Biodivers Conserv (2015) 24:1541–1548
DOI 10.1007/s10531-014-0849-5

CrossMark

COMMENTARY

The peril of PCR inhibitors in environmental samples: the case of *Didymosphenia geminata*

Leandro R. Jones · Noelia M. Uyua · Julieta M. Manrique

Received: 4 September 2014 / Revised: 4 November 2014 / Accepted: 9 December 2014 /
Published online: 16 December 2014
© Springer Science+Business Media Dordrecht 2014

Abstract Since the introduction of polymerase chain reaction (PCR) biodiversity study has been significantly influenced by the chance of generating unprecedented amounts of molecular data. Although it is a robust technique, those applications requiring high sensitivity and reproducibility, that is PCR detection and quantitative PCR, can be negatively affected by PCR inhibition. This is particularly challenging for diverse kinds of samples included the environmental ones, which usually contain complex mixtures of a variety of inhibitory substances. The problem of PCR inhibition can be overcome, or ameliorated, by implementing adequate protocols of nucleic acids purification, internal controls and modern analytical approaches focused on PCR kinetics. Herein, we remark these procedures and describe the general techniques that can be used to optimize DNA extraction protocols for PCR applications. In addition, we show that PCR inhibition might have negative consequences in molecular studies of *Didymosphenia geminata*, an invasive microalga that have recently developed massive blooms in temperate regions worldwide, and provide general guidelines for dealing with this problem.

Keywords Didymosphenia · Rock snot · Molecular detection · PCR

The polymerase chain reaction (PCR) allows for generating enormous amounts of copies of a specific DNA target. In essence, it consists of a liquid phase hybridization technique in which the need for oligonucleotides' hybridization for polymerase priming, or annealing, enables the amplification of highly specific sequence targets from a genome or a complex mix of DNA molecules. As successful oligonucleotides' hybridization is revealed by the

Communicated by David Hawksworth.

L. R. Jones (✉) · N. M. Uyua · J. M. Manrique
Laboratorio de Virología y Genética Molecular, Facultad de Ciencias Naturales sede Trelew, Universidad Nacional de la Patagonia San Juan Bosco, Trelew, Chubut, Argentina
e-mail: ljones@conicet.gov.ar

detection of the amplified product, the technique also rely on the efficiency of the enzymatic polymerization. Beside of its useful capabilities and technical simplicity, the PCR is very robust upon very rough DNA extracts. For example, a simple treatment with alkali can be enough to obtain PCR-ready DNA preparations from very complex samples such as stool or plant tissues (Allard et al. 1990; Xin et al. 2003). Thus, relatively impure DNA templates can be used in qualitative applications that are unaffected by the reaction efficiency, e.g. target DNA production for sequencing and engineering into plasmids. Conversely, for those applications in which PCR efficiency is critical, such as sensitive PCR-based detection/genotyping and quantitative PCR (qPCR), the presence of PCR inhibitors can be very harmful, since it can lead to false negative results and quantitative errors of several orders of magnitude, along with serious misinterpretations of these results (Bar et al. 2012; Rutledge and Stewart 2008). In particular, the complex chemical composition and characteristics of environmental samples are especially challenging, thus the selection of adequate protocols is an essential issue for environmental studies (Green and Field 2012; Pontiroli et al. 2011).

Herein, we comment on general procedures used for dealing with PCR inhibition. In addition, we present the case study of *D. geminata* (aka *rock snot* or *didymo*), a highly invasive diatom that pose potential nuisance problems with negative ecological and economic consequences. It is though that the didymo blooms reported in recent years to have become widespread in temperate regions could be due to dispersal of the algae by fishermen and fishing paraphernalia, but this hasn't been demonstrated yet. General surveys are needed to establish to which extent these blooms result from just a few, or perhaps even one, new genetic variant(s), or are a response of local populations to the influence of changing environmental factors (Taylor and Bothwell 2014; Whitton et al. 2009). Other aspects of *D. geminata* biology are unknown. For instance, it is uncertain whether colonies originate from a single cell by vegetative multiplication or by aggregation of free cells (Whitton et al. 2009) and it has been suggested that mats' development might depend on the extracellular matrix bacterial community structure (Kuhajek and Wood 2013). The taxonomic position of the species is rather unclear. While morphological studies support a proximity to genera with asymmetrical frustules such as *Gomphonema* and *Gomphoneis*, molecular phylogenies based on the 18S ribosomal gene suggest a closer relation to *Cymbella* species (Kermarrec et al. 2011). Molecular studies will help elucidate these and other open questions. As discussed in the following sections, the recalcitrance of *D. geminata* samples places this alga as an extraordinary example of the significant efforts required for optimizing molecular protocols used for the study of some environmental samples.

It is useful to classify PCR inhibition factors based on the interference source. Extrinsic factors are those derived from sample processing. For example, phenol employed at organic extractions as well as alcohols and salts applied for DNA precipitation or protein salting out, can have negative repercussions on enzyme stability and activity. Likewise, several transport media and extraction buffers incorporate ethylenediaminetetraacetic acid (EDTA), which, if improperly removed from DNA extracts, can sequester the magnesium ions required as polymerase cofactors. The majority of these drawbacks can be surpassed by implementing proper laboratory practices combined with careful analyses of the principles and reagents used during the extraction protocols (Uyua et al. 2014). Intrinsic factors, matrix composition and complexity, are more troublesome to deal with due to the diverse chemical nature of PCR inhibitors and the characteristics of the samples that difficult DNA release. In addition, many inhibitory mechanisms are poorly or not understood. Therefore, general quantitative and qualitative procedures are used to deal with the

Table 1 General procedures used to evaluate DNA extracts

Method	Parameter	Indicator
Qualitative		
UV spectroscopy	Purity	Absorbancies at 230, 260, 280 and 320 nm (A_{230}, A_{260}, A_{280} andA_{320}, respectively); A_{260}/A_{280} ratio
Gel electrophoresis	Integrity	Shearing, DNA/RNA contamination, microparticulate material (well fluorescence)
Enzymatic tests (e.g. restriction, PCR amplification)	Efficiency	Homogeneous and thorough digestion as opposite to incomplete or "banded" degradation; kinetics (i.e. an enzyme unit must digest a precise amount of DNA in a given time); set point PCR yield; PCR kinetics
Quantitative		
UV spectroscopy	DNA Mass	A_{260}
Fluorometry	DNA Mass	Intensity of fluorescence as measured with a fluorometer or by densitometry analysis of gel electrophoresed DNA

problem (Table 1). Quantitative performance is directly related to DNA recovery rate and yield and thus to assay sensitivity, whereas DNA qualitative assessment has more to do with the persistence of PCR inhibitors. However, DNA quantification can be distorted by the persistence of undesired compounds in DNA preparations. For example, no correlation is observed among the DNA concentration and absorbance at 260 nm (A_{260}) of extracts obtained with diverse protocols from benthic samples of *Didymosphenia geminata* (Fig. 1). This is due to the co-extraction of compounds that absorb at the same wave length than DNA, and thus indicates that spectrophotometry isn't a suitable quantitative method for these samples. The data depicted in Fig. 1 also show that the DNA extraction protocol used can affect assay sensitivity due to significant variations in the corresponding DNA recovery rates. Despite DNA extraction protocols can be optimized, many potential PCR inhibitors, such as heavy metals, can be difficult to abolish and/or difficult to monitor. Furthermore, the thorough analysis of DNA extracts used in high throughput applications is generally impractical. Thus, besides using fine tuned DNA extraction methods, it is highly advisable for quantitative applications to monitor PCR interference by spiking samples with internal amplification controls (IAC) (Hoorfar et al. 2004). Internal controls should be similar to the target molecules in terms of amplification kinetics. Furthermore, the optimal amount of IAC used must be determined with precision and standardized properly to minimize competition with the target sequence and avoid the heterodimeric DNA phenomenon (Fig. 2) (Bar et al. 2012; Becker-Andre and Hahlbrock 1989).

The complexity and composition of environmental samples of *D. geminata* may challenge PCR-based studies (Uyua et al. 2014). The A_{260}/A_{280} ratios of the majority of DNA preparations obtained from benthic samples of the algae are out of the 1.8–2.0 range expected for pure DNA, independently of the extraction method used. While this could be attributed to extrinsic factors such as phenol and salts, the most plausible cause in this case is the high polysaccharide content and the complexity of the microbial mats. Didymo cells secrete a mucilaginous substance that serves the alga to fix to the substrate and is the major component of the typical large colonies formed during the blooms. This extracellular matrix is composed mainly of polysaccharides and accumulates significant amounts of heavy metals (Bothwell et al. 2012; Gretz 2008; Sundareshwar et al. 2011), two substances that are known to inhibit enzymatic reactions and to be present in many environmental

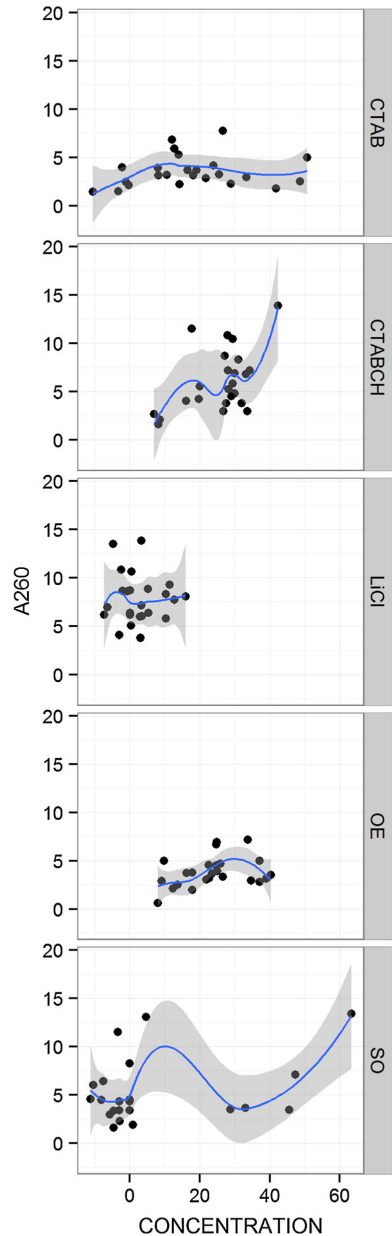

Fig. 1 Paired DNA concentration (ng/µl) and 260 nm absorbance (A_{260}) data corresponding to independent DNA preparations (n = 24) obtained by standard organic extraction, OE (Sambrook and Russel 2001), Cetyltrimethylammonium Bromide, CTAB (Uyua et al. 2014), Cetyltrimethylammonium Bromide plus Chelex 100, CTABCH (Uyua et al. 2014), and two salting out techniques, SO (Miller et al. 1988) and LiCl (Kelly 2009). A_{260} was obtained using a nanospectrophotometer (*Nanovue Plus*, GE healthcare); DNA yields were estimated by densitometry analysis against DNA mass standards (*High* and *Low* DNA mass ladders, Invitrogen). The *fitted lines* and confidence bounds were obtained by local regression (Wickham 2009)

samples (Aldrich and Cullis 1993; Monteiro et al. 1997; Pandey et al. 1996; Schrader et al. 2012). The use of buffers containing Cetyltrimethylammonium bromide (CTAB) and the differential solubility and precipitation of DNA and polysaccharides can help to circumvent or ameliorate this problem (Uyua et al. 2014; Wilson 1997). In addition, the incorporation of selective chelating agents, such as Chelex resins, can help improve the extraction methods in these cases (Uyua et al. 2014).

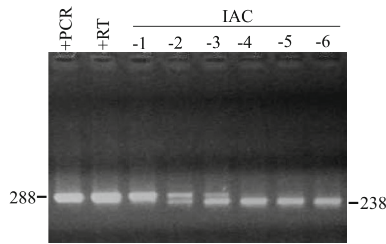

Fig. 2 RT-PCR co-amplification of a genomic viral target (288 bp) and an internal control (238 bp). One hundred µl of a *Pestivirus* viral stock containing ~4.35E6 viral plaque forming units (PFU) were subjected to RNA extraction as described elsewhere (Jones et al. 2001) and the obtained RNA suspension (5 µl final volume) was submitted to six serial 1:10 dilutions. Five µl of each dilution (−1, −2, −3, −4, −5 and −6) were spiked with one unit of an internal amplification control as described elsewhere (Golemba et al. 2008) and subjected to RT-PCR amplification with pan-pestivirus primers 324 and 326 (Vilcek et al. 1994). This RT-PCR technique has a sensitivity of ~5 PFU. Lanes marked +PCR and +RT correspond to external amplification controls. The absence of IC amplification in the lower dilutions obey to competence with the viral template

Recently, a qPCR assay to detect and enumerate didymo cells in complex environmental samples has been developed (Cary et al. 2014). Given the limitations posed by microscopic techniques currently used, combined with the fact that didymo cells cannot be easily cultured, the proposed methodology represents a potentially valuable tool for future studies. However, qPCR can be impaired by PCR inhibition, which may impact on the accuracy and sensitivity of the technique (Bar et al. 2012; Rutledge and Stewart 2008). Indeed, comparing different DNA extraction methods we have observed that, for the majority of the tested protocols, amplicon quantities at PCR set points did not correlate with template amounts, which indicate the persistence of enzymatic inhibitors in DNA extracts obtained from benthic samples (Fig. 3) (Uyua et al. 2014). Furthermore, DNA extracts obtained from cells isolated by mouth pipeting also displayed the presence of PCR inhibitors, demonstrating the recalcitrance of *D. geminata* samples, independently of the sampling method (Fig. 4). Cary et al. estimated amplification efficiencies (E) from the slope of standard curves performed by dilution series. This approach rely on the assumption that E is constant until the threshold cycle, implying an exponential mode of DNA amplification in which at cycle C the number of target molecules (N_C) is given by the equation:

$$N_C = N_0 \times (1 + E)^C \tag{1}$$

where N_0 is the initial number of template molecules. This assumption has been challenged by data supporting the concept that PCR amplification is a sigmoidal rather than exponential process (Rutledge and Stewart 2008). Under the sigmoidal model, E is maximal (E_{MAX}) at the onset of the amplification reaction and progressively decreases as the reaction progresses, following the equation:

$$E_C = \Delta E + E_{MAX} \tag{2}$$

where ΔE is the rate of loss of amplification efficiency. In addition, it has been shown that E estimations obtained from classical standard curves (E_{SLOPE}) correspond to E_{MAX} rather than to E at the threshold cycle (Rutledge and Stewart 2008). Thus, E_{SLOPE} values above 100 %, as those observed by Cary et al., may reflect the fact that serial template dilutions used in standard curves generation result also in dilution of PCR inhibitors. Improved methods have been developed that, combined with the use of IACs, may help to monitor

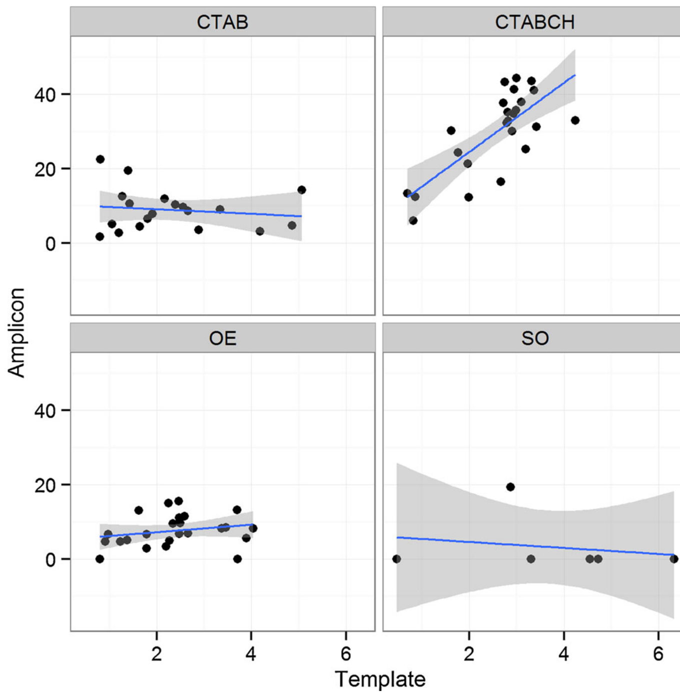

Fig. 3 Set point (Amplicon) and initial (Template) DNA amounts, observed among PCR amplifications of didymo DNA preparations obtained with different extraction protocols. Independent DNA extractions (n = 24) obtained by standard organic extraction, OE (Sambrook and Russel 2001), Cetyltrimethylammonium Bromide, CTAB (Uyua et al. 2014), Cetyltrimethylammonium Bromide plus Chelex 100, CTABCH (Uyua et al. 2014) and salting out, SO (Miller et al. 1988), were subjected to PCR under conditions described elsewhere (Uyua et al. 2014). Template and amplicon amounts were estimated by densitometry analysis against DNA mass standards (*high* and *low* DNA mass ladders, Invitrogen). The lines and confidence bands correspond to fitted linear models. Eighteen of the SO extracts and five of the CTAB ones displayed DNA amounts that fell out of the standard mass range used for quantification and thus the corresponding yields were not plotted

PCR inhibition in *D. geminata* studies (Bar et al. 2012; Green and Field 2012). This techniques, namely kinetics outlier detection (KOD), are based on detecting amplification curve anomalies by comparing kinetics parameters of test and reference reactions. Thus, KOD do not interfere with accuracy and sensitivity, can be easily automated and do not require much extra materials and labour. In addition, KOD analyses help to distinguishing PCR inhibition from DNA recovery problems and are more sensitive than traditional methods based on quantification threshold cycle.

In summary, PCR inhibition can jeopardize the success and significance of molecular studies of environmental samples. For the case of *D. geminata*, it would be of worth that molecular protocols consider the incorporation of IACs, standardized DNA extraction methods aimed to minimize PCR inhibition and KOD techniques. It would be advisable, for DNA extraction, to use a combination of surfactants, high saline concentrations, chelating agents and proper washing steps (Uyua et al. 2014). Internal controls should be spiked at the time of PCR amplification, since the effect of losing material prior to PCR is difficult to differentiate from PCR inhibition. Furthermore, the amount of IC used must be carefully optimized to preserve accuracy and sensitivity (Bar et al. 2012). As the DNA

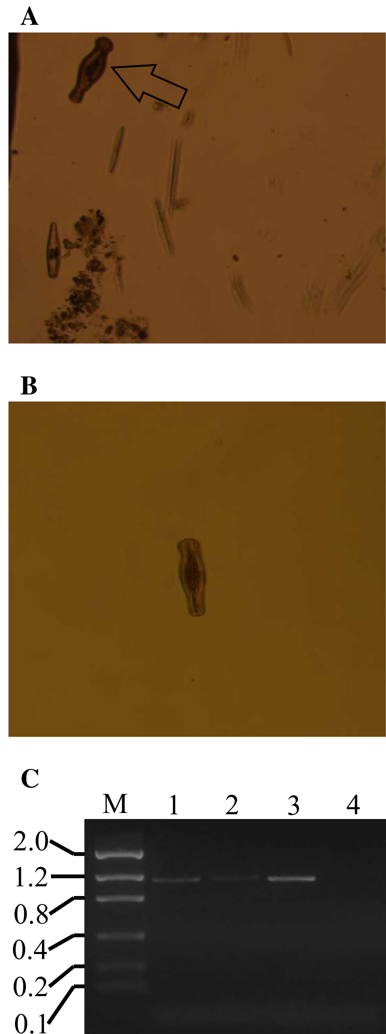

Fig. 4 PCR amplifications performed from *D. geminata* cells isolated from a complex environmental sample using mouth pipeting. *Panels A* and *B* depict the sample before and after didymo isolation. Ten didymo cells (*arrow in panel A*) were taken, gently washed with molecular grade water (UltraPure DNase/RNase-Free Distilled Water, Invitrogen) and submitted to nucleic acids extraction using a CTAB based protocol that have proven useful for other complex environmental samples (Manrique et al. 2012). The obtained DNA was suspended in 10 µl of molecular grade water and PCR reactions where made using either 1 (*Panel C*, *lane 1*) or 9 (*Panel C*, *lane 2*) µl of the suspension as template. An external control was included (*Panel C*, *lane 3*), consisting of a DNA extract obtained as described in Uyua et al. (2014). *Lane 4* in *panel C* corresponds to a negative control. The bands in the included DNA mass ruler correspond, from *top* to *bottom*, to 100, 60, 40, 20, 10, and 5 ng of DNA (*lane M* in *panel C*; *Low* DNA Mass Ladder, Invitrogen). Numbers close to each band correspond to band sizes, in Kbp. PCR conditions were as described elsewhere (Uyua et al. 2014)

yields observed for *D. geminata* samples are similar to the ones observed for recalcitrant DNA sources such as mummified and herbarium plant tissues (Uyua et al. 2014), it would be also advisable to asses for the effects of the matrix on the overall method's recovery.

Acknowledgments LRJ and JMM are members of the scientific career of the National Council for Scientific and Technical Research (CONICET, Argentina). NMU is a CONICET doctoral fellow.

References

Aldrich J, Cullis CA (1993) RAPD analysis in flax: optimization of yield and reproducibility using KlenTaq 1 DNA polymerase, Chelex 100, and gel purification of genomic DNA. Plant Mol Biol Report 11:128–141

Allard A, Girones R, Juto P, Wadell G (1990) Polymerase chain reaction for detection of adenoviruses in stool samples. J Clin Microbiol 28:2659–2667

Bar T, Kubista M, Tichopad A (2012) Validation of kinetics similarity in qPCR. Nucleic Acids Res 40:1395–1406

Becker-Andre M, Hahlbrock K (1989) Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res 17:9437–9446

Bothwell ML, Kilroy C, Taylor BW, Ellison ET, James DA, Gillis C-A et al (2012) Iron is not responsible for *Didymosphenia geminata* bloom formation in phosphorus-poor rivers. Can J Fish Aquat Sci 69:1723–1727

Cary CS, Coyne KJ, Rueckert A, Wood SA, Kelly S, Gemmill CEC et al (2014) Dvelopment and valiation of a quantitative PCR assay for the early detection and monitoring of the invasive diatom *Didymosphenia geminata*. Harmful Algae 36:63–70

Golemba MD, Parreño V, Jones LR (2008) Simple procedures to obtain exogenous internal controls for use in RT-PCR detection of bovine pestiviruses. Mol Cell Probes 22:212–214

Green HC, Field KG (2012) Sensitive detection of sample interference in environmental qPCR. Water Res 46:3251–3260

Gretz MR (2008) The stalks of didymo. In: Bothwell ML, Spaulding SA (eds) Proceedings of the 2007 international workshop on *Didymosphenia geminata*, vol 2795

Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P (2004) Practical considerations in design of internal amplification controls for diagnostic PCR assays. J Clin Microbiol 42:1863–1868

Jones LR, Zandomeni R, Weber EL (2001) Genetic typing of bovine viral diarrhea virus isolates from Argentina. Vet Microbiol 81:367–375

Kelly SR (2009) The origin, genetic diversity and taxonomy of the invasive diatom *Didymosphenia geminata* (bacillariophyceae) in New Zealand, The University of Waikato

Kermarrec L, Ector L, Bouchez A, Rimet F, Hoffmann L (2011) A preliminary phylogenetic analysis of the Cymbellales based on 18S rDNA gene sequencing. Diatom Res 26:305–315

Kuhajek J, Wood S (2013) Didymo phase 4: the influence of water chemistry and biofilm composition on Didymosphennia geminata establishment, Report No. 2347, Cawthron Institute, New Zealand

Manrique JM, Calvo AY, Jones LR (2012) Phylogenetic analysis of Ostreococcus virus sequences from the Patagonian Coast. Virus Genes 45:316–326

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:3

Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Viral R et al (1997) Complex Polysaccharides as PCR Inhibitors in Feces: *Helicobacter pylori* Model. J Clin Microbiol 35:995–998

Pandey RN, Adams RP, Flournoy LE (1996) Inhibition of random amplified polymorphic DNAs (RAPDs) by plant polysaccharides. Plant Mol Biol Report 14:17–22

Pontiroli A, Travis ER, Sweeney FP, Porter D, Gaze WH, Mason S et al (2011) Pathogen quantitation in complex matrices: a multi-operator comparison of DNA extraction methods with a novel assessment of PCR inhibition. PLoS One 6:e17916

Rutledge RG, Stewart D (2008) Critical evaluation of methods used to determine amplification efficiency refutes the exponential character of real-time PCR. BMC Mol Biol 9:96

Sambrook J, Russel DW (2001) Molecular cloning. A laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schrader C, Schielke A, Ellerbroek L, Johne R (2012) PCR inhibitors—occurrence, properties and removal. J Appl Microbiol 113:1014–1026

Sundareshwar PV, Upadhayay S, Abessa M, Honomichl S, Berdanier B, Spaulding SA et al (2011) *Didymosphenia geminata*: Algal blooms in oligotrophic streams and rivers. Geophys Res Lett 38:1–5

Taylor BW, Bothwell ML (2014) The origin of invasive microorganisms matters for science, policy, and management: the case of *Didymosphenia geminata*. Bioscience 64:531–538

Uyua NM, Manrique JM, Jones LR (2014) An optimized DNA extraction protocol for benthic *Didymosphenia geminata*. J Microbiol Methods 104C:12–18

Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ (1994) Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch Virol 136:309–323

Whitton BA, Ellwood NTW, Kawecka B (2009) Biology of the freshwater diatom Didymosphenia: a review. Hydrobiologia 630:1–37

Wickham H (2009) ggplot2: elegant graphics for data analysis. Springer, New York

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741–3751

Xin Z, Velten JP, Oliver MJ, Burke JJ (2003) High-throughput DNA extraction method suitable for PCR. Biotechniques 34(820–4):826