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Dinoflagellate community analysis of a fish kill using denaturing gradient gel electrophoresis

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Abstract

A molecular method using the polymerase chain reaction (PCR) amplification of small subunit gene sequences (18S rDNA) and denaturing gradient gel electrophoresis (DGGE) was used to determine both the population complexity and species identification of organisms in harmful algal blooms. Eighteen laboratory cultures of dinoflagellates, including *Akashiwo*, *Gymnodinium*, *Heterocapsa*, *Karenia*, *Karlodinium*, *Pfiesteria*, and *Pfiesteria*-like species were analyzed using dinoflagellate-specific oligonucleotide primers and DGGE. The method is sensitive and able to determine the number of species in a sample, as well as the taxonomic identity of each species, and is particularly useful in detecting differences between species of the same genus, as well as differences between morphologically similar species. Using this method, each of eight *Pfiesteria*-like species was verified as being clonal isolates of *Pfiesteria piscicida*. The sensitivity of dinoflagellate DGGE is approximately 1000 cells/ml, which is 100-fold less sensitive than real-time PCR. However, the advantage of DGGE lies in its ability to analyze dinoflagellate community structure without needing to know what is there, while real-time PCR provides much higher sensitivity and detection levels, if probes exist for the species of interest, attributes that complement DGGE analysis. In a blinded test, dinoflagellate DGGE was used to analyze two environmental fish kill samples whose species composition had been previously determined by other analyses. DGGE correctly identified the dominant species in these samples as *Karlodinium micrum* and *Heterocapsa rotundata*, proving the efficacy of this method on environmental samples. Toxin analysis of a clonal isolate obtained from the fish kill samples confirmed the presence of KmTx2, corroborating the earlier genetic identification of toxic *K. micrum* in the fish kill water sample.

Keywords: Community structure analysis; Denaturing gradient gel electrophoresis; *Karlodinium micrum*; *Pfiesteria piscicida*; *Pfiesteria shumwayae*

1. Introduction

Harmful algal blooms (HABs) are becoming more prevalent over the world, posing a threat to both marine organisms and human health. The consequences of HABs include mass mortality of fish and shellfish, as well as marine mammals, seabirds and other animals. These mortalities have adverse effects on the environment which both indirectly and directly affect human health often through the consumption of contaminated seafood. HABs also present great economic impacts on public health, commercial fishery, recreation and tourism, as well as monitoring and management costs (Anderson et al., 2000).

An essential component in the management and remediation of HABs is the quick and accurate

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assessment of the species populating the bloom. HAB species are taxonomically diverse and it is common for blooms to be composed of more than one species. Light microscopy does not have sufficient resolution to enable identification to the species level for all HAB organisms, especially as many species of dinoflagellates, for example, are morphologically similar to one another. Historically, dinoflagellate species differentiation requires more time-consuming procedures such as scanning electron microscopy (SEM) and careful examination of the arrangement of dinoflagellate thecal plates (Steidinger et al., 1996). This method is not useful, however, for all dinoflagellates, especially non-thecate or weakly thecate species. Nevertheless, plate tabulation analysis by SEM remains one of the most reliable methods for the taxonomic identification of dinoflagellates.

The advent of modern molecular techniques has facilitated more rapid detection and differentiation of HAB species. Chief among the molecular techniques is the polymerase chain reaction (PCR). PCR is frequently used to amplify regions of the small ribosomal subunit gene (18S rDNA) that are then used to establish phylogenetic relationships through nucleotide sequence analysis and comparison to known sequences in the databases. There is a strong correlation between the phylogenetic relationships established through thecal plate structure analysis and 18S rDNA homology (Litaker et al., 1999; Steidinger et al., 2001; Taylor, 1999).

PCR combined with species-specific 18S rDNA oligonucleotide primers has been widely used to detect and identify dinoflagellates. For example, PCR of 18S rDNA has been used by Rublee et al. (1999) to detect the presence of *Pfiesteria piscicida* in estuarine water samples. Saito et al. (2002) used primers to the non-transcribed spacer (NTS) region of the ribosomal gene of *P. piscicida* (Saito et al., 2002) and determined that their method was specific to only *P. piscicida* and was capable of detecting a single *P. piscicida* zoospore in 1 ml of water.

Of the PCR methods, the heteroduplex mobility assay (HMA) has received wide attention as a means of detection and differentiation of dinoflagellate DNA signatures, specifically *Pfiesteria* species (Oldach et al., 2000). In this method, whose principle is based on the ability of 18S rDNA fragments from different dinoflagellate species to form a DNA heteroduplex that migrates more slowly through the polyacrylamide gel than does a homoduplex, denatured "target" PCR-amplified 18S rDNA fragment is mixed with a single-stranded "driver" DNA of known origin. After annealing, the duplex DNA molecules are separated by electrophoresis, resulting in a distinct pattern dependent upon the sequence of each DNA strand. Thus, distinct band patterns are produced based on the sequence differences between DNAs, and the pattern of bands is unique for each species of dinoflagellate.

Denaturing gradient gel electrophoresis (DGGE) also uses PCR amplification of 18S rDNA and can detect differences in the melting behavior of the resulting DNA fragments (200–700 bp) that differ by as little as a single base substitution (Muyzer, 1999; Muyzer et al., 1993). In this sense DGGE is similar to HMA, however unlike the latter method, DGGE is a simpler technique to perform and does not require driver DNA or pre-run annealing to form a heteroduplex. Instead, the principle behind DGGE lies in the physical nature of double stranded DNA such that when a DNA fragment is subjected to an increasingly denaturing physical environment, it partially melts. The oligonucleotide primers used in DGGE contain a long GC-rich stretch at the 5′-end that is thermally stable, so that rather than dissociating into single strands, the DNA melts in a step-wise process. When a double-stranded fragment migrates by electrophoresis into a gradient of increasingly denaturing conditions, it partially melts and undergoes a sharp reduction in mobility because it changes shape. The denaturation process is not gradual, but instead occurs in defined steps caused by 'domains' within the DNA that suddenly dissociate at the same time. Once the dissociation occurs, migration through the gel dramatically slows relative to the original double-stranded DNA molecule. Slight changes in bp composition, often as little as 1 bp, will shift these domain boundaries and thereby alter the conditions needed for domain dissociation. Hence, fragments of similar size with even one base pair difference will often migrate to a different position in a gradient denaturation gel based on shifts in the melting domain boundaries (Litaker and Tester, 2002). The resolution of the acrylamide gel systems is such that many molecules with even slight to moderate sequence differences can be detected in a single lane. Thus, each DNA migrates to a defined point, dependent on its nucleotide sequence.

These DNA fragments (represented as bands on the gel) may be excised from the polyacrylamide and their sequence determined to yield taxonomic data, further increasing the power and versatility of DGGE.

In microbial ecology, DGGE is frequently used in the analysis of community structure. Both the complexity of the community, as represented by the number of DNA bands in a sample, as well as the phylogenetic affiliation of each member (as determined by the nucleotide sequence of each constituent DNA band) can be found using DGGE (Diez et al., 2001; Muyzer et al., 1993, 1995). Despite having been used to assess prokaryotic and eukaryotic populations (Muyzer et al., 1993, 1995), DGGE methodology has not been applied to the study of HABs. Here, we report results from an analysis of both laboratory cultures and bloom samples using dinoflagellate-specific oligonucleotide primers and DGGE for the detection and species discrimination of dinoflagellates. Chemical identification of a species-specific toxin was then used to corroborate the taxonomic identification derived from DGGE analysis.

2. Materials and methods

2.1. Dinoflagellate cultures

The following dinoflagellates species were used in this study: *Cryptoperidiniopsis* sp. (CCMP 1828), *Gymnodinium catenatum* (CCMP1937), *G. mikimotoi* (currently known as *Karenia mikimotoi* CCMP429), *G. sanguineum* (currently known as *Akashiwo sanguinea* CCMP1321), *Heterocapsa triquetra* (CCMP448), *Karlodinium micrum* (CCMP1974), *P. piscicida* (CCMP 1831, 1834, 1902, and 1921), *Pfiesteria shumwayae* (a gift from Patricia Tester, NOAA), *Pfiesteria*-like sp. (CCMP 1829, 1880, 1882, and 1929), *Prorocentrum lima* (CCMP1743), and *P. minimum* (CCMP695). Dinoflagellate strains other than *P. shumwayae* were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). A clonal isolate of *K. micrum* (JW020205-B4) from the Mount Pleasant (North Carolina) fish kill (Kempton et al., 2002) was provided by Jennifer Wolny (FIO-Florida Marine Research Institute) and has been deposited at CCMP.

The dinoflagellates were cultured in *f*/2 medium (Guillard, 1975) lacking silica and supplemented with 15 psu of artificial sea salts (ASW, Instant Ocean synthetic sea salt, Aquarium Systems, Mentor, Ohio) at 23 °C with a light–dark cycle of 14 h light (white-fluorescent; 150 μeinstein/(m^2 s)) and 10 h dark, as described by Alavi et al. (2001). The heterotrophic cultures were maintained by feeding with a *Rhodomonas* sp. (Cryptophyceae, CCMP 768) every 3–4 days.

2.2. DNA manipulation

Standard methods were used for the manipulation of DNA (Ausubel et al., 1988) unless specified otherwise. Water samples were centrifuged at 10,000 × *g* for 20 min at 4 °C to pellet the cells. Total DNA was extracted from each cell pellet using a cetyltrimethylammonium bromide (CTAB)–phenol–chloroform–isoamyl alcohol extraction (Ausubel et al., 1988) with only minor modifications. Environmental DNA from glass fiber filters collected on February 5 and 6, 2002 from the fish kill (Kempton et al., 2002) were extracted as described above.

2.3. Polymerase chain reaction (PCR) for DGGE

The oligonucleotide primers for the specific PCR amplification of dinoflagellate 18S rDNA were EUK4618R (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) and Dino (Oldach et al., 2000) with a 5′ GC-rich 40 base extension called DINOFGC (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCGATTGAGTGATCCGGTGAATAA-3′). These primers (lacking the GC-rich addition) have been used by Oldach et al. to amplify dinoflagellate 18S rDNA and have been reported to be specific to the majority of dinoflagellate genera (Oldach et al., 2000). PCR amplification was carried out with Hotstart™ *Taq* DNA polymerase (Qiagen, Valencia, CA) using a PTC200 thermal cycler (MJ Research, Waltham, MA). Each 50 μl reaction contained approximately 100 ng of template DNA, 2.5 units Hotstart *Taq* DNA polymerase, 0.5 pmol of each primer, 50 μM of each deoxynucleotide and 1× PCR buffer provided with the Hotstart *Taq* polymerase. PCR reaction cycling for dinoflagellate PCR was performed as described by Oldach et al. (2000). PCR products were detected

using 1.5% agarose gel electrophoresis in 1× TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM Na_2EDTA, pH 8.2).

2.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using the Dcode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA), following the recommendations of the manufacturer, and using polyacrylamide gels (8%, acrylamide: bisacrylamide 37.5:1) containing a vertical linear denaturing gradient formed with urea and deionized formamide. A 100% denaturing gradient is defined as 40% (v/v) formamide plus 7 M urea. Denaturing gradients ranged from 40 to 50% for dinoflagellate DGGE. The gel image was analyzed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and prominent DNA bands were then identified and excised from the gel using sterile razor blades Excised bands were analyzed by a second PCR-DGGE to ensure that a single band was obtained from each DNA fragment prior to nucleotide sequence analysis.

2.5. DNA sequence and phylogenetic analyses

The double-stranded DNA fragments excised and purified from DGGE gels were used as templates for nucleotide sequencing following the recommended procedures of the Prism Ready Reaction Dye Deoxy Termination Kit (Applied Biosystems, Foster City, CA), in conjunction with *Taq* polymerase and a model 373A DNA sequencer (Applied Biosystems, Foster City, CA). Oligonucleotide primers used for sequencing were identical to those used for dinoflagellate DGGE, but lacked the G–C rich clamp sequence. Nucleotide acid sequences were analyzed with the BLAST family of programs (Altschul et al., 1990).

2.6. Quantitative real-time PCR

Quantitative assessment of template DNA abundance was accomplished using species-specific primers and fluorescently labeled oligonucleotide probes. Dinoflagellate cultures were grown as described and the cell density as cells per ml was measured using a hemacytometer (Hausser Scientific Company, Horsham, PA). Total DNA was extracted from 30 ml of a fresh *P. piscicida* culture as described and serially diluted to yield a standard curve for real-time PCR. PCR reactions were run, detected, and analyzed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The thermocycler program included 2 min at 50 °C, 15 min at 95 °C and 40 cycles with 15 s at 95 °C and 1 min at 60 °C using the method and oligonucleotide primers as described by Bowers et al. (2000, 2002). Primers 107f (5′-CAGTTAGATTGTCTTTGGTGGTCAA-3′) and 320r (5′-AGCTGATAGGTCAGAAAG-TGATATGG-TA-3′) and *P. piscicida* specific probe (5′-FAM-CATGCACCAAAGCCCGAC-TTCTCG-TAMRA-3′) (Operon, Alameda, CA) were used to measure the abundance of *P. piscicida*.

2.7. Toxin analysis

The methods described by Deeds et al. (2002) were used with minor changes. The filtrate from the clonal isolate (JW020205-B) was tested for hemolytic and ichthyotoxic activity and shown to be positive. The filtrate was passed through a Sep-Pak Plus tC_{18} disposable cartridge (Waters Corp., Milford, MA), and the 80% MeOH fraction collected and concentrated to dryness. The dehydrated material was resuspended in MeOH and an aliquot (25 μl) was injected for HPLC analysis on a LiChroDART 125-4/RP-8 (5 μm) reversed phase HPLC column (Waters Corp., Milford, MA) equilibrated with 30% MeOH/70% H_2O and eluted with a linear gradient to 95% H_2O/5% MeOH over 20 min, at a flow rate of 1 ml/min (Deeds et al., 2002). The retention time of the major component, measured at 230 nm adsorbance, was compared to purified toxin (KmTx2) from the fish kill.

2.8. Nucleotide sequence accession numbers

Sequences of the partial 18S rDNA obtained from this study have been submitted to GenBank under accession no. AY295771 (clone SC1; *K. micrum*) and AY295772 (clone SC2; *Heterocapsa rotundata*).

3. Results

3.1. Molecular detection of dinoflagellates

DGGE was conducted using dinoflagellate-specific oligonucleotide primers derived from Dino and

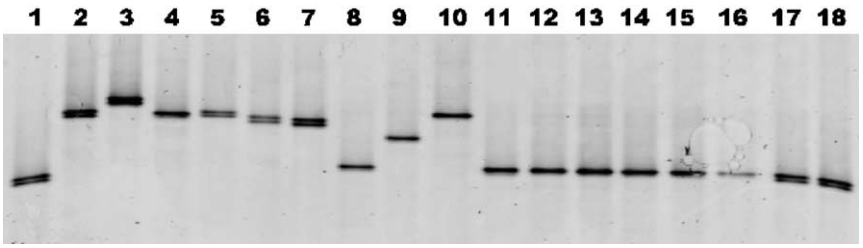

Fig. 1. Identification and species-specific differentiation of harmful dinoflagellate species and *Pfiesteria*-like dinoflagellate strains by DGGE. As described in Section 2, DNA was extracted from a cell pellet of each of 18 laboratory cultures of dinoflagellates. PCR amplification with dinoflagellate-specific oligonucleotide primers produced an amplicon containing a fragment of the 18S rDNA, which was then separated by DGGE. The lanes contain: (1) *G. catenatum*; (2) *K. micrum*; (3) *G. mikimotoi*; (4) *G. sanguineum*; (5) *Prorocentrum lima*; (6) *P. minimum*; (7) *H. triquetra*; (8) *P. piscicida*; (9) *P. shumwayae*; (10) *Cryptoperidiniopsis* sp. (CCMP 1828); (11) CCMP 1829; (12) CCMP 1831; (13) CCMP 1902; (14) CCMP 1921; (15) CCMP 1929; (16) CCMP 1834; (17) CCMP 1880; (18) CCMP 1882. Lanes 11–18 are *P. piscicida* cultures.

EUK4618R (Oldach et al., 2000). This primer pair has been used to amplify a 142 bp portion of 18S ribosomal DNA from a broad sampling of dinoflagellate taxa, but does not amplify DNA from a variety of other estuarine plankton and metazoan species (Oldach et al., 2000). The efficacy of this detection method was tested on a battery of laboratory cultures of dinoflagellates, including *K. micrum*, *P. piscicida* and *P. shumwayae*, three *Gymnodinium* species, two *Prorocentrum* species, *H. triquetra*, *Cryptoperidiniopsis* sp. (CCMP 1828), and eight *Pfiesteria*-like dinoflagellates (Fig. 1). As shown in Fig. 1, PCR amplification using the prescribed oligonucleotide primers results in a DNA product from all of the dinoflagellates tested. Furthermore, separation of these PCR products was possible using a 40–50% gradient, which provided unique migration patterns for each of the dinoflagellate species tested. For example, using these conditions, *P. piscicida* (lane 8) and *P. shumwayae* (lane 9) can be easily distinguished from each other and from other non-*Pfiesteria* dinoflagellates. Similarly, *K. micrum* (Fig. 1, lane 2) produces a band that is distinguishable from *P. piscicida*, *P. shumwayae*, or other look-alike dinoflagellates.

DGGE was also useful in establishing the clonal efficacy of the eight *P. piscicida* or *Pfiesteria*-like strains obtained from the CCMP. As shown in Fig. 1 lanes 11–18, the PCR product from each of these dinoflagellate cultures generated a product in DGGE that co-migrated with a clonal culture of *P. piscicida*, indicating that these strains are true monocultures. The occurrence of a single major band in each of the *P. piscicida* cultures also is strong evidence that these are monocultures and are not co-inhabited by other morphologically similar dinoflagellate species. The appearance of a lighter band immediately under the major PCR product in some lanes is an artifact of this method (see Section 4).

3.2. Sensitivity and specificity of DGGE compared to quantitative real-time PCR

DGGE data are, at best, semi-quantitative and prone to PCR and other biases, therefore the band densities observed in Fig. 1 cannot, by themselves, be used to determine the abundance of any dinoflagellate species. However, we can infer relative differences in abundance when a sample contains more than one PCR product, i.e., when multiple dinoflagellate species are present, by the intensity of each respective DNA band in corroboration with other data. As a foundation of support for this, the sensitivity and selectivity of DGGE for dinoflagellate community structure analyses was compared to a quantitative technique, real-time PCR (Bowers et al., 2000), using *P. piscicida* as a test organism.

The sensitivity of DGGE versus real-time PCR was evaluated using a dilution series of *P. piscicida* ranging from 1×10^5 to 1×10^0 cells/ml. The dinoflagellates were prepared and DNA extracted as described in Section 2. As shown in Fig. 2A, PCR amplification and DGGE was capable of detecting a DNA product from *P. piscicida* at cell concentrations ranging from 1×10^5 to 1×10^3 cells/ml (Fig. 2A).

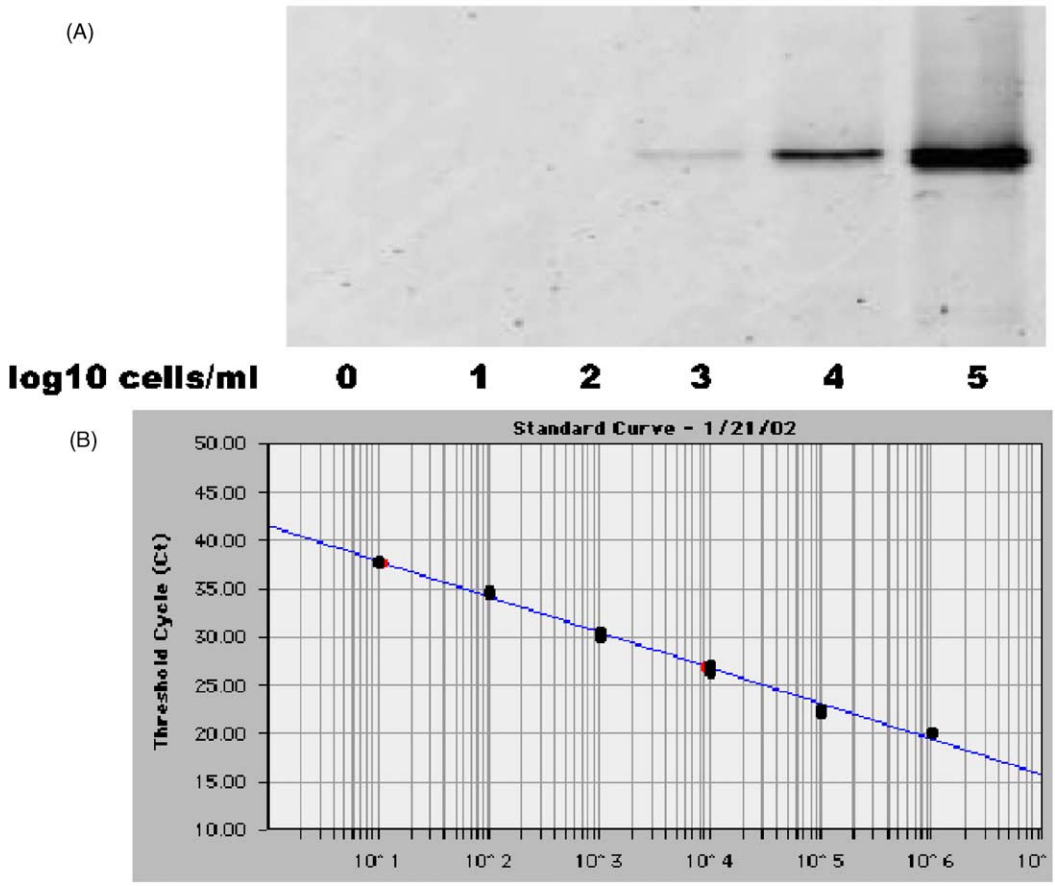

Fig. 2. Sensitivity of DGGE compared to quantitative real-time PCR. (A) DGGE sensitivity as measured by a 10-fold dilution series of a *P. piscicida* DNA template corresponding to cell densities from $1 \times 10^5 (5 \log_{10})$ to $1 \times 10^0 (0 \log_{10})$ cells/ml. The limit of detection by DGGE with the dinoflagellate-specific primers is ca. 1000 cells/ml. (B) A standard curve of C_t (threshold cycle) values from quantitative real-time PCR using the identical 10-fold dilution series of *P. piscicida* DNA. The correlation coefficient for the quantitative real-time PCR standard curve is 0.993 and the limit of detection is ca. 10 cells/ml.

At lower concentrations of *P. piscicida* (Fig. 2A), the PCR produced either a very light band or no DNA that could be detected by DGGE.

In contrast to DGGE, we found real-time PCR to be a very sensitive method for detecting dinoflagellate DNA. As shown in Fig. 2B, a significant CT value was generated even at concentrations of *P. piscicida* averaging 10 cells per ml. This is approximately equal to three cells per PCR reaction. Using species-specific oligonucleotides as probes for *P. piscicida*, we found that many dinoflagellates, such as *G. catenatum*, *P. lima* and *P. minimum* were not amplified at all by the *P. piscicida* specific primers and probe, while others, such as *K. micrum* and *G. sanguineum*, were amplified only very late during the PCR cycles, a result that can be regarded as an artifact due to signal "drift" that registers as a weak positive, albeit below the threshold value of significance.

3.3. DGGE analysis of dinoflagellate species in environmental samples from a fish kill event

DGGE was used to analyze the dinoflagellate population in environmental samples taken from a fish kill event that occurred on 5 February 2002 in a brackish water retention pond in

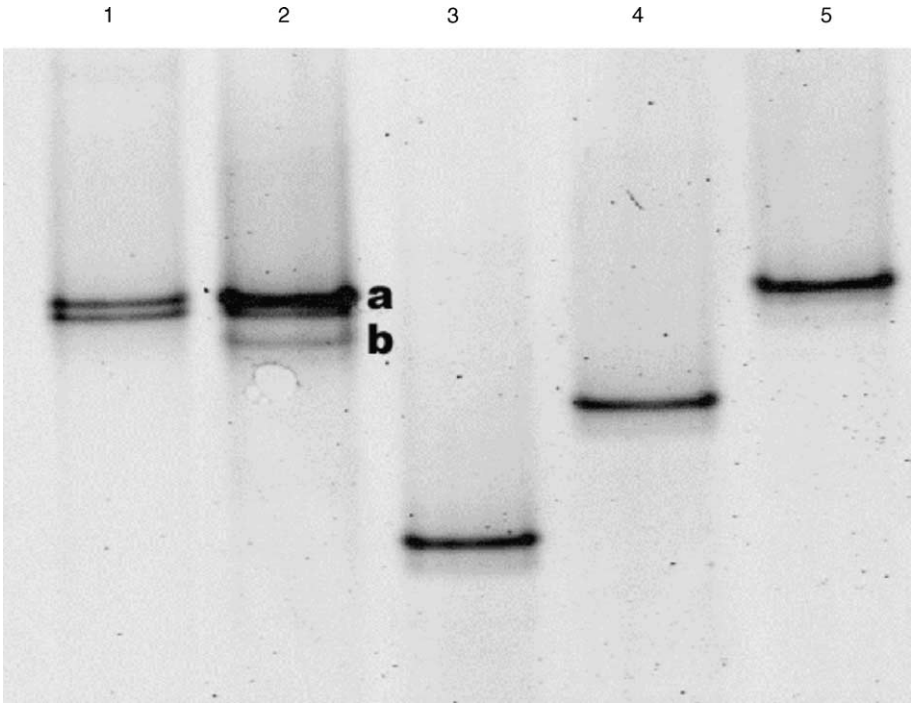

Fig. 3. DGGE analysis of environmental samples from a fish kill event. Surface water samples were collected on two consecutive days, 5 and 6 February 2002, during a fish-killing event in an aquaculture pond in South Carolina. Total DNA was extracted from these samples and amplified by PCR using dinoflagellate-specific primers, as described in Section 2, and the resulting DNA separated by DGGE. Both samples (lanes 1 and 2 representing 5 and 6 February, respectively) produced a PCR product (band "a") that co-migrated with a *K. micrum* control culture. In addition, DGGE identified a second DNA (labeled "b") from the 6 February sample (lane 2) that did not co-migrate with any of the control dinoflagellates. The nucleotide sequence of band "b" is similar to that of a *Heterocapsa* spp. The lanes are: (1) 5 February sample; (2) 6 February sample; (3) *P. piscicida*; (4) *P. shumwayae*; and (5) *K. micrum* reference controls.

Mount Pleasant, South Carolina (near Charleston). Light and epifluorescence microscopic analyses on collected water independently revealed the presence of high concentrations (68,280 cell/ml) of one dinoflagellate morphotype in these samples that was identified as *K. micrum* (Kempton et al., 2002). Kempton et al. (2002) also found in this sample relatively high concentrations of the marker pigments, chlorophyll c3 and gyroxanthin diester, consistent with a dominance of bloom algal biomass of *K. micrum*, and peridinin, contributed by *H. rotundata*. This information was not divulged during the DGGE analyses, which were run in a blind trial. These PCR amplifications used the same oligonucleotide primer pair (DinoFGC and EUK4816R) as used to discriminate the laboratory cultures of dinoflagellates (Fig. 1). As shown in Fig. 3, two distinct dinoflagellate-specific DNA bands were amplified from the DNA obtained from these samples. The dominant DNA band, co-migrated with the control *K. micrum* sample, was present in both samples, while a second dinoflagellate-specific DNA appeared in the second sample (6 February). The nucleotide sequence analysis of the band co-migrating with *K. micrum* DNA was a 100% match to sequences in the database corresponding to *K. micrum* (= *Gymnodinium galatheanum*; 100% identity [117/117 bases] with an e-value score of 9e−59), while the second DNA band was a nearly identical match to a *H. rotundata* (99% [114/115 bases] identity with an e-value score of 9e−59). The DGGE analysis thus independently confirmed the observations of Kempton et al. (2002) showing the presence of *K. micrum* (formerly *G. galatheanum*) by its nucleotide sequence and co-migration with a known standard, and strongly implicates the occurrence of *H. rotundata*, through nucleotide sequence

homology, in the water samples. No *Pfiesteria* species were detected in these two samples by DGGE (Fig. 3) or by real-time quantitative PCR (data not shown).

3.4. Confirmation of the identification as K. micrum by toxin determination

The identification of a major band in DGGE does not *a priori* indicate that the dinoflagellate producing that DNA also produced the toxin causing the HAB or fish deaths. It is just as likely that a minor component of the HAB may be the major producer of the toxin. Therefore, it is important to correlate specific toxins with specific species so that the DGGE diagnosis is meaningful in the context of the bloom. Such a chemical analysis was done for the environmental samples found by DGGE to contain a DNA phylogenetically identified as belonging to *K. micrum*.

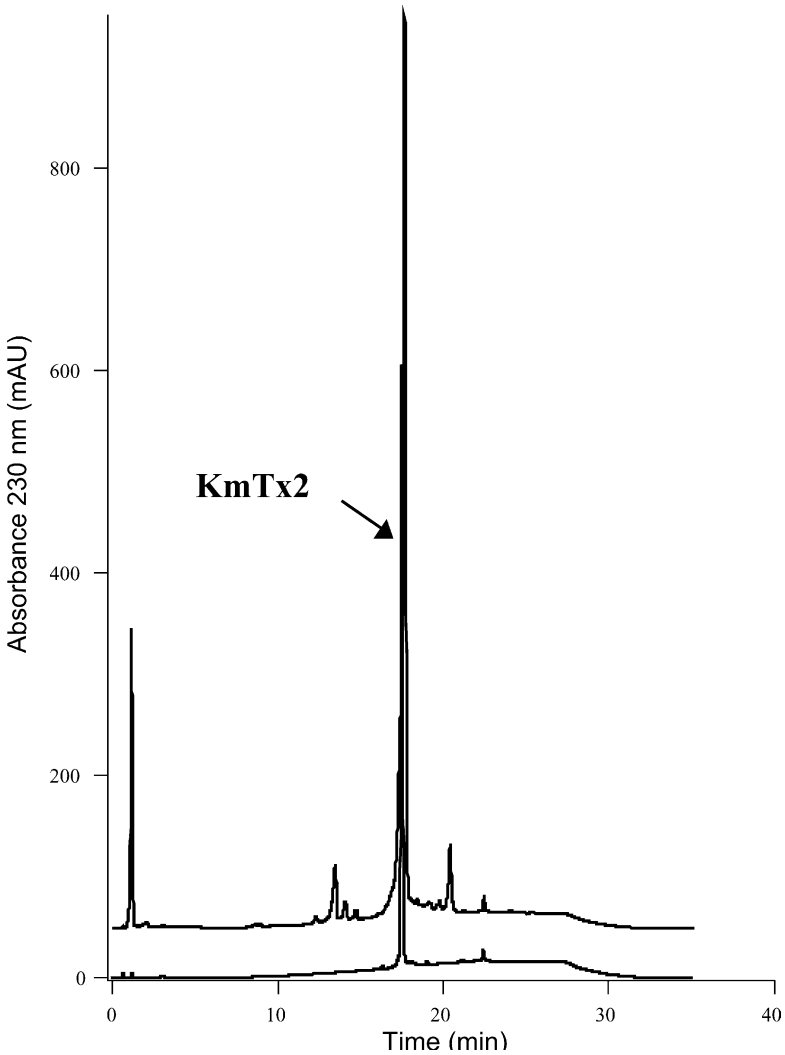

Fig. 4. Reversed phase HPLC elution profiles of toxin purified from clonal Mount Pleasant fish kill *K. micrum* JW020205-B compared to toxin directly extracted from the fish kill water. Detection of the toxin was monitored at 230 nm for a 25 μl injection of a concentrated 80% MeOH tC18 elution of filtrate of the clonal Mount Pleasant fish kill isolate (JW020205-B) using a flow rate of separation of 1 ml/min. The upper trace is the chromatogram of toxin from clonal *K. micrum* JW020205-B, while the lower trace is purified KmTx2 toxin from the fish kill.

At the time of the fish kill in 2002, a comparison of toxin isolated from the kill with that produced by two isolates from a Hilton Head pond (CCMP 2282 and CCMP 2283) showed the same toxin to be produced (Deeds et al., 2002). Since that time, an isolate from the Mount Pleasant pond (JW20205-B4) has been successfully isolated and the toxin produced by it has been compared with the purified toxin from the fish kill. As can be seen in Fig. 4, the HPLC chromatogram retention time is identical for the major peak in both samples, suggestive of molecular identity. Under laboratory growth conditions, we estimate that this isolate has a toxin content of ~0.5 pg per cell. These data confirm that a clonal isolate derived from the fish kill produces KmTx2, a toxin currently known to be produced solely by *K. micrum* and corroborate the taxonomic identification obtained through DGGE.

4. Discussion

The accurate identification of the causative organism associated with harmful algal blooms and the deaths of fishes is of paramount importance in enacting rapid, effective efforts to safeguard human health and remediate the effects of the bloom. Of the currently available techniques, light microscopy, while simple and fast, is not capable of differentiating look-alike organisms with the statistical confidence required. Scanning electron microscopy combined with swollen suture or other similar techniques used to morphologically examine dinoflagellate thecal plates is useful, but time-consuming and laborious, and not applicable to non-thecate or weakly thecate species. Further, both microscopy techniques benefit greatly by starting with pure monocultures of the dinoflagellate species, and suffer greater inaccuracy as the sample community complexity increases.

In contrast to microscopy, molecular techniques for the identification of harmful algal species often provide a more rapid and accurate way to identify these organisms. The application of several sets of species specific PCR primers has enabled the development of PCR-based detection methods for HAB species, such as the heteroduplex mobility assay (HMA) (Oldach et al., 2000), real-time quantitative PCR (Bowers et al., 2000) and PCR combined with agarose gel electrophoresis (Rublee et al., 1999; Saito et al., 2002; Zhang and Lin, 2002). While these molecular techniques are indeed powerful, they share a common weakness in that, while they can effectively detect the presence of a single species, they lack the resolving power to detect the co-occurrence of other dinoflagellates or other microorganisms at the site of the bloom. As is shown here, DGGE with dinoflagellate-specific primers significantly minimizes this weakness, providing a scientist with the ability to assess multiple dinoflagellate species within a single environmental sample.

Microbial community analysis using PCR amplification of ribosomal DNA in combination with DGGE has become a well-established molecular tool to compare the diversity of microbial communities and to monitor population dynamics (Muyzer, 1999). DGGE allows the simultaneous analysis of multiple samples and, most importantly, identification of community members through nucleotide sequencing of the excised DNA bands. DGGE also has the advantage that it cannot only detect the presence of a target organism, but also assess the clonality of the culture. Further, DGGE has the ability to identify specific DNA sequences within complex assemblages, e.g. environmental samples that comprise diverse dinoflagellate species, as shown in our analysis of the environmental sample containing both *K. micrum* and *H. rotundata* (Fig. 4). The same dinoflagellate-specific primer pair (without the GC clamp required for DGGE) has been successfully used in HMA, which is also based on the 18S rDNA sequence differences between dinoflagellate species. In HMA (Oldach et al., 2000), the definitive identification of sequences in complex template mixture is problematic because some heteroduplex bands are lost with increasing complexity. Such problems have not been encountered thus far with the DGGE method described in this report. Indeed, one of the advantages of DGGE is that co-migrating bands can be further resolved by excising, re-amplifying by PCR, followed by electrophoresis using a shallower gradient gel to achieve better band separation (Casamayor et al., 2000).

The power of DGGE for dinoflagellate analysis can be seen in Fig. 1. For example, the three morphologically similar dinoflagellates *P. piscicida*, *P. shumwayae*, and *Cryptoperidiniopsis* sp. CCMP 1828 (Fig. 1, lanes 8–10) were easily distinguishable from each other as well as from other non-*Pfiesteria*

dinoflagellates. Similarly, DGGE-based analysis is also very useful in determining the clonal status of a culture. This can be observed in Fig. 1, where 8 cultures tentatively identified by CCMP as *Pfiesteria*-like were examined (Fig. 1, lanes 11–18). As can be observed, all the putative CCMP *P. piscicida* strains were verified as being monoclonal cultures that resulted in DNA products that co-migrated with known *P. piscicida*. The species identity of these 8 cultures was confirmed upon DNA sequence analysis. These results differ slightly from reports of the analysis of the same strains by HMA (Oldach et al., 2000). Both methods agree that CCMP 1829 (Fig. 1, lane 11) is *P. piscicida* negative, however the HMA analysis (Oldach et al., 2000) determined that the CCMP 1834 culture was composed of at least two species of dinoflagellates other than *P. piscicida*, while the current data (Fig. 1, lane 16) indicate that it is a *P. piscicida* monoculture. This discrepancy suggests that the CCMP 1834 cultures used in Oldach et al. (2000) and in the current study are different in composition.

With all its power, DGGE does have several limitations that a user must be aware of when analyzing the data. Not all dinoflagellates, for example *K. micrum*, *G. sanguineum*, and *Prorocentrum lima* (Fig. 1, lanes 2, 4, and 5), produce easily distinguishable patterns on a 40–50% denaturing gradient, and often require a second DGGE analysis using a shallower DGGE gel gradient for greater separation of their product bands. Also, as observed in Figs. 1 and 3, a single DNA template species can on occasion give rise to doublet bands. These doublet artifacts are dependent on the amount of DNA template, as more template appeared to result in a great chance of doublet occurrence during these experiments (data not shown). While numerous attempts were made to correct this artifact by modifying the reaction and gel conditions in this study, a complete elimination of these doublets was not possible. Further, nucleotide sequence analysis of some of the doublets suggests that they may result from *Taq* polymerase producing a partial product that is missing one or two nucleotides at the 5′-end of the amplicon (data not shown). Thus, we speculate that the doublets observed in some of the lanes are due to *Taq* PCR artifacts and not the result of an amplification of two disparate template DNAs.

Analysis of the data obtained from DGGE must also take into consideration that the PCR products generated are relatively small (100–150 bp). This imposes limitations on ‘downstream’ analyses of taxonomic relatedness. Moreover, specific sized bands from environmental samples will always have to be sequenced in order to achieve a definitive identification. This two-step approach is more costly in terms of reagents and labor than real-time PCR and may be a disadvantage in routine monitoring programs for HABs. However, the power of DGGE lies in its ability to analyze dinoflagellate community structure without needing to know what is there, while real-time PCR provides much higher sensitivity and detection levels, if probes exist for the species of interest, attributes that complement DGGE analysis. So, as a tool for characterizing bloom communities, DGGE is superior to real-time PCR, while the latter technique is clearly better as a monitoring tool. As the two methods serve complementary roles in the investigation of HAB dynamics, the ideal situation may be to use DGGE to characterize the communities and once the toxic species have been identified, use real-time PCR or other technologies for more in-depth analysis.

An undisputed shortcoming of any 18S rDNA phylogenetic approach is the inability to know if the organism is producing a toxin, specifically the toxin causing a fish kill or other environmental perturbation. For example, the dominant species identified by the DGGE may not be the ones producing the toxin. Hence, it is important to correlate specific toxins with specific species so that the DGGE diagnosis becomes more informative. In this report, a chemical analysis of the toxin produced by *K. micrum* was done to corroborate the taxonomic identification and confirm toxicity of the dinoflagellate. While it is not possible to overcome this shortcoming when amplifying 18S rDNA sequences, our method is adaptable towards using alternative genes, such as those thought to be required for toxicogenesis. For example, recent reports by Snyder et al. (2003) call attention to the possibility of designing oligonucleotide primers to amplify the genes responsible for polyketide synthases (PKSs) as one choice to use in future DGGE analyses of HABs. As the vast majority of dinoflagellate toxins, such as the brevetoxins, okadaic acid, and amphidinolide J, are derived via PKS (Snyder et al., 2003), the use of PKS DGGE analysis may provide a remarkably effective tool in the analysis of toxin pro-

duction potential and community structure in bloom events.

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