Response of the Ubiquitous Pelagic Diatom *Thalassiosira weissflogii* to Darkness and Anoxia

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**Abstract**

*Thalassiosira weissflogii*, an abundant, nitrate-storing, bloom-forming diatom in the world’s oceans, can use its intracellular nitrate pool for dissimilatory nitrate reduction to ammonium (DNRA) after sudden shifts to darkness and anoxia, most likely as a survival mechanism. *T. weissflogii* cells that stored 4 mM $^{15}$N-nitrate consumed 1.15 (±0.25) fmol NO$_3^-$ cell$^{-1}$ h$^{-1}$ and simultaneously produced 1.57 (±0.21) fmol $^{15}$NH$_4^+$ cell$^{-1}$ h$^{-1}$ during the first 2 hours of dark/anoxic conditions. Ammonium produced from intracellular nitrate was excreted by the cells, indicating a dissimilatory rather than assimilatory pathway. Nitrite and the greenhouse gas nitrous oxide were produced at rates 2-3 orders of magnitude lower than the ammonium production rate. While DNRA activity was restricted to the first few hours of darkness and anoxia, the subsequent degradation of photopigments took weeks to months, supporting the earlier finding that diatoms resume photosynthesis even after extended exposure to darkness and anoxia. Considering the high global abundance of *T. weissflogii*, its production of ammonium and nitrous oxide might be of ecological importance for oceanic oxygen minimum zones and the atmosphere, respectively.

**Introduction**

Diatoms are a key group of the eukaryotic phytoplankton of the world’s oceans from polar to tropical latitudes. Pelagic diatoms form massive phytoplankton blooms [1] and may sink to the seafloor in vast abundances [2]. Diatoms are responsible for 40% of the marine primary production, or 20% of the Earth’s primary production [3,4]. Thus, they play a key role in the oceanic C-cycle and their productivity supports large-scale coastal fisheries [5]. Diatoms can also survive for decades buried deep within the dark, O$_2$-depleted sediment layers at the seafloor, where neither photosynthesis nor aerobic respiration is possible [6,7]. The survival mechanism under these non-phototropic conditions is still poorly understood. Only recently, the dissimilatory use of NO$_3^-$ by the benthic diatom *Amphora coffeaeformis* was discovered as a possible survival mechanism in darkness and anoxia [8]. The study revealed that *A. coffeaeformis* stored NO$_3^-$ intracellularly and used it for Dissimilatory Nitrate Reduction to Ammonium (DNRA; NO$_3^-$ NO$_2^-\mathrm{N}\mathrm{H}_4^+$) after sudden exposure to darkness and anoxia. Briefly, dissimilatory NO$_3^-$ reduction is an energy-generating pathway where NO$_3^-$ is taken as electron acceptor instead of O$_2$ in respiratory processes. It preferentially occurs in environments in which O$_2$ is scarce or in which steep O$_2$ gradients exist. In the marine realm, coastal sediments, oceanic Oxygen Minimum Zones (OMZs), and suspended aggregates (“marine snow”) are prominent (micro)environments characterized by O$_2$ shortage (e.g.[9-12]). Besides DNRA, denitrification (NO$_3^-\mathrm{N}\mathrm{O}_2^-\mathrm{N}\mathrm{N}_2$) and anammox (oxidation of $\mathrm{NH}_4^+$ to $\mathrm{N}_2$ with NO$_3^-$ as the electron acceptor) are important dissimilatory NO$_3^-$ reduction pathways. Dissimilatory NO$_3^-$ reduction has important implications for the marine N-cycle and is not least due to increasing use of synthetic fertilizers and subsequent pollution of rivers, estuaries, and coastal waters well studied (e.g. 13-17). However, our knowledge is almost exclusively based on prokaryotic studies; research on dissimilatory NO$_3^-$ reduction by eukaryotes and its quantitative impact on marine N-cycling is still in its infancy. The seminal work on marine eukaryotes that dissimilatorily reduce NO$_3^-$ was done by Risgaard-Petersen et al. [18]. The authors discovered that the foraminifer *Globobulimina pseudospinescens* store NO$_3^-$ in large quantities, and use it for complete denitrification under anoxic conditions. In following studies on diverse benthic foraminifera and a few gromidia from different benthic habitats, denitrification capacity was found for all analyzed species that
contained intracellular NO$_3^-$ [19-21]. In some foraminifera, denitrification is likely carried out by endobionts [22]. The storage of NO$_3^-$ might be a prerequisite for eukaryotes that can switch between O$_2$ and NO$_3^-$ respiration, because NO$_3^-$ can be taken up and stored under favorable,oxic conditions for the usage in habitats that can be temporarily exposed to anoxic conditions.

So far, all marine eukaryotes that have been found to dissimilatorily reduce NO$_3^-$ originate from benthic habitats in which anoxic conditions are common. This study addresses the response of the pelagic, NO$_3^-$-storing diatom *Thalassiosira weissflogii* to darkness and anoxia with respect to dissimilatory NO$_3^-$ reduction and stability of photopigments. Pelagic diatoms may be exposed to anoxic or hypoxic conditions in algal blooms, if O$_2$ consumption by the community exceeds O$_2$ production, e.g. at night. After the blooms, diatoms might also pass through the anoxic water layers of OMZs [12] and further sink towards the seafloor onto dark/anoxic sediments [2,23].

The occurrence and viability of *Thalassiosira* species in marine sediments is indeed well known (e.g. 24-26). We hypothesize that a survival mechanism must exist that is energized by dissimilatory NO$_3^-$ reduction. To test this hypothesis, we cultured an axenic *T. weissflogii* strain and followed the consumption of intracellularly stored $^{15}$NO$_3^-$ after a sudden shift to dark/anoxic conditions as well as the production of end products, by-products, and intermediates of denitrification and DNRA. We further investigated the stability of photopigments after exposure to darkness and anoxia as an indicator of the dark survival potential of *T. weissflogii*.

**Materials and Methods**

**Strain and Cultivation**

An axenic strain of the marine pelagic diatom *T. weissflogii* (CCMP 1336) was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA; formerly CCMP). The diatoms were cultured in F/2 medium plus silicate [27] 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$^{-1}$. *T. weissflogii* was frequently checked for possible contaminations with bacteria by careful phase-contrast microscopy and by plating out subsamples of the cultures on nutrient agar plates. Additionally, all *T. weissflogii* cultures used in the experiments were checked by DAPI staining of cell suspensions immobilized on polycarbonate membrane filters (0.2 μm; Osmonics). A contamination of the diatom strain with prokaryotes was never detected.

**Consumption and Production of Inorganic N-compounds in Dark/Anoxic versus Light/Oxic Conditions**

The time courses of intracellular NO$_3^-$ concentrations in *T. weissflogii*, and NO$_3^-$ and NH$_4^+$ concentrations in the growth medium under dark/anoxic versus light/oxic conditions were followed in a non-labeling experiment and a $^{15}$N-stable isotope labeling experiment (see below). For the non-labeling experiment, the cells were washed with sterile NaCl (salinity 35) and centrifuged (10 min at 1000g) three times to remove NO$_3^-$ from the medium, and transferred into NO$_3^-$-free artificial seawater. The cell number was determined (see below), and the experiment was started by dividing the culture for (a) the dark/anoxic incubation, and (b) the light/oxic control. For the dark/anoxic incubation, 20 mL of the diatom suspension was transferred into a dark serum bottle (wrapped in aluminum foil), flushed with N$_2$ for 20 min to remove O$_2$, sealed with a gas-tight rubber stopper, and incubated at 15°C. For the light/oxic control, the culture was kept under light/oxic culture conditions (see above). At time intervals of 0, 1, 2, 3, 4, 5, 6, and 7 h, 2 mL diatom suspension each was taken and transferred into a sample tube for centrifugation (10 min at 1000g). To assure anoxia, the dark serum bottle was flushed with N$_2$ after each sampling for 2 min. NO$_3^-$ and (non-labeled) NH$_4^+$ were determined in the cell-free supernatant and the diatom pellet was used for measurements of intracellular NO$_3^-$.

NO$_3^-$ was measured with an NO$_3$ analyzer connected to a reaction chamber (CLD 66s plus a Liquid NO Setup; Ecophysics). In the reaction chamber, acidified VCl$_2$ (0.1 M) reduces NO$_3^-$ plus NO$_2^-$ to NO at 90°C, which is then measured by a chemiluminescence detector [28]. If not noted differently, the results of the NO$_3^-$ plus NO$_2^-$ analyses are reported as NO$_3^-$ concentrations throughout, because NO$_2^-$ concentrations were $<<$ NO$_3^-$ concentrations. For intracellular NO$_3^-$ measurements, the diatom pellet was directly injected into the reaction chamber where cells burst and release the stored NO$_3^-$.

Intracellular NO$_3^-$ concentrations were calculated from the difference of NO$_3^-$ concentrations in the medium and the cell pellet, the cell numbers in the pellet, and the average cell concentration. Ammonium was measured by photometric absorbance determination at λ = 640 nm with a Genesys 10S spectrophotometer (Thermo Scientific; USA) following the sodium-nitroprusside-catalyzed reaction of NH$_4^+$ ions with salicylate and hypochlorite [29].

**Final Products of Dissimilatory Nitrate Reduction**

The time courses of intracellular NO$_3^-$ consumption and the possible products of dissimilatory NO$_3^-$ reduction, i.e. NH$_4^+$ for DNRA and N$_2$ for complete denitrification, were investigated with a $^{15}$N-stable isotope labeling experiment. Prior to the experiment, the (non-labeled) intracellular NO$_3^-$ pools of *T. weissflogii* were depleted by a starvation procedure. The cells were separated from the NO$_3^-$-containing culture medium via gentle centrifugation (10 min at 1000g), transferred into NO$_3$-free artificial seawater [30], and exposed to dark/anoxic conditions for six days. After this pre-incubation, (non-labeled) intracellular NO$_3^-$ had been completely consumed. For the subsequent storage of intracellular $^{15}$N-labeled NO$_3^-$ (98 atom %; Cambridge Isotope Laboratories), the NO$_3^-$-starved cells were harvested, re-inoculated into sterile, $^{15}$NO$_3^-$-containing F/2 medium plus silicate in artificial seawater, and cultured under...
Figure 1. Non-labeling experiment. Time courses of total intracellular NO$_3^-$ (expressed in μmol L$^{-1}$ of growth medium), and extracellular NO$_3^-$ and NH$_4^+$ concentrations in an axenic *T. weissflogii* culture in response to (A) dark/anoxic conditions, and (B) light/oxic conditions. Dark/anoxic conditions were initiated at time point 0.

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Figure 2. **15N-stable isotope labeling experiment.** Time courses of total intracellular $^{15}$NO$_3^-$ (expressed in μmol L$^{-1}$ of growth medium), and extracellular $^{15}$NH$_4^+$ and N$_2$$^{15}$N concentrations in an axenic *T. weissflogii* culture in response to (A) dark/anoxic conditions, and (B) light/oxic conditions. Dark/anoxic conditions were initiated directly after time point 0. Some error bars, which indicate standard deviation (n=3), are smaller than the symbols. The NO$_3^-$ was measured with a non-labeling sensitive technique and is consequently not plotted as $^{15}$NO$_3^-$. However, the only NO$_3^-$ source in the experiment was $^{15}$NO$_3^-$ (see Materials and Methods).

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optimal growth conditions for three days (see above). The cells were then washed via gentle centrifugation with sterile NO\textsubscript{3}\textsuperscript{-} free artificial seawater (salinity 35; 10 min at 1000g) to remove 16NO\textsubscript{3}\textsuperscript{-} from the medium, and transferred into NO\textsubscript{3}\textsuperscript{-}free artificial seawater enriched with 200 µM Na-acetate and 25 µM non-labeled NH\textsubscript{4}\textsuperscript{+}. Thus, the only NO\textsubscript{3} source during the 15N-stable isotope labeling experiment was 16NO\textsubscript{3}\textsuperscript{-} stored intracellularly by the diatoms. The cell density was obtained and the experiment was started by dividing the culture for (a) the dark/anoxic incubation, and (b) the light/oxic control. For the dark/anoxic incubation, ca. 200 mL of the diatom suspension was transferred into a dark bottle (wrapped in aluminum foil) and flushed with He for 30 min to remove O\textsubscript{2} and then transferred into 24 replicate 6 mL Labco-exetainers\textsuperscript{®} wrapped in aluminum foil. At time intervals of 1, 2, 3, 4, 5, 6, 8, and 10 h, a He headspace of 3 mL was set in three Labco-exetainers\textsuperscript{®} each, and the diatom cells in the remaining 3 mL were killed with 100 µL ZnCl\textsubscript{2} (50%). The Labco-exetainers\textsuperscript{®} were stored upside down at room temperature until measurement of 16N-labeled N\textsubscript{2} by gas chromatography-isotope ratio mass spectrometry (GC-IRMS, VG Optima; Isotech). The cell suspension collected during setting the headspace was filled into 15-mL tubes and centrifuged (10 min at 1000g). Part of the cell-free supernatant was used for immediately measuring the extracellular NO\textsubscript{3}\textsuperscript{-} concentrations, while the pellet was used for intracellular NO\textsubscript{3}\textsuperscript{-} determination (see above). Further, 1 mL cell-free supernatant was frozen at -20°C until 15NH\textsubscript{4}\textsuperscript{+} analysis using the hypobromite assay [31], followed by N\textsubscript{2}-15N analysis using GC-IRMS. The hypobromite assay actually measures the sum of 15NH\textsubscript{4}\textsuperscript{+} and 15N-labeled volatile N compounds such as methyl amines [32]. For the light/oxic control, the culture was kept under light/oxic conditions, and at time intervals of 0, 1, 2, 5, and 10 h, 3 mL cell material each was taken and processed exactly like the material that was obtained during setting the headspace in the dark/anoxic treatment. The sample collected at time point zero was used for both, the dark/anoxic incubation and the light/oxic control.

### Table 1. Cell-specific consumption (neg. values) rates of NO\textsubscript{3} by axenic *T. weissflogii* cultures in response to different experimental conditions for the non-labeling experiment.

| Experimental conditions | Time interval (h) | NO\textsubscript{3} (fmol N cell\textsuperscript{-1} h\textsuperscript{-1}) |
|-------------------------|------------------|--------------------------|
| Dark/anoxic             | 0 – 2            | -7.47                   |
|                         | 2 – 7            | -1.54                   |
| Light/oxic              | 0 – 2            | -12.38                  |
|                         | 2 – 7            | -0.29                   |

Rates were calculated from the time course of NO\textsubscript{3} presented in Figure 1 for linear concentration changes in the given time intervals. Cell densities were 435 cells µL\textsuperscript{-1} for dark/anoxic conditions, and 525 cells µL\textsuperscript{-1} for light/oxic conditions; the initial intracellular NO\textsubscript{3} concentration was 20 mM.

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### Intermediates and By-Products of Dissimilatory Nitrate Reduction

The time courses of N\textsubscript{2}O and NO\textsubscript{2} as possible intermediates or by-products of dissimilatory NO\textsubscript{3} reduction were measured during the 15N-stable isotope labeling experiment. Nitrous oxide was measured in the headspace of the Labco-exetainers\textsuperscript{®} from the dark/anoxic incubation experiment after N\textsubscript{2}O-15N analysis had been completed (see above; the gas volume removed for N\textsubscript{2}O-15N measurements was taken into account for the subsequent calculation of N\textsubscript{2}O concentrations), using a GC 7890 (Agilent Technologies) equipped with a CP-PoraPLOT Q column and a 63Ni electron capture detector. Nitrite was determined in the supernatant of the medium from the dark/anoxic and the light/oxic incubation with an NO\textsubscript{3} analyzer as described for the NO\textsubscript{3} determination, except that the reaction chamber contained acidified NaI (2 M) that reduces NO\textsubscript{2} to NO at 20°C.

### Degradation of Photopigments in Response to Dark/Anoxic Conditions

Chlorophyll a and fucoxanthin were determined in cultures of *T. weissflogii* that were first exposed to favorable growth conditions (i.e., with light and O\textsubscript{2} time 0) and then to dark/anoxic conditions for a time period of 46 weeks. To adjust dark/anoxic conditions, diatom cultures were transferred into gas-tight, dark bottles (wrapped in aluminum foil), flushed with N\textsubscript{2} for 30 min and kept at 15°C until sampling. At each time point, 2 mL of the cell suspension was taken in 3 replicates and cell numbers were counted. The samples were freeze-dried for 2 days and 5 mL ice-cold acetone was added for extraction of photopigments. After vigorous mixing and sonication for 5 min, the samples were left over night at -20°C, mixed again, and centrifuged for 5 min at 3000g. The supernatants were filtered (Acrodisc\textsuperscript{®} CR 4 mm, 0.45 µm Versapor\textsuperscript{®}; Gelman Laboratory) and the extracted photopigments were separated by means of HPLC (Waters 2695; U.S.A.) and analyzed by a photodiode array detector (Waters 996; U.S.A.) as described in Stief et al. [26]. In the chromatograms, chlorophyll a and fucoxanthin were identified according to their specific retention time and absorption spectra and the respective peaks were integrated with the Millenium\textsuperscript{®}32 software (Waters, U.S.A.). Calibrations were made with serial dilutions of chlorophyll a and fucoxanthin stock solutions (DHI, Denmark). All procedures were made under dark conditions and using HPLC-grade chemicals.

### Results and Discussion

#### Dissimilatory Nitrate Reduction to Ammonium by *T. weissflogii*

Our results strongly indicate that the ubiquitous pelagic diatom *T. weissflogii* is able to perform DNRA, similar to the benthic diatom *Amphora coffeaeformis*, which was the first phototrophic eukaryote shown to dissimilatorily reduce NO\textsubscript{3} under dark/anoxic conditions [8]. Consumption of intracellular NO\textsubscript{3} and simultaneous production of NH\textsubscript{4} in response to dark/anoxic vs. light/oxic conditions have been followed in two separate experiments: (a) a non-labeling experiment in which NH\textsubscript{4} was measured photometrically (Figure 1) and (b) a 15N-
stable isotope labeling experiment (Figure 2). In both experiments, the rapid consumption of intracellular NO$_3^-$ and $^{15}$NO$_3^-$ by T. weissflogii was accompanied by the production and release of NH$_4^+$ and $^{15}$NH$_4^+$, respectively, only under dark/anoxic conditions, but not in the presence of light and O$_2$ (Figures 1,2). In the $^{15}$N-stable isotope labeling experiment, the initial $^{15}$NH$_4^+$ concentration was 2 µM because the hypobromite assay actually measures the sum of $^{15}$NH$_4^+$ and $^{15}$N-labeled volatile N compounds such as methyl amines [32]. The concentration of NO$_3^-$ in the medium, i.e. extracellular NO$_3^-$, only decreased under light/oxic conditions, but remained constant after exposure to dark/anoxic conditions (Figure 1). This constant (and not increasing) extracellular NO$_3^-$ concentration indicates that the intracellular NO$_3^-$ (expressed in µmol L$^{-1}$ of growth medium) was indeed consumed by T. weissflogii rather than released from the cells into the medium.

Intracellular NO$_3^-$ was also consumed under light/oxic conditions, even at a higher rate than under dark/anoxic conditions (Tables 1,2), most probably because NO$_3^-$ was used for assimilation by photosynthetically active diatoms [33-35]. For N-assimilation, NO$_3^-$ is also reduced to NH$_4^+$, but NH$_4^+$ is not released from the cells.

In the absence of O$_2$, intracellular NO$_3^-$ can be used for dissimilation by sulfur bacteria [36-39] and only a few unicellular eukaryotes and fungi (e.g. [8,18,20,40,41]). The ubiquitous diatom T. weissflogii can now be added to the short list of eukaryotes that dissimilatorily reduce NO$_3^-$. Notably, T. weissflogii is the first marine pelagic eukaryote shown to have an anaerobic NO$_3^-$ metabolism, whereas all known eukaryotic NO$_3^-$ reducers thrive in stratified waters, sediments and soils in which anoxic conditions occur in subsurface layers. So far it is not known, whether DNRA in T. weissflogii is respiratory or fermentative. Briefly, in respiratory DNRA, ATP is generated by an electrochemical proton potential across a cell membrane, at which electrons are transferred from the donor to the acceptor NO$_3^-$, and in fermentative DNRA, ATP is generated by substrate-level phosphorylation [16,42-44]. In prokaryotes, the electron donor and acceptor for respiratory DNRA usually originate from an external source and not from cell metabolism, but may be either organic or inorganic, whereas the electron donor in fermentative DNRA is usually organic [45]. So far, the electron donor used by diatoms for DNRA is not known. In our labeling experiment, acetate was added as a potential electron donor. However, it needs to be further investigated, if T. weissflogii can perform DNRA also with intracellularly stored...
electron donors, like polysaccharides (e.g. chrysolaminarin), and if the external supply of acetate indeed influences the rate of DNRA.

Our experiments revealed that the rate of NO$_3^-$ consumption after exposure to dark/anoxic conditions depends on the concentration of intracellularly stored NO$_3^-$. In the non-labeling experiment, the initial intracellular NO$_3^-$ concentration was 20 mM, and in the labeling experiment only 4 mM, resulting in a 6 times lower rate of NO$_3^-$ consumption (Tables 1,2). In the labeling experiment, the production of $^{15}$NH$_4^+$ (plus N$_2$O and NO$_2^-$) by T. weissflogii balanced the consumption of intracellular $^{15}$NO$_3^-$ within the bounds of accuracy (Figure 2; Table 2), whereas in the non-labeling experiment, the net production of NH$_4^+$ did not balance the consumption of intracellular NO$_3^-$ (Figure 1). On average, less than half of the NO$_3^-$ was found back as NH$_4^+$ in the culture medium; further, the NH$_4^+$ concentration first increased and then decreased slightly with time (Figure 1). This decrease of the NH$_4^+$ concentration in the non-labeling experiment is explained by an uptake of NH$_4^+$ by T. weissflogii under dark/anoxic conditions that has also been confirmed in other experiments (data not shown). A dark NH$_4^+$ uptake and assimilation, respectively, is generally known for phytoplankton [34,46] and was recently also confirmed by gene expression analysis in Thalassiosira pseudonana [47]. This dark NH$_4^+$ uptake is not apparent in the labeling experiment, because the addition of non-labeled NH$_4^+$ as background concentration (see Materials and Methods) obscures the putative uptake of $^{15}$NH$_4^+$. However, under the light/oxic conditions of the labeling experiment, $^{15}$NH$_4^+$ also decreased after 5 h because the (non-labeled) background NH$_4^+$ was completely taken up (data not shown). The labeling approach did not reveal a production of N$_2$-$^{15}$N by T. weissflogii (Figure 2), which further supports that DNRA and not denitrification is used as a dissimilatory NO$_3^-$ reduction pathway by T. weissflogii.

**Release of Nitrous Oxide and Nitrite during Nitrate Dissimilation**

The production of N$_2$O and NO$_2^-$ in response to dark/anoxic conditions has been followed during the $^{15}$N-stable isotope labeling experiment (Figure 3). Both, N$_2$O and NO$_2^-$ were produced and released from the cells in the same time pattern that has been observed for $^{15}$NH$_4^+$, and their production apparently mirrors the consumption of intracellular NO$_3^-$ (Figures 1,2,3). However, the production rates of N$_2$O and NO$_2^-$ were about 1000 and 100 times, respectively, lower than the production rate of $^{15}$NH$_4^+$ (Table 2). Thus, N$_2$O and NO$_2^-$ are not
Table 2. Cell-specific consumption (neg. values) and production (pos. values) rates of N compounds by axenic T. weissflogii cultures in response to different experimental conditions for the 15N-stable isotope labeling experiment.

| Experimental conditions | Time interval (h) | NO3- (fmol N cell⁻¹ h⁻¹) | 15NH4⁺ (fmol N cell⁻¹ h⁻¹) | N2O (fmol N cell⁻¹ h⁻¹) | NO2⁻ (fmol N cell⁻¹ h⁻¹) |
|-------------------------|------------------|--------------------------|---------------------------|------------------------|--------------------------|
| Dark/anoxic             | 0 – 2            | -1.150 (±0.253)          | +1.571 (±0.212)           | +0.007 (±0.002)        | +0.049 (±0.047)           |
|                         | 2 - 10           | -0.261 (±0.026)          | +0.313 (±0.159)           | +0.002 (±0.001)        | +0.042 (±0.006)           |
| Light/oxic              | 0 – 2            | -1.508 (±0.283)          | -0.066 (±0.233)           | ND                     | ND                       |
|                         | 2 - 10           | -0.224 (±0.035)          | -0.442 (±0.141)           | ND                     | ND                       |

Rates were calculated from the time course of N compounds presented in Figures 2 and 3 for linear concentration changes in the given time intervals. Cell densities were 407 cells μL⁻¹; the initial intracellular NO3- concentration was 4 mM. Means (±SE) for n=3 are shown; ND: not determined; *Rate calculated for 1 - 2 h only.

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Slow Degradation of Photopigments in Darkness and Anoxia

To estimate how long T. weissflogii cells retain the ability to operate photosynthesis after exposure dark/anoxic conditions, the fate of the photopigments chlorophyll a and fucoxanthin was followed. Notably, the degradation of the photopigments did not temporally coincide with DNRA by T. weissflogii in response to dark/anoxic conditions. While DNRA activity peaked during the first few hours of dark/anoxic conditions, the major decrease in cellular photopigment contents occurred during the first 3 days (Figures 2, 4). After one week of dark/anoxic incubation, the cellular pigment contents had reached a low, but constant level that was maintained for at least 7.5 weeks (Figure 4). These observations are in good agreement with the hypothesis that diatoms use DNRA to enter a resting stage with low metabolic activity, and that T. weissflogii was found to survive at least for 6 weeks after adjusting them to dark/anoxic conditions [8]. Diatoms are known to start photosynthesis and growth very fast after (re)adjusting them to favorable growth conditions, i.e. light and fresh growth medium, even after extended periods of darkness [24,56,57]. To maintain at least low cellular contents of photopigments must be a prerequisite for this. Our experimental design, i.e. that no O2 and prokaryotes were present in the T. weissflogii culture, further led to a decreased rate of degradation, as O2-dependent pigment alteration and grazing-induced cell disruption could not occur [58]. Interestingly, the chloroplasts of T. weissflogii cells showed an autofluorescence even after more than 1 year under dark/anoxic conditions (pictures not shown), which might originate from photopigment degradation products that are still poorly understood [59].

Ecological and Evolutionary Perspectives

After the benthic diatom A. coffeaeformis was discovered as the first phototrophic eukaryote that dissimilatorily reduces NO3-, it was interesting to ask whether this metabolism also occurs in pelagic diatoms: and indeed, we found T. weissflogii as the so far only marine pelagic eukaryote showing this metabolic trait. The respiration of NO3- by diatoms might be widespread in marine ecosystems and could have so far overseen implications on the marine N-cycle. For benthic foraminifera, Piña-Ochoa et al. [20] calculated a contribution for the removal of fixed N from marine ecosystems that may be

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final products of dissimilatory NO3- reduction, but the congruent time patterns indicate that T. weissflogii releases N2O and NO3- as by-product and intermediate, respectively, of DNRA. In prokaryotes, N2O is a well-known by-product and intermediate of nitrification and denitrification, respectively, and there are some indications that N2O is also released as a by-product of DNRA, which might have been overseen in some organisms [16,48]. In higher plants, N2O is emitted from leaves by plant NO3 assimilation, strictly speaking during photoassimilation of NO3- in the chloroplast [49]. Recently, N2O production was also found in axenic, illuminated cultures of the green algae Chlorella vulgaris [50]. A release of N2O by phototrophic eukaryotes under darkness and anoxia has to our knowledge not been documented so far. Even though the rate of N2O released by T. weissflogii during DNRA might seem low (see above, Table 2), this finding can be of environmental importance because diatoms are highly abundant in the world’s oceans (e.g. [3,51]), hypoxic and anoxic marine environments are spreading [52], and N2O is a particularly strong greenhouse gas [53]. The production of N2O under dark/anoxic conditions has recently also been confirmed for the benthic diatom A. coffeaeformis [8], and it might be worth to screen other benthic and pelagic diatom species for N2O emission under these conditions.

The NO3- release during DNRA by T. weissflogii could be due to cell leakage or excretion that is frequently observed in marine phytoplankton, including diatoms [54,55], but has not been linked to a response of phytoplankton to darkness and anoxia so far. The observed NO3- release might be supported by a slightly higher rate of NO3- reduction than NO2- reduction throughout the incubation. Further, there might be a time delay in NO3- reduction to NH4+ because of constitutive expression of the NO3-reductase gene, whereas the (dissimilatory) NO2-reductase gene first needs to be induced by the production of NO2-. T. weissflogii is not able to take up the released NO2- again under dark/anoxic conditions, which is indicated by the observation that the medium NO2- concentration is not decreasing during the incubation (Figure 3). Additionally, intracellular NO2- storage does not occur in T. weissflogii (data not shown), probably because of the toxic effects of NO2- [54].
equally important to eukaryotic denitrification in the seafloor. DNRA will not directly remove fixed N, but in anoxic or hypoxic environments, the produced NH$_4^+$ can serve as electron donor for anammox that might be especially important in OMZs with high abundances of anammox bacteria [60]. Further research on NO$_3^-$ respiration by diatoms might also reveal that certain species are capable of other pathways than DNRA, like denitrification as shown for foraminifera [18,20]. Additionally, the exact ambient O$_2$ concentration in the (micro)environment of the diatoms may trigger different dissimilatory NO$_3^-$ reduction pathways as known from fungi [45].

To date, genes involved in dissimilatory NO$_3^-$ reduction have not been identified in NO$_3^-$-respiring diatoms, foraminifera or gromidiidae. In contrast, several functional genes have been identified in the denitrifying fungus *Fusarium oxysporum*: a copper-containing NO$_3^-$ reductase (nirK) and a nitric oxide reductase (P450nor) have been sequenced and characterized [41,61]. Intriguingly, NO$_3^-$-respiring fungi may use enzymes that are normally involved in assimilatory NO$_3^-$ reduction in a dissimilatory mode instead [62]. This could also hold true for diatoms. Assimilatory NO$_3^-$ reductases, multiple transporters for NO$_3^-$, and components of a NO$_3^-$-sensing system have only recently been discovered in diatom genomes [63,64]. First insights into diatom genomes and the ensuing ecophysiological studies revealed a fascinating evolutionary history of diatoms. An unexpected combination of genes by endosymbiotic gene transfer from two secondary endosymbionts to the exosymbiotic nucleus, and also horizontal gene transfer led to several additional inclusions from Bacteria and Archaea genomes [63-66]. The diverse assortment of genes results in novel biochemical pathways like the urea cycle [63,65,67-71] that formerly was not known for photosynthetic organisms and congruently makes diatoms for Armbrust et al. [51] to be neither plants nor animals. Further work on diatom genomes could lead to the identification of functional genes involved in dissimilatory NO$_3^-$ reduction. This would not only convey genetic evidence of dissimilatory NO$_3^-$ reduction by eukaryotes, but would also provide genetic markers for the cultivation-independent detection of so far unrecognized dissimilatorily NO$_3^-$ reducing diatoms directly in the environment.

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**Author Contributions**

Conceived and designed the experiments: AK PS DdB. Performed the experiments: AK PS JK. Analyzed the data: AK PS JK. Wrote the manuscript: AK PS DdB.
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