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NOTE

RECOVERY AND IDENTIFICATION OF *PSEUDO-NITZSCHIA* (BACILLARIOPHYCEAE) FRUSTULES FROM NATURAL SAMPLES ACQUIRED USING THE ENVIRONMENTAL SAMPLE PROCESSOR[1]

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Many species within the diatom genus *Pseudo-nitzschia* are difficult to distinguish without applying molecular analytical or microscopy-based methods. DNA, antibody and lectin probes have previously been used to provide rapid and specific detection of species and strains in complex field assemblages. Recently, however, well-documented cryptic genetic diversity within the group has confounded results of DNA probe tests in particular. Moreover, the number of species descriptions within the genus continues to increase, as do insights into toxin production by both new and previously described species. Therefore, a combination of classical morphological techniques and modern molecular methodologies is needed to resolve ecophysiological traits of *Pseudo-nitzschia* species. Here, we present an approach to recover and identify frustules from sample collection filters used for toxin analysis onboard the Environmental Sample Processor (ESP), an in situ sample collection and analytical platform. This approach provides a new and powerful tool for correlating species presence with toxin detected remotely and in situ by the ESP, and has the potential to be applied broadly to other sampling configurations. This new technique will contribute to a better understanding of naturally occurring *Pseudo-nitzschia* community structure with respect to observed domoic acid outbreaks.

Key index words: **frustules; *Pseudo-nitzschia*; scanning electron microscopy**

***Abbreviations*: DA, domoic acid; ESP, Environmental Sample Processor; GuSCN, guanidinium thiocyanate; SEM, scanning electron microscopy**

Pseudo-nitzschia H. Peragallo 1899 is a cosmopolitan diatom genus currently comprised of nearly forty species, approximately 30 % of which have been documented to produce domoic acid (DA; Lelong et al. 2012, Trainer et al. 2012, Teng et al. 2015), a neurotoxin that can accumulate in finfish, shellfish, and other invertebrates, and be transferred throughout the food web (e.g., Lefebvre et al. 2002, Kvitek et al. 2008, Trainer et al. 2012). Consumption of aquatic species capable of concentrating toxic *Pseudo-nitzschia* can lead to illness in humans (amnesic shellfish poisoning; Perl et al. 1990, Bates et al. 1993) and marine wildlife (DA poisoning; Work et al. 1993, Scholin et al. 2000). Toxic species have been recorded from coastlines on six continents (Trainer et al. 2012 and references therein), and although current routine monitoring programs help thwart major human illness events, many coastal economies continue to be severely impacted by extensive closures of shellfish harvesting (e.g., Hoagland et al. 2002, Fehling et al. 2004, Bill et al. 2006, Ajani et al. 2013).

Monitoring naturally occurring *Pseudo-nitzschia* in field assemblages can be challenging for several reasons. First, many species cannot easily be delineated via light microscopy, thereby requiring electron microscopy for definitive morphological characterization based on detailed morphometric analysis of the siliceous frustules. Second, several species historically considered nontoxic have now been confirmed as producing DA (e.g., Orsini et al. 2002, Trainer et al. 2009, Amato et al. 2010, Trick et al. 2010), supporting the hypothesis that all *Pseudo-nitzschia* species may be able to produce toxin given the right conditions (Parsons et al. 1999, Wells et al. 2005). Third, genetic, morphological, and physiological insights continue to support the description of new species within the genus (e.g., Lundholm et al. 2012, Lim et al. 2013), shedding light on confounding results from previous studies where regional differences in molecular probe specificity were noted (e.g., Parsons et al. 1999, Scholin et al. 1999). Resolving these challenges requires an integrative approach to fully characterize native *Pseudo-nitzschia* assemblages toward a better understanding of their

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ecology with respect to their toxin producing capabilities.

Here, we demonstrate the feasibility of identifying *Pseudo-nitzschia* species using scanning electron microscopy (SEM) analysis of frustules recovered from sample collection filters used on the Environmental Sample Processor (ESP) for DA testing. The ESP is a submersible autonomous robotic device that collects native seawater and utilizes DNA and protein probe arrays to detect numerous HAB species (Greenfield et al. 2006) and DA (Doucette et al. 2009), respectively. The ESP platform can be deployed for weeks to months at a time to detect a variety of *Pseudo-nitzschia* species and DA remotely in near real-time. We determined that frustules from two *Pseudo-nitzschia* size classes persist through an aqueous methanol (AqMeOH) extraction protocol used for DA analyses onboard the ESP, thereby offering an opportunity to directly correlate DA detected in situ with species identified via SEM after the ESP is recovered from a deployment. We also assessed recovery and identification of frustules from guanidinium thiocyanate (GuSCN) extracted filters used for DNA probe arrays onboard the ESP, as well as from column extractions used in a commercial DNA extraction kit in order to assess feasibility of frustule recovery from a broader range of sample filters processed using a variety of methods.

CULTURES

Representative species were chosen from two *Pseudo-nitzschia* size classes: *P. hasleana* Lundholm (Lundholm et al. 2012) and *P. arenysensis* Quijano-Scheggia, Garcés, Lundholm (Quijano-Scheggia et al. 2009) have cell widths less than 3 μm and represent the "*P. delicatissima* size class," while *P. fraudulenta* (Cleve) Hasle has a cell width greater than 3 μm and represents the "*P. seriata* size class" (Hasle and Syvertsen 1997). Cultures were established from single chain isolations and maintained in 0.2 μm-filtered *f*/2 medium (Guillard 1975) made with Monterey Bay water amended with 106 μM $NaSiO_3$. Growth was supported at 15°C under a 13:11 h light:dark photoperiod. For culture identifications, DNA was extracted from an aliquot of pelleted cells using the DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA, USA). Primers D1R-For (Scholin et al. 1994) and D3B-Rev (Nunn et al. 1996) were used to amplify the D1-D3 domain of the LSU rDNA (large subunit of the ribosomal DNA). PCR was carried out using AmpliTaq Gold® 360 Master Mix, and products were sequenced with the same primers. Supporting information and GenBank accession numbers for strains used in this study is outlined in Table 1.

ESP FILTERS

The ESP concentrates cells by filtration prior to extraction with GuSCN or AqMeOH for subsequent DNA probe array or DA immunoassay analyses, respectively. Here, we sought to use SEM to evaluate the quality and approximate quantities of *Pseudo-nitzschia* frustules left on filters following their extraction. To that end, we: (i) assessed frustule integrity following exposure to GuSCN or AqMeOH; (ii) estimated the lower limit of cells that would have to be sampled in order to allow their detection on a spent extraction filter; and (iii) evaluated the stability of frustules following extraction and prolonged storage. In order to test integrity of frustules after the two extraction methods used on the ESP platform, 10000–40000 cells from two *P. hasleana* cultures (strains 135 and 208) were spiked into duplicate 500 mL volumes of whole Monterey Bay water (0.2 μm-filtered or nonfiltered) that did not contain *Pseudo-nitzschia* (as determined by phytoplankton community counts via light microscopy). Samples were collected by an ESP configured on the benchtop to mimic in situ sampling (Roman et al. 2007). Briefly, samples were pulled into the intake line and passed through a 25 mm, 0.65 μm Durapore® filter (PVDF; EMD Millipore, Billerica, MA, USA) housed in a titanium puck. Cells were treated with 3 M GuSCN or with 50% AqMeOH/0.01% Tween20 for DNA and DA extraction prior to array processing, respectively. Collection pucks containing filters were retrieved and stored in a desiccator until examined using SEM. To assess the methodology at low cell densities, 50 or 100 cells of *P. hasleana* (strain 117b) were spiked into 50 mL of 0.2 μm-filtered seawater and processed as described above for DA arrays. To test the integrity of frustules following exposure to 3 M GuSCN and long-term storage in a humid (>90%) N_2-saturated environ-

TABLE 1. *Pseudo-nitzschia* species, strain designation, isolation date, depth, and GenBank accession number (large ribosomal subunit) for strains used in this study. Strains were established from the San Pedro Shelf area (southern California, USA) during two research cruises.

Species	Strain designation	Date	Depth	Accession number
Pseudo-nitzschia hasleana	135	March 15, 2013	2 m	KT189134
P. hasleana	208	March 15, 2013	10 m	KT189135
P. hasleana	117b	April 12, 2014	20 m	KT189136
P. arenysensis	2b	April 14, 2014	Surface	KT189137
P. fraudulenta	339b	April 17, 2014	18 m	KT189138

ment (matching conditions during an ESP deployment), one hundred thousand cells of *P. arenysensis* (strain 2b) were spiked into five 100 mL replicates of 0.2 μm-filtered seawater. After sample lysis, one filter was immediately recovered and frustules were removed into an Eppendorf tube using 1 mL wash with molecular biology grade (MBG) water. A second filter was immediately washed onboard the ESP with a flush solution (0.05% Tween20 v/v; Sigma, St. Louis, MO, USA) to evaluate whether this step would improve frustule integrity during long-term deployment scenarios. This and all remaining filters were recovered from the ESP, and stored within their puck housings in a humid, N_2-saturated benchtop chamber for 1–3 weeks. Finally, to assess frustule stability after storage in the ESP followed by long-term storage in a desiccator, we processed a DA filter from an April 2014 field deployment that was onboard the ESP for 11 days after sampling, and then housed in a desiccator for ~3 months before processing for SEM.

DNA EXTRACTION KIT FILTERS

We explored the feasibility of recovering frustules from DNA extraction columns using a commercial kit. DNA from pelleted *P. fraudulenta* (strain 339b) cells was extracted using the DNeasy® Blood and Tissue kit (Qiagen). After the elution step, two aliquots of 400 μL of MBG water were used to wash frustules from the DNeasy Mini Spin column by pipetting up and down several times. These water washes were immediately moved to an Eppendorf tube and stored at 4°C until processing for SEM. To assess the methodology at low cell densities, 10–30 cells of *P. hasleana* (strain 117b) were sterile pipetted into three 100 μL aliquots of MBG water. Each aliquot was passed through a 0.65 μm filter at low vacuum (5 mmHg) and the filter was processed with the DNeasy kit. At the end of the protocol, frustules were recovered from the DNeasy Mini Spin column as described above.

SCANNING ELECTRON MICROSCOPY

Recovered pucks were secured in a custom housing assembly mounted on a ringstand and the filter was washed three to five times by pulling 2 mL MBG water (with 2 min incubations in between) through the puck. The filter was then removed from the puck and transferred sample side facing inward to an Eppendorf tube. One ml of MBG water was washed over the filter by pipetting up and down several times, and the entire wash volume was then applied to a 1.3 μm Isopore™ polycarbonate filter (RTTP; EMD Millipore) secured in a modified filter rack (Miller and Scholin 1998). The sample was filtered and frustules retained on the filter were prepared for SEM using a series of filtration steps under low vacuum: (i) 2 mL aliquots of MBG water were sequentially filtered three times, with no incubation time in between; (ii) three to four drops of saturated $KMnO_4$ were added to the filter and incubated for 1 h to digest organic matter; (iii) after filtration, 1 mL concentrated HCl was filtered through three times, or until $KMnO_4$ was completely removed; (iv) 2 mL aliquots of MBG water were sequentially filtered three times, with no incubation time in between. The entire process (steps 1–4) was repeated, and after the final filtration with MBG water the filter was transferred to an 18 mm pin mount SEM stub with nonconductive adhesive (Ted Pella, Redding, CA, USA). Stubs were allowed to air dry completely, stored in a desiccation chamber, and coated with gold prior to imaging on a Quanta™ 3D field emission microscope (FEI; Hillsboro, OR, USA).

The integrity of morphological features used for species-level identification was maintained after AqMeOH extraction from filters used for DA analysis (Fig. 1, a and b). Frustules were suitable for common morphometric descriptions used for identifying *Pseudo-nitzschia* species (e.g., cell width and length; number of interstriae in 10 μm, fibulae in 10 μm, band striae in 10 μm, and poroids in 1 μm; number of sectors in poroids). Whole frustules were observed alongside broken frustules on all stubs examined in this study, however, it is possible that acquiring measurements of cell length could be problematic for a species in low abundance where all frustules were broken during the preparation.

Although accurate quantitative data for number of cells recovered are problematic because frustules often break apart, we noted high recovery rates from a known cell number as low as 50. This cell density is far below relevant numbers for field monitoring programs (50,000 cells $\cdot$ L^{-1}; Andersen 1996, Ajani et al. 2013, Taylor et al. 2013). We acknowledge that a more exhaustive evaluation of effects from background matrices, such as varying densities of *Pseudo-nitzschia* and other diatom species, would be valuable.

Given the robust recovery, dilutions of filter washes with separate SEM preparations would be advisable when cells are abundant. Conversely, the 3 M GuSCN extraction used for the DNA probe arrays routinely resulted in distorted frustules (Fig. 2, a and b) and much lower recovery. In order to assess whether the distortion effects from GuSCN could be overcome, a series of filters was tested that included a wash step onboard the ESP and long-term storage mimicking conditions during a deployment. Despite filtering one hundred thousand cells, and washing post-GuSCN lysis, recovery was less than 0.01%; most of these remaining frustules were compromised and only a few endured relatively intact (Fig. 2c). Distortion and loss were not a cumulative result of exposure to both GuSCN and potassium permanganate, since washing filters directly after GuSCN extraction yielded the same

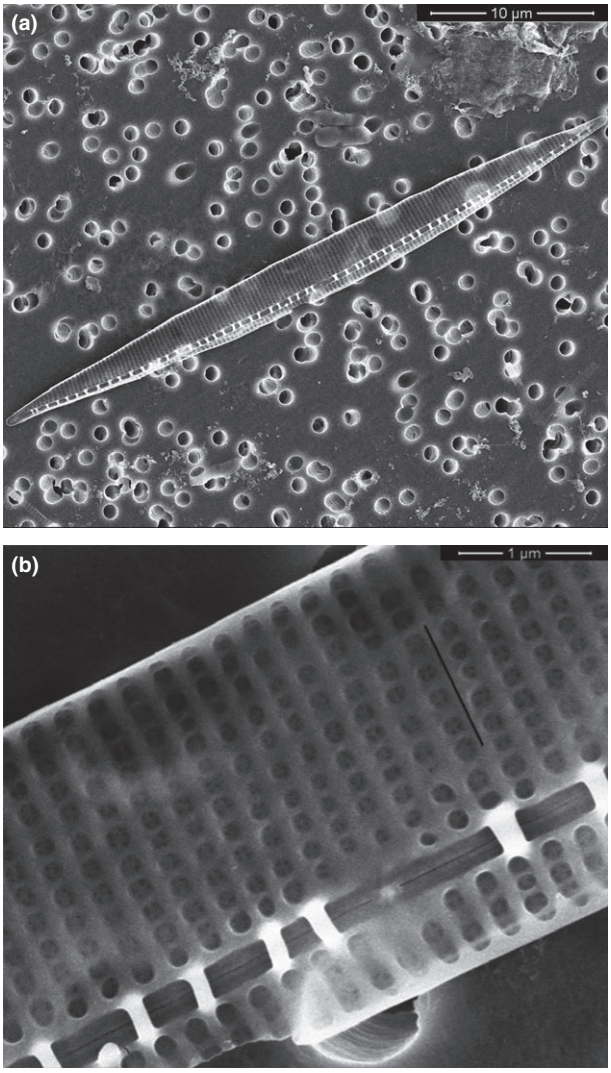

FIG. 1. Integrity of frustules after aqueous methanol extraction on DA array filters. (a) *Pseudo-nitzschia hasleana* (strain 135). (b) Detailed view of frustule in (a); black bar indicates six poroids per 1 µm, with approximately four sectors within each poroid. These are two of several metrics used to identify *P. hasleana* (Lundholm et al. 2012).

results (data not shown). Furthermore, these effects were observed consistently across all experiments and across all three species used in this study.

A filter that was used for DA extraction during an ESP deployment, and then stored following recovery of the instrument, was examined to determine if a typical ESP operational scenario would be conducive to downstream recovery and identification of frustules using SEM. Diatom frustules suitable for identification were observed after a total storage time (ESP and desiccator) of more than 3 months (Fig. 3).

Finally, we were interested in testing the feasibility of recovering *Pseudo-nitzschia* frustules from a commercially available kit and method often used for

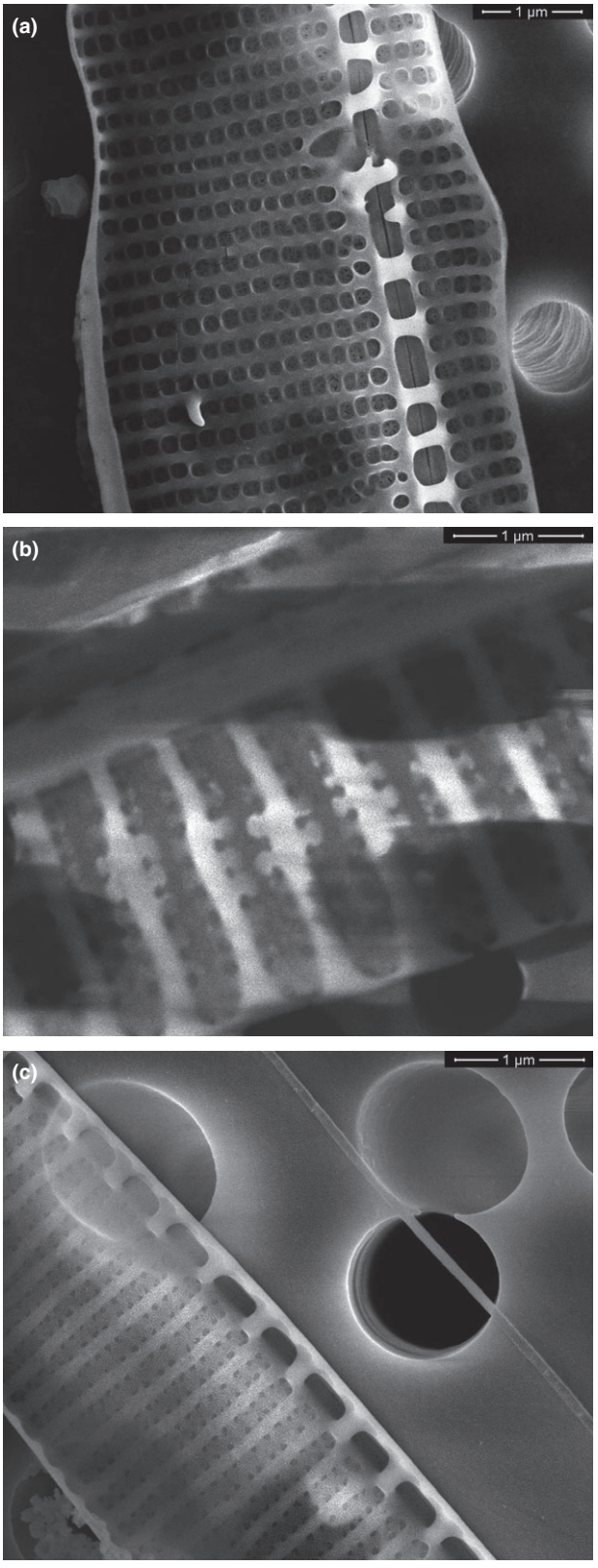

FIG. 2. (a and b) Integrity of frustules after GuSCN extraction on DNA probe array filters from two different preparations of *Pseudo-nitzschia hasleana* (strain 208). (c) While the overall majority of frustules across all species tested were distorted, some frustules endured, as shown with an intact *P. arenysensis* cell (strain 2b).

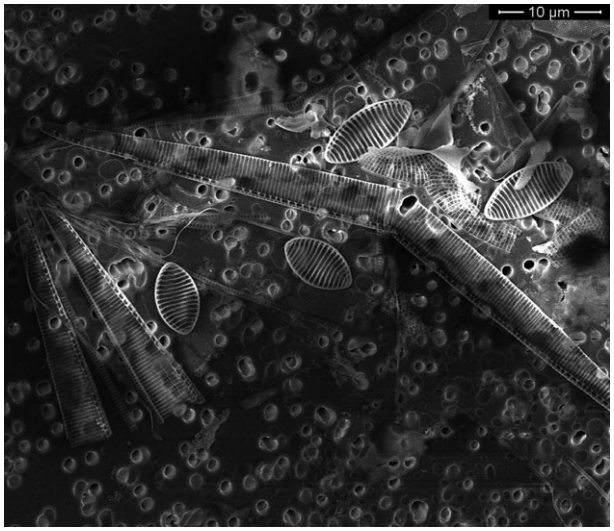

FIG. 3. Integrity of frustules after long-term dry storage (>3 months) of a methanol extracted DA filter from an ESP deployment in San Pedro, CA.

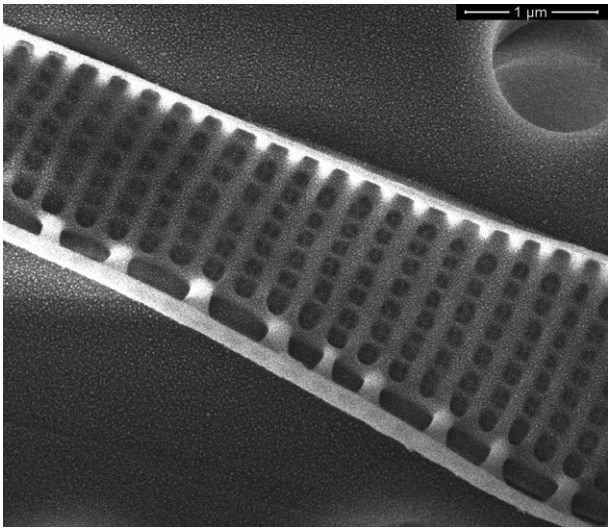

FIG. 4. Recovery of frustules (*Pseudo-nitzschia hasleana* strain 117b) from a DNA extraction column in a commercial kit (DNeasy® Blood and Tissue Kit; Qiagen).

extracting DNA from environmental samples. Frustule morphology remained intact (Fig. 4), and recovery of frustules from low cell abundances (less than 30 cells) was observed. We suspect that this likely reflects the less caustic nature of the commercial kit lytic buffers and protocol compared to that currently employed with the ESP.

The success of downstream frustule recovery and identification from the same filters used to measure DA remotely in the field offers a powerful new tool for assessing *Pseudo-nitzschia* community structure in relation to DA outbreaks. This approach could serve to correlate patterns in toxicity with species presence, as well as alert researchers to potential new species and further guide species-specific probe development. The frustule recovery technique was not as robust for filters used downstream of ESP DNA probe arrays; while there is some opportunity for comparisons similar to that done for DA testing, the method is far less reliable. Overall, the technique presented in this study provides a supplementary avenue for ground-truthing of molecular-based cell and toxin detection assays. Such approaches continue to expand the known number of species for many groups of organisms, including *Pseudo-nitzschia*. Furthermore, the direct coupling of frustule identification to remote measurements of DA is greatly beneficial. When combined with the diversity of supporting environmental contextual data that the ESP system and other in situ platforms afford (e.g., chlorophyll, temperature, salinity, etc.), it is possible to utilize sampling strategies that span spatial and temporal scales that are not possible or practical to apply using methods demanding a human presence. Fully automated platforms like the ESP, embedded in a larger ocean observing system, offer a holistic approach to understanding community structure and related environmental factors that may lead to HABs and their associated impacts.

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DISCLOSURE

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