

IDENTIFICATION AND ENUMERATION OF CULTURED AND WILD *PSEUDO-NITZSCHIA* (BACILLARIOPHYCEAE) USING SPECIES-SPECIFIC LSU rRNA-TARGETED FLUORESCENT PROBES AND FILTER-BASED WHOLE CELL HYBRIDIZATION¹

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ABSTRACT

Efforts to understand the ecologic and environmental parameters that govern harmful algal blooms (HABs) require rapid and specific identification of causative species. Traditional methods of species identification using light and electron microscopy are useful in this regard but are also time consuming, making routine analysis of a large number of samples difficult. Techniques that speed and ease the detection of HAB species as they occur in natural populations are therefore desirable. In this paper, we continue efforts to develop species-specific large subunit ribosomal RNA (LSU rRNA)-targeted fluorescent DNA probes for a variety of *Pseudo-nitzschia* H. Peragallo species, a group of marine pennate diatoms that includes representatives linked to production of domoic acid and amnesic shellfish poisoning (ASP). A custom filter tube and filtration manifold that has utility for both whole cell (in situ) hybridization as well as preparing samples for scanning electron microscopy (SEM) is described. Filter-based whole cell hybridization was used to identify a variety of newly isolated *Pseudo-nitzschia* clones, and probe results were confirmed using SEM. Some isolates of *P. pungens* (Grunow) Hasle exhibited variable (intraclonal) reactivity toward the *P. pungens*-specific probe. Three isolates of *P. subpacificum* (Hasle) Hasle were found to cross-react with probes designed for *P. fraudulenta* (Cleve) Hasle and *P. heimii* Manguin. Four isolates did not react with any species-specific probes; this group comprised three distinct morphotypes whose fine-scale morphologic features did not agree with published descriptions of *Pseudo-nitzschia* species. Evaluation of the filter method using cultured cells added to natural (whole water) samples indicated quantitative recovery of target species. Confirming results of probe assays using SEM was difficult when the target species was less than 10^4 cells·L⁻¹ in the presence of greater than 10^6 cells·L⁻¹ of other nontarget diatom species. A variety of *Pseudo-nitzschia*, including *P. australis* Frenquelli, *P. fraudulenta*, *P. heimii*, *P. pseudodelicatissima* (Hasle) Hasle, *P. pungens*, *P. multiseriata* (Hasle) Hasle, and *Nitzschia americana* Hasle, were identified using whole cell hybridization in a variety of field samples containing mixed assemblages of plankton, and these results were confirmed using SEM. The filter tube method of applying probes was used onboard ship for near real-time identification and enumeration of a variety of *Pseudo-nitzschia* species.

Key index words: amnesic shellfish poisoning (ASP); Ba-

cillariophyceae; domoic acid; fluorescent oligonucleotide; in situ hybridization; LSU rRNA; *Pseudo-nitzschia*

Recent concerns over the apparent increase in geographic distribution, frequency, and intensity of harmful algal blooms (HABs) has focused attention on the need to develop a better understanding of the ecologic and environmental parameters that govern HAB events (Anonymous 1995, Hallegraeff 1995, Boesch et al. 1997). The same concerns also highlight the importance of monitoring for causative species and the toxins they may produce to mitigate economic and public health impacts that may arise locally during the course of a harmful bloom (Shumway 1990, Smayda 1990, Taylor 1990, Hallegraeff 1993, 1995, Anderson 1994). A fundamental component of improving our understanding of HABs at both global and local scales is identifying and enumerating particular microalgal species collected in discrete water samples—a task that relies traditionally on microscopy-based observations. Although the latter methods are extremely valuable in this regard, they can be difficult to implement when rapid determinations of the presence and abundance of particular species in a large number of samples are required routinely. Nucleic-acid-, antibody- and lectin-based probe assays have been suggested as potential means to circumvent these difficulties (Bates et al. 1993, Anderson 1995, Vrieling and Anderson 1996). Here, we describe a continuation of our efforts to develop species-specific ribosomal RNA (rRNA)-targeted, fluorescently labeled DNA probes for identification and enumeration of HAB species using *Pseudo-nitzschia* H. Peragallo as a model target group. These commonly occurring, frequently abundant marine pennate diatoms are found worldwide in coastal, offshore, and oceanic plankton (Cupp 1943, Bolin and Abbott 1963, Hasle 1972, Fryxell et al. 1991, Hallegraeff 1993, 1994, Villac et al. 1993a, b, Lange et al. 1994, Hasle et al. 1996). A number of species within this genus are associated with production of domoic acid, a neuroexcitatory amino acid responsible for a human illness known as amnesic shellfish poisoning (ASP; Bates et al. 1989, Subba Rao et al. 1989, Martin et al. 1990, Buck et al. 1992, Dickey et al. 1992, Fritz et al. 1992, Garrison et al. 1992, Horner and Postel 1993, Todd 1993, Villac et al. 1993a, b, Work et al. 1993, Lundholm et al. 1994, Villareal et al. 1994, Walz et al. 1994).

Pseudo-nitzschia are strongly elongated cells, which

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form distinctive stepped chains by overlapping cell ends—features that make the genus readily recognizable under the light microscope. However, individual species within the genus are much harder to discriminate, requiring detailed morphologic observations of valve fine structure (Hasle and Syvertsen 1996). Characters such as cell length, valve outline, and width in valve view may be observed in fresh or preserved material under the light microscope, but the finest features, which in many cases are diagnostic for making species determinations, are only revealed under scanning or transmission electron microscopy (SEM or TEM). Thus, identification and enumeration of particular *Pseudo-nitzschia*, especially in natural populations harboring potentially toxic and nontoxic species, is difficult and time consuming. In turn, correlating the abundance and distribution of particular *Pseudo-nitzschia* with manifestation of domoic acid in the plankton or food web is problematic.

In previous work, we and others compared large subunit ribosomal RNA (LSU rRNA) gene sequences from cultured isolates of *P. australis* Frenguelli, *P. pungens* (Grunow) Hasle, *P. multiseriis* (Hasle) Hasle, *P. fraudulenta* (P. T. Cleve) Heiden, *P. heimii* Mangin, *P. delicatissima* (P. T. Cleve) Heiden, and *N. americana* (Hasle) Fryxell (Scholin et al. 1994, 1996). These sequences revealed unique nucleotide “signatures” for each species examined—a result that agreed with designations based on conventional morphologic criteria (Miller and Scholin 1996). Putative species-specific fluorescently labeled DNA probes were designed to target these unique sequences in an effort to speed and ease species identification. Fifteen such probes were tested against cultures of the species listed above, as well as *P. pseudodelicatissima* (Hasle) Hasle, and a subset of probes was identified that discriminate each species tested (Miller and Scholin 1996). This set of probes was then used to identify newly isolated *Pseudo-nitzschia* cultures, and species identifications based on probing results were later confirmed using morphologic criteria. These preliminary studies indicated that whole cell hybridization was a useful tool for discriminating among a variety of cultured *Pseudo-nitzschia* species and was both faster and less labor intensive than SEM. Nevertheless, a number of challenges remained, including the need to test the probes against additional *Pseudo-nitzschia* clones to examine for possible cross-reactions or inconsistencies in label strength and the need to assess the utility of the labeling method for identifying and quantifying target species collected from natural populations. With regard to the latter, a further challenge was to determine the lower limits of detection for target species, particularly when they are in low abundance relative to the rest of the plankton assemblage, and to devise a strategy for verifying results of the probe-based assays using SEM. To meet these needs, we developed a custom filter tube and filtration manifold that is useful for preparing sam-

TABLE 1. Cultures used in developing filter-based whole cell probing, SEM sample preparation methodology, and cell quantification experiments. USCG, United States Coast Guard breakwater, Monterey, California; SCW, municipal wharf, Santa Cruz, California; CMB, central Monterey Bay.

Species ^a	Clone designation ^b	Isolation date	Isolation locale
<i>Pseudo-nitzschia australis</i>	au19	Nov. 1994	USCG
<i>P. australis</i>	au22	May 1995	SCW
<i>P. australis</i>	au26	Mar. 1996	SCW
<i>P. australis</i>	au28	Oct. 1996	SCW
<i>P. australis</i>	au33	Mar. 1997	USCG
<i>P. fraudulenta</i>	fr10	May 1995	SCW
<i>P. pungens</i>	pu16	Mar. 1996	SCW
<i>P. multiseriis</i>	mu1	Apr. 1995	SCW
<i>Skeletonema</i> sp.	1B	Feb. 1997	CMB
<i>Asterionella</i> sp.	2B	Feb. 1997	CMB
<i>Chaetoceros</i> sp.	4C	Feb. 1997	CMB

^a Species identifications of *Pseudo-nitzschia* follow descriptions by Hasle (1964, 1965, 1993, 1994, 1995, Hasle et al. 1996, Hasle and Syvertsen 1996).

^b Clone designations as used by MBARI and UCSC culture collections.

ples for both whole cell hybridization and SEM. The filter tube system also significantly reduces sample processing time for both techniques relative to methods we used previously (Miller and Scholin 1996). Here, we describe the application of this apparatus and protocols for its use to identify and quantify a variety of *Pseudo-nitzschia* collected from both cultured and field samples.

MATERIALS AND METHODS

Culturing. Cultures used in this study (Table 1) were isolated from Monterey Bay, California, using a 35- μ m mesh net size and were established from single cells or a single chain of cells. Cultures are currently maintained at the University of California at Santa Cruz and the Monterey Bay Aquarium Research Institute under previously described conditions (Miller and Scholin 1996).

Construction of filter tubes and filtration manifold. The custom filter manifold used for both whole cell hybridization and SEM sample preparation is shown in Figure 1. Filter tubes were constructed from standard 15- or 50-mL polypropylene conical-bottom centrifuge tubes that were welded to 13- or 25-mm polypropylene Swinnex in-line filter holders (Millipore), respectively. This was accomplished by removing a portion of the inlet side of the filter housings and by cutting away the tips of the conical-bottom tubes such that the diameters of the filter housings and tubes matched. The halves were joined by heating the surfaces on a hot plate until molten, and pressing together until solid. A U-shaped 6-12 place vacuum manifold was constructed of threaded 3/8-inch schedule 80 PVC pipe and fittings. Each place in the manifold was threaded to fit a female Luer fitting, which connected to a male locking Luer stopcock (Cole-Parmer). The opposite, female, end of the stopcocks accommodates the male Luer slip end of the filter tubes. Vacuum applied to each filter tube can be regulated individually.

SEM. Samples were prepared for SEM using a simplified KMnO_4/HCl oxidation method (Round et al. 1990, Simonsen 1974). Filter tubes (described above) were fitted with 0.2–3.0- μ m pore size Isopore polycarbonate membranes (Millipore). To process samples on 13-mm filters fitted on 15-mL filter tubes, 0.5 mL or less of midexponential-phase cultures were added per tube and for net samples as large a volume as possible while avoiding clogging the filters (e.g. 1 mL). Samples were rinsed three times using 2 mL dH_2O and brought to near dryness using gentle vacuum before adding one or two drops of saturated KMnO_4 (enough to completely cover the filter). Within one to several minutes after

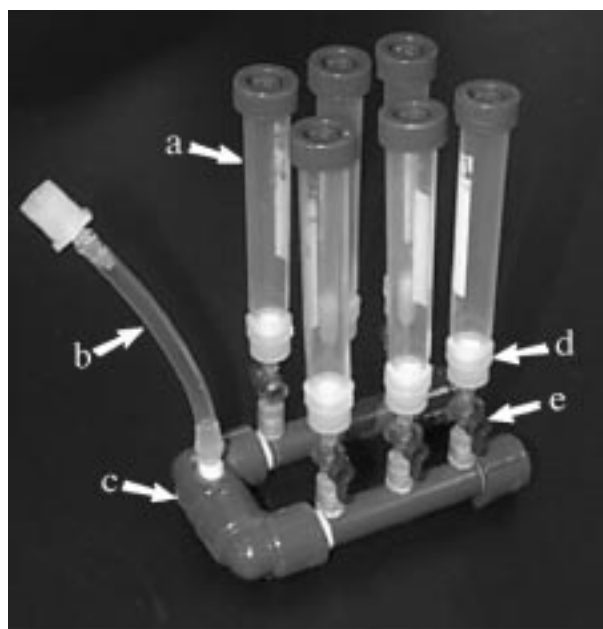


FIG. 1. Custom filter tubes and filtration manifold used for filter-based whole cell hybridization and for SEM sample preparation. Filter tubes are made by joining polypropylene centrifuge tubes (a) to Swinnex in-line filter housings (d; see text for construction details). The PVC manifold (c) is attached to vacuum source at (b), and vacuum to each tube is controlled individually by valves (e). The filter manifold can be easily configured to accommodate the desired number of filter tubes, and the entire apparatus fits easily into a water bath or dry incubator to control hybridization temperature.

adding the KMnO_4 , 1 mL of concentrated HCl was added at room temperature, filtered, and repeated as many times as necessary until samples were completely cleared of KMnO_4 . Samples were rinsed as above, and the KMnO_4 and HCl steps repeated once. After rinsing the samples three times with 5 mL dH_2O , filters were removed from filter tubes and bonded to aluminum stubs using colloidal graphite (Ted Pella, Inc., Redding, California), dried at 60°C , sputter-coated with gold-palladium, and viewed using an ISI WB6 scanning electron microscope. Species identifications fol-

lowed criteria established by Hasle (1964, 1965, 1972, 1993, 1994, 1995, Hasle et al. 1996, Hasle and Syvertsen 1996).

Whole cell probing. Approximately 0.25–1 mL of midexponential culture or net tow, or 10 mL of whole (unconcentrated) seawater, were added to 15-mL filter tubes configured with 1.2- μm pore size, 13-mm diameter Isopore (Millipore) or Cyclopore (Whatman) polycarbonate membranes. A slight vacuum (100 mm Hg) was used to filter samples until dry. Five milliliters of freshly prepared saline ethanol fixative (Scholin et al. 1996) were added to each tube and allowed to stand for 1–2 h at room temperature. Afterward, samples were filtered and rinsed once with 1 mL of $5\times$ SET hybridization buffer (Miller and Scholin 1996), resuspended in 0.5 mL of $5\times$ SET hybridization buffer, to which was added 2.5 μg of the desired probe (Table 2). The filter tube assembly was placed in an incubator or immersed in a water bath to maintain temperature, and hybridization reactions were allowed to proceed for 1–2 h. Excess unbound probe was removed by filtering and rinsing for several minutes with 1 mL of $5\times$ SET hybridization buffer at room temperature. Filters were removed from the filter tubes and placed on microscope slides sample side up, and 20 μL SlowFade Light (Molecular Probes, Eugene, Oregon) were added before mounting cover slips. Samples were viewed using a Zeiss Axioskop fitted with a fluorescein band-pass filter set (excitation 465–495 nm; emission 515–555 nm) and a 50 W light source. Photos were taken using an Olympus 10AD 35-mm camera system and Kodak Gold 400 ASA film. Epifluorescence images were recorded using a constant exposure time of 32 s.

Assessing lower limits of detection using filter-based whole cell hybridization and utilization of SEM to verify results of probe assays. The cell density of *P. australis* (clone au33, Table 1) was estimated by averaging three or more replicate counts of cells per 5 μL , and the culture was then serially diluted to theoretical densities of 1250, 2500, 5000, and 10,000 cells $\cdot\text{L}^{-1}$. In the first experiment, filtered seawater alone was used as the diluent. In a second experiment, the diluent consisted of a mixture of filtered seawater to which was added a total of 5.5×10^6 cells $\cdot\text{L}^{-1}$ of cultured *Skeletonema* sp. Greville, *Asterionellopsis* sp. Castracane, and *Chaetoceros* sp. Ehrenberg (Table 1). Six replicates of all treatments were analyzed using filter-based whole cell hybridization, as described above, by adding 10-mL aliquots to separate filter tubes and probing with the *P. australis*-specific probe auD1 (Table 2). The entire surface of each filter was scanned using epifluorescence microscopy, and labeled cells were counted. Counts for each treatment were averaged and multiplied by 100 to estimate the number of target cells per liter. At the same time samples were processed using whole cell probing, additional 10-mL aliquots of each treatment were prepared for SEM analysis using the filter tube method as described above.

Enumeration of cultured *Pseudo-nitzschia* species added to natural seawater. Cell densities of exponential phase cultures of *P. australis*

TABLE 2. Oligonucleotide probes, sequences, hybridization conditions, and targeted species.

Probe ^a	Sequence (5'-3') ^b	Hybridization conditions ^c		Species targeted
		T ^o C	SET	
uniC	GWATTACCGCGGCKGCTG	45	5 \times	positive control
uniR	CAGCMGCGCGGUAUWC	45	5 \times	negative control
NA1	AGTGCAACTCCCACCA	45	5 \times	<i>Alexandrium tamarense</i>
auD1	AAATGACTCACTCCACCAGG	45	5 \times	<i>P. australis</i>
puD1	ATGACTCACTTTACCA	45	5 \times	<i>P. pungens</i>
muD1	ATGACTCACTCTGCCA	45	5 \times	<i>P. multiseriata</i>
muD2	AAGCCCACAGCGCCAAGCC	55	5 \times	<i>P. multiseriata</i> , <i>P. pseudodelicatissima</i>
heD2-2	TATCCACAGCGCCACCA	45	5 \times	<i>P. heimii</i>
frD1	AAAGACTCATTCTACCAGG	45	5 \times	<i>P. fraudulenta</i>
deD1	AGACTCACTCTACCA	45	5 \times	<i>P. delicatissima</i>
amD1	ATGACTCATTACGCCA	45	5 \times	<i>Nitzschia americana</i>

^a uniC = Positive control, SSU-targeted universally conserved sequence (519r; Field et al. 1988, Embley et al. 1992); uniR is the complement of uniC. NA1 has also been used as a negative control when probing for *Pseudo-nitzschia* (Scholin et al. 1996).

^b rRNA complement.

^c Hybridization buffer = $5\times$ SET, 0.1% (v/v) Nonidet P-40, 25 $\mu\text{g}\cdot\text{mL}^{-1}$ polyadenylic acid (poly A). $25\times$ SET = 3.75 M NaCl, 25 mM EDTA, 0.5 M Tris, pH 7.8 (Miller and Scholin 1996).

TABLE 3. Probe-based identification of new unicellular isolates compared to SEM-based identification.

Culture ^a	Probe reactivity ^b										SEM identification
	pos	neg	auD1	muD1	puD1	frD1	heD2-2	deD1	muD2	amD1	
mu3	+	-	-	+	-	-	-	-	+	-	<i>P. multiseriis</i>
pu12	+	-	-	-	+ ^c	-	-	-	-	-	<i>P. pungens</i>
pu14	+	-	-	-	+ ^c	-	-	-	-	-	<i>P. pungens</i>
pu15	+	-	-	-	+ ^c	-	-	-	-	-	<i>P. pungens</i>
pu16	+	-	-	-	+ ^c	-	-	-	-	-	<i>P. pungens</i>
pu18	+	-	-	-	+	-	-	-	-	-	<i>P. pungens</i>
pu19	+	-	-	-	+	-	-	-	-	-	<i>P. pungens</i>
pu20	+	-	-	-	+	-	-	-	-	-	<i>P. pungens</i>
pu21	+	-	-	-	+	-	-	-	-	-	<i>P. pungens</i>
pu22	+	-	-	-	+	-	-	-	-	-	<i>P. pungens</i>
pu23	+	-	-	-	+	-	-	-	-	-	<i>P. pungens</i>
au23	+	-	+								<i>P. australis</i>
au24	+	-	+								<i>P. australis</i>
au25	+	-	+								<i>P. australis</i>
au26	+	-	+								<i>P. australis</i>
au27	+	-	+	-	-	-	-	-	-	-	<i>P. australis</i>
au28	+	-	+	-	-	-	-	-	-	-	<i>P. australis</i>
au33	+	-	+	-	-	-	-	-	-	-	<i>P. australis</i>
fr11	+	-	-	-	-	+	-	-	-	-	<i>P. fraudulenta</i>
fr12	+	-	-	-	-	+	-	-	-	-	<i>P. fraudulenta</i>
fr13	+	-	-	-	-	+	-	-	-	-	<i>P. fraudulenta</i>
fr14	+	-	-	-	-	+	-	-	-	-	<i>P. fraudulenta</i>
sp1	+	-	-	-	-	+ ^d	+	-	-	-	<i>P. subpacifica</i>
sp2	+	-	-	-	-	+ ^d	+	-	-	-	<i>P. subpacifica</i>
sp3	+	-	-	-	-	+ ^d	+	-	-	-	<i>P. subpacifica</i>
ps9	+	-	-	-	-	-	-	-	+	-	<i>P. pseudodelicatissima</i>
ps12	+	-	-	-	-	-	-	-	+	-	<i>P. pseudodelicatissima</i>
M1 7/12 1C	+	-	-	-	-	-	-	-	-	-	not determined
M1 7/12 3B	+	-	-	-	-	-	-	-	-	-	not determined
M1 7/12 3A	+	-	-	-	-	-	-	-	-	-	not determined
M1 7/12 4B	+	-	-	-	-	-	-	-	-	-	not determined

^a Culture designation assigned upon SEM analyses.

^b Blank spaces indicate reaction not tested.

^c Tests of puD1 against these *P. pungens* clones initially gave negative results but were later retested and results were positive, see Discussion.

^d Reaction using probe frD1 gave a weak positive label.

(au26, au28), *P. multiseriis* (mu1), and *P. pungens* (pu16; Table 1) were estimated as above, and each culture was serially diluted to theoretical densities of 5000, 10,000, and 25,000 cells·L⁻¹ using whole unfiltered seawater that contained a variety of plankton species as well as organic and inorganic matter. Ten-milliliter aliquots of each dilution, including whole seawater without added culture, were analyzed in triplicate or greater using filter-based whole cell hybridization and the auD1, muD1, or puD1 probes (Table 2) for detection of *P. australis*, *P. multiseriis*, or *P. pungens*, respectively. Labeled cells were counted from the entire surface of each filter, averaged, and multiplied by 100 to estimate cells per liter.

Detection and enumeration of naturally occurring Pseudo-nitzschia species. A net tow (35- μ m mesh size) sample was collected from Santa Cruz, California, municipal wharf and returned to the laboratory for immediate analysis. Of this dense sample, 750- μ L aliquots were added to filter tubes fitted with Whatman Cyclo-pore 3- μ m pore size polycarbonate filters. Whole cell hybridizations were carried out as above using the auD1, frD1, muD1, muD2, and amD1 probes, as well as a control having no probe added. A portion of the net sample was preserved using acid Lugol's iodine (Sournia 1978) and later cleaned for SEM analysis as described above.

Filter-based whole cell hybridization was used to enumerate *Pseudo-nitzschia* species collected from Monterey Bay surface whole water samples while onboard the R/V *Western Flyer* in March 1997. Immediately after sample collection, 10-mL aliquots were analyzed as described above using the auD1, muD1, puD1, and NA1 probes, as well as positive- and no-probe controls (Table 3). The number of cells per liter was estimated by counting all labeled cells per filter and multiplying by 100. Whole water samples were also collected and

preserved using acid Lugol's iodine for SEM analysis to verify probe results.

RESULTS

Preliminary trials using the filter-based method of whole cell probing gave results similar to those described earlier (Miller & Scholin 1996, Scholin et al. 1996) when samples were processed in centrifuge tubes and cells were pelleted to facilitate exchange of fixative, hybridization, and wash solutions. For example, cultured *P. australis* and *P. fraudulenta*, hybridized with probes auD1 and frD1, respectively, as well as the positive control probe, typically showed a uniform distribution of bright fluorescence throughout the cell except for the nuclear region where fluorescence was confined to nucleoli. Treatments with negative control probe, no probe, or other *Pseudo-nitzschia* species-specific probes yielded samples that were consistently dark, demonstrating little to no nonspecific binding or autofluorescence (e.g. Fig. 2). The same filter tubes and manifold used for whole cell probing were also useful for preparing both cultured and field samples for SEM. Examples of the

TABLE 4. Experiments in which a known number of cultured *P. australis* cells were serially diluted using A) filtered sea water only (FSW) or B) FSW plus 5.5×10^6 cells·L⁻¹ nontarget cultured diatom species. Species-specific probe auD1 was applied using the filter manifold system (Fig. 1) and fluorescently labeled cells counted in replicate samples.

	Expected no. cells·L ⁻¹ ^a	Expected no. per filter	No. of replicates examined	Range (no. cells per filter)	Average no. cells per filter ± SD ^b	Calculated abundance (cells·L ⁻¹ ± SD) ^c	% Difference between expected and actual ^d
FSW only as diluent	1250	12.5	6	2–21	15.5 ± 7.1	1550 ± 710	+24
	2500	25	6	0–31	17.0 ± 11.7	1700 ± 1170	-32
	5000	50	6	38–82	54.3 ± 16.6	5430 ± 1660	+8.6
	10,000	100	6	89–116	100.5 ± 10.5	10,050 ± 1050	+0.5
FSW with nontarget species as diluent	1250	12.5	6	2–43	13.5 ± 15.5	1350 ± 1550	+8.0
	2500	25	6	21–70	36.2 ± 17.5	3620 ± 1750	+45
	5000	50	6	43–78	61.0 ± 15.3	6100 ± 1530	+22
	10,000	100	6	35–127	96.0 ± 33.7	9600 ± 3370	-4

^a Expected number of cells, based on the original density of the culture and the dilutions used, as described in Materials and Methods.

^b The entire area of the filter was counted (= cells·[10 mL]⁻¹).

^c Average of cell counts × 100 = cells·L⁻¹.

^d [(Number of cells counted - theoretical number added)/(theoretical number added)] × 100; value is negative if fewer cells are counted than were theoretically added, positive if more cells counted.

latter are shown in Figures 2 and 3e and f, respectively.

A variety of filter membranes are available for use with the filter tube system shown in Figure 1. The best choice of membrane depends on how one intends to visualize the sample. The near optical transparency of Whatman Cyclopore membranes make them particularly useful for viewing samples using epifluorescence along with transmitted light microscopy (e.g. Fig. 3). However, if samples were being processed for either SEM or epifluorescence microscopy alone, where membrane transparency is not important, then we found that the Millipore Isopore membranes are a good choice because they allow filtration of a greater sample volume before clogging, compared to the Whatman Cyclopore membranes.

Thirty-one newly established and as yet unidentified cultures from Monterey Bay, California, each a distinct clone isolated from a single chain of cells,

were tested with the suite of probes listed in Table 2 to determine species identifications. Results indicated that one was *P. multiseriis*, 10 were *P. pungens*, seven were *P. australis*, four were *P. fraudulenta*, two were *P. pseudodelicatissima*, three were labeled by both the frD1 and heD2-2 probes, and four were not labeled by any of the species-specific probes. For *P. multiseriis*, *P. pungens*, *P. australis*, *P. fraudulenta*, and *P. pseudodelicatissima*, probe-based species designations were confirmed using SEM (e.g. Fig. 2). SEM analysis of the three cultures labeled by both frD1 and heD2-2 probes determined that they were *P. subpacifici* (Hasle) Hasle. SEM of the four cultures not identified by probes revealed three distinct morphotypes which did not agree with published descriptions of known *Pseudo-nitzschia* (data not shown).

Pseudo-nitzschia australis was diluted to theoretical densities of 1250, 2500, 5000, and 10,000 cells·L⁻¹ using either filtered seawater (FSW) or FSW to which

TABLE 5. Experiments in which known numbers of cultured *P. australis*, *P. multiseriis*, and *P. pungens* cells were serially diluted using whole seawater, species-specific probes applied using the filter manifold system (Fig. 1), and fluorescently labeled cells counted in replicate samples.

Species	Expected no. cells·L ⁻¹ ^a	Expected no. per filter	No. of replicates examined	Range (no. cells per filter)	Average no. cells per filter ± SD ^b	Calculated abundance (cells·L ⁻¹ ± SD) ^c	% Difference between expected and actual ^d
<i>P. australis</i>	5000	50	9	29–60	50.0 ± 10.0	5000 ± 1000	no difference
	10,000	100	9	66–152	109.6 ± 33.20	10,960 ± 3320	+9.6
	25,000	250	9	175–323	251.7 ± 49.18	25,170 ± 4918	+0.68
<i>P. multiseriis</i>	5000	50	3	30–79	50.3 ± 25.5	5030 ± 2550	+0.6
	10,000	100	3	73–96	82.3 ± 12.1	8230 ± 1210	-17.7
	25,000	250	3	146–230	196.3 ± 44.41	19,630 ± 4441	-21.5
<i>P. pungens</i>	5000	50	3	31–77	52.0 ± 23.2	5200 ± 2320	+4.0
	10,000	100	3	74–93	86.3 ± 10.7	8630 ± 1070	-13.7
	25,000	250	3	179–318	239.3 ± 71.3	23,930 ± 7130	-4.3

^a Expected number of cells, based on the original density of the culture and the dilutions used, as described in Materials and Methods.

^b The entire area of the filter was counted (= cells·[10 mL]⁻¹).

^c Average of cell counts × 100 = cells·L⁻¹.

^d [(Number of cells counted - theoretical number added)/(theoretical number added)] × 100; value is negative if fewer cells are counted than were theoretically added, positive if more cells counted.

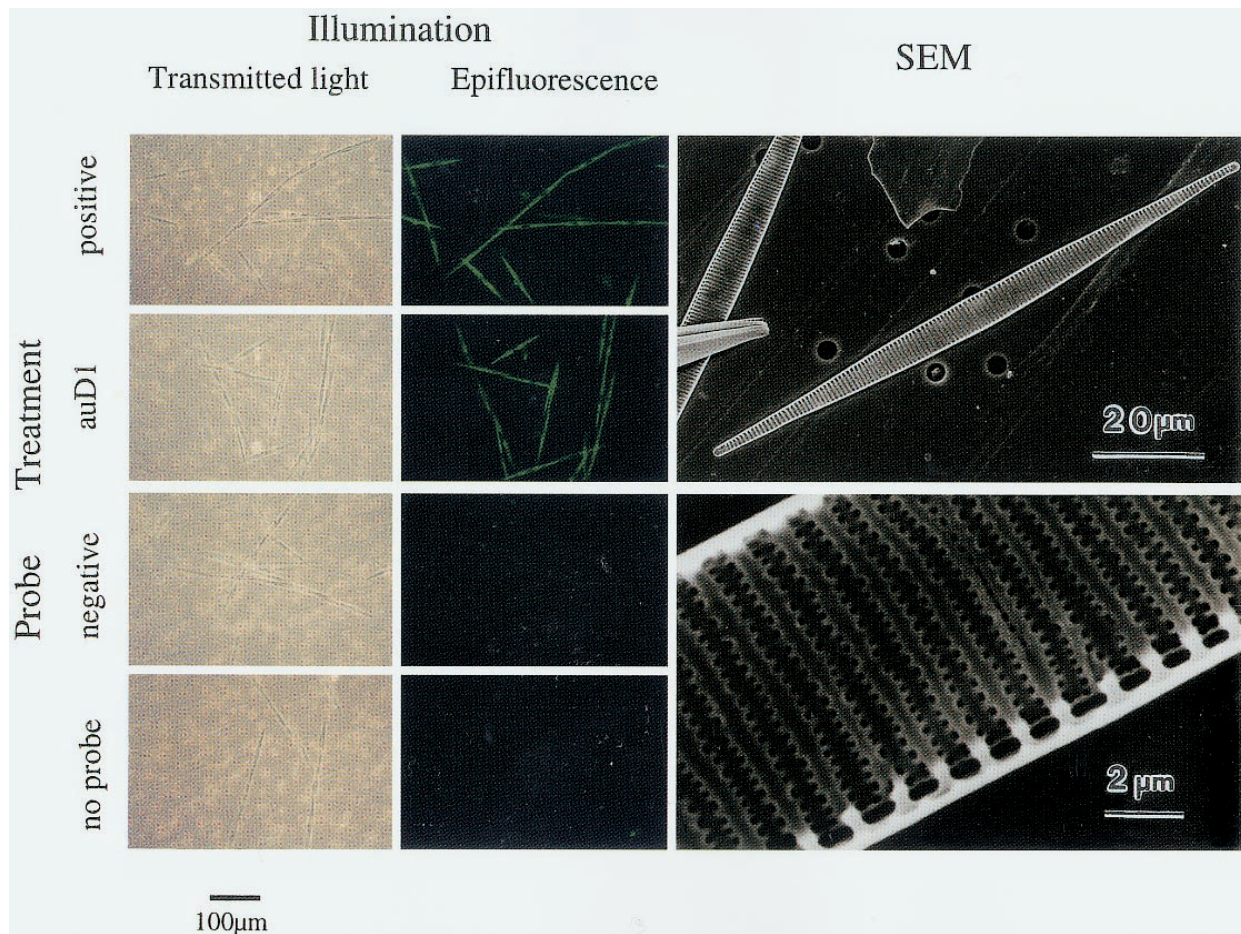


FIG. 2. Example of results obtained using filter-based whole cell probing and filter-based SEM preparation to identify a pure culture of *P. australis*. Transmitted and epifluorescence light micrographs were taken using constant exposure times to allow comparison of treatments. The epifluorescence images illustrate the range of fluorescence intensities observed from cells hybridized with positive and negative control probes and species-specific probe auD1 and a treatment with no probe added. Probe-based results are confirmed using traditional morphologic criteria, as shown in the scanning electron micrographs.

was added approximately 5.5×10^6 cells·L⁻¹ of cultured *Skeletonema* sp., *Asterionellopsis* sp., and *Chaetoceros* sp. (hereafter referred to as nontarget spp.). Ten-milliliter aliquots of each dilution (six replicates each) were incubated with the auD1 probe using the filter tube system. Labeled *P. australis* cells were then enumerated using epifluorescence microscopy. In addition, single 10-mL aliquots of the same dilutions were also processed for SEM using the filter tube system.

For those treatments in which FSW was used as the diluent (Table 4), agreement between expected and actual cell densities was excellent for densities of 10,000 and 5000 cells·L⁻¹ with the difference between expected and actual ranging from 0.5%–8.6%. For trials with 2500 and 1250 cells·L⁻¹, the difference was 32% fewer and 24% greater, respectively. For those treatments in which FSW plus nontarget spp. was used as the diluent, agreement between actual and expected was similar to the above (Table 4). The results for the 10,000 cell·L⁻¹ dilution was 4% fewer cells counted than expected, and for the 5000, 2500 and 1250

cells·L⁻¹ dilutions, there was 22%, 45%, and 8.0%, respectively, more cells counted than expected.

SEM analysis of samples diluted with FSW alone showed that samples diluted to 10,000 cells·L⁻¹ had sufficient cleaned valves distributed on the filter to make detection relatively easy (e.g. detection of valves within less than 1 min of scanning the filter using 500× magnification). Valves were also detectable on filters from samples that were diluted further; however, valves were increasingly sparse with greater dilution. Likewise, *P. australis* valves were seen in each of the samples that also contained a background of nontarget species, but detection in this case was considerably more difficult and required higher magnification and far more careful observation (e.g. using 1000× magnification it took over 10 min to find any *P. australis* valves at the greatest dilution and several minutes at the 10,000 cells·L⁻¹ dilution).

To determine if targeted cells were recovered quantitatively when using natural (unfiltered) seawater as a

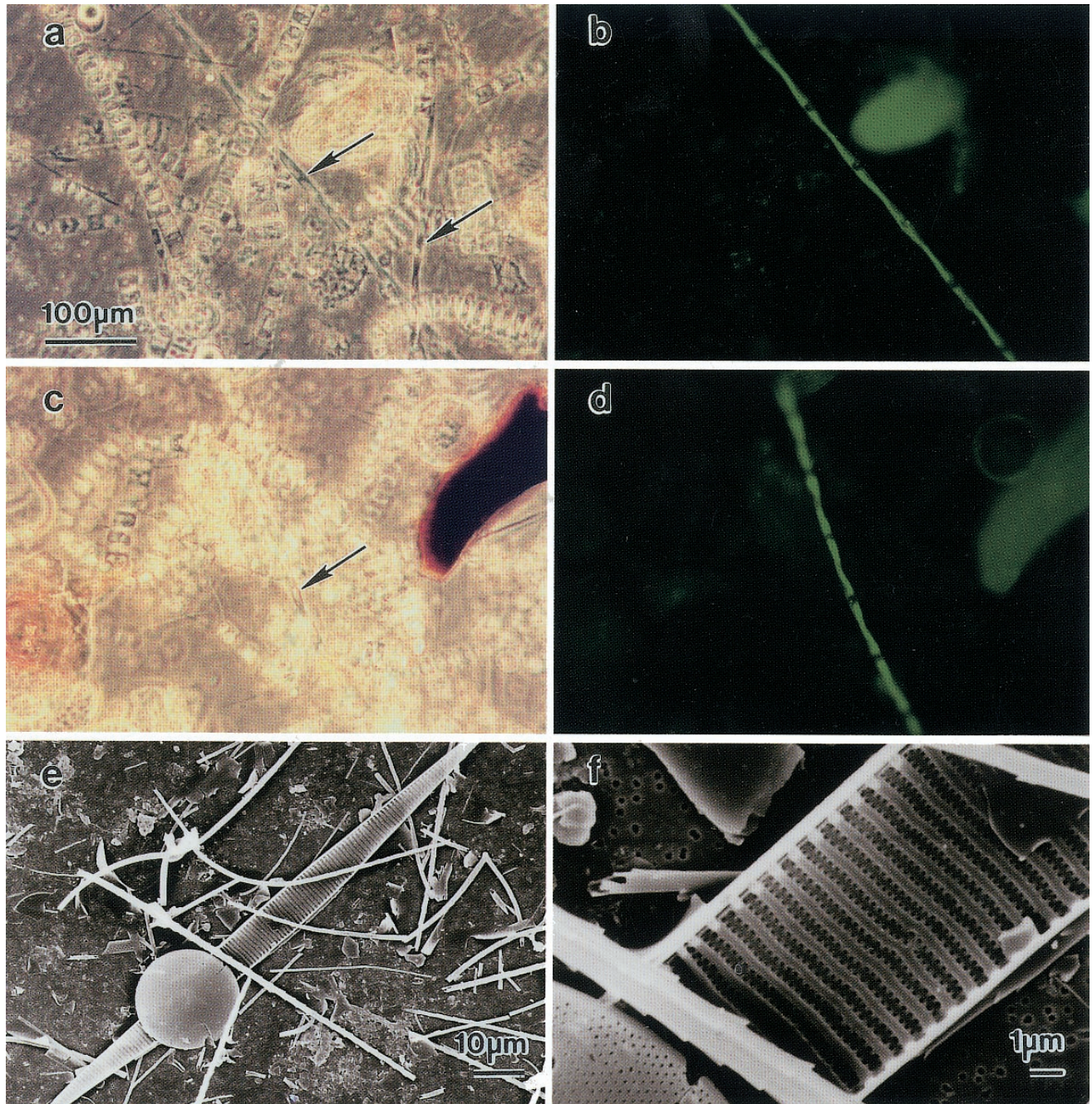


FIG. 3. Example of the type of results obtained using the filter tube system for whole cell probing and SEM analysis of a field sample. a) Transmitted light micrograph of a sample that has been treated with *P. australis* species-specific probe auD1 and shows two chains of *Pseudo-nitzschia* (arrows) in a mixed assemblage of plankton. Note in the corresponding epifluorescence micrograph (b) the single chain of labeled *P. australis* and the lack of label in the nontarget chain. c) Another region of the same sample that has such an abundance of material layered above the filter membrane that it almost entirely buries a chain of *Pseudo-nitzschia* (arrow). Despite being obscured by the material above it in the transmitted light micrograph, the chain was visible using epifluorescence (d). e, f) SEM image of the same sample confirming the presence of *P. australis*.

diluent, cultures of *P. australis*, *P. multiseriis*, and *P. pungens* were each serially diluted in whole seawater to theoretical densities of 5000, 10,000, and 25,000 cells·L⁻¹, and actual densities were then estimated by collecting 10-mL aliquots and applying the auD1, muD1, and puD1 probes using the filter tube system.

For each trial, three replicates, with no culture added, were probed to verify absence of target *Pseudo-nitzschia* from the whole seawater used as the diluent. In all cases, there was good agreement between the density of cells expected versus that determined empirically (Table 5). The difference between theoretical and es-

TABLE 6. Examples of using a subset of the probes listed in Table 2 for near real-time identification and enumeration of *Pseudo-nitzschia* species from surface whole water samples while underway onboard ship. Aliquots of each sample were preserved and later processed for SEM to confirm probe-based results. Analysis of numerous samples collected during the course of this cruise showed *Pseudo-nitzschia* to be largely absent from Monterey Bay and adjacent waters (see text).

Sample	Number of cells counted for each probe treatment ^a					Species detected using SEM
	uniC ^b	NA1	auD1	muD1	puD1	
1	126	0	35	0	0	<i>P. australis</i> , <i>P. cf. pseudodelicatissima</i>
2	35	0	0	0	0	<i>P. fraudulenta</i> , <i>P. cf. pseudodelicatissima</i>

^a Ten milliliters of whole water filtered per sample; the entire surface of the filter was examined and labeled cells counted.

^b Universal, positive control probe used to count total *Pseudo-nitzschia*.

timated abundance of cells for all trials ranged from approximately 21% fewer to 10% greater than expected.

A net tow sample taken from the Santa Cruz, California, municipal wharf during the 1996 spring bloom was observed under the stereoscope to contain a relatively small number of *Pseudo-nitzschia* spp. mixed with an abundance of other diatoms (including *Chaetoceros*, *Ditylum* J. W. Bailey, *Asterionellopsis*, *Eucampia* Ehrenberg, *Skeletonema*, and others), dinoflagellates, copepods, and larval forms. Analysis using the filter tube system and species-specific probes auD1, frD1, amD1, muD1, and muD2 revealed several chains of *P. australis*, two chains of *P. fraudulenta*, very abundant *N. americana* (epiphytic on *Chaetoceros* setae), and a small number of *P. pseudodelicatissima*, as judged by epifluorescence microscopy. An example of the type of results obtained for one probe treatment is shown in Figure 3a–d of *P. australis*. SEM analysis of this sample verified the presence of *P. australis*, *P. fraudulenta*, and *N. americana*, but not *P. pseudodelicatissima*. In addition to these species, a small number of *P. pungens*, for which we did not probe, were detected by SEM. Figures 3e and f show an SEM micrograph of this sample revealing *P. australis* as was predicted by probe analysis.

Surface whole water samples were collected while at sea during March 1997 onboard the R/V *Western Flyer*, and 10-mL aliquots were processed immediately using the auD1, muD1, puD1, NA1, and uniC probes and no probe as well; aliquots were also preserved for subsequent SEM analyses (Table 6). A variety of *Pseudo-nitzschia* species were identified among an otherwise predominantly mixed assemblage of centric diatoms; no *Alexandrium* (Halim) Balech were identified in any of the samples. In one sample, 126 *Pseudo-nitzschia* were identified in the positive control treatment, and 35 were identified as *P. australis* by the probe auD1. Later, SEM analysis verified the presence of *P. australis* and also confirmed the presence of another species that was not

probed for in these trials, *P. cf. pseudodelicatissima*. In another sample, 35 *Pseudo-nitzschia* were counted in the positive control treatment, and none were detected by any of the species-specific probes. Observation of this sample by SEM confirmed the absence of species probed for, but did reveal a small number of *P. fraudulenta* and *P. cf. pseudodelicatissima*. Numerous other samples showed *Pseudo-nitzschia* species absent from the surface waters over the course of the cruise.

DISCUSSION

The results presented here support our previous conclusions (Miller and Scholin 1996, Scholin et al. 1996) that whole cell hybridization is a useful technique for discriminating among cultured *Pseudo-nitzschia* species. The custom filter tube and vacuum manifold system (Fig. 1) improved the speed and ease of applying probes to detect and quantify *Pseudo-nitzschia* species collected from cultured or natural samples (see also Scholin et al. 1997). Recently, a similar approach was adopted by Lim et al. (1996) to facilitate quantitative identification of marine and freshwater nanoplanktonic protists. Specific advantages of the apparatus described here are that it is cheap, easy to construct, expandable, and also facilitates SEM sample preparation of cultured and natural samples. In particular, here and in a related study (Scholin et al. 1997), we have demonstrated that it is possible to discriminate and quantify in near real-time a variety of *Pseudo-nitzschia* species collected from natural populations. For toxic representatives, such as *P. australis*, rapid detection was possible at target cell levels well below those of public health concern. In the discussion that follows we address each of these points in detail, as well as the relative strengths and weaknesses of using fluorescence *in situ* hybridization for detecting and enumerating *Pseudo-nitzschia* species in natural samples.

Filter-based whole cell hybridization reduced the labor associated with sample processing compared to our original method (Miller and Scholin 1996, Scholin et al. 1996) by eliminating the need for multiple centrifugation steps, and repetitive transfers of samples to and from centrifuge tubes. In addition, samples were retained within a single tube for fixation, washing, hybridizing, and rinsing steps, reducing sample loss that was associated with each step of the original protocol and enabling enumeration of target species. Similarly, the filter tubes also improved processing for SEM by eliminating many cumbersome centrifugation steps and sample manipulations. Moreover, filter-based SEM sample preparations were consistently well cleaned (e.g. Fig. 2, 3e, f), and the method appeared to be less destructive to lightly silicified frustules compared to methods we used previously (Scholin et al. 1994, Miller and Scholin 1996). This resulted in fewer broken or eroded valves, improving our ability to identify a wide range of

Pseudo-nitzschia species. For example, delicate structures such as girdle bands were frequently lost when we applied methods requiring centrifugation and aspiration steps; using filter-based sample preparation these structures were retained to a much greater extent. The filter-based method also made it possible to work with very small sample volumes (100 μL or less of midexponential culture, 250 μL of net tow, or 10 mL of whole water), reducing the need for collecting relatively large sample volumes prior to SEM analysis. These attributes along with those discussed below suggest that the filter tube system of the type shown in Figure 1 has broad application for both whole cell probing and SEM.

Newly isolated clones of *Pseudo-nitzschia* that had not yet been identified to species provided an opportunity to test the specificity of the probes. Using the new isolates as "unknowns" we correctly identified *P. multiseriata*, *P. pungens*, *P. australis*, *P. fraudulenta*, and *P. pseudodelicatissima* and verified these predictions using SEM analysis. Four of the *P. pungens* isolates initially were not identified by probes (however, see below) and later were identified by SEM as *P. pungens*. Three isolates were weakly labeled by probe frD1 but strongly labeled by heD2-2 (see Miller and Scholin 1996 for examples of labeling strength); SEM analysis revealed these clones to be *P. subpacificae*, a species for which we have neither sequence data nor specific probe. Four isolates were not identified by any of the probes used. Three distinct morphotypes comprise the latter group, but their species designations are still unknown as their fine-scale morphology does not appear to conform to any of the established criteria for described *Pseudo-nitzschia* species. Speciation of these organisms requires further study, in which the additional resolution provided by TEM will be used to view diagnostic poroid and girdle band structures (Fryxell, pers. commun., Lange, pers. commun.). These results show that the probes are useful tools for identifying their intended targets in cultured samples. Instances when novel cross-reactions occurred (i.e. frD1 and heD2-2 reacting with *P. subpacificae*), or when no species-specific reactions were noted, provided a basis for predicting that a new species previously not held in our collection had been isolated. We expect that as studies like this are expanded to include species and strains of *Pseudo-nitzschia* from other regions of the globe, additional cross-reactions will likely be noted. However, it is noteworthy that so far *Pseudo-nitzschia* species defined by LSU rRNA sequences, whether by sequencing or by application of probes, continue to show a positive correlation with the organisms defined by morphologic criteria (see also: Douglas et al. 1994, Scholin et al. 1994, Manhart et al. 1995).

In previous work (Miller and Scholin 1996), we noted that the *P. pungens*-specific probe puD1 exhibited variability in labeling strength when applied

to different clones of *P. pungens*, as well as in repeated application to the same clone. In all cases, the positive control labeled the cultures brightly. The reason for this difference in reactivity of puD1 was not known. When testing four of the 10 new isolates of *P. pungens* described here (Table 3), we at first obtained negative results after treatment with the *P. pungens*-specific probe. However, testing the same isolates again we discovered that they were indeed labeled specifically with puD1, giving an intensity similar to that of the positive control. The reasons for the observed differences in probe reactivity are not understood at present. Differences in cell membrane permeability can not account for this observation given that the positive control probe always labeled cells brightly. Changes in the physiologic state of cells may be related to differential intracolon reactivity, but we cannot prove this to be the case.

In an effort to estimate a lower limit of detection for a target species, we diluted cultured *P. australis* cells first with FSW alone, as a control, and second with FSW to which had been added a high density of background nontarget diatom species that frequently cooccur with *Pseudo-nitzschia* in nature. In addition, *P. australis*, *P. multiseriata*, and *P. pungens* were also diluted in natural, unfiltered seawater. In all of the probe treatments, and for each of the dilutions, target cells were readily identified and estimates of cell abundance were within the expected range (Tables 4 and 5). However, there were at times significant variations in the numbers of cells counted between replicate treatments. The variable nature of *Pseudo-nitzschia* chain formation contributes to this error. For example, if *P. australis* was present at 5000 cells $\cdot\text{L}^{-1}$ and occurred in lengths of 10 cells per chain, then a random 10-mL sample of that population might capture on average five chains of cells; plus or minus only a single chain in that same sample volume would contribute a 20% error in abundance estimates. In the results shown in Table 4, for example, the *P. australis* clone had chains of cells varying from 2 to 18 cells, contributing to stochastic errors in estimating the cell density of the original culture and preparing the dilutions and in all subsequent analytical steps. This highlights the importance of replicate treatments for a given sample if accurate estimates of cell density are desired. Moreover, if chains are not evenly distributed over the filter and if the entire filter is not examined, then estimates of cell density could be grossly under or over estimated, particularly as average chain length increases. Nevertheless, based on the results shown in Tables 4 and 5, the filter method of whole cell hybridization appears useful for estimating cell densities of *Pseudo-nitzschia* to at least 1250–2500 cells $\cdot\text{L}^{-1}$ —a level well below that of bloom conditions. Additional results of dilution experiments in which natural seawater was used as a diluent (Table 5) support the notion that the filter

system is indeed a promising means by which one might quantify target species in natural assemblages.

In contrast to whole cell probing, where labeled cells stand out against a dark background, SEM images instead reveal a uniform mass of material in which valve shape alone is the key to identifying a particular species. When target species are present in low numbers, it is difficult to discriminate them from nontarget species, making verification of probe results problematic. SEM examination of samples shown in Table 4 provided a controlled experiment in which to examine this situation. Whereas it was relatively easy to find and identify *P. australis* valves in all dilutions made with FSW alone, it was far more difficult and time consuming when the same density of target cells was combined with other nontarget species, especially in those dilutions having less than 10,000 cells·L⁻¹ of the target.

An example of detecting *Pseudo-nitzschia* in a field sample when it was a minor component of a mixed assemblage of species is shown in Figure 3. Observation under the stereo microscope of the fresh net sample used for this experiment revealed an abundance and diversity of centric diatoms mixed with dinoflagellates, various invertebrate larval forms, and a small number of *Pseudo-nitzschia* chains. Despite their low numbers relative to other species, *P. australis*, *P. fraudulenta*, and *P. pseudodelicatissima* were readily detected using fluorescent probes. *Nitzschia americana*, formerly *Pseudo-nitzschia americana* (Hasle 1994, Hasle and Syvertsen 1996), was also abundant and occurred primarily as an epiphyte on *Chaetoceros* setae. The presence of *P. australis*, *P. fraudulenta*, and *N. americana* was confirmed by SEM, but *P. pseudodelicatissima* was not. The low absolute and relative abundance of total *Pseudo-nitzschia* in this sample made finding them by using SEM a difficult and tedious process, as predicted by our earlier controlled experiments; it is not surprising under these circumstances that *P. pseudodelicatissima* was not detected. A species we did not probe for, *P. pungens*, was also detected by SEM, albeit in low numbers. Using this same approach, we have also identified a variety of other *Pseudo-nitzschia* species in natural populations, including *P. pungens*, *P. multiseriis*, and *P. heimii* (data not shown). Therefore, it is possible to use the probes shown in Table 2 to identify a wide range of *Pseudo-nitzschia* species collected from natural populations. Independent tests conducted by other workers have confirmed this observation (e.g. L. Rhodes, M. Parsons, pers. commun.), although label intensity has been noted to vary depending on the sample (see below).

An oceanographic cruise during March 1997 gave us the opportunity to test the feasibility of applying the probes in near real-time while working onboard ship. Surface whole water samples were collected by bucket, filtered, fixed, and probed as described above, and filters were examined immediately to enumerate target species. Using the positive control probe, it was

possible to make generic-level identifications of *Pseudo-nitzschia* based on their distinctive stepped chain formation. This enabled counting total *Pseudo-nitzschia* prior to enumerating individual species using species-specific probes. Although *Pseudo-nitzschia* were generally not very abundant in the large number of samples collected, in one sample we did observe 126 *Pseudo-nitzschia* cells in 10 mL of whole water. Species-specific probes (auD1, muD1, puD1, and NA1; Table 2) applied to replicates of the same sample revealed 35 labeled cells (i.e. ca. 3500 cells·L⁻¹) of *P. australis*, but no *P. multiseriis*, *P. pungens*, or *A. tamerense*. SEM analysis of this sample confirmed the presence of *P. australis* and the absence of *P. multiseriis* and *P. pungens* and also revealed a large number of *P. cf. pseudodelicatissima*, a species we did not attempt to identify. A second sample was found to have 35 *Pseudo-nitzschia*, but none of the four species-specific probes labeled any cells. This observation was verified by SEM analyses, which revealed a small number of *P. fraudulenta* and *P. cf. pseudodelicatissima* but not *P. australis*, *P. multiseriis*, or *P. pungens*. These results indicate that whole cell probing is useful for enumerating particular *Pseudo-nitzschia* species in near real-time simultaneously with shipboard hydrographic mapping. By using a combination of the positive control and species-specific probes, one can enumerate the total and relative abundance of congeners and confirm such observations using traditional SEM-based techniques at a later time.

One factor that influences label intensity of cells collected from natural populations is the proportion of material in the sample that binds probe nonspecifically. For instance, we have found that during times of significant terrestrial run-off, the amount of fine clay and other organic/inorganic debris retained on the filters increases and appears to bind a substantial amount of probe. When this occurs, brightly labeled particles are observed in all of the probe treatments but are noticeably absent from the "no probe" control. We suspect that under these conditions, the large amount of charged matter in the samples titrates the blocking agents (poly A and NP-40) beyond their capacity. Sites not blocked attract probe nonspecifically yielding a "milky way" of highly fluorescent particles that obscure labeled target cells. In addition, nonspecific adsorption of the probe appears to reduce the effective probe concentration, limiting the amount of free probe available for intended target sites. In turn, the fluorescence of target species may appear weak. We are currently testing other blocking agents in an effort to mitigate this effect. Cellular physiologic state likely influences the intensity of labeling as well. For example, in natural populations during earlier stages of a bloom, target species have been noted to label brightly, and as the bloom dissipates and cells begin to die the label intensity wanes with time so significantly that cells may not be visualized using the probes (V. Trainer, M. Parsons, L. Rhodes, pers. commun.).

For relatively low numbers of target cells (e.g. 5000–25,000 cells·L⁻¹), it is necessary to view the entire surface of the filter, because chains of cells do not seem to be distributed randomly enough that transects of the filter would provide accurate estimates of species presence and abundance. Additionally, at least three replicate samples should be analyzed if quantitative estimates are required. As cell densities drop below 5000 cells·L⁻¹ and/or chain length increases, we would expect the variation in counts between replicates to increase, signaling a less robust estimate of cell concentrations. Nevertheless, trials using the filter apparatus and probe methodology indicate that detection of potentially toxic *Pseudo-nitzschia* species (e.g. *P. australis*) in whole water samples is possible at a level an order of magnitude below which they might be considered a public health concern (e.g. >50,000 cells·L⁻¹; Andersen 1996). Should it prove desirable to enumerate cells at lower concentrations, we would advise using a larger filter tube assembly configured with a 25-mm diameter filter that would allow filtering a larger sample volume.

Although the filter tube system has dramatically improved our ability to apply *Pseudo-nitzschia* species-specific probes, a few methodologic drawbacks still remain. Field samples should be processed within a day after fixation or label intensity will diminish. Once hybridization reactions are completed, samples are stable for 2–3 days if kept at 4° C, after which time label intensity decreases. Presently, we are evaluating new methods to prolong sample stability after initial collection and fixation as well as after hybridization. Whole cell hybridization has improved our ability to detect particular *Pseudo-nitzschia* in field samples, but it has not eliminated the need for microscopic observations. Therefore, analysis of a large number of samples is still relatively tedious, particularly when examining a single sample for many species. Nevertheless, current methodology does offer an advantage over traditional means of species identification, especially when screening multiple samples and prioritizing accordingly for more rigorous analyses (e.g. SEM, toxin analysis).

In conclusion, filter-based whole cell hybridization is a simple and rapid technique for the identification of a variety of cultured *Pseudo-nitzschia*. Using current methodology, one can process 12 filters in approximately 4 h (from live sample through microscopic examination). The method is also useful for identifying *Pseudo-nitzschia* collected in field samples and can be employed onboard ship for near real-time species enumeration. This enables rapid identification of patches of water containing target species in order to effect more comprehensive sampling of those sites, as opposed to taking samples only along a predetermined grid and analyzing them much later. Because many organisms that cooccur with *Pseudo-nitzschia* remain intact throughout the probing procedure, it is also possible to document the presence and abundance of targeted spe-

cies in the context of a mixed assemblage. Variable label intensities have been observed for some cultured species, particularly *P. pungens*, and for a variety of species collected from natural populations. Variations in label intensity may arise as a result of the sample matrix (i.e. particles retaining the probe nonspecifically) or may reflect alterations in the physiologic condition of targeted cells. Last, the filter tube system has also proven useful for speeding and easing sample preparation for SEM, which in turn facilitates verification of probe results using traditional morphologic criteria.

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