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PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages

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Abstract

Cultured strains and individually isolated dinoflagellate cells from field samples were preserved in different fixatives to find a method of cell preservation that could provide DNA template in PCR reactions and preserve cell morphology for microscopic studies. Lugol's solution and various ethanol concentrations all showed shortcomings, whereas an initial formalin preservation step followed by storage in 100% methanol fulfilled both demands. Cells could be stored up to 1 year and still provide functional DNA template for positive PCR reactions. The amplified fragment was approximately 700 bp of the D1/D2 region of the LSU rDNA, which is to our knowledge significantly longer than the low-molecular-weight DNA typically reported from formalin preserved samples. By cloning and sequencing the PCR product and subsequently aligning the sequences with previously sequenced fragments of the same or similar species, we confirmed that no base pair alteration had taken place during the time that the cells were fixed and frozen. In another experiment it was demonstrated that the growth phase of cultured *Alexandrium minutum* did not have any influence on the result of PCR reactions. This was true for extracted DNA from cultures and for direct PCR with a small number of disrupted cells. Phenol/chlorophorm/isoamylalcohol extraction proved to be an unpredictable method for DNA extraction, whereas direct PCR on isolated cells was more reliable. Extracted DNA purified with a commercial DNA cleaning kit always rendered a positive PCR. The environmental condition for cells to be used as DNA template in PCR is discussed.

Keywords: Dinoflagellate; Dinophyceae; Growth stage; PCR; Preservatives

1. Introduction

Advanced nucleic acid techniques have during recent years revolutionised the possibility to extend the range of organisms that are accessible to molecular investigations. They have made it possible to directly study the genetic structure of natural populations of single-celled organisms such as protists and algae. Moreover, accurate detection, enumeration and identification of algal species or strains that constitute only a minor component in plankton communities are now possible using different molecular methods (Anderson, 1995; Scholin and Anderson, 1998; Uribe et al., 1999; Tengs et al., 2001). Identification and detection of microalgae by oligonucleotide probes targeting ribosomal RNA (rRNA) and antibody probes (monoclonal antibodies, MAb, or polyclonal antibodies, PAb), have the advantage over traditional,

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non-quantitative polymerase chain reaction (PCR) detection because they can be quantitative techniques. However, a significant level of variability in labelling intensity in the two probe types because of environmental conditions and growth stages has been demonstrated (Vrieling et al., 1997; Anderson et al., 1999; Peperzak et al., 2000).

Collections of individual algal cells for identification purposes or DNA-based phylogenetic studies have usually been achieved using laboratory cultures, or by picking specimens from live natural plankton samples (e.g. Bolch et al., 1999; Rehnstam-Holm et al., 2002). In the latter instance, cells are typically isolated under the microscope by capillary manipulation within hours or days of the original sampling. Since single cell isolation is time consuming and requires practice and significant taxonomic skills, this strategy is not always suitable. This is especially a problem when historical bloom events have to be analysed in hindsight, or when samples have to be transported to other research experts for morphological and molecular identification or other kinds of genetic studies. This has led to an obvious need for new and better methods for preserving cells without destroying the DNA needed as template in molecular experiments. In addition, the applied methods should maintain both intact cell morphology and cell numbers, which are of uttermost importance in studies utilizing environmental samples of algae.

In this study we outline a fixation method which retains the ability to amplify, clone and sequence genes from marine algae without the need for live specimens. The technique was tested on preserved dinoflagellate cultures and individually isolated dinoflagellates from field samples. We believe that this method can form a sound basis for further investigations of this group of algae on the genetic level. In another experiment we have tested whether detection and identification of potential harmful algal species by PCR is dependent on the growth status of the cells.

2. Methods

2.1. Cultures and field sample collection

The following microalgal cultures were used in the experiments: *Prorocentrum minimum* (Pavillard) Schiller (CCMP1329) and *Alexandrium minutum* Halim (CCMP113) obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). *Alexandrium catenella* (Whedon *et* Kofoid) Balech (ACQH01), obtained from S. Hall, *A. fundyense* Balech (GTCA28) and *A. excavatum* (Braarud) Balech et Tangen (GEV1) are maintained at D.M. Anderson's laboratory at the Woods Hole Oceanographic Institution. The strains were cultured in f/2 medium (Guillard, 1975) without added silicate. Field samples were collected with a 10 μm plankton net from Perch Pond, Falmouth, MA, USA.

2.2. Cell fixation procedures and the outcome of PCR, cloning and sequencing

A serial dilution of ethanol (75–95%), acid Lugol's solution (Willén, 1962), and 5% formalin + 100% methanol (F/M fixation) were tested as preservatives on cultures of *Alexandrium fundyense* and *Prorocentrum minimum* harvested in mid log phase according to Table 1.

Ten millilitres of cell culture was pelleted by mild centrifugation (3000 rpm, 5 min) and resuspended into 10 ml of 75, 80, 85, 90, and 95% of ice-cold ethanol. From cultures fixed in 1% acid Lugol's solution, 10 ml was extracted and pelleted as for ethanol treatment. The supernatant was withdrawn and the cells were resuspended in 10 ml sterile sea water. In addition to the cultures of *A. fundyense* and *P. minimum*, cultures of *A. catenella* and *A. excavatum* and field samples were used in the F/M fixation test. For the F/M fixation, 10 ml of cultures or 50 ml of field samples were spun down and resuspended in a buffered 5% formalin solution, stored on ice for 5–30 min and thereafter pelleted once more. The formalin solution was withdrawn and the cells were resuspended in 10 ml of 100% ice-cold methanol.

Single cells of *Dinophysis acuminata* Claparède *et* Lachmann, collected by micropipetting under the microscope, were isolated from the F/M-fixed field samples. The cells were washed twice in sterile water and thereafter directly used as templates in PCR reactions.

Alexandrium catenella and *A. excavatum* cultures were F/M-fixed and stored in methanol, at −80 °C for 1 year before they were used in PCR reactions, cloning and sequencing.

Table 1
Result of the amplification of LSU rRNA from preserved dinoflagellate strains

Strains	Lugol	Ethanol 75%	Ethanol 80%	Ethanol 85%	Ethanol 90%	Ethanol 95%	F/M[a]
Prorocentrum minimum	−	+	+	−	−	+	+
Alexandrium fundyense	−	+	+	+	−	+	+

[a] F/M: formalin/methanol fixation (see text).

One hundred microlitres of ethanol, Lugol and F/M-fixed cultures were collected by mild centrifugation (3000 rpm for 5 min) and the pellet was washed once in 100 μl 1 × TE (10 mM Tris–HCl pH 7.5, 1 mM ethylenediaminetetra acetate, EDTA). The cells were diluted into 10 μl of sterile Milli-Q water (Millipore Corp., Molsheim, France). One to ten microlitres of the sample was directly added to a mixture of 5 μl PCR buffer, 5 μl dNTP mix (1 mM each of the nucleotides dATP, dCTP, dGTP and dTTP), 2.5 μl $MgCl_2$ (25 mM), 1.5 μl (1 μM) each of the 28S rRNA specific primers D1R (forward, *Prorocentrum micans* position 24-43, 5′-ACCCGCTGAATTTAAGCATA) and D2C (reverse, *P. micans* position 733-714, 5′-CCTTGGTCCGTGTTTCAAGA) (Scholin et al., 1994). Sterile Milli-Q water was added to the reaction mixture to a final volume of 50 μl. The mixture was heated to 95 °C for 5 min and the primers were annealed at 45 °C for 1 min, after which three units of Taq DNA polymerase (Promega, Madison, WI, USA) was added. The PCR reaction was run for 30 cycles consisting of repetitive rounds of 95 °C, 1 min; 45 °C, 1.5 min and 72 °C, 1 min, followed by a final elongation step at 72 °C for 5 min. The PCR product was loaded together with dye buffer onto a 1% TAE (Tris–acetate–EDTA) low melt agarose gel (Ausubel et al., 1987). Ethidium-bromide stained gels were studied under UV-transillumination. Weak positive DNA fragments were cut out from the gel and extracted with Micropure™ Separator and Gel Nebulizer (Amicon, Danvers, MA, USA). Cleaned DNA (1–10 μl) was used as template in a new PCR round until the concentration of the product was approximate 100 ng.

For cloning, and thereafter sequencing, of the PCR products the Original TA cloning® kit from Invitrogen (Carlsbad, CA, USA) was used. Gel slices containing DNA fragments were cut from the gel, melted and ligation was performed directly with 3 μl of agarose–PCR product (approximately 1–10 ng), 1 μl of 10 × ligation buffer, 2 μl (25 ng μl^{-1}) pre-cut PCR 2.1 vector, 1 μl T4 ligase and Milli-Q water to a final volume of 10 μl. The ligation reaction was incubated at 16 °C for 18 h. Transformation was performed with competent *Escherichia coli* INVαF′ cells according to the manufacturer. At least 10 positive (white) colonies were picked for further analysis. Isolated colonies were grown overnight at 37 °C in 3 ml Luria Broth (LB) containing 50 μg ml^{-1} of ampicillin. Wizard miniprep kit (Promega, WI, USA) was used for plasmid isolation. Purified plasmids were analysed by *Eco*RI restriction enzyme digestion to determinate correct inserts.

Nucleotide information was obtained using M13 primers (M13 forward −20 and M13 reverse −40) and the SequiTherm EXEL TM Long-Read DNA sequencing kit LC (Epicenter Technologies, Chicago, IL, USA) for automated sequencing. Reaction products were run on a LI-COR R Sequencer (LI-COR Biosciences, NE, USA).

2.3. Growth status and PCR

Cultured *Alexandrium minutum* was quantified in a Zeiss Axiovert 135 inverted microscope. Forty-six 50 ml Nunc polystyrene flasks were prepared with 1 ml of *A. minutum* culture of known density and filled to final volume of 40 ml with f/2 media, silica excluded. The bottles were kept at 18 °C, 55 μE m^{-2} s^{-1} in L:D 16:8.

At each day of sampling one flask was taken for analyses and treated as follows: (1) In order to determine cell abundance, 0.2 ml was extracted from the flask and the volume was adjusted to 10 ml with filtered seawater (0.22 μm, 26 PSU). This volume was thereafter fixed in acid Lugol's solution and poured into an Utermöhl-type sedimentation chamber (Utermöhl, 1931). After a minimum of 12 h, the number of *A. minutum* cells was counted at a magnification of 200×. (2) Thirty millilitres from the flask was used

for DNA extraction. This volume was centrifuged at 3000 rpm for 5 min and thereafter the supernatant was discarded. The algal pellet was redissolved in 420 μl sterile autoclaved Milli-Q water and stored at −80 °C until analysed within 2 months. (3) 1–250 μl from the sampled flask was taken for direct PCR and pipetted directly into a PCR tube. Two replicate samples were taken. We wanted to have approximately 50 cells/tube for the direct PCR. The volume to extract was calculated from the preceding sample's cell abundance ($N_{t+1} = R^t \times N_t$, where N is the cell abundance (cells ml^{-1}), t the time (days) and R the growth rate). The PCR tubes were centrifuged at 3000 rpm for 5 min and the supernatant was carefully removed. Thereafter the cells were washed in 100 μl sterile Milli-Q water, again centrifuged at 3000 rpm for 5 min and the supernatant was discarded. The sample was stored at −80 °C and analysed within 2 months.

The algal pellet used for DNA extraction was thawed and 20 μl 250 mM EDTA (pH 8.0), 50 μl 10% sodium dodecyl sulphate (SDS), 5 μl 1 M Tris–HCl (pH 7.5), and 5 μl 1 M NaCl was added. DNA were extracted with buffered phenol/chloroform/isoamylalcohol (24:24:1). The mixture was vortexed for 1 min and centrifuged at 15000 rpm for 3 min. The upper aqueous phase was extracted twice with phenol/chloroform/isoamylalcohol and thereafter the DNA was precipitated at −20 °C with 1/10 volume of 3 M Na-acetate (pH 4.5), and 2 volumes of absolute ethanol for a minimum of 1 h. DNA was pelleted by centrifugation at 15000 rpm for 30 min. The pellet was washed with cold 70% ethanol, centrifuged again at the same speed and the new pellet was dried for 5 min at 49 °C (Ausubel et al., 1987). Before the DNA was used in the PCR reaction it was re-suspended in 25–50 μl of sterile Milli-Q water. In cases when the PCR was negative the DNA extracts were further purified using FlexiPrep Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) following the protocol of the manufacturer. Concentration and purity of DNA was checked on a GeneQuant II, RNA/DNA Calculator (Pharmacia Biotech, Piscataway, NJ, USA) at wavelengths 260 and 280 nm. DNA samples were stored at −20 °C.

The PCR tubes containing *A. minutum* cells, which were to be used directly as templates in the PCR reactions, were moved from liquid nitrogen (−196 °C) to 96 °C in order to disrupt the cells within the tubes. The tubes were exposed to 4 × 5 min at each extreme.

Extracted DNA and crude DNA from disrupted cells were amplified using primers designed for *A. minutum* identification (Godhe et al., 2001). The PCR reactions were run in total volumes of 25 μl each, consisting of Ready-To-Go™ PCR-beads (Amersham Pharmacia Biotech), 0.25 μl of each primer, 1 μl template DNA (in the case of samples with extracted DNA) and sterile Milli-Q water. Amplification was carried out in a thermal cycler (Perkin-Elmer, GeneAmp PCR System 2400) as follows: initially 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min. After the cycles, extension was completed at 72 °C for 7 min. Ten microlitres of the PCR product was loaded together with 2 μl dye buffer onto 0.8% agarose gel in 0.5 × TAE buffer (Ausubel et al., 1987). Ethidium-bromide stained gels were studied under UV-transillumination.

3. Results

One of our goals was to achieve positive amplification of DNA fragments from preserved cells. Initially cells preserved in different ethanol dilutions (75–95%) were tested to see if they were suitable as DNA templates (Table 1). Positive PCR products could always be achieved from cells preserved in low concentrations of ethanol (75–80%), whereas amplification was more variable for cells preserved in higher ethanol concentrations. No intact cells could be observed in samples preserved in 75% ethanol, and there was approximately a 10% loss of cells in the 95% ethanol preserved sample after 2 weeks of storage. Samples fixed in Lugol's solution never generated a positive PCR product. A short (10–30 min) initial formalin preservation step followed by a transfer to 100% methanol was also tested. Both cultured dinoflagellates and dinoflagellates isolated from field samples preserved according to this method, kept their morphological features and could be used successfully as templates in amplification reactions. Positive PCR products were achieved in 87.5% of the reactions when cultured dinoflagellates preserved in this manner were used as the DNA template. For the reactions with individually isolated cells from field samples, 17.3%

of the reactions were positive. All positive reactions rendered full size (~700 bp) fragments of the D1/D2 region of large subunit ribosomal RNA (LSU rRNA).

In order to check both purity of the amplified DNA, and to determine if any base alteration had occurred, positive PCR products from *Alexandrium catenella* and *A. excavatum*, preserved in formalin and stored in methanol for 1 year, were cloned and sequenced. Alignment of *A. excavatum*, strain GEV1 (GenBank accession number AY056824) showed 100% sequence identity with the same unpreserved strain previously sequenced (*A. excavatum* strain GE1V, GenBank accession number L38632). The *A. catenella* (GenBank accession number AY056823) strain has not previously been sequenced, but showed, as predicted, high similarity to other strains within the "*Alexandrium tamarense*-complex". The D1/D2 region of the LSU rRNA gene from formalin/methanol-fixed environmental isolates of *Dinophysis acuminata* cells were also successfully amplified, cloned and sequenced. The results from this analysis are reported in Rehnstam-Holm et al. (2002).

In the *Alexandrium minutum* growth experiment, the first sampled flask was taken on the 5th day after the preparation of the 46 Nunc flasks (day 0), and the last flask was sampled on day 459. The density of the culture ranged from 75 cells/ml on day 0 to a maximum of 114176 cells/ml on day 84 (Table 2, Fig. 1A). On the last day of sampling, day 459, the density of the culture was 3950 cells/ml. The last flask contained numerous empty thecae but the vital cells appeared normal. Since R ($N_{t+1} = R^t \times N_t$) was fluctuating somewhat from one sampling occasion to the other, the volumes of 1–250 μl extracted for direct PCR represented 16–451 cells. Hence this spread of number of cells for direct

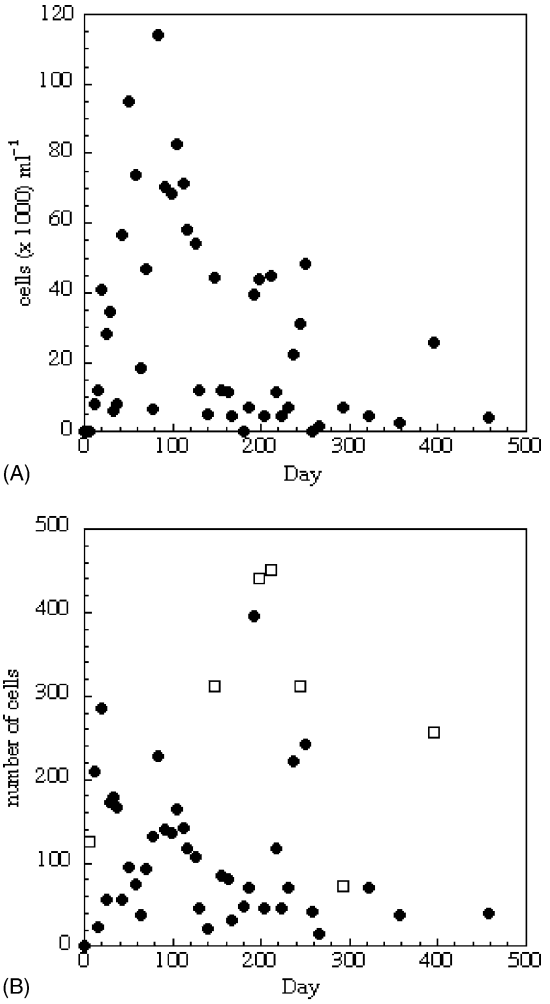

Fig. 1. (A) Cell yield of cultured *Alexandrium minutum* during prolonged batch culture. (B) Number of *A. minutum* cells used for direct PCR amplification (● denotes positive direct PCR, □ denotes negative direct PCR).

Table 2
Details on PCR results of cultured *Alexandrium minutum*

Days sampled	5–459
Percentage of positive direct PCR	85
Percentage of positive PCR–phenol/chloroform extract	39
Percentage of positive PCR–gene clean kit	100
Range of DNA conc. yielding positive PCR (μg μl^{-1})	0.0037–0.79
Range of R ($N_{t+1} = R^t \times N_t$)	0.72–1.74
Range of number of cells/ml in culture	503–114176
Range of number of cells used for direct PCR	16–451

PCR (Table 2). Direct PCR with primers designed for *A. minutum* detection and disrupted *A. minutum* cells constituting the DNA template, yielded positive results in 85% of the samples (Table 2, Fig. 1B). On seven sampling occasions, the direct PCR failed to give a positive result (day 5, 147, 197, 211, 244, 293, 396), representing 73–451 disrupted cells. On five of the seven occasions, the volume extracted for direct PCR contained a high number of cells. In the PCR reactions where DNA extraction was used to produce a template, 39% of the phenol/chlorophorm extracted

DNA yielded positive PCR reactions. In cases of negative PCR results, the DNA extracts were further purified with a DNA cleaning kit. These extracts always yielded a positive PCR result (Table 2). The concentration of DNA did not influence whether or not the PCR was positive or negative using extracted DNA as the template. The results of the PCR on directly disrupted cells or on DNA extracts were also independent of the age of the culture, i.e. growth stage.

4. Discussion

We found that cultured or natural samples of thecate dinoflagellates fixed in formalin for a maximum of 30 min and thereafter stored in 100% methanol kept their morphology intact and could be used as template DNA in PCR reactions (Table 1). Full size (~700 bp) amplified fragments of the D1/D2 region of LSU rDNA were amplified. This is, to our knowledge, significantly longer than the low-molecular-weight DNA typically reported from formalin preserved samples. Further, cloning, sequencing and alignment with published sequences from the same clone or related strains demonstrated that no base pair alteration had taken place following this fixation and extended storage. It should be stressed that this preservation method might not be suitable for preserving non-thecate dinoflagellate species, since they are known to alter their morphology when fixed (Takayama et al., 1998).

Glutaraldehyde and pure formalin-fixed algal cells have previously been reported as inadequate as templates in DNA amplification reactions (e.g. Marín et al., 2001). Therefore these fixatives were excluded in this study. Lugol's solution (iodine-based) is one of the most commonly used fixatives in phytoplankton research. Iodine is a strong, halogenating agent that probably not only acts on proteins, but also may bind to nucleic acids, thereby making DNA inaccessible to amplification. We were never able to get any amplified product from algal cells preserved with Lugol's solution (Table 1). This is in accordance with the result obtained by Marín et al. (2001). However, Tengs et al. (2001) reported that Lugol's-fixed field samples containing the target organism could be used as template in real-time PCR. Likewise, Bowers et al. (2000) successfully extracted DNA from 1% Lugol's-fixed cultures or field samples spiked with the target organisms and used this DNA as a template in real-time PCR with a positive result. The Lugol's preserved samples required 10 × higher cell concentration for positive PCR result, compared to unpreserved DNA extracts. In our case, the poor success with Lugol's preserved material may reflect the inadequate amount of biomass or extracted DNA we were amplifying.

Some studies have revealed that formalin does not interfere with molecular reactions. It has been shown in various techniques, e.g. pulse-field electrophoresis typing, that a treatment with formalin for 1 h neutralizes DNase activity and allows typing of strains (Wilson, 1997). Formalin (3.7%) fixed cultures and field samples, stored for a maximum of 3 days, have also been used in quantitative in situ hybridisation experiments with oligonucleotide probes targeting nanoplankton (Lim et al., 1996). But, whole cell hybridisation of various *Pseudo-nitzschia* species fixed in formalin for 1 h and thereafter stored in ethanol (70%), methanol (90%) or saline ethanol did not fluorescence well. The result was dim and gave an uneven distribution of labels. A saline ethanol solution gave the best result (Miller and Scholin, 2000).

Cultures and field samples preserved in different concentrations of ethanol and methanol have been successfully used in PCR experiments (Marín et al., 2001). PCR products of predicted length were also obtained in our study from cells preserved in different concentrations (75–95%) of ethanol (Table 1). This preservation method may be preferred when fixing pure cultures, or when cell morphology and cell numbers in a given sample are not desired. However, the significant cell-loss that was observed with these levels of ethanol eliminates ethanol fixation as suitable for preservation and long-term storage of environmental dinoflagellate samples.

One concern has been whether the stage of the growth cycle and nutritional status are important for the lysis of cells and hence DNA extraction for use in PCR (Wilson, 1997), but thus far, these factors have been little studied. In the experiment where we determined whether *Alexandrium minutum* cells could be used in PCR in different growth stages, we found that the age of the culture, i.e. the growth stage, did not matter. This was true both for the extracted DNA and the direct PCR (Table 2). The nutrient conditions in the culture flasks were not monitored during the experiment, but it is reasonable to expect that during 461

days, without any addition of new culture medium, the nutritional status of the cells had dramatically changed. Furthermore, an extensive production of secondary metabolites, change of pH, etc. may also have taken place in this very old culture. None of these factors seemed to influence PCR results. The direct PCR was positive in 85% of the samples. In five of the seven samples where the PCR failed the number of cells were high (Fig. 1B). Hence, we suspect that this high number of cells used for the reaction could have generated an inhibitory effect. However, the occasions displaying negative results of direct PCR are not correlating with the age of the culture.

Only 39% of the phenol/chlorophorm/isoamylalcohol extracted DNA samples generated a positive PCR, whereas when these extracts were further purified, they always generated a product of expected length. Studies examining the pit-falls of PCR have found that many phenol/chlorophorm extracted DNA samples fail to amplify. Phenolic compounds may act as inhibitors by denaturing the lytic enzymes and thereby failing to expose the DNA, or by inhibiting the reaction by binding to or denaturating the polymerase (Wilson, 1997).

The PCR reactions were never dependent on the cell concentration or the growth stage of the culture. As few as 16 cells were needed for successful amplification in direct PCR (Table 2, Fig. 1B), though this probably greatly exceeds the threshold number of cells required for a positive reaction. Our results demonstrate clearly that PCR can be performed on a small number of cells without the need for DNA extraction. This high sensitivity of PCR has previously been demonstrated by several authors (Penna and Magnani, 1999; Bowers et al., 2000; Godhe et al., 2001; Marín et al., 2001; Tengs et al., 2001).

5. Conclusion

In this report we have outlined a formalin/methanol preservation technique for algal samples that allows us to use the cells directly as templates in amplification reactions. We were able to amplify and sequence the whole D1/D2 region of LSU rDNA (∼700 base pairs) from formalin/methanol-fixed *Alexandrium* strains that had been stored at −80 °C for 1 year. We thereby also showed that no sequence alterations had occurred in the fixed cells during this prolonged storage period by comparing the result with previously reported LSU rDNA sequences. PCR products of predicted length were also achieved from cells preserved in different concentrations of ethanol (75–95%), but in those cases, the cell morphology was aberrant.

We have performed PCR on extracted DNA and disrupted cells from a culture of *Alexandrium minutum* during an extended culturing interval. The results show that the growth stage of the culture did not influence the reactions. Phenol/chlorophorm/isoamylalcohol extraction of DNA proved to be an unpredictable method for DNA extraction, whereas direct PCR was more reliable. Extracted DNA purified with a commercial DNA cleaning kit always rendered a positive PCR.

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