

Development of a qualitative PCR method for the *Alexandrium* spp. (Dinophyceae) detection in contaminated mussels (*Mytilus galloprovincialis*)

Luca Galluzzi^{a,b}, Antonella Penna^c, Elena Bertozzini^a, Maria Grazia Giacobbe^d,
Magda Vila^e, Esther Garcés^e, Silvia Prioli^f, Mauro Magnani^{g,*}

^a Center of Biotechnology, University of Urbino, via Campanella 1, 61032 Fano (PU), Italy

^b Istituto Zooprofilattico Sperimentale Umbria e Marche, via Salvemini 1, 06126 Perugia, Italy

^c Centro Biologia Ambientale, University of Urbino, V.le Trieste 296, 61100 Pesaro (PU), Italy

^d Istituto per l'Ambiente Marino Costiero, CNR, Via San Raineri 86, 98122 Messina, Italy

^e Institut de Ciències del Mar, Passeig Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain

^f Mare Soc. Coop. arl, via Toti 2, 47841 Cattolica (RN), Italy

^g Institute of Biological Chemistry "G. Fornaini", University of Urbino, via Saffi 2, 61029 Urbino (PU), Italy

Received 13 June 2004; received in revised form 22 November 2004; accepted 31 January 2005

Abstract

Paralytic shellfish poisoning (PSP) is a syndrome caused by the consumption of shellfish contaminated with neurotoxins produced by organisms of the marine dinoflagellate genus *Alexandrium*. *A. minutum* is the most widespread species responsible for PSP in the Western Mediterranean basin. The standard monitoring of shellfish farms for the presence of harmful algae and related toxins usually requires the microscopic examination of phytoplankton populations, bioassays and toxin determination by HPLC. These procedures are time-consuming and require remarkable experience, thus limiting the number of specimens that can be analyzed by a single laboratory unit. Molecular biology techniques may be helpful in the detection of target microorganisms in field samples. In this study, we developed a qualitative PCR assay for the rapid detection of all potentially toxic species belonging to the *Alexandrium* genus and specifically *A. minutum*, in contaminated mussels. *Alexandrium* genus-specific primers were designed to target the 5.8S rDNA region, while an *A. minutum* species-specific primer was designed to bind in the ITS1 region. The assay was validated using several fixed seawater samples from the Mediterranean basin, which were analyzed using PCR along with standard microscopy procedures. The assay provided a rapid method for monitoring the presence of *Alexandrium* spp. in mussel tissues, as well as in seawater samples. The results showed that PCR is a valid, rapid alternative procedure for the detection of target phytoplankton species either in seawater or directly in mussels, where microalgae can accumulate.

© 2005 Elsevier B.V. All rights reserved.

Keywords: *Alexandrium minutum*; ITS; *Mytilus galloprovincialis*; PCR; rDNA

* Corresponding author. Tel.: +39 0722 305211; fax: +39 0722 320188.

E-mail address: magnani@uniurb.it (M. Magnani).

1. Introduction

Paralytic shellfish poisoning (PSP) is a syndrome exhibiting neurological symptoms predominantly caused by the consumption of contaminated shellfish. PSP is a serious problem for human health and fishing industry resources along temperate coasts throughout the world (Hallegraeff, 1993; Chang et al., 1997; Harvell et al., 1999). In the Mediterranean Sea, PSP toxins are produced by dinoflagellates such as *Alexandrium* spp. *A. minutum* is the most widespread toxic species in the Western Mediterranean basin (Giacobbe and Maimone, 1994; Forteza et al., 1998; Vila et al., 2001). This species has been responsible for toxic blooms in the northern region of the French Brittany coast (Le Doux et al., 1990), along the Catalan coast (Spain) and Island of Mallorca (Forteza et al., 1998), in the South Tyrrhenian Sea (Giacobbe, 2001, personal communication), as well as along the northwestern coast of the Adriatic Sea (Italy), where mussel farms have been contaminated (Honsell et al., 1996). Shellfish monitoring for toxin contamination normally requires both bioassays and toxin determination. The bioassays (toxicity test) are usually performed following the Association of Analytical Communities (AOAC) standard procedures with acidified extracts of shellfish meat (Hollingworth and Wekell, 1990), while toxin determination is performed by HPLC analysis (Oshima, 1995).

Coastal water monitoring programmes to detect the presence of toxic algae could provide an essential tool to access bloom formation and consequently potential shellfish contamination. Usually, monitoring involves accurate morphological identification and the enumeration of target phytoplankton species in the seawater samples by using standard microscopy procedures. These methods are time-consuming and require a remarkable taxonomic experience, since identification is based on the recognition of morphological characteristics (Steidinger and Tangen, 1997). In addition, the species of interest may represent only a minor component in a mixed phytoplankton population. In particular, *A. minutum* is difficult to distinguish from other species of the same genus because it is characterized by minute details of its thecal plates (Taylor et al., 1995); consequently, the morphological identification of this species

requires taxonomic skills and well-trained personnel. Therefore, a suitable, highly sensitive method for the rapid and specific identification of this HAB species would be especially useful. PCR-based methods are attractive as diagnostic tools because they can rapidly detect limited numbers (as low as one cell) of specific eukaryotic and/or prokaryotic organisms in mixed ecological scenarios. Different regions of the rDNA cluster have been selected as targets for PCR amplification in microalgae. These regions include the small subunit (SSU) gene (Scholin and Anderson, 1994), the large subunit (LSU) gene (Zardoya et al., 1995; Rehnstam-Holm et al., 2002), the 5.8S subunit gene, two internal transcribed spacers (ITS1 and ITS2) (Penna and Magnani, 1999; Kooistra et al., 2001) and the non-transcribed spacer (NTS) regions (Saito et al., 2002). The internal transcribed spacer (ITS) regions of the rDNA have been used as molecular targets for the identification of microbial species because they are often variable enough to be used as a species-specific target regions (Nazar et al., 1991; Zechman et al., 1994). In particular, the ITS region sequence has been used to distinguish closely related species of the genus *Alexandrium* (Adachi et al., 1996) and recently Litaker et al. (2003) used ITS sequences for the development of specific PCR assays to detect *Pfiesteria piscicida* and *Pfiesteria*-like organisms. The PCR amplification technique of target ribosomal DNA regions has been successfully employed for the detection of various toxic dinoflagellates in seawater samples (Anderson et al., 1999; Bowers et al., 2000; Penna and Magnani, 2000; Coyne et al., 2001; Guillou et al., 2002; Saito et al., 2002). Godhe et al. (2001) described a PCR-based method for the detection of *A. minutum* in field samples using primers designed on the SSU rDNA region. In this case the primers were species-group-specific since the PCR yielded a product also from two closely related species. Here, we try to improve the specificity and sensitivity of the assay by designing new primers to target the ITS1–5.8S rDNA regions of *A. minutum*. Moreover, this assay is used to detect *A. minutum* cells in contaminated mussels, along with natural seawater samples, in order to provide a rapid method for monitoring the presence of toxin-producing algal species in mussel tissues.

2. Materials and Methods

2.1. Algal cultures

The *A. minutum* strain CNR-AMI-A4 was used for experimental shellfish contamination. *A. minutum* cultures were maintained in f/2 medium (Guillard, 1975) at 17 ± 1 °C. Light was provided by cool-white fluorescent bulbs (photon flux of $100 \mu\text{E m}^{-2} \text{s}^{-1}$) on a standard 14 h light–10 h dark cycle. Algal cell density was determined by counting Lugol's iodine stained cells (Throndsen, 1978) using a hemacytometer (Neubauer, Hausser Scientific, Horsham, PA).

2.2. Phytoplankton sample collection and species identification

Phytoplankton samples were collected from different locations in the Mediterranean Sea (Table 1). All water samples except number 9 were collected at the subsurface (0.5 m) using sampling bottles. Sample 9 was collected in a non-quantitative manner using a 10 μm -mesh plankton net. All samples were fixed with Lugol's iodine solution (0.4% final concentration). 50 mL volumes of the preserved samples were settled in Utermöhl chamber for 24 h and counted using an inverted microscope. Fixed samples were stored in the dark at +4 °C until needed. For

Alexandrium species identification, the samples were dyed with calcofluor white (Fritz and Triemer, 1985) and observed under an epifluorescence microscope. The identification was based greatly on thecal plate tabulation (Balech, 1995) as well as on cell shape and size. The amount of *A. minutum* cells were related to the total amount of phytoplankton cells to determine the percentage of these cells in field samples.

2.3. Mussel contamination

Mussel stocks of *Mytilus galloprovincialis* were collected along the Northern Adriatic coast in November 2002. A total of 30 individuals were acclimatized to laboratory conditions in 60 L aerated and circulating seawater under a 12:12 L/D cycle for a 7-day period, before the contamination experiment. After the 7-day period, the seawater was contaminated with *A. minutum* cells to reach a final concentration of 10^6 cells L^{-1} , in order to simulate the bloom condition (day 0). Two mussels were removed 7, 72 and 168 h after this contamination. To confirm that cells were filtered by mussels, algal cell concentration was determined each time the mussels were removed. At day 3, after the 72 h mussel sampling, a further amount of 2.5×10^6 *A. minutum* cells were added in the water. This second contamination was done in order to increase the algal biomass in the aquarium and sustain

Table 1
Details of natural plankton sample collection

Sample number	Date	Sampling site	<i>A. minutum</i> (%) ^b	<i>A. minutum</i> cysts (%) ^b	Other phytoplankters (%) ^b	Dominant species/taxa
1	18 February 2002	Arenys harbour, Catalan Sea, Spain	97.7	0	2.3	<i>A. minutum</i>
2	18 February 2002	Arenys harbour, Catalan Sea, Spain	97.6	0	2.4	<i>A. minutum</i>
3	18 February 2002	Arenys harbour, Catalan Sea, Spain	99.6	0	0.4	<i>A. minutum</i>
4	10 March 2003	Verde pond, Tyrrhenian Sea, Italy	52.8	0.4	46.8	<i>A. minutum</i>
5	18 April 2003	Verde pond, Tyrrhenian Sea, Italy	3.1	0	96.9	Flagellates
6	15 April 2003	Porto Rosa, Tyrrhenian Sea, Italy	1.5	4.6	93.9	Flagellates
7	2 April 2003	Siracusa, Ionian Sea, Italy	12	1	87	<i>Prorocentrum triestinum</i>
8	2 April 2003	Porto Marmoreo-Siracusa, Ionian Sea, Italy	8.1	0.3	91.6	<i>Prorocentrum triestinum</i>
9 ^a	20 May 2003	Saronicos Gulf, Aegean Sea, Greece	n.a.	n.a.	n.a.	n.a.
10	17 June 2003	Vulcano Island, Tyrrhenian Sea, Italy	0	0	100	<i>A. taylori</i>
11	17 June 2003	Vulcano Island, Tyrrhenian Sea, Italy	0	0	100	<i>A. taylori</i>
12	12 March 2003	Porto Rosa, Tyrrhenian Sea, Italy	0	0	100	Flagellates

n.a.: not available.

^a Sample collected with a 10 μm plankton net.

^b The percentage values are calculated from the phytoplankton cell number per liter of water.

the bloom-like condition. The environmental conditions in the aquarium were maintained and monitored during the whole experiment: water temperature was between 20 and 23 °C, pH was between 7.3 and 7.8, dissolved oxygen was between 5.1 and 6.7 mg L⁻¹. Mussels collected from the aquarium were immediately processed: they were opened, drained and the digestive tissues (or hepatopancreas) were extracted from the rest of the body (mantle, gills, foot, adductor muscles). Both the hepatopancreas and the animal remains were fixed separately in 10 mL 95% ethanol and stored at -20 °C.

2.4. DNA extraction and purification

Cells from phytoplankton samples were either collected by mild centrifugation (samples 1–4) or harvested onto 25 mm-diameter hydrophilic Durapore membranes (5 µm pore size; Millipore) by vacuum filtration (samples 5–12). Filters were transferred to tubes containing absolute ethanol and stored at +4 °C. Prior to DNA extraction, filters were air dried and transferred into clean tubes. DNA was extracted using PUREGENE Genomic DNA purification Kit (Gentra Systems, Minneapolis MN, USA) or DNeasy Plant mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. In order to extract DNA from mussels, no more than 0.5 g of dry mussel tissue were mechanically disrupted in 10 mL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1% sodium dodecyl sulfate; 25 mM EDTA; 100 µg mL⁻¹ proteinase K) using a Dounce homogenizer. The disrupted tissue was incubated at 55 °C for 4 h and the nucleic acids were isolated by extracting the completely digested tissue twice with phenol/chloroform/isoamil alcohol (25:24:1) and once with chloroform. The DNA was precipitated with 95% ethanol, washed with 70% ethanol, air-dried and resuspended in 5–6 mL of TE (10 mM Tris-HCl; 1 mM EDTA). DNA purity and concentration were determined by absorbance at 260/280 nm. Alternatively, purified DNA was quantified on agarose gel using serially diluted DNA marker 2 (MBI Fermentas, Germany) and a gel-doc apparatus (Bio-rad, Hercules, CA, USA).

2.5. Primer design

The PCR primers were designed using Oligo 6 primer analysis software and an alignment of multiple

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 5.8S region is well conserved among the *Alexandrium* species. Primers 5.8S-5' (5'-GCAADGAATGTCT-TAGCTCAA-3') and 5.8S-3' (5'-GCAMACCTT-CAAGMATATCCC-3') were designed to target consensus sequences specific for the genus *Alexandrium*. Primer ITS1m (5'-CATGCTGCTGTGTTGAT-GACC-3') was designed to a target sequence specific to the *A. minutum* species and was used along with the reverse primer 5.8S-3' in the PCR assays. Oligonucleotides were purchased from Sigma-Genosys Ltd. (USA).

2.6. Specificity and sensitivity test of the primers

The sequence specificity of the primers was confirmed using BLAST2 (Basic Local Alignment Search Tool) (http://www.infobiogen.fr/services/analyseq/cgi-bin/blast2_in.pl). The primers' specificity for the genus *Alexandrium* was also tested by amplification of a representative sample of the genomic DNA of other dinoflagellates and diatoms including *Gyrodinium corsicum*, *Karlodinium micrum*, *Coolia monotis*, *Ostreopsis* spp., *Dinophysis sacculus* and *Pseudonitzschia pseudodelicatissima*. To test the species-specificity of the primers for *A. minutum*, we performed the PCR assay using ITS1–5.8S–ITS2 rDNA-cloned sequences and genomic DNA from *A. minutum*, *A. catenella*, *A. tamarense*, *A. taylori* and *A. andersoni* as template. Moreover, also the genomic DNA from *G. corsicum*, *K. micrum* and the ITS1–5.8S–ITS2 rDNA-cloned sequence of *D. sacculus* were used as template. The PCR assays sensitivity was tested using a plasmid containing the ITS1–5.8S–ITS2 rDNA sequence of *A. minutum* as template, with or without 100 ng of background DNA purified from uncontaminated mussels. Moreover, the PCR assays sensitivity was also tested using purified genomic *A. minutum* DNA.

2.7. rDNA cloning and sequencing

The 5.8S rDNA and flanking ITS regions (ITS1 and ITS2) were amplified from purified *A. minutum* genomic DNA and cloned into the pMOSBlue vector

(Amersham, UK) as previously described (Penna and Magnani, 1999). Several clones were sequenced using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, CA, USA) in an ABI PRISM 310 instrument. Plasmid DNA was purified using Qiaprep Spin Miniprep kit (Qiagen). The *A. minutum* strain CNR-AMI-A4 ITS1–5.8S–ITS2 sequence has been deposited in GenBank database (accession number AJ318460). Either genus- or species-specific PCR products obtained from field samples 5 and 7 were purified using the MinElute Gel Extraction kit (Qiagen) and directly sequenced. Sequencing was performed using approximately 15 ng of DNA and 3 pmol of primer.

2.8. PCR assays

For marine samples, the PCR mixtures contained 1 ng of template DNA in reaction buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂), 0.3 µM of each primer, 200 µM each of dATP, dCTP, dTTP, dGTP and 1.25 units of Taq Gold DNA polymerase (Applied Biosystems) in a total volume of 50 µL. For mussels, the PCR mixture was the same while the amount of template DNA was between 10 ng and 1 µg. Samples were processed in a GeneAmp PCR System 2400 (Applied Biosystems) as follows: 7 min at 95 °C, 40 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. Positive controls were spiked with 10⁴ copies of ITS1–5.8S–ITS2 *A. minutum* cloned sequence. Negative controls (no-template) were included in each PCR reaction. Ten microliters of PCR product were run in 2% agarose gel (w/v) and visualized with ethidium bromide. A 100-bp double-stranded DNA ladder (MBI Fermentas) was included on the gels as a size standard.

3. Results

3.1. Specificity and sensitivity of PCR assay

Selected primers for the conserved 5.8S rDNA region of *Alexandrium* genus and primers specific for *A. minutum* species gave PCR products of 135 and 212 bp, respectively. Molecular weight of the amplified product was as expected and no other bands were visible when *Alexandrium* genomic DNA was used as

template. The specificity of all primers was confirmed in silico using BLAST: the sequences of primers 5.8S–5' and 5.8S–3' matched exactly only with the 5.8S sequences belonging to the *Alexandrium* genus and the sequence of primer ITS1m matched exactly only with the ITS1 sequences belonging to the *A. minutum* species. The specificity of the primers for the genus *Alexandrium* was also tested by amplification of genomic DNA purified from several other dinoflagellates and diatoms, as described in materials and methods. The amplification products were undetectable in reactions containing DNA of these microalgae (data not shown). Moreover, using the primers specific for *A. minutum* and the ITS1–5.8S–ITS2 cloned sequences from *A. minutum*, *A. catenella*, *A. tamarense*, *A. taylori* and *A. andersoni* as template, only the PCR reaction containing *A. minutum* rDNA yielded the expected 212 bp amplicon. None of the other species' DNA was amplified in this PCR assay (Fig. 1). The same results were obtained using genomic DNA from *A. minutum*, *A. catenella*, *A. tamarense*, *A. taylori*, *A. andersoni*, *G. corsicum*, *K. micrum* and the ITS1–5.8S–ITS2 rDNA-cloned sequence of *D. sacculus* as template. The PCR assays resulted sensitive enough to detect 10 copies of ITS1–5.8S–ITS2 rDNA-cloned sequences, with or without 100 ng of background DNA purified from uncontaminated mussels, whether using genus-specific or species-specific primers (Fig. 2a and b). However, when analyzing the band intensities with a gel-doc apparatus, the signal of the PCR product obtained with species-specific primers was approximately 1 log

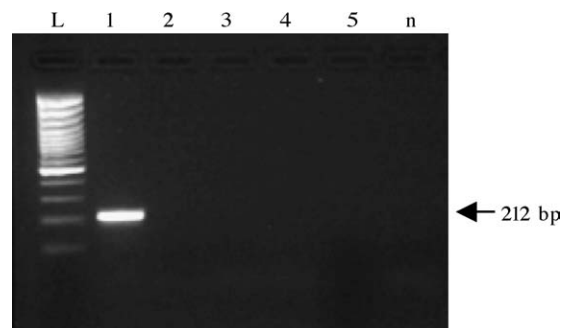


Fig. 1. Specificity of *A. minutum* amplification. ITS1–5.8S–ITS2 rDNA-cloned sequences from *A. minutum* (1), *A. catenella* (2), *A. taylori* (3), *A. tamarense* (4) and *A. andersoni* (5) were used in PCR assays with primers ITS1m and 5.8S–3'. L, 100-bp DNA ladder; n, negative control.

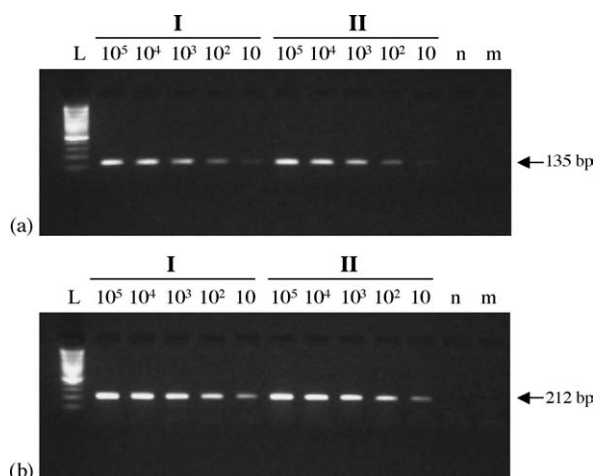


Fig. 2. (a) Sensitivity of PCR assay specific for the *Alexandrium* genus. (b) Sensitivity of PCR specific for the *A. minutum* species. The plasmid containing the ITS1–5.8S–ITS2 *A. minutum* sequence was serially diluted from 10⁵ to 10 copies per reaction tube and the PCR was performed without (I) or with (II) 100 ng of genomic mussel DNA as background. L, 100-bp DNA ladder; n, negative control; m, genomic mussel DNA only.

greater compared with the PCR product obtained with genus-specific primers. This observation could be, at least in part, a consequence of the difference in size between the two PCR products. Furthermore, the PCR assays resulted sensitive enough to detect 0.1 pg of purified *A. minutum* genomic DNA either using genus-specific or species-specific primers (data not shown). Referring to our estimation on DNA and rDNA content per *A. minutum* cell (Galluzzi et al., 2004), 0.1 pg of genomic DNA should be equivalent to about 10 copies of rDNA.

3.2. Detection of *A. minutum* cells in seawater samples

First, we analyzed three *A. minutum* natural monospecific bloom samples (referred as no. 1–3 in Table 1) fixed with Lugol's solution by PCR. In these bloom samples up to 99.6% of the phytoplankton population was represented by *A. minutum* while other species were not significantly represented. After cell identification and counting by microscopy, the concentration was 2.45×10^6 , 1.88×10^6 and 10.82×10^6 cells L⁻¹ in samples 1, 2 and 3, respectively. Genomic DNA was purified from 147,000, 93,000 and 113,000 total cells for samples 1, 2 and 3, respectively. After DNA amplification using *A. minutum* species-specific primers, PCR products of

the expected size were clearly visible in all samples (Fig. 3) confirming the amplifiability of the genomic DNA and the applicability of this PCR assay to fixed natural seawater samples. Once the feasibility of the PCR assay applied to natural samples was established, we analyzed several seawater samples containing *Alexandrium* cells in mixed phytoplankton populations. The genomic DNA was amplified either with *Alexandrium* genus- or *A. minutum* species-specific primers and the amplification products, of 135 and 212 bp, respectively, were visualized on 2% agarose

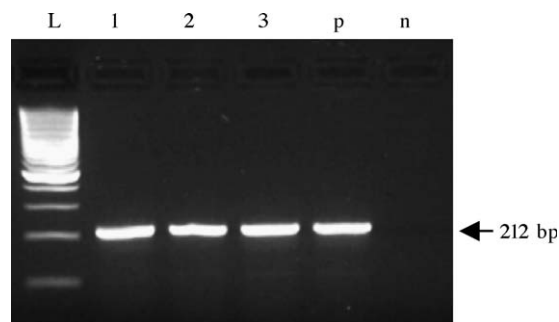


Fig. 3. *A. minutum* identification by PCR in natural monospecific bloom seawater samples fixed with Lugol's solution. Purified DNA from samples 1, 2 and 3 was amplified with *A. minutum* species-specific primers. L, 100-bp DNA ladder; p, positive control (10,000 copies of plasmid containing ITS1–5.8S–ITS2 *A. minutum* rDNA); n, negative control.

gels. The analyzed samples contained various percentages of *A. minutum* cells compared to other dinoflagellates and small flagellates, ranging from 52.8% in sample 4 to 0% in samples 10, 11 and 12 (Table 1). Samples 10 and 11 consisted of monospecific blooms of *A. taylori* and were used as negative controls for *A. minutum*-specific PCR assays. Sample 12 consisted of a bloom of *D. sacculus* in which *Alexandrium* cells were not detectable using LM techniques. The DNA extracted from this phytoplankton sample was used as a negative control for *Alexandrium* genus-specific PCR. This DNA was also spiked with 10^5 copies of a plasmid containing the ITS1–5.8S–ITS2 rDNA sequence of *A. minutum* in order to demonstrate the lack of significant inhibitors in the PCR reactions. The results, summarized in Table 2, show that the PCR assays were able to detect *Alexandrium* cells in all seawater samples in which these cells were distinguishable using LM techniques. In this table, the number of cells from which the DNA was effectively extracted, corresponding to the various sample volumes processed (ranging from 10.5 mL of sample number 3 to 187.5 mL of sample number 6), is also indicated. The PCR products from samples 5 and 7 were sequenced as described in the materials and methods in order to confirm the specificity of the assays. The four sequences (two obtained with genus-specific primers and two with species-specific primers) were 100% identical to the *A. minutum*

ITS1–5.8S rDNA sequences already described by Vila et al. (in press), confirming the specificity of the PCR product.

3.3. Detection of *A. minutum* cells in contaminated mussels

In order to establish the possibility of detecting microalgal DNA in mussel tissues, total genomic DNA purified from mussels 7, 72 and 168 h after *A. minutum* contamination was used as template in the PCR assays. The hepatopancreas was separated from the rest of the body to evaluate the possible dissimilar distribution of microalgal DNA in digestive or non-digestive tissues. In particular the gills, which have also the function of harvesting food particles toward the mouth, were always included in DNA extractions. The DNA yield varied between 1 and 3 mg but the amount of DNA purified from non-digestive tissues was always greater when compared to DNA purified from hepatopancreas. Algal cell concentration in the aquarium was determined before every mussel drawing: after 7 h from the beginning of the experiment, the cell concentration was $\sim 1 \times 10^5$ cells L^{-1} , after 72 h it was 14×10^3 cells L^{-1} and after 168 h it was 2.5×10^3 cells L^{-1} . In order to establish the largest amount of DNA usable in the reactions, enhancing the sensitivity without exerting inhibitory effects, three different amounts of DNA template

Table 2
Details of PCR-amplified natural seawater samples

Sample number	<i>Alexandrium</i> species (cells L^{-1}) ^a	<i>Alexandrium</i> cell number in the processed sample ^a	<i>Alexandrium</i> genus-specific PCR	<i>A. minutum</i> species-specific PCR
1	<i>A. minutum</i> (2,450,000)	147,000	n.a.	+
2	<i>A. minutum</i> (1,880,000)	93,000	n.a.	+
3	<i>A. minutum</i> (10,820,000)	113,000	n.a.	+
4	<i>A. minutum</i> (175,700 + 1,341 ^b)	4,390 + 33 ^b	+	+
5	<i>A. minutum</i> (87,400)	4,370	+	+
6	<i>A. minutum</i> (2,680 + 8,048 ^b)	503 + 1,509 ^b	+	+
7	<i>A. minutum</i> (804,600)	40,230 + 3,352 ^b	+	+
8	<i>A. minutum</i> (177,600)	2,664 + 98 ^b	+	+
9	<i>A. minutum</i> (n.a.)	100	+	+
10	<i>A. taylori</i> (8,314,200)	415,710	+	—
11	<i>A. taylori</i> (2,809,200)	140,460	+	—
12	Absent	Absent	—	—

n.a.: not available.

^a From microscope observation.

^b *A. minutum* cysts.

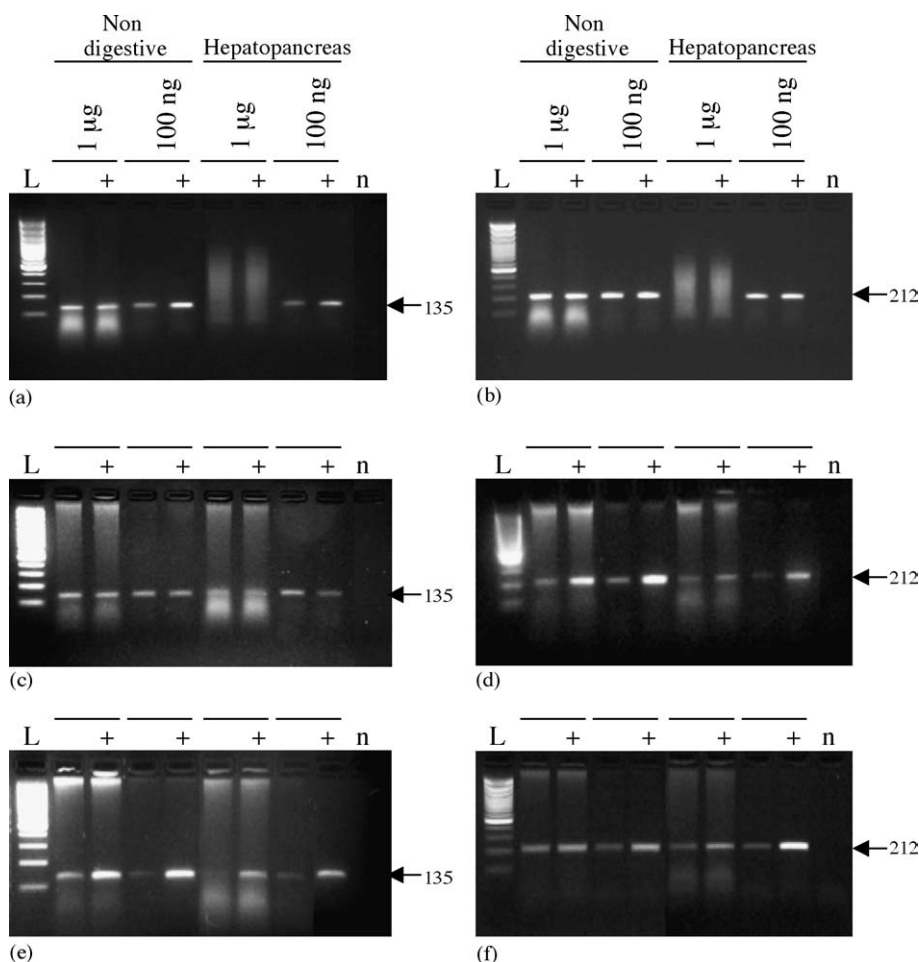


Fig. 4. Identification of *Alexandrium* cells in contaminated mussels. Mussel total genomic DNA purified from hepatopancreas or from the non-digestive tissues (gills) was used in *Alexandrium* genus-specific (a, c, e) and *A. minutum* species-specific (b, d, f) PCR reactions. PCR assays were performed at 7 h (a, b), 72 h (c, d) and 168 h (e, f) after the first *A. minutum* contamination using 1 µg and 100 ng of purified mussel DNA. +, samples spiked with 10,000 copies of plasmid containing ITS1–5.8S–ITS2 *A. minutum* rDNA; L, 100-bp DNA ladder; n, negative control.

(1000, 100 and 10 ng) were used in the PCR assays. With 10 ng of template DNA, the PCR amplified products were visible until 72 h, when using either DNA purified from hepatopancreas or from non-digestive tissues (data not shown). Using 1000 or 100 ng of DNA template, the amplified products were clearly visible until 168 h after the first contamination, when cell concentration in the aquarium was 2.5×10^3 cells L^{-1} (Fig. 4). Positive controls spiked with 10^4 copies of ITS1–5.8S–ITS2 *A. minutum* cloned sequence were included in each PCR reaction to check DNA amplifiability. Generally the DNA purified from non-digestive tissues (gills) appeared

more amplifiable than DNA purified from hepatopancreas. In one case (Fig. 4a and b), 1000 ng of DNA obtained from hepatopancreas completely inhibited the PCR reaction, probably because of poor DNA quality.

4. Discussion

The results obtained in this study demonstrate that these PCR assays are potentially useful in monitoring the presence of *Alexandrium* cells and in particular the *A. minutum* species, in both seawater samples and

shellfish tissues. The species-specificity of the PCR assay was improved compared to that obtained in the study carried out by Godhe et al. (2001) because we have designed the *A. minutum*-specific primer (ITS1m) to target the highly variable ITS1 rDNA region. The ITS regions, which are located between the small subunit (SSU), 5.8S and large subunit (LSU) genes, diverge more rapidly during speciation and are more variable among different species (Bargues et al., 2000). Therefore, these sequences, compared to the SSU, 5.8S or LSU genes, are more suitable for developing species-specific PCR assays (Connel, 2002). The PCR assays described in this study amplified 10 copies of ITS1–5.8S–ITS2 *A. minutum* cloned sequence, even in the presence of 100 ng of unrelated DNA as background. We recently established through real-time PCR that the ITS1–5.8S–ITS2 rDNA content in *A. minutum* is about 1000 copies/cell (Galluzzi et al., 2004). Taken together, these data mean that the PCR assays can amplify a theoretical equivalent amount of 0.01 cells in a 50 μ L reaction tube. While sensitivity is not a problem when high biomass and monospecific blooms are monitored, it could become an important problem in routine work, when collected samples contain mixed phytoplankton populations and the species of interest is not highly represented. Moreover, the DNA extraction yield is critical when high sensitivity is required and the purification method should be as quantitative as possible. We have shown that *Alexandrium* cells can be detected in natural samples containing mixed phytoplankton population, even at low cell concentration and when the target species is not a dominant member of the natural plankton community (Table 1). For example, in sample 9 the *A. minutum* cell number was estimated to be only 100 in a net haul. This method of collection was not quantitative and we don't have any information regarding *A. minutum* cell concentration in the seawater however, considering the cell number present on the net, this value must have been much lower than 100 cells L^{-1} .

Sensitivity is also important for the detection of microalgal DNA in mussel tissues. In fact, unlike PSP toxins, DNA can be metabolized by mussels and therefore we expected to find a limited quantity of genomic algal DNA in the hepatopancreas. For this reason we also analyzed gills that, as well as taking in oxygen, function to move food particles toward the mouth. The results showed that algal DNA can be

detected in both tissues 1 week after initial contamination, at which time 2500 *A. minutum* cells L^{-1} were still present in the water. The presence of algal DNA in mussel tissues could not possibly be due to contamination by the water since mussels were drained, tissues were fixed in 10 mL of 95% ethanol, no more than 0.5 g of dry tissue were used for DNA extraction and the DNA used in the PCR (100 ng) was at least 1/10,000 of total DNA extracted. Even in the hypothesis that the cut drained tissues contained 1 mL of water (equivalent to 2.5 cells) per 0.5 g of dry weight, after dilution in ethanol and DNA extraction the theoretical number of cells in the PCR reaction would have been 2.5×10^{-5} (equivalent to 0.025 rDNA copies), which would be undetectable in PCR.

In this study, we show that *Alexandrium* DNA detection by PCR in natural samples using ITS1–5.8S rDNA regions as targets is feasible, sensitive and specific. Moreover, we demonstrate that this PCR assay could be used for the detection of *Alexandrium* DNA directly in mussels, where accumulation of PSP toxins may cause serious problems for human health. Further experiments will be needed to assess the effective toxicity of mussels in which *A. minutum* cells were detected by PCR. In fact, the presence of the *A. minutum* DNA does not necessarily indicate the presence of the toxin in mussels. However, because all *A. minutum* strains can be potentially toxic, as we registered in the Mediterranean Sea (Vila et al., in press), the detection of DNA of these dinoflagellates in mussels could give an “early warning” for the possible presence of PSP toxin. This will be then eventually confirmed by conventional toxicity assays. Certainly, the availability of a PCR detection method for the monitoring of *A. minutum* in mussels together with a parallel monitoring of the PSP toxin presence will provide conclusive evidence for the usefulness of the molecular method reported here, that would greatly improve the speed of toxicological investigations, if properly validated. These new studies will be the subject for future investigations in our and other laboratories.

Acknowledgements

This work was partially supported by the EU STRATEGY project contract no. EVK3-CT-2001-

00046, EU CHEMAG project PPC00-3045 (2000) and Ministero delle Politiche Agricole e Forestali (MiPAF) project 6C18.

References

- Adachi, M., Sako, Y., Ishida, Y., 1996. Analyses of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *J. Phycol.* 32, 424–432.
- Anderson, D.M., Kulis, D.M., Keafer, B.A., Berdalet, E., 1999. Detection of the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and antibody probes: variability in labeling intensity with physiological conditions. *J. Phycol.* 35, 870–883.
- Balech, E., 1995. The Genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station, Cork (Special publication).
- Bargues, M.D., Marcilla, A., Ramsey, J.M., Dujardin, J.P., Schofield, C.J., Mas-Coma, S., 2000. Nuclear rDNA-based molecular clock of the evolution of triatominae (Hemiptera: Reduviidae), vectors of Chagas disease. *Mem. Inst. Oswaldo Cruz* 95, 567–573.
- Bowers, H.A., Tengs, T., Glasgow Jr., H.B., Burkholder, J.M., Rublee, P.A., Oldach, D.W., 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. *Appl. Environ. Microbiol.* 66, 4641–4648.
- Chang, F.H., Anderson, D.M., Kulis, D.M., Till, D.G., 1997. Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. *Toxicon* 35, 393–409.
- Connel, L., 2002. Rapid identification of marine algae (Raphidophyceae) using three-primer PCR amplification of nuclear internal transcribed spacer (ITS) regions from fresh and archived material. *Phycologia* 41, 15–21.
- Coyne, K.J., Hutchins, D.A., Hare, C.E., Cary, S.C., 2001. Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. *Aquat. Microb. Ecol.* 24, 275–285.
- Forteza, V., Quetglas, G., Delgado, M., Reyero, I., Fraga, S., Franco, J.M., Cacho, E., 1998. Toxic *Alexandrium minutum* bloom in Palma de Mallorca harbour (Balearic islands, western Mediterranean). In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), *Harmful Algae*. Xunta de Galicia and IOC of UNESCO, Santiago de Compostela, pp. 58–59.
- Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. *J. Phycol.* 21, 662–664.
- Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garcés, E., Magnani, M., 2004. Development of real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (dinoflagellate). *Appl. Environ. Microbiol.* 70, 1199–1206.
- Giacobbe, M.G., Maimone, G., 1994. First report of *Alexandrium minutum* Halim in a Mediterranean Lagoon. *Cryptogamie Algol.* 15, 47–52.
- Godhe, A., Otta, S.K., Rehnstam-Holm, A.-S., Karunasagar, I., Karunasagar, I., 2001. Polymerase chain reaction in detection of *Gymnodium mikimotoi* and *Alexandrium minutum* in field samples from Southwest India. *Mar. Biotechnol.* 3, 152–162.
- Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), *Culture of Marine Invertebrate Animals*. Plenum Press, New York, pp. 26–60.
- Guillou, L., Nezan, E., Cuffe, V., Erard-Le Denn, E., Cambon-Bonavita, M.A., Gentien, P., Barbier, G., 2002. Genetic diversity and molecular detection of three toxic dinoflagellate genera (*Alexandrium*, *Dinophysis*, and *Karenia*) from French coasts. *Protist* 153, 223–238.
- Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia* 32, 79–99.
- Harvell, C.D., Kim, K., Burkholder, J.M., Colwell, R.R., Epstein, P.R., Grimes, D.J., Hofmann, E.E., Lipp, E.K., Osterhaus, A.D., Overstreet, R.M., Porter, J.W., Smith, G.W., Vasta, G.R., 1999. Emerging marine diseases—climate links and anthropogenic factors. *Science* 285, 1505–1510.
- Hollingworth, T., Wekell, M.M., 1990. Paralytic shellfish poison. In: Helrich, K. (Ed.), *Official Methods of Analysis of the Association of Official Analytical Chemists*, 15th ed., vol. 2. Association of Official Analytical Chemists Inc, Arlington, pp. 881–882.
- Honsell, G., Poletti, R., Pompei, M., Sidari, L., Milandri, A., Casadei, C., Viviani, R., 1996. *Alexandrium minutum* Halim and PSP contamination in the Northern Adriatic Sea (Mediterranean Sea). In: Yasumoto, T., Oshima, T., Fukuyo, Y.T. (Eds.), *Harmful and Toxic Algal Blooms*. UNESCO, Paris, pp. 77–83.
- Le Doux, M., Frémy, J.M., Nézan, E., Erard, E., 1990. Recent occurrences of paralytic shellfish poisoning (PSP) toxins from the northwestern coasts of France. *J. Shellfish Res.* 8, 486.
- Litaker, R.W., Vandersea, M.W., Kibler, S.R., Reece, K.S., Stokes, N.A., Steidinger, K.A., Millie, D.F., Bendis, B.J., Pigg, R.J., Tester, P.A., 2003. Identification of *Pfiesteria piscicida* (Dinophyceae) and *Pfiesteria*-like organisms using internal transcribed spacer-specific PCR assays. *J. Phycol.* 39, 754–761.
- Kooistra, W.H.C.F., de Boer, M.K., Vrieling, E.G., Connell, L.B., Gieskes, W.W.C., 2001. Variation along ITS markers across strains of *Fibrocapsa japonica* (Raphidophyceae) suggests hybridisation events and recent range expansion. *J. Sea Res.* 46, 213–222.
- Morgenstern, B., Werner, T., Dress, A.W.M., 1996. Multiple DNA and protein sequence alignment based on segment-to-segment comparison. *Proc. Natl. Acad. Sci. U.S.A.* 93, 12098–12103.
- Nazar, R.N., Hu, X., Schmidt, J., Culham, D., Robb, J., 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* with pathogens. *Physiol. Mol. Plant. Pathol.* 39, 1–11.
- Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. *J. AOAC Int.* 78, 528–532.
- Penna, A., Magnani, M., 1999. Identification of *Alexandrium* (Dinophyceae) species using PCR and rDNA-targeted probes. *J. Phycol.* 35, 615–621.
- Penna, A., Magnani, M., 2000. A PCR immunoassay method for the detection of *Alexandrium* (Dinophyceae) species. *J. Phycol.* 36, 1183–1186.

- Rehnstam-Holm, A.S., Godhe, A., Anderson, D.M., 2002. Molecular studies of *Dinophysis* (Dinophyceae) species from Sweden and North America. *Phycologia* 41, 348–357.
- Saito, K., Drgon, T., Robledo, J.A., Krupatkina, D.N., Vasta, G.R., 2002. Characterization of the rRNA locus of *Pfiesteria piscicida* and development of standard and quantitative PCR-based detection assays targeted to the nontranscribed spacer. *Appl. Environ. Microbiol.* 68, 5394–5407.
- Scholin, C.A., Anderson, D.M., 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). RFLP analysis of SSU rRNA genes. *J. Phycol.* 30, 744–754.
- Steidinger, K.A., Tangen, K., 1997. Dinoflagellates. In: Tomas, C.R. (Ed.), *Identifying Marine Phytoplankton*. Academic Press, Florida, pp. 387–584.
- Taylor, F.J.R., Fukuyo, Y., Larsen, J., 1995. Taxonomy of harmful dinoflagellates. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), *Manual of Harmful Microalgae*. IOC UNESCO, Paris, pp. 283–317.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Thronsdon, J., 1978. Preservation and storage. In: Sournia, A. (Ed.), *Phytoplankton Manual*. UNESCO, Paris, pp. 69–74.
- Vila, M., Camp, J., Garcés, E., Masó, M., Delgado, M., 2001. High resolution spatio-temporal detection of HABs in confined waters of the NW Mediterranean. *J. Plankton Res.* 23, 497–514.
- Vila, M., Giacobbe, M.G., Masó, M., Gangemi, E., Penna, A., Sampedro, N., Azzaro, F., Camp, J., Galluzzi, L. A comparative study on recurrent blooms of *Alexandrium minutum* in two Mediterranean coastal areas. *Harmful Algae*, in press.
- Zardoya, R., Costas, E., Lopez-Rodas, V., Garrido-Pertierra, A., Baustista, J.M., 1995. Revised dinoflagellate phylogeny inferred from molecular analysis of large-subunit ribosomal RNA gene sequences. *J. Mol. Evol.* 41, 637–645.
- Zechman, F.W., Zimmer, E.A., Theriot, E.C., 1994. Use of ribosomal DNA internal transcribed spacers for phylogenetic studies in diatoms. *J. Phycol.* 30, 507–512.