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ORIGINAL ARTICLE

Capture probe conjugated to paramagnetic nanoparticles for purification of *Alexandrium* species (Dinophyceae) DNA from environmental samples

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Abstract

Aims: To develop a rapid, cost-effective and selective *Alexandrium* DNA extraction procedure from environmental samples in order to provide good-quality template for the downstream PCR-based detection assay.
Methods and Results: In this study, we tested a DNA extraction method based on silica-coated, superparamagnetic nanoparticles conjugated to a DNA-capture sequence (probe) complementary to a specific region of 5·8S rDNA of the genus *Alexandrium*. Cultured *Alexandrium catenella* cells were used as the harmful algal bloom species for the DNA extraction. Then, a PCR assay was performed with primers specific for the genus *Alexandrium* to assess the specificity and sensitivity of the nucleic acid extraction method. This method was applied to both cultured and field samples, reaching in both cases a detection limit of one *A. catenella* cell.
Conclusions: The results suggest that the use of probe-conjugated paramagnetic nanoparticles could be effective for the specific purification of microalgal DNA in cultured or environmental samples, ensuring sensitivity and specificity of the subsequent PCR assays.
Significance and Impact of the Study: The DNA extraction method optimized in this study represents a progress towards the rapid and efficient direct detection of *Alexandrium* cells in seawater monitoring. In fact, this method requires no other equipment than a magnet and a hybridization oven and, in principle, can be adapted to different toxic microalgal species and can be automated, allowing the processing of a high number of samples.

Introduction

The occurrence of harmful algal blooms (HABs) in the Mediterranean Sea is a serious and increasing problem involving both human health and fishing industry. In fact, algal toxins can accumulate in mussels and, when consumed, can cause syndromes such as paralytic shellfish poisoning (PSP) or diarrhoeic shellfish poisoning in humans. Dinoflagellates belonging to the genus *Alexandrium* are the most widespread toxic microalgae responsible for PSP intoxication in the Mediterranean basin (Vila *et al.* 2001). An important contribution in the understanding of the HAB phenomena is represented by the detection and enumeration of target phytoplankton species found in seawater samples. Presently, this task is accomplished through microscope-based cell identification methods that are time consuming and require remarkable taxonomic skills to discriminate

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morphological features indicative of the HAB species. Several molecular methods have been developed in the last decade for the detection of genetically distinct HAB species, including PCR techniques and DNA probes against ribosomal DNA (rDNA) such as the large subunit, small subunit, 5·8S and internal transcribe spacer regions. These innovative methods could represent a rapid and sensitive alternative to standard methods (Godhe *et al.* 2001; Guillou *et al.* 2002; Saito *et al.* 2002). A PCR-based method for the detection of dinoflagellates belonging to the genus *Alexandrium*, targeting the 5·8S rDNA region, has been previously developed (Galluzzi *et al.* 2005). Two drawbacks in testing environmental samples by PCR are the presence of enzymatic inhibitors and the limited volume of the sample that can be processed (Ma *et al.* 1995). In this context, an effective and selective DNA extraction method that can be applied to a mixed phytoplankton population sample could be useful in increasing the efficiency of a standard PCR assay. In fact, a large quantity of nontarget phytoplankton cell genomic DNA in the sample to be analysed may reduce the sensitivity of the PCR towards the DNA of the target phytoplankton species, as the quantity of total DNA per reaction tube represents a limiting factor. Indeed, an excess of template DNA in the reaction tube could lead to PCR inhibition. To address this issue, we developed a rapid, sensitive and cost-effective DNA isolation procedure based on silica-coated paramagnetic nanoparticles conjugated to a DNA-capture sequence complementary to a specific region of the rDNA of the genus *Alexandrium*. In this approach, the target sequences are hybridized to the specific probe and concentrated by a magnetic separation process. Target sequences isolated in this manner may be amplified directly in the PCR assay. Additional advantages inherent to the sequence-capture approach include the simultaneous elimination of the PCR inhibitory substances (Jacobsen 1995) and nonspecific DNA. This procedure has been applied successfully to improve the PCR-based detection of various viral and bacterial nucleic acids in clinical or environmental samples (Heermann *et al.* 1994; Millar *et al.* 1995; Mangiapan *et al.* 1996; Regan and Margolin 1997; Miyachi *et al.* 2000; Maibach *et al.* 2002; Tanaka *et al.* 2004).

In this study, NH_2-modified DNA probes were coupled to a new class of silica-coated magnetic nanoparticles harbouring NH_2 groups on the surface and used to capture the target gene. These nanoparticles were substantially smaller than the commonly used Dynabeads (Invitrogen, Carlsbad, CA, USA), providing a higher density of amino groups per mass unit of support available for the coupling of the capture probe and for the recovery of DNA. The captured target was subsequently amplified directly by conventional PCR with primers specific for *Alexandrium* spp. in order to assess the specificity and sensitivity of the method. We examined the efficacy of rDNA recovery both from cultures and from marine field samples preserved with Lugol's solution, using *A. catenella* as model phytoplanktonic micro-organism.

Materials and methods

Algal cultures and seawater sample collection

The dinoflagellate *A. catenella* strain CNR-S1 (kindly provided by Dr M.G. Giacobbe, CNR, Messina, Italy) and the diatom *Navicula* spp. strain CBA-1 were grown in batch cultures in f/2-enriched seawater medium (Guillard 1975) for 28 days at 18°C. Light was provided by cool-white fluorescent tubes at a photon flux of 100 μmol m^{-2} s^{-1} on a 14:10 LD cycle. Algal cell concentration was determined by counting Lugol-solution-stained cells (Throndsen 1978) using a haemacytometer (Neubauer; Hausser Scientific, Horsham, PA, USA). Field samples were collected from the Adriatic Sea (Italy) using a 10-μm-mesh plankton net. A volume of 250 ml was fixed with Lugol's iodine solution and 10 ml preserved sample was counted in Utermöhl chambers using an inverted microscope.

Probes and primers design

The primers and the capture probe (AP) specific for the genus *Alexandrium* were selected in the 5·8S rDNA region which is known to be highly conserved in this genus. The forward primer 5·8S-5′ (5′-GCA ADG AAT GTC TTA GCT CAA-3′) and the reverse primer 5·8S-3′ (5′-GCA MAC CTT CAA GMA TAT CCC-3′) were designed as previously described (Galluzzi *et al.* 2005). The capture probe, AP (5′-TYG GGA TAT KCT TGA AGG TDT G-3′), was designed using Oligo 6 primer analysis software and a multiple alignment of 5·8S rDNA *Alexandrium* sequences available in the EMBL database. The alignment was constructed using DIALIGN 2.0 (Morgenstern *et al.* 1996) and CLUSTALW 1.8 (Thompson *et al.* 1994). The capture probe sequence partially overlapped the primer 5·8S-3′. The sequence specificity of the probe was confirmed using Basic Local Alignment Search Tool (BLAST). Two probe structures were tested in hybrid-capture experiments. One was modified with a 12-carbon spacer amino group at the 5′ end, while the other contained six extra cytidine residues (to minimize inhibitory effects including steric hindrance) and was modified with a six-carbon spacer amino group at the 5′ end. A fluorescent probe complementary to the AP sequence (ACP; 5′-CAH ACC TTC AAG MAT ATC CCR A-3′) was also used to test the capture efficiency. All the oligonucleotides were purchased from MWG Biotech AG (Ebersberg, Germany).

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Magnetic nanoparticle modifications

The amino-modified, silica-coated magnetic nanoparticles (30–100 nm diameter) were produced and coupled to the amino-modified capture probe, AP, via glutaraldehyde as previously described in details by Del Campo *et al.* (2005). A scheme of the coupling mechanism is presented in Fig. 1. Briefly, 5 mg modified nanoparticles was resuspended in 0·5 ml of a 5% v/v glutaraldehyde solution in 1X SSC pH 7·3. After 3 h incubation at 18°C, the material was washed three times with 1 ml 1X SSC pH 7·3 to remove excess of glutaraldehyde. A 0·5-ml volume of a 10-μmol l^{-1} solution of 5′ amino-modified oligonucleotide in 1X SSC pH 7·3 was added, and the mixture was incubated overnight while shaking. The oligo-modified nanoparticles were then washed once with 1X SSC pH 7·3 and placed in 0·5 ml $NaBH_3CN$ solution (0·03% w/v in 1X SSC pH 7·3) for 30 min at 18°C. The material was then washed three times with 0·8 ml 1X SSC pH 7·3 and resuspended in 0·5 ml the same buffer. The AP-modified nanoparticles were either used in this form (unblocked) or after further four washing steps with a blocking solution [phosphate-buffered saline (PBS) pH 7·4 containing 40 mmol l^{-1} glycine and 0·5% bovine serum albumin (BSA)] and resuspension in storage buffer (PBS pH 7·4 containing 0·1% BSA) at a final concentration of about 10 mg ml^{-1} (blocked nanoparticles). The AP-modified nanoparticles were washed and resuspended in distilled water before use. Three different batches of modified nanoparticles were produced to test the standardization of the method and the reproducibility of the results in our marine application.

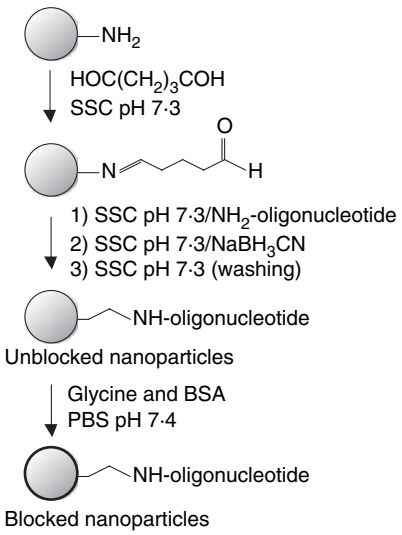

Figure 1 Coupling of amino-modified capture probe to amino-modified silica paramagnetic nanoparticles via glutaraldehyde (adapted from Del Campo *et al.* 2005). See text for details.

Capture efficiency test

The capture efficiency of the two different probe structures (see Probes and Primers Design) immobilized on the surface of the nanoparticles was evaluated using a complementary fluorescent probe (ACP) as previously described (Del Campo *et al.* 2005). Briefly, 300 pmol of fluorescent probe ACP in 13X SSC/0·05% BSA were added to 1 mg AP-modified nanoparticles. After 30 min at room temperature with gentle shaking, the nanoparticles were washed three times with 1 ml 13X SSC and resuspended in 200 μl distilled water. The nanoparticles suspension was heated to 85°C for 4 min to obtain dehybridization, and the supernatant was removed. The amount of fluorescent ACP selectively captured and recovered was determined using a spectrofluorophotometer RF-5301PC (Shimadzu, Kyoto, Japan). A negative control experiment was also performed with a noncomplementary fluorescent oligonucleotide.

DNA sequences capture

The cultured and natural samples containing various quantities of *Alexandrium* cells were harvested by centrifugation at 3000 ***g*** for 10 min. Cell pellets were washed twice with artificial sea water (0·4 mol l^{-1} NaCl, 10 mmol l^{-1} KCl, 20 mmol l^{-1} $MgSO_4$, 20 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $NaHCO_3$, 0·4 mmol l^{-1} H_3BO_3) and were frozen at −80°C to facilitate cellular lysis. Frozen pellets were resuspended in 0·3 ml lysis buffer (50 mmol l^{-1} Tris–HCl pH 8·0, 50 mmol l^{-1} EDTA pH 8·0, 0·5% Triton X-100) and incubated at 55°C for 1 h with 5 μl of 20 mg ml^{-1} proteinase K. The samples were boiled for 15 min to allow the denaturation of DNA and inactivation of proteinase K; then they were immediately cooled on ice. Afterwards, 333 μl 20X SSC buffer (3 mol l^{-1} NaCl, 0·3 mol l^{-1} sodium citrate, pH 7·3), 5 μl of 10 mg ml^{-1} AP-modified nanoparticles and distilled water to reach the final volume of 1 ml were added to each sample. The AP-modified nanoparticles were incubated at 80°C for 4 min before use to stretch out the probe and eliminate possible hairpin structures. The hybridization was performed at 52°C for 1 h under constant rotation. After hybridization, the magnetic nanoparticles/DNA complex was immobilized on a magnetic support and washed three times with the washing buffer (1 mol l^{-1} NaCl, 10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA pH 7·4). Finally, the magnetic nanoparticles/DNA complex was resuspended in 20 μl distilled water, and 2 μl of this suspension (equivalent to 5 μg of nanoparticles) was used directly in the PCR.

The sensitivity experiments were performed mixing different quantities of *A. catenella* cells with *Navicula* spp. cells

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Table 1 Mixed samples of cultured *Alexandrium catenella* and *Navicula* spp. cells used for the sensitivity experiments

Sample	*A. catenella* cells	*Navicula* spp. cells	Percentage of *A. catenella* cells in the sample
A	100	900	10
B	100	9900	1
C	100	99 900	0·1
D	100	250 000	0·04
E	100	999 900	0·01
F	10	990	1
G	10	9990	0·1
H	10	99 990	0·01
I	10	250 000	0·004
L	1	250 000	0·0004

(diatoms) serving as the cellular background. The percentage of *A. catenella* cells in the samples ranged from 10 to 0·0004% of the total cells, while the absolute number of *A. catenella* cells in the samples varied from 100 to 1. All the experiment conditions considered are summarized in Table 1. While 100 and 10 cells were added in the samples after accurate counting and dilution of *A. catenella* cell culture, the single cells were picked up using an inverted microscope and a micropipette in a volume of 1 μl and were added manually to the sample pellet. Each condition of these experiments was performed in triplicate. In all experiments, a sample containing only *Navicula* spp. cells was used as a negative control to check for possible contamination of *Alexandrium* spp. nucleic acids during the DNA extraction. A 50-ml volume of fixed seawater net samples was also contaminated with 100, 10 and one *A. catenella* cells in order to confirm the sensitivity of the method when applied to natural samples.

DNA purification

Microalgal genomic DNA was purified using the DNEasy plant mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions, and a phenol–chloroform-based method as previously described (Galluzzi *et al.* 2005). DNA purity and concentration were determined by absorbance at 260/280 nm. Alternatively, purified DNA was quantified on agarose gel using serially diluted λDNA/*Hin*dIII (Fermentas GmBH, St. Leon-Rot, Germany) and a gel-doc apparatus (Bio-Rad, Hercules, CA, USA).

PCR assay optimization

The PCR specificity and sensitivity and the PCR conditions have been described previously (Galluzzi *et al.* 2005). Each PCR tube contained reaction buffer (67 mmol l^{-1} Tris–HCl, pH 8·8, 16·6 mmol l^{-1} $(NH_4)_2SO_4$, 0·01% Tween 20, 3 mmol l^{-1} $MgCl_2$), 0·4 μmol l^{-1} of each primer, 200 μmol l^{-1} each of dATP, dCTP, dTTP, dGTP and 0·1 units of Hot-Rescue DNA polymerase (Diatheva srl, Fano, Italy), in a final volume of 50 μl. The potential inhibitory effects of the AP-modified nanoparticles in the PCRs were evaluated by adding various amounts of these nanoparticles, ranging from 2·5 to 12·5 μg, in PCR tubes containing 0·1 ng *A. catenella* genomic DNA as template and measuring the band intensities after agarose gel electrophoresis in a gel-doc apparatus (Bio-Rad). We evaluated the inhibitory effects of these materials using either nanoparticles treated with a blocking solution (see Magnetic Nanoparticle Modifications) or untreated nanoparticles. Before the PCR assay, the nanoparticles were washed and resuspended with distilled water to a final concentration of 2·5 mg ml^{-1}. Positive controls were spiked with 10^4 copies of *A. catenella* 5·8S rDNA-cloned sequence. Alternatively, the *A. catenella* 5·8S rDNA-cloned sequence containing an insertion of 82 bp was used as positive control to avoid false-positive results because of plasmid contamination. Negative controls (no template) were included in each PCR. Twenty microlitres of PCR product were run in 1·8% (w/v) agarose gel and visualized with ethidium bromide.

In order to test the potential inhibition of AP-modified magnetic nanoparticles in a real-time PCR assay, 10^7 copies of a plasmid containing the 5·8S rDNA sequence of *A. minutum* were amplified in duplicate. One of the reactions was spiked with the 5-μg AP-modified magnetic nanoparticles, and the two amplification plots were compared either in the exponential phase or at the end point.

Results

Hybrid-capture efficiency

The hybrid-capture efficiency test was performed using three different batches of nanoparticles. The results obtained were very similar indicating the reliability of the modified magnetic support used for the experiments. Moreover, no significant differences were observed using the two probe structures described in Materials and Methods. For this reason, we used both probe structures (always indicated as AP in the capture experiments) without distinction. In the tested conditions, 120 ± 16 pmol of fluorescein-labelled probe ACP were selectively captured and recovered on 1 mg of AP-modified nanoparticles. This value was obtained from six different experiments using three different batches of nanoparticles. No sequence capture was observed when a noncomplementary fluorescent oligonucleotide was used in the experiments (data not shown).

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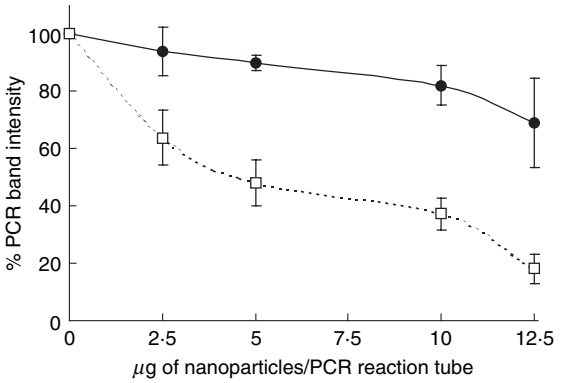

Figure 2 Evaluation of the inhibitory effects of AP-modified nanoparticles in the PCRs. 2.5–12.5 μg of the blocked (black circles) or unblocked (white squares) AP-modified nanoparticles were added to PCR tubes containing 0.1 ng of *Alexandrium catenella* genomic DNA as template. The PCR products were analysed by agarose gel electrophoresis, and the band intensities were compared using a gel-doc apparatus.

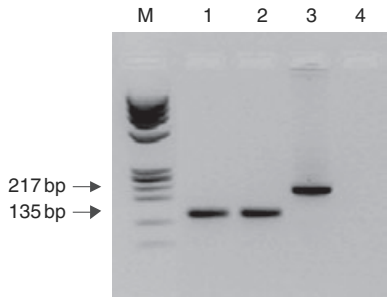

Figure 3 PCR amplification of DNA recovered using the AP-modified nanoparticles from two samples consisting of 300 000 *Alexandrium catenella* cultured cells. M: DNA size marker. 1 and 2: PCR amplification of captured DNA. 3: PCR-positive control (*A. catenella* 5·8S rDNA-cloned sequence containing an insertion of 82 bp). 4: PCR-negative control without template DNA.

PCR assay optimization

We evaluated the potential inhibitory effects of the AP-modified nanoparticles in the PCRs as described in Materials and Methods, in order to evaluate the possibility of using these materials directly in the PCR assay avoiding elution of bound DNA. Nanoparticles treated with glycine and BSA (blocked) and untreated (unblocked) were used (see Materials and Methods). The treatment with glycine and BSA blocked the aspecific interactions between nanoparticles and nontarget molecules, reducing the inhibitory effects of these materials. Results showed that up to 5 μg of blocked AP-modified nanoparticles can be added to a PCR tube without marked inhibition of the PCR (Fig. 2). This quantity of nanoparticles represented an acceptable compromise between PCR inhibitory effects and quantity of DNA template. For this reason, a volume equivalent to 5 μg of blocked AP-modified nanoparticles was added directly to the PCR tube, avoiding the DNA elution step. We also established that by adding 0·2 μg μl^{-1} BSA in the PCR, the inhibitory effects of the nanoparticles could be even further reduced. This will permit the use of up to 12·5 μg of nanoparticles per PCR, improving the robustness of the method in terms of sensitivity.

Sensitivity of DNA capture

First, we tested the capture procedure using lysates obtained from 300 000 *A. catenella* cultured cells to assess the feasibility of the method. We easily amplified DNA recovered from these samples as shown in Fig. 3. Then, we used paramagnetic nanoparticles modified with the capture probe to selectively isolate *Alexandrium* DNA from samples containing different amounts of *A. catenella* cells mixed with different amounts of *Navicula* spp. cells (diatoms) which served as background (Table 1). The results revealed that the target 5·8S rDNA could be recovered and amplified by PCR from all samples containing *A. catenella* cells. Remarkably, the target DNA was recovered and amplified even from samples containing only one *A. catenella* cell in a background of 250 000 diatom cells, indicating the high sensitivity, specificity and selectivity of the method based on AP-modified nanoparticles. A representative PCR result is shown in Fig. 4.

The sensitivity experiments using 100, 10 and one *A. catenella* cells in a mixed sample of 250 000 *Navicula* spp. cells were also performed with genomic DNA purified using Dneasy plant mini kit (Qiagen) and a phenol–chloroform-based method. Approximately 10 ng of purified DNA was used in the PCRs. Results revealed that the detection limit of one cell in a background of

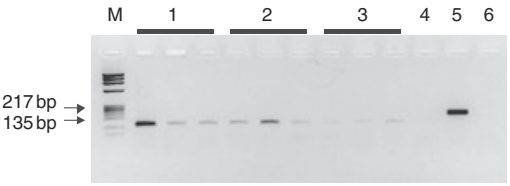

Figure 4 Sensitivity of the DNA-capture method. A total of 250 000 *Navicula* spp. cells (Diatoms) were spiked with 100, 10 and one *Alexandrium catenella* cells. The recovered DNAs were amplified in triplicate by PCR, and the PCR products were analysed by agarose gel electrophoresis. M: DNA size marker. 1–3: PCR amplification of captured DNA from samples spiked with 100, 10 and one *A. catenella* cells. 4: Negative control containing only *Navicula* spp. DNA. 5: PCR-positive control (*A. catenella* 5·8S rDNA-cloned sequence containing an insertion of 82 bp). 6: PCR-negative control without template DNA.

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250 000 was also reached using genomic DNA purified with these two methods (data not shown).

Recovery of target DNA from field samples

To assess the applicability of the magnetic capture hybridization method on field samples, this procedure was applied to a Lugol-fixed net sample from the Adriatic Sea (Italy) consisting in a bloom of dinoflagellates belonging to the genus *Gonyaulax*, in which *Alexandrium* cells were not detected after a first qualitative examination using a light microscope. However, dinoflagellates belonging to the genus *Alexandrium* are sometimes present, even if at a low concentration, in the Adriatic Sea (Boni *et al.* 1989; Honsell *et al.* 1996). As a result of the limited number, these cells are not easily detected with conventional methodologies. Three 50 ml aliquots of the seawater sample were spiked with 100, 10 and one *A. catenella* cells as described in Materials and Methods; another aliquot was processed without adding *A. catenella* cells to check for *Alexandrium* spp. presence in the seawater sample using the molecular methods described here. After PCR and agarose gel analysis, the amplification product was detectable in all spiked samples, confirming the applicability of this method on field samples (Fig. 5). Moreover, a PCR product was also visible in the unspiked sample (Fig. 5, lane 4), indicating the presence of *Alexandrium* cells in the sea water. To confirm this result, 20 ml (twice the volume used for the previous analysis) of the same field sample was re-examined with a light microscope: this time a few *Alexandrium* cells were found, confirming the data obtained by PCR. In the attempt to identify the *Alexandrium* species present in our sample, we performed five additional PCRs using five primer pairs previously designed in our laboratory, specific for *A. catenella*, *A. andersoni*, *A. taylori*, *A. tamarense* and *A. minutum*. The amplification product was detectable only in reactions containing primers specific for *A. taylori* and *A. tamarense* (data not shown), indicating the presence of these *Alexandrium* species in the seawater sample.

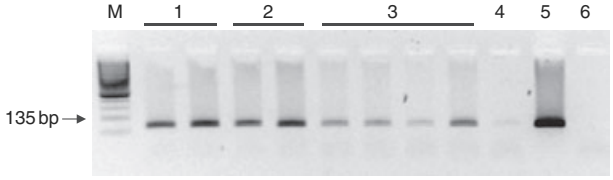

Figure 5 PCR-based detection of target DNA from environmental samples. Three aliquots of Lugol-fixed net samples consisting of a bloom of dinoflagellates belonging to the genus *Gonyaulax* were spiked with 100, 10 and one *Alexandrium catenella* cells; another aliquot of the same sample was processed without adding *A. catenella* cells. M: DNA size marker. 1–3: PCR amplification from field samples spiked with 100, 10 and one *A. catenella* cells. 4: PCR amplification from the unspiked field samples. 5: PCR-positive control (*A. catenella* 5·8S rDNA-cloned sequence). 6: PCR-negative control without template DNA.

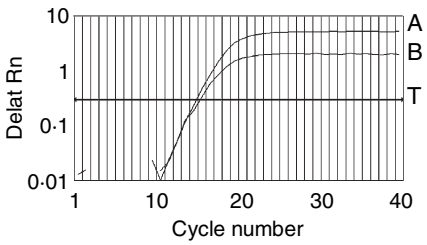

Figure 6 Applicability of AP-modified magnetic nanoparticles in real-time PCR. 1×10^7 copies of a plasmid containing *Alexandrium* 5·8S rDNA-cloned sequence were amplified without (a) or with (b) 5 μg of AP-modified nanoparticles in a real-time PCR assay previously developed (Galluzzi *et al.* 2004). One representative experiment is shown here. The cycle number is plotted *vs* the Delta Rn, which represents the normalized reporter signal (Rn) minus the baseline signal established in the early PCR cycles. The no template control (NTC) signal is undetectable. T: threshold.

Potential application in real-time PCR

The applicability of AP-modified magnetic nanoparticles has also been evaluated in a real-time PCR assay previously described (Galluzzi *et al.* 2004). The inhibitory effects of these nanoparticles in this particular application were variable, depending on whether the PCR results were analysed during the exponential phase or at the end point. In the first case, the amplification plot of the reaction containing the nanoparticles was slightly delayed (<1 cycle), while in the second, the fluorescence of the tube containing the nanoparticles diminished approximately 73% compared with the unspiked control (Fig. 6). Considering the limited inhibition during the exponential phase of the reaction, these materials could potentially be used for the qualitative detection of *Alexandrium* cells in real-time PCR.

Discussion

An important contribution to the understanding of the HAB phenomena is provided by detection and enumeration of target phytoplankton species found in seawater samples. Molecular methods, in particular the PCR, could be helpful in accomplishing this task. For this reason, an efficient and affordable method for DNA purification from marine samples is of great interest. This study was undertaken to develop a rapid and cost-effective method for the isolation of *Alexandrium* DNA from environmental samples, in order to remove PCR inhibitors and permit the analysis of a larger sample volume. Nucleic acid capture using magnetic nanoparticles was shown to

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accomplish these objectives without employing hazardous reagents. This method has been successfully applied to detect a variety of viruses and bacteria in clinical and environmental samples during the last decade. In this study, we used a new type of modified paramagnetic nanoparticles to recover the rDNA of toxic dinoflagellates belonging to the genus *Alexandrium*, from cultured and seawater samples. The same type of materials was successfully used to isolate *Listeria* DNA from milk samples (Amagliani *et al.* 2006). Contrary to previous approaches (Maibach *et al.* 2002; Shah *et al.* 2003), target DNA capture was performed by adding the AP-modified paramagnetic nanoparticles directly to the raw lysate samples, avoiding the previous DNA purification step and therefore shortening the entire procedure. Moreover, considering the elevated number of samples that have to be processed in coastal monitoring programmes, the rapidity of this method is comparable to that of the DNeasy plant kit from Qiagen, because of the elimination of centrifugation steps during the DNA purification. Compared with the phenol–chloroform DNA extraction method, the magnetic capture hybridization method offered not only a greater simplicity in the procedures but also the important advantage of eliminating steps that require the use of hazardous chemicals. In terms of sensitivity and amplificability of recovered DNA from cultured diatoms and *A. catenella* cells, all methods gave the same results. This was probably due to the fact that rDNA genes are present in multiple copies per cell, meaning that the presence of some target sequence in the amount of template used in the downstream PCR was highly probable, even if the purification of target DNA was not selective. Nevertheless, the AP-modified, magnetic nanoparticle-based approach presents the advantage of selectively recovering the target DNA molecules from a heterogeneous sample and allows the easy handling of these molecules in solution. In this way, the purified molecules can be detected not only by PCR as described here but also using a different and more direct approach, for example, the use of fluorescent specific probes complementary to an adjacent sequence of the target. The feasibility of this approach for dinoflagellate identification in terms of sensitivity is currently being evaluated in our laboratory. However, the use of the high-copy rDNA as a target suggests that a satisfactory level of sensitivity could be reached. Finally, the magnetic nanoparticle-based method requires no other equipment than a magnet and a hybridization oven and, in principle, can be automated allowing the processing of a high number of samples (Miyachi *et al.* 2000). We observed that an excessive quantity of nanoparticles in the PCR mix leaded to the inhibition of the amplification reaction. This problem was overcome treating the nanoparticles with glycine and BSA, source of primary amines, which block the aspecific interactions of nanoparticles with nontarget molecules in the sample and in the PCR mixture. We established that up to 5 μg of blocked AP-modified nanoparticles could be added to a PCR tube without substantial inhibition of the PCR. It is noteworthy that the amino-modified probe used for the capture experiments was partially complementary to the reverse primer 5·8S-3′. This feature and the close proximity with the forward primer could make this system suitable for the recovery and subsequent amplification of a damaged and fragmented DNA. In fact, degraded low-molecular-weight DNA is sometimes present in marine samples not properly fixed or stored for a long period of time prior to laboratory analysis. During the PCR assay, the capture probe could also bind to the reverse primer withdrawing it from the reaction, but this possibility is minimized by the fact that the primer 5·8S-3′ in the 50 μl PCR is greatly in excess when compared with the capture probe (about 15 and 0·6 pmol, respectively).

In the seawater sample obtained from the NW Adriatic Sea, the species-specific PCR assays revealed the presence of the dinoflagellates species *A. taylori* and *A. tamarense*, confirming previous observations through microscopy (Boni *et al.* 1989; M. Pompei, personal communication) and PCR analyses (A. Penna, unpublished data). This fact explains the positive PCR result obtained with DNA captured from the unspiked field sample. Moreover, the difference in band intensities between the samples spiked with one *A. catenella* cell and the unspiked sample (Fig. 5, lanes 3 and 4) could be explained by the fact that several tens of thousands of copies of rDNA are present in *A. catenella* cells, while *A. taylori* contains 'only' about 1500 copies of rDNA per cell (L. Galluzzi, unpublished data). We have no information regarding *A. tamarense* rDNA content.

These results indicate that the use of probe-conjugated paramagnetic nanoparticles could be effective for the specific purification of microalgal DNA in mixed samples, ensuring sensitivity and specificity of the following PCR. Because of its simplicity, this DNA-capture method could be easily adapted to other toxic dinoflagellates or harmful algae. The technique described in this article is not able to distinguish between toxic and nontoxic strains of *Alexandrium*, and no molecular markers utilizable as target for toxicity are known at the moment. However, the detection of these dinoflagellates in marine water could give an early warning for the possible presence of toxic species. This could be then confirmed by conventional microscope analysis and/or toxicity assays.

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