New applications of a biogenic silica deposition fluorophore in the study of oceanic diatoms

Karine Leblanc* and David A. Hutchins
College of Marine Studies, University of Delaware, Lewes, DE 19958, USA

Abstract

Silicon (Si) availability is known to be one of the main factors controlling the productivity and distribution of diatoms (Dugdale et al. 1995), but current chemical and microscopic methods do not allow discrimination of the key species responsible for Si biominalization in mixed natural diatom assemblages. In 2001, new insights into biological silicification became available when studies with diatom cultures established that PDMPO [2-(4-pyridyl)-5-[4-dimethylaminoethyl-aminocarbamoyl]-methoxy[phenyl]oxazole] selectively binds to polymerizing silica and emits an intense fluorescence under ultraviolet (UV) excitation wherever newly formed Si is deposited (Shimizu et al. 2001). Here, we focus on adapting the PDMPO method to the study of Si use in natural diatom communities, including identification of individual cells carrying out new Si deposition. Experiments determined a simple and reproducible way to label actively silicifying diatoms, to preserve stained samples and identified the best optical tools to visualize the fluorescence properties of Si-bound PDMPO. The application of confocal multiphoton microscopy to the PDMPO method revealed unprecedented high-resolution three-dimensional (3D) imaging of new Si deposition within diatom cells, which can be used to study biogenic silica deposition in relation to the cell cycle. The quantitative aspects of this method were further explored and resulted in a protocol allowing simultaneous measurement of newly deposited biogenic silica and PDMPO incorporation in the same sample. This aspect was successfully tested on both cultures and field samples. Toxicity assays were also run on a mixed natural diatom community to ensure that the probe presented no deleterious effect on diatom growth, biogenic silica deposition, or silicic acid uptake rates over 24 h incubations and at the recommended concentration. There is also great potential for a better understanding of the biogeochemistry of Si in diatoms through coupling of this method to other quantifying tools, such as UV flow cytometry and image analysis.

*E-mail: leblanc@com.univ-mrs.fr

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The Si biogeochemical cycle has an impact on global CO₂ concentrations through chemical weathering processes of silicate minerals, which transfer carbon dioxide from the atmosphere to the lithosphere (Wollast and Mackenzie 1983). Si also affects carbon cycling through biological processes, as the siliceous phytoplankton group known as diatoms contributes significantly to atmospheric CO₂ drawdown (Tréguer and Pondaven 2000). Diatoms contribute from 25% to 75% of the ocean’s total primary productivity (Nelson et al. 1995), and they are the only ecologically dominant phytoplankton group requiring silicic acid [Si(OH₄)] for growth. Despite being the third most abundant element on earth, Si is one of the limiting nutrients in vast regions of the world oceans (Dugdale et al. 1995). This is mainly due to biological uptake by diatoms, which strips silicic acid out of the surface waters and exports it to depth as particulate biogenic silica.

The Si and C cycles are further linked by the ability of diatoms to form large blooms, which then sediment en masse at high sinking rates (Smetacek 2001), potentially pumping more organic C toward the deep ocean than most other phytoplankton groups. It has been hypothesized that changes in the supply of Si and trace metals (mainly iron) to the ocean’s surface through aeolian and riverine inputs could modify the dominance of diatoms versus other major groups such as coccolithophorids (calcifying organisms) and have large implica-
tions for the atmospheric CO₂ variations associated with glacial/interglacial transitions (Martin et al. 1991; Harrison 2000). A better knowledge of the control exerted by silicic acid availability on diatom growth is therefore a key to understanding major shifts in phytoplankton community composition, their impacts on CO₂ drawdown from the atmosphere, and, ultimately, to building realistic models that can predict future climate changes.

Methods available for studying the silicon biogeochemistry and silicon requirements of diatoms include chemical and isotopic determination of bulk parameters such as biogenic silica, silicic acid concentrations, and biological silicic acid uptake rates. The recent development of multi-elemental, multifunctional-group biogeochemical models emphasizes the importance of being able to attribute the flux of the main nutrients (Si, N, P) to particular phytoplankton groups (Doney 1999; Moore et al. 2002; Aumont et al. 2003), in some cases down to the level of ecologically important species. In an effort to link biogeochemical fluxes to specific phytoplankton taxa in order to improve these biogeochemical models, a current trend in oceanography is the search for new tools capable of tracing the activity of key functional groups, as for instance N₂ fixation by diazotrophs, calcification by coccolithophorids, or silicification by diatoms.

Thus, the application of the fluorophore PDMPO to silicification studies may lead to a better understanding of the way Si is used by different diatom species in natural communities. PDMPO is a specific tracer of new silica deposition in diatom frustules, yielding unique new information about the diatom Si cycle (Shimizu et al. 2001). The use of PDMPO in labeling biomineralization of silica in diatoms was first described for cultures of *Thalassiosira weissflogii* under controlled laboratory conditions. Here, we describe modifications of the method initially published by Shimizu et al. (2001) allowing its application to field work on natural diatom communities collected in diverse coastal and open ocean environments. We further present developments of the method for the study of both qualitative and quantitative aspects of the Si deposition process in diatoms.

**Materials and procedures**

*Molecular probes*—The fluorescent probe PDMPO [2-(4-pyridyl)-5-[4-dimethylaminoethylamino-carbamoyl]-methoxy]-phenyl]oxazole], also named LysoSensor™ Yellow/Blue DND–56, was obtained from Molecular Probe (L-7545) in a 1-mM dimethylsulfoxide matrix. The probe was divided into 50 µL aliquots of 1 mM PDMPO and stored at −20°C in the dark. Working stocks of 100 µM were obtained by diluting each stock solution in 500 µL MilliQ water immediately before use.

*Spectral characteristics and microscopy equipment*—PDMPO is excited under UV light (λ<sub>ex</sub>: 357-377 nm) and has a dual emission fluorescence in the blue spectrum (λ<sub>em</sub>: 417-483 nm) and in the yellow/green spectrum (λ<sub>em</sub>: 490-530 nm) (Diwu et al. 1999; Shimizu et al. 2001). Stained samples were analyzed on a direct light Olympus BX51 epifluorescence microscope equipped with a Spot RT-color CCD camera. We found that a standard 4’,6-diamidino-2-phenylindole (DAPI) filter cube (Chroma 31000, exciter D360/40X, dichroic 400 DCLP, emitter D460/50m) was compatible with parts of PDMPO’s spectral characteristics and allowed us to see stained cells in the blue part of the visible spectrum. Rotations between the DAPI and Chlorophyll a (Chl a) filter cubes (Chroma 31017a) allowed us to visualize new biogenic silica deposition (in blue) and Chl a content of cells (in red) (Fig. 1a to 1c).

However, visualization of stained cells over PDMPO’s entire emission range yielded much better results in terms of fluorescence intensity and photostability and can be achieved by using a long-pass DAPI filter (exciter D360/40X, dichroic 400 DCLP, emitter E420lpv2). The use of this filter results in stained cells fluorescing in the yellow/green spectrum and allows discrimination between what appears to be organic debris stained by the probe (bright blue fluorescence), and the biogenic silica-PDMPO complex (yellow-green fluorescence). Custom-made filter cubes can also be constructed to look at PDMPO-labeled diatoms and should aim at the following spectral characteristics: exciter D375/40X, dichroic 400 DCLP, emitter D530/40m.

Particularly detailed pictures of stained cells can also be obtained using a multiphoton confocal microscope (Zeiss LSM 510 NLO), which allows visualization of samples in several layered sections and reconstruction of three-dimensional (3D) images as well as video animations of labeled diatoms. The generated images were acquired using the Zeiss Laser Scanning Microscopy (LSM) Image browser, which was also used to construct the 3D and z-stack animations (sequential visualization of two-dimensional (2D) images along the depth axis of the sample) (Fig. 2).

*Quantitative analysis by spectrofluorophotometry*—The quantitative applications of this method were tested on a Shimadzu RF-1501 scanning UV spectrofluorophotometer. The spectrofluorophotometer is equipped with a xenon lamp and was set to excite at 375 nm. Emission scans over the 350 to 700 nm range were run at λ<sub>ex</sub> = 375 nm in order to determine the exact fluorescence emission spectrum of PDMPO-stained intact or digested cells.

*Labeling protocol*—In the protocol previously described for *T. weissflogii* cultures (Shimizu et al. 2001), the authors followed the manufacturer recommendations to use PDMPO at a final concentration in solution of 1 µM. Working with both cultures and natural communities, we found that a final concentration as low as 0.125 µM still produced a consistent and long lasting staining of newly formed silica in diatom cells. Typically, for natural samples, volumes of 150 mL of seawater were incubated in the presence of PDMPO (final concentration 0.125 µM) in shipboard flow-through incubators for 24 h or in laboratory incubators when working on cultures. The seawater was subsequently filtered under low-vacuum pressure (<13 × 10⁵ Pa) in a glass filter tower onto black polycarbonate filters (0.22 µm, 25mm). The filters were then immediately placed on a drop of
Fig. 1. Images of new silicon deposition and chlorophyll $a$ (Chl $a$) of coastal and sub-arctic diatoms under fluorescence microscopy. A DAPI filter cube was used to visualize PDMPO-stained diatom valves in the blue emission spectrum (a to c), while the natural autofluorescence of cells was observed using a standard Chl $a$ filter cube. Chl $a$ fluorescence allows visualization of the entire cell contours and illustrates how only the half-valves of newly divided adjacent cells were stained with PDMPO. A long-pass DAPI filter cube was then used to visualize the entire fluorescence spectrum (d to h). Newly deposited valves exhibit an intense yellow-green fluorescence, and Chl $a$ may in some cases be visible in red simultaneously. (a-d) Natural diatom community from the Bering Sea. (a, d. Cf Paralia sp., b. Proboscia alata, c. Chaetoceros sp.). (e-h) Delaware Bay sample (e. Rhizosolenia sp., f. Odontella cf sinensis, g. Actinoptychus senarius, h. Bacillaria paxilllera). PDMPO binding to polymerizing Si is clearly illustrated in these examples, where the fine structure of valve areolae and striae becomes perfectly visible under UV light. These pictures were taken between 1 and 2 y after the samples were mounted on glass slides and demonstrate the persistence of the PDMPO fluorescence properties.
immersion oil on a glass slide and sealed under a glass coverslip with another drop of oil above the filter. Glass slides were preserved at –20°C in the dark. Contrary to the protocol described by Shimizu et al. (2001), no rinsing agents like monensin or nigericin were used to clean the cells. Filters collecting the live, stained cells were simply rinsed with 0.2 µm filtered seawater to eliminate any unbound PDMPO before preservation. Mounted slides of natural diatom communities sampled in the Bering Sea and Delaware Bay were used to assess the qualitative aspect of the PDMPO staining, while quantitative assays were run in the laboratory on cultures of cold water diatom isolates from the Southern Ocean and the Bering Sea as well as on field samples from the Delaware Bay.

**Assessment**

*Quality of the staining procedure and sample preservation—*

The fluorophore PDMPO shows first a fast accumulation into the silicon deposition vesicles and is then selectively incorporated into diatom cell walls and codeposited with Si into the frustule crystalline matrix (Shimizu et al. 2001). The unique property of this probe is to produce an intense fluorescence under UV excitation spanning the blue-green light spectrum wherever silicic acid is polymerized to biogenic silica during the frustule formation process (Shimizu et al. 2001). The fluorescent probe rhodamine 123 has previously been used in a similar manner to trace Si deposition in diatoms (Brzezinski and Conley 1994). However, the red fluorescence of rhodamine interferes with chlorophyll autofluorescence, requiring rinsing treatments and cell fixation. Furthermore, the low accumulation efficiency of this probe in the frustule also presents some visualization caveats (Shimizu et al. 2001).

In contrast, a major advantage of the PDMPO stain is its high accumulation efficiency in the frustule, which allows easy detection of newly deposited silica after only a few hours of incubation. The PDMPO labeling thus allows visualization...
of the sequential Si deposition process in live diatoms, such as following the formation of first one valve and then the other (Fig. 1a-f, Fig. 2b). The superposition of both PDMPO and Chl a fluorescence for several species (Fig. 1a,b,f; Fig. 2a) clearly demonstrates the excellent labeling of newly formed biogenic silica during cell division, with often only one valve or one frustule element like the cingulum fluorescing.

Live, stained cells can be observed directly without any fixation procedures and virtually no bleaching visible to the eye when using a DAPI long-pass filter. Samples examined in blue light with a regular DAPI filter were much fainter and bleached somewhat rapidly after illumination. Following the simplified glass-mount protocol on natural diatom communities from the Bering Sea and Delaware Bay (Fig. 1), we found that the intense yellow/green fluorescence of the samples was still perfectly visible after more than 2 years if kept in the dark at –20°C.

A bleaching test was conducted on a stained sample from the Delaware Bay, which had been previously stored at –20°C for 2 weeks by exposing the same area of the glass slide to continuous UV light under the epifluorescence microscope for 45 min (Fig. 3) using a long-pass DAPI filter. The image of a stained chain of Skeletonema costatum was acquired every minute for the first 12 min of UV exposure, followed by two snapshots after 21 and 45 min constant exposure. Each image was captured under the exact same conditions (same area, light intensity, exposure time, etc.) and acquired in gray scale with Image Pro. The image was then converted to a color scale, which allows a better visual comparison of the variations in fluorescence intensity. While virtually no bleaching of the sample was visible during the first 3 min of exposure, the successive image capture and color conversion showed some slight bleaching of the stained Skeletonema costatum in the following 10 min of continuous UV exposure, while consequent bleaching was observed after 45 min (Fig. 3a). However, the PDMPO-labeled parts of the frustule were still perfectly visible under direct observation after 45 min. Furthermore, the fluorescence intensity of the same diatom colony recovered very well after storing the sample at –20°C and in the dark again for several days (Fig. 3b), even after having been exposed to constant UV light for 45 min, which is much longer than the time that would be normally required to analyze a specific area of the sample.

Preserved samples stored up to 2 y in the same conditions were also observed on a confocal multiphoton microscope, which allowed us to visualize the newly deposited frustule in

### Table 1. List of the main diatom species present in the spring bloom water collected in Delaware Bay in March 2005*

<table>
<thead>
<tr>
<th>Species</th>
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<tr>
<td><em>Skeletonema tropicum</em></td>
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<td><em>Skeletonema costatum</em></td>
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<tr>
<td><em>Thalassiosira nordenskioeldii</em></td>
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<tr>
<td><em>Thalassiosira rotula</em></td>
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<tr>
<td><em>Thalassiosira sp.</em></td>
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<td><em>Ditylum brightwellii</em></td>
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<td><em>Asterionellopsis japonica</em></td>
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<td><em>Lauderia sp.</em></td>
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<tr>
<td><em>Chaetoceros decipiens</em></td>
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<td><em>Chaetoceros sp.</em></td>
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<td><em>Navicula sp.</em></td>
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<tr>
<td><em>Thalassiosema nitzschioides</em></td>
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<tr>
<td><em>Rhizosolenia setigera</em></td>
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<tr>
<td><em>Rhizosolenia alata</em></td>
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<tr>
<td><em>Guinardia delicatula</em></td>
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<tr>
<td><em>Asteromphalus sp.</em></td>
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<td><em>Pseudonitzschia sp.</em></td>
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<td><em>Striatella sp.</em></td>
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*Skeletonema tropicum* represented on average 55% of the total diatom abundance, followed by *Skeletonema costatum* (30%) and *Thalassiosira nordenskioeldii* (10%). All other species combined represented less than 5% of the total abundance.
3D down to a level of detail not possible with epifluorescence microscopy (Fig. 2). The images generated in Fig. 2 again demonstrate the excellent photostability of this fluorescent probe, after 2 y storage. The 3D reconstructions can further be visualized as video animations, allowing observation of newly deposited Si from different angles (our video animations are available online at http://www.ocean.udel.edu/cms/dhutchins/leblanc/pdmppo.html). This equipment can be used to study the fine details of PDMPO incorporation and sequential deposition into diatom frustules during the cell division cycle at an excellent resolution. The use of this probe in confocal and epifluorescence microscopy could, in some cases, be a good tool for identifying diatom species, as the fine structures of the frustule labeled with PDMPO become visible with a resolution often close to that of SEM (Scanning Electron Microscopy) images (see Figs. 1a, 1f-h, 2b). Furthermore, the 3D reconstructions of the cells obtained with this tool may overcome the problematic orientation of certain species of diatoms like pennates, which tend to get oriented valves side-up on filters and sometimes render the observation of newly deposited girdle bands difficult.

Toxicity assay—In their initial study of the applications of PDMPO to silicification processes, Shimizu et al. (2001) did not report any deleterious effect of the probe on diatom growth at their recommended working concentrations of 1 µM. In order to substantiate this assertion, we conducted an experiment on natural diatom communities collected from Delaware Bay (13 March 2005) during the diatom spring bloom to test the effects of increasing concentrations of PDMPO (from 0 to 1 µM) on cell growth and biogenic silica deposition.

Equal amounts (200 mL) of the diatom bloom water were split in 6 clear polycarbonate bottles and amended with

Fig. 4. Toxicity assay on a natural diatom community collected during the spring bloom in the Delaware Bay (March 2005). The growth of the three dominant species, and the increase in the total diatom abundance was monitored over 96 h for a range of increasing PDMPO concentrations (from 0 to 1 µM). Triplicate counts were done for each time point, and the error bar represents the standard deviation of the cell count (n = 3). The star above a bar indicates when the data are significantly different from the control for the same time point (statistical t test).
PDMPO in increasing concentrations (0.01, 0.125, 0.25, 0.5, and 1 µM) except for the control, which received no PDMPO. The potential toxicity of PDMPO on diatom growth was assessed by sampling for cell counts every 2 d (in triplicate) and biogenic silica every day for a period of 96 h, even though we usually only use PDMPO for 24 h incubations. In vivo fluorescence was also measured in parallel every day to get a quick estimate of cell density in each bottle. The water samples collected in the Delaware Bay contained 18 main diatom species (Table 1), but 3 of them dominated the community: *Skeletetonema tropicum* represented on average 55% of the total diatom abundance, followed by *Skeletonema costatum* (30%), and *Thalassiosira nordenskioeldii* (10%). All other species combined represented less than 5% of the total abundance. Thus, the cell number increase was represented over time for these three dominant species as well as for the total diatom abundance for the different PDMPO concentrations tested (Fig. 4).

The results of this experiment demonstrate that PDMPO has no deleterious impacts on diatom growth for at least 48 h of incubation no matter what concentration was used. After 96 h, the first significant decreases in cell numbers are observed for *Skeletonema tropicum* and for the total diatom abundance for the two highest concentrations used (0.5 and 1 µM). However, a decrease in the average number of cells for all species, even if not statistically significant due to the large error bars, are observed for the highest concentrations of PDMPO (0.5 and 1 µM) after 96 h.

Due to a limited volume of water available, only one sample (15 mL) was analyzed for biogenic silica (BSi) according to Nelson et al. (1989) at each time point (Fig. 5a). Some variability in BSi concentrations is obvious from the measurements at the beginning and throughout the length of the incubation, but no consistent deleterious effect can be inferred from the data. The BSi content in the control sample was equal to or less than the samples amended with PDMPO except at 48 h, where the BSi content appeared lower in the control than in all PDMPO treated samples. However, after 72 h and 96 h, the BSi content in the control reached very similar values to what was measured for all PDMPO-treated samples. The in vivo fluorescence measurements showed a similar trend, with a fluorescence intensity in the control lower than in the stained samples for the first 48 h, whereas there was no difference between the control and the stained samples after 72 and 96 h (Fig. 5b).

This experiment demonstrates that PDMPO used at the recommended final concentration of 0.125 µM has no negative impacts on diatom growth or on biogenic silica deposition over a period of 96 h, while concentrations more than 4 times higher (> 0.5 µM) may start affecting growth rates of some species after 48 and 96 h. Use of recommended PDMPO concentrations and incubation times (24 h) thus appears to pose no risk of toxic effects on diatom growth.

Assessment of the quantitative measurement of PDMPO incorporation—A series of tests were run on a UV spectrofluorophotometer to assess whether a quantification of the frustule-bound PDMPO was possible on stained samples. Preliminary tests performed on suspensions of labeled cells gave poor results in terms of reproducibility. Cells were filtered and resuspended in PDMPO-free filtered seawater prior to the reading. This procedure resulted in a visible fluorescence signal but with emission peaks shifting inconsistently in different samples between 440 and 510 nm.
Subsequently, frustule solubilization methods were tested on culture samples and allowed a successful quantification of PDMPO incorporation using either of two standard methods for digesting particulate silica: hot NaOH digestion or hydrofluoric acid (HF) leaching. We showed that solubilized PDMPO formerly retained in the frustule exhibits one clear yellow-green emission peak centered on 530 nm, in accordance with the results obtained in fluorescence microscopy (Fig. 1d-h). The optimal excitation wavelength was determined to be 375 nm for digested samples. Time-course experiments consisting of sample readings under continuous UV illumination showed that the fluorescence signal was stable for several minutes with a negligible bleaching (~1.2% after 5 min).

Several species of cultured diatoms (Nanoneis haslaea, Thalassiosira sp., Chaetoceros sp., Cylindrotheca sp.) were inoculated with 0.125 mM of PDMPO (final concentration) and incubated for 24 h. Samples of dense cultures were filtered onto 0.6 µm polycarbonate filters (Nucleopore, 47 mm), and filters were rinsed thoroughly with filtered seawater to eliminate any extracellular PDMPO. Filters were then placed in 15 mL Nalgene tubes and digested in 4 mL hot NaOH (0.2 N) for 1 h according to Nelson et al. (1989). After digestion, samples were cooled in an ice bath and neutralized with 1 mL HCl (1N). Large amounts of organic matter or disintegration of the filter may interfere with and alter the reading, thus we recommend either centrifugation or filtration of the samples in order to get consistent measurements. A few milliliters of the supernatant can then be read directly on a spectrofluorophotometer.

Samples exhibited various fluorescence intensities and a standard curve for PDMPO in a NaOH-HCl matrix similar to the samples can then be used to calculate the amount of PDMPO initially incorporated into the frustules (Fig. 6a). The HF digestion method was then tested on a new set of culture samples and yielded very similar results with an emission peak centered on 530 nm. Filters were extracted in 0.2 mL HF (2.5 N) for 24 h and then neutralized with 9.8 mL saturated boric acid (H₃BO₃). A fraction of the supernatant was used for the fluorescence reading, and no difference in the wavelength of the emission peaks were observed as compared with the
NaOH digestion (Fig. 6b). However, the fluorescence intensity (in arbitrary units) varied and a different standard curve for this extraction was made in a HF-H₃BO₃ matrix, yielding a similarly linear relationship. The results of these preliminary experiments demonstrated that the chemical dissolution of biogenic silica through a strong base or acid treatment did not alter the PDMPO fluorescence properties and further allowed a quantitative measurement using UV spectrofluorophotometry.

Once we determined that both the NaOH and HF digestions successfully solubilized frustule-bound PDMPO, we then compared the extraction efficiency of both methods. A dense culture of *Thalassiosira* sp. was inoculated with PDMPO (final concentration 0.125 µM) and incubated for 24 h. At the end of the incubation, 10 replicate samples were filtered and digested following the NaOH method, while 10 other replicates were assayed through HF digestion as described above. The supernatants were used to measure PDMPO concentrations on a UV spectrofluorophotometer and yielded very similar results.

The average PDMPO concentration obtained with the HF extraction method (0.43 ± 0.02 nM) was slightly higher (+11%) than with the NaOH digestion (0.38 ± 0.05 nM) but this increase was not statistically significant. This slight difference was attributed to the greater efficiency of the HF treatment in dissolving biogenic silica, which was confirmed by a similar increase in BSi concentrations (+14%). However, the strong acid HF will also remineralize lithogenic silica (mineral silica) in natural samples and, furthermore, is quite hazardous to handle both at sea and in the laboratory. Given the similar results obtained with both methods, we thus recommend the NaOH extraction for simultaneous BSi and PDMPO measurements.

Next, we assessed the potential interference of PDMPO staining with BSi analysis and investigated whether the BSi mea-
measurements performed on PDMPO-stained samples were still accurate. A dense culture of *Thalassiosira* sp. was split in two identical volumes and incubated in the same conditions, except that one culture was inoculated with PDMPO (0.125 µM) while the other remained unaltered. After 24 h, 10 replicate samples were filtered from each culture and assayed for biogenic silica following the NaOH digestion method. BSi concentrations for the stained samples amounted to $138.1 \pm 3.2 \text{ µmol L}^{-1}$ while BSi in the unstained samples was $142.1 \pm 7.2 \text{ µmol L}^{-1}$. Thus, no significant differences in the BSi measurements were observed, which indicates that PDMPO does not interfere with the colorimetric measurement of silicic acid at 810 nm. Thus, we conclude that both particulate biogenic silica and frustule-bound PDMPO concentrations can be measured very simply.
from the same labeled water sample through one standard BSi extraction procedure. The detection limit of the method was considered to be twice the height of the baseline noise of non-stained samples at 530 nm and corresponded to a detection level of 2 nM of PDMPO (Fig. 6a).

Quantitative assays on culture and field samples—We then investigated the proportionality of PDMPO and Si deposition to estimate whether PDMPO measurements can be used as a quantitative proxy for new Si deposition on both cultures and field samples. Two diatom cultures isolated from the Southern Ocean (Thalassiosira sp. and Chaetoceros sp.) were incubated with PDMPO and subsampled for BSi and PDMPO analysis over a 96-h time-course experiment. At each time point, 5 replicate volumes were filtered onto 1 µm polycarbonate membranes and rinsed 3 times thoroughly with 0.2 µm filtered seawater to eliminate any adsorbed PDMPO without damaging the cells. A second set of 5 replicates were filtered simultaneously, but were covered with 10% HCl for 2 min and subsequently rinsed with MilliQ water in order to osmotically lyse the cells and release all the intracellular Si and PDMPO that were not bound in the frustule. Thus we were able to compare the total intracellular (frustule bound + vacuolar) with the solely frustule-bound Si and PDMPO contents. The results of this experiment showed a linear proportional increase of PDMPO deposition with BSi for both species (Fig. 7), and this relationship remained consistent for both total cellular contents (Fig. 7a and 7c) and frustule contents (Fig. 7b and 7d). The proportion of PDMPO retained in the cell vacuoles, which is not bound in the frustule, is likely to vary for each species depending on cellular volume. Indeed, the percentage of intracellular PDMPO (as calculated from the difference between total PDMPO and frustule-bound PDMPO) varied from 19% to 63% for Thalassiosira sp. (calculated biovolume = 5000 µm³) whereas it varied between 0% and 7% for Chaetoceros sp. (calculated biovolume = 500 µm³). Thus, the best way to obtain an accurate measurement of PDMPO uptake in the frustule is to systematically osmotically lyse the cells (10% HCl and MilliQ rinse) prior to analysis.

The efficiency of PDMPO incorporation in the frustule was calculated for both species, yielding an average of 2800 molecules of Si deposited for each molecule of PDMPO incorporated. This shows a much better incorporation efficiency than Rhodamine 123, which was reported to be incorporated in a ratio of 17:1 million molecules of Si to R123 in Thalassiosira weissflogii (Brzezinski and Conley 1994).

To test the validity of the quantitative aspect of the method on natural diatom communities, a second experiment was carried out on water collected during the diatom spring bloom in the Delaware Bay on 13 March 2005, on a natural mix of diatom species (Table 1). The experiment was designed to investigate if the uptake of PDMPO in diatom frustules was proportional to BSi and diatom abundance in a mixed diatom assemblage and also aimed at comparing a number of measurements on stained and unstained samples to reveal any potential toxic effects of PDMPO on a natural community.
Whole water samples were diluted with 0.22 µm filtered seawater (collected at the same site) to get several samples with increasing diatom density. Five dilutions of the bloom water were prepared in polycarbonate bottles for a final volume of 2 L: 100% (non diluted water), 75%, 50%, 25%, and 10%. For each dilution, triplicate cell counts, biogenic silica, and silicic acid samples were collected at the start of the incubation (T0). Immediately after sampling, the remaining water for each dilution was divided into four replicate 250 mL polycarbonate bottles. Two of these replicates were inoculated with 0.125 µM PDMPO whereas the two others were used as controls. One control and one stained sample were inoculated with 32Si (2630 Bq) for Si uptake rate measurements according to Tréguer et al. (1991) and Leynaert (1993). Twenty polycarbonate bottles were then incubated in a flow-through incubator with circulating surface seawater for 24 h from dawn-to-dawn. After 24 h, 10 samples inoculated with 32Si were filtered onto 0.6 µm polycarbonate membranes, rinsed thoroughly with 10% HCl and MilliQ water to osmotically lyse the cells, and the filters were stored away in scintillation vials. For the remaining 10 bottles, triplicate samples were taken for cell counts, BSi/PDMPO, and silicic acid analysis. BSi filters were similarly rinsed with 10% HCl and MilliQ water and analyzed as described previously for the culture experiments. One milliliter of digested NaOH/HCl solution of each sample was used to measure BSi concentrations, while the remaining 4 mL were used to measure PDMPO uptake on a spectrofluorimeter.

Results show that the dilution method adequately reproduced a linear gradient of biogenic silica and diatom abundance (Figs. 8 and 9). The increase in BSi over the 24-h incubation period as well as the decrease in Si(OH)₄ were proportional to the dilution degree of the initial dense bloom water (Fig. 8a,b). The final values of BSi and Si(OH)₄ measured after 24 h for both stained and unstained samples at each dilution were very similar and suggest that PDMPO had no effect on either bulk BSi deposition or silicic acid uptake. This was confirmed by a parallel measurement of Si uptake rate through the 32Si method, which showed identical absolute and specific Si uptake rates (Fig. 8c,d) in both the controls and the PDMPO-labeled samples, except for 1 value in the 25% dilution, which actually shows a slightly higher Si uptake rate in the PDMPO sample than in the control (by 17%). PDMPO uptake and subsequent deposition in the frustule measured after 24 h showed an excellent linearity with biogenic silica increase ($r^2 = 0.98$) and total diatom abundance increase ($r^2 = 0.97$) over the studied range of dilutions (Fig. 8e,f). The increase in cell density for the two dominant species as well as for the total number of diatoms again showed no evidence of any deleterious effect of PDMPO on cell growth over that time period (Fig. 9).

In summary, the experiment carried out on field samples consisting of a mix of several diatom genera such as the bloom-forming Skeletonema sp. showed that over 24 h and at a final concentration of 0.125 µM, PDMPO uptake remained proportional to the amount of biogenic silica and diatom

Fig. 10. Comparison of chlorophyll a (Chl a) and PDMPO fluorescence (observed here with a standard DAPI filter cube) for a natural chain of the centric diatom *Paralia* sp. from the Bering Sea (a and b) and for a culture of *Thalassiosira weissflogii* (c and d). Note that only 6 of 10 colonial cells of *Paralia* visible by their Chl a fluorescence are dividing and depositing a new siliceous valve, visible in blue by PDMPO fluorescence (b). Similarly, the comparison between Fig. 10c and 10d shows that only 30% of the *Thalassiosira* cells enumerated by Chl a autofluorescence are depositing silica, which is visible in blue.
abundance. PDMPO also had no impact on Si uptake rates, which were identical for both stained and unstained samples, and PDMPO did not significantly modify biogenic silica deposition, Si(OH)$_4$ decrease, or specific diatom growth rates (either of individual species or of total diatom abundance).

Furthermore, the average incorporation ratio of BSI to PDMPO calculated for this mixed diatom community was 3045 ± 230, which is very close to the average value calculated for the *Thalassiosira* and *Chaetoceros* culture experiments (2800 ± 780). PDMPO seems to be incorporated in the cell in fairly constant proportions to BSI for the different species studied, as the average ratio of BSI:PDMPO (mol:mol) for all experiments where BSI and PDMPO were measured in parallel amounted to 3230 ± 660. Thus, we infer that PDMPO measurements can be used as a proxy for the amount of new BSI deposited over time in mixed diatom communities.

**Discussion**

The main application of this technique is to allow the distinction between diatom cells that are actively depositing biogenic silica and those that are not, due to senescence or simply to a resting phase. This was already achieved by the use of $^{32}$Si in autoradiography by Shipe and Brzezinski (1999), which allows visualization of where biogenic silica was deposited in the frustule and thus inference of species-specific new biogenic silica deposition. However, this technique requires between 4 months to a year exposure time of the samples to a photographic emulsion and necessitates the use of the expensive radioisotope $^{32}$Si. The use of the fluorescence probe PDMPO presents the advantages of a very simple protocol achievable at a lower cost, together with immediate visualization of the labeled cells. By a simple inoculation with the PDMPO tracer, it becomes possible to identify diatoms actively depositing Si in mixed natural assemblages, or the percentage of cells depositing silica within a monospecific culture. Such distinctions could previously not be made under regular light microscopy or by bulk analysis methods. The standard chemical quantification of biogenic silica, for instance, does not discriminate between live and dead cells.

This method can thus be used to correct for Si uptake kinetic parameters, which are usually normalized to total biogenic silica. It is known that Si uptake, transport, and deposition patterns are intrinsically linked to the diatom cell cycle and are decoupled during the different phases of cell division (Li et al. 1989; Brzezinski et al. 1990). When Si uptake rates are measured in natural diatom communities using isotopic techniques ($^{32}$Si, $^{30}$Si), it is assumed that cells take up Si at a continuous rate, which is almost never the case in nature (Brzezinski and Conley 1994). Specific Si uptake rates, which are used to calculate the uptake kinetic parameters $V_{\text{max}}$ and $K_s$ (maximum specific uptake rate and half-saturation constant) are usually normalized to BSI, a measurement of the bulk biomass constituted of both live and dead, and active and inactive cells. As a result, the kinetic constants of live cells can be substantially underestimated when the total amount of BSI is used (Brzezinski and Conley 1994).

A simple experiment carried out on a laboratory monoculture of *Thalassiosira weissflogii* using PDMPO demonstrated, for instance, that only 30% of the cells at this particular life stage were actively depositing silica over a 24-h period, which is typically used for Si uptake experiments (Fig. 10c,d). In this example, the maximum specific $V_{\text{max}}$ value, corrected for the nonactive siliceous biomass, would be diminished by as much as 70%. The asynchronous division rate of diatoms is also obvious from the observation of natural communities of PDMPO-labeled diatoms, where cells within the same colony and living in the same physical and chemical environment do not always simultaneously deposit biogenic silica. This is demonstrated in Fig. 10a and 10b, where only 6 of 10 cells from the same colony are forming a new valve. Nonetheless, synchronous division rates have been observed in some instances in the field for chains of *Rhizosolenia* sp. in the Central North Pacific using the $^{32}$Si autoradiographic method (Shipe and Brzezinski 1999).

The maximum specific Si production rates ($V_{\text{max}}$) can be considered as a measure of the maximal biomass yield under favorable nutrient conditions. However, the current estimates available for different oceanic systems are likely to be largely underestimated when a variable fraction of the diatom community is inactive with regards to Si deposition. Production measurements carried out in parallel with PDMPO staining will allow normalization of the Si uptake flux to a truly active siliceous biomass and thus estimate more realistic values of $V_{\text{max}}$, which is an essential parameter to biogeochemists and modelers for predicting phytoplankton productivity.

Microscopic cell counts of the PDMPO-stained species relative to the total diatom community will allow biogeochemists to rapidly identify the key species playing the most prominent role in the Si production flux and discriminate between a residual diatom biomass and rapidly growing cells. The unique fluorescence properties of the PDMPO-Si complex allowing for the quantification of frustule bound PDMPO can also be used as a proxy for the rate of newly deposited silica over time.

A serious limitation of the quantitative aspect of this method may be that PDMPO measurements run on samples collected on different occasions and stored for varying lengths of time might not be intercomparable, as the fluorescence properties of the Si-PDMPO may decrease over time. However, sample sets stored for the same amount of time and analyzed in the same conditions would still be comparable and yield relative information about species-specific silicification rates. Hence, PDMPO may be used as a quantitative proxy for Si deposition for relative comparison of similarly treated samples, but not as an absolute quantitative measurement of new biogenic silica formation.

Furthermore, epifluorescence and confocal multiphoton microscopy can be used to study the deposition pattern of Si at a subcellular level over both time and space, which
ultimately will help better understand the control exerted by Si availability over diatom growth rates and frustule formation. In other Si-related studies, PDMPO labeling was, for instance, used to identify silicic acid transporters in marine sponges using digital fluorescence microscopy (Schröder et al. 2004). This method could also offer promising applications for studying the intracellular Si transport processes in diatoms, which are still under investigation (Hildebrand et al. 1997; Martin-Jézéquel et al. 2000). This new molecular probe thus presents a number of advantages, from the simplicity and the high efficiency of the staining procedure to the subsequent easy treatment, preservation, and photostability of the samples, which makes it suitable for work at sea and in the laboratory.

Comments and recommendations

The full potential of the PDMPO method has yet to be fulfilled in the study of the marine Si biogeochemical cycle, and further progress is now needed in the development of this technique to benefit marine biogeochemists studying diatom silicification. Development of sophisticated image analysis software coupled to epifluorescence microscopy will make it possible to quantify and normalize the amount of fluorescence at the level of individual diatom cells. This will allow determination of the relative contribution of each species within a mix of diatoms to the total Si production, for instance. If coupled with quantitative measurements of the total Si production (using $^{32}$Si), this technique will further allow researchers to determine kinetic parameters ($K_s$, $V_{max}$) and accurate Si production rates for each species present instead of a global community flux.

Further development of this method will help to identify key species responsible for the largest Si fluxes in diverse oceanic environments, which is the next critical step in understanding the importance of diatoms in the biological pump. The development of image analysis of PDMPO-labeled samples will also yield more information about the degree of silicification of diatoms. It is known that diatoms may reduce or increase the thickness of their frustule in response to various environmental factors such as Si or Fe availability (Brzezinski et al. 1990; Hutchins and Bruland 1998). Modification of the silicification rate of diatoms has profound consequences for their ultimate fate in the water column, as more heavily silicified diatoms will sink faster out of the euphotic layer and potentially increase the C export to depth. Thus the comparison of the fluorescence intensity of the frustule-bound PDMPO for the same species subjected to different incubation conditions will help biogeochemists understand the silicification processes in relation to changing nutritional environments.

Developments in UV-flow cytometry are also required to refine this method and will be suitable for non-chain-forming diatoms from cultures or natural samples. Quantification of fluorescence in flow cytometry should allow not only a total measurement of PDMPO deposition, but would also yield a relative fluorescence value for each diatom cell or group, which ultimately will tell us about the degree of silicification of various diatom groups within a natural community.

References


Leblanc and Hutchins Si deposition probe


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