

A Novel Immunofluorescence Flow Cytometry Technique Detects the Expansion of Brown Tides Caused by *Aureoumbra lagunensis* to the Caribbean Sea

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During the past 3 decades, brown tides caused by the pelagophytes *Aureococcus anophagefferens* and *Aureoumbra lagunensis* have caused ecological and economic damage to coastal ecosystems across the globe. While blooms of *A. lagunensis* had previously been confined to Texas, in 2012, an expansive brown tide occurred on Florida's East Coast, causing widespread disruption within the Indian River and Mosquito Lagoons and generating renewed interest in this organism. A major impediment to detailed investigations of *A. lagunensis* in an ecosystem setting has been the absence of a rapid and reliable method for cell quantification. The combination of their small size (3 to 5 μm) and nondescript extracellular features makes identification and enumeration of these cells with conventional methods a challenge. Here we report the development of an immunological-based flow cytometry method that uses a fluorescently labeled antibody developed against *A. lagunensis*. This method is species specific, sensitive (detection limit of 1.5×10^3 cells ml^{-1}), precise (1% relative standard deviation of replicated samples), and accurate ($108\% \pm 8\%$ recovery of spiked samples) over a wide range of cell concentrations. Furthermore, this method effectively quantifies *A. lagunensis* in both glutaraldehyde- and formalin-preserved samples, yields a high throughput of samples (~ 35 samples h^{-1}), and is cost-effective, making it an ideal tool for managers and scientists. This method successfully documented the recurrence of a brown tide bloom in Florida in 2013. Bloom densities were highest in June ($>2.0 \times 10^6$ cells ml^{-1}) and spanned >60 km from the Ponce de Leon inlet in the northern Mosquito Lagoon south to Titusville in the Indian River Lagoon. Low levels of *A. lagunensis* cells were found >250 km south of this region. This method also quickly and accurately identified *A. lagunensis* as the causative agent of a 2013 brown tide bloom in Guantanamo Bay, Cuba, and thus should prove useful for both quantifying the dynamics of ongoing blooms of *A. lagunensis* as well as documenting new outbreaks of this harmful alga.

The proliferation of certain harmful algal blooms (HABs) has become a common occurrence in many coastal ecosystems around the world, leading to significant damage to human health, ecosystems, and economies. During the past several decades, the geographic distribution of several HABs has expanded, a trend driven by climate change, ballast water transport, eutrophication, and improved detection capabilities (1–6).

One group of HABs that has displayed a recent expansion in distribution is brown tides caused by the pelagophytes *Aureococcus anophagefferens* and *Aureoumbra lagunensis* (7). Brown tides caused by *A. anophagefferens* were first documented in the northeastern United States in 1985 (8) but were subsequently detected in the mid-Atlantic United States and South Africa during the 1990s (9, 10) and more recently in the Bohai Sea in northeastern China (11). In contrast, blooms of *A. lagunensis* had been confined to Laguna Madre and Baffin Bay in Texas for >20 years (12–14). However, in 2012, the Indian River Lagoon (IRL) on Florida's East Coast experienced an intense brown tide bloom, resulting in a 20-fold increase of phytoplankton biomass over historical means (15). Genetically identified as *A. lagunensis*, this bloom persisted for several months and represented a noteworthy expansion of this alga to the East Coast of the United States (15). Negative ecosystem impacts associated with the brown tides in Florida included widespread mortality of finfish and cultured and native shellfish populations (15) as well as a $>60\%$ loss of sea grass coverage in the affected area (L. Morris, St. Johns River Water Management District [SJRWMD], personal communication). The economic costs associated with the loss of seagrass-based com-

mercial and recreational fisheries have been estimated to be \$237 million to \$470 million (Ed Garland, IRL National Estuary Program, personal communication). Analyses of environmental data suggested that anomalously high salinities, low nitrate concentrations, and elevated levels of dissolved organic nitrogen (DON) may have contributed to the occurrence of the *A. lagunensis* bloom in 2012 (15). Although the initial brown tide in Florida ended in October 2012 (15), brown tides in Texas have been known to persist for up to seven consecutive years (16).

Due to their small size and morphologically nondescript surface, positive identification of small pelagophytes such as *A. anophagefferens* and *A. lagunensis* within environmental samples under a light microscope, even at high magnifications, is virtually impossible. When the first brown tides occurred in New York during the late 20th century, Anderson et al. (17) pioneered the use of a polyclonal, immunofluorescent antibody to quantify *A. anophagefferens*. Later, Caron et al. (18) developed an enzyme-

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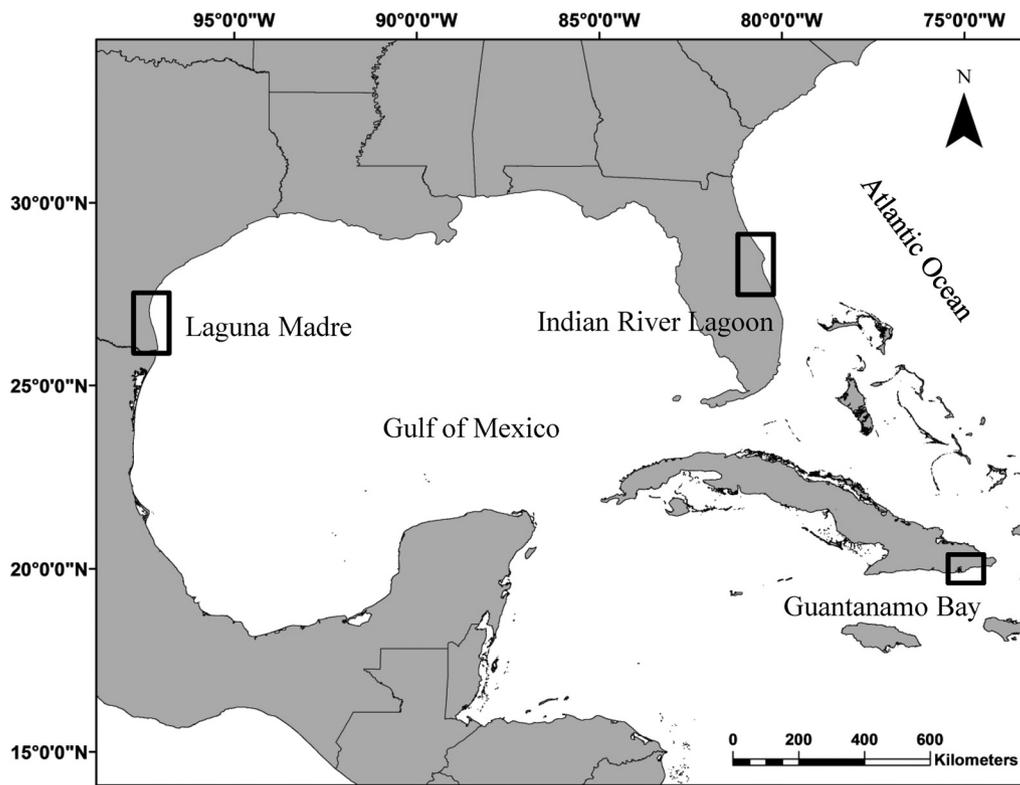


FIG 1 Locations of the only recorded blooms of *A. lagunensis* (highlighted areas) in Laguna Madre, TX; Guantanamo Bay, Cuba; and the Indian River Lagoon system in Florida.

linked immunosorbent assay (ELISA)-based immunofluorescent approach by using a monoclonal antibody to quantify *A. anophagefferens*, and Stauffer et al. (19) adapted the monoclonal antibody (18) for use on a flow cytometer (FCM), permitting even more accurate and rapid enumeration of *A. anophagefferens*. Lopez-Barrero et al. (20) developed a polyclonal antibody for *A. lagunensis* and used it in a microscopic format to detect and enumerate this alga, a method later used by Villareal et al. (14) to reveal the distribution of *A. lagunensis* throughout the coastal waters of the Gulf of Mexico, from southern Texas through southern Florida. This original method for quantifying *A. lagunensis*, however, is laborious, potentially imprecise, and impractical for timely enumeration using large quantities of sample required for monitoring and/or research purposes.

The objective of this study was to develop a method that quickly identifies and accurately enumerates *A. lagunensis* within environmental samples when present at both bloom and background concentrations ($>2 \times 10^6$ and $<1 \times 10^3$ cells ml^{-1} , respectively). This was achieved by using an existing polyclonal antibody, conjugating it to a fluorescein dye, and analyzing the labeled samples on a flow cytometer. In addition, we document the distribution of *A. lagunensis* along Florida's East Coast and present the seasonal dynamics of the 2013 Florida brown tide bloom. Finally, we highlight the utility of this method by positively identifying *A. lagunensis* as the source of a 2013 brown tide outbreak in Cuba.

MATERIALS AND METHODS

Environmental samples for this study were obtained from Laguna Madre, TX (10 February 1999 and 3 March 2013) (Fig. 1); Guantanamo Bay, Cuba

(18 May 2013) (Fig. 1); and the Indian River Lagoon, FL (Fig. 1), the only sites with reported blooms of *A. lagunensis* to date (7).

Development of a fluorescence-labeled antibody against *A. lagunensis*. A polyclonal antibody developed against *A. lagunensis* from rabbit blood serum by Lopez-Barrero et al. (20) was used to develop the flow cytometric method. This original rabbit serum was purified by Harlan Laboratories, Inc., using protein G chromatography and was diluted to a final concentration of 2.66 mg antibody ml^{-1} , as determined by using a spectrophotometer with a 1-cm light path and an extinction coefficient of 1.0 at an absorbance of 280 nm. The purity of the antibody was $>83\%$, as determined via polyacrylamide gel electrophoresis (PAGE). The antibody was then conjugated with fluorescein isothiocyanate (FITC) at an FITC/antibody molar ratio of 4.79, filtered to 0.2 μm , and frozen at -80°C until use. Cultures of *A. lagunensis* and other phytoplankton used to test this new antibody were either obtained from the National Center for Marine Algae and Microbiota (NCMA) or isolated from Guantanamo Bay and the Indian River Lagoon via serial dilution with GSe medium (21).

To determine the ideal concentration of antibody to quantify *A. lagunensis* in environmental samples, 800 μl of phosphate-buffered saline (PBS)-Tween 20 (salinity of ~ 11 ; Sigma-Aldrich) was added to 200 μl of 1% (final concentration) glutaraldehyde-preserved cultures of *A. lagunensis* from Texas (CCMP1510), *A. lagunensis* from Florida (15), *A. anophagefferens* (CCMP1984), and the pelagophytes *Pelagococcus subviridis* (CCMP1429) and *Pelagomonas calceolata* (CCMP1756). In addition, glutaraldehyde-preserved field samples were collected from a brown tide of *A. anophagefferens* in Shinnecock Bay (SB), NY, on 18 June 2013 as well as from the Indian River Lagoon, Laguna Madre (12 June 2013), and Guantanamo Bay, Cuba (20 May 2013). For all samples, 2.7, 5.4, 13.3, 19.9, and 26.6 $\mu\text{g ml}^{-1}$ of the conjugated antibody were added. The optimal incubation time was determined by placing a subset of samples that

received $13.3 \mu\text{g ml}^{-1}$ of antibody in the dark for 10, 15, 20, 30, and 60 min.

Optimization of flow cytometric analysis. Samples were analyzed with a FACSCalibur FCM (Becton, Dickinson, San Jose, CA) equipped with a 15-mW, 488-nm, air-cooled blue argon-ion laser and a 635-nm red diode laser. Cytometric measurements included forward-angle light scatter, side scatter (SSC), and four channels for the detection of emitted light: FL1 (green light, 500 to 560 nm), FL2 (orange light, 543 to 627 nm), FL3 (dark red light, >670 nm), and FL4 (red light, 645 to 677 nm). The manufacturer's FACSFlow sheath fluid (product no. 342003; Becton, Dickinson) was used since it mirrored the salinity of PBS-Tween-diluted samples. A speed setting of $35 \mu\text{l min}^{-1}$ was used, calibrated by the addition of 2- μm Sphero fluorescence-calibrated glass beads (RFP-20-5; Spherotech, Lake Forrest, IL) at a known concentration. The instrument was optimized for antibody-labeled *A. lagunensis* populations by gating a SSC-versus-green-light (FL1) window, thereby eliminating signal from nonlabeled particles. All data files were acquired by using manufacturer-supplied Cell Quest software (Becton, Dickinson) and analyzed by using Cyflogic software (CyFlo Ltd., Finland).

While glutaraldehyde is the ideal preservative for enumeration of another brown tide-forming pelagophyte, *A. anophagefferens* (19), archived samples from monitoring programs are often preserved in buffered formalin or Lugol's iodine solution. To establish the efficacy of the new method for quantifying *A. lagunensis* in different preservatives, samples of a monoclonal culture of *A. lagunensis* (CCMP1530) and from water from Laguna Madre, TX (collected on 3 March 2013), were preserved in 1% (vol/vol, final concentration) glutaraldehyde, 1% phosphate-buffered formalin, and 1% Lugol's iodine solution (22) and stored in either glass or plastic scintillation vials at both 4°C and 25°C for a period of 3 months. All samples were subsequently analyzed by using the method described here.

Comparison of methods. The ability to quantitatively enumerate *A. lagunensis* in seawater was evaluated by adding 4.7×10^6 , 2.9×10^6 , 1.8×10^6 , and 1.1×10^6 cells ml^{-1} of an *A. lagunensis* culture (Florida isolate) (15) to water from Shinnecock Bay, NY, during an *A. anophagefferens* brown tide. Parallel samples were added to filtered seawater. All samples were preserved in 1% glutaraldehyde and analyzed on a flow cytometer as described above and on a Beckman Coulter Multisizer 3 Coulter counter with a 50- μm aperture (23, 24). The methodological detection limit was established by incubating the antibody with 0.2- μm -filtered seawater samples ($n = 20$) from *A. lagunensis* blooms as well as from whole seawater samples ($n = 20$) with no *A. lagunensis* (i.e., during an *A. anophagefferens* bloom in New York). In each instance, the region where antibody-labeled *A. lagunensis* typically appeared within the flow cytometric images was gated within the imaging software, particles inside the gated region were quantified, and the detection limit of the method was defined as $3 \times$ the standard deviation of these counts (25). The efficacy of this method compared to those of other quantification methods was established by preserving replicates of an *A. lagunensis* culture (Florida isolate) with 1% (final concentration) glutaraldehyde, as described above, and analyzing them in parallel on a Beckman Coulter Multisizer 3 Coulter counter with a 50- μm aperture (23, 24), under a light microscope by using a hemocytometer and by using the immunoassay on a flow cytometer as described above. For the light microscope counts, a minimum of 200 organisms or 100 grids were counted per sample (26). In addition, *A. lagunensis* densities in 64 glutaraldehyde-preserved field samples from the Indian River Lagoon system (Fig. 1) during June to September 2013 were analyzed in tandem with a light microscope by using a hemocytometer and by using the new immunoassay on a flow cytometer.

Application of the new method. As part of the NOAA Harmful Algal Bloom Event Response program, water samples were collected from several stations (Fig. 1 and Table 1) from June to November 2013 with the help of the SJRWMD to establish temporal and spatial bloom dynamics of *A. lagunensis* in the IRL. Water was collected in acid-cleaned (10% HCl), 1-liter amber polyethylene bottles that were brought to the Marine Discovery Center in New Smyrna Beach, FL, and preserved in 1% glutaralde-

hyde. *A. lagunensis* abundances were enumerated by using the immunoassay method described above. In addition, on 25 September 2013, 500 km of Florida's East Coast from Ormond Beach to Key Largo was surveyed for the presence of *A. lagunensis* by collecting samples as described above.

Identification of the "Cuban brown tide." In early 2013, brown water was observed in Guantanamo Bay, an event that led to repeated short-term shutdowns of the desalination plant supplying all freshwater to the U.S. Naval Base in Guantanamo Bay. Brown tide bloom water collected by Arthur N. Torley, III, U.S. Navy, on 20 May 2013 from Guantanamo Bay, Cuba (Fig. 1), was shipped to the United States, preserved with 1% glutaraldehyde, and treated with antibodies specific to *A. anophagefferens* (19, 24, 27) and *A. lagunensis* (this study) in order to quickly identify the causative organism. As further verification, bloom water was pelleted and frozen, and a culture was established and preserved for molecular analysis as described previously by Gobler et al. (15). Culture isolates were obtained by serially diluting bloom water into polystyrene cell culture plates containing GSe culture medium (21), and an antibiotic-antimycotic solution (Mediatech, Inc., Herndon, VA) was added at a 1% concentration (final concentrations of 100 IU ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin, and $0.25 \mu\text{g ml}^{-1}$ amphotericin B) to ensure that cultures remained free of bacteria and fungi. Cells were cultured in sterile GSe medium supplemented with the same antibiotic-antimycotic solution at 22°C in an incubator with a 14-h–10-h light-dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of $\sim 100 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ to cultures.

For genetic analyses of bloom water and clonal isolates, 50 ml of cultured cells or bloom water was pelleted by centrifugation at 5,000 rpm for 2 min. The majority of the medium was removed by aspiration, leaving behind $\sim 150 \mu\text{l}$ with the cell pellet. The cell pellet was frozen at -20°C overnight and then at -80°C for 48 h, transferred into a 1.5-ml Eppendorf tube, and spun at 10,000 rpm for 3 min. The cell pellet was then resuspended and serially diluted (1:10) in LoTE buffer (3 mM Tris-HCl [pH 8.0]–0.2 mM EDTA [pH 8.0]). Culture aliquots (1 μl) of the diluted series were used as the PCR template (see below). For field samples, 50 ml of bloom water was collected on a 1- μm polycarbonate filter, placed into a 1.5-ml Eppendorf tube, and frozen at -80°C . LoTE buffer (500 μl) was added to the tube, which was then vortexed and mixed via vigorous pipetting. Resuspended cells (50 μl) were removed and serially diluted (1:10) in LoTE buffer. Aliquots (1 μl) of diluted field sample series were used as the PCR template. PCR mixtures contained 1 μl of cellular resuspension, 12.5 μl $2 \times$ GoTaq Green (Promega), 1 μM 18S rRNA forward primer (Euk A [5'-AACCTGGTTGATCCTGCCAGT-3']), 1 μM 18S rRNA reverse primer (329R [5'-TGATCCTTCYGCAGGTTAC-3']), and distilled, deionized water in a total volume of 50 μl . Reactions were performed with the following cycling parameters: 94°C for 5 min followed by 31 cycles of 94°C for 45 s, 55°C for 60 s, and 72°C for 3 min, followed by a final extension step at 72°C for 5 min. Sequencing was performed directly on the unmodified PCR product by using 50 ng of PCR product and 3.2 pM primer on an ABI3730 genetic analyzer using a BigDye Terminator cycle sequencing kit (Applied Biosystems) at the Stony Brook University DNA Sequencing Facility. Sequences were analyzed with Geneious version 5.6.6, created by Biomatters.

Data analysis. One-way analysis of variance (ANOVA) or Student's *t* test was used to establish differences between preservatives and enumeration methods. A *P* value of 0.05 was used to establish significant differences for all tests.

Nucleotide sequence accession numbers. The nucleotide sequences of the *Aureoumbra lagunensis* 18S rRNA genes have been deposited in GenBank under accession numbers KJ193856 and KJ193857.

RESULTS

The newly developed immunoassay technique was accurate, precise, and efficient for enumerating *A. lagunensis* over a wide range of cell densities.

Optimization of the immunoassay. The addition of the FITC-

TABLE 1 Distribution of *A. lagunensis* along Florida's East Coast^a

Station	Latitude (degrees)	Longitude (degrees)	Salinity (PSU)	Mean <i>A. lagunensis</i> abundance (cells ml ⁻¹) ± SD
Ormond Beach	29.287033	-81.053786	15.5	1,750 ± 276
Daytona Beach	29.216244	-81.019236	16.6	2,600 ± 453
New Smyrna Beach	29.027339	-80.921097	36.3	5,180 ± 1,050
IRLV05	29.007472	-80.91014	35.2	<DL
CM65	28.940028	-80.86800	35.4	2,230 ± 147
IRLV11	28.95295	-80.84417	37.0	39,800 ± 8,040
Governer's Cut	29.287033	-81.053786	35.6	2,530 ± 257
National Park Service	28.926744	-80.825042	37.0	17,400 ± 1,850
IRLV17	28.877583	-80.83883	35.8	14,900 ± 945
IRLML169	28.837889	-80.79522	39.1	90,900 ± 7160
CM29	28.797063	-80.77354	39.4	128,000 ± 12,000
CM43	28.74664	-80.74761	39.4	128,000 ± 15,500
IRLML02	28.73181	-80.71733	40.6	57,200 ± 9,730
Southbiolab	28.69800	-80.69944	43.8	31,200 ± 5,200
Scotts Moor	28.77028	-80.83088	32.8	143,000 ± 13,200
CM1	28.72347	-80.76719	39.2	117,000 ± 31,100
IRLI02	28.73553	-80.79794	35.6	132,500 ± 4,800
27010785	28.68717	-80.81267	35.1	140,000 ± 9,480
IRLI06	28.63608	-80.80222	34.4	83,100 ± 10,300
IRLI07	28.603472	-80.79839	33.6	91,700 ± 14.0
IRLSR50	28.563	-80.7785	33.2	68,600 ± 12,100
IRLI10	28.41095	-80.742933	32.7	4,730 ± 602
Banana River	28.404572	-80.644414	17.6	<DL
Rockledge	28.301117	-80.700556	28.4	<DL
Aurora St.	28.136089	-80.63015	24.5	<DL
Malabar	27.987272	-80.554111	28.0	<DL
Sebastian	27.855286	-80.491247	32.4	<DL
Wabasso Bridge	27.754531	-80.426478	27.9	<DL
Vero Beach	27.650239	-80.374744	27.7	<DL
Fort Pierce	27.457383	-80.323594	31.8	<DL
S. Indian River Dr.	27.345881	-80.277814	25.7	1,900 ± 233
St. Lucie River	27.204717	-80.259981	23.3	<DL
N. Swells Point	27.209022	-80.2029	24.0	2,280 ± 259
Jupiter	26.977228	-80.086075	36.1	<DL
E. Blue Heron Blvd.	26.783947	-80.042419	36.0	<DL
Lake Worth	26.615722	-80.044533	19.0	<DL
Boca Raton	26.339681	-80.078058	16.9	<DL
E. Oakland Blvd., Ft. Lauderdale	26.167669	-80.104517	11.2	<DL
Broad Causeway	25.888303	-80.146431	32.3	<DL
Matheson Hammock County Park	25.678617	-80.259739	31.1	<DL
Card Sound Rd., Key Largo	25.287217	-80.368292	31.4	<DL
Overseas Hwy., Key Largo	25.1958	-80.4139	30.7	<DL

^a For each station coordinate, salinity and abundance of *A. lagunensis* are given. *A. lagunensis* densities were determined by using the new immunoassay flow cytometry method described in this paper. Abundances are depicted as means ± standard deviations ($n = 3$), while <DL denotes values below the detection limit of 1,500 cells ml⁻¹.

conjugated, *A. lagunensis*-specific antibody successfully labeled cultures and field populations of *A. lagunensis* at all concentrations tested (Table 2). With the exception of *P. calceolata* and *P. subviridis*, which cross labeled at the highest concentration tested, populations of the other pelagophytes, clearly visible via autofluorescence of chlorophyll *a*, were not labeled with the antibody (Fig. 2). Based on this and the separation of the labeled populations from the nonlabeled populations, the addition of 13.3 μg ml⁻¹ (5 μl ml⁻¹) of antibody was found to provide optimal labeling of populations (data not shown). Incubation times of between 10 and 60 min all provided strong, similar fluorescence intensities of labeled populations, and subsequently, a 15-min incubation time was chosen.

There was no significant difference between cell densities

quantified in 1% formalin- and glutaraldehyde-preserved cultures (means ± standard deviations, 371 × 10⁴ ± 35.1 × 10⁴ and 381 × 10⁴ ± 4.8 × 10⁴ cells ml⁻¹, respectively) or environmental samples (75.2 × 10⁴ ± 6.3 × 10⁴ and 83.1 × 10⁴ ± 1.8 × 10⁴ cells ml⁻¹, respectively) ($P > 0.05$) (Fig. 3; see also Fig. S1 in the supplemental material). While the standard deviations of formalin-preserved samples were generally higher, there were also no significant differences between samples stored in glass and those stored in plastic or between preservation at 4°C and preservation at 25°C (see Fig. S1 in the supplemental material). In addition, 14-year-old, formalin-preserved samples from Laguna Madre, TX, were labeled and yielded FITC intensities comparable to those of the samples collected from the same region in 2013. Preservation of samples with Lugol's iodine solution produced an abnor-

TABLE 2 Responses of different pelagophyte cultures and field populations to various concentration of *A. lagunensis* antibody^a

Species (isolate or location)	Response at antibody concn of:				
	2.7 $\mu\text{g ml}^{-1}$	5.4 $\mu\text{g ml}^{-1}$	13.3 $\mu\text{g ml}^{-1}$	19.9 $\mu\text{g ml}^{-1}$	26.6 $\mu\text{g ml}^{-1}$
<i>Aureococcus anophagefferens</i> (CCMP1850)	–	–	–	–	–
<i>Aureococcus anophagefferens</i> (CCMP1984)	–	–	–	–	–
<i>Pelagomonas calceolata</i> (CCMP1756)	–	–	–	–	+
<i>Pelogococcus subviridis</i> (CCMP1429)	–	–	–	–	+
<i>Aureoumbra lagunensis</i> (CCMP1510)	+	++	++	++	++
<i>Aureoumbra lagunensis</i> (FL) culture	+	++	++	++	++
<i>Aureoumbra lagunensis</i> (FL) field sample	+	+	++	++	++
<i>Aureoumbra lagunensis</i> (TX) field sample	+	+	++	++	++
<i>Aureoumbra lagunensis</i> (TX, 1999) field sample	+	+	++	++	++
Cuban brown tide field sample	+	++	++	++	++

^a “–” signifies that the antibody did not label the population, while “+” and “++” denote labeled and strongly labeled populations, respectively. The Florida and Cuban cultures were established by the Gobler laboratory, while field samples from Florida, Texas, and Texas in 1999 were collected on 6 June 2013, 3 March 2013, and 10 February 1999, respectively.

mal fluorescent signature and indistinguishable populations by using the antibody method (Fig. 3).

The percent recovery of cultures added to seawater at environmentally relevant densities (10^6 cells ml^{-1}) was $108\% \pm 8\%$, and the detection limit was 1.5×10^3 cells ml^{-1} , based on $3 \times$ the standard deviation of blank samples. A comparison of the various methods for quantifying *A. lagunensis* in cultures demonstrated

that the novel flow cytometric method was significantly more precise than the other methods tested. The mean values of cultures analyzed via light microscopy, the use of a Coulter counter, and flow cytometry ($4.89 \times 10^6 \pm 0.44 \times 10^6$, $4.68 \times 10^6 \pm 0.16 \times 10^6$, and $4.89 \times 10^6 \pm 0.03 \times 10^6$ cells ml^{-1} , respectively) were not significantly different, but there were large differences in the variances of the three methods (relative standard deviations of 9.1, 3.5,

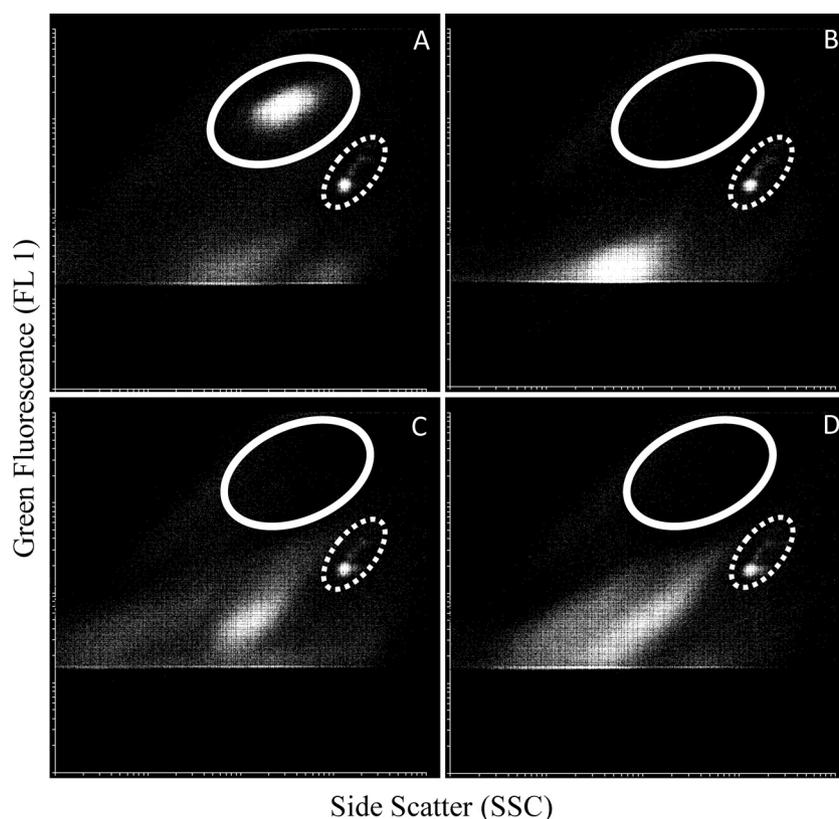


FIG 2 Data output from a FACSCalibur instrument showing side scatter (SSC) versus green fluorescence (FL1). Both scales are relative log-based scales and depict cultures of *Aureoumbra lagunensis* (A), *Aureococcus anophagefferens* (B), *Pelagococcus subviridis* (C), and a naturally occurring plankton community collected from Shinnecock Bay, NY (D) (see Materials and Methods), incubated with $13.3 \mu\text{g ml}^{-1}$ of the *A. lagunensis* antibody. Two-micrometer fluorescent glass beads were added to all samples as a size standard and are visible in each panel as the population surrounded by the dashed line. The antibody clearly distinguishes the *A. lagunensis* culture (population surrounded by a solid line in panel A) from the background, while the other pelagophytes and SB seawater did not label and appear low on FL1.

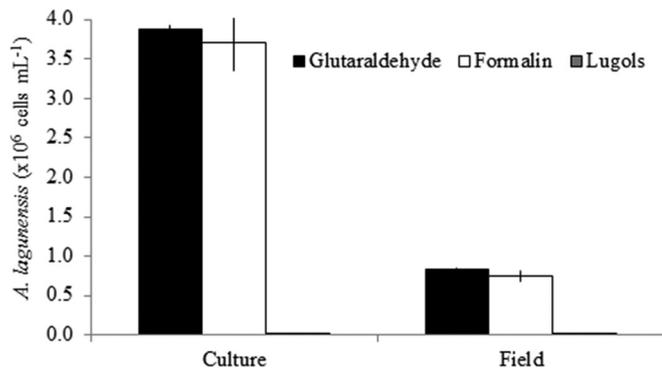


FIG 3 Effects of various preservatives on the efficacy of the new immunoassay method. Samples were preserved from a monoclonal culture of *A. lagunensis* (CCMP1530) and from water collected on 3 March 2013 from Laguna Madre, TX, and preserved with either 1% (vol/vol) glutaraldehyde, 1% formalin, or 1% Lugol's iodine solution. Values represent means \pm standard deviations ($n = 3$).

and 0.7%, respectively) (Fig. 4). A comparison of *A. lagunensis* abundances obtained via the immunoassay described in this study and counts by light microscopy from water collected from 64 stations in the IRL from June to September 2013 revealed a poor relationship between the two methods during times of high *A. lagunensis* abundance (Fig. 5A). In contrast, during times of relatively low abundances, there was a general, linear relationship between the two approaches ($r^2 = 0.42$), although light microscopy overestimated *A. lagunensis* by 53% compared to the flow cytometric counts (Fig. 5B).

Bloom dynamics of the 2013 IRL brown tide bloom. *A. lagunensis* returned to the East Coast of Florida in 2013, with brown water being reported by the end of May (L. Hall, SJRWMD, personal communication), originating near the Hallover Canal near station IRLI02 (Fig. 6). By June, the bloom had expanded to cover most of the northern part of the Indian River Lagoon and all of the Mosquito Lagoon (Fig. 6). Cell densities ranged from $6.01 \times 10^3 \pm 0.3 \times 10^3$ cells ml^{-1} (mean \pm standard deviation) ($n = 3$) near Port St. John (IRLSR50) to $2.1 \times 10^6 \pm 0.21 \times 10^6$ cells ml^{-1} at IRLV05, in the northern Mosquito Lagoon (Fig. 6). By 8 August, the bloom had declined, with concentrations an order of magni-

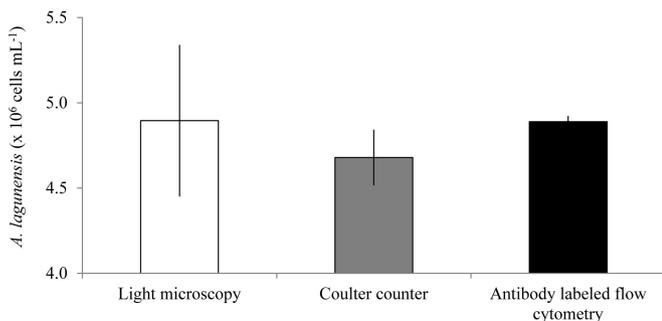


FIG 4 Comparison of various methods of enumerating *Aureoumbra lagunensis*. A culture of the newly established clonal isolate of *A. lagunensis* from Florida was enumerated with a light microscope, on a Coulter counter, and by using the antibody method described in this study. Values represent means \pm standard deviations ($n = 6$). The relative standard deviations for each method were 9.1, 3.5, and 0.7% for light microscopy, Coulter counter analysis, and antibody-labeled flow cytometry, respectively.

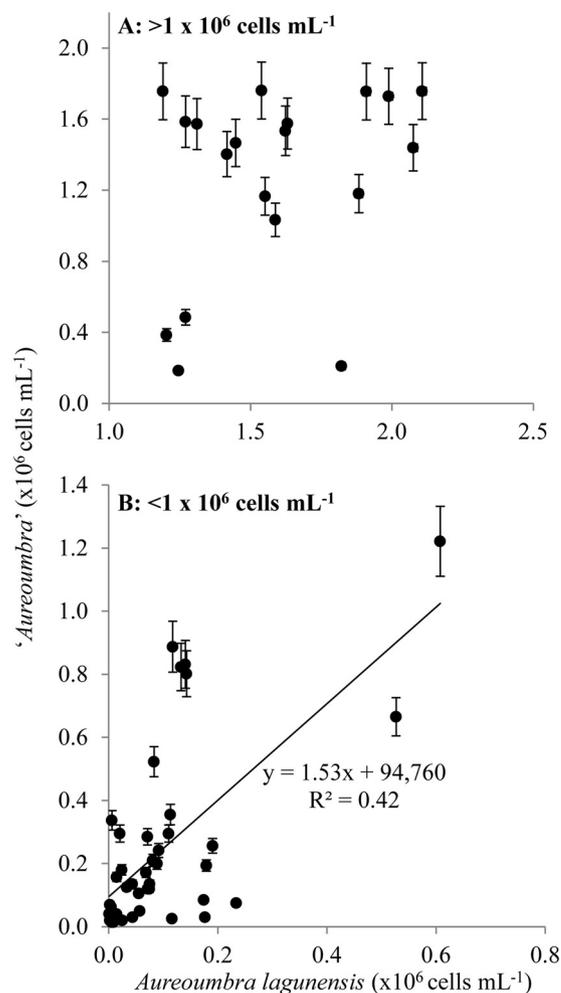


FIG 5 Comparison of methods of *Aureoumbra* enumeration using the new immunofluorescence flow cytometry method (x axis) and inverted light microscopy (y axis) for high *A. lagunensis* abundances ($>1 \times 10^6$ cells ml^{-1}) (A) and low *A. lagunensis* abundances ($<1 \times 10^6$ cells ml^{-1}) (B). Values represent the means \pm standard deviations.

tude lower in the northern Indian River Lagoon and southern Mosquito Lagoon than the densities present in June ($1.57 \times 10^5 \pm 0.15 \times 10^5$ and $1.36 \times 10^5 \pm 0.22 \times 10^5$ cells ml^{-1} for stations IRLI06 and CM43, respectively) (Fig. 6). While densities on 25 August were similar to those observed earlier in the month ($1.40 \times 10^5 \pm 0.09 \times 10^5$ and $1.24 \times 10^5 \pm 0.22 \times 10^5$ for 27010785 and CM43, respectively), concentrations in the northern Mosquito Lagoon declined to below the detection limit of our method (1.5×10^3 cells ml^{-1}). From September to November, *A. lagunensis* abundances in the northern Indian River Lagoon and southern Mosquito Lagoon increased from $1.13 \times 10^5 \pm 0.98 \times 10^5$ and $0.02 \times 10^5 \pm 0.01 \times 10^5$ cells ml^{-1} on 25 September to $1.87 \times 10^5 \pm 0.28 \times 10^5$ and $0.27 \times 10^5 \pm 0.01 \times 10^5$ cells ml^{-1} on 13 October and to $2.35 \times 10^5 \pm 0.21 \times 10^5$ and $0.55 \times 10^5 \pm 0.01 \times 10^5$ cells ml^{-1} on 13 November at IRLI06 and IRLML02, respectively (Fig. 6). The 500-km survey of Florida's East Coast lagoons and bays detected *A. lagunensis* cells from Ormond Beach south to station IRLI10, near Port St. John, and detected low concentrations of cells near the St. Lucie inlet ($\sim 2 \times 10^3$ cells ml^{-1}), 250 km south of Ormond Beach (Table 1).

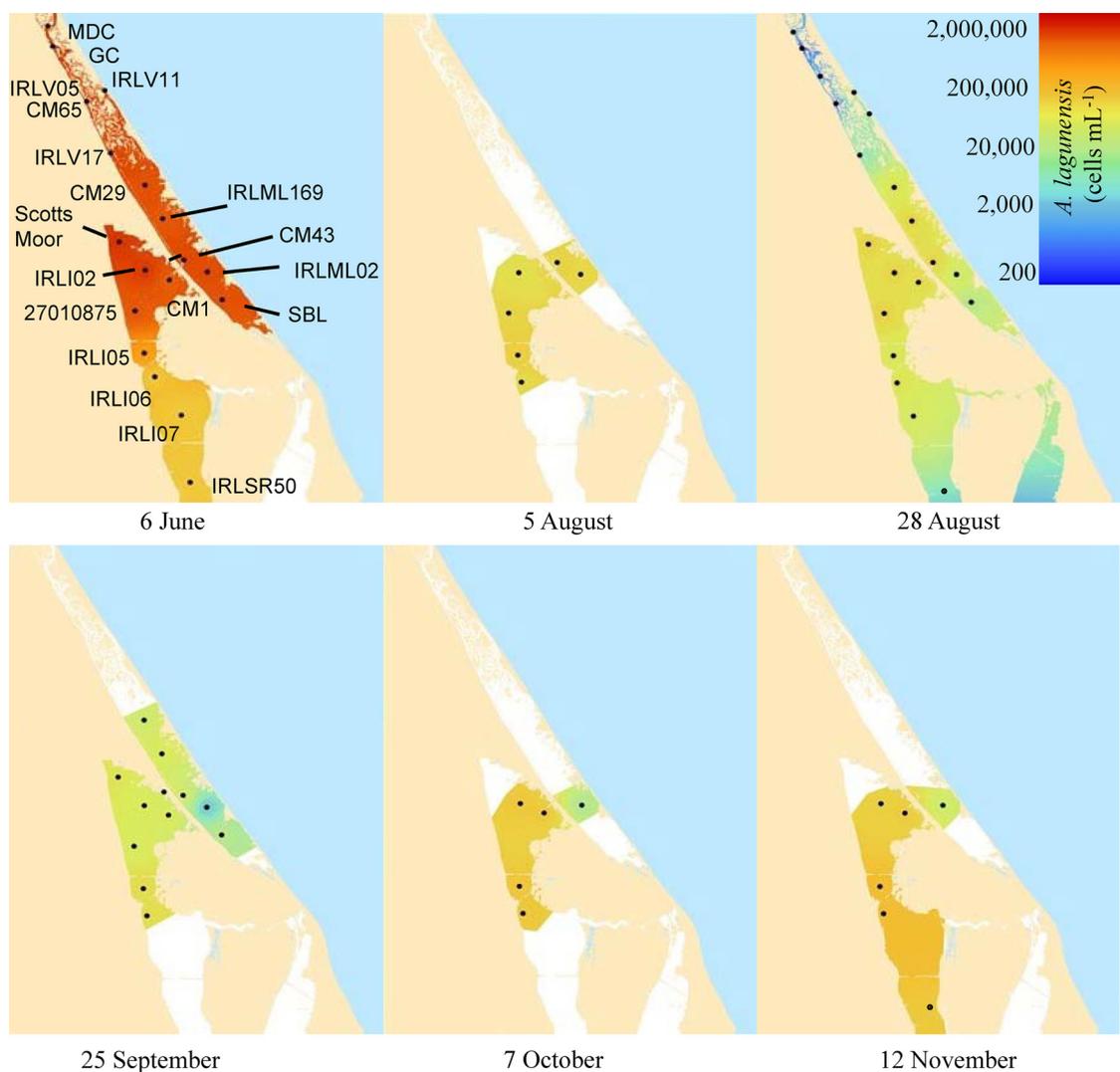


FIG 6 Spatial and temporal dynamics of the 2013 Florida brown tide bloom in the Indian River and Mosquito Lagoons. Samples were collected by the SJRWMD as part of routine monthly water quality monitoring. Values are plotted on a log scale. (Created by using ArcGIS 10 [ESRI, Redlands, CA].)

Identification of *A. lagunensis* as the causative organism of brown tides in Cuba. The *A. lagunensis* immunoassay described in this study successfully labeled the population of picoplankton responsible for causing the 2013 brown tide bloom in Guantanamo Bay, Cuba (Fig. 7A), while concurrent assays with the *A. anophagefferens* antibody did not label the samples (Fig. 7B). Cell densities of *A. lagunensis* in samples from Guantanamo Bay obtained during May 2013 were $1.1 \times 10^6 \pm 0.05 \times 10^6$ cells mL^{-1} . In addition, our sequencing of the 18S rRNA genes from samples from Guantanamo Bay (KJ193856 and KJ193857) showed 100% identity to GenBank sequences of *Aureoanra lagunensis* isolates from Texas (GenBank accession numbers HQ710573 and HQ710574) (Fig. 7C).

DISCUSSION

Unlike *A. anophagefferens*, which, since its initial appearance (28), has bloomed throughout the mid-Atlantic United States (29–31) as well as South Africa and China (9, 11), *A. lagunensis* blooms have been confined to coastal bays in and around Laguna Madre,

TX. Partly as a result of this difference in distribution, *A. anophagefferens* has been studied far more extensively than *A. lagunensis* (7, 24, 27, 32–36). However, the recent expansion of *A. lagunensis* from Texas to Florida's East Coast and now Cuba has begun to generate renewed interest in this brown tide species (15, 37). While culture studies of this species have been common (12, 15, 38–40), field and monitoring studies of *A. lagunensis* have been partly hampered by an inability to accurately and rapidly enumerate this species. This constraint is common in the study of pico- and nano-eukaryotic phytoplankton species in general, since their small size makes them difficult to identify and quantify by using conventional light microscopy. To address this issue, studies have increasingly turned to species-specific immunological and genetic approaches.

While several methods for the detection and enumeration of *A. anophagefferens* have been developed (17–19, 41), only one detection method had been established for *A. lagunensis* prior to this study (20). While this method had a low detection limit ($\sim 10^2$ cells mL^{-1}), it required filtration and multiple incubations of sam-

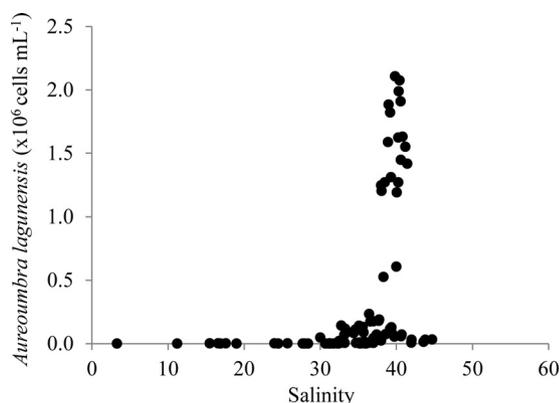


FIG 8 Relationship between salinity (PSU) and *A. lagunensis* densities of samples collected from the Indian River Lagoon system ($n = 95$). *A. lagunensis* densities are reported as millions of cells mL^{-1} .

small coccoid cells via light microscopy. Consistent with our culture work, variabilities in the microscope counts were consistently an order of magnitude higher during blooms of *A. lagunensis* than by flow cytometric quantification, likely leading to the lack of correlation between the two methods.

Consistent with the findings of Lopez-Barreiro et al. (20), samples preserved with Lugol's iodine solution had no reactivity to the antibody for *A. lagunensis*, likely due to degradation of the cell surface antigen in this solution. This is in contrast to the results reported previously by Anderson et al. (43), who used an antibody raised against *A. anophagefferens* to enumerate Lugol's iodine-fixed samples. While the preservation of *A. lagunensis* samples in glutaraldehyde yielded higher background fluorescence when used with the original method (20), this was not observed for the purified and FITC-conjugated antibody developed in this study. Moreover, preservation in glutaraldehyde resulted in less variance among our replicates than with formalin. Notably, Stauffer et al. (19) recommended the use of 1% glutaraldehyde for preservation followed by subsequent storage in the dark in glass vials. Regardless, our preservation comparison indicated that formalin-preserved samples can be used to assess the historical distribution of *A. lagunensis* from archived samples, as we quantified cell densities on par with the counts originally recorded for 15-year-old samples from Laguna Madre, TX.

The *A. lagunensis* quantification method described here was highly useful for documenting the temporal and spatial dynamics of the 2013 Florida brown tide. Compared to 2012, the bloom peaked early (June versus August) and was less intense (2×10^6 versus 4×10^6 cells mL^{-1}) but displayed a similar geographic distribution (15). While the exact causes of these blooms are still unknown, hypersalinity was proposed to be one of the factors facilitating the 8-year *A. lagunensis* bloom in Texas (13, 44) and to be a factor in the initiation of the 2012 Florida brown tide bloom (15). An analysis of the relationship between salinity and *A. lagunensis* abundance in Florida from all of the sample collections of 2012 and 2013, including a survey of lagoons along the majority of Florida's East Coast, indicates that the bloom was confined to hypersaline conditions, specifically at salinities above 35 PSU (Fig. 8). Buskey et al. (44) were able to demonstrate that microzooplankton abundance and copepod size in Laguna Madre and Baffin Bay, TX, were negatively correlated with salinity, suggesting

that a decrease in grazing pressure under hypersaline conditions contributes to the success of *A. lagunensis* (7, 45). In 2013, the annual peak rainfall in the Indian River Lagoon occurred in June (~ 30 cm [<http://trmm.ksc.nasa.gov/>]), which was likely responsible for the lower salinities observed in the study area in early August than in June (36.6 versus 39.1) and could have contributed to the order-of-magnitude decline in *A. lagunensis* cell densities via the above-discussed top-down controls. Elevated salinities (>39 practical salinity units [PSU]) returned to the IRL in the fall of 2013 and may have contributed to the increasing cell densities in November. Hypersalinity may also favor *A. lagunensis* via changes in the nitrogen supply. It has been suggested that *A. lagunensis* is not able to grow on nitrate (38) but is able to utilize regenerated and organic forms of nitrogen (39). Similarly to *A. anophagefferens*, this ability, coupled with the lower-than-average nitrate concentrations associated with hypersalinity in Florida lagoons (15) as well as a massive influx of organic nitrogen and ammonium from the decaying benthic aquatic vegetation (L. Hall, SJRWMD, personal communication), likely favors *A. lagunensis* over other phytoplankton that are less able to utilize more refractory sources of nitrogen (5, 46, 47).

The antibody method described in this study was able to positively identify the causative agent of a brown tide in Guantanamo Bay, Cuba, in 2013 as *A. lagunensis*. While the detailed dynamics of the brown tide in Cuba are not clear, the bloom was reported to have begun in late 2012 and persisted through most of 2013 (Arthur N. Torley, III, U.S. Navy, personal communication). In a manner consistent with the brown tide in Florida, Guantanamo Bay was hypersaline during the brown tide (salinity, >38 PSU). Guantanamo Bay is the largest embayment on the south shore of Cuba (~ 100 km^2), and the U.S. Naval Base from which the samples were obtained is located at the southern extent of this system, where the bay exchanges with the open Caribbean Sea. As such, it is likely that the more enclosed portions of Guantanamo Bay, north of this region, likely hosted even higher densities than the measured density of 1.1×10^6 cell mL^{-1} and even more hypersaline water, given that the salinity of Caribbean Sea in the vicinity of Guantanamo Bay during May 2013 was ~ 36 PSU (<http://ocean.jpl.nasa.gov/epo/>).

The outbreaks of brown tide in Cuba and Florida are troubling occurrences, since these blooms have the ability to fundamentally change the ecosystem through a series of positive feedbacks (45). Indeed, this study and a previous study by Gobler et al. (15) suggest a possible ecosystem phase shift from one dominated by microplankton and seagrass (48, 49) to one dominated by cyanobacteria, pico- and nanoplankton, and macroalgae in Florida (15; SJWMD, personal communication). The appearance of blooms in both Cuba and Florida in 2012 and 2013 points toward either a recent introduction in the region or a regional physiochemical disturbance that favored the development of blooms from an endemic background population of *A. lagunensis*. Studies have highlighted the importance of ballast water transport and other physical introductions in the spread of HABs (50–52), and this hypothesis seems reasonable in the case of Guantanamo Bay, which hosts a deep-water harbor and vessels originating from various locations across North America. In contrast, however, the Indian River Lagoon lacks deep-water ports, and as such, the hypothesis proposed previously by Baas Becking (53), that “everything is everywhere but the environment selects,” may be applicable in Florida. Villareal et al. (14) detected background levels of *A.*

lagunensis from Texas to southern Florida, and the water quality monitoring data from the IRL showed a clear deviation from 16-year mean conditions, especially in terms of salinity and nitrate concentrations (15), further supporting the concept of a fundamental ecosystem shift prior to and during the outbreak of brown tide in this system. With a changing climate leading to increases in droughts in the Gulf of Mexico and the Caribbean (54), hypersaline conditions in these systems will likely become more widespread, increasing the potential for brown tides of *A. lagunensis* to occur. Finally, for both locations, a recent natural introduction cannot be ruled out. If this possibility is considered for Cuba, the prevailing surface currents travel from the Lesser Antilles and the northern coast of South America toward the Yucatan Peninsula. While HAB monitoring in these regions is minimal, the rapid turnaround time of samples and the antibody's specificity for *A. lagunensis*, along with its relatively low detection limit and cost, make it an ideal tool for not only monitoring current blooms but also identifying systems from where blooms may have originated.

In summary, the expansion of brown tide blooms caused by *A. lagunensis* and the documented adverse effects of such HAB events necessitate better monitoring and more research regarding its ecology and bloom dynamics. The method presented in this study was instrumental in detecting the spread of brown tides to the Caribbean Sea and offers a powerful new tool for managers and researchers alike.

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