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An optimized DNA extraction protocol for benthic *Didymosphenia geminata*

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ABSTRACT

Didymosphenia geminata mats display few cells in relation to extracellular material and contain polysaccharides and heavy metals that interfere with molecular studies. We describe an optimized DNA extraction protocol that help to overcome these difficulties. Our protocol outperformed five previously described DNA extraction techniques.

1. Introduction

Didymosphenia geminata is a freshwater diatom capable of forming colonial mats consisting, primarily, of attachment stalks that are composed mainly of sulfated polysaccharides, uronic acids and proteins (Blanco and Ector, 2009; Gretz, 2008; Whitton et al., 2009). Due to the greenish and viscous aspect of the colonies, these algae have earned the common name of "rock snot" (Segura, 2011). The natural range of *D. geminata* is thought to be restricted, mostly, to cool temperate waters from the Holartic region (Blanco and Ector, 2009; Spaulding and Elwell, 2007; Whitton et al., 2009). Early reports of mass developments of the algae date back to the 19th century (Blanco and Ector, 2009). However, the increased frequency of nuisance, massive blooms observed over the last decade has attracted the attention of scientists and management authorities (Beltrami et al., 2008; Bergey et al., 2009; Bhatt et al., 2008; Blanco and Ector, 2008; Falasco and Bona, 2013; Kawecka and Sanecki, 2003; Kilroy, 2004; Kilroy et al., 2009; Kirkwood et al., 2007, 2009; Pite et al., 2009; Tomás et al., 2010; Whitton et al., 2009).

One of the most accepted hypothesis for explaining the nuisance blooms reported in recent times, is the dispersal by fishermen and fishing paraphernalia such as felt-soled waders (Bothwell et al., 2009; Segura, 2011). However, the actual causes underlining the invasive behavior observed in some places haven't been scientifically determined (Taylor and Bothwell, 2014). Indeed, deeper studies will be needed to determine whether the recent, uproarious blooms are due to the overall dispersion of a new genetic variant or to the growth of local populations (Kilroy, 2004; Taylor and Bothwell, 2014). Many other aspects of *D. geminata* biology are also unknown. For instance, it is not known whether colonies develop clonally (*i.e.* originate from a single cell by vegetative multiplication) or are formed by aggregation of free cells (Whitton et al., 2009). Likewise, molecular analyses based on the 18S ribosomal gene grouped *D. geminata* together with *Cymbella* species, whereas frustules morphology suggests a closest relationship with genera harboring asymmetrical frustules with respect to the transapical axis, such as *Gomphonema* and *Gomphoneis* (Kermarrec et al., 2011).

Molecular studies will help to elucidate many of these open questions, as well as to achieve a deeper understanding of other aspects of *D. geminata* ecology, as well as the extent of economical and ecological risks represented by this species. Obtaining high yield as well as high purity nucleic acids form environmental samples is a critical step for these studies. *D. geminata* mat samples pose a challenge due to their high proportion of stalks in relation to cells, as well as the high stalks' content of polysaccharides, which are known to be potent enzymatic inhibitors (Monteiro et al., 1997; Pandey et al., 1996). Purification of nucleic acids from *D. geminata* has been reported before (Cary et al., 2006, 2007, 2014; Kelly, 2009; Kermarrec et al., 2011). In particular Cary et al. (2007) compared six commercial DNA extraction kits from

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four different companies, observing acceptable but variable rates of DNA amplification success. Comparisons of the performance achieved by standard DNA extraction techniques, as well as development of new approaches specifically designed for *D. geminata* mats are lacking.

In this work, five standard nucleic acid extraction methods were applied directly to *D. geminata* benthic samples obtained by simple field environmental sampling protocols. These techniques have been used previously to obtain DNA in diverse genetic studies involving a wide variety of organisms such as diatoms (Iwatani et al., 2005), dinoflagellates (Richlen and Barbar, 2005), copepods (Simonelli et al., 2009), bacteria, viruses and picoeukaryotes (Manrique et al., 2012; Manrique and Jones, 2014), spiders (Casquet et al., 2012) and human nucleated cells (Miller et al., 1988). All the considered techniques use broadly available chemicals, and can be reproduced at any laboratory. In addition, a new protocol was designed based on physical and chemical characteristics of *D. geminata* mats as well as the results obtained for the other protocols studied. Being able to count with a standardized, fast and simple method that allows the purification of high molecular weight DNA from any sample submitted to the laboratory (*i.e.* from simple water samples to complex mats ones harboring potentially enzyme inhibitors) will help to gain advantageous molecular information promoting the advancement in the scientific understanding of this invasive algae, which should allow the implementation of improved management policies.

2. Materials and methods

2.1. Environmental sampling

Benthic samples were collected in April 2013 (autumn season at the southern hemisphere) from selected sites of the Futaleufú River, where visual exploration had determined the presence of *D. geminata* colonies. A large sample of benthic didymo was obtained by scraping from a big, submerged rock using a sterile scalpel (*i.e.* opened at the sampling site). The scraped material was collected in 50 ml tubes containing molecular grade 70% ethanol. All the tubes were sealed after sampling using Parafilm to ensure there was no spillage and refrigerated for its transportation to the laboratory. Sample handling was performed according to the safety guidelines for invasive species. Once at the laboratory, samples were stored at −20 °C until used. Immediately upon arriving, microscopic observations using a 400× magnification were done to corroborate the presence of *D. geminata* cells (Olympus CKX41 microscope). Cells were photographed using an Olympus Evolt E-330 camera.

2.2. DNA extraction

Five direct nucleic acid extraction protocols were used to obtain total DNA from mat samples. For each DNA extraction, a 50 mg portion of *D. geminata* mat sample was centrifuged and the supernatant was removed. The obtained pellet was washed once with 150 μl of pure water (BioPack; Ruta 9 Km 105 — Zárate — Buenos Aires — Argentina) to remove residual ethanol, before immediate processing. All the protocols studied here are based on a range of physico-chemical principles and chemical compounds: i) organic extraction combined with a proteinase *K* digestion and nucleic acid precipitation (Sambrook and Russel, 2001); ii) treatment with a chelating ion exchange resin (Chelex® 100, BioRAd) that has proven to be useful in many difficult applications samples; iii) treatment with a high concentration of an anionic surfactant (cetyltrimethylammonium bromide, CTAB); iv) high concentration of proteinase K treatment, followed by an organic extraction and a salting out step; and v) the salting out technique, combined with proteinase K treatment. The major characteristics of these five methods are given in Table 1. Furthermore, based on the fact that *D. geminata* stalks are known to accumulate significant amounts of metals (Bothwell et al., 2012; Sundareshwar et al., 2011) and the performance displayed by the CTAB protocol described in the following sections, an improved procedure was developed combining CTAB and Chelex-100. The details of the six protocols are outlined below.

2.3. Cetyltrimethylammonium bromide, CTAB

(Manrique et al., 2012): The pellets were incubated at 60 °C for 1 h in 700 μl of pre-heated CTAB buffer (2% CTAB Sigma, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl pH 8.0) with 0.3 μg/μl of proteinase K. Afterwards, the samples were centrifuged for 4 min at maximum speed and the obtained supernatants were transferred to fresh tubes. After treatment with chloroform: isoamyl alcohol (24:1), RNA materials present at the suspensions were digested with RNase A (Sigma-Aldrich) at a final concentration of 10 μg/mL for 1 h at 37 °C. The DNA material was precipitated with cold isopropanol for 1 h at 4 °C and then centrifuged at 21,000 ×*g* for 30 min at 4 °C (Sorval Legend Micro 17 R, Thermo Scientific). The DNA pellets were washed with 70% ethanol, air-dried and resuspended in 10 μl of ultrapure, DNase free water (Invitrogen).

2.4. Salting out, SO

(Miller et al., 1988): The sample pellets were resuspended in 600 μl of buffer (10 mM Tris–HCl pH 8.2, 400 mM NaCl and 2 mM Na_2EDTA), 40 μl of 10% SDS and 100 μl of a proteinase K solution (1 mg/ml proteinase K in 1% SDS, 2 mM Na_2EDTA), and incubated at 37 °C overnight. After incubation, 200 μl of a saturated NaCl solution (~6 M) was added to each tube, shaken vigorously for 15 s and centrifuged for 15 min at 16,873 ×*g* (Centrifuge 5418, Eppendorf). The supernatant was transferred to a fresh tube, and room temperature ethanol 100% was added. The tubes were inverted several times until the DNA precipitated, and then centrifuged at 16,873 ×*g* for 3 min. The DNA pellets were air-dried and resuspended in 10 μl of TE buffer (10 mM Tris–HCl, 0.2 mM Na_2EDTA, pH 7.5).

2.5. Chelex 100, CH

A 10% Chelex-100 (Bio-Rad) suspension was prepared as suggested by the manufacturer. Three hundred microliters of this suspension were added to each sample and the obtained mixtures were vigorously mixed by vortex for approximately 5 s and briefly centrifuged. The samples were then heated at 100 °C for 10 min, mixed by vortexed and centrifuged again, and finally stored at −20 °C until used.

2.6. Lithium chloride, LiCl

(Kelly, 2009): The samples were incubated at 55 °C for 2 h and then at 37 °C overnight in a mix containing 300 μl of lysis buffer (700 mM Tris–HCl, pH 9; 1% SDS; 50 mM EDTA, pH 8; 100 mM NaCl) and 25.5 μl of a 20 mg/ml proteinase K solution. Then, the samples were treated with 300 μl of 5 M LiCl and 645 μl of chloroform for 45 min. The DNA was precipitated by adding one volume of room temperature isopropanol followed by centrifugation at 16,873 ×*g* for 10 min. The obtained pellets were air dried and resuspended in 10 μl of ultrapure, DNase free water (Invitrogen).

2.7. Organic extraction, OE

(Sambrook and Russel, 2001): The samples were incubated at 37 °C overnight in a mix containing 400 μl of proteinase K buffer (10 mM Tris–HCl pH 7.8, 5 mM EDTA, 0.5% SDS) and 1 μl of 20 μg/μl proteinase K solution. Then, nucleic acids were extracted by adding one volume of phenol: chloroform: isoamyl alcohol (25:24:1), shaken vigorously and centrifuged at 16,873 ×*g*. Any trace of phenol was removed by a second extraction with chloroform. The nucleic acids contained in the aqueous phase were precipitated by adding sodium acetate (3 M; pH 5.2) and ethanol 100%, before centrifugation at 16,873 ×*g* for 5 min. The obtained

Table 1
Comparison of the five published DNA extraction methods[a] studied.

	SO	OE	CTAB	CHELEX	LiCl
Chelator	–	–	–	Chelex 100	–
Salting out	~1.5 M NaCl	–	1.4 M NaCl	–	2.5 M LiCl
Surfactant	–	–	Cationic	–	–
Digestion	PK (~0.1 μg/μl)	PK (~0.05 μg/μl)	PK (0.3 μg/μl) and RNase A	–	PK (~1.5 μg/μl)
Solvents[b]	–	P:C:I + C	C:I	–	C-LiCl
Salt[c]	~1.5 M NaCl	0.3 M NaAc	1.4 M NaCl	–	2.5 M LiCl
Alcohol[d]	Ethanol	Ethanol	Isopropanol	–	Isopropanol
Washing	–	70% ethanol	70% ethanol	–	–
Storing buffer	TE	H_2O	H_2O	–	H_2O

[a] Salting out SO; organic extraction OE; cetyltrimethylammonium bromide CTAB; Chelex-100 Chelex; lithium chloride LiCl.
[b] Solvents used in organic extractions. C:I: Chloroform:isoamyl alcohol; C-LiCl: chloroform combined with LiCl; P:C:I + C: phenol:chloroform:isoamyl alcohol plus a second extraction with chloroform.
[c] Salt providing cations in nucleic acid precipitation.
[d] Alcohol used for nucleic acid precipitation.

pellets were washed in 70% ethanol, air dried and resuspended in 10 μl of ultrapure, DNase free water (Invitrogen).

2.8. Cetyltrimethylammonium bromide — Chelex-100, CTABCH (this study)

This protocol has been derived from the CTAB and Chelex-100 protocols described before. Briefly, samples were incubated at 60 °C for 1 h in 700 μl of pre-heated CTAB buffer (2% CTAB Sigma, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl pH 8.0), 10% Chelex-100 (Bio-Rad) and 0.3 μg/μl of proteinase K. After digestion, the samples were centrifuged for 4 min at maximum speed and the obtained supernatants were transferred to fresh tubes. The rest of the protocol remains the same as the CTAB one described above.

2.9. Analysis of DNA suspensions

The quality of the obtained nucleic acid preparations was assessed by electrophoresis in agarose gel and spectrophotometric measures at different wave lengths (230, 260, 280 and 320 nm) using a *Nanovue Plus* (GE healthcare) spectrophotometer. Electrophoreses were performed with 0.8% agarose in TAE buffer (40 mM Tris–Acetate, 1 mM EDTA, pH 8), stained with ethidium bromide (0.5 μg/ml final concentration) and visualized under UV light on a transilluminator (Elettrofor). For DNA quantitation, images were digitized using a camera-based gel documentation equipment (Biodynamics, KodaK, DOC-6490), and DNA yield was estimated by densitometry analysis against standards of 10, 20, 30, 40, 60 and 100 ng of DNA (High DNA Mass Ladder, Invitrogen) using the ImageJ software (Abramoff et al., 2004).

2.10. DNA amplification conditions

Partial amplification of the 18S rDNA gene of *D. geminata* was obtained using specific primers 602F and D1670R, that were designed to amplify a 1068 bp region (Cary et al., 2006, 2014). Optimal PCR conditions were initially determined by analyzing product amplification in an increasing temperature gradient PCR experiment, in order to maximize specificity and amplicon yield. All the amplifications were carried out in a MyCycler Thermal Cycler with gradient option (BioRad). The PCR reactions were carried out a in 50 μl reaction mixtures containing 1 μl of diluted DNA extract, 1.25 units of GoTaq® DNA Polymerase (Promega), 10 μl of 5× GoTaq Buffer and 1 μM of each specific primer. Cycling conditions were; initial step at 94 °C 1 min followed by 30 cycles of 94 °C for 30 s, 52.1 °C for 30 s and 72 °C for 90 s; and a final extension step of 72 °C for 5 min was applied. All the obtained products were analyzed by electrophoresis in 2% agarose gels with 1× TAE buffer as described before. The visualized PCR products were further analyzed by densitometry as described above.

2.11. Experimental setup and statistical analyses

For each extraction method, twenty four independent DNA extractions were performed using 50 mg of the same mat sample obtained as described above (subsection DNA extraction). In order to confirm the successful separation of the cell content from frustules, all samples were analyzed by microscopic observation after the corresponding treatments. Statistical analyses were carried out using the R statistical package version 2.15.1 (R development Core Team, 2013). Statistical comparisons were made by the Kolmogorov–Smirnov test (Hollander and Wolfe, 1999). This test assumes two random samples $X_1, \ldots, X_m$ and $Y_1, \ldots, X_n$ drawn from continuous, mutually independent populations with distribution functions F and G, respectively. The aim is to evaluate the existence of any difference between the X and Y probability distributions, which is to say testing the null hypothesis:

$$H_0 : F(t) = G(t), \text{for every } t,$$

against the most general alternative possible:

$$H_0 : F(t) \neq G(t), \text{for at least one } t.$$

3. Results

Microscopic observations showed that all the studied DNA extraction methods successfully allowed the detachment of cellular contents from frustules (Fig. 1). For the CTAB, CTABCH and OE methods, total DNA could be detected by gel electrophoresis analysis in all the samples processed. For the LiCl and SO, 23 and 21 samples had DNA detectable in agarose gels (not shown). In all cases, the DNA could be visualized as high molecular weight DNA (>10 kpb fragment), with minimal shearing effects. There was no detectable DNA in the suspensions obtained by the CH method.

In general, spectrophotometric analyses indicated that DNA purities were slightly below the optimal ranges, as most of 260 to 280 absorbance ratios (A_{260}/A_{280}) were below 1.7, and the majority of the extracts displayed considerable absorptions when measured at wave lengths of 230, 280 and 320 nm (Fig. 2; Table 2). Notwithstanding, many of the preparations obtained by the OE, CTAB and CTABCH techniques displayed average A_{260}/A_{280} ratios that were close to or above 1.7 (Table 2; Fig. 2). The rest of the methods showed significantly lower A_{260}/A_{280} ratios, indicating lower DNA purities relative to the OE, CTAB and CTABCH techniques ($p < 0.035$, Kolmogorov Smirnov test; Table 2, Fig. 2).

The amounts of DNA obtained by the CTAB, CTABCH and OE protocols were significantly higher than the DNA amounts obtained by LiCl and SO ($p < 5e\text{-}4$, Kolmogorov–Smirnov test; Fig. 3). The DNA yields obtained by the OE and CTAB techniques were statistically indistinguishable. In comparison to these two techniques, the CTABCH method resulted in slightly higher amounts of DNA ($p < 0.05$, Kolmogorov–

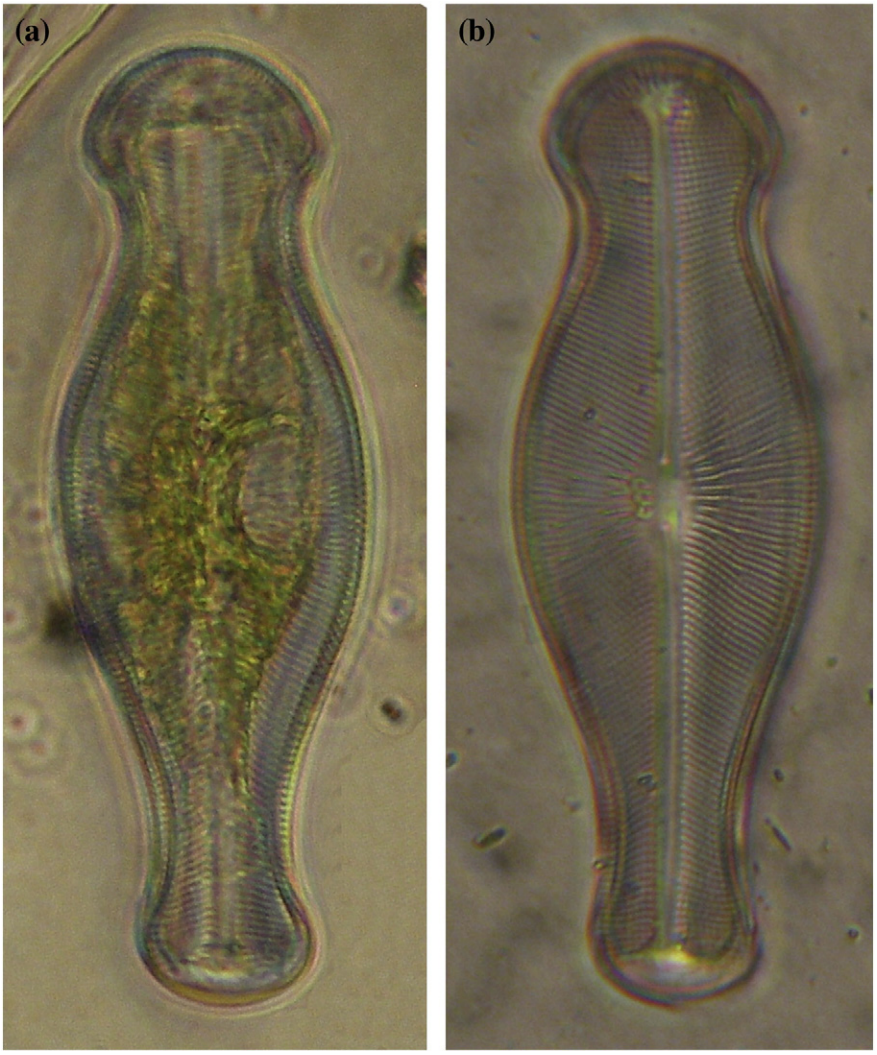

Fig. 1. Microscope images of *D. geminata* frustules before (a) and after (b) the DNA extraction procedure. The frustule shown in b corresponds to a Chelex-100 treated sample. The rest of the techniques produced equivalent results (not shown). Magnification: 400×.

Smirnov test; Fig. 3). As mentioned above, the preparations obtained by CH presented undetectable amounts of genetic material, precluding DNA quantification. Based on the spectrophotometric results, for the samples studied here, A_{260} was an unsuitable indicator of the amount of DNA present in the extracts. Indeed, regression and correlation analyses indicated that A_{260} absorptions did not correlate to the DNA concentrations estimated by densitometry analyses (Fig. 4; Table 3). Thus, DNA yield determinations based on A_{260} were dismissed.

Under the experimental conditions described, the DNA samples obtained by the LiCl and CH methods resulted in no detectable PCR products. Eleven of the samples obtained by the SO technique also resulted in undetectable amplification products, while the rest of amplifications performed from these extracts gave a minimum of 91.5 ng of and a maximum 1246.9 ng of PCR product. The mean PCR yield obtained from the SO extracts was 376.3 ng, with a standard deviation of 448.4. The average PCR yield observed for the DNA preparations obtained by the OE protocol was 379.9 ng, with a standard deviation of 21.09. Only one of the OE replicates resulted in undetectable amplification products. The highest PCR yield observed for this technique was of 777.8 ng. All the extracts obtained by the CTAB and CTABCH protocols resulted in positive PCR reactions. The DNA suspensions obtained by CTAB displayed a mean PCR yield of 382.4 ng, with a standard deviation of 282.1, and minimum and maximum yields of 49.8 ng and 1125 ng, respectively. The suspensions obtained by CTABCH had an average

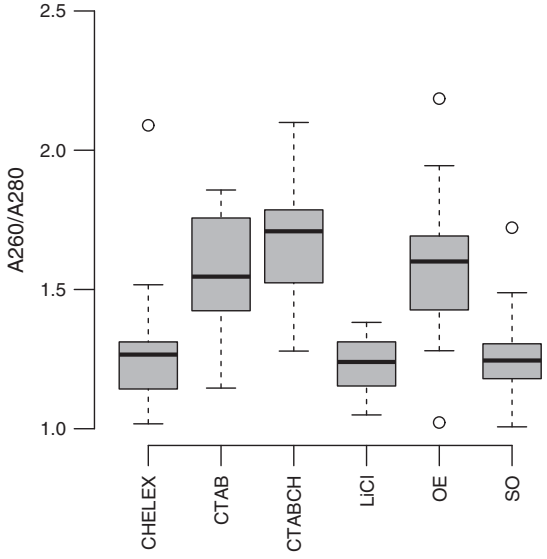

Fig. 2. Box plots of the 260/280 absorbance ratios observed for DNA preparations obtained by the six methods studied. CH: Chelex-100, CTAB: cetyltrimethylammonium bromide, CTABCH: cetyltrimethylammonium bromide and Chelex-100, OE: organic extraction, LiCl: lithium chloride protocol, and SO: salting out.

Table 2
Absorbance of the extracts obtained by the six DNA extraction methods[a] studied.

Method	Absorbance[b]			
	A230	A260	A280	A320
SO	5.91 (14.5)	4.99 (8.2)	3.99 (7.4)	2.42 (5.5)
EO	2.29 (1.2)	3.46 (1.5)	2.21 (1.0)	0.62 (0.6)
CTAB	3.24 (1.2)	3.22 (1.6)	2.9 (1)	0.70 (0.6)
CHELEX	3.44 (4.3)	2.06 (4.2)	1.64 (4.2)	1.30 (3.7)
LiCl	9.73 (3.3)	7.78 (2.5)	6.35 (2.2)	4.35 (1.8)
CTABCH	3.92 (1.8)	6.09 (3.2)	3.57 (1.8)	0.95 (1.2)

[a] Salting out SO; organic extraction OE; cetyltrimethylammonium bromide CTAB; Chelex-100 Chelex; lithium chloride protocol LiCl; cetyltrimethylammonium bromide and Chelex-100 CTABCH.
[b] Mean (standard deviation); n = 24.

PCR yield of 1491.0 ng and a standard deviation of 554.8. The smaller and larger amounts of DNA obtained by PCR from the CTABCH extracts were 298.6 ng and 2215.0 ng, respectively. The PCR yields observed for the improved protocol described here were significantly higher than the yields observed for the rest of protocols ($p < 0.05$, Kolmogorov-Smirnov test; Fig. 5).

The amounts of template DNA used in the PCR reactions did not correlate with the corresponding PCR yields (Fig. 6), with the exception of the PCR amplifications performed from CTABCH extracts ($p = 3.085e\text{-}5$, $R^2 = 0.74$, Pearson's product moment correlation coefficient test; Fig. 6).

4. Discussion

A detailed comparison of six direct DNA extraction protocols was performed in terms of their ability for obtaining good yields of high purity DNA extracts, and their efficacy as well as their efficiency in PCR amplifications.

The DNA preparations obtained by OE, CTAB and CTABCH methods displayed the highest DNA yields along with the finest DNA grades. However the average A260/A280 ratio was 1.65; with only 29.1% of these extracts displaying ratios among 1.7 and 1.9, which correspond to high quality DNA preparations (Sambrook and Russel, 2001). An

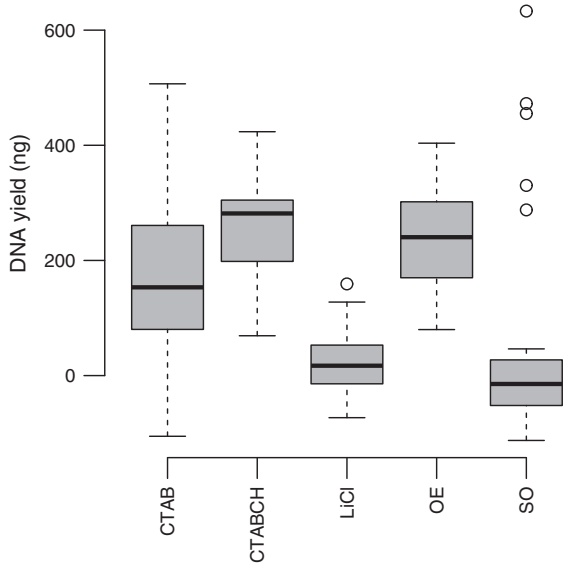

Fig. 3. Box plots of the DNA concentrations of nucleic acid preparations obtained by the DNA purification techniques evaluated in this study (N = 24). OE: Organic extraction, CTAB: cetyltrimethylammonium bromide, CTABCH: cetyltrimethylammonium bromide and Chelex-100, LiCl: lithium chloride protocol, and SO: salting out. Note that the presence of negative values obey to the fact that some samples presented DNA amounts falling outside the range of the mass standard used for calibration.

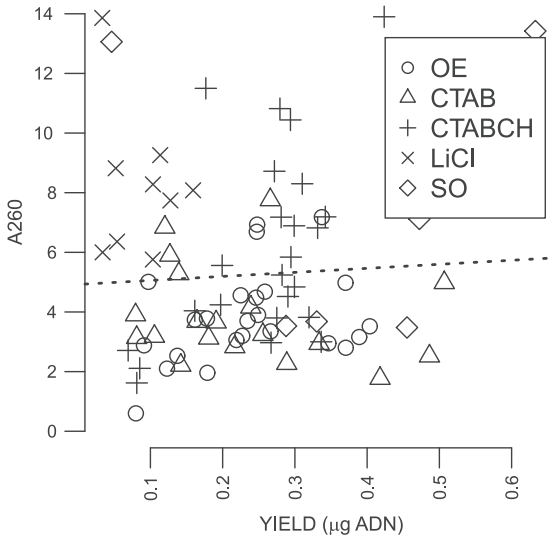

Fig. 4. Scatter plot of DNA yield *versus* absorbance at 260 nm (A260) of the DNA extracts obtained by the six methods studied here. OE: Organic extraction, CTAB: cetyltrimethylammonium bromide, CTABCH: cetyltrimethylammonium bromide and Chelex-100, LiCl: lithium chloride protocol, and SO: salting out. Samples that presented DNA amounts falling outside the range of the mass standard used to calibrate the curve were dismissed. Dashed lines correspond to fitted linear models (please see also Table 3).

equivalent scenario was observed for the values obtained at wave lengths of 230 and 320 nm, which indicates the presence of interfering salts or solvents, and particulate material respectively (Table 2). These data are in agreement with the ones observed for DNA preparation obtained from complex environmental samples containing high amounts of contaminant materials or extracellular polymeric substances (Bey et al., 2010; Sharma et al., 2014). In addition, although all the tested methods allowed for separating cellular contents from frustules (Fig. 1), 33% of the extracts displayed no visible amount of DNA in agarose gels, whereas the yield per mg of stalk for the rest of extracts ranged from 9.8 pg to 12.64 ng (mean 4.2 ng; standard deviation 2.69). These DNA yields are comparable to the ones observed for recalcitrant materials such as some herbarium and mummified plant tissues, leaves with high levels of secondary compounds and microbial mats, and are much lower than DNA yields obtained with tissues more amenable to DNA extraction (Aljanabi and Martinez, 1997; Bey et al., 2010; Cota Sanchez et al., 2006; Jackson et al., 1990; Porebski et al., 1997; Rogers and Bendich, 1985). The most likely explanation for the low yields observed could be the high proportion of stalks in relation to cells present in *D. geminata* mats, although other problems might be involved as discussed below.

Performance in enzymatic reactions such as PCR is a paramount consideration in evaluating the adequacy of DNA preparations for molecular biology applications. In this work, PCR inhibition was quite frequent for several of the studied protocols, with 41.6% of the bulk

Table 3
Relationship[a] between the amounts of DNA present in DNA suspensions obtained by the methods studied here[b] and absorbance at 260 nm.

	SO	OE	CTAB	LiCl	CTABCH
$\hat{\beta}$	1E-3 (0.95)	0.019 (0.12)	−0.016 (0.41)	−0.003 (0.58)	0.014 (0.01)
R^2	9E-4	0.103	0.040	0.044	0.26
S	0.031 (0.95)	0.103 (0.12)	0.040 (0.41)	0.044 (0.58)	0.260 (0.01)

[a] Estimated slope (p-value; t-test); R-squared; Pearson's product moment correlation coefficient (p-value; F-statistic).
[b] Salting out SO; organic extraction OE; cetyltrimethylammonium bromide CTAB; Chelex-100 CH; lithium chloride protocol LiCl; cetyltrimethylammonium bromide plus Chelex-100 CTABCH. It was not possible to determine the DNA amounts obtained by the Chelex-100 method (please see the text for further explanation).

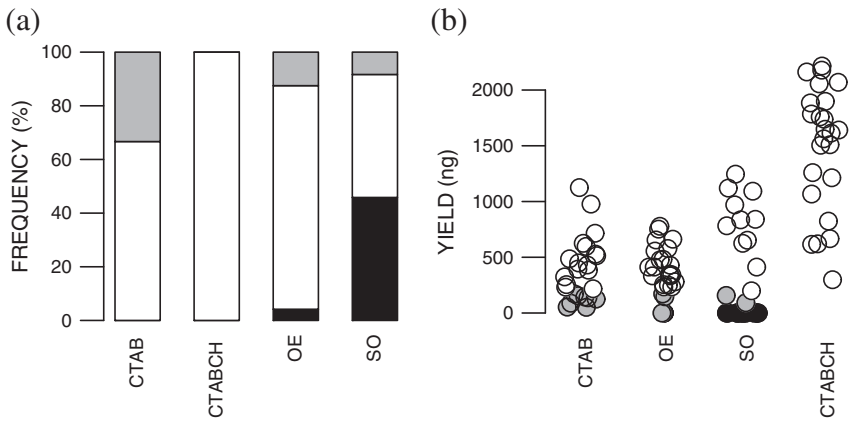

Fig. 5. Amplification performance of DNA samples obtained by cetyltrimethylammonium bromide (CTAB), organic extraction (OE), salting out (SO) and the protocol that combines the cetyltrimethylammonium bromide and Chelex-100 (CTABCH) methods. PCR efficacy rates are given in panel a. White: PCRs that yielded more than 20 ng of DNA per 5 μl; Black: no detectable amplification; gray: PCRs yielding less than 20 ng of DNA per 5 μl. Panel b displays strip chart plots corresponding to amplification efficiencies.

samples resulting in no amplification products. All the PCR reactions performed with the DNA extracts obtained by CTAB and the CTABCH protocol developed here, were successful. However, the PCR product yields observed for the CTAB samples did not correlate to the corresponding template amounts (Fig. 6). This was not the case for the samples obtained by our improved protocol, for which a clear correlation was observed between template amounts and the corresponding PCR yields ($p = 3.08e\text{-}05$, Pearson's product moment correlation coefficient test; Fig. 6), although R-squared coefficient was of 0.5332, indicating a poor fit of the data to the model. These results, combined with the absence of an evident plateau in PCR yields (Fig. 6), support the idea that the most likely reason for PCR failure is the persistence of PCR inhibitors in the DNA extracts. We believe that one factor that could explain the presence of enzymatic inhibition is the high stalks' polysaccharide content, as polysaccharides can be potent enzymatic inhibitors (Monteiro et al., 1997; Pandey et al., 1996). Protocols that use buffers with high concentrations of salts are known to facilitate the removal of polysaccharides (Porebski et al., 1997), which might explain the differences in performance observed among the OE protocol and the CTAB and CTABCH ones (Table 1; Figs. 5 and 6).

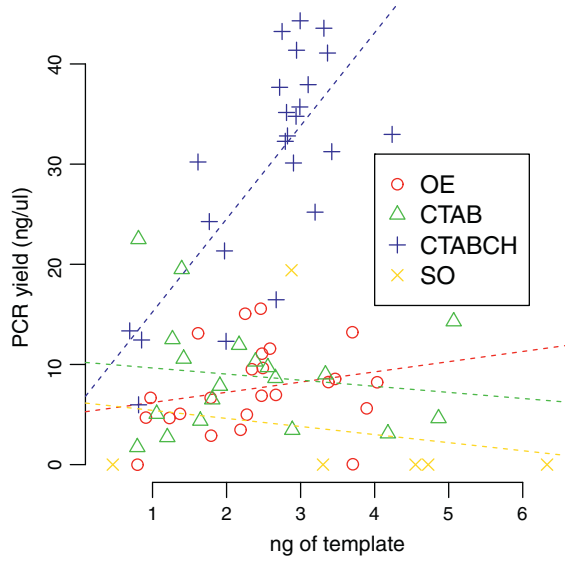

Fig. 6. Relationship among template amounts used in each PCR reaction and the corresponding DNA yields. The dashed lines correspond to fitted linear models for each DNA extraction protocol.

By the other side, it has been shown that *D. geminata* stalks accumulate significant amounts of iron (Bothwell et al., 2012; Sundareshwar et al., 2011). Iron compounds, and heavy metals in general, are thought to interfere with amplification reactions by inhibiting enzymatic activity or damaging DNA. This has led other authors to include Chelex 100, which can sequester heavy metal cations, in their DNA extraction protocols (Abramoff et al., 2004; Aldrich and Cullis, 1993). Indeed, the combined CTABCH method described here outperformed the standard CTAB protocol in PCR amplifications, which we attribute to iron clean up (Figs. 5 and 6). This could seem to contradict what we observed for the CH protocol. However, the failure of the CH protocol is better explained by the relatively small amounts of template DNA present in CH extracts relative to the other protocols (*i.e.* for CH protocol the DNA stayed in a volume of 300 μl, whereas for the rest of protocols it was resuspended in 10 μl). It has been suggested that the standard Chelex treatment is too mild for efficient lyses of the silicate thecae of diatoms (Simonelli et al., 2009), but this disagree with our observations (Fig. 1). As in CH protocol, the DNA extracts obtained by LiCl could not be amplified. In comparison to SO, CTAB and CTABCH, the LiCl protocol uses higher amounts of salt for the salting out step and uses lithium, instead of sodium, as the monovalent cation for DNA precipitation. In addition, it does not include a washing step after DNA precipitation (Table 1). Thus, we hypothesize that the high saline strength used in the salting out step of the LiCl protocol could interfere with proteinase k treatment, perhaps by causing its precipitation. Besides, the absence of a washing step could cause salts or other reagents used in the purification procedure to persist in the DNA extracts with an evident interference in downstream enzymatic amplification (Al-Soud and Rådström, 2001).

Our results support the concept that *D. geminata* benthic samples are recalcitrant DNA sources in terms of both DNA yield and the potential persistence of enzymatic inhibitors. Indirect DNA extraction methods involving cell separation before nucleic acid extraction (Bey et al., 2010) or selective sampling procedures (Cary et al., 2014) are frequently used for this sort of complex environmental samples. Indirect procedures allow the exclusion of matrix background and contaminants, as well as the presence of other microorganisms present in the sample. In this sense, other sources of DNA are present in *D. geminata* mat samples, a fact that could be interpreted as a disadvantage for *D. geminata* molecular studies due to the presence of spurious DNA. However, the use of specific primers, along with an optimization of the PCR reaction, as performed in this study, can surpass this obstacle. This might place the DNA extracts obtained by our improved method in an advantageous place, since having metagenomic DNA form the mats could potentially contribute to perform in-deep studies. Indeed, recent reports have suggested that bacterial structure in biofilm communities of *D. geminata* may be

associated with the establishment and survival of the invasive alga (Kuhajek and Wood, 2013).

In this study, it was shown that direct DNA extraction protocols that (*i*) use lysis buffers including anionic surfactants such as CTAB; (*ii*) implement buffers with high saline concentrations in order to facilitate the removal of polysaccharides, (*iii*) include washing steps after DNA precipitation and (*iv*) incorporate chelating agents, could help to overcome or at least to ameliorate the difficulties posed by benthic *D. geminata* for molecular applications. The optimized DNA extraction protocol that was developed based on these points outperformed the rest of protocols studied here, and has the potential of constituting a fundamental tool for future genetic studies of benthic *D. geminata* and the associated biota.

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