Diatoms respire nitrate to survive dark and anoxic conditions

Anja Kamp[1], Dirk de Beer, Jana L. Nitsch, Gaute Lavik, and Peter Stief

Department of Biogeochemistry, Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany

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Diatoms survive in dark, anoxic sediment layers for months to decades. Our investigation reveals a correlation between the dark survival potential of marine diatoms and their ability to accumulate NO_3^- intracellularly. Axenic strains of benthic and pelagic diatoms that stored 11–274 mM NO_3^- in their cells survived for 6–28 wk. After sudden shifts to dark, anoxic conditions, the benthic diatom *Amphora coffeaeformis* consumed 84–87% of its intracellular NO_3^- pool within 1 d. A stable-isotope labeling experiment proved that $^{15}NO_3^-$ consumption was accompanied by the production and release of $^{15}NH_4^+$, indicating dissimilatory nitrate reduction to ammonium (DNRA). DNRA is an anaerobic respiration process that is known mainly from prokaryotic organisms, and here shown as dissimilatory nitrate reduction pathway used by a eukaryotic phototroph. Similar to large sulfur bacteria and benthic foraminifera, diatoms may respire intracellular NO_3^- in sediment layers without O_2 and NO_3^-. The rapid depletion of the intracellular NO_3^- storage, however, implies that diatoms use DNRA to enter a resting stage for long-term survival. Assuming that pelagic diatoms are also capable of DNRA, senescing diatoms that sink through oxygen-deficient water layers may be a significant NH_4^+ source for anammox, the prevalent nitrogen loss pathway of oceanic oxygen minimum zones.

eukaryotic microbiology | marine ecology | N-cycle | nitrate ammonification | microalgae

Diatoms are eukaryotic phototrophs that are ubiquitous in both the pelagic and benthic zones of aquatic ecosystems. Approximately 40% of marine primary production is due to diatoms, making them key players in the global carbon cycle (1). Many diatoms take up and store NO_3^- intracellularly in concentrations of up to a few 100 mM (2–4), which exceeds ambient NO_3^- concentrations by several orders of magnitude. It is well documented that diatoms use intracellular NO_3^- for assimilatory NO_3^- reduction (2); however, the intracellular NO_3^- pool also might be used for dissimilatory NO_3^- reduction.

Mass sinking of pelagic diatom blooms is triggered by nutrient depletion in the surface layer of the water column (5). However, silicate depletion, not nitrate depletion, is the prevalent environmental cue for increased sinking rates (6). Therefore, the intracellular NO_3^- pool might not be depleted in diatoms that sink from the nutrient-poor surface layer to nutrient-rich deeper layers or the sediment surface. In fact, Lomstein et al. (7) found high intracellular NO_3^- pools in phytoplankton freshly deposited on the seafloor. A considerable fraction of the settled diatoms survive for months to decades as vegetative or resting cells in dark, anoxic sediment layers, in which neither photosynthesis nor aerobic respiration can occur (8–10). Benthic diatoms experience shifts to dark, anoxic conditions due to vertical migration behavior in the sediment (11) and burial by bioturbating animals (12). The occurrence of NO_3^--storing diatoms in deep sediment layers increases the concentration of cell-bound NO_3^-, which has potential implications for nitrogen cycling (7, 13–15). However, the energy-providing metabolism that allows diatoms to survive in dark, anoxic sediments is not known, and the fate of the intracellular NO_3^- is unclear.

Dissimilatory NO_3^- reduction is common in many anaerobic prokaryotes, and was recently found in several eukaryotic taxa as well. The anaerobic protozoan *Loxodes* spp. respires NO_3^- to NO_2^- (16), and the two fungi *Fusarium oxysporum* and *Cylindrocarpon tonkinense* respire NO_3^- to N_2O (17, 18). Complete denitrification of NO_3^- to N_2 has been reported for NO_3^--storing benthic foraminiferans (19, 20). Dissimilatory NO_3^- reduction also can lead to NH_4^+ formation in a pathway known as dissimilatory nitrate reduction to ammonium (DNRA), a process well documented in prokaryotes, such as large sulfur bacteria (21, 22). The only eukaryotes known to be capable of DNRA are fungi (23, 24). DNRA is involved in anaerobic energy generation via a two-step reaction sequence: NO_3^- reduction to NO_2^- is coupled to electron transport phosphorylation, whereas NO_2^- reduction to NH_4^+ is coupled to substrate-level phosphorylation (25). The second reduction step has only a small ATP yield and can thus be considered an electron sink that serves to regenerate nicotinamide adenine dinucleotide (26).

We hypothesize that dissimilatory NO_3^- reduction is a metabolism used by diatoms to survive darkness and anoxia. To test this hypothesis, we anexically cultured three pelagic and three benthic diatom strains to investigate the maximum storage capacity for intracellular NO_3^- and the correlation between the intracellular NO_3^- concentration and survival in darkness and anoxia. We further investigated the benthic diatom *A. coffeaeformis* with respect to the consumption of intracellularly stored NO_3^- after a sudden shift to dark/anoxic conditions and the production of intermediates and end products of denitrification and DNRA.

Results

Intracellular NO_3^- Accumulation. The maximum intracellular NO_3^- concentrations reached during cultivation of diatom strains under optimal conditions (i.e., availability of light, O_2, and nutrients) ranged from 0.4 mM (for *Cylindrotheca closterium*) to 274 mM (for *A. coffeaeformis*) (Table 1). The lack of substantial intracellular NO_3^- storage in *C. closterium* might have resulted from competition with bacteria contaminating the culture. The maximum intracellular NO_3^- concentration was not correlated with the habitat type of the different diatom species (pelagic or benthic). The residual NO_3^- concentrations in the growth medium at the time of cell harvesting were always lower than the intracellular NO_3^- concentrations (Table 1). The resulting enrichment factors (i.e., intracellular over extracellular NO_3^- concentration) ranged from 5 (for *Ditylum brightwellii*) to 391 (for *A. coffeaeformis*; Table 1). The maximum intracellular NO_3^- contents were not correlated with the cell volume (Table 1); that is, large diatom cells generally did not store more NO_3^- than small cells.

Survival Under Dark/Anoxic Conditions. The maximum time of surviving dark/anoxic conditions was significantly positively cor-

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[1]To whom correspondence should be sent. E-mail: akamp@mpi-bremen.de.

Table 1. Intracellular NO_3^- accumulation in cultures of six diatom species from benthic and pelagic habitats

Species name	Habitat type	Maximum intracellular NO_3^- content, fmol*	Cell volume, pL	Maximum intracellular NO_3^- concentration, mM†	NO_3^- concentration in growth medium, mM‡	Enrichment factor
D. brightwellii	Pelagic	111.6 (32.2)	24.80	4.5 (1.3)	0.9 (0.2)	5 (0.3)
S. costatum	Pelagic	3.7	0.33	11.1	0.1	111
T. weissflogii	Pelagic	113.1 (6.7)	1.22	92.9 (5.5)	0.9 (0.1)	103 (5.4)
C. closterium	Benthic	0.1	0.15	0.4	< 0.1	NA
N. punctata	Benthic	4.5 (0.3)	0.11	40.6 (2.5)	0.9 (0.1)	45 (2.2)
A. coffeaeformis	Benthic	128.8 (41.1)	0.47	273.7 (87.4)	0.7 (0.1)	391 (69.0)

NA, not applicable.
*Maximum intracellular NO_3^- content reached during cultivation under light/oxic conditions. Values are mean (SD) of three replicate culture tubes; *S. costatum* and *C. closterium* were analyzed only once.
†Calculated from maximum intracellular NO_3^- content and cell volume.
‡Measured at the time of cell harvesting for analysis of intracellular NO_3^- content.

related with the maximum intracellular NO_3^- concentration in the various diatom species (Fig. 1). In contrast, survival time was not correlated with the habitat of the diatom species. In single culture tubes of *Nitzschia punctata* and *A. coffeaeformis*, a maximum survival time of 28 wk was recorded. Cell counts did not reveal growth in any of the six tested diatom species incubated under dark/anoxic conditions. In contrast, all six species were actively growing before the start of the survival experiment.

Consumption of Intracellular NO_3^- in Response to Dark/Anoxic Conditions. Intracellular NO_3^- was rapidly consumed in response to dark/anoxic conditions, with much of the consumption occurring during the first 8 h of the experiment (Fig. 2). Within 21 h of the experiment, 87% of the intracellular NO_3^- was consumed. The stable NO_3^- concentration in the growth medium indicated that intracellular NO_3^- was indeed consumed by *A. coffeaeformis* rather than lost to the growth medium (Fig. 2). The axenic *A. coffeaeformis* culture did not grow under dark/anoxic conditions (Fig. 2), whereas the culture was actively growing before the experiment.

Dissimilatory NO_3^- Reduction in Response to Dark/Anoxic Conditions. Axenic *A. coffeaeformis* cultures grown under light/oxic conditions with 1 mM $^{15}NO_3^-$ or $^{14}NO_3^-$ were harvested, washed, and transferred into NO_3^--free artificial seawater enriched with 200 μM Na-acetate as an electron donor. Thus, for the subsequent experiments, the sole NO_3^- source was intracellularly stored $^{15}NO_3^-$ or $^{14}NO_3^-$. Initial intracellular NO_3^- concentrations and cell densities differed slightly between the two pretreatments. When exposed to dark/anoxic conditions, axenic *A. coffeaeformis* cultures simultaneously consumed intracellular NO_3^- and produced NH_4^+ that was released into the growth medium (Fig. 3*A*). In contrast, when exposed to light/oxic conditions, axenic *A. coffeaeformis* cultures also consumed intracellular NO_3^-, but did not release NH_4^+ (Fig. 3*B*). *A. coffeaeformis* cultures exposed to light/oxic conditions were growing (Fig. 3*B*), whereas those exposed to dark/anoxic conditions were not (Fig. 2). Thus, the intracellular NO_3^- pool is used for assimilation during growth conditions and energy generation under dark/anoxic conditions. The production of $^{46}N_2O$ and $^{30}N_2$ from intracellular $^{15}NO_3^-$ under dark/anoxic conditions was negligible (Fig. 3*A* and Table 2).

The rates of simultaneous NO_3^- consumption and NH_4^+ production under dark/anoxic conditions were higher in the early phase of the experiment (0–2.5 h) than in the late phase (8–192 h) (Table 2). Within 24 h of dark/anoxic incubation, 84% of the intracellular NO_3^- was consumed (Fig. 3*A*). Axenic *A. coffeaeformis* cultures preincubated with $^{15}NO_3^-$ released $^{15}NH_4^{+*}$ into the growth medium at about twice the rate of NH_4^+ release (Fig. 3*A* and Table 2). $^{15}NH_4^{+*}$ denotes isotopically labeled *N* compounds measured with the hypobromite assay (see *Materials and Methods* for details). At the end of the experiment, the total amounts of NH_4^+ and $^{15}NH_4^{+*}$ released corresponded to 56% ± 5% and

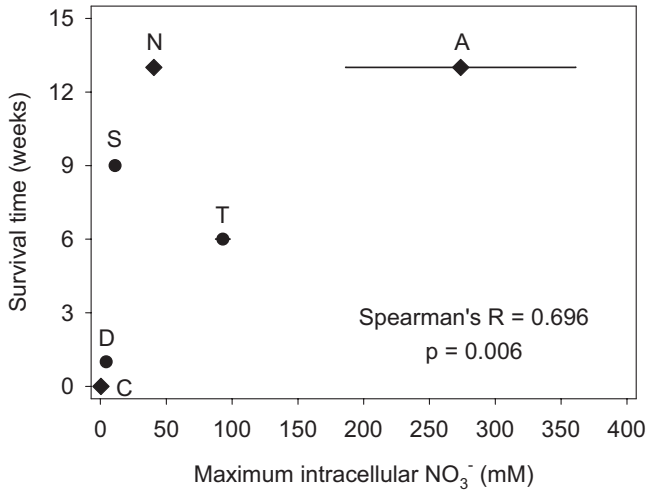

Fig. 1. Correlation between maximum intracellular NO_3^- concentration and survival time under dark/anoxic conditions in the diatom species *D. brightwellii* (D), *S. costatum* (S), *T. weissflogii* (T), *C. closterium* (C), *N. punctata* (N), and *A. coffeaeformis* (A). Circles denote pelagic species, diamonds denote benthic species. Values are mean concentration ± SD of three replicates; Spearman's *R* and probability *P* for nonlinear correlations are given.

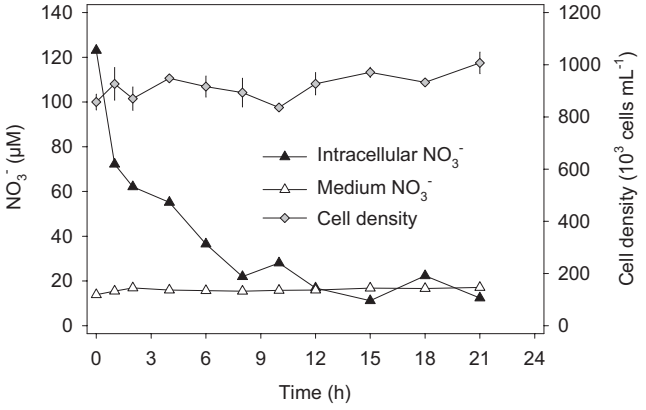

Fig. 2. Consumption of intracellular NO_3^- (expressed in μmol/L of growth medium) in an axenic *A. coffeaeformis* culture in response to dark/anoxic conditions. Time courses of NO_3^- concentration in the growth medium and of the mean cell density (± SE of four repeated counts) are also shown. Dark/anoxic conditions were initiated at 0 h.

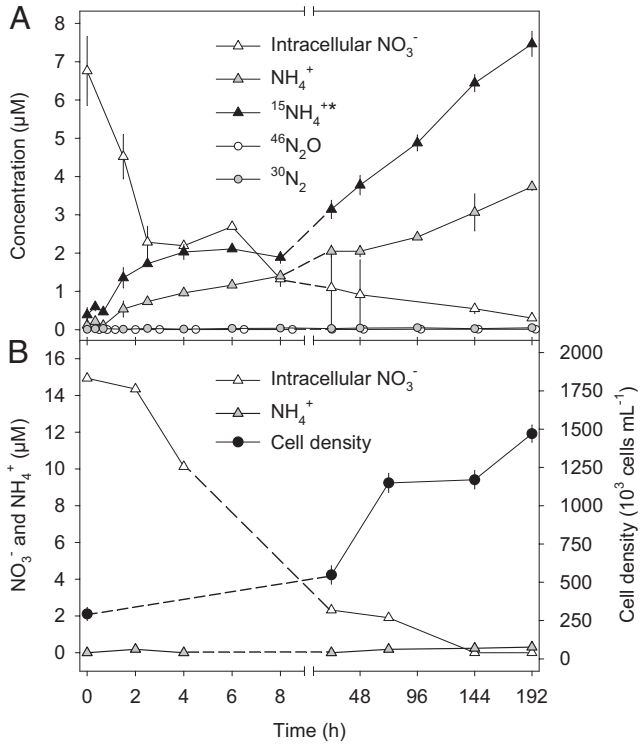

Fig. 3. Time course of *N* compounds in an axenic *A. coffeaeformis* culture in response to dark/anoxic conditions (*A*) and light/oxic conditions (*B*). Cells were preincubated with $^{15}NO_3^-$ (*A*) or $^{14}NO_3^-$ (*B*) in the growth medium under light/oxic conditions. Experimental conditions were initiated at 0 h. $^{15}NH_4^{+*}$ denotes isotopically labeled *N* compounds measured with the hypobromite assay. Intracellular NO_3^- concentrations are expressed in μmol/L of growth medium. Values are mean ± SD of three replicate culture tubes are shown.

110% ± 10%, respectively, of the total amount of intracellular NO_3^- consumed (calculated from data presented in Fig. 3*A*).

Discussion

Intracellular NO_3^- Aids Survival of Dark/Anoxic Conditions. Viable diatom cells are buried in sediments at depths in which light and O_2 are absent (8–10). These cells show only negligible signs of pigment degradation and start photosynthesizing immediately after being reexposed to light (10, 27, 28). The metabolism that enables diatoms to survive in this low-energy environment was previously not known. Our study results reveal a correlation between the ability of diatoms to survive dark/anoxic conditions and the capacity to store NO_3^- intracellularly. This finding suggests that NO_3^--storing diatom species possess a nonphotosynthetic, anaerobic metabolism that involves NO_3^- as an electron acceptor. In fact, dissimilatory NO_3^- reduction to NH_4^+ (i.e., DNRA) was found in *A. coffeaeformis*, the diatom species with the highest intracellular NO_3^- concentration in the present study.

Most of the intracellular NO_3^- pool in *A. coffeaeformis* (84–87%) was consumed within 1 d after the experimental shift to dark/anoxic conditions. This could indicate that dissimilatory NO_3^- reduction is largely used for the energy-demanding transition of the diatoms from the growing to the resting stage, whereas the ability to store NO_3^- intracellularly is linked to long-term survival. Synthesis of enzymes and structural proteins that are essential for the transition to the resting stage requires energy and nitrogen equivalents that might both be provided by NO_3^- reduction. Once the resting stage has been reached, the energy demand of the cells should be much lower. In fact, the consumption rate of intracellular NO_3^- decreased by a factor of ~300 only a few hours after the onset of dark/anoxic conditions in the stable-isotope labeling experiment (Table 2). *A. coffeaeformis* cultures grew in none of the experiments under dark/anoxic conditions. This observation suggests that intracellular NO_3^- is used during the transition to a resting stage in sediments or to sustain cell metabolism during temporary stays in dark/anoxic water layers.

DNRA, Not Denitrification, Mediates Dissimilatory NO_3^- Reduction. Three lines of evidence indicate that in *A. coffeaeformis*, dissimilatory NO_3^- reduction under dark/anoxic conditions proceeds as DNRA rather than denitrification: (*i*) The consumption of intracellular $^{15}NO_3^-$ resulted in the concomitant and equimolar release of $^{15}NH_4^{+*}$ (i.e., ^{15}N-labeled NH_4^+ and organic *N* compounds) into the growth medium; (*ii*) the consumption of intracellular $^{15}NO_3^-$ resulted in the release of only trace amounts of ^{15}N-labeled N_2O and N_2 into the growth medium; and (*iii*) in the presence of light and O_2, consumption of intracellular NO_3^- did not result in the release of NH_4^+ into the growth medium.

The cellular release of NH_4^+ under dark/anoxic conditions merits particular attention because the growth medium did not initially contain any NH_4^+. Under such NH_4^+-limited conditions, NH_4^+ normally remains inside the cell for *N* assimilation (29, 30). This was indeed the case under light/oxic conditions when the *A. coffeaeformis* cultures were growing, but not under dark/anoxic conditions when the cultures were not growing. In addition, the total amount of $^{15}NH_4^{+*}$ released balanced the total amount of intracellular $^{15}NO_3^-$ consumed (see the next paragraph). We conclude that in *A. coffeaeformis*, NO_3^- reduction under dark/anoxic conditions is dissimilatory, probably using the added acetate as the electron donor. However, only half of the $^{15}NH_4^{+*}$ released was actually NH_4^+, indicating that the other half of the $^{15}NO_3^-$ was reduced to organic *N* compounds that also were released by the cell. These *N* compounds should not be considered assimilation products, given that they are excreted by the cell completely. More likely, they represent unidentified byproducts of DNRA (possibly methyl amines or urea) that are also measured by the hypobromite assay (31). The cell-specific DNRA rates of *A. coffeaeformis* measured at 0–2.5 h and 8–192 h after the shift to

Table 2. Cell-specific rates of *N* conversion by axenic *A. coffeaeformis* cultures in response to different experimental conditions

Experimental conditions	Time interval	NO_3^- consumption, fmol $cell^{-1}$ h^{-1}	NH_4^+ production, fmol $cell^{-1}$ h^{-1}	$^{15}NH_4^{+*}$ production, fmol $cell^{-1}$ h^{-1}	$^{46}N_2O$ production, fmol $cell^{-1}$ h^{-1}	$^{30}N_2$ production, fmol $cell^{-1}$ h^{-1}
Dark/anoxic	0–2.5 h	−9.123 (2.631)	1.365 (0.394)	2.958 (0.853)	0.007 (0.002)	0.025 (0.007)
	8–192 h	−0.027 (0.008)	0.058 (0.017)	0.148 (0.043)	<0.001	<0.001
Light/oxic	0–2.5 h	−4.101 (0.602)	<0.001	ND	ND	ND
	8–192 h	−0.016 (0.002)	0.001	ND	ND	ND

ND, not determined.

Rates were calculated from the time course of *N* compounds presented in Fig. 3 for the linear changes in concentration between 0 and 2.5 h and between 8 and 192 h of incubation. Cell densities were 194 ± 56 × 10^3 cells mL^{-1} (0–192 h) in the dark/anoxic incubation and 293 ± 43 (0–2.5 h) and 1,263 ± 163 × 10^3 cells mL^{-1} (8–192 h) in the light/oxic incubation. Values are mean (SD) for rates >0.001 fmol $cell^{-1}$ h^{-1}.

dark/anoxic conditions correspond to 0.246 and 0.012 nmol $^{15}NH_4^{+*}$ per mg of protein per min, respectively (cellular protein content data from ref. 32). For comparison, the DNRA rate of sulfide-oxidizing *Thioploca* spp. is 1 nmol $^{15}NH_4^{+*}$ per mg of protein per min (21).

The time courses of total intracellular $^{15}NO_3^-$ and extracellular $^{15}NH_4^{+*}$ were not perfectly correlated (Fig. 3*A*). In contrast, the consumption rate of total intracellular $^{15}NO_3^-$ was initially higher and later lower than the release rate of $^{15}NH_4^{+*}$ (Table 2). By the end of incubation, however, the total amount of intracellular $^{15}NO_3^-$ consumed balanced the total amount of $^{15}NH_4^{+*}$ produced. This observation suggests the transient accumulation of an unidentified intermediate product that was further reduced to NH_4^+, which was then released to the medium (33).

DNRA in Eukaryotic Organisms. *A. coffeaeformis*, a eukaryotic phototroph, is capable of dissimilatory NO_3^- reduction via the DNRA pathway. So far, DNRA activity has been identified only in prokaryotes (reviewed in ref. 33) and in fungi (23, 24). Intriguingly, some fungi use their assimilatory NO_3^- and NO_2^- reductases for generating ATP by dissimilatory NO_3^- and NO_2^- reduction (24, 34). Such a dual use of enzymes could be also realized in diatoms. In addition, analysis of the first two diatom genomes (35, 36) revealed that these eukaryotic organisms have incorporated prokaryotic genes by horizontal gene transfer from various marine bacteria (37). Thus, diatom species may possess combinations of metabolisms that were never previously found together in one organism (38). In the case of *A. coffeaeformis*, these are obviously photosynthesis and dissimilatory NO_3^- reduction.

Significance of DNRA in the Life Cycle of Diatoms. Dissimilatory NO_3^- reduction may have important functions in the life cycle of diatoms by providing a physiological means to (*i*) prepare the resting stage of a cell or (*ii*) bridge the temporary absence of light, O_2, or nutrients. Under nutrient-replete conditions, diatoms take up NO_3^- for *N* assimilation, but they also may store NO_3^- and use it as an electron acceptor under dark/anoxic conditions (Fig. 4). In sediments, diatoms can be exposed to darkness and anoxia for short or long periods due to their own vertical migration (11), the activities of bioturbating animals (12), and the advective water flow through permeable sediments (39) (Fig. 4). DNRA activity may explain why the cellular metabolism of many diatoms remains relatively high in the absence of light and nutrients (28), and also why the recovery of diatoms from adverse conditions is immediate (27, 28). Thus, DNRA activity by buried diatoms may play a thus-far overlooked role in the functioning of sediments as seed banks for future diatom blooms (9).

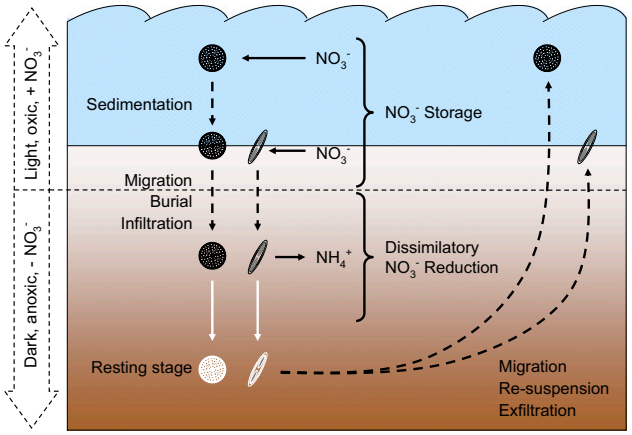

Fig. 4. Conceptual model of intracellular storage and dissimilatory reduction of NO_3^- by benthic and pelagic diatoms. Black arrows indicate cellular uptake or release of *N* compounds, black dashed arrows indicate transport of cells, and white arrows indicate physiological transition to resting stage. Block arrows on the left side and horizontal dashed line through the sediment delineate zones in which light, O_2, and extracellular NO_3^- are present or absent. Diatom cells are stylized, with centric and pennate cells representing pelagic and benthic diatoms, respectively.

DNRA probably does not sustain the metabolism during long-term survival of diatoms under dark/anoxic conditions. Survival times of diatoms range from months to decades (summarized in refs. 8 and 9) and thus greatly exceed the turnover time of the intracellular NO_3^- pool determined in the present study. Even if this time span would be elongated by renewal of the NO_3^- pool through uptake from the sediment porewater (which has not yet been investigated), it seems unlikely that DNRA activity by diatoms could persist in deep sediment layers for decades.

Implications for Nitrogen Cycling. Diatoms represent a massive and often overlooked cellular pool of NO_3^- in benthic aquatic ecosystems. Intertidal microphytobenthos harbors up to 10 times more NO_3^- than the sediment porewater (15), and the sedimentation of phytoplankton blooms further increases the cellular NO_3^- pool in sediments (7). The present study suggests that diatoms use this NO_3^- for DNRA under dark/anoxic conditions in the sediment (Fig. 4). The direct effect of DNRA by diatoms on the sedimentary nitrogen inventory may seem small, because NH_4^+ is rarely limiting in aquatic sediments. Nevertheless, DNRA by diatoms will retain fixed nitrogen in the sediment and thereby stimulate nitrification and indirectly denitrification and anammox as nitrogen removal pathways. If some of the diatoms die (e.g., due to mechanical disruption) before they have used up their intracellular NO_3^-, then other sediment microorganisms may use the NO_3^- directly for denitrification and anammox.

The NO_3^- storage capacity and dark survival potential of pelagic diatoms suggests that they are capable of DNRA as well. Assuming this, oceanic oxygen minimum zones (OMZs) are potentially important sites of DNRA activity by diatoms. Senescing diatom blooms sink out of the surface layer and pass deeper layers that are O_2-poor, but NO_3^--rich (6). Thus, sinking diatoms could exhibit DNRA activity in the dark/anoxic part of the water column at the expense of intracellular or extracellular NO_3^-. The prevalent nitrogen loss pathway in most OMZs is anammox, which requires NH_4^+ (40), which recently has been shown to be supplied by DNRA activity (41). Given their high abundance in the oceans (1), pelagic diatoms thus could be major transporters of NO_3^- and producers of NH_4^+ in the OMZs that are responsible for 30–50% of the nitrogen loss from the ocean (42, 43).

Materials and Methods

Strains and Cultivation. Axenic strains of the marine diatoms *D. brightwellii*, *Skeletonema costatum*, *Thalassiosira weissflogii*, *C. closterium*, *N. punctata*, and *A. coffeaeformis* were obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton. The diatoms were cultured in F/2 medium plus silicate (44) prepared with filtered (0.45 μm) and autoclaved North Sea seawater (salinity 35). The cultivation temperature was 15 °C, the light:dark cycle was 10:14 h, and the light intensity was 160 μmol photons m^{-2} s^{-1}. The diatom strains maintained under these optimal growth conditions were frequently checked for possible contamination with bacteria by careful phase-contrast microscopy. All culture materials used in the experiments were also checked by DAPI staining of cell suspensions immobilized on polycarbonate membrane filters (0.2 μm; Osmonics). These checks were negative for all diatom strains except *C. closterium*. In the *A. coffeaeformis* experiments (see below), the possible contamination of culture materials by bacteria was also checked by PCR using bacteria domain-specific 16S rRNA gene primers (45); the results were negative.

Intracellular NO_3^- Accumulation. The maximum intracellular NO_3^- concentration reached during the growth phase was determined for each strain by cultivation under optimal growth conditions. The cultures were repeatedly subsampled, after which the cells were washed three times with filtered and autoclaved NaCl solution (salinity 35), and then directly injected into the reaction chamber connected upstream to an NO_x analyzer (CLD 86; Eco-

Physics). At 90 °C, the acidified VCl_3 (0.1 M) in the reaction chamber reduces both NO_3^- and NO_2^- that are liberated from the bursting cells to NO, which is measured by a chemiluminescence detector (46). For simplicity, the results of the NO_x analyses are reported as NO_3^- concentrations throughout. The intracellular NO_3^- concentration was calculated from the total amount of NO_3^- in the injected subsample divided by the total cell volume of the respective diatom strain (see below). The NO_3^- concentration in the growth medium was determined after separation of cells and medium by gentle centrifugation (2 min at 100 × *g*) to avoid bursting of cells.

Determination of cell density in the subsamples was needed to monitor growth of the cultures and to calculate the intracellular NO_3^- concentration. This was done using a Fuchs–Rosenthal counting chamber and phase-contrast microscopy at 400× magnification. The strain-specific cell volume was determined by taking light micrographs of randomly chosen cells at 1,000× magnification ($n > 100$). Cell width and length were measured using UTHSCSA ImageTool (University of Texas Health Science Center). The strain-specific cell volume was estimated from these dimensions assuming a cylindrical cell shape.

Survival Under Dark/Anoxic Conditions. Cultures of the five axenic diatom strains and the contaminated *C. closterium* strain (~50×10^3 cells mL^{-1} for *D. brightwellii* and ~500×10^3 cells mL^{-1} for all other strains) were transferred into sterile, completely dark Hungate bottles (50 mL) with a gas-tight septum in the lid. Through this lid, the headspace was flushed with N_2 for 10 min to remove O_2. Every few weeks, a subsample of each culture was obtained with a sterile syringe and transferred into a new culture flask containing fresh growth medium. As a measure of survival under optimal growth conditions (illuminated and oxic), the cultures were checked for growth or no growth within 1–2 wk of transfer. The last sampling of cells was carried out after 13 wk (in single cases after 28 wk), after which the Hungate bottles were opened to measure the O_2 concentration in the cultures with a microsensor (47). O_2 was not detected in any culture.

Consumption of Intracellular NO_3^- in Response to Dark/Anoxic Conditions. *A. coffeaeformis* cells were grown under light/oxic conditions in F/2 medium plus silicate to allow uptake and intracellular storage of NO_3^-. After 2 d, the cells were harvested and washed three times with filtered and autoclaved NaCl solution (salinity 35; 10 min at 170 × *g*) to remove NO_3^- from the medium. After washing, the cells were transferred into artificial seawater (48) enriched with 200 μM Na-acetate, a commonly used electron donor for dissimilatory NO_3^- reduction (19) that promotes heterotrophic growth of many marine diatoms (49). Artificial seawater does not contain any NO_3^-, so the only source of NO_3^- in this experiment was NO_3^- stored intracellularly by the diatoms. Then 25 mL of the *A. coffeaeformis* cell suspension in artificial seawater was transferred into a sterile 50-mL Hungate bottle. The first subsample (0 h) was obtained before the Hungate bottle was sealed with a gas-tight septum and completely darkened with aluminum foil. The cell suspension was flushed with N_2 for 15 min to remove O_2. Subsamples were obtained with a sterile syringe at set time intervals over a 21-h period. For each subsample, 1 mL of well-mixed cell suspension was used to measure intracellular and extracellular NO_3^- concentrations as well as cell density (see above). To ensure that no O_2 penetrated into the medium during sampling, the cell suspension was flushed again with N_2 for several minutes after each sampling. All work was done under sterile conditions.

Dissimilatory NO_3^- Reduction in Response to Dark/Anoxic Conditions. The pathway of dissimilatory NO_3^- reduction was studied with a stable isotope labeling experiment. Before the experiment, the intracellular NO_3^- pools of *A. coffeaeformis* cells were depleted by a starvation procedure. Axenic cells were washed three times with sterile NaCl solution (salinity 35; 10 min at 170 × *g*), transferred into artificial seawater (48), and exposed to dark and anoxic conditions for 2 d (see above). After this preincubation, no NO_3^- was detectable in the medium, and the intracellular NO_3^- concentration was <0.5 mM. A subsample of these cells was cultured under optimal growth conditions in F/2 medium plus silicate in artificial seawater enriched with 1 mM $^{15}NO_3^-$ (98 atom%; Cambridge Isotope Laboratories). After 5 d, a subsample of this culture was transferred into fresh F/2 medium plus silicate in artificial seawater, enriched with 1 mM $^{15}NO_3^-$, for 3 d. The cells were then harvested, washed three times with sterile NaCl solution (see above) to remove $^{15}NO_3^-$ from the medium, and transferred into ($^{15}NO_3^-$-free) artificial seawater enriched with 200 μM Na-acetate. Thus, the only NO_3^- source during this experiment was $^{15}NO_3^-$ stored intracellularly by the diatoms.

The experiment was started by flushing the culture with He for 60 min to remove O_2 and then transferring the anoxic culture into 33 culture tubes (12 mL; Labco) wrapped in aluminum foil. At set time intervals over a 192-h period, three culture tubes each were killed to obtain subsamples for measuring cell density, intracellular and extracellular NO_3^- concentrations, and concentrations of NH_4^+, $^{15}NH_4^+$, ^{15}N-N_2O, and ^{15}N-N_2 in the growth medium. Cell density and intracellular and extracellular NO_3^- concentrations were determined as described above. Subsamples (2.5 mL) for NH_4^+ and $^{15}NH_4^+$ analysis were centrifuged (10 min at 170 × *g*), and the cell-free supernatant was frozen at −20 °C for later analysis. NH_4^+ was measured photometrically according to the method of Kempers and Kok (50), and $^{15}NH_4^+$ was measured using the hypobromite assay of Warembourg (51), followed by N_2 analysis by gas chromatography–isotope ratio mass spectrometry (VG Optima; Isotech). The hypobromite assay actually measures the sum of $^{15}NH_4^+$ and ^{15}N-labeled volatile *N* compounds such as methyl amines (31), which we denote by $^{15}NH_4^{+*}$. Subsamples (9.5 mL) for ^{15}N-N_2O and ^{15}N-N_2 analysis were killed by adding 100 μL of saturated $HgCl_2$ solution. ^{15}N-N_2O and ^{15}N-N_2 were measured by gas chromatography–isotope ratio mass spectrometry. Increases in ^{15}N-N_2O concentration with time indicate incomplete denitrification activity, whereas increases in ^{15}N-N_2 indicate complete denitrification activity.

As a negative control, an axenic culture of *A. coffeaeformis* pregrown in F/2 medium plus silicate enriched with 1 mM $^{14}NO_3^-$ was washed three times with sterile NaCl solution to remove NO_3^- from the medium (see above) and then transferred into NO_3^--free artificial seawater enriched with 200 μM Na-acetate. The culture (200 mL) was incubated under light/oxic conditions. At set time intervals over a 192-h period, subsamples were obtained to measure intracellular and extracellular NO_3^- concentrations as well as the concentration of NH_4^+ in the growth medium (see above). Cell density was determined throughout the experimental incubation.

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