Development of a Real-Time PCR Assay for Rapid Detection and Quantification of *Alexandrium minutum* (a Dinoflagellate)

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The marine dinoflagellate genus *Alexandrium* includes a number of species which produce neurotoxins responsible for paralytic shellfish poisoning (PSP), which in humans may cause muscular paralysis, neurological symptoms, and, in extreme cases, death. *A. minutum* is the most widespread toxic PSP species in the western Mediterranean basin. The monitoring of coastal waters for the presence of harmful algae also normally involves microscopic examinations of phytoplankton populations. These procedures are time consuming and require a great deal of taxonomic experience, thus limiting the number of specimens that can be analyzed. Because of the genetic diversity of different genera and species, molecular tools may also help to detect the presence of target microorganisms in marine field samples. In this study, we developed a real-time PCR-based assay for rapid detection of all toxic species of the *Alexandrium* genus in both fixative-preserved environmental samples and cultures. Moreover, we developed a real-time quantitative PCR assay for the quantification of *A. minutum* cells in seawater samples. *Alexandrium* genus-specific primers were designed on the 5.8S rDNA region. Primer specificity was confirmed by using BLAST and by amplification of a representative sample of the DNA of other dinoflagellates and diatoms. Using a standard curve constructed with a plasmid containing the ITS1-5.8S-ITS2 *A. minutum* sequence and cultured *A. minutum* cells, we determined the absolute number of 5.8S rDNA copies per cell. Consequently, after quantification of 5.8S rDNA copies in samples containing *A. minutum* cells, we were also able to estimate the number of cells. Several fixed *A. minutum* bloom sea samples from Arenys Harbor (Catalan Coast, Spain) were analyzed using this method, and quantification results were compared with standard microscopy counting methods. The two methods gave comparable results, confirming that real-time PCR could be a valid, fast alternative procedure for the detection and quantification of target phytoplankton species during coastal water monitoring.

The occurrence of harmful algal bloom (HAB) events in marine waters along temperate coasts throughout the world is an important and expanding threat to human health, fishery resources, and tourism industries (2, 23, 53). Several phytoplankton species have the capacity to produce toxins that can be accumulated through the food web. The consumption of shellfish and other grazers that have accumulated these algal toxins causes a variety of poisoning syndromes in humans. The global increase in HAB phenomena has involved the western Mediterranean basin in enclosed sea sites such as harbors, beaches, and near-shore waters along the coasts (40, 58). In these HAB episodes, several dinoflagellates (which include the genus *Alexandrium*) proliferated, with a number of species producing potent neurotoxins responsible for paralytic shellfish poisoning, which in humans may cause neurological disorders and, in extreme cases, death (3, 54). Three species of *Alexandrium* (*A. minutum*, *A. catenella*, and *A. taylori*) have often caused bloom events in the western Mediterranean over the last decade (M. Masó, E. Garcés, M. G. Giacobbe, A. Penna, M. Vila, G. Basterretxea, A. Orfila, J. Camp, and J. Tintore, Abstr. Xth Int. Conf. Harmful Algae, abstr. 189,

2002). *A. minutum* is the most widespread toxic species in the Mediterranean basin. This species formed toxic blooms along the northern part of the French Brittany coast (32), along the Catalan coast (Spain) (M. Vila, E. Garcés, M. Masò, and J. Camp, Abstr. Xth Int. Conf. Harmful Algae, abstr. 291, 2002), in the South Tyrrhenian Sea, and along the northwestern coast of the Adriatic Sea (Italy), where it contaminated mussels with paralytic shellfish poisoning toxin (27). Widespread blooms of *A. catenella* and *A. taylori* were described during recent years along the Spanish, French, and Italian coasts (15, 18, 34, 59). *A. taylori* is not a toxin producer, but it can proliferate, with high biomass densities.

Monitoring coastal waters for the presence of HAB species is essential in assessing the potential for bloom formation. Normally, this type of monitoring involves accurate morphology identification and enumeration of target phytoplankton species by using light or electron microscopy examinations in addition to toxicity tests of shellfish. Unfortunately, these identification methods can be difficult for long-term monitoring, because the microscopic analyses are time consuming and require a great deal of taxonomic experience to identify the species (47).

Because of the genetic diversity of different genera and species (30, 33, 38, 41), molecular tools may be very useful for the detection of a microorganism, both in seawater and in sedi-

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ment (5, 22, 43, 49, 51). Because of their rapidity, PCR-based methods are used more and more. The PCR amplification technique of targeting ribosomal DNA (rDNA) regions has been successfully employed for the detection of various toxic dinoflagellates in seawater samples (8, 11, 19, 44, 48). The rDNA genes have been the regions of choice for the development of molecular assays because these sequences are repeated in tandem in high copy numbers and are highly conserved (24, 36, 37, 50, 55). Different regions of the rDNA cluster have been selected as targets for PCR amplification. These regions include the small subunit, the large subunit, the 5.8S region, two internal transcribed spacers (ITS1 and ITS2), and the nontranscribed spacer (7). The choice of a particular region as the target was based mostly on the level of variability of each region within a particular species of interest and the requirements of assay specificity and sensitivity (1, 9, 10, 16, 29, 35).

Real-time PCR offers all the advantages of conventional PCR, such as high sensitivity and specificity, and also allows for the quantification of PCR product formation during the exponential phase of the reaction. The detection of PCR products is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye (25) to double-stranded (dsDNA). Even though real-time PCR was originally developed for clinical applications, it has recently been applied to microbial ecology. For example, it has been used to detect small-subunit rRNA in natural microbial communities (56) and for rapid detection of *Pfiesteria piscicida* in culture and environmental water samples (8).

In this study, we developed a real-time PCR-based assay using the 5.8S region as the target for the detection of *Alexandrium* species and quantification of *A. minutum* cells in both fixative-preserved environmental samples and cultures. Based on several rDNA sequences obtained from different *Alexandrium* spp. strains in GenBank, genus-specific primers were designed and used in real-time PCR assays, first on *Alexandrium* species cultures and then on field samples from one bloom spatial series collected in winter inside the Arenys harbor (Catalan coast).

MATERIALS AND METHODS

Algal cultures, sample collection, and species identification. The *A. minutum* strain used in this study (CNR-AMI-A4) was kindly provided by M. G. Giacobbe (CNR, Istituto per l'Ambiente Marino Costiero, Messina, Italy). *A. minutum* cultures were maintained in f/2 medium (21) at $17 \pm 1°C$. Light was provided by cool-white fluorescent bulbs (photon flux of 100 $\mu E \ m^{-2} \ s^{-1}$) on a standard 14-h-light, 10-h-dark cycle. Between 7 and 17 days after inoculation, when cultures were in exponential growth phase, algal cell density was accurately determined by enumeration of Lugol's iodine-stained cells (57) by using a hemacytometer (Neubauer; Hausser Scientific, Horsham, Pa.). To ensure reliability, the counts were repeated several times until the total number of cells counted was greater than 100. The average of these values was considered the concentration of cells in culture. Samples of culture equivalent to 1×10^5 or 5×10^4 cells were harvested by centrifugation at $4,000 \times g$ for 15 min. Cell pellets were carefully washed with 800 μl of sterile artificial seawater and stored at $-80°C$.

Field samples were collected from Arenys Harbor (Spain) during a huge *A. minutum* bloom in February 2002 (E. Garcés, I. Bravo, M. Vila, R. I. Figueroa, M. Masó, and N. Sampedro, submitted for publication) and another one in April 2003. A volume of 150 ml was fixed with Lugol's iodine solution; a 50-ml volume of preserved samples was settled in Utermöhl chambers for 24 h and counted by using an inverted microscope. After being counted, cells were concentrated by centrifugation, resuspended in 2 to 5 ml of seawater, and stored in the dark at 4°C. Cells were also counted before real-time PCR. For the identification of *A.*

minutum, the samples were dyed with calcofluor white (14) and observed under an epifluorescence microscope. Identification was based on cell shape and size but especially on the thecal plates tabulation (6).

DNA preparation from cultures and Lugol-fixed samples. Frozen pellets of 1×10^5 or 5×10^4 cells, respectively, were resuspended in 800 μl or 400 μl of lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.5% Nonidet P-40, 0.5% Tween 20, 0.1 mg of proteinase K/ml), briefly sonicated with Ultrasonic Homogenizer LABSONIC L with needle probe tip 40T (B. Braun Biotech International) for 10 seconds at 50 to 100 W, and incubated at 60°C for 3 h. The samples were vortexed every 30 min. The samples were then incubated at 100°C for 5 min to inactivate proteinase K and centrifuged at $12,000 \times g$ for 1 min at room temperature. The supernatants containing DNA were immediately used in real-time PCR assays. Alternatively, the supernatants were aliquoted, stored at $-80°C$, and used for PCR within 2 to 3 weeks.

Lugol-fixed samples were centrifuged at $4,000 \times g$ for 15 min at room temperature, and pellets were washed with 10 ml of sterile artificial seawater (0.4 M NaCl, 10 mM KCl, 20 mM MgSO$_4$, 20 mM MgCl$_2$, 10 mM CaCl$_2$, 2 mM NaHCO$_3$, 0.4 mM boric acid). DNA was prepared by resuspending the pellets in 400 μl of lysis buffer and following the procedure described above.

Alternatively, DNA was extracted by using a Dynabeads DNA DIRECT Universal kit (Dynal) or DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions.

DNA quantification. Purified DNA and DNA contained in crude extract were quantified by using a PicoGreen dsDNA quantification kit (Molecular Probes) and a spectrofluorophotometer RF-5301PC (Shimadzu). The standard curve was made with the bacteriophage λ DNA provided in the kit. PicoGreen is an ultrasensitive fluorescent nucleic acid stain for the quantification of dsDNA in solution with minimal interference by RNA, single-stranded DNA, nucleotides, salts, proteins, and detergents. PicoGreen provides a linear correlation between dsDNA concentration and fluorescence, with a detection range from 25 pg to 1 μg of dsDNA/ml (52). For the quantification of DNA in cell lysates, to avoid denaturation of DNA we did not boil the samples after incubation at 60°C. Alternatively, purified DNA was quantified on agarose gel by using serially diluted DNA marker 2 (MBI Fermentas) and a Gel Doc apparatus (Bio-Rad).

rDNA cloning and sequencing. The 5.8S rDNA and flanking internal transcribed spacers (ITS1 and ITS2) were amplified from purified *A. minutum* total DNA and cloned into the pMOSBlue vector (Amersham) as previously described (44). Several clones were sequenced by using the BigDye Terminator cycle sequencing kit (Applied Biosystems) in an ABI PRISM 310 instrument. Plasmid DNA was purified by using a Qiaprep Spin Miniprep kit (QIAGEN).

Primer design. The primers were designed by using Primer Express software (Applied Biosystems) and an alignment of all available ITS1-5.8S-ITS2 ribosomal DNA sequences for the genus *Alexandrium*. Sequences were either downloaded from GenBank or obtained from our laboratory. The alignment was constructed by using DIALIGN 2.0 (42) and CLUSTALW. The alignment included sequences of several strains of *A. catenella*, *A. minutum*, *A. taylori*, and *A. tamarense* (Table 1). The 5.8S region is very conserved among these species. Primers 5.8S-b5′ (5′-YGATGAAGAATGCAGCAAMATG-3′) and 5.8S-b3′ (5′-CAAGCAHACCTTCAAGMATATCC-3′) were designed to target consensus sequences specific for the genus *Alexandrium*. In order to confirm the sequence specificity, we used BLAST (Basic Local Alignment Search Tool) to search in the EMBL and GenBank databases for published sequences identical to the primers 5.8S-b5′ and 5.8S-b3′. At present, the databases contain about 480 5.8S sequences belonging to the *Dinophyceae* class and more than 800 belonging to the group Alveolata. The primers were purchased from Sigma-Genosys Ltd. The primers and PCR conditions were tested in a qualitative PCR assay to ensure the specificity of the reaction.

Theoretical basis of real-time PCR. In real-time PCR, reactions are analyzed during the initial exponential phase rather than at the end point. By using SYBR Green (an intercalating fluorescent dye) in the PCR mixture, it is possible to detect double-stranded PCR product formation after each cycle. For each reaction tube, the fluorescence signal of the reporter dye (SYBR) is divided by the fluorescence signal of the passive reference dye (ROX) to obtain a ratio defined as the normalized reporter signal (Rn). The higher the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The threshold cycle parameter (C_t) is defined as the fractional cycle number at which the fluorescence crosses a fixed threshold above the baseline. The amount of target sequence in an unknown sample is calculated by plotting the C_t value on the standard curve.

Real-time PCR. Real-time PCR assays were performed on an ABI PRISM 7000 SDS (Applied Biosystems) in a final volume of 25 μl containing 1$\times$ SYBR Green PCR Master Mix (Applied Biosystems) and primers at a final concentration of 300 nM. Two microliters of each appropriately diluted sample (standard

TABLE 1. Isolates and strains of *Alexandrium* species, collection sites, and EMBL ITS1-5.8S-ITS2 rDNA sequence accession numbers

Species	Strain	Collection site	EMBL accession no.
A. catenella	CSIC[a]-4	Catalan Sea, Mediterranean, Spain	AJ298900
A. catenella	M17	Harima Nada, Japan	AB006990
A. taylori	CSIC[a]-AV8	Catalan Sea, Mediterranean, Spain	AJ251654
A. taylori	CNR[b]-AT4	Ionian Sea, Mediterranean, Italy	AJ251653
A. taylori	CBA[c]-ATAYB2	Tyrrhenian Sea, Mediterranean, Italy	AJ300451
A. taylori	CYSTS	Tyrrhenian Sea, Mediterranean, Italy	AJ291785
A. tamarense	CCMP[d] 116	European Atlantic, Spain	AJ005047
A. tamarense	AT-A	Chinhae Bay, Korea	AF374224
A. tamarense	AT-2	Chinhae Bay, Korea	AF374227
A. tamarense	AT-6	Chinhae Bay, Korea	AF374228
A. tamarense	AT-B	Chinhae Bay, Korea	AF374225
A. minutum	CNR[b]-AMIA4	Ionian Sea, Mediterranean, Italy	AJ318460
A. minutum	LAC-27	Adriatic Sea, Mediterranean, Italy	AJ005050

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curve points and algal lysates) was added to 23 μl of the PCR master mixture. The following quantification cycling protocol was used: 95°C for 10 min, followed by 35 or 40 cycles at 95°C for 15 s and 60°C for 1 min. At the end of each run, a dissociation protocol was performed to ensure that only the specific PCR product was present. Experiments were performed with duplicates for each lysate sample and with triplicates for each standard curve point. Each PCR run included the standard curve, established by serially diluted plasmid containing the target sequence, and a no-template control.

Standard curve construction. The standard curve was constructed with 10-fold serial dilutions of plasmid containing the ITS1-5.8S-ITS2 sequence of the target. The curve range was from 10^6 to 10 copies. Dilutions were freshly prepared for each experiment from one aliquot of plasmid stock solution stored at −20°C.

RESULTS

***A. minutum* identification in field bloom samples.** The algal cells in field samples were rounded to ovoid and small sized (average dimensions, 21 to 24 μm wide and 22.5 to 24 μm long; $n = 110$). Plate 1′ was narrow and contacted the apical pore plate directly. It showed a small ventral pore on the anterior right margin. Plate 6″ was narrow, and the posterior sulcal plate was slightly wider than it was long. All these features are typical for the species *A. minutum* (6).

Specificity and sensitivity of real-time PCR assay. Selected primers for the conserved 5.8S rDNA region of the *Alexandrium* genus gave a 111-bp PCR product. Because real-time PCR was carried out in the presence of SYBR Green, a fluorescent dsDNA intercalating dye, it was important to avoid unspecific PCR product or primer dimer formation. The specificity of the primers and the absence of unspecific products or primer dimers were tested by analyzing the reactions in 2% agarose gel stained with ethidium bromide. The molecular weight of the amplified product was as expected, and no other bands were visible when DNA from *A. minutum*, *A. taylori*, or *A. catenella* was used (Fig. 1). Moreover, all real-time PCRs were followed by a dissociation protocol consisting of measuring fluorescence in a temperature gradient from 60 to 95°C. This protocol allowed establishment of the melting temperature of the PCR product, at the same time excluding the presence of other (shorter or longer) unspecific amplicons. The melting temperature of the amplicon was in agreement with the theoretical melting temperature calculated by using Primer Express software. The primers' specificity was also con-

firmed in silico by using BLAST: the results of the BLAST search showed that the sequences of selected primers matched exactly only with the 5.8S sequences belonging to the *Alexandrium* genus. Furthermore, the primers' specificity was tested by real-time amplification of representative DNA samples from other dinoflagellates and diatoms. Table 2 shows a list of DNAs screened and their phylogenetic affinities. Only the PCRs containing DNA from the genus *Alexandrium* were positive, while amplification products were undetectable in reactions containing the DNA of other dinoflagellates and diatoms. Real-time PCR sensitivity was tested by using a plasmid containing the ITS1-5.8S-ITS2 rDNA *A. minutum* sequence (see below). This assay was sensitive enough to detect 10 copies of 5.8S rDNA sequences in less than 35 amplification cycles.

Standard curve and dynamic range of real-time PCR. The standard curve was constructed with 10-fold serial dilutions of plasmid containing the ITS1-5.8S-ITS2 sequence of *A. minutum*. The dynamic range was wide: from 10^6 to 10 copies (up to 5 orders of magnitude); also, a strong linear relationship between the C_t and the log of the starting copy number was always demonstrated ($R^2 \geq 0.996$). The efficiency of the reaction (E) calculated by the formula $E = 10^{(1/m)} − 1$, where m is the slope of the standard curve, ranged from 80 to 90% in the different assays (Fig. 2).

Total DNA quantification in cells of *A. minutum*. In general, DNA content per algal cell is very heterogeneous, varying from 0.1 pg for *Navicula pelliculosa* to 200 pg for *Gonyaulax poliedra* (26). Nothing is known about the DNA content in *Alexandrium* species cells. We tried to establish the DNA content in *A. minutum* to support and validate data obtained with real-time PCR (see below). At first, we purified DNA from cultured algal cells by using commercial kits (QIAGEN or Dynal). DNA yields were variable, ranging from 89 to 144 ng per 100,000 cells. Due to the variability and poor DNA yield that result using commercial kits, we tried to quantify DNA directly in cell lysates. Because of its sensitivity and specificity, we used Pico-Green dye for quantification of DNA in crude cell lysates. Different scalar dilutions of cell lysate (from 1:50 to 1:5,000) were done to ensure the absence of inhibitors during quantification. We prepared the lysates as described in Materials and
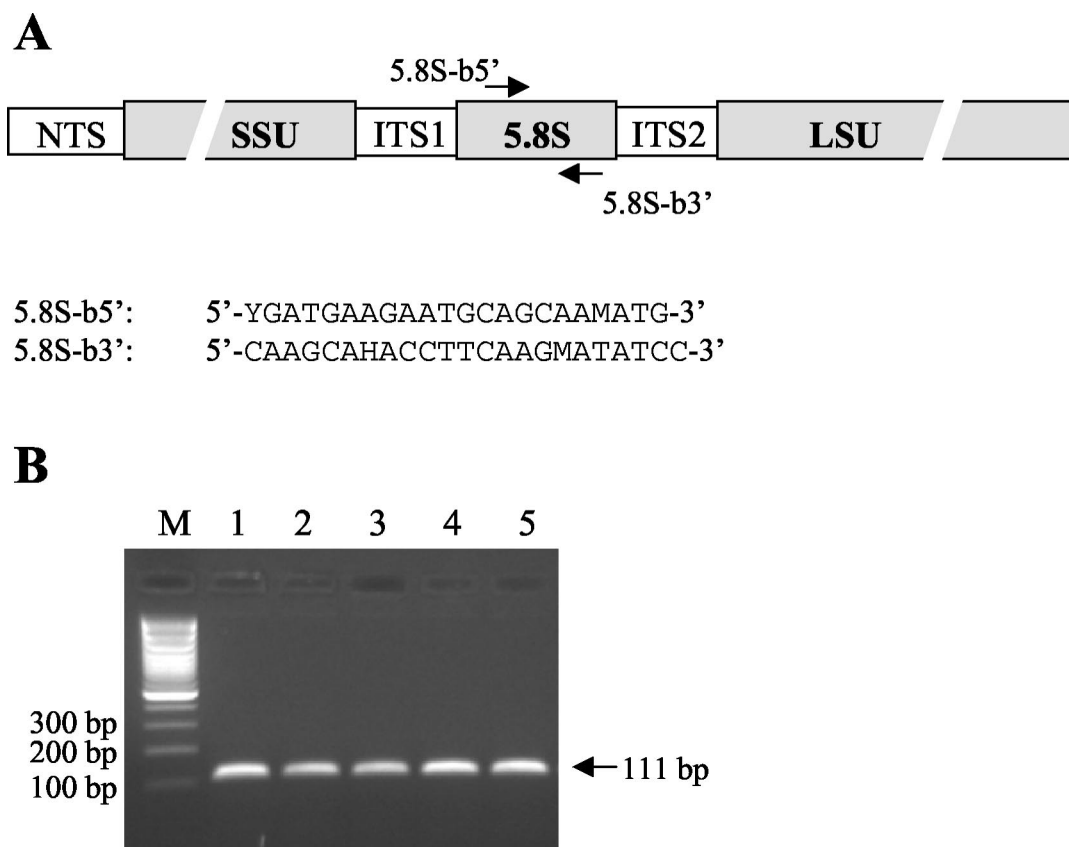
FIG. 1. (A) Organization of *Alexandrium* species rDNA and location of primers for the *Alexandrium* genus-specific PCR. (B) Amplification of target DNA in real-time PCR followed by analysis on standard agarose gel. Lanes: 1, template DNA from *A. catenella* CSIC-4; 2, template DNA from *A. taylori* CBA-1; 3, template DNA from *A. minutum* CNR-AMIA4; 4, template plasmid containing *A. catenella* ITS1-5.8S-ITS2 rDNA; 5, template plasmid containing *A. taylori* ITS1-5.8S-ITS2 rDNA; M, 100-bp ladder molecular size marker. A clean no-template control was included in the real-time assay (not shown on the gel). NTS, nontranscribed spacer; SSU, small subunit; LSU, large subunit.

Methods, but we did not boil the samples after incubation at 60°C, to avoid denaturation of DNA and loss of sensitivity of PicoGreen, which binds exclusively to dsDNA. In fact, it was observed that the quantity of DNA in the boiled samples was underestimated by about three times compared to that for the nonboiled samples (data not shown). The PicoGreen excitation-emission wavelength does not interfere with chlorophyll *a*, but excitation at 480 nm could interfere with beta carotene and xanthophyll absorbance. In order to assess the possible interference from pigments or other cell components, we compared the fluorimetric dosages of cell lysate diluted 1:20 with or without the PicoGreen component. The fluorescence intensity of the lysate without PicoGreen was about 0.2% compared to that for lysate with PicoGreen, suggesting that lysate autofluorescence was completely negligible.

The values obtained from the fluorimetric quantifications were expressed in pg/μl. Dividing these values for cell concentration in the lysate (125 cells/μl), we calculated the amount of

TABLE 2. Specificity of real-time PCR

Species	Strain	Phylogenetic association	Amplification[e]
Pseudonitzschia pseudodelicatissima	IEO[a]-PS 50V	Diatom (*Bacillariaceae*)	−
Gyrodinium corsicum	GC1 CSIC[b]-1	Dinoflagellate (*Gymnodiniaceae*)	−
Karlodinium micrum	KM1 CSIC[b]-1	Dinoflagellate (*Gymnodiniaceae*)	−
Coolia monotis	IEO[a]-CM7C	Dinoflagellate (*Ostreopsidaceae*)	−
Ostreopsis sp.	IEO[a]-OSBR3	Dinoflagellate (*Ostreopsidaceae*)	−
Dinophysis sacculus (ITS1-5.8S-ITS2 cloned sequence)	No strain	Dinoflagellate (*Dinophysidae*)	−
Alexandrium minutum	CNR[c]-AMIA4	Dinoflagellate (*Gonyaulacaceae*)	+
Alexandrium catenella	CSIC[b]-4	Dinoflagellate (*Gonyaulacaceae*)	+
Alexandrium taylori	CBA[d]-1	Dinoflagellate (*Gonyaulacaceae*)	+

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[d] Centro Biologia Ambientale, Urbino University, Italy.
[e] −, amplification products not detected; +, amplification products detected.
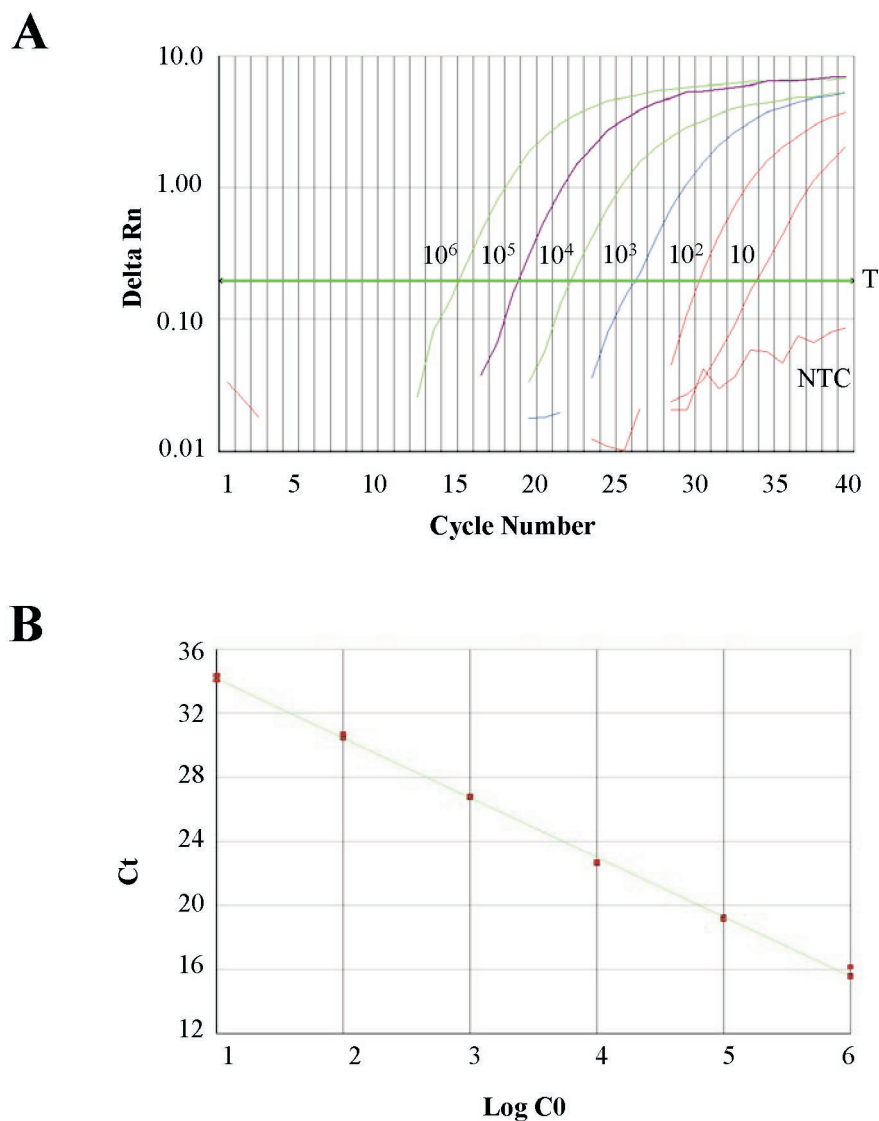
A

B

FIG. 2. Example of standard curve with plasmid containing ITS1-5.8S-ITS2 rDNA sequence of *A. minutum*. (A) Amplification plot for standard curve with plasmid copy number from 10^6 to 10. The cycle number is plotted versus the Delta Rn. The Delta Rn represents the Rn minus the baseline signal established in the early PCR cycles. Three replicates were performed for each reference DNA sample, but data for only one are shown here. (B) Calibration curve plotting log starting copy number (C_0) versus C_t. Slope, -3.71; correlation coefficient (R^2), 0.9986. NTC, no-template control; T, threshold.

DNA per cell. In this way we established that the DNA content per cell was 11.4 ± 2.6 pg for *A. minutum*. This average value was obtained from five experiments, in duplicate or triplicate, including two experiments in which we used the lysis buffer and protocol from a DNeasy Plant kit (QIAGEN) or a lysis buffer containing 1% SDS and 25 mM EDTA.

Application of real-time PCR for quantification of 5.8S rDNA in *A. minutum*. To avoid variability and low yields in DNA purifications, we used crude cell lysates (prepared as described in Materials and Methods) as DNA templates in PCRs. These lysates contained cell components which could interfere with PCRs. To find an amount of template which can be amplified without any inhibitory effect, cell lysates containing DNA were diluted fivefold until quantification results were proportional to sample dilutions. We found that *A. minutum*

lysate dilutions equal to or higher than 1:50 (corresponding to five cells per reaction) were always amplified without inhibition. This approach was possible because of the abundance of 5.8S rDNA sequences in dinoflagellate cells and because of the sensitivity of this PCR. The amount of 5.8S rDNA copies in the PCR mixtures was calculated for each diluted lysate sample by plotting the C_t values on a standard curve obtained using a known copy number of the plasmid containing the cloned target sequence (see Materials and Methods). The lysate dilutions used for the PCR amplifications were 1:50, 1:250, and 1:1,250, corresponding to 5, 1, and 0.2 cells per reaction, respectively. The number of 5.8S rDNA copies obtained for each sample after real-time PCR was normalized to that for sample dilutions corresponding to 1 cell per reaction. In this way, we found that the absolute number of 5.8S rDNA copies per cell of *A.*

TABLE 3. Quantification of *A. minutum* cells in bloom field samples from Arenys harbor (Spain): comparison between standard and real-time PCR counting methods

Sample ID[b]	Collection date	*A. minutum* cells/liter[a]	
		Standard method	Real-time method
2	April 2003	3,964,000 ± 313,500	3,276,450 ± 527,290
6	February 2002	2,500,000 ± 256,500	2,497,000 ± 74,500
9	February 2002	1,845,000 ± 156,000	2,475,000 ± 336,150
11	February 2002	2,125,000 ± 247,500	1,317,600 ± 153,250
12	February 2002	2,171,000 ± 239,000	2,255,000 ± 180,650
13	February 2002	2,950,000 ± 279,000	2,566,000 ± 353,140
1/1b	February 2002	1,832,000 ± 158,500	1,198,300 ± 99,000
3/0b	February 2002	286,000 ± 31,100	188,800 ± 20,750
4/2a	February 2002	1,894,000 ± 161,200	1,389,000 ± 137,950

[a] Values are ± standard deviations.
[b] ID, identification.

minutum was 1,084 ± 120.3. This value resulted from the average of 16 different determinations, ranging from 860 to 1,284, obtained from three different sample collections. Moreover, the absolute number of 5.8S rDNA copies normalized to 1 pg of purified DNA was also calculated, and the result was 104 ± 32.1. Total DNA was purified with Dynabeads from *A. minutum* cells either in the exponential growth phase (8 days after inoculation) or in the stationary phase (1 month after inoculation). The ratio between the number of 5.8S rDNA copies per cell and the number of 5.8S rDNA copies per picogram of purified DNA gave us the theoretical amount of total DNA per cell in *A. minutum*. The DNA content calculated in this manner was 10.42 pg/cell, in agreement with the DNA quantification with PicoGreen on cell lysates. Thus, we confirmed the real-time PCR data, and at the same time, we validated the DNA content per cell measured with PicoGreen.

Quantification of *A. minutum* in field bloom samples fixed with Lugol. Based on the number of 5.8S gene copies per cell, the concentrations of *A. minutum* in environmental field samples were determined by using the real-time PCR assay. *A. minutum* field bloom samples were collected and fixed with Lugol as described in Materials and Methods. The samples were stored at 4°C for more than 1 year prior to being analyzed. Lysates of Lugol-fixed bloom samples were diluted 10-fold (1:10 to 1:1,000) to eliminate inhibitory effects of fixative or cell components and used in real-time PCR assays. Dilutions of 1:10 partially inhibited the PCR and were not considered in the analysis of results. Quantification results were compared with those for standard counting methods (Table 3). The results showed that the two methods were comparable in all tested bloom samples. In fact, one-way analysis of variance showed that, with a *P* value of 0.01, the means of the standard and real-time counting methods for each sample were not significantly different. The cell concentration in sample 3/0b is much lower than that for other bloom samples because some of the vial content was lost during transportation.

DISCUSSION

A. minutum is a small and widely distributed toxin-producing species responsible for HAB events in the western Mediterranean basin (13, 17, 59). The real-time PCR-based assay de-

scribed in this study has proven to be specific and sensitive for the detection of *Alexandrium* spp. cells in cultured and environmental samples. Bowers et al. (8) previously developed a real-time PCR assay for rapid detection of *Pfiesteria piscicida* 18S rDNA, but no quantitative approaches were attempted due to the lack of information concerning the number of 18S gene copies per cell. In our study, which was focused on the 5.8S rDNA region of the genus *Alexandrium* (dinoflagellates), we attempted to overcome this limit by determining the number of rDNA copies per *A. minutum* cell.

DNA extraction from marine algae is hampered by the large quantity of polysaccharides and polyphenolics produced in many species. There are several DNA extraction methods that work well with marine algae, but DNA yield and reproducibility are sometimes poor. Because of this, for quantification purposes we decided to perform PCRs using crude cell lysates from *A. minutum* cultures and field bloom samples. These lysates were diluted until the potential inhibitory effects of contaminants were eliminated. This approach was possible because in most eukaryotes the rDNA gene family is repeated in tandem in a high copy number. For example, 100 to 200 copies are present in yeast, and 50 to 10,000 copies are present in mammals (49). Within the group Alveolata, the rDNA copy number ranges from 2 to >100. Two copies have been reported in *Theileria parva* (28) and *Cryptosporidium parvum* (31), 3 copies have been reported in *Babesia bovis* (12), 110 copies have been reported in *Toxoplasma gondii* (20), and 100 to 200 copies have been reported in *Pfiesteria piscicida* (49). Nothing is known about rDNA copies in the *Alexandrium* genus. In this work, for the first time we provided information regarding genome size and rDNA sequence amount in *A. minutum* cells. This information allowed us to determine the number of *A. minutum* cells in environmental seawater samples without doing laborious microscopic counting.

In oceanographic field work, fixation of samples is an important step in preserving archived material until laboratory processing. Fixation methodology should have minimal impact on downstream analysis. Lugol's solution is a widely used fixative that, like glutaraldehyde and formaldehyde, can introduce artifacts like reduction in the abundance of particular taxa and changes in phytoplankton cell size. In the dinoflagellates, for example, fixation could induce species-specific cell swelling or shrinking (39). In this work, we demonstrated that the real-time PCR approach can be applied as a quantitative assay to environmental *A. minutum* samples fixed with Lugol's solution even after 14 months of storage without significant loss of cell number and 5.8S rDNA sequence information. In samples 11, 1/1b, and 3/0b, the more evident variability between standard counting methods and real-time methods could be due to some cell loss during sample concentration from 50 ml to 2 to 5 ml and/or during long-term storage in Lugol's solution. However, after statistical analysis, the resulting values from the two counting methods were not significantly different. Generally, there are two main factors that could affect the variability between standard and real-time PCR-based counts. The first is the cellular DNA content during a bloom event. *A. minutum* cells are generally haploid (*n*), only the planozygotes/resting cysts being diploid (2*n*). It has been stated that one of the causes of the bloom decline might be sexual processes and subsequent encystment (4, 45, 46). The high percentage of

diploid cells in a bloom could lead to an overestimation of cell number when the real-time PCR-based count is used. However, during the intensive monitoring of the *A. minutum* bloom in Arenys Harbor in February 2002, it was observed that the encystment rate was very low (<1%) (E. Garcés et al., submitted). The second factor concerns the presence of species morphologically similar to the target in the phytoplankton community. In the Arenys area, *A. minutum* usually blooms together with other dinoflagellates such as *Prorocentrum micans*, *P. triestinum*, and *Scrippsiella* spp., among others. In some cases, the *Scrippsiella* species have the same size as *A. minutum*. In those cases, the position in which *Scrippsiella* is observed under light microscopy is critical for correct identification. The possible misidentification of some specimens could increase the counting error when these species are highly abundant. However, in Arenys Harbor during the bloom in February 2002, *Scrippsiella* spp. represented less than 0.3% of the dinoflagellate concentration (M. Vila, unpublished data).

In this work, we demonstrated the feasibility of detection and quantification of *A. minutum* in high-density bloom samples. It is noteworthy that this real-time PCR assay was able to amplify ≤ 0.2 cell equivalent of algal lysate and up to 10 copies of 5.8S rDNA. Therefore, the sensitivity level of this molecular method will allow detection of *Alexandrium* species not only at bloom concentrations but also in field samples containing a low number of cells, which will be extremely useful for long-term monitoring programs, during which high-density blooms could be exceptional events and low-density blooms could occur commonly.

The real-time PCR assay was optimized with primers specific for the genus *Alexandrium*. For this reason, we were able to quantify only *A. minutum* bloom samples, where other *Alexandrium* species were not significantly represented. In fact, up to 96% of the microphytoplankton abundance in the bloom samples collected for this study was *A. minutum*. Optimization of real-time PCR with *A. minutum*-specific primers will allow detection and quantification of these algae in environmental samples containing mixed *Alexandrium* species populations. Moreover, we established that *A. minutum* rDNA quantification data cannot be extended to other *Alexandrium* species. In fact, preliminary data indicate that the quantity of 5.8S rDNA in *A. catenella* and *A. taylori* is different from that in *A. minutum*, generally appearing proportional to the cell's DNA content. After we complete and validate these data, we may have the tools for fast monitoring of toxic and harmful *Alexandrium* algae in the Mediterranean sea.

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