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Quantitative PCR Coupled with Melt Curve Analysis for Detection of Selected *Pseudo-nitzschia* spp. (Bacillariophyceae) from the Northwestern Mediterranean Sea▽

Karl B. Andree,[1,3]* Margarita Fernández-Tejedor,[1,3] Laurence M. Elandaloussi,[1,3] Sonia Quijano-Scheggia,[4] Nagore Sampedro,[2,3] Esther Garcés,[2,3] Jordi Camp,[2,3] and Jorge Diogène[1,3]

Institut de Recerca i Tecnologia Agroalimentàries, Carretera Poble Nou Km 5.5, E-43540 Sant Carles de la Rápita, Spain[1]; Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CSIC, Passeig Marítim de la Barceloneta, 37-49, E-08003 Barcelona, Spain[2]; Xarxa de Referència de Recerca i Desenvolupament en Aqüicultura, Generalitat de Catalunya, Catalonia, Spain[3]; and Centro Universitario de Investigaciones Oceanológicas, Universidad de Colima, Carretera Manzanillo-Barra de Navidad Km 19.5, Colonia El Naranjo, C.P 28860, Manzanillo, Colima, Mexico[4]

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The frequency and intensity of *Pseudo-nitzschia* spp. blooms along the coast of Catalonia have been increasing over the past 20 years. As species from this genus that are documented as toxigenic have been found in local waters, with both toxic and nontoxic species cooccurring in the same bloom, there is a need to develop management tools for discriminating the difference. Currently, differentiation of toxic and nontoxic species requires time-consuming electron microscopy to distinguish taxonomic features that would allow identification as to species, and cryptic species can still remain misidentified. In this study, cells of *Pseudo-nitzschia* from clonal cultures isolated from seawater were characterized to their species identity using scanning electron microscopy, and subsamples of each culture were used to create an internal transcribed spacer 1 (ITS-1), 5.8S, and ITS-2 ribosomal DNA database for development of species-specific quantitative PCR (qPCR) assays. Once developed, these qPCR assays were applied to field samples collected over a 2-year period in Alfaques Bay in the northwestern Mediterranean Sea to evaluate the possibility of a comprehensive surveillance for all *Pseudo-nitzschia* spp. using molecular methods to supplement optical microscopy, which can discern taxonomy only to the genus level within this taxon. Total *Pseudo-nitzschia* cell density was determined by optical microscopy from water samples collected weekly and compared to results obtained from the sum of eight *Pseudo-nitzschia* species-specific qPCR assays using duplicate samples. Species-specific qPCR followed by melt curve analysis allowed differentiation of amplicons and identification of false positives, and results correlated well with the total *Pseudo-nitzschia* cell counts from optical microscopy.

The correct identification of species and characterization of their toxic potential are key aspects of harmful alga monitoring programs. However, the value of the data obtained from such work hinges on the key step of determining taxonomy, usually performed via the use of keys for morphological traits which, for some taxa, are not always invariant (8, 39), especially among the pennate diatoms that undergo a miniaturization of the frustules through repeated cell divisions (9, 50). Taxon delimitation is made somewhat more complicated by the additional need to identify mating type and verify sexual reproduction as recent studies suggest that there is a need to reevaluate the concept of species in diatoms using mating experiments as well as DNA sequence analyses (2, 7, 37, 39, 40, 46).

Amnesic shellfish poisoning is caused by certain species and/or strains of *Pseudo-nitzschia* due to the production of domoic acid (4). Further, high levels of genetic diversity have been found among particular species, with cryptic species being misidentified (1, 14, 15, 35, 38). For these reasons it is important to correctly identify the *Pseudo-nitzschia* species present and verify their genotypes to more clearly understand the toxic threat they may represent. In monitoring programs where optical microscopy is the tool used for species identification, *Pseudo-nitzschia* spp. can be characterized only at the genus level, meaning that differentiating potentially toxic from nontoxic species/strains is not possible.

Until 2001 only three species of the genus *Pseudo-nitzschia* were known from the Catalan coast (55). In a recent study, nine species of the genus were identified through the implementation of morphological keys, genetic data, and mating experiments (45, 47). Some of these now documented locally are known to be toxigenic (7, 8, 44, 48, 51).

Quantitative PCR (qPCR) has been used previously to quantify microalgae to the genus level for *Alexandrium* (23) and *Pseudo-nitzschia* (19). While coding regions of the ribosomal DNA (rDNA) cistron have proven useful for genus-level discrimination (26, 41, 52, 53), the noncoding external and internal transcribed spacers (ETS and ITS, respectively) have less selective pressure imposed on them, making them regions of high diversity between and within species (30). The ITS-2

* Corresponding author. Mailing address: Institut de Recerca i Tecnologia Agroalimentàries, Crta. de Poble Nou, Km 5.5, E-43540 San Carlos de la Rapita (Tarragona), Spain. Phone: 34 977 74 5427. Fax: 34 977 74 4138. E-mail: karl.andree@irta.es.

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sequence together with secondary structure has been found to be useful for taxonomic assignment at various levels, suggesting the existence of some selective pressure conserving the sequence of the ITS-1, 5.8S, and ITS-2 rRNA gene regions (11, 12). Further, the level of conservation has been useful for inter- and intraspecific population studies of *Pseudo-nitzschia* spp. (16, 31, 43).

PCR methodologies utilizing SYBR Green dye enables the use of melt curve analysis to confirm specificity of the amplified product without gel electrophoresis or sequencing. Recent improvements in PCR methods have shown melt curve analysis to be useful for single nucleotide polymorphism (SNP) detection and differentiation of hetero- and homozygotes, as well as species detection (13, 24, 49). Small changes in sequence of the ITS-1, 5.8S, and ITS-2 rRNA gene regions between different strains of the same species should also be detectable and facilitate species as well as strain identification of *Pseudo-nitzschia* spp.

Our harmful microalga monitoring program has operated for 20 years and has shown annual blooms of *Pseudo-nitzschia* spp. to be increasing in intensity throughout this period (18). To protect an important shellfish growing region in Alfaques Bay, weekly sample collections from five locations are analyzed for a variety of physical and chemical parameters, as well as description of the abundance of various microalga taxa, including *Pseudo-nitzschia* spp. The weekly survey includes only optical microscopy and toxin analyses for tracking the occurrence of these blooms. During surveillance for harmful algae, potentially toxic species may be detected in advance of detectable levels of toxin or cytotoxicity. Therefore, there needs to be a level of biodiversity detection below the genus level in "real time." In this study, we used standard survey methods for sample collection to obtain material for evaluation. The intent was to implement newly developed qPCR assays to examine and compare the efficacy of species-specific qPCR for detection and quantification with that of optical microscopy, which can discern *Pseudo-nitzschia* spp. only at the genus level.

MATERIALS AND METHODS

Alfaques Bay sample collection for monitoring. Water samples for this study within the monitoring program were collected from five points in Alfaques Bay each week during a 2-year period from April 2007 to February 2009. A total of 310 samples were analyzed in parallel using integrated water column samples collected using a silicone hosepipe 7 m long. The content of the hosepipe was mixed in a bucket, and two subsamples were retained for analysis. During extended periods in which there was an absence of *Pseudo-nitzschia* cells detected using microscopy, only a portion of these samples were tested using qPCR. Of the five sampling stations, one point is located inside the port area (PA), and four others are outside the port, i.e., in the exterior area of the bay (EA), interior bay area (IA), central bay area (CA), and central interior area (CIA). For qPCR 5 drops of Lugol's iodine were added to a 50-ml sample of seawater, and the entire biomass was collected by centrifugation at 2,500 rpm (990 × *g*) for 20 min using a swinging bucket rotor, with a second centrifugation of the bottom 2 ml at 5,000 rpm (2,300 × *g*) for 5 min in an Eppendorf microcentrifuge, after which the majority of supernatant was removed by pipetting. These pellets were either processed immediately or frozen at −20°C for later analysis.

DNA extraction. Genomic DNA from cultures and seawater samples was extracted in the same manner as described below. The cell pellet was resuspended in 200 μl of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6) and transferred to a 2-ml screw-cap cryotube containing approximately 50 mg of 0.5-mm diameter zirconium glass beads (Biospec). Twenty-five milliliters of 10% dodecyltrimethylammonium bromide (DTAB) and 200 μl chloroform were added, and cellular material in the seawater samples was disrupted using a BeadBeater-8 (BioSpec) pulsed for 40 s at full speed. The beads and cell debris were pelleted, and organic and aqueous phases were separated by centrifugation at 2,000 × *g* for 5 min, after which 100 μl of the aqueous supernatant was transferred to a fresh tube. The DNA was extracted from this solution using a GeneClean Kit (MP Biomedicals, LLC) for genomic DNA isolation following the protocol adapted by Fawley and Fawley (17).

Optical microscopy. A 150-ml subsample of each integrated sample was fixed with 1% formalin for microscopic identification and enumeration using inverted microscopy. At arrival to the laboratory, 50-ml subsamples for microscopic analysis were acclimated to room temperature, and settled in 50-ml Hydro-Bios chambers for 24 to 48 h. Either five fields of 0.78 mm^2 each along one diameter of the settling chamber or the whole chamber was counted at a magnification of ×200 to ×400 in order to obtain sufficient cells for an accurate count.

Clonal cultures. Cells of *Pseudo-nitzschia* spp. were identified from live field samples collected from several locations along the Catalan coast (for details on sampling locations outside Alfaques Bay, see reference 45). After examination under an inverted microscope (Leica DM-IL), cells were isolated with a glass Pasteur pipette and transferred into a tissue culture flask filled with silicate-containing f/2 or L1 medium (27, 28). These flasks were subsequently maintained at 19 to 21 ± 1°C using a 12:12-h light-dark cycle. Illumination was provided by fluorescent tubes (Gyrolux, Sylvania, Germany) with a photon irradiance of 100 μmol of photons m^{-2} s^{-1}.

Morphometric characteristics in SEM. Lugol-fixed natural and clonal culture samples were processed for scanning electron microscopy ([SEM] Hitachi S-3500N microscope operated at 5 kV) to distinguish the fine structure of the frustules and confirm species identification (for more details on morphology and electron microscopy images, see reference 45). This analysis was performed on all clonal cultures obtained from seawater but on none of the field samples collected for routine monitoring.

Cloning and sequencing. rRNA gene sequences were obtained from all the clonal cultures isolated from seawater, the majority of which were *Pseudo-nitzschia calliantha* and *Pseudo-nitzschia delicatissima* (for details, see reference 45). Primers MicroSSU (3′ end of the 18S rRNA gene) and DINO E (5′ end of the 24S rRNA gene) were used to amplify the intervening ITS-1, 5.8S, and ITS-2 rRNA gene regions. Amplified rDNA products or their plasmid clones, were sequenced bidirectionally (Sistemas Genómicos, LLC; Valencia, Spain) using the same primers as those used in the initial amplification (MicroSSU and DINO E) (Table 1). Plasmids were produced from a subset of the sequences obtained and purified using QiaPrep kits (Qiagen, Valencia, CA). A plasmid clone was retained for each of eight species of *Pseudo-nitzschia* obtained in the study, and each clone was representative of the consensus sequence for all strains of that species (Table 2). These plasmids were checked for quality and quantity by agarose gel electrophoresis and spectrophotometry and used later as internal calibrator samples in each qPCR experiment. All sequences were manually edited and aligned using BioEdit, version 7.0.5.2 (29).

Primer design and qPCR. The aligned sequences were examined for areas unique to each species in either the ITS-1 or ITS-2. Each ITS primer was then paired with the appropriate 5.8S primer (either sense or antisense) to amplify products of similar size to maintain similar efficiencies of amplification for each assay (Table 1). Primer sequences were checked using an *in silico* PCR program (Amplify, version 3.1; University of Wisconsin, Madison, WI) and by BLAST analysis before the *in vitro* PCR cross-testing was begun using plasmid DNA and genomic DNA from clonal cultures.

For detection of each species a hemi-specific assay was used containing one genus-specific 5.8S primer and one species-specific ITS-1 or ITS-2 primer (Table 1). Amplifications were performed on an ABI 7300 in 20-μl volumes extended for 45 cycles following a standard two-step protocol of 94°C for 30 s followed by primer annealing/extension at 65°C for 30 s. Reaction mixtures contained SYBR Green dye for amplification detection and melt curve analysis. The thermal profile for melt curve determination began with an incubation of 1 min at 60°C with a gradual increase in temperature (1°C/15 s) to 95°C, during which time changes in fluorescence were monitored.

Preliminary cross-testing was done using plasmid clones and genomic DNA from cultures to determine primer specificity. Each primer set was tested using each *Pseudo-nitzschia* spp. genomic DNA and a sample of *Protoceratium reticulatum* genomic DNA, (as an example of nontarget species) individually and as a mixture of all nine genomic DNA samples. This same cross-testing was performed using the plasmid clones of the ITS-1, 5.8S, and ITS-2 rRNA genes for each species to ensure the reliability of the clones as calibration samples within each experimental plate.

To improve reproducibility among replicates, it was found necessary to dilute DNA from field samples at 1:50. Higher dilutions caused the threshold cycle (C_T) values to increase without further improvement in reproducibility; therefore, dilutions of field samples (1:50) became part of the standard protocol. Many of

TABLE 1. Primers used for amplification of the ITS-1, 5.8S, and ITS-2 rDNA regions, the genus-specific 5.8S primers, and the specific-specific ITS primers

Species and/or target (specificity)[a]	Primer	Sequence (5′–3′)	Size (bp)[b]
18S rDNA (sense)	MicroSSU	GTGAACCTGCGGAAGGATC	~800
23S rDNA (antisense)	DINO E	CCKSTTCAYTCGCCRTTAC	~800
Pseudo-nitzschia spp. 5.8S rDNA (sense)	5.8S	CAGCGGTGGATGTCTAGGTTC	~230
Pseudo-nitzschia spp. 5.8S rDNA (antisense)	5.8SR	GAACCTAGACATCCACCGCTG	~180
P. brasiliana ITS-1 (sense)	QPbra	CCATCCCGACGCCAACTTAACG	185
P. calliantha ITS-2 (antisense)	QPcal3	GCTGGCTACTGGAGCAGCAAC	236
P. delicatissima ITS-1 (sense)	QPdelRa2	GTGCAATACTTTGCTTGGGTTTCG	182
P. arenysensis ITS-2 (antisense)	QPdelRa3	AACCAGCTACCGGTATAGAATACG	205
P. fraudulenta ITS-2 (antisense)	QPfrau	CCGCTGCTAGAGCGGTCAGAG	225
P. galaxiae ITS-1 (sense)	QPgal	AGTCTCACTAGCATACTGTCGTCA	185
P. multistriata ITS-1 (sense)	QPmult	CACTGGCAGAGCTAATGCATTCT	185
P. pungens ITS-1 (sense)	QPpung	GTTGCTGCCATTCTTTACGATTGG	185

[a] The strand specificity (sense or antisense) of the primers is indicated.
[b] The size of the specific amplicon for qPCR when paired with the appropriate genus-level primer is shown.

the samples were collected prior to completion of the optimization tests, and these were stored as cell pellets at −20°C for testing at a later date. No apparent difference was observed in results obtained from samples processed fresh versus frozen.

The quantity of cells in the qPCR seawater samples was extrapolated from C_T values calibrated to external standard curves. Standard curves were first prepared using serial dilutions of plasmids, and later cell culture samples were used. Genomic DNA standard curves from clonal cultures were developed using samples serially diluted prior to counting and DNA extraction. However, cultures from some of the species had died before good results for standard curves could be obtained. Reliable standard curves were obtained from cultures of four different species (*P. calliantha*, *P. delicatissima*, *Pseudo-nitzschia multistriata*, and *Pseudo-nitzschia pungens*) using cell samples diluted from 10^5 to 10^2 cells. Every qPCR experimental plate had high-copy-number (10^5) and low-copy-number (10^2) plasmid positive controls for confirmation of the melt curve analysis and internal calibration, in addition to negative controls containing only the PCR reagents.

The cell totals obtained from the two counting methods (optical microscopy for genus-level detection and the sum of eight qPCR assays for species-level detection of *Pseudo-nitzschia*) were compared by linear regression using statistical analysis software (SigmaStat, version 9.0; SPSS, Richmond, VA).

RESULTS

Sequence analysis. The initial species identified were *P. calliantha*, two genotypes of *P. delicatissima*, *Pseudo-nitzschia fraudulenta*, *P. multistriata*, and *Pseudo-nitzschia pungens* (47).

TABLE 2. Designations of strains discussed in this study and accession numbers of the submitted sequences[a]

Plasmid	Species	Strain	ICM no.	GenBank accession no.
pCal24	*P. calliantha*	24	119	DQ990359
pDelRa2	*P. delicatissima*	Ra2	101	DQ990362
pDelRa3	*P. arenysensis*	Ra3	102	DQ990363
pFrauPO2	*P. fraudulenta*	PO2	178	DQ990364
NA[b]	*P. fraudulenta*	AR2	104	DQ990365
NA	*P. fraudulenta*	AR3	105	DQ990366
pMultCM3	*P. multistriata*	CM3	115	DQ990369
pPungPO3	*P. pungens*	PO3	108	DQ990370
pBra172	*P. brasiliana*	172	172	EU327364
pGal173	*P. galaxiae*	173	173	EU327368

[a] Most of the strains used in preparing this study were shared with the Instituto de Ciencias Marinas (ICM), and their strain designation numbers are also provided. The plasmids used as positive controls and internal calibration samples for each of the species tested are also indicated.
[b] NA, not applicable.

Late in the study two additional species were identified: *Pseudo-nitzschia brasiliana* (36) and *Pseudo-nitzschia galaxiae* (37). For all clonal cultures, species identifications were performed using morphometric analysis (SEM) and confirmed by genetics (sequencing of the ITS-1, 5.8S, and ITS-2 rRNA gene regions). The sequencing of the ITS-1, 5.8S, and ITS-2 rRNA gene regions found these regions of the genome to contain nonconserved regions useful for designing probes or primers to distinguish between species. Where genotypic differences between strains of the same species seemed too large in one of the ITS regions, the other ITS region was chosen for the species-specific primer design. Even the more "conserved" ITS region in a few strains had single nucleotide point mutations (SNPs), but primers were designed outside these areas. A conserved region in the 5.8S rRNA gene was chosen as the site from which to design a second primer to pair with the species-specific primers. In this way each assay amplifies approximately the same size product to ensure that assays have similar amplification efficiencies.

Assay development. External standard curves were established using dilutions of plasmid clones representative of a consensus sequence for each species ITS-1, 5.8S, and ITS-2 rRNA gene region. Correlation between *Pseudo-nitzschia* quantification by optical microscopy and qPCR using standard curves derived from plasmid DNA was poor (data not shown). A likely explanation for this is a change in the copy number of the rRNA cistron. Dilutions of cell culture genomic DNA were used for establishing standard curves as well. The slopes of the standard curves (an indicator of PCR efficiency) were all similar, ranging from −3.2 to −3.7, demonstrating that the assays should be able to detect each species with similar efficiencies (average efficiency, 93%). Using these standards, a good correlation was established with the optical microscopy cell counts.

Cross-testing of species-specific primers with each species ITS-1, 5.8S, and ITS-2 rRNA gene plasmid clone and genomic DNA samples from cultures showed that primers do not hybridize with the DNA samples of nonidentical species. Nor did tests using mixtures of nine genomic DNA samples (from eight *Pseudo-nitzschia* species and *Protoceratium reticulatum*) indicate significant impairment of specific amplification in each assay. The efficacy in estimation of copy number in mixed

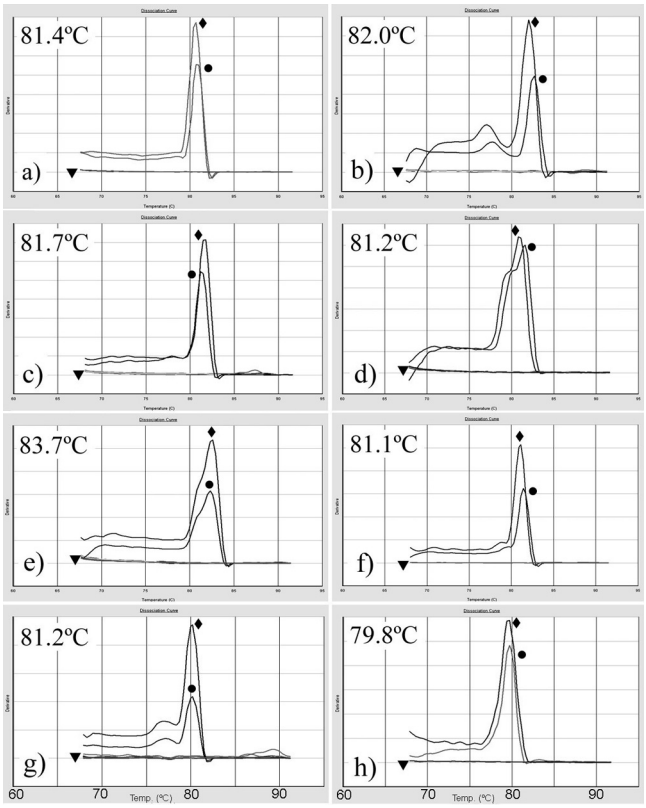
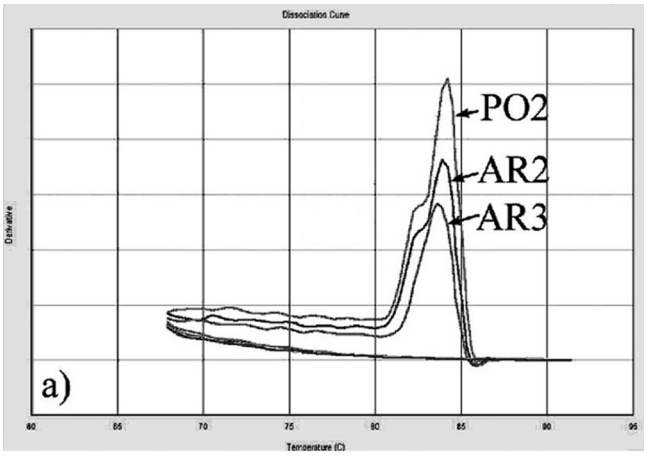


FIG. 1. Species-specific primers tested to demonstrate melt curve profiles and melting temperatures for each of the *Pseudo-nitzschia* species examined: *P. arenysensis* (a), *P. brasiliana* (b), *P. calliantha* (c), *P. delicatissima* (Ra2) (d), *P. fraudulenta* (PO2) (e), *P. galaxiae* (f), *P. multistriata* (g), and *P. pungens* (h). In the experiment shown within each panel, plasmid clone (◆) and genomic DNA (●) for the target species are tested together with the nontarget species (▼). The shapes of the curves are characteristic for strains with these genotypes. The melt curves for *P. arenysensis*, *P. calliantha*, and *P. galaxiae* have similar melt curve profiles and melting temperatures; however, primer specificity allowed clear differentiation of species. Temperature (°C) is shown on the *x* axis at the bottom.



b)

ITS-1

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....|....| ....|....| ....|....| ....|....| ....|
          80         90        100        110
PO2-Pfraud  TTGCCTCAAA AG-------- ---------- -ACTTGTATA CAGGT//
AR2-Pfraud  TTGCCTCAAA AGTCA----- ---------- -ACTTGTATA CAGGT//
AR3-Pfraud  TTGCCTCAAA AGTCAACTTG TATACAGGTC AACTTGTATA CAGGT//
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ITS-2

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....|....| ....|....| ....|....| ....|....| ....|..
          510        520        530        540
PO2-Pfraud  //GCCCAGTT GCTGGTTGCT ATGGCTCTGA CCGCTCTAGC AGCGG//
AR2-Pfraud  //GCCCAGTT GCTGGTTGCT ATGGCTCTGA CCGCTCTAGC AGCGG//
AR3-Pfraud  //GCCTAGTT GCTGGTTGCT ATGGCTCTGA CCGCTCTAGT AGCGG//
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FIG. 2. (a) An example of strain differentiation by melt curve analysis using three strains of *P. fraudulenta*. PO2 and AR2 have an inflection in the curve which is lacking in AR3. Different quantities of genomic DNA were used for each sample to enable clear separation of melt curves. (b) Portions of the ITS-1 and ITS-2 regions from three strains of *P. fraudulenta*. The ITS-2 region shown is a fragment of the amplicon for qPCR detection. Gaps (−) and areas of discontinuous sequence (//) in this figure are indicated. The strain AR3 has a T-C transition mutation shown highlighted in gray. A sample of the larger variability in the ITS-1 region is shown for comparison.

genomic DNA samples in each cross-testing experiment was not significantly different from that of single-template reaction mixtures. Melt curve analysis was also performed to examine genomic DNA and plasmid clones from each species. The melt curve profiles were nearly the same between the genomic sample and the plasmid clone for each species, yet differences between most species were identifiable (Fig. 1). While differences in melt curve profiles between *Pseudo-nitzschia arenysensis* and *P. calliantha* were negligible, the primer specificity was clearly demonstrated. The melt curve profiles together with the specificity of the primers seemed to offer reliable discrimination between species. Using the primers for *P. fraudulenta*, we were also able to identify two distinct strains by a change in the melt curve profile (Fig. 2). This was a reproducible result dependent on a single SNP (T → C transition mutation) within the amplicon.

Field testing. Most of the samples in which qPCR detected *Pseudo-nitzschia* spp. in 2007 (the beginning of field sampling) had a high abundance of *P. calliantha* although some *P. pungens* cells were also detected (Fig. 3 and 4). During 2007 and early 2008 a good correlation was seen between the two detection methods ($P < 0.016$). During this time the species composition shifted from primarily *P. calliantha* to *P. delicatissima*. In the later part of 2008, the species composition shifted back to *P. calliantha*, with the additional appearance of *P. galaxiae*. There was a brief appearance of *P. arenysensis* during 3 weeks near the middle of 2008. Only on a few occasions and at very low densities were *P. brasiliana*, *P. fraudulenta*, and *P. multistriata* detected. On several occasions multiple species were detected in the same water sample; moreover, the total cell abundance that was detected using multiple qPCR species-specific assays was in good agreement with the optical microscopy results (Table 3). The overall trend for both methods of diatom detection (qPCR and optical microscopy) showed significant statistical correlation ($P < 0.05$) when a 10-week period during 2008 was excluded. During this period, a bloom of very high density was detected by optical microscopy, but this analysis was able to provide total cell counts only of the genus *Pseudo-nitzschia* (Fig. 4). The qPCR method detected some *Pseudo-nitzschia* species during this period but at much lower densities and therefore did not account for the majority of this particular bloom.

In only 29 of the samples tested (9.6%) was there a complete

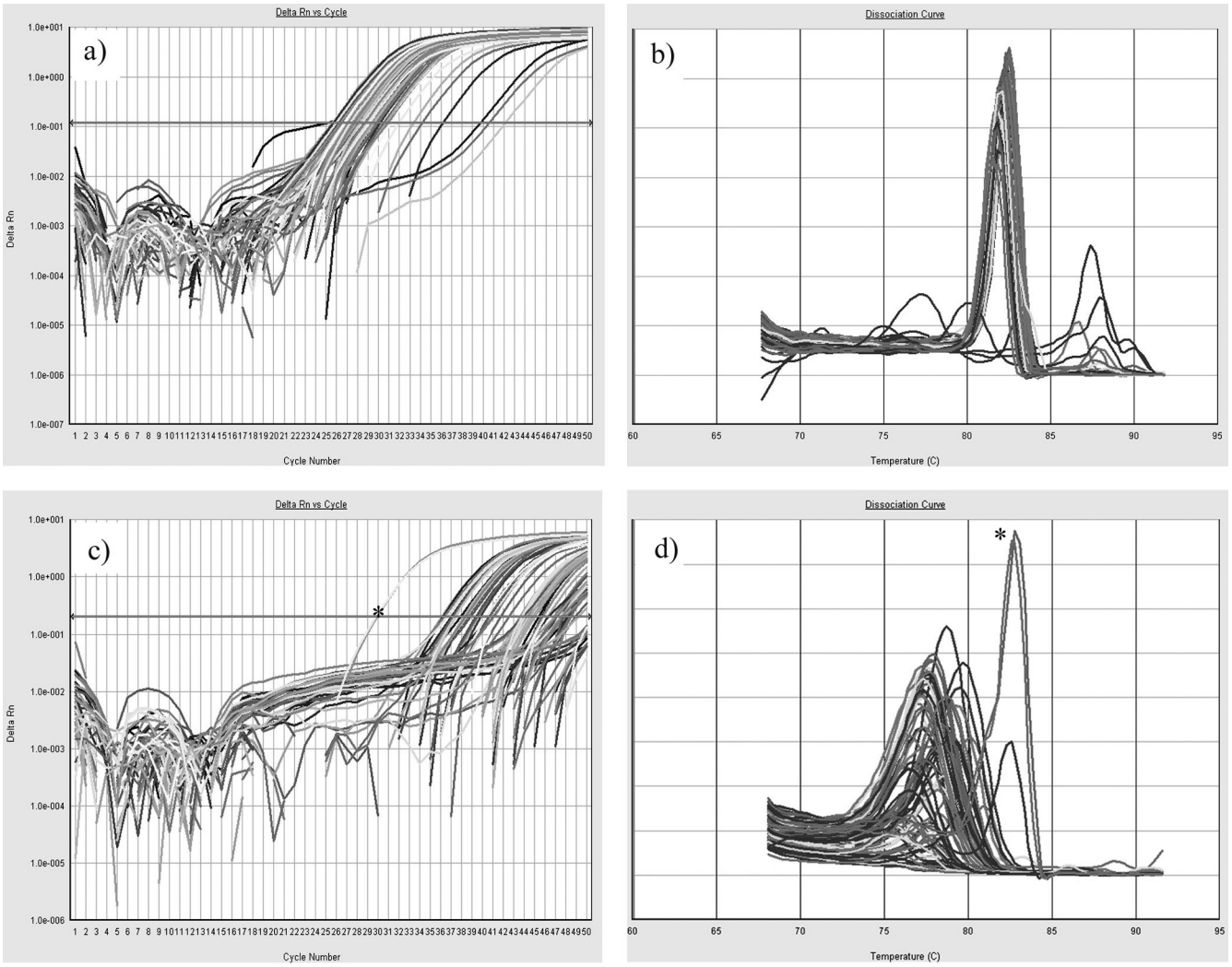

FIG. 3. Amplification plots and melt curves for samples from 20 August 2007 to 8 October 2007. The results from the assay for *P. calliantha*, as an example of positive results, are shown in panels a and b while the results from the *P. fraudulenta* assay, as an example of negative results, are shown in panels c and d. The plasmid positive controls (pFrauPO2) used for calibration of the *P. fraudulenta* assays are indicated (*).

absence of *Pseudo-nitzschia* spp. observed using microscopy, and from these samples low cell densities were detected in six of them using qPCR (*P. calliantha*, four samples of <200 cells/liter; *P. multistriata*, two samples of <480 cells/liter); the remaining samples were negative by qPCR for all species.

On six occasions during weeks 21, 22, 25, 26, 60, and 61 there was significant overabundance of *Pseudo-nitzschia* spp. detected using qPCR, and this was attributed to the *P. delicatissima* (Ra2) assay (Fig. 4).

DISCUSSION

Pseudo-nitzschia identification has become a more serious concern within the framework of the local monitoring program as the increased intensity of blooms over the past 20 years (18), together with the occurrence of mixed-species blooms, including toxic and nontoxic species within the same body of water, has put emphasis on the need for species and/or strain level discrimination for public health monitoring purposes (5, 6, 20, 34, 56).

An additional aspect that may improve microalga monitoring is having real-time results rather than next-day results, which can be a factor enabling more appropriate management responses. In developing these assays several requirements were adhered to: (i) protocols needed to be rapid so that sample processing and final results would be available on the same day; (ii) assays needed to have biogeographic relevance (address specific local biodiversity rather than addressing every species and strain known); and (iii) reagent costs needed be kept to a minimum. The protocol developed requires minimal time, with only 5 h needed to complete the analysis. In comparison, standard microscopy requires that the water samples be held overnight for the cells to settle before counting and does not differentiate below the genus level. Using the SYBR Green reagent reduces costs over a TaqMan probe assay but does not allow multiplexing. However, TaqMan assays provide

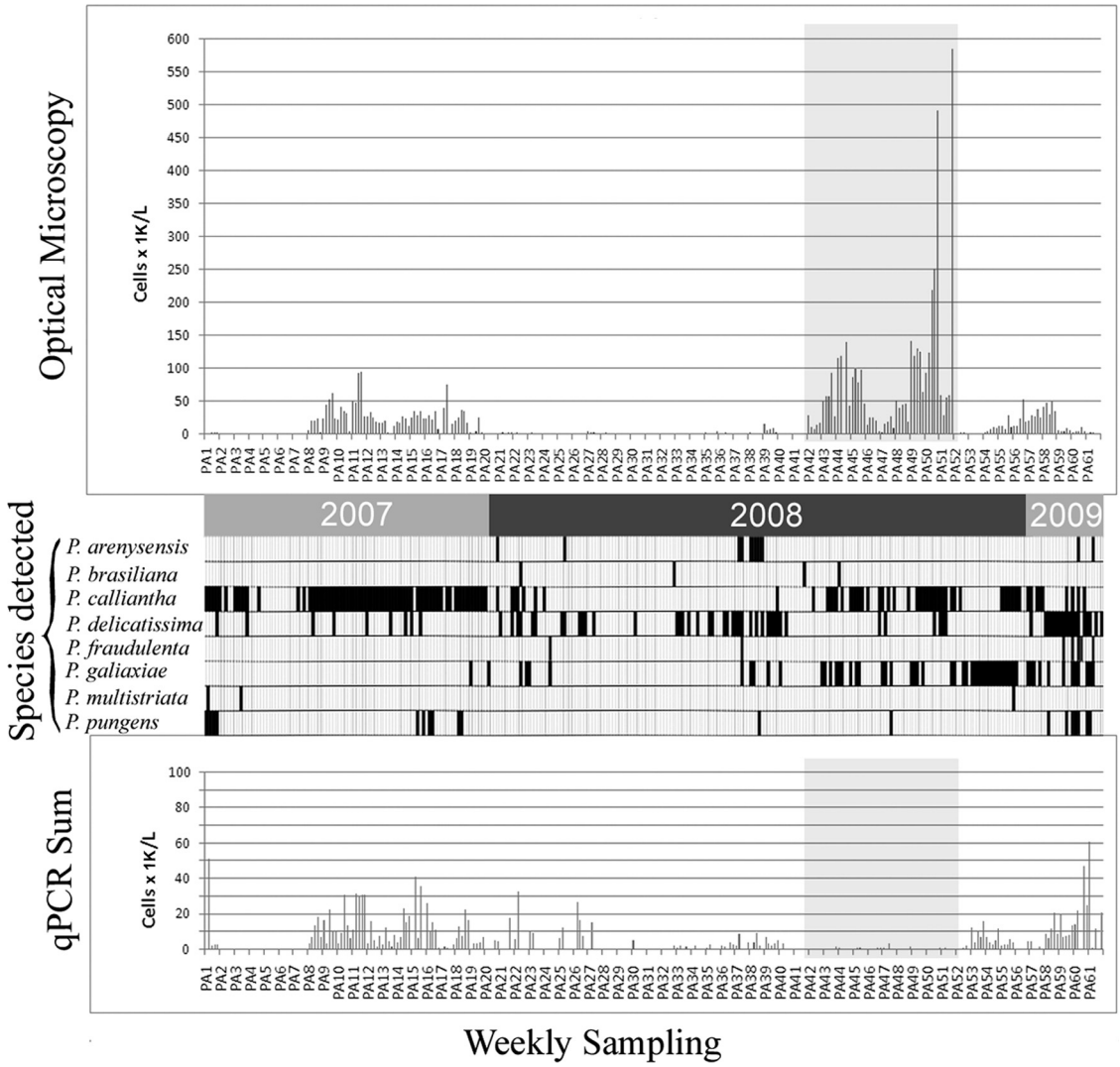

FIG. 4. Comparison of optical microscopy results with species-specific qPCRs from parallel samples collected weekly in Alfaques Bay. Samples are listed chronologically and within each week are in the order PA, EA, CIA, IA, and CA. The sum of all species detected from eight species-specific qPCR assays is shown in the bottom graph, with all species detected in each sample indicated in the central chronogram. Good statistical correlation between the two methods was found during 2007 and until the middle of 2008 ($P < 0.016$). Significant statistical correlation ($P < 0.05$) was found for the entire survey period when the gray-shaded sample group is excluded. Species with the highest overall abundance during the survey were *P. calliantha*, *P. delicatissima* (Ra2), *P. galaxiae*, and *P. pungens*.

no possibility for melt curve analysis, which is a main advantage of this protocol, as discussed further below.

The entire spectrum of species within a genus is rarely to be found in a given geographic region. Some genera are more diverse than others, and within a species many strains can be found, as exemplified with *P. delicatissima* (38). To make an assay like this useful and relevant, it is necessary to address only those species which are to be found locally. Of the 11 species documented from the coast of Catalonia, sequences were obtained from multiple strains of eight species and used in this project for the development of the species-specific qPCR assays.

In this study, we documented 20 sequences from *P. calliantha* clonal cultures (two strains with SNPs in the amplicon). Likewise, we obtained sequences from 20 *P. delicatissima* clonal cultures (two strains with SNPs in the amplicon), and three of these were later reassigned as a new species (*P. arenysensis* sp. nov.) due to mating compatibility and significant rRNA gene sequence differences (46). Of the three clonal cultures of *P. fraudulenta* identified, one of them had an SNP within the amplicon.

For the purpose of simplifying possible future development of automated systems, the hemi-specific primer combinations used during this project were intended to provide the needed specificity while at the same time minimizing the number of primers required. Having one primer in a conserved region, such as the 5.8S rRNA gene, ensures that in each assay one strand is always polymerized, strengthening the overall efficiency of amplification for all species and strains encountered. A similar strategy has been described for quantifying both diatom and dinoflagellate biomasses in seawater using quantitative PCR (25). Further, due to the high degree of variability

TABLE 3. A selection of samples from the survey period in which multiple species were detected using qPCR and there was still a good agreement with optical microscopy

Sample	Cell total for all species by:		No. of cells counted using each species-specific assay							
	Microscopy	qPCR	*P. arenysensis*	*P. brasiliana*	*P. calliantha*	*P. delicatissima* (Ra2)	*P. fraudulenta*	*P. galaxiae*	*P. multistriata*	*P. pungens*
CIA1	3,240	3,547	0	0	821	0	0	0	0	2,727
IA1	3,300	2,949	0	0	2,820	0	0	0	0	130
CA1	2,895	2,842	0	0	1,684	461	0	0	0	697
CIA3	140	177	0	0	162	0	0	0	15	0
CA11	26,638	31,192	0	0	29,529	1,663	0	0	0	0
CIA14	27,009	23,011	0	0	21,112	1,900	0	0	0	0
IA15	34,653	35,503	0	0	10,856	0	0	0	0	24,647
PA16	23,824	26,001	0	0	5,718	0	0	0	0	20,282
CA18	17,759	16,546	0	0	15,930	0	0	616	0	0

in the ITS region, unanticipated sequence differences may be encountered in some undescribed strains. The applicability of melt curves would have more relevance for differentiating such strains with SNPs within the amplicon without the need for new amplification primers or probes (see below).

The original standard curves developed for these qPCR assays used serially diluted plasmid clones, which give a very accurate estimate of copy number when extremely pure, high-quality DNA is used, such as that obtained from plasmid clones. However, using these standard curves leads to an underestimation of the number of cells in the field samples. It is documented that rRNA gene copy number is not invariant in some organisms but is likely dependent on the cell cycle. This system of copy number control has been best studied in *Saccharomyces cerevisiae* (10, 21, 32). The variable copy number of rRNA genes has also been studied in microalgae, with differences in copy number being attributable to species differences by some investigators (57) while others see it as a variable trait within species and possibly controlled by the cell cycle (22). In other studies with *Pseudo-nitzschia* spp., stability of fluorescence signal from hybridization probes was seen as an indication of relatively stable rRNA copy number regardless of the stage of cell cycle, which is suggestive that rDNA copy number remains unchanged as well (53). Due to the underestimation of cell number using plasmid-based standard curves, new standard curves were developed using logarithmically growing cell cultures. Cell culture DNA extracts provided C_T values that likely represent an average copy number per cell, even without information about the actual copy number at any given stage of the cell cycle. The C_T value for each cell dilution thereby provides the needed correlation. Standard curves prepared in this way provided better accuracy without relying on the exact copy number per cell. If the copy number per cell does change during the cell cycle of a natural bloom, defining copy number per cell would not be practical for the present purpose since cells in a bloom are not in a synchronous cycle. While the copy number per cell may change during a bloom, influencing the calculated number of cells, the overall trend can give an indication of a bloom advancing or retreating and provide previously unavailable information (using optical microscopy) about species/population flux over time.

During the 2 years of sampling and analysis using the qPCR assays for *P. calliantha*, *P. galaxiae*, *P. delicatissima* (Ra2), *P. pungens*, *P. arenysensis*, *P. multistriata*, *P. brasiliana*, and *P. fraudulenta*, significant blooms were detected using only the assays specific for the first four of these species. The assay for *P. calliantha*, in particular, detected large blooms during the later months of the year in both 2007 and 2008, making up the majority of the *Pseudo-nitzschia* biomass during these months. Additionally, linear regression analysis of the data obtained showed that there was good agreement between optical microscopic counts of *Pseudo-nitzschia* cells and the total cell numbers obtained using eight species-specific qPCR assays during much of this period ($P < 0.016$).

One interesting result from this survey is the lack of concordance between qPCR and microscopy during 10 weeks of a very large bloom in 2008. There was some detection of *P. calliantha*, *P. delicatissima* (Ra2), and *P. galaxiae* during this time, but the amount detected was not sufficient to account for all the cells observed by optical microscopy. This gap in detection would seem to indicate the presence of some undocumented species. Prior to the work by Quijano-Scheggia (45), only three species were known from Catalan coastal waters, and the possibility of the existence of other undocumented species is quite likely. It is worth noting that the lack of amplification during this period provides additional support to the apparent specificity of each of these eight qPCR assays while also pointing to the possible existence of new species.

Nonspecific amplifications were present in many of the assays but were easily discounted by examination of the melt curves and comparison to the profile of the plasmid controls (Fig. 3). This type of artifact amplified very late during the reaction as well (usually cycle 36 or later) and is difficult to avoid without the use of TaqMan probes and when the matrix is as complex as seawater, where microalgae, zooplankton, bacteria, viruses, and other detritus are present. The full catalogue of the ocean's biodiversity is not yet described, and for this reason designing primers that will not accidentally amplify DNA of undescribed species is difficult to anticipate. Since this type of artifact shows up very late, it would seem that this does not represent strong competition with the actual targets of amplification. Further, this type of artifact should not affect the quantification of microalgae since in most cases in which *Pseudo-nitzschia* cells were present, the presence of specific target eliminated this type of noise from reactions (compare Fig. 3a and b).

Nonspecific amplification giving rise to melt curve profiles quite similar to the profile of the plasmid control was also apparent on several occasions (weeks 21, 22, 25, 26, 60, and 61) (Fig. 4) and was attributable to the *P. delicatissima* (Ra2) primers. This occurred only during the first weeks of January in 2008 and 2009 and may be a seasonal phenomenon since the *P. delicatissima* (Ra2) assay seemed to work well during the rest of the year. The *P. delicatissima* (Ra2) qPCR assay also contributed to an overestimate of cell abundance in a few samples although it is possible that DNA from *Pseudo-nitzschia* spp. was being detected in the gut of some type of predatory organisms that had been grazing on a bloom that was not part of the water sample collected and that this DNA "signature" was simply the residue from the digestive contents of a grazer (42, 54).

Detection of strains within species should also be possible using this methodology since melt curve analysis can identify SNPs in the amplicon, as has been shown previously in human studies (13, 24, 49). A DNA strand of approximately 200 bp has 4^{200} different possible sequences, but the melting temperature (T_m) varies over only a 40°C range. Because of the high proportion of conserved segments in ribosomal genes of the same species, small changes in sequence can invoke detectable differences. The T_m represents only one point on a DNA melting curve, and when the entire curve is considered, even more sequence discrimination is possible. Thus, a precisely determined melt curve is very characteristic of a given double-stranded DNA sequence (49). It is for this reason that we use the shape of the melt curve as well as the T_m for comparison. While small shifts in T_m were evident between the plasmid and genomic DNA samples for some species, the shape of the curve was consistently reproducible for the plasmids to suffice as a calibrator and internal control of each experimental plate. An SNP within the amplicon was found for one strain of *P. fraudulenta*. During cross-testing of genomic DNA from each species, we identified a strain of *P. fraudulenta* that displayed a melt curve profile lacking the inflection that was present in the other two strains, PO2 and AR2. This is due to a T-to-C transition mutation within the ITS-2 amplicon, and it corresponds to a bigger insertion-deletion (indel) in the ITS-1 region of the same strain, AR3 (Fig. 2). However, during field surveys only the C allele was detected.

The detection of species and strains of species for a better understanding of population flux within microalga blooms is possible only by careful biogeographic surveys of the genetic diversity to confirm what species and strains exist (33). Only after this has been done can careful primer design allow multiple strains of a species to be detected using their melt curve profiles. Work remains to improve this system and more fully explore the utility of melt curve analysis for use in strain differentiation. Newer hardware and software for high-resolution melt curve (HRM) analysis should advance this area. However, using melt curve analysis and species-specific qPCR shows potential as a practical adjunct tool for monitoring programs, allowing high-throughput analysis of microalga bloom samples where morphology is an imprecise character and/or is not practical for taxonomic identification and where local species genetic diversity has previously been characterized. The method is relatively inexpensive and rapid and provides same-day results for species identification of *Pseudo-nitzschia* spp. blooms. Continued surveillance and accumulation of this type of data correlated to other physical parameters may aid in development of models for prediction of blooms of certain species of *Pseudo-nitzschia*. This would be especially useful for those species which are known to be toxigenic.

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