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# Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes

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**Abstract**

Three different molecular methods were used with traditional brightfield microscope techniques to enumerate the toxic dinoflagellate *Alexandrium fundyense* in samples collected in the Gulf of Maine in 1998, 2000, 2001, and 2003. Two molecular probes were used in fluorescent whole-cell (WC) microscopic assays: a large-subunit ribosomal RNA (LSU rRNA) oligonucleotide probe (NA1) and a monoclonal antibody probe thought to be specific for *Alexandrium* spp. within the *tamarense*/*catenella*/*fundyense* complex. Cell abundance estimates also were obtained using the NA1 oligonucleotide probe in a semi-quantitative sandwich hybridization assay (SHA) that quantified target rRNA in cell lysates. Here we compare and contrast the specificity and utility of these probe types and assay approaches.

WC counts of the 1998 field samples demonstrated that *A. fundyense* cell densities estimated using the antibody approach were higher than those using either the NA1 oligonucleotide or brightfield microscopy due to the co-occurrence of *A. ostenfeldii* with *A. fundyense*, and the inability of the antibody to discriminate between these two species. An approach using cell size and the presence or absence of food vacuoles allowed more accurate immunofluorescent cell counts of both species, but small cells of *A. ostenfeldii* that did not contain food vacuoles were still mistakenly counted as *A. fundyense*. For 2001, a dual-labeling procedure using two oligonucleotide probes was used to separately enumerate *A. ostenfeldii* and *A. fundyense* in the WC format. In addition, the SHA was used in 2001 and 2003 to enumerate *A. fundyense*. Some agreement was observed between the two oligonucleotide methods, but there were differences as well. Not including samples with cell numbers below empirically determined detection limits of 25 cells $l^{-1}$, good correlation was observed for surface samples and vertical profiles in May 2001 and June 2003 when the SHA estimates were, on average, equivalent, and 1.5× the WC counts, respectively. The worst correlations were for virtually all samples from the June 2001 cruise where the SHA both over- and under-estimated the WC counts. Some differences were expected, since the SHA and the WC assays measure different, but related parameters. The former quantifies intact cells and particulate material that might contain non-viable cells or fragments, whereas the latter measures only intact cells that survive sample processing and are visible in a sample matrix. A variety of factors can thus affect results, particularly with the WC method, including variable uptake of the oligonucleotide probe due to cell permeability changes, cell lysis during sampling, preservation and processing; variable rRNA content or accessibility due to nutritional or environmental factors; and the variable detection of intact cells or cell fragments in fecal pellets and detritus. The SHA offers dramatic increases in sample throughput, but introduces uncertainties, such as those due to sample matrix effects (non-specific labeling and cross-reactions), variable rRNA levels in intact cells or to the possible presence of target rRNA in cell fragments, fecal pellets, or detritus. Molecular

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probes are powerful tools for monitoring and research applications, but more work is needed to compare and refine these different cell enumeration methods on field samples, as well as to assess the general validity of brightfield or fluorescent WC approaches.


*Keywords:* *Alexandrium fundyense*; *Alexandrium ostenfeldii*; Antibody; HAB; Molecular probes; Oligonucleotide

## 1. Introduction

Rapid and reliable species identification and enumeration are necessary elements of phytoplankton research and monitoring programs. Difficulties arise, however, when an organism is only a minor component of the plankton assemblage, or if morphological characteristics that distinguish it from the rest of the plankton community are lacking (Anderson, 1995; Vrieling and Anderson, 1996). To remedy these problems, molecular probes such as antibodies (Hiroshi et al., 1988; Nagasaki et al., 1989, 1991; Uchida et al., 1989; Adachi et al., 1993a; Bates et al., 1993; Vrieling et al., 1993; Costas and Lopez-Rodas, 1994; Chang et al., 1999), lectins (Costas and Lopez-Rodas, 1994; Cho et al., 1998; Rhodes et al., 1998) and oligonucleotides (DeLong et al., 1989; Adachi et al., 1996; Miller and Scholin, 1996; Scholin et al., 1996; John et al., 2005) have been developed. These have been used most extensively to aid in the identification and enumeration of individual algal species that cause toxic or harmful algal blooms (HABs) (Anderson, 1995).

There are similarities between these various probe-based approaches to cell identification and enumeration (e.g., they facilitate rapid identification and quantitation), but there are also significant differences with respect to the target molecules to which probe binding occurs, the specificity of those targets among species, the condition or form of the cells during analysis, and the preservation or fixation methods. Antibody probes, e.g., typically target cell surface antigens that are unique to the species or group of interest and thus the probes are used on intact cells (Campbell and Carpenter, 1987; Anderson et al., 1989; Shapiro et al., 1989; Bates et al., 1993). Since the antibodies are produced as part of the general immune response of an animal injected with whole-cells (WCs) or cell fragments of the algal species of interest, probe specificity must be achieved through careful selection of the antiserum or hybridoma cell line that binds most specifically to the target organism. Specificity of polyclonal or monoclonal antibodies can only be determined by empirical tests on multiple cultures or field samples (Adachi et al., 1993b).

Oligonucleotide probes are short segments of synthetic DNA designed to bind to complementary sequences of RNA or DNA from a specific species or group of species. Probe specificity depends on whether the targeted sequence is unique to a particular organism or shared among a group of organisms. Oligonucleotides can be fluorescently labeled and used to visualize intact cells (Adachi et al., 1996; Miller and Scholin, 1996), or they can be used to quantify the target nucleic acids in cell homogenates (Scholin et al., 1997, 1999; Bowers et al., 2000). With respect to probe design, specificity is dependent in part upon the number and taxonomic affiliations of sequences of the targeted gene available in databases, since this information provides a reference for defining probes that contain sequences found only in the organism(s) of interest. Even then, it is often necessary to obtain sequences from strains of the target species that inhabit the region of interest, as considerable genetic variability has been documented among geographically dispersed strains of the same species (Scholin et al., 1994). Common targets for oligonucleotide probes have been the small and large-subunit ribosomal RNA (LSU rRNA) genes since sequences for many different algal species and a host of other organisms are available. Even with this information, it is always possible that cross-reactions will occur with organisms whose sequences have not been entered into the database.

Until recently, most probe work with HAB species has been in the laboratory or developmental stage, but field applications are now emerging (see Scholin et al., 2002). The WC assay using oligonucleotide probes has been used for *Pseudo-nitzschia* spp. along the US west coast (Scholin et al., 2000), and for *Alexandrium* spp. on the east coast of the US, as reported here, as well as the west coast (Matweyou et al., 2004; Scholin, unpubl. data) and the North Sea (John et al., 2003). Antibodies and oligonucleotide probes are being used in New Zealand as part of that country's biotoxin

monitoring program (Rhodes et al., 1998). Oligonucleotide probes also have been used to identify and enumerate *Pfiesteria* species in the southeastern US (Rublee et al., 1999). The sandwich hybridization assay (SHA) has been applied in the field for *Pseudo-nitzschia* spp. (Scholin et al., 1997, 1999, 2000), for *Heterosigma akashiwo* (Tyrrell et al., 2001, 2002), and for *Alexandrium catenella* (Matweyou et al., 2004; Scholin, unpub. data). More recently, oligonucleotide probe procedures have been adapted for use in the Environmental Sampling Processor, a moored instrument that collects water samples and conducts WC and SHA chemistries autonomously (Scholin et al., 1998).

Given the variety in probe targets and specificities, differences are expected in cell abundance estimates obtained with different probe types and assay procedures. Typically, however, these methods are not compared with each other, but rather only against traditional light microscope cell counts. Here we compare four different enumeration methods that target *Alexandrium* spp. in samples collected during Ecology and Oceanography of HABs (ECOHAB)—Gulf of Maine (GOM) and Monitoring and Event Response for HABs (MERHAB) cruises conducted in 1998, 2000, 2001 and 2003. These were: traditional brightfield microscopy, oligonucleotide and antibody "WC" probing with epifluorescent microscope observation, and a SHA that quantifies rRNA in cell homogenates. Three different oligonucleotides were used in the fluorescent WC microscopic assays of intact cells—the NA1 probe that hybridizes to cells of the North American ribotype of the *A. tamarense* species complex (*A. tamarense, A. fundyense* and *A. catenella*) (Scholin et al., 1994), the AG probe, which reacts with all species within the genus *Alexandrium*, and the AOST01 probe designed to identify *A. ostenfeldii* (John et al., 2003). A monoclonal antibody probe (MAb M8751-1) reported to be specific for cell surface antigens on *Alexandrium* spp. (Adachi et al., 1993b) also was used in a WC microscope format. Additionally, analyses were performed using the SHA (Scholin et al., 1999) in which one oligonucleotide (a capture probe, based on the NA1 sequence) is reacted with nucleic acids in cell lysates, after which a second (signal) probe complementary to a different region of the rRNA target sequence is used to generate an enzymatic reaction leading to a colored product proportional to the target species' abundance. The initial target of cell count estimates was *A. fundyense*,[1] but it soon became clear that *A. ostenfeldii* co-occurred with *A. fundyense* in the GOM and needed to be enumerated separately, not only to provide accurate *A. fundyense* abundance, but because *A. ostenfeldii* can be toxic as well (Cembella et al., 2000).

Here we present a comparison and analysis of the results of these multiple approaches to cell counting, highlighting the benefits and disadvantages of each, as well as the uncertainties that remain with respect to their application on natural plankton samples from the GOM.

## 2. Materials and methods

### 2.1. Field sampling

Field samples used in this study were collected during ECOHAB and MERHAB cruises in the spring of 1998, 2000, 2001, and 2003. The study area comprised a large region between Penobscot Bay to the east and Casco Bay to the west (Fig. 1). Station sampling differed among years. In 1998, 14 stations were sampled each week for 10 weeks. In 2000, 25 stations were sampled each week for 8 weeks, and in 2001, 270 stations were sampled in May, and 172 stations in June. In June 2003, approximately 80 stations were sampled in three back-to-back cruises. Only data from Legs 1 and 2 of that cruise are presented here. The general study area for all of these cruises is shown in Fig. 1, which also highlights a few selected stations referred to elsewhere in the text. A distinction is made between two hydrographic domains in the study area—the eastern Maine region dominated by the eastern segment of the Maine Coastal Current (Lynch et al., 1997), and the western Maine region, dominated by the western segment of the Maine Coastal Current. Details of the hydrography and *Alexandrium* population dynamics of these domains can be found in Anderson (1997), Townsend et al. (2001),

[1]Both *A. tamarense* and *A. fundyense* occur in the Gulf of Maine (Anderson et al., 1994). *A. catenella* has never been observed in these waters. We consider *A. tamarense* and *A. fundyense* to be varieties of the same species (Anderson et al., 1994; Scholin et al., 1995). Neither antibody nor oligonucleotide probes can distinguish between them, and only detailed analysis of the thecal plates on individual cells can provide this resolution. This is not practical for large numbers of field samples. Accordingly, for the purpose of this study, the name *A. fundyense* is used to refer to both forms.

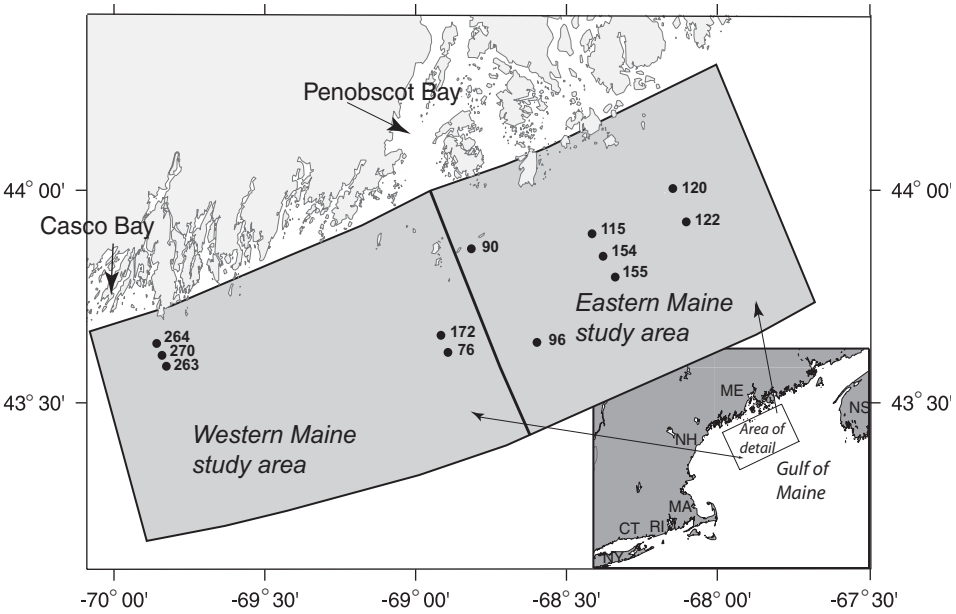


Fig. 1. Map of the study area. Locations of a few selected stations from the 1998 and 2000 cruises are highlighted here and in the text. These are only a small fraction of the total stations sampled.

Pettigrew et al. (2005), Janzen et al. (2005), Keafer et al. (2005a, b), and Churchill et al. (2005).

For WC and SHA samples, 8 l of seawater from a 10 l Niskin bottle were concentrated by filtration through a 20 μm Nitex sieve and backwashed with filtered seawater (FSW) to a volume of 20 ml. Two 5 ml subsamples of the concentrate were collected on separate 5 μm pore size, 25 mm diameter Millipore hydrophilic Durapore filters for SHAs. One replicate filter was either processed immediately during shipboard surveys, or both filters were frozen in liquid nitrogen for later SHA analysis. Each of the latter filters was placed separately in a 2.0 ml cryogenic vial and without delay, was either processed onboard or stored in liquid nitrogen. The two remaining 5 ml subsamples were pipetted into 15 ml disposable centrifuge tubes and brought to a volume of 14 ml with FSW and preserved with 0.75 ml formalin (5% v/v, final concentration = 1.9% formaldehyde). Both formalin-preserved subsamples were stored dark and on ice and then transferred to a laboratory refrigerator (4 °C) within 36 h. Subsamples for antibody labeling and traditional brightfield microscopic analysis were stored at 4 °C until counting, while subsamples for oligonucleotide labeling were immediately centrifuged ($5000 \times g$, 5 min, room temperature), the supernatant aspirated away, and the cell pellet resuspended in 14 ml of ice-cold methanol to extract chlorophyll and stabilize rRNA. These samples were stored at −20 °C.

### 2.2. Oligonucleotide whole-cell sample processing

*Probes and fluors*: For WC analysis, samples were hybridized with various oligonucleotide probes conjugated (at the 5′ end) with different fluorochromes as shown in Table 1. Most commonly, NA1 (Scholin et al., 1996) conjugated to FITC was used to enumerate *Alexandrium fundyense* (North American ribotype), but that probe does not detect *A. ostenfeldii*. Another probe (AG), which targets all species within the *Alexandrium* genus, was conjugated to FITC while the NA1 probe was conjugated to Texas Red in a dual-label approach. The difference between the abundance of AG-FITC labeled cells and NA1-Texas Red labeled cells provided an estimate of the abundance of *A. ostenfeldii* assuming that *A. ostenfeldii* was the only other *Alexandrium* species present, which we believe to be the case. On several samples collected during the year 2000, a fluorescent stain, Calcofluor White MR2 (Polysciences, Warrington, PA #4359), was used simultaneously with the NA1 Texas Red and the AG-FITC probes. Calcofluor White stains the thecal plates that are used for morphological

Table 1
Various oligonucleotide probes used in the WC format for microscopic identification and enumeration of *Alexandrium* spp. in the Gulf of Maine

| Target organism | Probe designation/sequence | Fluor | Filter sets |
|---|---|---|---|
| North American ribotype *Alexandrium fundyense/catenella/tamarense* | NA1; 5′-AGT GCA ACA CTC CCA CCA-3′ | FITC | Zeiss #09<br>Zeiss#09/ Chroma#HQ510LP[a]<br>Chroma #51009 |
| North American ribotype *Alexandrium fundyense/catenella/tamarense* | NA1; 5′-AGT GCA ACA CTC CCA CCA-3′ | Texas Red™ | Chroma #31004 |
| North American ribotype *Alexandrium fundyense/catenella/tamarense* | NA1; 5′-AGT GCA ACA CTC CCA CCA-3′ | Cy3™ | Chroma #41032<br>Chroma #51009 (dual band) |
| *Alexandrium ostenfeldii* | AOST01; 5′-ATT CCA ATG CCC ACA GGC-3′ | FITC | Zeiss#09/ Chroma#HQ510LP[a]<br>Chroma #51009 (dual band) |
| *Alexandrium* spp. | AG; 5′-ACC CAC TTT GCA TTC CCA TG-3′ | FITC | Zeiss #09 |

[a]The Zeiss #09 emission filter was replaced (in 2001) with a Chroma (HQ) emission filter to obtain better spectral qualities that reduced background autofluorescence. When coupled with a Zeiss Fluar 10 × objective, microscopic visualization of weakly stained target cells improved.

identification of armored dinoflagellates (Fritz and Triemer, 1985). In 2001, an *A. ostenfeldii* probe (AOST01; John et al., 2003) conjugated with FITC was used to unambiguously identify this species. A dual-label approach was used for all surface samples and some subsurface samples that year in which the NA1 probe, conjugated to Cy3™ was used simultaneously with the AOST01-FITC probe to allow separate enumeration of *A. fundyense* and *A. ostenfeldii*, respectively.

*Processing*: Subsamples ranging from 2.0 to 7.5 ml of the formalin/methanol-preserved material were collected by gentle vacuum filtration on a 25 mm Cyclopore membrane (Whatman Inc., 5 μm pore size) using a filtration manifold (Promega Corp., #A7231) fitted with custom filter holders (modification of the ones described by Scholin et al., 1997). Before adding the probes, 1.0 ml of pre-hybridization buffer [5 × SET (750 mM NaCl, 5 mM EDTA, 100 mM Tris–HCl [pH 7.8]), 0.1 μg ml$^{-1}$ polyadenylic acid, 0.1% Tergitol NP-40 (Sigma Chemical Co. #T-7631) and 10% ultra-pure formamide] was added to each filter tube and allowed to incubate for 5 min at room temperature. This solution was removed by vacuum filtration and replaced with 1.0 ml hybridization buffer (pre-hybridization buffer plus probe (see Table 1); final probe concentration 4.8 μg ml$^{-1}$). The filter tubes were capped to minimize evaporation and placed (along with the manifold) in a dark bag to minimize degradation of the fluorochrome. Samples were incubated for 1 h at 50 °C. Following hybridization, the sample was washed with 1.0 ml, 50 °C, 0.2 × SET for 10 min at room temperature, the wash solution was removed by vacuum filtration and the filter was mounted on a microscope slide. Twenty-five microliters of SlowFade Light (Molecular Probes Inc., Eugene, OR #S-7461) was placed on the filter and a cover glass added. The specimens were enumerated using a Zeiss epifluorescence microscope at 100 × magnification using the filter sets complementary for the probe/fluorochrome used (see Table 1).

When using two probes with different fluorochromes simultaneously in a dual-labeling approach, the protocol was the same. When calcofluor White MR2 was used, it was added (5 μl ml$^{-1}$) during the wash step and visualized using a standard Zeiss #02 filter set. Visualization using multiple probes requires multiple filter sets specific for each fluorochrome (Table 1). A dual band pass filter set (Chroma #51009; Table 1) was employed to simultaneously view FITC- and Cy3-labeled cells in a sample without switching filter sets as was necessary in 2000. Caution must be exercised, however, since emission from phycoerythrin-containing autofluorescent phytoplankton can

at times be very similar to Cy3stained cells. The Cy3 filter set (Chroma #41032; Table 1) has spectral qualities not discernable with the dual band filter set that can distinguish the subtle color differences of the fluorescent particles. In those cases, switching between two filter sets was preferable.

### 2.3. Antibody labeling protocol

First, 1 ml of blocking reagent [5% normal goat serum (NGS; Sigma Chemical Co. #9023) in 0.02 M phosphate buffered saline (PBS; 0.02 M $PO_4$, 0.15 M NaCl, pH 7.45)] was added directly to the sample on the filter, incubated for 30 min at RT, and removed by filtration. Second, 315 μl primary antibody (M8751-1 diluted 1:50 v/v in 5% NGS/PBS) was added, incubated for 30 min at RT and removed by filtration. The filtered sample and the adjoining chimney were then washed 3× with 5 ml 0.5% NGS/PBS. Third, 315 μl of the secondary antibody [Goat anti-mouse IgG conjugated to fluorescein (GAM-FITC; Molecular Probes Inc., #F-2761) diluted 1:300 v/v in 5% NGS/PBS] were added, incubated for another 30 min, and removed. Controls using only the blocking agent (NGS/PBS) and only the secondary antibody also were run. Finally, the filtered sample was again washed 3× (5 ml 0.5% NGS/PBS) and the filter was mounted on a glass microscope slide by placing 25 μl of 80% glycerol/PBS onto the filter and adding a cover glass. The sample was counted as described above using the FITC filter set.

*Brightfield microscopy*: Subsamples (2.5 ml) of the formalin-preserved material were settled on an inverted microscope counting chamber for 24 h. Cells were enumerated at 160× magnification with a Zeiss IM35 inverted microscope. All *Alexandrium* sp. cells were enumerated without attempting identification at the species level.

*Sandwich hybridization sample processing and calibration*: Samples frozen in liquid nitrogen were allowed to warm for several minutes to near room temperature and 1.0 ml of lysis buffer (Saigene Corp., Seattle, WA) was added. The cryovial was then heated to 85 °C, mixed briefly after 2.5 min then returned to the 85 °C block for an additional 2.5 min. During shipboard surveys, some samples were processed in the same manner immediately after concentrating onto the Durapore filter. The resulting sample lysate was filtered through a 0.45 μm pore size Millipore hydrophilic Durapore filter and allowed to cool to room temperature before dispensing 250-μl aliquots in triplicate into row H of a 96-well plate custom-manufactured by Saigene Corp. for the detection of the North American *Alexandrium tamarense/catenella/fundyense* ribotype. Automated sample processing was conducted using a robotic processor available from Saigene based on that described by Briselden and Hillier (1994) that was modified to accommodate two 96-well plates in which hybridization reagents are dispensed, two comb-like "prongs" that serve as solid supports on which the hybridization and detection reactions take place, and a heating block for maintaining temperature of the 96-well plates (see Scholin et al., 1999). The capture probe (NA1S) and signal probe (AlexS) sequences are given in Table 2. The NA1S probe is highly specific so it will capture only rRNA from North American members of the *tamarensis* species complex; the AlexS probe need not be as specific, since non-target material has been eliminated through washing by the time this probe is applied. Accordingly, this signal probe was designed to be *Alexandrium* genus specific.

SHAs performed in 1998, 2000 and 2001 used the same equipment and reagents as described by Scholin et al. (1999). In 2003, the SHA was modified as follows: the prong shape was changed; prongs were coated with biotin-dextran, and the biotinylated capture probe was pre-mixed with streptavidin (SA) in Assay Wash (Saigene) to yield a SA-capture probe complex that was dispensed when plates were manufactured; the volume of all reagents in the 96-well plate was 250 μl; fluorescein moieties on the signal probe were replaced with digoxigenin and the final concentration of signal probe adjusted to 100 ng $ml^{-1}$; the polyclonal anti-fluorescein HRP conjugate was replaced by anti-dig HRP (Pierce #31468) diluted 1:1000 in Guardian™ HRP Stabilizer Diluent Blocker (Pierce #37558); Assay Wash used in rows B, C and F was replaced by Tris-buffered saline with Tween-20 (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 v/v); the signal solution was replaced with 1Step™ Ultra TMB-ELISA (Pierce #34028). All SHA reagents and plates not from Pierce are now available from Orca Research, Inc. (Seattle, WA); Saigene no longer markets the SHA. The sample processing takes about an hour to complete and two plates can be processed simultaneously in a single run. In 1998 and 2000, each sample for SHA was archived at sea and processed in quadruplicate; three samples could thus be processed per plate. In the 2001 and 2003

Table 2
Capture and signal probes used for the sandwich hybridization assay (SHA) and the years they were deployed[a]

| SHA probe designation | Sequence (5′–3′) and modifications[a] | Field season |
|---|---|---|
| *1.1.1. Capture* | | |
| NA1S 3C9 B | 5′ Biotin-LC $(C9)_3$ GCA AGT GCA ACA CTC CCA CCA 3′ | 1998–2001 |
| *1.1.2. Capture* | | |
| NA1S 3C9 BON | 5′ Biotin-ON $(C9)_3$ GCA AGT GCA ACA CTC CCA CCA 3′ | 2003 |
| *Signal* | | |
| ALEX S 5′2FC9(IFC913)3′C9F | 5′ $(Fluor)_2$ (C9) BAM TTC AAA GTC CTT T*TC ATA TTT CCC (C9) Fluor 3′ | 2000 |
| *Signal* | | |
| ALEX S TRI F | 5′ Fluor (C9) TTC AAA GTC CTT T*TC ATA TTT CCC (C9) Fluor 3′ | 2001 |
| *Signal* | | |
| ALEX S TRI DIG | 5′ Dig (C9) TTC AAA GTC CTT T**TC ATA TTT CCC (C9) Dig 3′ | 2003 |

The capture probe targets North American ribotype *Alexandrium catenella/tamarense/fundyense* LSU rRNA, while the signal probe targets a LSU rRNA sequence common among all *Alexandrium* (Scholin et al., 1994).
[a]All probes were manufactured by Oligos etc. (Wilsonville, OR) with the following modifications:
5′ Biotin-LC $(C9)_3$ = 5′ end modification consisting of biotin "long chain" coupled to three, C9 spacers end-to-end.
Biotin-ON = replacement for biotin-LC (no longer available in 2003).
5′ $(Fluor)_2$ (C9) BAM = 5′ end modification consisting of two fluoresceins, each coupled to a C9 spacer and a branched amino modifier.
(C9) Fluor 3′ = 3′ end modification consisting of one, C9 spacer coupled to fluorescein.
5′ Fluor (C9) = 5′ end modification consisting of fluorescein coupled to one, C9 spacer.
5′ Dig (C9) = 5′ end modification consisting of digoxigenin coupled to one, C9 spacer.
(C9) Dig 3′ = 3′ end modification consisting of one, C9 spacer coupled to digoxigenin.
T* = T residue with amino modifier coupled to one, C9 spacer coupled to fluorescein (internal label).
T** = T residue with amino modifier coupled to one, C9 spacer coupled to digoxigenin (internal label).

large-scale mapping efforts, capacity was increased to four samples per plate by decreasing the number of replicates to three. In 2001, four samples from each station representative of 1, 5, 10, and 20 m depths were analyzed in a single plate. The subsequent station was processed as another single plate on a second processor, since the first processor was still in operation and not yet ready for new samples. By alternating the two processors, the study area could be mapped in near-real time with four depths per station. In 2003, mostly surface samples were collected, with only a limited number of samples from depth. In this case, sample analysis took at least 5 h since the processor was idle until a sufficient number of samples were collected to justify a processor run of four or eight samples. The second processor was not needed and only served as a back-up that year.

After completion of the sample processing, the plates were placed in a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA) and the optical density (OD) @650 nm of row "A" recorded. Plates were removed from the reader and 50 μl of 10% $H_2SO_4$ were added to each well in row "A". The sample and the acid were then mixed by repetitive pipetting using a multi-channel pipette, and the OD@450 nm recorded. Both the 650 and 450 values are proportional to the concentration of the target cell in a given sample (e.g., Scholin et al., 1999, 2000; Tyrrell et al., 2001, 2002).

Calibration curves for the SHA were generated using both laboratory cultures and field material. For the year 2000, *A. catenella* (strain A5 isolated from Santa Cruz, CA) was grown in replete f/2–Si medium (Guillard and Ryther, 1962) and harvested by filtration. Lugol's solution preserved subsamples were collected at the time of harvest to determine the cell concentration via traditional microscopic enumeration methods. A lysate prepared from the cells retained on the filter was serially diluted in lysis buffer to yield samples of known cell concentrations that were then analyzed using the SHA. To generate the most accurate standard curve, it was necessary to confidently determine the number of cells in a "known" sample given the variability in pipetting, cell counting, and filter processing. During preparation of samples for the standard curve, the culture was therefore counted multiple times

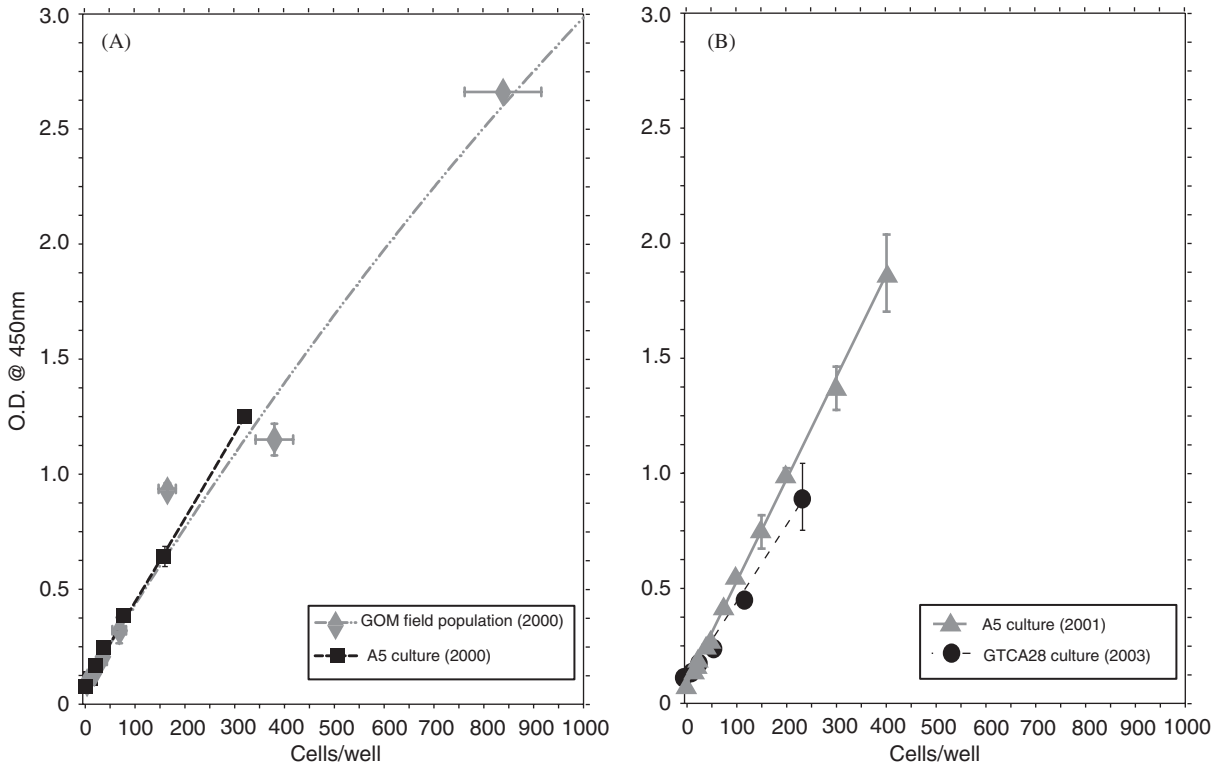


Fig. 2. Calibration data and regression lines for the SHA obtained using cultured *A. catenella* and *A. fundyense* cells or a dilution series of GOM field populations of *A. fundyense*. (A) Year 2000 calibrations using *A. catenella* culture A5 (–■–) and Gulf of Maine field sample of *A. fundyense* bloom (–◆–). (B) Year 2001 calibration using *A. catenella* culture A5 (–▲–) and 2003 calibration using *A. fundyense* culture GTCA28 (–●–).

(at least five). Furthermore, the cells from that "known" culture were filtered multiple times (at least three) and the lysate from each filter was combined to form a single lysate that was used for the dilution series. This processing helped to alleviate sample-to-sample variability in making standards for the calibration. Several identical liquid nitrogen-preserved standard filters also should be archived to serve as a periodic check on the performance of the SHA when the plates are used over extended periods during the 6-month shelf life of the reagents.

A similar calibration series also was performed during a bloom of *A. fundyense* in Casco Bay, Maine, in 2000. The bloom was detected using near real-time SHA and later determined to contain ca 2500 cells $l^{-1}$ by both oligonucleotide (NA1) and antibody (M8751-1) WC assays. A sample from the bloom was serially diluted 1:1 with FSW (filtrate from 20μm sieves) to yield 8 dilutions (range = 23–3000 cells $l^{-1}$). Triplicate aliquots for both oligonucleotide WC assays (preserved with modified saline ethanol and hybridized according to Miller and Scholin (1998, 2000) and SHA were collected from each of the dilutions. Similar results were obtained for calibrations in 2003 using a culture of *A. fundyense* (GTCA28) from the GOM. The resulting plots (Fig. 2) indicated that the field calibration using *A. fundyense* cells from the GOM was nearly identical to the laboratory calibrations using the *A. catenella* A5 strain and the GTCA28 strain of *A. fundyense*. It should be noted that there were slight differences from year to year in the probes (see Table 2), prong configuration, and reagent batches. The similarity in the curves indicated that the background was relatively stable within a range around 0.08 OD at 450 nm and provided fairly consistent results despite year-to-year differences in methodologies. Despite these similarities, a new standard curve must be generated with the arrival of each batch of reagent plates to ensure consistent quality.

## 3. Results

### 3.1. 1998 survey

In total, 121 samples from six cruises were analyzed with both the FITC-labeled M8751-1 antibody and the NA1 oligonucleotide probes using the WC format. Forty-five of these samples from four cruises were also examined with traditional brightfield microscope techniques. The M8751-1 antibody labels the *Alexandrium* cell wall, providing an easily observable bright green outline of the organism when viewed with epifluorescence microscopy (Figs. 3A and C). The oligonucleotide NA1 probe binds to rRNA within the cell, producing a green-labeled cytoplasm (Fig. 3B) that is also easily visualized.

Cell densities of *Alexandrium* spp. determined using brightfield microscopy ranged from 0 to 405 cells $l^{-1}$. In these counts, the objective was to enumerate *A. fundyense*, so only pigmented cells (Anderson et al., 1983; Anderson and Lindquist, 1985) with the morphology of *A. fundyense* vegetative cells and a size typical for this species of 30–40 μm were counted. For the MAb and oligonucleotide counts, all cells that showed the fluorescent label for each probe type were enumerated, regardless of size.

Comparison of the brightfield with the NA1 oligonucleotide and MAb WC counts showed

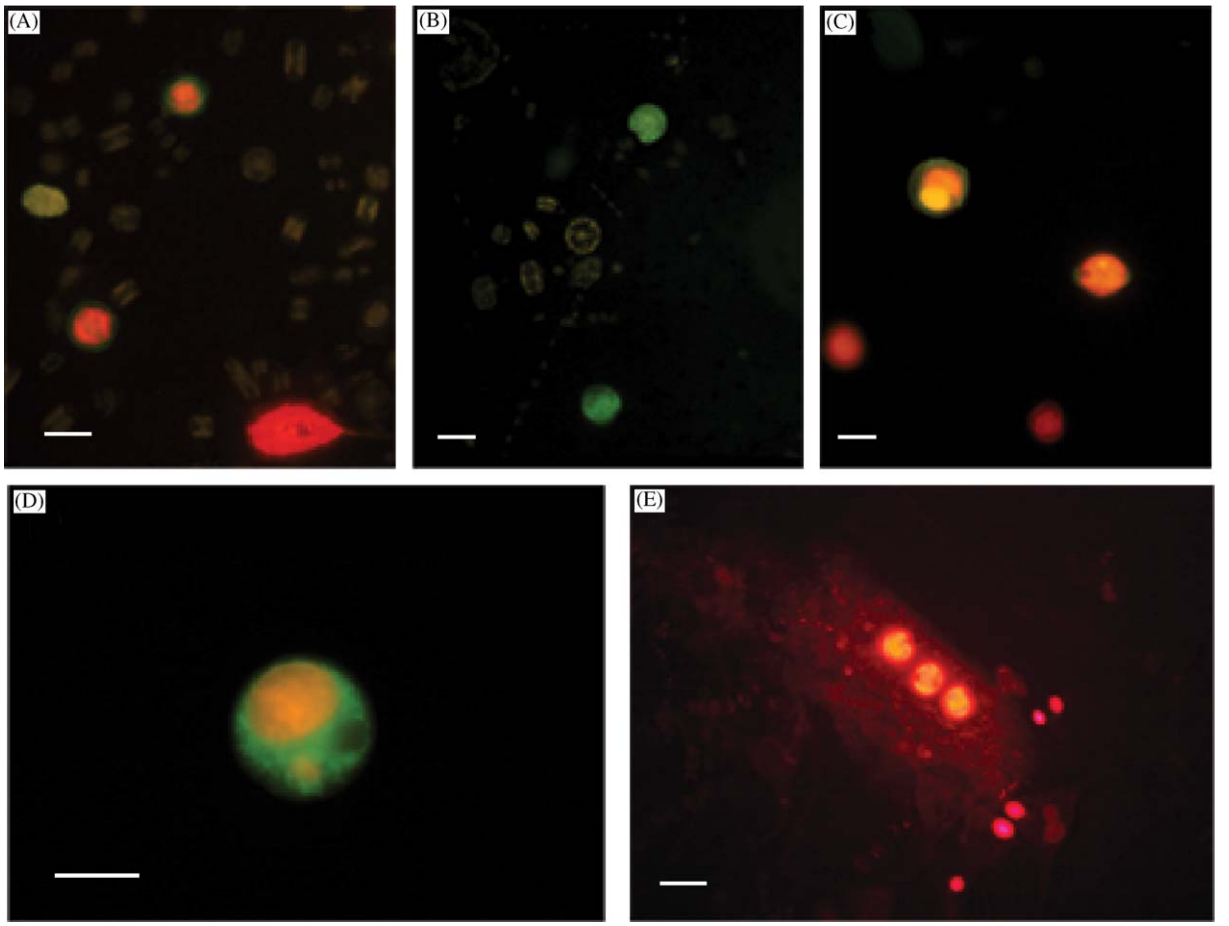


Fig. 3. Epifluorescent images of antibody- and oligonucleotide-probed cells and samples from the GOM. In the images, the red color is from chlorophyll autofluorescence, whereas the green is from either the monoclonal antibody probe or the NA1 oligonucleotide probe, both visualized with FITC. (A–D) Fluorescein (FITC)-labeled probes. (A) Monoclonal antibody (M8751-1) and (B) oligonucleotide (NA1) labeled *Alexandrium fundyense* cells which do not contain inclusion bodies. (C) Monoclonal antibody labeled and (D) oligonucleotide (AG) labeled *Alexandrium ostenfeldii* cells. Note large and small inclusion bodies in the cells which are used to help distinguish *A.* cf. *ostenfeldii* from *A. fundyense*; (E) epifluorescent image of fecal pellet containing Cy3-labeled *Alexandrium fundyense* cells.

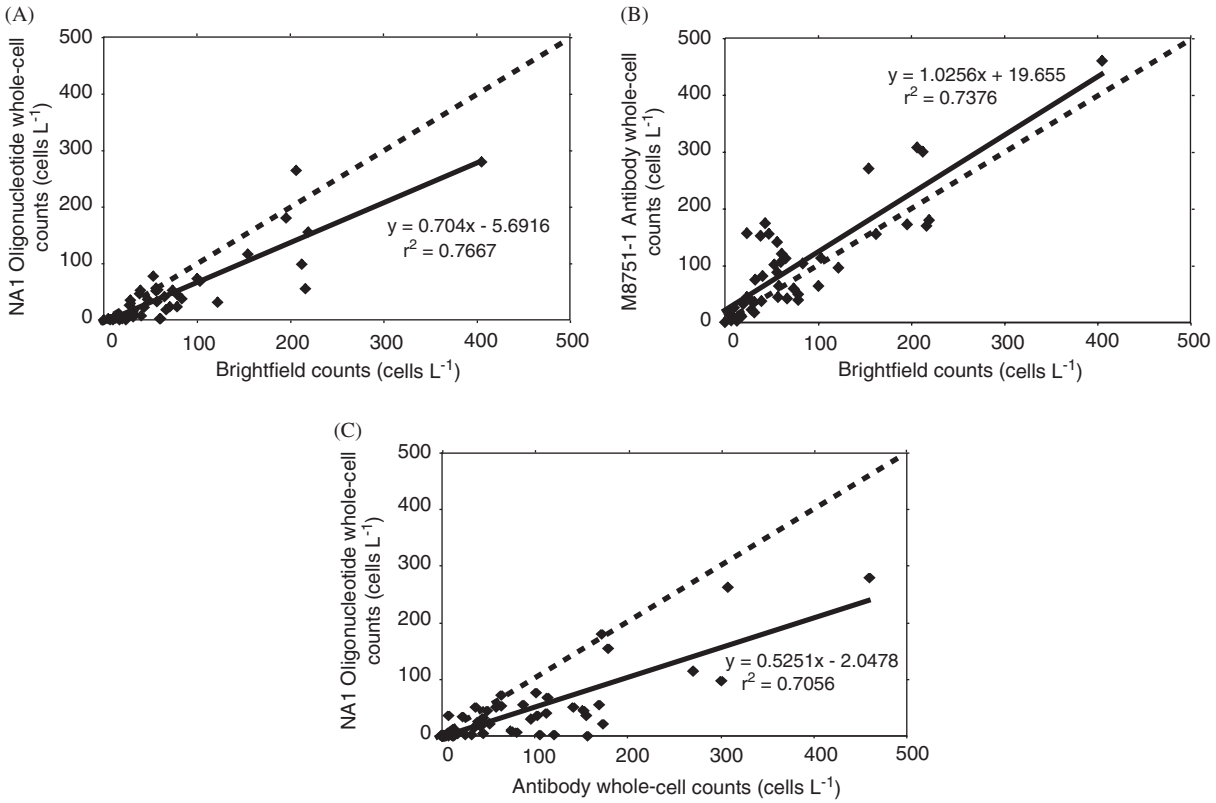


Fig. 4. Comparison of traditional brightfield and fluorescent microscope cell counts using molecular probes in the year 1998 GOM samples. (A) Oligonucleotide versus brightfield cell counts. (B) Antibody versus brightfield cell counts. (C) Oligonucleotide versus antibody cell counts. Solid line represents linear curve fit while the dotted line represents 1:1 correlation.

general agreement (Figs. 4A and B). The slope of the brightfield versus oligonucleotide regression (Fig. 4A) was 0.70 with an $r^2$ value of 0.77, whereas the slope of the brightfield versus MAb regression was 1.03 with an $r^2$ of 0.74 (Fig 4B). Linear regressions of the antibody versus the oligonucleotide counts gave an $r^2$ value of 0.71 and a slope of 0.53 (Fig. 4C). The slopes of these lines indicate that the brightfield counts were typically higher than those enumerated using the NA1 oligonucleotide, but lower than counts with the antibody. The antibody counts were roughly twice the oligonucleotide counts.

Autofluorescent food vacuoles or inclusion bodies were observed in some, but not all of the *Alexandrium* species, and thus were considered as a possible distinguishing feature for *A. ostenfeldii,* a mixotrophic species that has been previously described as containing food vacuoles (Jacobson and Anderson, 1996). On close inspection, it was found that the NA1 probe labeled only, but not all, the 30–40 μm *Alexandrium* cells that did not contain food vacuoles or inclusion bodies (Fig. 3B), while the M8751-1 antibody probe labeled all *Alexandrium* spp., regardless of size or food vacuole content (Figs. 3A and C). Food vacuoles were easily seen with fluorescence microscopy using an FITC filter set, but were difficult to discern using brightfield microscopy. Thus, they were not used as an identifying character in the brightfield counts. Food vacuoles within *Alexandrium* cells were variable in number, size and color. A cell could contain anywhere from one to several vacuoles ranging in size from barely observable at 100 × magnification to almost filling the cytoplasm of a cell (i.e., ranging 2–40 μm). Both large and small cells contained food vacuoles. Vacuole color, as viewed with a long-pass FITC emission filter, ranged from pale yellow, to orange to bright red in both formalin-preserved and methanol-extracted samples.

To help gain a better understanding of the discrepancy between the antibody and oligonucleotide WC counts, 11 previously counted samples

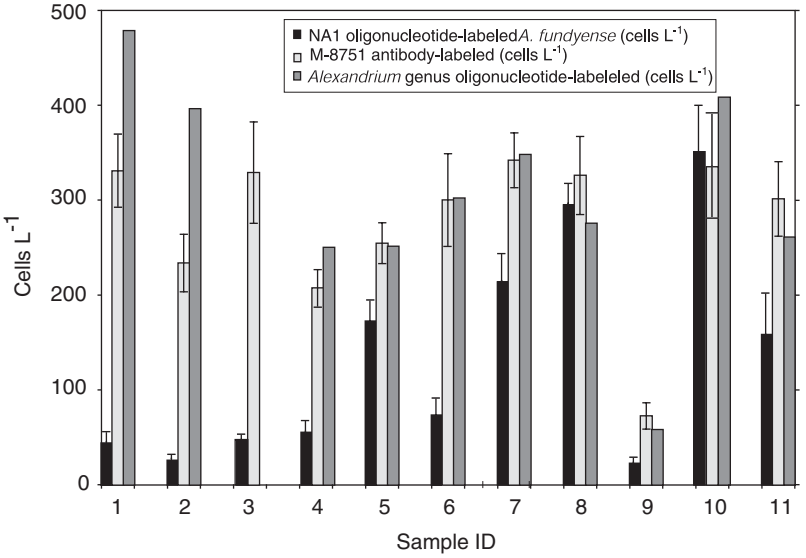


Fig. 5. Dual-labeled oligonucleotide versus antibody whole-cell counts of selected 1998 GOM samples. Comparison of NA1 oligonucleotide-labeled cells (Texas Red®), AG oligonucleotide-labeled *Alexandrium* genus cells (FITC), and all cells labeled with the M8751-1 antibody probe. Error bars represent 1 standard deviation.

were processed "blind" with both probe types and then counted by three individuals. The M8751-1 antibody counts included all *Alexandrium* cells that labeled green with the FITC-conjugated secondary antibody. For the samples processed using the antibody procedure, any *Alexandrium*-like cells with a green fluorescent label on the periphery of the cell were enumerated as *Alexandrium* sp. (e.g., Fig. 3A and C). Similarly, for samples processed using the FITC-conjugated NA1 oligonucleotide probe, any internally green-labeled *Alexandrium*-like cells were enumerated as *A. fundyense*. In addition, replicate oligonucleotide samples were simultaneously labeled with the FITC-conjugated *Alexandrium* genus (AG) probe and with a Texas Red®-conjugated NA1 probe. After the hybridization reaction, the samples were scanned using an FITC filter set. Whenever a green-labeled cell was encountered, it also was examined with the Texas Red® filter set to determine if it was labeled with the NA1 probe as well. No Texas Red®-labeled cells were ever observed that did not also label green, validating that the dual labeling worked. In effect, all of the *Alexandrium* spp. cells in the sample were identified and enumerated using the genus probe, and those that were labeled with the NA1 probe in this dual-labeling procedure were enumerated separately, as a subset of the genus counts.

All counts by the independent researchers were comparable to each other, as indicated by the small size of the error bars in Fig. 5. Differences were observed between the different probe types, however. The M8751-1 antibody and AG oligonucleotide probe labeled all *Alexandrium* cells, including those that did, and did not, have food vacuoles, and thus the counts were similar (Fig. 5). In contrast, none of the cells that were labeled with the NA1 probe contained food vacuoles, equating to lower cell concentrations than observed with the M8751-1 or *Alexandrium* genus labeling.

### 3.2. 2000 survey

Given the labeling results for 1998 in which multiple *Alexandrium* cell types were observed, some with food vacuoles and some without, three samples from year 2000 were triple labeled on filters using the AG probe (coupled to FITC), the NA1 probe (coupled to Texas Red®), and Calcofluor White added during the wash step to stain the cellulose theca. This procedure made it possible to determine which cells were labeled by each of the probes, which, if any, of those had the distinctive pore of *A. ostenfeldii* on the first apical plate (Balech, 1995), and which contained food vacuoles. The apical pore is easily seen when the cells have

been labeled with Calcofluor White and can be rolled over manually to expose the ventral epitheca. *A. ostenfeldii* did not always contain an observable fluorescent food vacuole, and ranged in diameter from 30–82 μm with an average size of 42 μm ($n = 519$) across the mid-latitudinal region of the protoplast. For the three samples examined in 2000, 0%, 16%, and 37% of the small *A. ostenfeldii* cells (<40 μm) did not contain food vacuoles (mean 17.7%, $n = 73$). *A. fundyense* cells (i.e., those labeled by both the AG and NA1 probes) never contained food vacuoles and ranged in size from 30 to 39 μm in diameter (mean 35 μm; $n = 66$).

Ninety-nine of the samples collected in the year 2000 were enumerated with the oligonucleotide and antibody WC methods. When counts of all cells labeled with the M8751-1 antibody were compared to the NA1 oligonucleotide counts, there was moderate agreement, with a linear regression $r^2$ of 0.75 and a slope of 0.90 (Fig. 6A). When the NA1 WC counts and M8751-1 antibody counts of cells without food vacuoles were compared, the correlation improved to an $r^2$ of 0.9 with a slope of 0.97 (Fig. 6B).

Seventy-one of the year 2000 field samples were analyzed using the SHA and the calibration data given in Fig. 2 based on an *A. fundyense* culture. A linear regression of the SHA and NA1 WC count estimates gave an $r^2$ of 0.63 and a slope of 0.54 (Fig. 7). The SHA thus yielded cell count estimates that were approximately one-half of those obtained using the WC approach with the same type of probe, although there is considerable scatter. The samples used for these comparisons were from surface waters in the western GOM (Fig. 1).

### 3.3. 2001 surveys

Two cruises were conducted in 2001—a 5-day cruise in May (May 6–11, 2001), and a 4-day cruise in June (June 6–10, 2001). Field samples, including both surface and deep samples, were again analyzed for *A. fundyense* with both the SHA and the WC. No antibody labeling was conducted since an oligonucleotide probe (AOST01) specific for *A. ostenfeldii* LSU rRNA had been obtained (John et al., 2003). Double labeling of samples using the AOST01 FITC-labeled oligonucleotide probe and the NA1 Cy3™ labeled probe proved to be ideal for discriminating between *A. fundyense* and *A. ostenfeldii*. Distribution and toxicity data for *A. ostenfeldii* in the GOM obtained using this new probe are presented by Gribble et al. (2005).

Approximately 1000 samples collected during the 2001 surveys were examined using both the NA1 WC approach and the SHA, calibrated with a new standard curve shown in Fig. 2B. In most stations in the western waters of the GOM near Casco Bay (Fig. 1), *A. fundyense* cell abundance was very low (<25 cells $l^{-1}$ according to the WC assay), and agreement was poor between the cell count estimates given by the two methods (data not shown). At stations further to the east and offshore, *A. fundyense* cells were more abundant. Samples from the May cruise with cell abundance >25 cells $l^{-1}$ as determined by the WC assay showed a reasonable correlation between the two enumeration methods, with an $r^2$ of 0.49 and a slope of 1.0 ($n = 34$) (Fig. 8A). Fig. 8B shows the SHA and NA1 WC data for all stations in the June 2001 cruise that were enumerated using both methods, again for samples with WC

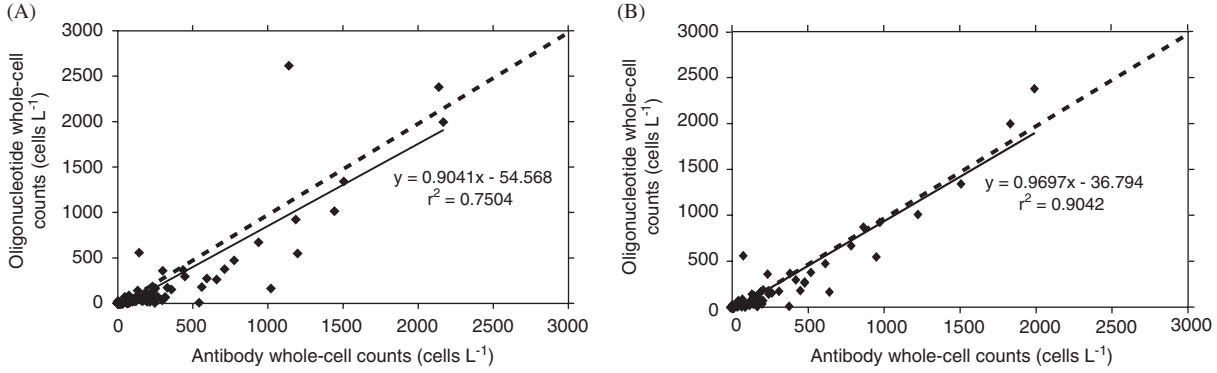


Fig. 6. Plots of year 2000 GOM *Alexandrium* spp. cells enumerated by whole-cell epifluorescent microscopic analysis of M8751-1 antibody- and NA1 oligonucleotide-labeled samples. (A) NA1 counts versus antibody counts of all labeled cells regardless of food vacuole content; and (B) NA1 counts versus antibody counts of labeled cells without food vacuoles. Solid line represents linear curve fit while the dotted line represents 1:1 correlation.

counts > 25 cells $l^{-1}$. The relationship between the two enumeration methods was poor, with a linear regression $r^2$ of 0.01 and a slope of 0.09 ($n = 122$).

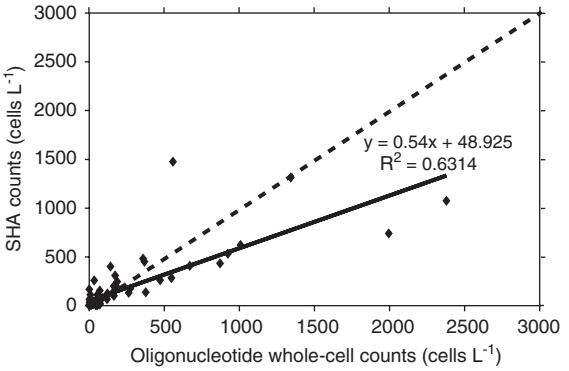


Fig. 7. Whole-cell NA1 oligonucleotide cell counts of year 2000 samples (all from surface waters in the western Gulf of Maine) versus SHA estimates of *A. fundyense* cell abundance. Solid line represents linear curve fit while the dotted line represents 1:1 correlation.

Fig. 9 presents vertical profiles of *A. fundyense* abundance at several stations in the study area in May and June 2001. At some of the stations in May (e.g., eastern Maine stations 115, 120, and 90; Fig. 9A), the SHA abundance estimates are in good agreement with the NA1 WC counts at all depths. One month later, however, samples from other stations showed agreement as well as disagreement between the SHA and the WC NA1 counts. In some cases, the SHA counts were considerably higher than the WC counts, while at other stations or depths, they were lower (Fig. 9B).

The distributions of *A. fundyense* determined using the SHA and the WC approach are shown in Fig. 10 for the May and June 2001 cruises. Both methods capture similar features of the bloom population, but there are differences in the distributions, especially in June (Fig. 10C and D).

The large variability of the SHA results compared to the WC measurements for the June samples raised the question of whether the assay was

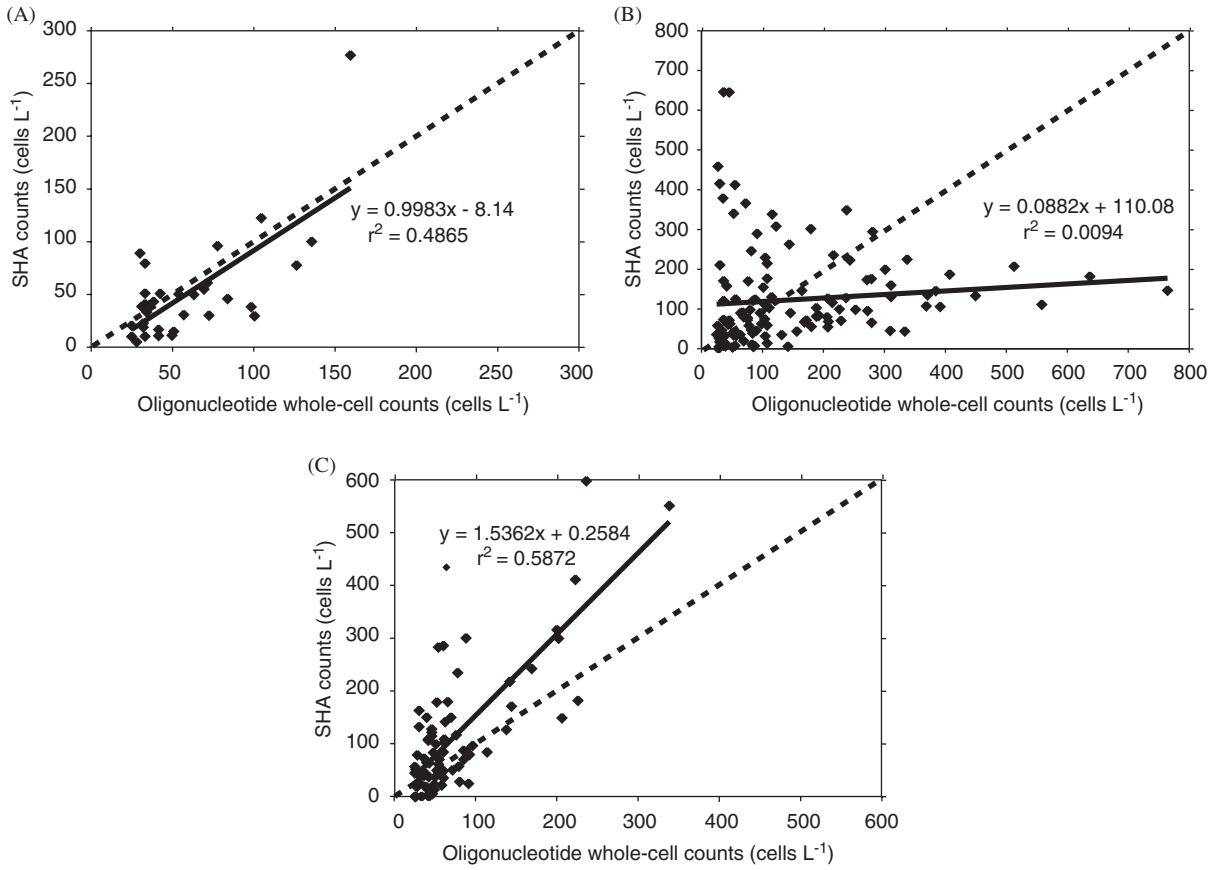


Fig. 8. Whole-cell NA1 oligonucleotide counts versus SHA estimates for years 2001 and 2003 samples from the Gulf of Maine. Only samples with WC counts > 25 are shown. Solid line represents linear curve fit while the dotted line represents 1:1 correlation. (A) Samples collected in May 2001; (B) samples collected in June 2001; (C) samples collected in June 2003.

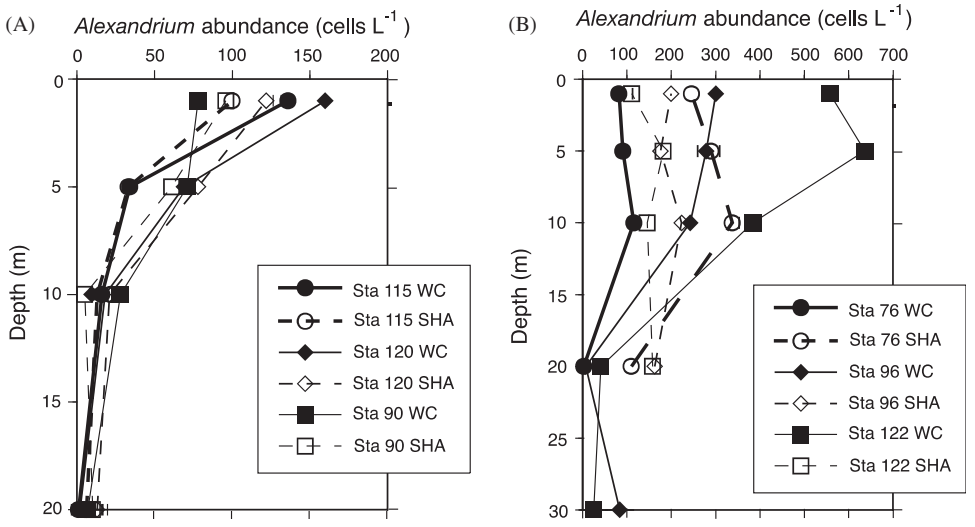


Fig. 9. Vertical profiles of *A. fundyense* abundance at selected stations. Station locations are indicated in Fig. 1. WC NA1 counts are represented by solid symbols; SHA counts are represented by open symbols. (A) Profiles from May 2001; (B) profiles from June 2003.

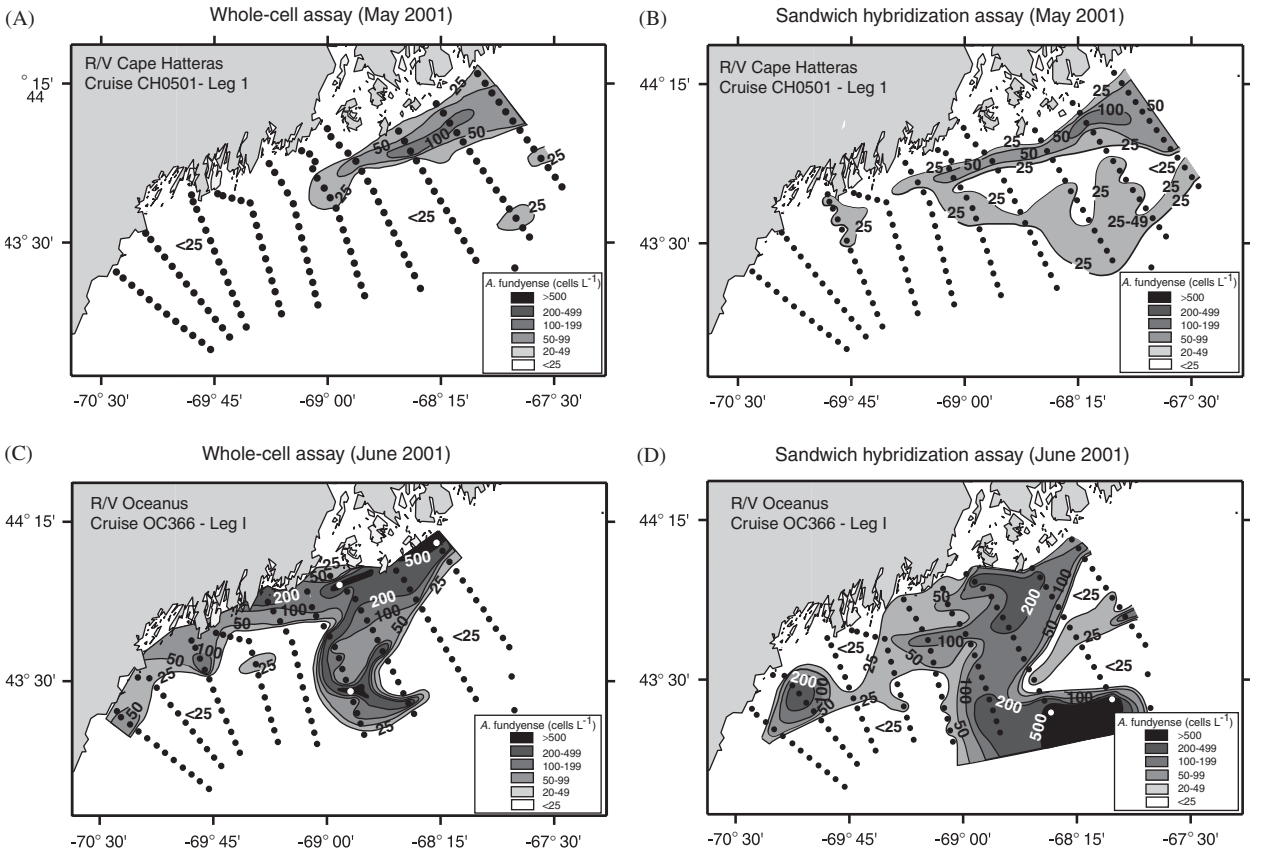


Fig. 10. Contour plots of SHA and WC abundance estimates (cells $l^{-1}$) for *A. fundyense* in GOM surface waters in May (panels A, B) and June 2001 (panels C, D). SHA results (panels A, C) and WC results (panels B, D).

working properly. Prior to the field season, the synthesized probes had passed the same quality control tests as those used in the May cruise, and standard curves were completed that showed the SHA responded properly. At sea, the assay also appeared to be working based on a reagent-check solution that employs an oligonucleotide complementary to both capture and signal probes (i.e., synthetic target), in lysis buffer. Following the cruise, cultured *A. fundyense* (strain GTCA28) was used to test the SHA response using the different batches of reagents and prongs used in the May and June cruises. The assay behaved as expected over a 3-fold dilution series, with no significant differences found between the reagents. Selected field samples from both high and low-SHA areas also were reanalyzed using replicate filters archived from the June cruise. Those replicates, which used the same reagents and plates tested with the dilution series, showed good agreement. Therefore, there is no evidence that might suggest the SHA was not working properly during the June cruise.

### 3.4. 2003 surveys

Three successive 3-day cruises in May/June (May 29–June 6) 2003 sampled approximately 80 stations per cruise. Samples from two of the three cruises were analyzed using both SHA and WC approaches. Most of the samples were from the surface, but some deep samples also were analyzed. Fig. 8C shows a regression of SHA versus WC counts for all samples analyzed with both methods for which the WC counts were greater than 25 cells $l^{-1}$. Agreement is moderate, with an $r^2$ of 0.59 and a slope of 1.5 ($n = 79$). The latter indicates that the SHA counts tended to be approximately 50% higher than the WC counts. No systematic difference was noted in the relative accuracy of deep versus surface samples. Contour plots of the two datasets are presented in Fig. 11 for the two cruise legs. Qualitatively, both methods reveal the same general features in the *A. fundyense* distributions (compare Fig. 11A versus 11B, and 11C versus 11D), but the cell concentration estimates are slightly higher with the SHA.

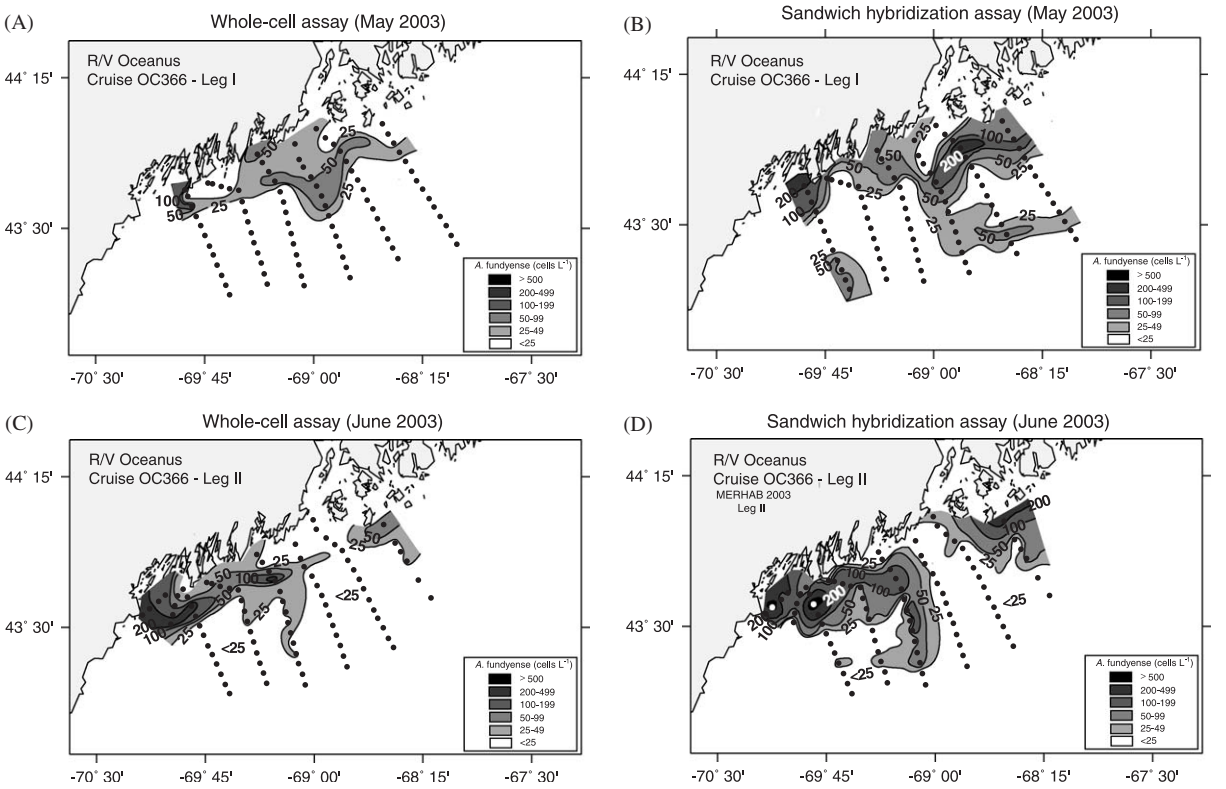


Fig. 11. Contour plots of WC and SHA abundance estimates (cells $l^{-1}$) for *A. fundyense* in GOM surface waters in June 2003. SHA results (panels A, C) and WC results (panels B, D).

## 4. Discussion

In the first large-scale evaluation of antibody- and oligonucleotide-based probe technologies for the enumeration of toxic *Alexandrium* species in the GOM, significant improvements were achieved compared to traditional light microscope counts, but a number of problems with the probe methodologies also were encountered. On the positive side, counts could be completed significantly faster, and, depending on the probe method used, with more accuracy than was possible with brightfield methods due to better resolution of morphologically similar species. The problems encountered were significant, however, and indicate that further work is needed to understand and define what the different probe types and assay formats are actually measuring. Care also must be taken to assess detection limits and the relative validity of these methods at low cell concentrations in samples representative of those found in natural waters. Another problem area reflected the co-occurrence of two *Alexandrium* species and the inability of an antibody probe targeting a cell surface antigen to discriminate between them. Yet another problem was evidenced by differences in the cell abundance estimates provided by two oligonucleotide probe assays, one targeting rRNA inside intact cells, and the other quantifying rRNA in cell lysates. The techniques used to enumerate cells using these approaches are very different, and thus differences in cell counts are to be expected. We remain convinced that molecular probes are highly valuable tools for HAB species detection and enumeration, but emphasize the need for further characterization and refinement of the methods and protocols on site-specific and species-specific bases.

### 4.1. Processing times and logistical issues

A key advantage in using molecular probes to enumerate phytoplankton microscopically is the relative ease and speed of counting fluorescently labeled cells. In our hands, 14 samples can be labeled with either the antibody or oligonucleotide probes in a period of about 2 h and all of those samples counted in the WC format at relatively low-power magnification in about 2–3 h. One individual can thus process and count up to 28 samples per day using WC methods. In contrast, brightfield counts of settled plankton samples take approximately 1 h per sample for counting. The time savings with the WC probe approach is approximately 3-fold.

Another advantage of the WC technique is that more sample can be concentrated (per unit area observed) as these methods utilize membrane filters for sample processing. Furthermore, fluorescently labeled cells are easily identified with little or no interference from the surrounding biomass, eliminating visibility problems that can confound a brightfield count. WC scanning can thus be done at low-power magnification, making the counting faster as well.

For some samples, however, rapid scanning of a single replicate can be problematic. High amounts of organic and inorganic particulate matter can make it difficult to observe and count the target species, and when the target species is rare it is advisable to prepare and analyze replicate samples, as recommended by Miller and Scholin (1998). In these instances, rapid, low-power epifluorescence scanning may not yield accurate estimates of target cell density.

There are logistical considerations with probe-based WC counts that offset some of the time savings. The primary reason only a limited number of field samples were collected for oligonucleotide probing in 1998 and 2000 was that extra steps are involved in sample preservation. After the initial formalin fixation, the sample must be centrifuged, the supernatant aspirated, and ice-cold methanol added to the cell pellet. Not only are these steps time consuming, but hazardous waste is generated and sample processing, via centrifugation, is typically not practical on board a small research vessel. Samples also must be stored between $-20$ and $-80\,^{\circ}C$, where they are stable for a year or more. Hybridized samples on slides can be stored cold and dark with the labeled cells being visible for several months. While the ethanol-SET fixation method of Miller and Scholin (2000) is amenable to shipboard use and does not use hazardous chemicals such as formalin and methanol, the preserved specimens have a shelf life of only 4–6 weeks. The antibody technique has the advantage of utilizing formalin-preserved material with a long storage life, while also providing a brighter signal than the oligonucleotide probe (Anderson et al., 1999). Note, however, that the signal intensity of the oligonucleotide probes can be increased, as occurred in 2001 through the use of the CY3 fluor and higher-quality filter sets.

Table 3
Attributes of the cell enumeration techniques used to count *Alexandrium fundyense*

| Assay method | Cellular target | Preservation method | Recommended storage/stability | Signal type/ intensity* | Comments |
|---|---|---|---|---|---|
| Whole-cell antibody | Cell surface antigens | 5% v/v formalin | 4 °C/several years | Epifluorescence + + + + | Simple field and lab protocol |
| Whole-cell oligonucleotide | LSU rRNA | Formalin/MeOH | −20 to −80 °C at least 1 year | Epifluorescence + + + | Field preservation cumbersome, |
| | | EtOH/SET | Room temp./at least 1 month | Epifluorescence + + + | simple lab protocol |
| Sandwich hybridization | LSU rRNA | Filter stored in $LN_2$ | $LN_2$/months | Absorbance @ 450 nm + + + + | $LN_2$, and sample processor |

*Signal intensity of method denoted by number of + marks. $LN_2$ = liquid nitrogen; MeOH = methanol; EtOH/SET = ethanol/SET.

Formalin-preserved samples for immunofluorescence counts can be stored at 4 °C and are stable for several years, and the labeled cells are visible for at least 1 year when stored on slides at 4 °C. Table 3 lists the fixation and storage details for the different counting methods used in this study. Development of alternative methods for preserving cells for WC probing and storage remains an area of continuing research, especially the development of new protocols that limit use of hazardous chemicals and that are amenable to field applications.

The SHA uses a robotic processor, plate reader and heating block, equipment that costs less than half that of a research-grade epifluorescence microscope. Compared to microscopy-based methods, the SHA requires less time to handle the sample and has the potential to be much faster. The SHA takes approximately 1 h to complete, from live sample to cell abundance estimates, with ~15 min of hands-on time for preparing samples and recording results. A single processor holds two plates, each of which may contain 3–4 samples depending on the number of replicate wells dedicated to each sample. A single person can easily run two processors at the same time, giving an overall sample throughput of ~12–16 samples per hour. Two processors run 6 times in a day thus allow a single person to process 72–96 individual samples (or half that amount if only a single processor is available). It is even possible for a single person to run three processors simultaneously, which would give a maximum sample throughput of ~108–144 samples per day under laboratory conditions. The user handles two solutions only (lysis buffer and, when added assay sensitivity is desired, 10% $H_2SO_4$), whereas WC probing requires at least five (fixative, pre-hybridization, probe, wash and anti-fade mounting solutions). The SHA can also be run at sea, even on relatively small vessels. Under those conditions we find that a single person can operate two processors simultaneously at roughly two-thirds the pace that is possible in the laboratory. For long cruises with round-the-clock operations, a two-person team can maintain a processing rate of ~8–10 samples per 1–1.5 h. That pace, at sea, is very hard work, but it is tractable and yields near real-time data while the ship is underway. Comparable sample analysis using WC methods is not possible given the protocols used in this study.

Storage of samples for SHA involves immersion in liquid nitrogen. This presents constraints with respect to transportation of archived samples from the field to the laboratory. The long-term stability of samples containing *Alexandrium* spp. is not yet known. Protocols for preserving samples for SHA are under active investigation. For example, Tyrrell et al. (2002) have shown that samples containing *H. akashiwo* can be preserved for SHA in acidic Lugol's iodine at room temperature for at least 3 months.

### 4.2. Co-occurring *Alexandrium* species

At the beginning of the ECOHAB-GOM program, the widespread occurrence and abundance of the dinoflagellate *A. ostenfeldii* in the GOM was not known. There had been isolated observations of the species in that region (e.g., Jacobson and Anderson, 1996) as well as in the Gulf of St. Lawrence (Levasseur et al., 1998), but there was no knowledge of the extent to which this species might co-occur with *A. fundyense* in the study area, or how abundant and widespread it might be. During our efforts to enumerate *A. fundyense* cells in field

samples collected in 1998, the agreement between cell abundance estimates obtained using the M8751-1 antibody, the NA1 oligonucleotide and traditional brightfield microscope counts was only moderate (Fig. 4). Linear regressions of these counts versus each other gave $r^2$ values of approximately 0.7, with the slopes of those regressions suggesting that the brightfield method counted more cells than the NA1 oligonucleotide, and less than the MAb. Other comparison counts (Figs. 5 and 6), as well as careful examination of thecal plate details, revealed that the reason for these differences was the presence of *A. ostenfeldii*, a species that is morphologically similar to *A. fundyense* under brightfield examination, and cross-reacts with the M8751-1 MAb. This antibody was thought to be specific for species within the *tamarense*/*catenella*/*fundyense* complex (Adachi et al., 1993b), but clearly is not. *A. ostenfeldii* spans a size range (30–82 μm) that overlaps with that of *A. fundyense* (30–39 μm), and has a generally similar morphology as well. The brightfield counts therefore would have included some, but not all *A. ostenfeldii* cells, missing only the largest cells since these would have exceeded the maximal size of *A. fundyense* vegetative cells (~40 μm). This then explains the generally higher *A. fundyense* abundance estimates from the MAb counts compared to the brightfield counts (Fig. 4B) and the strong agreement between counts of cells labeled with the *Alexandrium* genus probe and the MAb (Fig. 5). In effect, these two probes both labeled all *Alexandrium* cells in the samples, as there were no *Alexandrium* species present other than *A. fundyense* and *A. ostenfeldii*. The NA1 oligonucleotide does not label *A. ostenfeldii*, so those counts were lower than the MAb counts, and lower than the brightfield counts (Figs. 4A and C) since the latter would have included some of the smaller *A. ostenfeldii* cells.

A different approach to counting with the MAb was attempted for the year 2000 samples, relying on cell size and the presence or absence of food vacuoles in labeled cells to differentiate *Alexandrium ostenfeldii* from *A. fundyense*. *A. ostenfeldii* is a mixotrophic species that often contains food vacuoles (Jacobson and Anderson, 1996) that are fluorescent (Fig. 3D) and thus easily visible under epifluorescent illumination. We have not observed fluorescent vacuoles in *A. fundyense* during examination of thousands of cells labeled with the NA1 oligonucleotide probe. The comparisons between NA1 WC *A. fundyense* counts and the MAb counts of labeled cells improved considerably when the presence or absence of food vacuoles was used as an additional element of discrimination, yielding an $r^2$ of 0.9 and a slope of 0.97 (Fig. 6B).

Despite this improvement in the accuracy of counts with the MAb, it was not used in 2001 since an *A. ostenfeldii*-specific rRNA probe (AOST01) was generously supplied by U. John (John et al., 2003). This was used in a dual-labeling procedure with our *A. fundyense* specific NA1 probe, with each probe linked to a different fluor. The two species could be simultaneously viewed and enumerated with a dual band-pass filter set. Although we chose to rely exclusively on oligonucleotide probes for the two *Alexandrium* species, we note that relatively accurate cell counts of both *A. fundyense* and *A. ostenfeldii* are possible with the M8751-1 antibody if cell size and food vacuole content are taken into account. This can be tedious in some samples, and thus is not a preferred alternative when two *Alexandrium* species are present. One category of cells will still be miscounted—the *A. ostenfeldii* cells that are relatively small (<40 μm) and that do not contain food vacuoles. In three samples examined in detail, this cell type averaged 18% of the total *A. ostenfeldii* (range 0–37%). This is a one-time estimate of the extent to which *A. fundyense* cells would be overcounted and *A. ostenfeldii* undercounted in samples using the MAb label and food vacuoles as identifying features.

Clearly, the NA1 oligonucleotide, used in conjunction with an *A. ostenfeldii* specific probe is more accurate than the MAb, with a comparable level of sample processing and analysis. We note, however, that the MAb has one significant advantage over the oligonucleotide probe—it can be used to separate *A. fundyense* and/or *A. ostenfeldii* cells from plankton samples using an immunomagnetic bead isolation procedure (Aguilera et al., 1996, 2001). This technique was recently used to obtain species-specific urease activity estimates for field populations of *A. fundyense* that were relatively free of *A. ostenfeldii* cells (Dyhrman and Anderson, 2003). Probe-based WC separations or isolations are not possible using oligonucleotides.

### 4.3. Sandwich hybridization versus whole-cell comparisons

Results from the 1998 and 2000 field seasons highlighted the apparent accuracy and specificity of the oligonucleotide approach to cell enumeration

using the WC format. The next step in the development and application of this technology for field work was to use the rRNA probes in the semi-automated SHA format (e.g., Scholin et al., 1999). Comparisons across a broad range of conditions from many areas and many depths in the GOM for years 2001, and 2003 showed some agreement between SHA and WC estimates of *A. fundyense* abundance, as well as significant differences. Here we compare samples for which the WC counts were $>25\,\text{cells}\,l^{-1}$ because of large discrepancies between the methods at lower levels. Given this threshold, good agreement was achieved in May 2001 (Figs. 8A and 10A and B) and June 2003 (Figs. 8C and 11). In the former instance, the counts were comparable, with a 1:1 slope for the linear regression, suggestive of equivalence, on average. In the latter instance, the SHA counts were generally 50% higher than the WC counts. In marked contrast to the good correlations in May 2001, results from a cruise 1 month later showed very poor correlations. These included both underestimates and over-estimates (Fig. 8B).

Disagreement between counts is not surprising given the fundamental differences between these two assay types. A successful WC assay requires that the target cell survive treatment from sample collection through processing and that molecules inside these intact, recognizable cells must: (a) be accessible to the probe, and (b) be of sufficient quantity to visualize that cell above background. Results of a WC assay are thus operationally defined—even if a target cell is present it may not always be detected with this approach. Furthermore, anything that causes cells to clump or otherwise be hidden (e.g., large quantities of particulate organic matter or fecal pellets (Fig. 3E)) will affect results of a WC assay, as shown for *Pseudo-nitzschia* by Scholin et al. (1999) and *Heterosigma* by Tyrrell et al. (2001). Similarly, results of cell homogenate assays are operationally defined. The basic concept of the SHA is to detect molecules free from particulate matter, analogous to detection of algal toxins or DNA sequences in phytoplankton samples. Target cells, or even remnants thereof, need only survive the initial collection step. Successful detection then depends on: (a) extracting the target molecule, (b) a sufficient quantity of the target molecule to elicit a positive reaction, and (c) minimal interference (signal suppression/enhancement) from the sample matrix.

Several possible explanations for the observed discrepancies can be offered. For the WC approach, cellular uptake of the probe may vary (independent of rRNA concentration) due to cell permeability differences, such as those associated with life history transformations or nutritional condition. Temporary cysts, e.g., are readily formed by *Alexandrium* species when subjected to sudden mechanical or environmental stress (Anderson and Wall, 1978; Cembella et al., 2000). If the cysts are less permeable to the probes than vegetative cells, this could lead to weakly stained cells, and to lower WC counts. We re-examined some of the field samples that showed the largest counting discrepancies with this concern in mind, but did not observe the large number of temporary cysts that would be needed to reconcile the counts. This is not a likely explanation for observed differences.

Alternatively, cells may be more prone to lysis under certain physiological conditions, reducing the number of cells enumerated with the WC assay. Different processing and preservation procedures are followed for the two oligonucleotide-based assay methods. In particular, the formalin/methanol fixation used in WC assay may lyse more cells relative to the liquid nitrogen used in the SHA assay, as fragile cells are known to be deformed or destroyed in formalin. The extent of lysis may vary depending on physiological condition of cells and result in differences in counts depending upon environmental conditions. The validity of this explanation will be the subject of further studies.

Anderson et al. (1999) explored the variability and oligonucleotide labeling intensity in *A. fundyense* cells under different growth conditions using the WC assay. Labeling intensity (reflective of rRNA levels) tended to decrease with nutrient limitation. In this regard, we note that in May, *A. fundyense* populations in eastern Maine may have been relatively healthy, benefiting from the high-nutrient, high-light conditions of the eastern segment of the Maine Coastal Current (Townsend et al., 2001). The relatively good agreement between the two methods at that location and time (Fig. 8A) may reflect the absence of nutrient limitation during the early phase of the bloom. In June 2001, however, nutrient limitation was much more widespread than was the case in May (Poulton, 2000; Dyhrman and Anderson, 2003). A significant number of SHA counts were lower than the WC counts at that time (Fig. 8B), perhaps as a result of this nutrient limitation. This possibility was recently explored in the context of *Pseudo-nitzschia multiseries* grown in continuous cultures limited by

silicate and nitrate. SHA analysis of cultures limited by silicate tended to yield higher signals than those limited by nitrate for the same number of cells (Miller et al., 2004). A WC count would not be subject to this effect, since the amount of rRNA is not measured. Cells that are labeled are counted regardless of whether the intensity of that labeling varies between samples. The human eye tends to adjust to light intensity so as to obscure these types of intensity differences.

Yet another possibility is that the differing abundance estimates may be a reflection of grazing pressure. WC assays estimate the intact cells present in a dynamic food web, while the SHA estimates the total *A. fundyense*-specific rRNA released from particulate material. Therefore, when grazing pressure is low, WC assays and the SHA may tend to agree. In areas of intensive grazing (e.g., deep samples in vertical profiles), SHA estimates may be higher than WC counts due to rRNA in fecal pellets and detritus. Indeed, in June 2001, samples with high SHA counts relative to WC counts typically contained abundant copepods and fecal pellets, although this is a subjective observation. In addition, we have observed labeled cells in fecal pellets (Fig. 3E). Such images clearly show how *Alexandrium* cells could be entrained in material that would affect WC counts of free-living organisms. Consistent with this possibility, Durbin et al. (2002) have shown that North Atlantic right whales in the lower Bay of Fundy are exposed to *Alexandrium* spp. and PSP toxins via a zooplankton vector, *Calanus finmarchicus*. Thus, *Alexandrium* spp. along with molecules indicative of their presence are moving through the food web and this should give rise to discontinuities between WC and cell-free detection formats (e.g., SHA) as observed here. In support of this same concept, Nejstgaard et al. (2003) present preliminary evidence showing how DNA sequences specific for some phytoplankton can be recovered from copepods and their fecal pellets. These authors suggest that DNA can be used as a means of identifying prey items of zooplankton given their relatively neutral gut pH and short digestion times. It is not yet clear if this concept is applicable to detection of rRNA indicative of *Alexandrium* spp. in natural samples using the SHA, though this possibility has been raised previously in the context of *Heterosigma* (see Tyrrell et al., 2002). Indeed, O'Halloran and Silver (2003) have since shown that both the SHA (LSU rRNA-targeted) and a PCR assay (rDNA ITS-targeted; Connell, 2002) can indicate presence of *Heterosigma* in natural samples when intact cells are not visible microscopically.

A final explanation for the observed differences between *Alexandrium* spp. abundance estimates based on WC and SHA formats could rest with variable background in the SHA due to sample matrix effects. If the background OD is raised non-specifically due to non-specific binding or cross-reactivity, e.g., estimates of *A. fundyense* abundance will obviously be in error. Scholin et al. (1999) demonstrated how this could occur when those authors used multiple *Pseudo-nitzschia* probes simultaneously and grew suspicious of SHA results for multiple organisms that rose and fell in concert with one another. (OD values obtained using multiple probes are generally not similar and do not co-vary numerically.) The net effect of variable background OD depended a great deal on the slope of the standard curve for a given probe. Slight changes in background OD will have a large impact on estimates of cell abundance when the calibration slope is steep and the SHA reacts strongly against the target species, since it takes relatively few cells to elicit a positive response and change in the OD. This could present a serious problem for quantifying North American *A. fundyense* because the SHA is very sensitive to this species. The problem becomes even worse at low *A. fundyense* cell abundance, since in those instances, the signal from background could be large compared to the contribution from target cells. Since only a single species was targeted in this study using the SHA, it was not possible to "flag" questionable results as Scholin et al. (1999) did. Future applications of the *A. fundyense* SHA should utilize some form of negative control or utilize probes for more than one species so that potential problems like an increase in assay background can be readily identified. It is also clear from the lack of correlation we observed for samples where the WC counts were $<25$ cells $l^{-1}$ that extremely low cell densities may not yet be amenable to SHA analysis, as the concentration of water samples to capture more *A. fundyense* cells enhances background or matrix problems. Efforts to reduce background OD represent yet another area of active investigation.

### 4.4. Calibration issues

In this study, the SHA was calibrated using cultured cells. This approach was deemed appropriate

after comparison of standard curves obtained using cultures with those from California and Casco Bay, Maine bloom populations, which were essentially the same (Fig. 2). For situations in which *A. fundyense* abundances are needed in near real-time, such as during mapping efforts on-board ship, a new standard curve should be generated from serially diluted culture material using the same batch of probe and reagents intended for the cruise. Alternatively, if immediate analysis is not required, WC counts of subsamples collected from the same water sample used for the SHA can serve to "ground-truth" the initial calibration curve established from culture. These field subsamples should be chosen from both surface and deep waters and should include diverse areas of the sampling domain that might represent different growth conditions or areas containing potentially interfering biomass (matrix effects).

Even this approach is subject to errors, however, since as described here, WC assays from the field may not detect all cells due to the presence of detritus, grazing and the production of fecal pellets, and/or cell permeability issues, while the SHA could be influenced by interfering compounds or biomass. Given the inherent uncertainty in cell abundance estimates of any type, calibration using cultured cells is thus far from the approach of choice, yet other options are equally problematic. This is another area that deserves further study on a regional basis. Indeed, the same conclusions were reached by Matweyou et al. (2004) using the NA1 probe in both WC and SHA formats in studies of PSP outbreaks on Kodiak Island, Alaska. Those authors also found calibration of the SHA with respect to WC counts to be somewhat problematic and raised a similar series of possibilities as discussed here. Matweyou et al. (2004) nevertheless concluded that the SHA could serve as a valuable proxy for estimating the abundance of *Alexandrium* in water samples, especially with respect to predicting a toxic event in the water column prior to harvesting shellfish. In that regard, the most useful application of the SHA may be in providing a range of cell density estimates to be used as a risk assessment tool or for a general indication of cell abundance. The same conclusion was reached by Tyrrell et al. (2001) with respect to monitoring for blooms of *Heterosigma* and *Fibrocapsa* that could lead to a fish mortality event. We note the strong similarity between the cell distribution maps obtained using WC and SHA methods, as presented in Figs. 10 and 11. Despite the fundamental differences between the two counting methods, they do provide valuable distributional information in a rapid manner that can be of great value in management and research.

## 5. Conclusions

Molecular probes show great promise for HAB monitoring programs, yet more work is clearly needed to understand what different probe types and assay formats actually measure within particular regions—in our case in the GOM. No particular cell counting method can yet be considered the "gold standard" against which all other methods should be compared, and in reality, this is never likely to be the case. Each situation and location has unique requirements and constraints that have to be considered in choosing an enumeration method. In some cases, speed and sample throughput might be the dominant consideration, whereas in others, high-resolution discrimination among species and cell enumeration might be paramount. These considerations dictate different approaches. With regard to high sample throughput, brightfield microscope counts take much more time than the three probe approaches evaluated here, and this traditional procedure introduces errors due to the difficulty in discriminating among morphologically similar but taxonomically or toxicologically distinct taxa such as *A. fundyense* and *A. ostenfeldii*. Antibody probes increase sample throughput and facilitate species identification, but specificity issues can lead to errors, as seen here for the M8751-1 antibody and the mixed *Alexandrium* populations in the GOM. WC assays using the NA1 oligonucleotide probe (in conjunction with a similar probe specific for *A. ostenfeldii*) enhanced sample throughput and eliminated specificity concerns, but the WC count estimates may still be in error due to issues such as variability in cell permeability, rRNA accessibility, or cell lysis among samples. There is also the possibility that intact cells might be masked by detritus or fecal pellets. The SHA offers still more increases in sample throughput, but introduces its own uncertainties, such as those due to sample matrix effects, variable rRNA levels in intact cells or to the possible presence of target rRNA in cell fragments, fecal pellets, or detritus. These problems were most severe at extremely low cell densities that may not be appropriate for this method, at least until background OD can be reduced. More work is clearly needed to compare

and contrast these different methods on additional field samples, hopefully in parallel with efforts to incorporate these procedures in monitoring programs or in automated instruments designed to detect HABs. Molecular probes are highly useful tools for monitoring and research applications, but there is a clear need for further characterization and refinement of the methods and protocols.

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