

## The use of quantitative polymerase chain reaction for the detection and enumeration of the harmful alga *Aureococcus anophagefferens* in environmental samples along the United States East Coast

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### Abstract

*Aureococcus anophagefferens* is a small pelagophyte that has caused harmful brown tide blooms in New York, New Jersey, Rhode Island, and Maryland. It is visually indistinguishable from other small algae in environmental samples using standard light microscopy, and current species-specific immunofluorescent counting techniques are not sensitive enough to detect concentrations lower than approximately 5000 cells mL<sup>-1</sup>. A TaqMan molecular probe was developed that is specific for *A. anophagefferens* based on a unique 18S rDNA sequence to detect cells at low levels. Real-time quantitative PCR (qPCR) on an ABI Prism 7700 Sequence Detection System was used to generate a standard curve of cycle thresholds (Ct) with known concentrations of *A. anophagefferens* ( $R^2 = 0.98$ ). Cell concentrations of *A. anophagefferens* in environmental samples are determined by linear regression analysis, with a sensitivity of approximately 1 cell mL<sup>-1</sup>. Estuarine and coastal water samples were collected along the U.S. East Coast from Florida to Delaware, and during a transect from the Delaware River to offshore mid-Atlantic waters, then analyzed using qPCR. *Aureococcus* has a wide distribution at background concentrations as far south as Northern Florida, far outside the presently recognized range of the organism. It was also found in offshore Atlantic samples, suggesting a possible oceanic source for bloom inoculation. qPCR allows for detection of cells at prebloom levels, which is essential for effective management and prediction of brown tide blooms.

Harmful algal blooms appear to be increasing in frequency around the world, however, at least part of this apparent increase may be due to increased awareness (Hallegraeff 1993; Richardson 1997). Although nontoxic to humans, brown tide blooms caused by the pelagophyte *A. anophagefferens* have had

deleterious environmental and economic impacts in affected ecosystems. Shellfish populations have been reduced because the presence of *A. anophagefferens* interferes with the filter-feeding mechanisms of species such as the bay scallop, *Argopecten irradians* (Cosper et al. 1987); the hard clam, *Merccenaria mercenaria* (Bricelj et al. 2001); and the blue mussel, *Mytilus edulis* (Tracey 1988). The scallop industry in the Long Island, New York, USA, area has been completely devastated by the occurrence of brown tide blooms (Cosper et al. 1987). Brown tide blooms have decimated eelgrass beds (*Zostera marina*) because cell densities may reach concentrations as great as 10<sup>6</sup> cells mL<sup>-1</sup>, greatly attenuating light penetration (Cosper et al. 1987).

Brown tides caused by *Aureococcus anophagefferens* occur along the United States East Coast from Maryland to New York in shallow estuaries. Surveys conducted in 1988 and 1989 found *A. anophagefferens* from New Jersey to New Hampshire (Anderson et al. 1993). Samples taken in Delaware, Maryland, and Virginia at that time all tested negative for

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the presence of *Aureococcus*. Surveys conducted in 1998, however, demonstrated that *A. anophagefferens* was present in Little Assawoman Bay, Delaware, and in Assawoman Bay, Maryland (Popels and Hutchins 2002). Blooms have since occurred in Chincoteague Bay, Maryland, in 1999 and 2000 and Little Assawoman Bay, Delaware, in 2002. Blooms of *A. anophagefferens* are not confined to the U.S. They have also recently been noted in Saldanha Bay, South Africa (Naidoo 1999; Pitcher and Calder 2000; Probyn et al. 2001). It is possible that *A. anophagefferens* is present in many locations at background concentrations, creating the potential for blooms under favorable conditions. An extensive survey of the distribution of *A. anophagefferens* could help to determine if cells are present in background concentrations or if the alga has been introduced to new areas, increasing the possibility of widespread blooms.

*Aureococcus anophagefferens* cells are 2 to 3  $\mu\text{m}$  in size and morphologically indistinguishable from other small algae (Smayda and Villareal 1989). Direct microscopic cell counts are therefore not possible on environmental samples because cells cannot be positively identified as *A. anophagefferens*. A polyclonal antibody immunofluorescent technique was developed to detect and enumerate *A. anophagefferens* in environmental samples (Anderson et al. 1989). However, non-specific binding may occur with the polyclonal technique resulting in an overestimation of cells present in a sample (Anderson et al. 1989; Gobler unpub. data unref.). In addition, the method requires time-consuming microscopic cell counts. The new monoclonal antibody immunofluorescent technique (Caron et al. 2003) reduces non-specific binding and uses an efficient ELISA technique for detection and enumeration of *Aureococcus* cells in samples. Both immunofluorescent techniques work well at high cell concentrations, however, with a sensitivity of approximately 5,000 cells  $\text{mL}^{-1}$  (Caron et al. 2003), it is not possible to detect the presence of *A. anophagefferens* at pre-bloom background levels. The prediction and management of brown tide blooms require methods that are capable of detecting lower concentrations than is possible with the currently available methods.

Molecular techniques allow for the detection of harmful algal species at prebloom concentrations, as was demonstrated for *Pfiesteria piscicida* using PCR-fluorescent fragment detection (Coyne et al. 2001) and qPCR (Bowers et al. 2000). In the quantitative real-time polymerase chain reaction method, primers target the gene of interest and a molecular probe anneals between them. During amplification and elongation, a reporter and quencher are cleaved from the probe due to exonuclease activity of the DNA polymerase, allowing the reporter to fluoresce. The fluorescence is detected during the exponential phase of the amplification before any reagent becomes limiting. A cycle threshold value, Ct value, is set above background fluorescence during the exponential phase of the amplification. The cycle where the fluorescent signal crosses the Ct value is inversely proportional to the initial

amount of the gene present in the sample (Gibson et al. 1996; Heid et al. 1996; Tyrrell et al. 1997).

Quantitative PCR has been used to detect *Pfiesteria piscicida* in environmental samples (Bowers et al. 2000; Coyne et al. in prep. unref.) and quantify specific prokaryotic taxa in mixed microbial populations (Suzuki et al. 2000). Quantitative PCR is a relatively new technique and, before this work, it had not been used to detect *A. anophagefferens*. The method is particularly applicable to *A. anophagefferens* because of the alga's small size and the difficulties in detecting it at low levels. In this study, primers and a molecular TaqMan probe were developed targeting unique sequences in the 18S rRNA gene of *A. anophagefferens*. Quantitative real-time PCR was validated in the laboratory, then used to detect and enumerate *A. anophagefferens* in estuarine samples collected from Florida to Delaware along the U.S. East Coast, as well as in an onshore/offshore transect in Delaware coastal waters.

### Materials and procedures

The 18S rDNA sequence for *A. anophagefferens* was determined by sequencing laboratory cultures and comparing them with existing sequences. Priming and probe sites specific for *Aureococcus* were then determined from the sequences. A standard curve was developed using known concentrations of *Aureococcus* culture spiked into environmental water. Standards were collected on a filter, and then extracted and diluted to a concentration of 25 ng DNA  $\mu\text{L}^{-1}$ . Environmental samples for qPCR were filtered, extracted, and diluted to a concentration of 25 ng DNA  $\mu\text{L}^{-1}$ . Samples and standards were run on an ABI Prism 7700 Sequence Detector for analysis with qPCR using linear regression analysis to determine concentrations of the samples. Samples were then corrected for biomass using chlorophyll to get an estimate of cells  $\text{mL}^{-1}$  concentration.

*Determination of 18S rDNA sequence*—*Aureococcus anophagefferens* (CCMP 1708) was cultured at 18°C in growth medium as described in Popels and Hutchins (2002). The culture was sequenced as a standard protocol to confirm that the cultures we had matched the expected sequences in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Cells were filtered onto a 1- $\mu\text{m}$  polycarbonate filter and placed in 0.7 mL CTAB extraction buffer (100 mM Tris HCl [pH 8], 1.4 M NaCl, 2% [w/v] cetyltrimethylammonium bromide [CTAB], 0.4% [v/v]  $\beta$ -mercaptoethanol, 1% [w/v] polyvinylpyrrolidone, 20 mM EDTA [Dempster et al. 1999]). DNA was extracted as in Coyne et al (2001). The 18S rDNA gene was amplified by PCR using eukaryotic-specific primers, Euk A (5' AACCTGGTTGATCCTGCCAGT 3') and Euk B (5' GATCC(A/T)TCTGCAGGTTACCTAC 3') (Medlin et al. 1988). A 50  $\mu\text{L}$  total reaction volume was used for the PCR, containing 0.2 mM dNTPs, 0.5  $\mu\text{M}$  each primer, 1.25 mM  $\text{MgCl}_2$ , 1X Taq polymerase buffer (Promega), and 0.5 units Taq Polymerase (Promega). The reaction for PCR consisted of 35 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C, followed with a 5-min extension at 72°C. PCR products were cloned into TOPO II vector (Invitrogen) and sequenced using

Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI). The clones were confirmed to be *A. anophagefferens* based on comparisons of their sequence to existing sequences in GenBank (Accession nr AF119119).

**Molecular probe and primers**—Sequences were aligned in Genetic Data Environment (Smith et al. 1994) using Clustal (Thompson et al 1994) and inspected visually. Priming sites specific for *A. anophagefferens* were identified after comparing the sequences of *A. anophagefferens* (accession nrs AF119119, U40257, and AF117776) with to the aligned sequences of the pelagophyte *Aureoumbra lagunensis* (nr U40258) and an unidentified coccoid pelagophyte (nr U40927). Potential TaqMan probe sites were selected using the default parameters in Primer Express (ABI). The probes were then evaluated for specificity by comparing to sequences in GenBank using Blast.

A species-specific forward primer, Aa1685f (5' ACCTCCG GACTGGGGTT 3') was designed to anneal at base 1685 of the *Aureococcus anophagefferens* 18S rRNA gene sequence. The universal primer Euk B was used as a reverse primer. A TaqMan Probe with the sequence 5' GGCGAACGTACCTCGGGAAGT TGG 3' with a 6FAM fluorescent marker on the 5' end was designed to anneal at base 1714 of the 18S sequence.

A water sample that tested positive for *A. anophagefferens* from Roosevelt Inlet, Delaware, was amplified by PCR using Aa1685f and Euk B primers, and then cloned and sequenced as described previously. The sequence was run through GenBank to find all matching sequences that had been submitted to the database and ensure that the primer and probe set was accurately detecting *A. anophagefferens*.

**Quantitative PCR**—Primer and probe concentrations were optimized for qPCR on the ABI Prism 7700 Sequence Detection System using the cloned plasmid containing the sequence for *A. anophagefferens* 18S rDNA. Optimized total reaction volumes of 25  $\mu$ L were used for each sample, consisting of 12.5  $\mu$ L Universal Master Mix (ABI), 0.9  $\mu$ M forward primer Aa1685f, 0.9  $\mu$ M reverse primer Euk B, QH<sub>2</sub>O, 0.1  $\mu$ M probe, and 62.5 ng of the template, standard or sample. Plasmid concentrations of 0.1 ng  $\mu$ L<sup>-1</sup> were used as template for optimization. Standards and samples were kept at a concentration of 25 ng DNA  $\mu$ L<sup>-1</sup>. Standards need to be used to generate a standard curve each time samples are run. Therefore, with each set of samples, 6 standards in triplicate were run along with 2 negative controls.

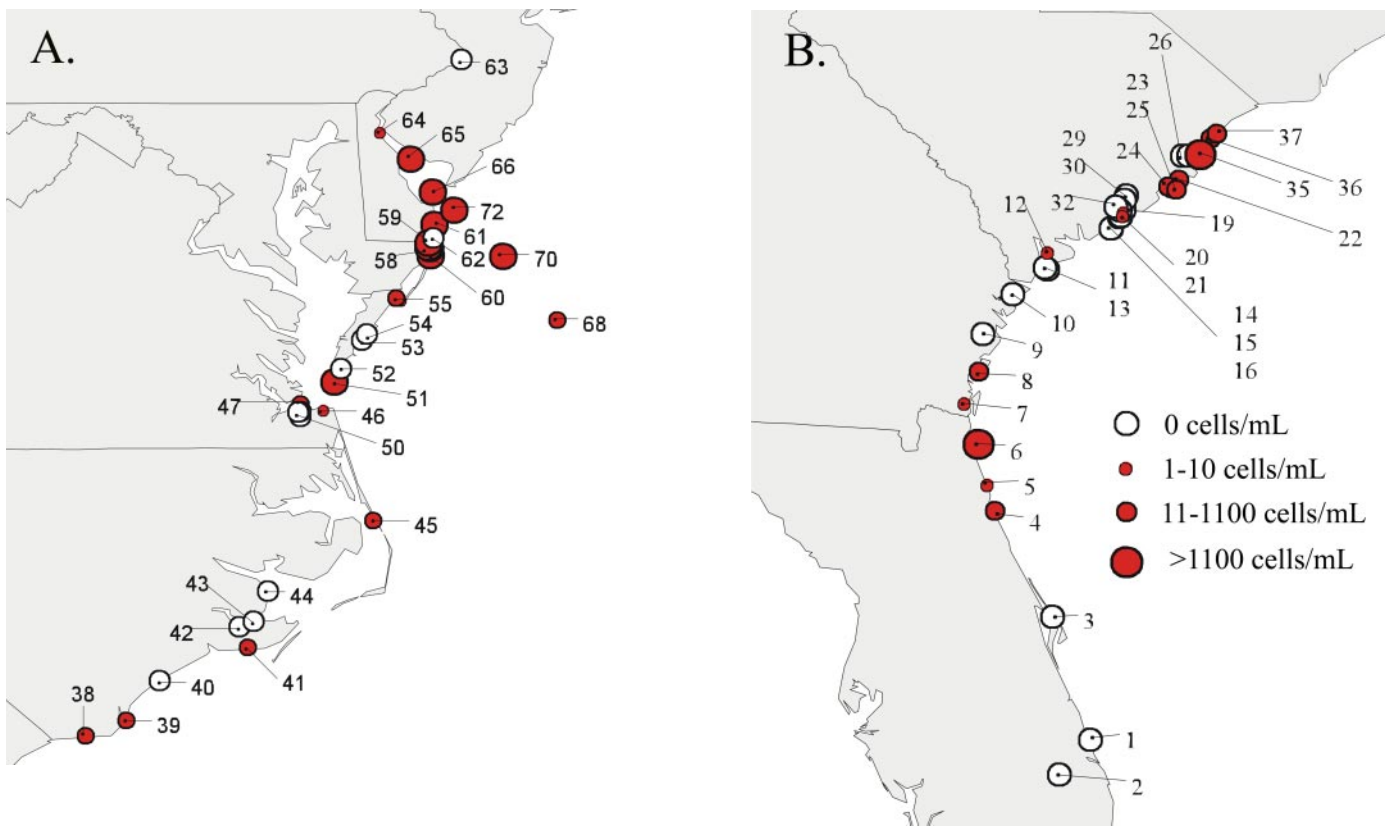
*A. anophagefferens* concentrations in cells mL<sup>-1</sup> were calculated by linear regression analysis. Some variability in biomass was presumed for equivalent volumes of field samples and spiked standards. The amount of DNA template used for each PCR reaction, however, was kept constant (62.5 ng) to minimize variability caused by fluctuating DNA concentrations or inconsistencies in extraction technique. For this reason, the ratio of chlorophyll *a* concentrations (sample:standard) was used as a dilution factor to more closely approximate the volume of field sample represented by 62.5 ng of PCR template. In this way, the outcome was not dependent upon a quantita-

tive extraction protocol, but rather on the proportion of targeted template DNA (*A. anophagefferens*) within the total DNA extracted. The chlorophyll concentration of the environmental water before it was spiked with *Aureococcus* was used for the calculation. We have found that there is little variability in total chlorophyll *a* values when concentrations below 10<sup>5</sup> cells mL<sup>-1</sup> of *Aureococcus* are added to a water sample. Therefore, we used the same total chlorophyll *a* value for all of the standard concentrations.

Although chlorophyll is commonly used by oceanographers as a proxy for algal biomass, there are unavoidable uncertainties associated with using it as a biomass correction factor, and this may introduce error into final calculated cell concentrations. When the raw data for cell concentration from the ABI7700 is corrected using a ratio of volumes filtered (standard:sample) for samples with equal DNA concentrations, there is very close agreement with the values obtained using the chlorophyll correction factors. For example, from the artificial community data, a concentration of 62 cells mL<sup>-1</sup> determined with the chlorophyll correction factor also gives 62 cells mL<sup>-1</sup> with the volume correction. Therefore, we believe that chlorophyll concentrations are a valid correction factor.

**Standard curve development**—Standards were made using cultured *A. anophagefferens* with cell concentrations of 0 (not spiked), 1, 10, 100, 1000, and 10,000 *Aureococcus* cells mL<sup>-1</sup>. The cell concentration of the cultures was determined by automated microscopic cell counts of culture samples preserved with glutaraldehyde to a final concentration of 2%, and then filtered onto a 0.22  $\mu$ m black polycarbonate filter. The software package Image Pro (Media Cybernetics, The Imaging Experts) was used to count 10 fields per filter using chlorophyll autofluorescence under epifluorescence microscopy and triplicate filters were counted (Popels and Hutchins 2002).

Red Mill Pond water (Lewes, Delaware: 38.751 N, 75.222 W) was prefiltered through a 20- $\mu$ m and a 5- $\mu$ m filter. Pond water was used because it contains a natural community with mixed DNA but it does not contain *A. anophagefferens*, in contrast to coastal seawater where the brown tide alga could potentially be present. One hundred milliliters of the 5- $\mu$ m filtrate was passed through a 1- $\mu$ m filter, and then the damp filter was spiked with an appropriate amount of *A. anophagefferens* culture. The fresh pond water was not spiked directly to avoid osmotic shock to the *Aureococcus* cells. The filter was placed in CTAB extraction buffer at 50°C for 15 min. Spiked standards were then stored at -80°C until extraction. DNA was extracted for each sample as described above and DNA concentration was determined by ultraviolet spectrophotometry using a Beckman DU 640B spectrophotometer with a wavelength accuracy of  $\pm$ 0.2 nm and a wavelength repeatability of  $\pm$ 0.1 nm. To quantify the small volume of DNA, 1  $\mu$ L was diluted to a final volume of 50  $\mu$ L, and then the concentration was read on the spectrophotometer. The DNA was then diluted to 25 ng  $\mu$ L<sup>-1</sup> with MilliQ water for use in the qPCR reaction.



**Fig. 1.** (A) Sampling sites from North Carolina to Delaware. (B) Sampling sites from Florida to South Carolina. The size of the symbols corresponds to the cell concentration. Note that not every station is pictured on the map. In some of the high-density sampling areas, the symbols for the stations overlap. In these situations, the higher cell concentrations are mapped.

**Environmental samples**—Fifty-six duplicate coastal and estuarine surface water samples were collected along the U.S. East Coast from Indian River Lagoon, Florida, to Chincoteague, Virginia, between 18 May and 31 May 2001. Samples from Maryland were collected 25 May 2002 and samples from Delaware Inland Bays were collected 29 and 30 June 2002. Samples were also collected at 10 stations along a transect from the freshwater portion of Delaware Bay to offshore continental shelf break waters in March 2002. Sample locations are presented in Fig. 1 and corresponding station names are noted in Table 1. From each water sample collected, chlorophyll *a*, salinity, and temperature were measured and approximately 300 mL of water was filtered for DNA. In some samples with very high biomass a smaller volume was filtered, and for offshore samples 1 L was filtered. Samples were pre-filtered through a 20- $\mu$ m and a 5- $\mu$ m filter and then collected onto a 1- $\mu$ m filter. The 1- $\mu$ m filter was then placed in 0.7 mL of CTAB buffer and heated at 50°C for 15 min. Filtered samples were stored on dry ice or at -20°C during the sampling trips, and then stored at -80°C until extraction. Extracted DNA was stored at -80°C until analysis with qPCR. Concentrations of DNA in samples were determined using a spectrophotometer and then samples were diluted to 25 ng  $\mu$ L<sup>-1</sup> and qPCR was conducted as described previously.

Amplified DNA from 7 stations (nr 22, 33, 35, 41, 56, 65, and 68) that tested positive using qPCR was sequenced to confirm that the primers and the probe were targeting *A. anophagefferens*. PCR was conducted using Aa1685f and Euk B to amplify the target DNA. The cycles of the PCR consisted of 35 cycles of 0.5 min at 94°, 0.5 min at 55°, and 1 min at 72°. The PCR product was purified using the Sigma GenElute PCR Clean-up Kit and sequenced using BigDye Terminator Cycle Sequencing Ready Reaction (ABI). Sequencing was conducted in both directions, and then the traces were brought into Sequence Navigator (ABI) for analysis, aligned, and visually checked. Sequences were then blasted using *blastn* on GenBank to find all matching sequences.

All samples that tested negative for the presence of *A. anophagefferens* were amplified again after being spiked with plasmid to make sure that PCR was not inhibited by a substance present in the sample. To do this, the regular qPCR reaction mix was used and the plasmid containing the DNA of interest was added to the sample. An equal volume of the plasmid was added to a positive control, and the values from the samples and the positive controls were compared to determine if there was inhibition.

In addition, samples from Florida to Virginia were collected for analysis with the polyclonal antibody method by preserv-

**Table 1.** Sample results. Sample numbers correspond to those on maps in Fig. 1.

Sample	Station Name	qPCR (cells mL <sup>-1</sup> )	N <sup>a</sup>	Polyclonal (cells mL <sup>-1</sup> )	Longitude	Latitude	Salinity	Chlorophyll <i>a</i> (µg/L)
1	Indian River Lagoon—Bear Point Sanctuary, FL	0 ± 0.0	2	149.63 ± 95.23	-80.282	27.43	38	8.8
2	Claude Edge Front Street Park—Indian River, FL	0 ± 0.1	2	299.03 ± 293.07	-80.599	27.08	30	19.9
3	Mosquito Lagoon, FL	0 ± 0.0	2	210.46 ± 162.63	-80.666	28.68	40	4.2
4	Crescent Beach, FL (Anastasia Island)	67 ± 3.2	2	3281.67 ± 1940.77	-81.25	29.75	36	4.8
5	Guana River State Park, FL	3 ± 1.0	2	1113.78 ± 562.54	-81.328	30.02	36	11.8
6	Little Talbot Island, FL	177 ± 39.5	2	216.97 ± 102.28	-81.424	30.43	36	6.5
7	Crooked River State Park, near Cumberland Island, GA	3 ± 1.8	4	180.81 ± 51.14	-81.559	30.85	31	10.4
8	St. Simons Island, GA	31 ± 4.8	4	180.81 ± 51.14	-81.413	31.17	28	15.0
9	Belleview Point, GA	0 ± 0.0	2	0.00 ± 0.00	-81.372	31.55	30	13.1
10	Skidaway Island, GA	0 ± 0.1	2	108.48 ± 51.14	-81.067	31.95	27	6.7
11	Hilton Head Island, SC, off SSR 245	0 ± 0.0	2	108.48 ± 51.14	-80.716	32.2	32	9.5
12	Port Royal Island, SC off 802	1 ± 1.0	4	0.00 ± 0.00	-80.717	32.38	35	6.9
13	Hilton Head, SC, off Gumtree Rd off 278,	0 ± 0.0	2	361.62 ± 204.56	-80.743	32.22	30	168.4
14	Kiawah Island, SC	0 ± 0.0	4	1048.69 ± 664.82	-80.069	32.62	20	183.1
15	Kiawah Island, SC	0 ± 0.0	2	253.13 ± 51.14	-80.071	32.62	30	22.7
16	Kiawah Island, SC	0 ± 0.0	2	795.56 ± 102.28	-80.07	32.62	27	32.8
17	Cross Creek, SC	0 ± 0.1	2	198.89 ± 180.81	-79.982	32.73	22	29.9
18	James Island Creek at Folley, SC	0 ± 0.0	2	0.00 ± 0.00	-79.97	32.75	26	24.8
19	New Market (downtown), SC	0 ± 0.0	2	277.24 ± 324.78	-79.942	32.81	8	10.6
20	City Marina, SC	4 ± 0.5	2	253.13 ± 51.14	-79.949	32.78	25	27.7
21	James Island Creek at Harborview, SC	1 ± 0.6	4	144.65 ± 0.00	-79.951	32.75	26	20.6
22	Intercoastal, SC	83 ± 44.0	4	0.00 ± 0.00	-79.389	33.11	33.3	19.6
23	Key Creek, SC	84 ± 8.4	2	216.97 ± 0.00	-79.455	33.03	36	11.3
24	Noname Creek, SC	23 ± 22.7	4	0.00 ± 0.00	-79.49	33.05	35.6	13.6
25	Romain River, SC	50 ± 15.6	4	253.13 ± 51.14	-79.411	33.02	31.4	20.1
26	3V Chemical, SC	0 ± 0.0	2	253.13 ± 153.42	-79.349	33.36	9	15.0
27	GT—Steelmill, SC	0 ± 0.2	2	216.97 ± 102.28	-79.285	33.36	13.2	24.0
28	Sampit River, SC	0 ± 0.0	2	0.00 ± 0.00	N/A	N/A	2.5	3.9
29	Flagg Creek, SC	0 ± 0.2	4	0.00 ± 0.00	-79.913	32.95	3	26.4
30	Mouth of Cooper Creek, SC	0 ± 0.0	2	506.26 ± 102.28	-79.932	32.91	6	16.8
31	Citadel Creek, SC	0 ± 0.1	2	0.00 ± 0.00	-79.977	32.8	24	16.6
32	Country Farm Landing, SC	0 ± 0.1	4	0.00 ± 0.00	-80.021	32.84	17	23.0
33	Clam Bake Bridge, SC	13 ± 9.1	4	72.32 ± 0.00	-79.205	33.34	35	8.6
34	Oyster Landing Pier, SC	79 ± 19.5	2	361.62 ± 204.56	-79.189	33.35	35	5.1
35	Debi Due Creek (in Debordieu), SC	116 ± 21.1	2	108.48 ± 51.14	-79.169	33.37	34	6.8
36	Huntington Beach State Park boat launch, SC	42 ± 2.2	2	216.97 ± 102.28	-79.062	33.53	34	3.1
37	Garden City, SC	47 ± 19.6	4	144.65 ± 0.00	-79.004	33.58	34	33.3
38	Near Holden Beach, just over 130 bridge, NC	16 ± 12.2	4	216.97 ± 102.28	-78.267	33.92	35	5.3

(Continued)

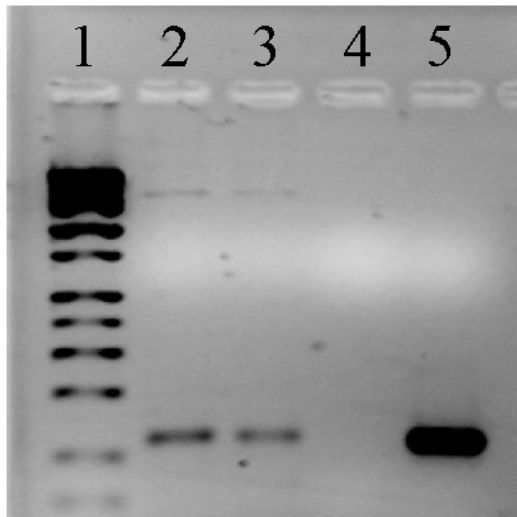
**Table 1.** Continued

Sample	Station Name	qPCR (cells mL <sup>-1</sup> )	N <sup>a</sup>	Polyclonal (cells mL <sup>-1</sup> )	Longitude	Latitude	Salinity	Chlorophyll <i>a</i> (µg/L)
39	Near Carolina Beach State Park— wildlife boat ramp, NC	35 ± 10.7	4	253.13 ± 153.42	-77.892	34.06	28	22.7
40	Watts Landing, NC	0 ± 0.1	4	0.00 ± 0.00	-77.584	34.42	36	2.8
41	Marina, Camp Glenn, NC	42 ± 15.2	2	108.48 ± 51.14	-76.782	34.73	32	7.9
42	Cahogue Creek, NC	0 ± 0.0	2	433.94 ± 102.28	-76.853	34.92	8	13.5
43	Minnesott Beach, NC	0 ± 0.0	2	433.94 ± 102.28	-76.722	34.97	10	13.3
44	Off Jones Bay, NC	0 ± 0.0	2	614.75 ± 51.14	-76.591	35.25	12	7.7
45	Roanoke Island, NC	11 ± 1.3	4	578.59 ± 306.84	-75.63	35.89	19	11.0
46	Lynhaven Inlet, VA	2 ± 1.4	4	397.78 ± 51.14	-76.086	36.91	22	1.4
47	Willoughby Harbor Marina, VA	16 ± 3.5	4	0.00 ± 0.00	-76.295	36.97	20	16.4
48	Nauticus Nautical Museum, VA	0 ± 0.0	4	542.42 ± 255.70	-76.295	36.85	20	8.8
49	52nd Street at Crittenton Hall, VA	0 ± 0.0	2	108.48 ± 51.14	-76.294	36.89	17	31.7
50	ODU sailing club dock— Powhattan, VA	0 ± 0.1	4	397.78 ± 460.26	-76.318	36.89	18	9.2
51	Kiptopeke Park, VA	155 ± 135.8	2	216.97 ± 102.28	-75.988	37.17	25	2.3
52	Oyster, VA	0 ± 0.1	2	325.45 ± 255.70	-75.923	37.29	32	6.6
53	Quinby, VA	0 ± 0.1	4	0.00 ± 0.00	-75.732	37.55	33	2.8
54	Wachapreague, VA	0 ± 0.0	2	108.48 ± 51.14	-75.687	37.6	29	3.5
55	Shell Bay (near Chincoteague), VA	15 ± 5.2	2	72.32 ± 0.00	-75.42	37.94	31	2.5
56	Chincoteague (by Chincoteague Shellfish Farms, Inc), VA	2 ± 0.3	4	180.81 ± 153.42	-75.372	37.92	31	2.3
57	Isle of Wight Bay, MD	91598 ± 11235.6	4	N/A	-75.108	38.39	30	18.0
58	Saint Martin River, MD	111114 ± 11002.6	4	N/A	-75.125	38.4	30	24.5
59	Gray's Creek, MD	122593 ± 23161.3	4	N/A	-75.126	38.44	22	46.4
60	West Ocean City Marina, MD	753 ± 233.1	4	N/A	-75.107	38.33	31	17.6
61	Indian River Marina, DE	139 ± 29.8	4	N/A	-75.072	38.61	26	6.3
62	Little Assawoman boat ramp, DE	0 ± 0.0	4	N/A	-75.077	38.49	30	43.1
63	Delaware River, STA 2, DE	0 ± 0.0	4	N/A	-74.818	40.13	0.1	3.8
64	Delaware Bay, STA 18, DE	1 ± 0.2	4	N/A	-75.562	39.46	8.7	19.5
65	Delaware Bay, STA 22, DE	140 ± 63.2	4	N/A	-75.288	39.21	15.8	23.6
66	Delaware Bay, STA 26, DE	206 ± 235.5	4	N/A	-75.085	38.91	28.5	4.8
67	Shelf Break, 10-m depth	15 ± 11.9	3	N/A	-73.942	37.74	35.2	1.5
68	Shelf Break, 30-m depth	8 ± 4.4	5	N/A	-73.942	37.74	35.2	1.5
69	On shelf 5-m depth	29 ± 15.6	2	N/A	-74.44	38.32	33.8	4.0
70	On shelf 20-m depth	1093 ± 165.9	2	N/A	-74.44	38.32	33.8	4.0
71	Offshore 5-m depth	360 ± 367.9	4	N/A	-74.887	38.74	31.9	3.4
72	Offshore 10-m depth	794 ± 25.9	4	N/A	-74.887	38.74	31.9	3.3

<sup>a</sup>N represents the number of replicates for a sample.

ing 45 mL to a final concentration of 1% glutaraldehyde using 10% glutaraldehyde in seawater. For the polyclonal antibody method, *A. anophagefferens* cells were enumerated by staining cells that were gently filtered (<5 kPa) onto a 0.8-µm black polycarbonate filter with a polyclonal immunofluorescent label, as described by Anderson et al. (1989, 1993). Modifications of the original technique included increasing the amount of primary and secondary antibody used by 2-fold, a step required to ensure accurate quantification of *Aureococcus* cells

in field samples (Gobler et al. 2002). A sample volume of 1 to 2 mL was filtered and a minimum of 30 fields were counted at a magnification of 400×. To ensure accurate results, the immunofluorescent technique was compared to counts performed with a hemacytometer on a light microscope. The two techniques yielded statistically identical results using *A. anophagefferens* clones 1708 and 1794 at cell densities of 10<sup>5</sup> cells mL<sup>-1</sup>. A relative standard deviation of 9% was obtained for replicate counts of the same sample (*n* = 6) at this cell density.



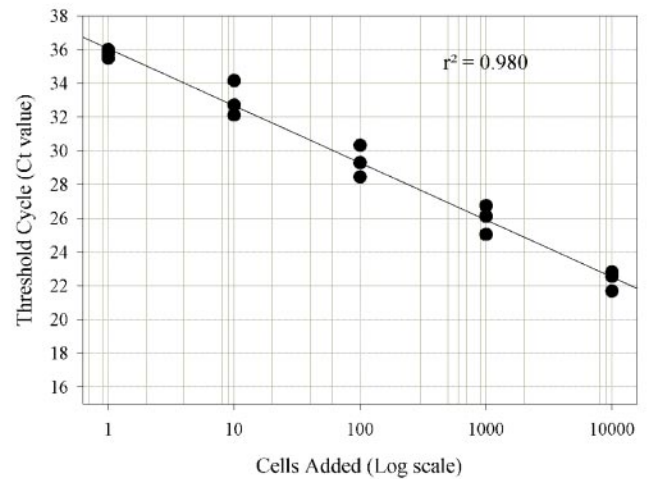
**Fig. 2.** PCR amplification of environmental sample: 1, HiLo molecular marker (Minnesota Molecular, Minneapolis, Minnesota); 2 and 3, amplification of *Aureococcus anophagefferens* in replicate environmental samples; 4, negative PCR control; 5, positive PCR control.

**Assessment**

To validate the specificity of the primers used, PCR was conducted on several environmental samples. Fig. 2 shows amplification of the samples taken from Roosevelt Inlet, Delaware. The amplified PCR products were sequenced and confirmed to be *A. anophagefferens*. Samples amplified from the East Coast survey were also confirmed to be *A. anophagefferens* by sequencing with 100% identity to the GenBank sequences (Accession nr AF117776, AF117777, AF117778, AF117779, AF118443, AF119119).

**Standard curve development**—A standard curve ( $r^2 = 0.98$ ) was developed using pond water spiked with known concentrations of *A. anophagefferens* cells (Figs. 3 and 4). The standard curve of threshold cycle (Ct) versus cells mL<sup>-1</sup> of *A. anophagefferens* in the spiked standards demonstrates linearity over 4 orders of magnitude for detection of *Aureococcus* in a mixed community with a sensitivity of 1 cell mL<sup>-1</sup>. As determined by our standards, it is possible to detect  $1.4 \pm 0.7$  cells in a single qPCR reaction. This was determined by multiplying the concentration of cells in the standard by the volume of water filtered and by the proportion of DNA used for the qPCR out of all of the DNA extracted from the standard.

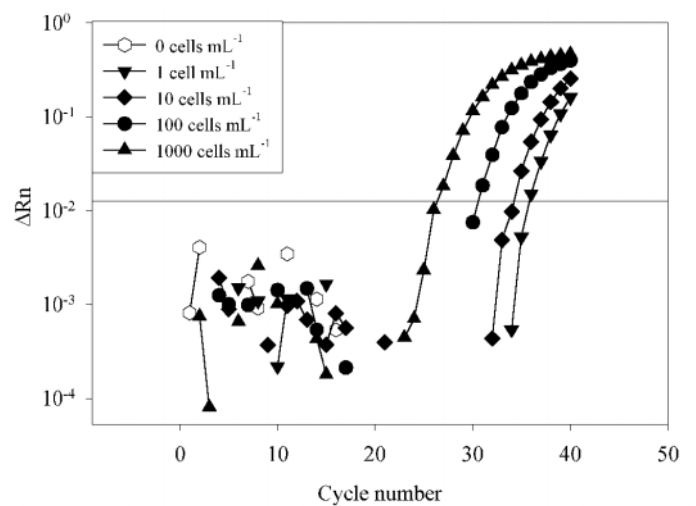
One assumption of this method is that the ribosomal gene copy number of *A. anophagefferens* is the same in cells grown under culture conditions and in the field. This assumption of invariant gene copy number underlies all qPCR enumerations, but further work is needed to confirm this and validate the use of cultured standards for quantifying environmental samples. Bailey and Anderson (1999) found few differences in the 18S rDNA sequence of 14 *Aureococcus* strains isolated from Long Island Sound, New York, USA. This may suggest relatively lit-



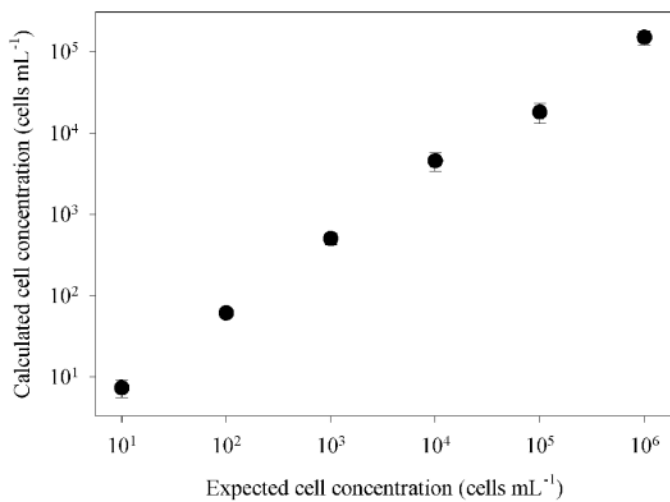
**Fig. 3.** Environmental water samples spiked with 1 to 10,000 cells mL<sup>-1</sup> of *Aureococcus anophagefferens* were amplified using qPCR. Standard curve of threshold cycle (Ct) versus cells mL<sup>-1</sup> demonstrates linearity over four orders of magnitude.

tle 18S genetic variability among different *Aureococcus* populations in the wild, but studies are needed to compare ribosomal gene copy number for different strains of this alga and in other species of interest for qPCR enumeration.

**Test samples using artificial community**—To confirm that the qPCR technique accurately quantified *A. anophagefferens* concentrations in seawater samples, an artificial community was made using laboratory cultures of marine phytoplankton and 0.2 μm filtered seawater. The cultures used were the centric



**Fig. 4.** Amplification plot of standards from qPCR run on ABI7700 of  $\Delta Rn$  versus cycle number.  $\Delta Rn$  is a measure of the fluorescence intensity. The straight horizontal line marks the fluorescence threshold that is used to determine the Ct value.



**Fig. 5.** Plot of calculated versus expected cell concentrations for *Aureococcus anophagefferens* in artificial communities. Calculated concentrations were determined using qPCR analysis of samples from laboratory cultures spiked with known concentrations of *Aureococcus* cells. Expected concentrations are the number of *Aureococcus* cells determined by microscopic cell counts and dilutions.

diatom *Thalassiosira weissflogii* (CCMP 1336) at a density of 3100 cells mL<sup>-1</sup> in the artificial community, the coccolithophorid *Emiliania huxleyi* (CCMP 373) with a concentration of 8000 cells mL<sup>-1</sup>, and the unicellular cyanobacterium *Synechococcus* sp. (CCMP 1334) at 86,000 cells mL<sup>-1</sup>. The seawater with the artificial community was divided into 1.1 L aliquots and spiked with known concentrations of *Aureococcus* cells, as determined by microscopic cell counts with Image Pro. Ten-fold dilutions of the *Aureococcus* cultures ranging from 10 to 10<sup>6</sup> cells mL<sup>-1</sup> were prepared in the artificial community. Triplicate samples of 300 mL each were filtered onto a 1- $\mu$ m filter for each cell concentration, except for the concentration of 10<sup>6</sup> cells mL<sup>-1</sup> where only 170 mL could be filtered because of the density of cells in the sample. Non-spiked samples were also filtered to use as negative controls. The filter towers were rinsed with deionized water between samples and the filters were placed into CTAB buffer as described above.

The artificial community samples demonstrate that the qPCR method accurately quantifies *A. anophagefferens* in mixed community samples within the correct order of magnitude. Fig. 5 shows the expected cell concentrations versus the cell concentration determined using qPCR, with error bars representing the standard deviation among replicates. There is fairly good agreement between calculated and expected values up to 10<sup>4</sup> cells mL<sup>-1</sup>, with errors between 25% to 54%. The error associated with the cell counts to make the artificial community samples and differences in DNA extraction efficiency may account for the discrepancies in the values. The standard curve demonstrated linearity to a cell concentration of 10<sup>4</sup> cells mL<sup>-1</sup>. Above 10<sup>4</sup> cells mL<sup>-1</sup>, qPCR begins to under-

estimate the number of cells mL<sup>-1</sup> in a sample. There is approximately 82% to 85% error for 10<sup>5</sup> and 10<sup>6</sup> cells mL<sup>-1</sup>. Therefore, the dynamic range of the method is from 0 to 10<sup>4</sup> cells mL<sup>-1</sup>. It may however be possible to dilute bloom range concentrations of cells to bring the samples within the dynamic range of the method. Samples with no *Aureococcus* added were negative, suggesting that it may be sufficient to rinse filter towers with deionized water between filtrations.

**Environmental samples**—qPCR was successfully used to enumerate *A. anophagefferens* in environmental samples. *A. anophagefferens* was present in water samples at background concentrations from Florida to Delaware (Fig. 1, Table 1). In total, 32 of 72 samples tested were negative for the presence of *A. anophagefferens*; 9 had concentrations of less than 10 cells mL<sup>-1</sup>; 18 had concentrations in the range of 10 to 100 cells mL<sup>-1</sup>; 9 had concentrations in the range of 100 to 1000 cells mL<sup>-1</sup>; and 4 had concentrations >1000 cells mL<sup>-1</sup>. The highest value detected in the survey, ~120,000 cells mL<sup>-1</sup> (sample 59), was collected from Gray's Creek in Maryland during bloom conditions. This sample was, however, outside the dynamic range of the technique. The sample with the highest concentration within the dynamic range of the method, sample 70, with 1093 cells mL<sup>-1</sup> was collected from the shelf off the coast of Delaware at 20-m depth. The concentration was also high just off the coast of Delaware at sample site 72.

The survey provided a snapshot view of the distribution of *A. anophagefferens*. For this study, each location was only sampled on one occasion. Temporal variability is therefore not included in this study. Station 62, Little Assawoman Bay, Delaware, is evidence of this because the sample tested negative for *Aureococcus* on this particular survey; however, on other occasions, it has been found in the bay (Popels and Hutchins 2002, Coyne unpubl. data unref.).

There is error inherent in the qPCR method because of differences in extraction efficiency and the possibility of substances present in the samples that may inhibit the qPCR reaction. As discussed previously, the use of chlorophyll to measure algal biomass in a water sample could also introduce variability in enumeration. The method that we used for DNA extraction is widely used and the presence of polyvinylpyrrolidone removes many inhibitors (Dempster et al. 1999); however, there still may potentially be inhibitors present in the samples that could interfere or inhibit the reaction.

These problems may be eliminated or reduced by the use of an internal standard, such as that used by Saito et al. (2002) to test for false-negative results. The internal standard would allow for a correction factor because of differences in extraction efficiency, and it would test for PCR inhibitors. It would no longer be necessary to correct the samples for biomass using chlorophyll, and instead, sample values could be corrected using volume filtered. We tested for the presence of false negatives by adding the plasmid containing the DNA of interest to each sample that tested negative for the presence of *Aureococcus*. None of the negative samples in this study had

substances present that inhibited the qPCR reaction because there were no differences in amplification between the positive control and the spiked negative samples.

There is no evident correlation between *A. anophagefferens* counts using qPCR and the polyclonal technique from samples taken on the East Coast sampling trip (Table 1). The low cell concentrations present in the samples probably account for the lack of agreement. Although there is unavoidable error associated with both of these techniques, at extremely low cell concentrations, there is an especially large amount of intrinsic error associated with the polyclonal counts. This is due to the nature of microscopic cell counts, in which multiple fields containing highly variable numbers of only a few (or no) cells are enumerated and then averaged to obtain an overall cell concentration, yielding high analytical standard deviations. When cells are counted, the accuracy varies inversely with the square root of the number of cells counted (Lund et al. 1958). Accuracy is defined as "a quantitative measure of the magnitude of error" (IEEE 1990). Therefore, if 100 cells are counted, the accuracy is 20% and the range of cells in the sample is 80 to 120. However, if 400 cells are counted, the accuracy is now 10% and the range is 360 to 440. Non-specific binding and aging-related variability of the antibody stock also contribute to uncertainties with the polyclonal method (Gobler unpub. data unref.). At some stations, such as sample 1, there were no cells detectable by qPCR, yet there were 150 cells mL<sup>-1</sup> detected with the polyclonal antibody. This result could be caused by non-specific binding of the polyclonal antibody. In other stations, such as stations 22 and 24, qPCR detected the presence of *Aureococcus* at 83 and 23 cells mL<sup>-1</sup> respectively, yet no cells were detected with the polyclonal antibody method. It is possible that the cells were simply not present in the fields used for the polyclonal cell counts because of the very low concentrations. The two techniques are useful at different cell concentrations for environmental samples, with qPCR being much more reliable at low, prebloom levels, and the polyclonal technique yielding more accurate values at bloom concentrations (>10<sup>4</sup> cells mL<sup>-1</sup>).

One drawback for qPCR is the high start-up costs for the technique. An Applied Biosystems Sequence Detection System is currently approximately U.S. \$50,000 to \$130,000. The ABI 7700 that was used in this study is no longer available, but there are other models, such as the ABI 7000 or the ABI 9700, that can be used in its place. Other real-time PCR thermocyclers, such as the Stratagene MX3000P, the Cepheid Smart Cycler, and the BioRad iCycler IQ RT-PCR machine, are available in the price range of \$25,000 to \$48,500. The new technology is becoming less expensive and more available for widespread use. Once the initial equipment is purchased, however, it only costs approximately \$2 per replicate for analysis, including the cost of the Universal Master Mix, the 96 well plates, primers, and the probe. It costs approximately \$40 to run the standards and the negative controls alone, so as many samples as possible should be run on the plate each time to make this procedure more cost effective.

**Statistical properties**—qPCR is sensitive over 4 orders of magnitude, in agreement with the range reported by Suzuki et al. (2000). For the artificial community trial, triplicate samples were each run twice on the ABI 7700. The coefficient of variation for each sample (a total of six replicates) was less than 0.28 for all samples with an average of 0.21 ( $n = 7$ ), again in agreement with Suzuki et al. (2000). Variability is likely because of differences in extraction efficiency, pipetting error, and concentration of DNA (deviations from exactly 25 ng  $\mu$ L<sup>-1</sup>). For the analytical duplicates of each artificial community sample, the average coefficient of variation was 0.11, with a high value of 0.19 and a low of 0.009. For the field samples, the average coefficient of variation for replicate samples was 0.52 and the analytical coefficient of variation was 0.21. The larger variation in the field samples is possibly due to differences in successive duplicate samples taken from the same location or to differences in extraction efficiency between samples.

## Discussion

*Aureococcus anophagefferens* is a small organism that is difficult to differentiate from other small algae in environmental samples. A method for detecting cells at prebloom levels is necessary to predict potential bloom sites, to determine possible environmental factors that influence or initiate a bloom, and to evaluate potential sources of low-level cell inoculations through natural or anthropogenic transport mechanisms (see Popels and Hutchins 2002). qPCR is useful for detecting cells at extremely low concentrations in environmental samples. Here a primer and probe set were successfully developed to enumerate *A. anophagefferens* in natural samples. The method is quantitative over a wide range of low concentrations, as demonstrated by the development of the standard curve, although microscopy appears to be more accurate at very high bloom levels. qPCR allows for rapid sample analysis with high throughput processing. Samples can also be archived for at least a year and still provide the same Ct values (data not shown).

There is some introduced error in the method, most likely due to differences in DNA extraction efficiency between replicates and samples, the presence of qPCR inhibitors, chlorophyll normalizations, and pipetting error. To minimize problems caused by pipetting error, it is necessary to run several replicates of the same sample. Chlorophyll measurements are used to correct for biomass differences at stations to reduce variability in extraction efficiency between samples. Chlorophyll normalizations could also introduce error into counts, due to light- or nutrient-driven variability in chlorophyll:biomass ratios. There is sure to be some variability in successive samples taken at the same location because of the patchiness of the water column, as reflected by large differences in the replicate samples from the same location. However, the average analytical coefficient of variation for samples is well within the range reported by Suzuki et al. (2000).

There is no correlation between the polyclonal counts and the qPCR counts from the survey. This is most likely because the two methods are valid within different ranges, with qPCR being more accurate at low cell concentrations and the polyclonal technique more accurate at high cell concentrations. As described previously, the nature of cell counts may cause a lot of error at low cell concentrations. For this reason, it may be possible to get a negative result for the presence of *Aureococcus* using the polyclonal technique, yet a positive result with qPCR. The non-specific binding of the polyclonal antibody paired with the inherent error in cell counts at low concentrations could be the reason that some stations tested positive with the polyclonal technique, yet they were negative when tested with qPCR. All of the negative samples were spiked with a positive control to ensure that there were no PCR inhibitors present in the sample.

This novel method has allowed us to demonstrate a dramatic extension of the known range of the brown tide organism on the East Coast of the U.S. *Aureococcus* was not previously known to exist south of Maryland. The newly defined range of *A. anophagefferens* extends from Florida northward to New Hampshire (Anderson et al. 1989; this study). Note that since the brown tide first occurred in New York and Rhode Island in 1985, blooms have subsequently appeared in estuaries further south in New Jersey and Maryland (Gastrich et al. 2002; Glibert et al. 2001). While blooms have not yet been reported south of Maryland, the organism is widespread at background concentrations, and there is potential for future blooms to continue their propagation south along the East Coast of the United States.

Our Delaware transect work also found significant background levels of cells in Delaware Bay ( $1$  to  $>200$  cells mL<sup>-1</sup>). This bay is a large, deep, and well-mixed tidal estuary where blooms have not and are not likely to occur, due to unsuitable physical conditions for bloom development. Note that the organism was present in waters with salinities too low to support growth (growth does not occur at salinities  $< 22$ , [Casper et al. 1989]), such as at stations 64 (salinity  $\sim 9$ ) and 65 (salinity  $\sim 16$ ). We suggest that these positive samples may represent coastal populations advected into the tidal portion of the Delaware estuary. No *Aureococcus* cells were found, however, at the non-tidal freshwater station (63).

*Aureococcus* was also detected in coastal and offshore waters along this Delaware transect. This lends support to the hypothesis that the organism is an offshore species that is periodically moved into coastal areas by currents, where it opportunistically forms blooms (Yentsch et al. 1989). This of course does not preclude additional human-assisted introductions via recreational boats and ballast water, as suggested by Popels and Hutchins (2002). The availability of this species-specific, low-level qPCR detection and enumeration method should prove invaluable in assessing the potential for both natural and anthropogenic introductions into previously unaffected areas.

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