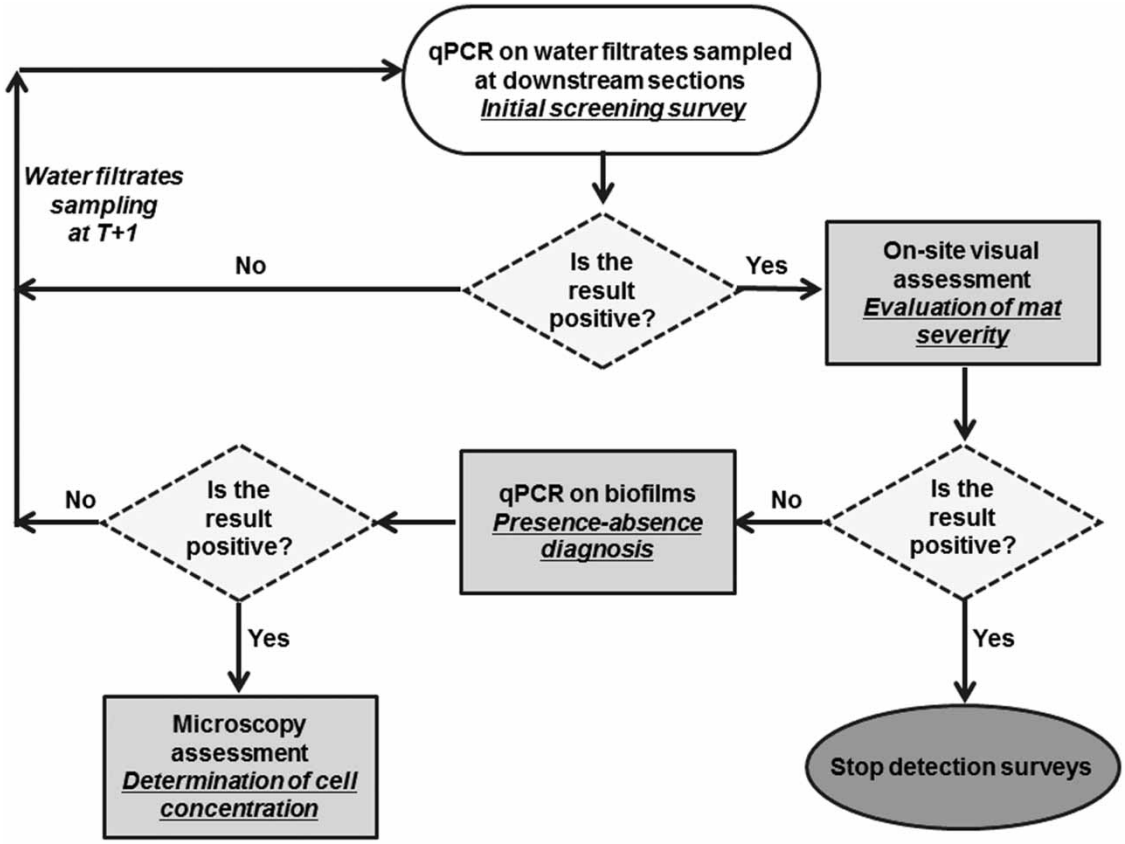

Figure 4 | Flow chart proposed for the survey of *D. geminata*. Water filtrates for the initial screening survey could be sampled at determined frequencies and during a specific period for example every month during ice-off.

While the use of a calibrator sample (where *D. geminata* cell density in the calibrator sample is estimated under the microscope) has been suggested by Cary *et al.* (2014) (relative quantification), calibration curves may also be developed based on samples containing different densities of *D. geminata* cells (absolute quantification) manually isolated as described by Khan-Bureau *et al.* (2016).

To conclude based on our findings, we propose the flow chart presented in Figure 4 for the survey of *D. geminata*. We recommend the use of the qPCR approach on water filtrate samples in downstream sections of rivers for initial screening surveys to detect the presence of *D. geminata* at the watershed scale and pinpoint areas of watersheds that might have algal accrual. Upon positive diagnosis, we recommend an on-site visual assessment (i.e. Standing Crop Index (SCI)) as described in Kilroy & Bothwell (2011) to assess the severity of *D. geminata* within a given reach. Upon negative diagnosis regarding the visual assessment, additional molecular based investigations of *D. geminata* cells in biofilms can be conducted, if these results are positive, then further microscopic assessments can follow in order to determine the cell concentration.

These combined data would yield an accurate map of *D. geminate* distribution and mat severity, and would help identify reach scale and broad scale controlling factors for cellular presence and mat accrual. Overall, *D. geminata* presence-absence assessment by qPCR has a great potential to assist in sound management and conservation strategies of vulnerable rivers and may help maintain the ecological goods and services they provide.

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