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IDENTIFICATION AND EXPRESSION ANALYSES OF THE NITRATE TRANSPORTER GENE (*NRT2*) FAMILY AMONG *SKELETONEMA* SPECIES (BACILLARIOPHYCEAE)[1]

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High-affinity nitrate transporters are considered to be the major transporter system for nitrate uptake in diatoms. In the diatom genus *Skeletonema*, three forms of genes encoding high-affinity nitrate transporters (*NRT2*) were newly identified from transcriptomes generated as part of the marine microbial eukaryote transcriptome sequencing project. To examine the expression of each form of *NRT2* under different nitrogen environments, laboratory experiments were conducted under nitrate-sufficient, ammonium-sufficient, and nitrate-limited conditions using three ecologically important *Skeletonema* species: *S. dohrnii*, *S. menzelii*, and *S. marinoi*. Primers were developed for each *NRT2* form and species and Q-RT-PCR was performed. For each *NRT2* form, the three *Skeletonema* species had similar transcriptional patterns. The transcript levels of *NRT2:1* were significantly elevated under nitrogen-limited conditions, but strongly repressed in the presence of ammonium. The transcript levels of *NRT2:2* were also repressed by ammonium, but increased 5- to 10-fold under nitrate-sufficient and nitrogen-limited conditions. Finally, the transcript levels of *NRT2:3* did not vary significantly under various nitrogen conditions, and behaved more like a constitutively expressed gene. Based on the observed transcript variation among *NRT2* forms, we propose a revised model describing nitrate uptake kinetics regulated by multiple forms of nitrate transporter genes in response to various nitrogen conditions in *Skeletonema*. The differential *NRT2* transcriptional responses among species suggest that species-specific adaptive strategies exist within this genus to cope with environmental changes.

Key index words: **diatom; gene expression; nitrate transporter gene; Nitrogen; *NRT2* gene family; *Skeletonema***

***Abbreviations*: *EFL*, translation elongation factor-like gene; MMETSP, Marine Microbial Eukaryote Transcriptome Sequencing Project; *NRT2*, nitrate transporter gene; Q-RT-PCR, quantitative reverse-transcription polymerase chain reaction**

Diatoms contribute ~40% of annual marine primary production (Nelson et al. 1995, Field et al. 1998). Nitrogen is likely a major limiting nutrient controlling their growth in the ocean (Howarth and Marino 2006). However, diatoms are a diverse group of phytoplankton (Kooistra et al. 2007, Armbrust 2009), and different species possess different strategies for survival in environments with various nitrogen sources and availability (Dortch 1990, Lomas and Glibert 1999). For example, several diatom species (e.g., *Skeletonema costatum*) are able to take up and store nitrate in excess of growth demands (Dortch et al. 1984, Lomas and Glibert 2000), while other species (e.g., *Chaetoceros affinis*) exhibit nitrogen uptake rates that are closely coupled to growth rates (Collos 1982, Dortch 1982). Differences in nitrate uptake dynamics among species lead to differing competitive advantages under different nitrate concentrations providing a plausible mechanism for diatoms' geographic distribution patterns and species succession (Kang et al. 2015).

Nitrate is the most stable and abundant form of inorganic nitrogen in seawater and has been regarded as the main nitrogen source for diatoms. For decades, studies of nitrate uptake have guided our understanding of how diatom cells utilize nitrate for growth (e.g., Eppley and Thomas 1969, Eppley et al. 1969, Falkowski 1975, Morel 1987). The classic way to represent diatom uptake is based on the Michaelis–Menten functional form for the uptake rate (Dugdale 1967, Droop 1973). Since diatom cells are able to adjust their kinetic parameters in response to environmental changes, more complex models have been proposed (Morel 1987, Aksnes and Egge 1991, Smith et al. 2009, Bonachela et al. 2011). However, the basic mechanism of

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nitrate transport across diatom cell membranes remains unclear.

High-affinity nitrate transporters are membrane-associated proteins and considered to be the major transporter system responsible for nitrate uptake in marine diatoms (Song and Ward 2007). Multiple nitrate transporter genes, *NRT2*s, have been identified in numerous diatom species (Hildebrand and Dahlin 2000, Song and Ward 2007, Kang et al. 2011), haptophytes (Kang et al. 2007, Song and Ward 2007, Charrier et al. 2015), and eukaryotic picophytoplankton (McDonald et al. 2010). Generally, the mRNA level of *NRT2* in diatoms changes rapidly and sensitively in response to different nitrogen conditions, where *NRT2* transcript levels are low in the presence of ammonium, moderately expressed under nitrate-sufficient conditions, and highly expressed under nitrate-deprived conditions (Song and Ward 2007, Kang et al. 2009). Although it has been noted that there are multiple forms of *NRT2* genes in diatoms (e.g., Armbrust et al. 2004, Bowler et al. 2008, Bender et al. 2014), comparative gene expression among different forms has been investigated only in *Thalassiosira pseudonana*. Differential gene expression among three different forms of *NRT2* in *T. pseudonana* revealed form-specific expression in response to different nitrogen sources (Kang et al. 2009) suggesting that nitrate uptake in diatoms may prove to be more complex than anticipated, with at least one form behaving like a constitutively expressed gene. Therefore, exploring the function and regulation of multiple *NRT2* genes may provide additional information for understanding how diatoms adjust their nitrate uptake kinetics to cope with environmental changes.

Skeletonema is a common diatom genus, especially in coastal, estuarine, and upwelling regions where it often forms dense blooms (Cloern et al. 1985, Kooistra et al. 2008, Borkman and Smayda 2009). Our recent study reported that both *Skeletonema* abundance and *Skeletonema NRT2* expression were closely related to the depth of the nutricline, which suggested that nitrate supply has a strong bottom-up influence on the distribution of *Skeletonema* spp. in coastal regions (Kang et al. 2015). Recently, the genus *Skeletonema* was shown to consist of several distinct species based on both morphological and genetic variation (Sarno et al. 2005, 2007, Zingone et al. 2005). There appear to be differences in geographic distributions among species (Kooistra et al. 2008) as well as temporal species succession within individual locations (Canesi and Rynearson 2016) suggesting that there are significant ecological differences among these closely related species. Variation in nutrient metabolism among *Skeletonema* species may help to explain species succession and geographic distributions in the ocean.

In this study, we examined high-affinity nitrate transporters in the *NRT2* gene family among *Skeletonema* species using transcriptomes generated as part of the Marine Microbial Eukaryote Transcriptome Sequencing Project, MMETSP (Keeling et al. 2014), and identified three forms of *NRT2* genes. We examined their transcriptional patterns under various nitrogen conditions in three cultured strains: *S. dorhnii*, *S. marinoi,* and *S. menzelii*. These species were chosen because they have different seasonal cycles, with *S. marinoi* and *S. dohrnii* being most common during winter and spring and *S. menzelii*, during summer (Kaeriyama et al. 2011, Canesi and Rynearson 2016).

MATERIALS AND METHODS

Phylogenetic analysis of Skeletonema NRT2 genes. *NRT2* sequences from the *Thalassiosira pseudonana* (JGI12741, 26974, and 39592) and *Phaeodactylum tricornutum* (JGI54101, 26029, 54560, 12032, and 12171) genomes were used for homology-based searches to identify *Skeletonema* sequences from the MMETSP transcriptome database (https://www.imicrobe.us/project/view/104) using BlastX (E-value cut-off of 1E-20, ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/). For species with multiple transcriptomes in the MMETSP database, *NRT2* sequences with >98% identity were combined for phylogenetic analysis. Transcript sequences were translated to deduced amino acid sequences using Bioedit Sequence Alignment Editor (version 7.2.5; Department of Microbiology, North Carolina State University, Raleigh, NC, USA). The corresponding NRT2 sequences in the genomes of other diatom species were obtained from GenBank and the diatom genome database (Joint Genome Institute, http://genome.jgi.doe.gov). The amino acid sequences were aligned using ClustalW (Thompson et al. 1994) and trimmed to include the conserved region (421 amino acid positions) spanning 12 predicted transmembrane domains (Hildebrand and Dahlin 2000). The resulting alignment files were used to construct phylogenetic trees with PHYLIP software (the PHYLogeny Inference Package version 3.69, Felsenstein 1989). Maximum likelihood trees were generated with the PROML program in PHYLIP. Bootstrap values were obtained with 100 bootstrap replicates.

Culture conditions. *Skeletonema menzelii* strain CCMP793 and *Skeletonema dohrnii* strain CCMP3373 were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). *Skeletonema marinoi* strain Skel F was isolated from Narragansett Bay RI, USA by D. Roche. All strains were maintained at 14°C in f/2-enriched seawater medium (Guillard and Ryther 1962) under a 12:12 h light:dark photoperiod with a light intensity of 100 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} and were confirmed axenic at the beginning of the experiment.

NRT2 *mRNA abundance of NO_3^--sufficient, NH_4^+-sufficient, and N-limited cells.* For each species, cells in nitrate-sufficient, ammonium-sufficient, and nitrogen-limited conditions were prepared in duplicate for the detection of *NRT2* mRNA abundance. Nitrate-sufficient cells were grown in batch cultures using f/2 medium with an initial nitrate concentration of 883 μM as the nitrogen source. After inoculation, cells were harvested daily during the 3 d of the early- to mid-log phase for cell counts and RNA isolation (~5.1×10^6 to 1.8×10^8 cells). Ammonium-sufficient cells were grown in modified f/2 medium (h/2; Guillard and Ryther 1962) with nitrate replaced by ammonium at an initial concentration of 500 μM as the nitrogen source. The ammonium-sufficient cells were also harvested daily during the 3 d of the early- to mid-log phase (~2.4×10^6 to 1.4×10^7 cells). N-limited cells were prepared by collecting ammonium-sufficient cells via

centrifugation at 3,000*g* for 5 min at 4°C, followed by two washes with nitrogen-free (N-free) medium. The washed cells were then resuspended in the N-free medium, and incubated for 2 and 3 d to reach a nitrogen-limited status before harvest (~3.6×10^7 to 2.0×10^8 cells). To ensure nitrogen-free status, the N-free f/2 medium was prepared with surface Sargasso seawater. For all treatments, cell concentrations were determined with a haemocytometer (Hausser Scientific, Horsham, PA, USA). Growth rates (in doublings $\cdot$ d^{-1}) were calculated from cell counts using the following equation:

$$k = \frac{\log_2\left(\frac{N_t}{N_0}\right)}{t_2 - t_0} \quad (1)$$

where k is the doublings per day and N_t and N_0 are the cell concentrations at t_2 and t_0, respectively. Algal cells for RNA isolation were harvested via centrifugation at 3,000 *g* for 10 min at 4°C, resuspended in RLT buffer (Qiagen, Valencia, CA, USA) with 1% v/v of β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and then stored at −20°C until further processing.

RNA isolation. Samples for RNA isolation were thawed on ice, followed by supersonic disruption (Digital Sonifier 450; Branson, Danbury, CT, USA). Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The RNase-Free DNase Set (Qiagen) was applied for on-column digestion of residual DNA during RNA purification. RNA concentrations were determined with a spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA) at the wavelengths of 260 and 280 nm. Total RNA (0.1 μg) was reverse-transcribed into cDNA using random hexamers and Quantiscript Reverse Transcriptase (QuantiTect Reverse Transcription Kit; Qiagen).

Primer design. The PCR primer sets for real-time quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) were specifically designed to amplify a 100–150 bp fragment from each of the *NRT2* and *EFL* (translation elongation factor-like) genes in each *Skeletonema* species (Table S1 in the Supporting Information). *EFL* was used as the reference gene (Kang et al. 2012). To separately determine the transcript levels of each *NRT2* gene, each primer contained at least four mismatching nucleotides compared to homologous segments belonging to the other two forms of *NRT2* and a single mismatch at the 3′ terminal. The amplification efficiencies of these primer sets were evaluated using serially diluted cDNA obtained from each species. Controls without reverse transcriptase were performed to confirm absence of genomic DNA.

Real-time Q-RT-PCR. Quantitative PCRs were initiated by adding 5 μL of 10-fold diluted cDNA to a mixture containing 1X QuantiFast SYBR Green PCR Master Mix (Qiagen) and 300 nM forward and reverse primers. Real-time PCR analysis was conducted using an Mx3005P QPCR System (Stratagene; Agilent Technologies Inc., Santa Clara, CA, USA). Thermocycler settings were 95°C for 5 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The specificity of the Q-RT-PCR products was confirmed by melting temperature analysis from 55 to 95°C. The fluorescence intensity from the complex formed by SYBR Green and the double-stranded PCR product was continuously monitored from cycles 1 to 40. The threshold cycle (C_T) at which the fluorescence intensity exceeded a preset threshold was used to calculate the gene transcript levels of each gene. The amount of target-gene cDNA (X) normalized to that of a reference gene (R) was calculated according to the absolute ratio method described by Chung et al. (2005) as follows:

$$\log\left(\frac{X_0}{R_0}\right) = \log\left(\frac{M_R}{M_X}\right) + \frac{C_{T,X}}{b_X} - \frac{C_{T,R}}{b_R} \quad (2)$$

where X_0 and R_0 are the target and reference molecules in the original sample, M_R and M_X are the molecular weights of the target and reference amplicons, $C_{T,X}$ and $C_{T,R}$ are the C_T values of X and R, and b_X and b_R are the standard curve slopes of X and R, respectively. Each Q-RT-PCR was performed in duplicate to enable the calculation of a standard deviation for tube replicates:

$$\mathrm{Std}_{\log(X_0/R_0)} = \sqrt{\frac{\mathrm{Var}(C_T, X)}{{b_X}^2} + \frac{\mathrm{Var}(C_T, R)}{{b_R}^2}} \quad (3)$$

where $\mathrm{Var}(C_{T,X})$ and $\mathrm{Var}(C_{T,R})$ are the variances of $C_{T,X}$ and $C_{T,R}$, respectively.

Statistical analyses. To compare differential expression among treatments and forms, transcript levels of each *NRT2* form were monitored during the first 3 d of the experiment. An analysis of variance (ANOVA) test was conducted on each treatment, species and target gene to confirm that no significant differences in gene expression occurred over the 3-d period and where appropriate, a post hoc Fisher's least significant difference (LSD) was applied to determine days where gene expression remained stable. Because transcript levels of each *NRT2* form in nitrogen-replete cultures were not significantly different (Figs. S1–S3 in the Supporting Information), average expression levels were calculated using duplicates for days 1–3 in ammonium-sufficient ($n = 6$) and nitrate-sufficient ($n = 6$) conditions. For cells transferred into N-free medium, average expression levels were calculated using only days 2 and 3 ($n = 4$), based on the results of the ANOVA and LSD tests (Figs. S1–S3) showing that for some species and genes, expression stabilized only after 48 h in the N-free media. This response is consistent with previous literature showing that *Skeletonema* can take up and store nitrogen in excess of growth demands (Dortch et al. 1984, Lomas and Glibert 2000). To test for differences in transcript levels among *NRT2* forms and nitrogen conditions among species, ANOVA and post hoc LSD tests were used. Differences were considered statistically significant with $P < 0.05$.

RESULTS

Identification of NRT2 *genes in* Skeletonema. Homology-based searches of the MMETSP database using *NRT2* genes from *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* genomes yielded 52 homologous amino acid sequences belonging to 11 *Skeletonema* strains. These amino acid sequences were aligned and verified via manual inspection (Tables S2 and S3 in the Supporting Information). Three forms of NRT2 were identified in *Skeletonema* (Fig. 1): all were classified in the same protein family PF07690 (Pfam)/IPR004737 (interpro) and there were no differences in domains among NRT2 forms. Phylogenetic analyses revealed that each NRT2 form comprised a distinct clade with strong bootstrap support (100%). The sequences in Clade I shared an average identity of 95.7% with the first discovered NRT2 sequence in *S. costatum* (NRT2_AAL85928; Fig. 1; Song and Ward 2007). NRT2 in this clade was thus named NRT2:1. Clade II shared an average identity of 74.7% with Clade I, and these two clades

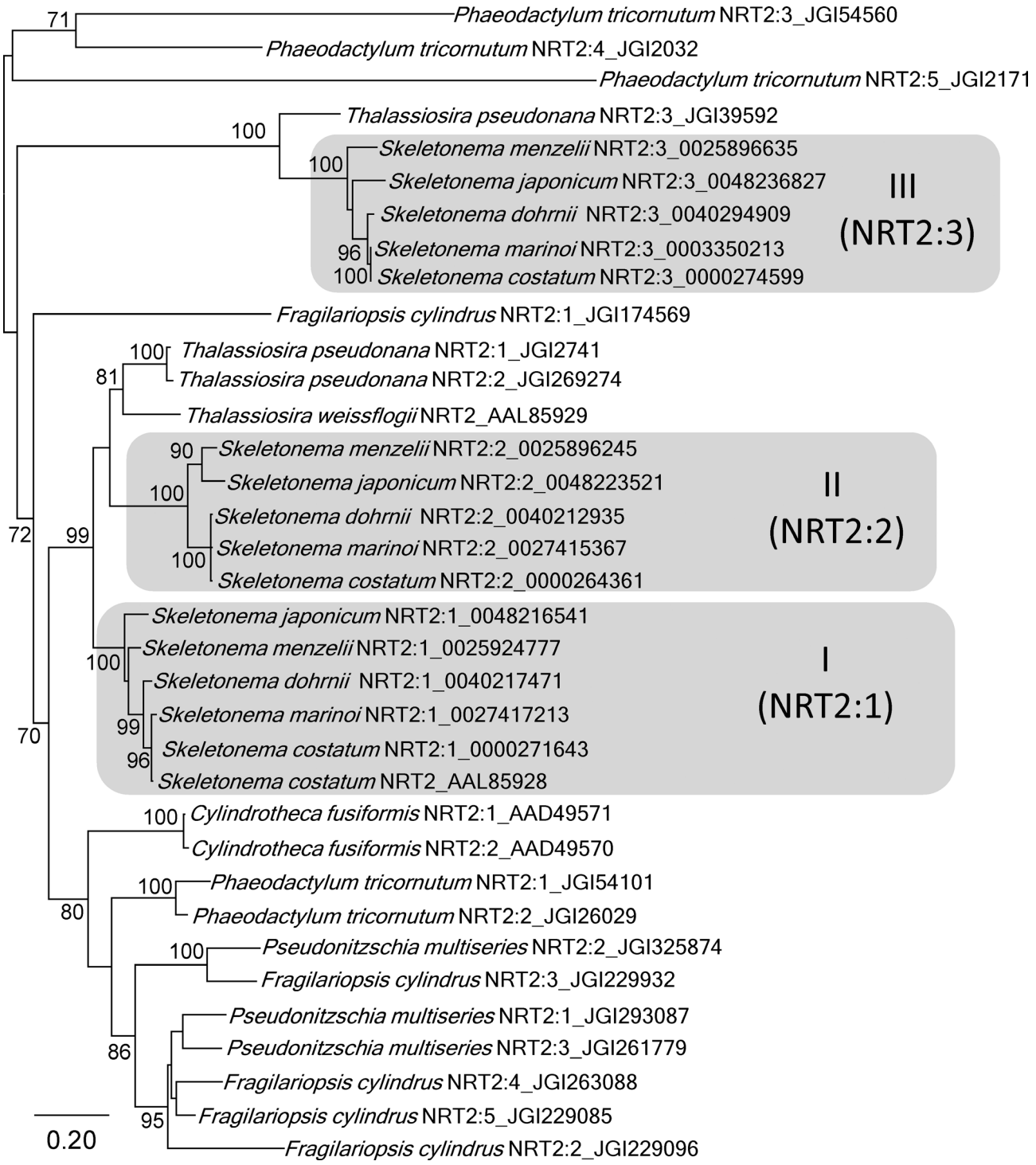

FIG. 1. Phylogenetic tree of nitrate transporter (NRT2) amino acid sequences in diatoms. This phylogenetic tree is a condensed version of a detailed analysis including homologous *NRT2* sequences from the MMETSP. To reduce the sequences from multiple transcriptomes among different strains and treatments, sequences (spanning the conserved transmembrane regions) with shared identities of >0.98 were combined and the most-conserved sequence was selected as a representative. The numbers at the nodes are bootstrap values based on 100 resamplings, and only values of > 70% are shown. Sequence accession numbers are from GenBank or JGI. Sequences from MMETSP are followed by the CAMNT number.

were closely related to the TpNRT2:1 and TpNRT2:2 which were considered as the primary nitrate transporters in *Thalassiosira pseudonana* (Kang et al. 2009). Therefore, NRT2 sequences in Clade II were named NRT2:2. Finally, the sequences in Clade III differed greatly from Clade I and II and

shared only an average identity of 51.8%. This distinct clade was clustered with TpNRT2:3, thus, sequences in this clade were named NRT2:3.

Effect of nitrogen sources on the growth of Skeletonema *species.* The growth characteristics of cells maintained in nitrate or ammonium as the sole nitrogen source were compared in *Skeletonema dohrnii* CCMP3373, *S. menzelii* CCMP793, and *S. marinoi* Skel F (Fig. 2). When grown in either nitrate or ammonium media, *S. dohrnii* exhibited significantly faster growth rates than the other species (Fig. 2; Fisher LSD test, $P < 0.05$). The growth rates of *S. marinoi* and *S. menzelii* were similar when maintained in nitrate media. In media supplemented with ammonium, *S. marinoi* had the slowest growth rates of the three species (Fig. 2; Fisher LSD test, $P < 0.05$). Maximum biomass obtained by *S. dohrnii* and *S. menzelii* in media with nitrate or ammonium was similar, with an average of $4.2 \pm 1.5 \times 10^6$ cells $\cdot$ mL^{-1}. In *S. marinoi*, the maximum biomass of cells grown with nitrate was not significantly different compared to the other two species, but when grown with ammonium its maximum biomass was significantly reduced ($9.7 \pm 1.2 \times 10^4$ cells $\cdot$ mL^{-1}; Fig. 2, Fisher LSD test, $P < 0.05$), revealing significant interspecific variation in physiological responses to different nitrogen sources.

Expression patterns of NRT2 *genes under various nitrogen conditions.* For each of the three *Skeletonema* species, the transcript levels of multiple *NRT2* genes were determined by Q-RT-PCR and their expression patterns were compared by normalizing to a reference gene (*EFL*). The amplification efficiency of each primer set was high, ranging from 91.6% to 110%, with strong linearity ($r^2 > 0.993$) in each species (Table 1). To compare transcriptional patterns of multiple *NRT2* genes among treatments and species, the transcript levels measured under the same nitrogen conditions were averaged for each treatment and species (Figs. 3; S1–S3).

Transcript levels of *NRT2:1* in *Skeletonema dohrnii*, were significantly different among treatments (Fig. 3; Fisher LSD test, $P < 0.05$) with the lowest average level of 0.19 mmol $\cdot$ (mol *Efl*)$^{-1}$ in ammonium-sufficient cells and the highest in nitrogen-limited cells (12.7 mmol $\cdot$ (mol *Efl*)$^{-1}$). In *S. menzelii* and *S. marinoi,* transcriptional patterns of *NRT2:1* among treatments were similar to *S. dohrnii*, except that nitrogen-limited cells had transcript levels approximately two orders of magnitude higher (Fig. 3). Like *NRT2:1, NRT2:2* had the lowest transcript levels in the ammonium-sufficient treatment in all three species. Unlike *NRT2:1,* transcript levels in *NRT2:2* for nitrate-sufficient and nitrogen-limited cells were not significantly different in *S. menzelii* and *S. marinoi* (Fisher LSD test, $P < 0.05$), and were higher in nitrate-sufficient than nitrogen-limited cells in *S. dohrnii*. In contrast, transcript levels of *NRT2:3* did not vary significantly under different nitrogen conditions in *S. dohrnii* (Fig. 3; Fisher LSD test, $P < 0.05$), and were only slightly enhanced under nitrogen starvation in *S. menzelii* and *S. marinoi*.

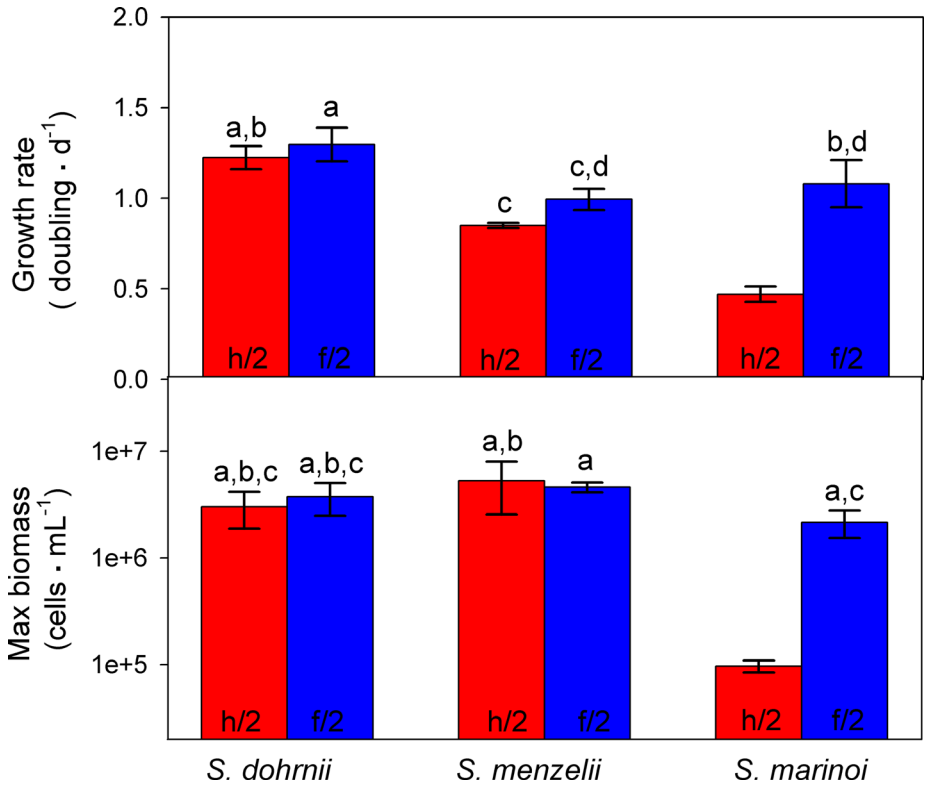

FIG. 2. Growth rates and maximum biomass for the three species of *Skeletonema* grown with ammonium (h/2) or nitrate (f/2) as the nitrogen source. Error bars represent ±1 SD from replicate experiments. Identical letters above the columns indicate no significant difference between means of growth rates or biomass concentrations ($P < 0.05$, ANOVA with Fisher's least significant difference test).

TABLE 1. Properties of standard curves generated by each primer set using serial dilution of cDNA from each species as a template.

Target species	Target gene	Primer set	E%[a]	r^2
Skeletonema dohrnii	*EFL*	SxEfl	105	0.999
	NRT2:1	SdNC3	102	0.998
	NRT2:2	SNB2	103	0.999
	NRT2:3	SNA1	99.5	0.993
Skeletonema menzelii	*EFL*	SxEfl	106	0.999
	NRT2:1	SNC1	106	0.999
	NRT2:2	SmNB3	109	0.997
	NRT2:3	SmNA4	104	0.997
Skeletonema marinoi	*EFL*	SxEfl	101	0.999
	NRT2:1	SNC1	98.9	0.998
	NRT2:2	SNB2	99.6	0.997
	NRT2:3	SNA1	91.6	0.998

[a]Amplification efficiency (E%) = $[10^{(-1/\text{slope})} - 1] \cdot 100\%$.

DISCUSSION

Three forms of NRT2 *genes in* Skeletonema. Diatom genomes commonly contain multiple *NRT2* genes. For example, the genomes of *Thalassiosira pseudonana* and *Pseudonitzschia multiseries* have at least three copies, *Phaeodactylum tricornutum* and *Fragilariopsis cylindrus* have at least five copies, and it has been proposed that in diatoms, these genes can be divided into at least two orthologous groups (Armbrust et al. 2004, Bowler et al. 2008, Bender et al. 2014). The three NRT2s we identified in *Skeletonema* each formed a distinct and strongly supported clade and were most closely related to the NRT2s in *T. pseudonana* (Fig. 1). Within each of the three NRT2 clades, the topology was principally consistent with the phylogenetic relationship among species inferred from rDNA sequences (Sarno et al. 2005, 2007), suggesting that the three forms of *NRT2s* evolved prior to speciation of the *Skeletonema* species investigated here. Within each NRT2 clade, sequence identity among *Skeletonema* species was high, further suggesting that the NRT2s in the same clade might possess similar uptake kinetic properties.

Patterns and significance of NRT2 *gene expression in* Skeletonema. Many phytoplankton, including *Skeletonema,* are known to have high rates of nitrate uptake after short periods of nitrogen deprivation (e.g., Serra et al. 1978, Martinez 1991). An active transporter has long been suggested to be responsible for the active transport of nitrate at extremely low concentrations (Falkowski 1975, Raven 1980). In nitrogen-depleted conditions, only *NRT2:1* had significantly higher transcript levels across all species compared to nitrogen-sufficient conditions, suggesting that it is an active transporter of nitrate (Fig. 3). High gene expression of *NRT2:1* under nitrogen starvation can be considered as an adaptive strategy for the eventual synthesis of NRT2 protein when nitrate concentrations increase. In contrast, the orders of magnitude lower transcript levels of *NRT2:1* in the presence of ammonium is also consistent with the fact that ammonium strongly inhibits the activity of this nitrate transporter (Serra et al. 1978). *NRT2* genes with similar expression patterns have been repeatedly observed in diatoms and haptophytes (Kang et al. 2007, 2009, Song and Ward 2007, Bender et al. 2014, Charrier et al. 2015), suggesting that *NRT2:1* was the major gene responsible for nitrate uptake under low nitrate conditions. The expression patterns of *NRT2:1* observed in *Skeletonema* further support the suggestion that *NRT2:1* may serve as a good indicator of nitrogen status across a broad range of diatom species (Kang et al. 2009, 2015).

Similar to *NRT2:1, NRT2:2* transcript levels were also repressed by the presence of ammonium, and transcript levels increased under nitrate-sufficient and nitrogen-limited conditions in *Skeletonema*. However, the response was less extreme, with a maximum response of 21-fold (Fig. 3). Interestingly in *S. dohrnii, NRT2:2* exhibited the highest transcript levels in nitrate-sufficient conditions compared to both the other two conditions, and the other two forms of *NRT2* genes (Fig. 3). Since *S. dohrnii* exhibited a higher growth rate than the other species when grown with nitrate as a nitrogen source (Fig. 2), these results imply that *NRT2:2* could be the main gene responsible for nitrate uptake under nitrate-sufficient conditions. A biphasic or nonsaturating character of nitrate uptake kinetics in high nitrate concentrations has been described in laboratory cultures and natural communities of *Skeletonema* (Serra et al.1978, Collos et al. 1992, 1997, 2005, Lomas and Glibert 2000). *NRT2:2*, with its higher capacity working under higher external nitrate concentrations, could be the second nitrate uptake mechanism that has been considered as a possible explanation for the occurrence of *Skeletonema* in eutrophic waters such as coastal, estuarine, and upwelling areas (Collos et al. 1992, 2005, Flynn 1999).

In contrast to *NRT2:1* and *NRT2:2*, the transcript levels of *NRT2:3* did not vary notably under different nitrogen conditions, and behaved more like constitutively expressed genes that maintain a stable transcript level under different growth conditions (Fig. 3). These results are consistent with the constitutive expression of *NRT2:3* in *Thalassiosira pseudonana* (Kang et al. 2009). Since the expression of *NRT2:3* is not repressed by ammonium, its protein may still function even in the presence of ammonium. Past studies showed that while ammonium significantly repressed nitrate uptake in diatoms, they were still able to take up trace amounts of nitrate when nitrate and ammonium were simultaneously provided as N sources (Dortch and Conway 1984, Lund 1987, Dortch 1990, Dortch et al. 1991, Yin et al. 1998). This suggests that NRT2:3 in diatoms could function to take up nitrate in the presence of ammonium, albeit with low capacity.

A revised model for nitrate uptake in Skeletonema. Models of phytoplankton nutrient uptake

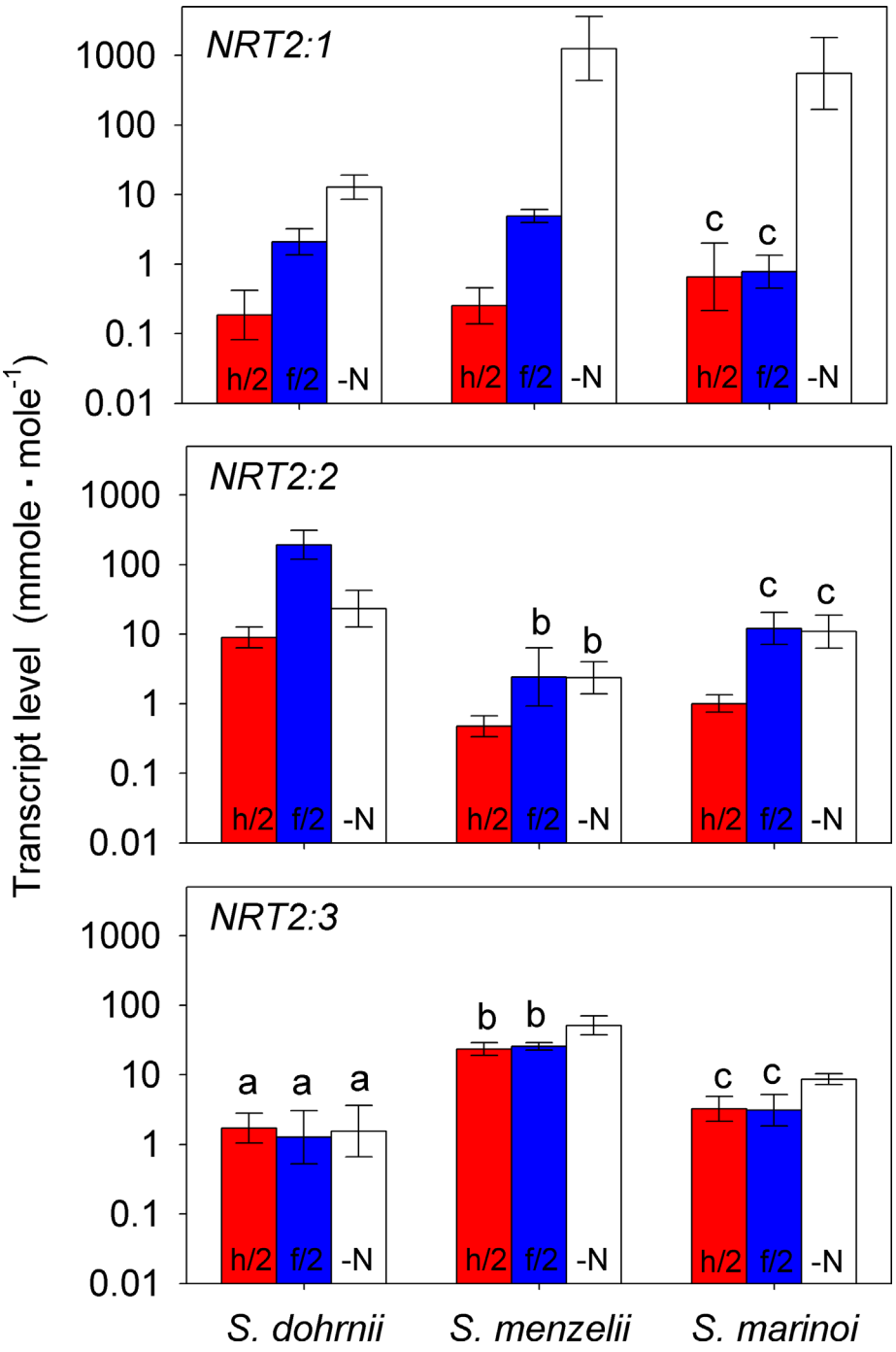

FIG. 3. *NRT2* transcript levels for the three species of *Skeletonema* grown under three nitrogen conditions. The growth conditions tested include ammonium-sufficient cells (h/2), nitrate-sufficient cells (f/2), and nitrogen-limited cells (−N). Error bars represent ±1 SD of replicate experiments. Identical letters above the columns indicate no significant difference between means of transcript levels ($P < 0.05$, ANOVA with Fisher's least significant difference test).

kinetics have taken into consideration the fact that phytoplankton are able to regulate the number of uptake sites (transporters) in response to changes in nutrient availability (Aksnes and Egge 1991, Smith et al. 2009, Bonachela et al. 2011). Given the three forms of nitrate transporters identified here and their gene expression patterns under different nitrogen conditions, we propose a revised model based on the nitrate uptake kinetics of *Skeletonema costatum* (Collos et al. 1992, Lomas and Glibert 2000) and the regulation of multiple forms of transporters and their expression levels measured here (Fig. 4). We hypothesize that when ambient nitrate concentration is low, nitrate is mainly taken up via the high affinity, low capacity nitrate transporter, NRT2:1. This is supported by Martinez (1991) who observed that nitrogen-limited cells generated large numbers of transporters to take up nitrate from the environment and is consistent with high transcript levels of *NRT2:1* under nitrogen-limited conditions observed here. With increasing ambient nitrate concentrations, there is a reduction

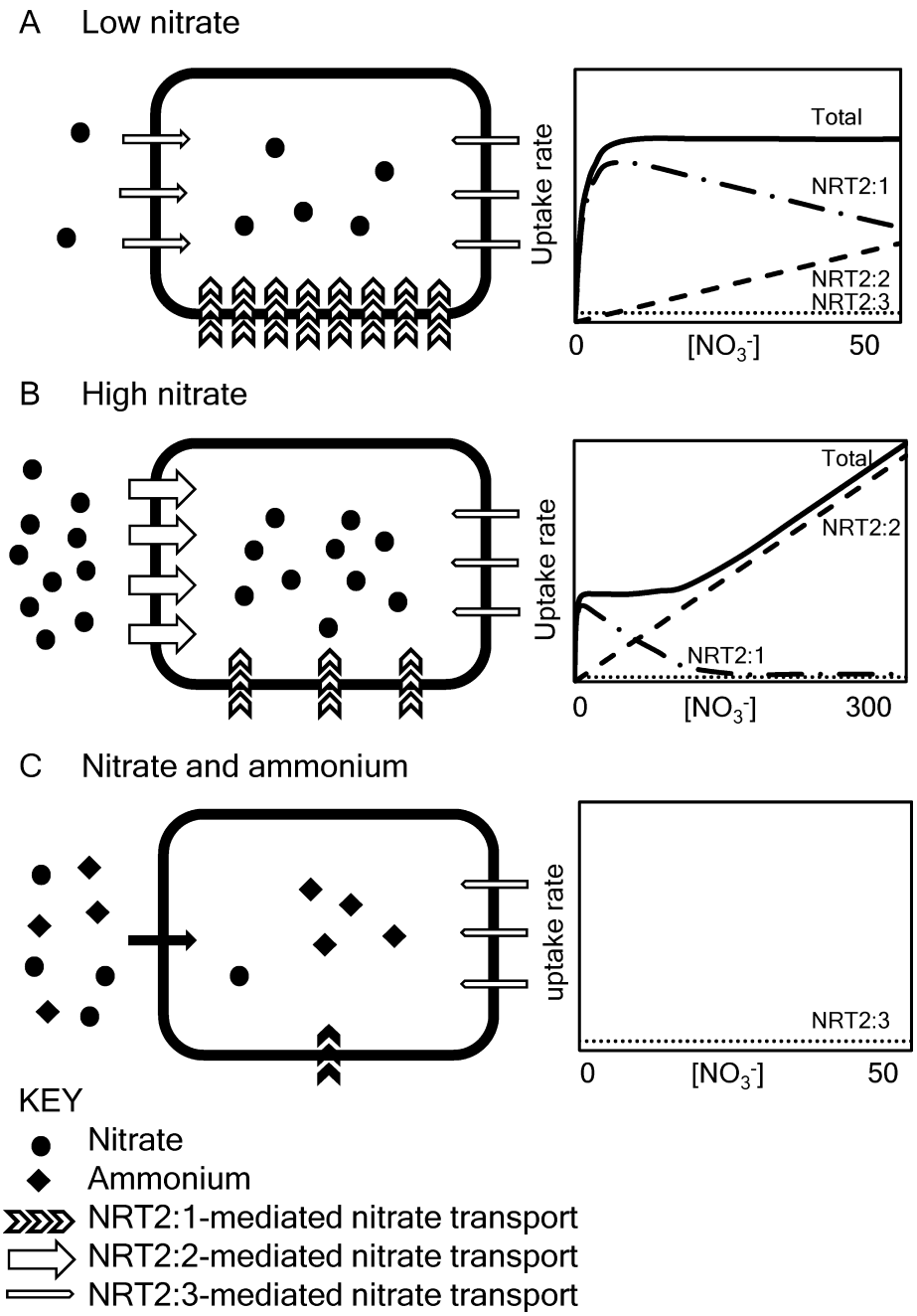

FIG. 4. Proposed hypothesized model of nitrate uptake in *Skeletonema* grown under low nitrate concentrations (A), high nitrate concentrations (B), and in the presence of nitrate and ammonium (C). In the images in the left column, a rectangular box represents a diatom cell. Filled circles represent nitrate and diamonds represent ammonium. Arrows denote direction of transport with magnitude indicate by their thickness. Filled arrows (panel C, NRT2:1 and NRT2:2) indicate that the transport may be blocked by ammonium. Stylized graphs of hypothesized uptake kinetics are shown in the right column. Note the difference in *x*-axis scale for scenario B.

in transcript levels of *NRT2:1* in *Skeletonema* as well as other diatom species (Liu et al. 2013), and we hypothesize that nitrate uptake is gradually mediated by another high-capacity transporter, NRT2:2. Early kinetic studies have observed the biphasic or nonsaturable kinetics of nitrate transport in several diatom species including *S. costatum* (Collos et al. 1992, 1997, Lomas and Glibert 2000). A possible explanation involves the participation of multiple transporters with different uptake kinetics, such as NRT2:1 and NRT2:2 in *Skeletonema*. In higher nitrate concentrations (>60 μM), *Skeletonema* exhibited increased nitrate uptake rates compared to low nitrate concentrations (Lomas and Glibert 2000). These increased uptake rates were likely related to NRT2:2 instead of NRT2:1 (Fig. 4B). In the presence of ammonium, the transcript levels of *NRT2:1* and *NRT2:2* are significantly repressed suggesting that ammonium might inhibit NRT2:1 and NRT2:2. In this scenario, we hypothesize that NRT2:3 constitutive expression results in the uptake of trace nitrate, even in the presence of ammonium. This revised model is a generalized model for the genus *Skeletonema*, and each species apparently has a different arrangement of these transporters reflecting various nitrate uptake characteristics. Overall, there is a

positive correlation of gene expression with protein amount and enzyme activity in diatoms (see for example Dyhrman et al. 2012) but no specific evidence exists for *NRT2*. Since the precise functions of NRT2 proteins in diatoms are still unclear, future functional studies, such as nitrate uptake into *Xenopus* oocytes (Zhou et al. 2000, Orsel et al. 2006, Kotur et al. 2012), are needed to confirm the role of each NRT2 in nitrate uptake.

Ecology. Phylogenetic similarity among species in the genus *Skeletonema* does not appear to reflect physiological similarity. For example, two of the most closely related species in this genus, *S. dohrnii* and *S. marinoi* (Sarno et al. 2005, Ellegaard et al. 2008), also had high amino acid identities within each NRT2 form (Fig. 1) but showed significant differences in growth rate under different nitrogen sources. The growth rate of *S. marinoi* was repressed under high ammonium compared to high nitrate conditions while *S. dohrnii* growth rates did not differ (Fig. 2). Furthermore, patterns of transcriptional responses to nitrogen conditions varied between the two species in each *NRT2* gene (Fig. 3) suggesting that the two closely related species may have evolved different strategies of transcriptional regulation to cope with their environments. Together the growth and transcriptional responses may provide some insights into the types of environments each species inhabits. The increase in *NRT2:1* transcript levels in all three species under nitrogen starvation suggests that all species are able to acclimate to a nitrate-limited environment with a pulsed nutrient supply, as is commonly found in coastal regions (Nixon 1995). Under high nitrate conditions, the data suggests that *S. dohrnii* would be the most successful species given its elevated growth rates and unique ability to significantly increase transcript levels of *NRT2:2*.

Based on field observations, it has been proposed that genetic and phenotypic variation among species in the genus *Skeletonema* influence their temporal and spatial distributions (Kooistra et al. 2008, Canesi and Rynearson 2016). In the dynamic coastal and estuarine ecosystems that *Skeletonema* inhabits, there may be a trade-off among species as environmental conditions shift between habitats or throughout an annual cycle. For example, in Narragansett Bay, six *Skeletonema* species comprise and often numerically dominate different blooms over the course of a year when dissolved inorganic nitrogen concentrations range from 0.14 to 18.94 μM (Canesi and Rynearson 2016). Environmentally variable ecosystems inhabited by multiple *Skeletonema* species present an opportunity to examine *Skeletonema* gene expression in situ to further examine the relationship between nitrogen conditions and nitrogen uptake, to define the physiological characteristics among species and to better understand the process of species succession.

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Aksnes, D. L. & Egge, J. K. 1991. A theoretical model for nutrient uptake in phytoplankton. *Mar. Ecol. Prog. Ser.* 70:65–72.

Armbrust, E. V. 2009. The life of diatoms in the world's oceans. *Nature* 459:185–92.

Armbrust, E. V., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putnam, N. H., Zhou, S. et al. 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306:79–86.

Bender, S. J., Durkin, C. A., Berthiaume, C. T., Morales, R. L. & Armbrust, E. V. 2014. Transcriptional responses of three model diatoms to nitrate limitation of growth. *Front. Mar. Sci.* 1:10.3389/fmars.2014.00003.

Bonachela, J. A., Raghib, M. & Levin, S. A. 2011. Dynamic model of flexible phytoplankton nutrient uptake. *Proc. Natl. Acad. Sci. USA* 108:20633–8.

Borkman, D. G. & Smayda, T. J. 2009. Multidecadal (1959–1997) changes in *Skeletonema* abundance and seasonal bloom patterns in Narragansett Bay, Rhode Island, USA. *J. Sea Res.* 61:84–94.

Bowler, C., Allen, A. E., Badger, J. H., Grimwood, J., Jabbari, K., Kuo, A., Maheswari, U. et al. 2008. The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature* 456:239–44.

Canesi, K. L. & Rynearson, T. A. 2016. Temporal variation of *Skeletonema* community composition from a long-term time series in Narragansett Bay identified using high-throughput DNA sequencing. *Mar. Ecol. Prog. Ser.* 556:1–16.

Charrier, A., Bérard, J. B., Bougaran, G., Carrier, G., Lukomska, E., Schreiber, N., Fournier, F. et al. 2015. High-affinity nitrate/nitrite transporter genes (*Nrt2*) in *Tisochrysis lutea*: identification and expression analyses reveal some interesting specificities of Haptophyta microalgae. *Physiol. Plant.* 154:572–90.

Chung, C. C., Hwang, S. P. L. & Chang, J. 2005. Cooccurrence of *ScDSP* gene expression, cell death, and DNA fragmentation in a marine diatom, *Skeletonema costatum*. *Appl. Environ. Microbiol.* 71:8744–51.

Cloern, J., Cole, B., Wong, R. & Apine, A. 1985. Temporal dynamics of estuarine phytoplankon: a case of San Francisco Bay. *Hydrobiologia* 129:153–76.

Collos, Y. 1982. Transient situations in nitrate assimilation by marine diatoms. III. short-term uncoupling of nitrate uptake and reduction. *J. Exp. Mar. Biol. Ecol.* 62:285–95.

Collos, Y., Siddiqi, M. Y., Wang, M. Y., Glass, A. D. M. & Harrison, P. J. 1992. Nitrate uptake kinetics by two marine diatoms using the radioactive tracer ^{13}N. *J. Exp. Mar. Biol. Ecol.* 163:251–60.

Collos, Y., Vaquer, A., Bibent, B., Slawyk, G., Garcia, N. & Souchu, P. 1997. Variability in nitrate uptake kinetics of phytoplankton communities in a Mediterranean coastal lagoon. *Estuar. Coast. Shelf Sci.* 44:369–75.

Collos, Y., Vaquer, A. & Souchu, P. 2005. Acclimation of nitrate uptake by phytoplankton to high substrate levels. *J. Phycol.* 41:466–78.

Dortch, Q. 1982. Effect of growth conditions on accumulation of internal nitrate, ammonium, amino acids, and protein in three marine diatoms. *J. Exp. Mar. Biol. Ecol.* 61:243–64.

Dortch, Q. 1990. The interaction between ammonium and nitrate uptake in phytoplankton. *Mar. Ecol. Prog. Ser.* 61:183–201.

Dortch, Q., Clayton, J. R. Jr, Thoresen, S. S. & Ahmed, S. I. 1984. Species differences in accumulation of nitrogen pools in phytoplankton. *Mar. Biol.* 81:237–50.

Dortch, Q. & Conway, H. L. 1984. Interactions between nitrate and ammonium uptake: variation with growth rate, nitrogen source and species. *Mar. Biol.* 79:151–64.

Dortch, Q., Thompson, P. A. & Harrison, P. J. 1991. Variability in nitrate uptake kinetics in *Thalassiosira psuedonana* (Bacillariophyceae). *J. Phycol.* 27:35–9.

Droop, M. R. 1973. Some thoughts on nutrient limitation in algae. *J. Phycol.* 9:264–72.

Dugdale, R. C. 1967. Nutrient limitation in the sea: dynamics, identification, and significance. *Limnol. Oceanogr.* 12:685–95.

Dyhrman, S. T., Jenkins, B. D., Rynearson, T. A., Saito, M. A., Mercier, M. L., Alexander, H., Whitney, L. P. et al. 2012. The transcriptome and proteome of the diatom *Thalassiosira pseudonana* reveal a diverse phosphorus stress response. *PLoS ONE* 7:e33768.

Ellegaard, M., Godhe, A., Harnstrom, K. & McQuoid, M. 2008. The species concept in a marine diatom: LSU rDNA-based phylogenetic differentiation in *Skeletonema marianoi/dohrnii* (Bacillariophyceae) is not reflected in morphology. *Phycologia* 47:156–67.

Eppley, R. W., Rogers, J. N. & McCarthy, J. J. 1969. Half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. *Limnol. Oceanogr.* 14:912–20.

Eppley, R. W. & Thomas, W. H. 1969. Comparison of half-saturation constants for growth and nitrate uptake of marine phytoplankton. *J. Phycol.* 5:375–9.

Falkowski, P. G. 1975. Nitrate uptake in marine phytoplankton: comparison of half-saturation constants from seven species1. *Limnol. Oceanogr.* 20:412–7.

Felsenstein, J. 1989. PHYLIP- Phylogeny Inference Package (Version 3.2). *Cladistics* 5:164–6.

Field, C. B., Behrenfeld, M. J., Randerson, J. T. & Falkowski, P. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. *Science* 281:237–40.

Flynn, K. J. 1999. Nitrate transport and ammonium-nitrate interactions at high nitrate concentrations and low temperature. *Mar. Ecol. Prog. Ser.* 187:283–7.

Guillard, R. R. L. & Ryther, J. H. 1962. Studies on marine planktonic diatom. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8:229–39.

Hildebrand, M. & Dahlin, K. 2000. Nitrate transporter genes from the diatom *Cylindrotheca fusiformis* (Bacillariophyceae): mRNA levels controlled by nitrogen source and by the cell cycle. *J. Phycol.* 36:702–13.

Howarth, R. W. & Marino, R. 2006. Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: evolving views over three decades. *Limnol. Oceanogr.* 51:364–76.

Kaeriyama, H., Katsuki, E., Otsubo, M., Yamada, M., Ichimi, K., Tada, K. & Harrison, P. J. 2011. Effects of temperature and irradiance on growth of strains belonging to seven *Skeletonema* species isolated from Dokai Bay, southern Japan. *Eur. J. Phycol.* 46:113–24.

Kang, L. K., Gong, G. C., Wu, Y. H. & Chang, J. 2015. The expression of nitrate transporter genes reveals different nitrogen statuses of dominant diatom groups in the southern East China Sea. *Mol. Ecol.* 24:1374–86.

Kang, L. K., Hwang, S. P. L., Gong, G. C., Lin, H. J., Chen, P. C. & Chang, J. 2007. Influences of nitrogen deficiency on the transcript levels of ammonium transporter, nitrate transporter and glutamine synthetase genes in *Isochrysis galbana* (Isochrysidales, Haptophyta). *Phycologia* 46:521–33.

Kang, L. K., Hwang, S. P. L., Lin, H. J., Chen, P. C. & Chang, J. 2009. Establishment of minimal and maximal transcript levels for nitrate transporter genes for detecting nitrogen deficiency in the marine phytoplankton *Isochrysis galbana* (Prymnesiophyceae) and *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 45:864–72.

Kang, L. K., Tsui, F. H. & Chang, J. 2012. Quantification of diatom gene expression in the sea by selecting uniformly transcribed mRNA as the basis for normalization. *Appl. Environ. Microbiol.* 78:6051–8.

Kang, L. K., Wang, H. F. & Chang, J. 2011. Diversity of diatom nitrate transporter genes from isolated single-cells and mixed samples for the establishment of mRNA quantification in the East China Sea. *Appl. Environ. Microbiol.* 77:122–30.

Keeling, P. J., Burki, F., Wilcox, H. M., Allam, B., Allen, E. E., Amaral-Zettler, L. A., Armbrust, E. V. et al. 2014. The marine microbial eukaryote transcriptome sequencing project (MMETSP): illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing. *PLoS Biol.* 12:e1001889.

Kooistra, W. H. C. F., Gersonde, R., Medlin, L. K. & Mann, D. G. 2007. The origin and evolution of the diatoms: their adaptation to a planktonic existence. *In* Falkowski, P. G. & Amsterdam, A. H. K. [Eds.] *Evolution of Primary Producers in the Sea.* Elsevier, Academic Press, Amsterdam, pp. 207–49.

Kooistra, W. H. C. F., Sarno, D., Balzano, S., Gu, H. F., Andersen, R. A. & Zingone, A. 2008. Global diversity and biogeography of *Skeletonema* species (Bacillariophyta). *Protist* 159:177–93.

Kotur, Z., Mackenzie, N., Ramesh, S., Tyerman, S. D., Kaiser, B. N. & Glass, A. D. M. 2012. Nitrate transport capacity of the *Arabidopsis thaliana* NRT2 family members and their interactions with AtNAR2.1. *New Phytol.* 194:724–31.

Liu, Y., Song, X., Han, X. & Yu, Z. 2013. Influences of external nutrient conditions on the transcript levels of a nitrate transporter gene in *Skeletonema costatum*. *Acta Oceanol. Sin.* 32:82–8.

Lomas, M. W. & Glibert, P. M. 1999. Interactions between NH_4^+ and NO_3^- uptake and assimilation: comparison of diatoms and dinoflagellates at several growth temperatures. *Mar. Biol.* 133:541–51.

Lomas, M. W. & Glibert, P. M. 2000. Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. *J. Phycol.* 36:903–13.

Lund, B. A. 1987. Mutual interference of ammonium, nitrate, and urea on uptake of ^{15}N sources by the marine diatom *Skeletonema costatum* (Grev.) Cleve. *J. Exp. Mar. Biol. Ecol.* 113:167–80.

Martinez, R. 1991. Transient nitrate uptake and assimilation in *Skeletonema costatum* cultures subject to nitrate starvation under low irradiance. *J. Plankton Res.* 13:499–512.

McDonald, S. M., Plant, J. N. & Worden, A. Z. 2010. The mixed lineage nature of nitrogen transport and assimilation in marine eukaryotic phytoplankton: a case study of *Micromonas*. *Mol. Biol. Evol.* 27:2268–83.

Morel, F. M. M. 1987. Kinetics of nutrient uptake and growth in phytoplankton. *J. Phycol.* 23:137–50.

Nelson, D. M., Tréguer, P., Brzezinski, M. A., Leynaert, A. & Quéguiner, B. 1995. Production and dissolution of biogenic silica in the ocean: Revised global estimates, comparison with regional data and relationship to biogenic sedimentation. *Global Biogeochem. Cycles* 9:359–72.

Nixon, S. W. 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. *Ophelia* 41:199–219.

Orsel, M., Chopin, F., Leleu, O., Smith, S. J., Krapp, A., Daniel-Vedele, F. & Miller, A. J. 2006. Characterization of a two-component high-affinity nitrate uptake system in *Arabidopsis*; Physiology and protein-protein interaction. *Plant Physiol.* 142:1304–17.

Raven, J. A. 1980. Nutrient transport in microalgae. *Adv. Microb. Physiol.* 21:47–226.

Sarno, D., Kooistra, W. H. C. F., Balzano, S., Hargraves, P. E. & Zingone, A. 2007. Diversity in the genus *Skeletonema* (Bacillariophyceae): III. Phylogenetic position and morphological variability of *Skeletonema costatum* and *Skeletonema grevillei*, with the description of *Skeletonema ardens* sp. nov. *J. Phycol.* 43:156–70.

Sarno, D., Kooistra, W. H. C. F., Medlin, L. K., Percopo, I. & Zingone, A. 2005. Diversity in the genus *Skeletonema* (Bacillariophyceae). II. An assessment of the taxonomy of *S. costatum*-like species with the description of four new species. *J. Phycol.* 41:151–76.

Serra, J. L., Llama, M. J. & Cadenas, E. 1978. Nitrate utilization by the diatom *Skeletonema costatum*: II. Regulation of nitrate uptake. *Plant Physiol.* 62:991–4.

Smith, S., Yamanaka, Y., Pahlow, M. & Oschlies, A. 2009. Optimal uptake kinetics: physiological acclimation explains the

pattern of nitrate uptake by phytoplankton in the ocean. *Mar. Ecol. Prog. Ser.* 384:1–12.

Song, B. & Ward, B. B. 2007. Molecular cloning and characterization of high-affinity nitrate transporters in marine phytoplankton. *J. Phycol.* 43:542–52.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673–80.

Yin, K., Harrison, P. J. & Dortch, Q. 1998. Lack of ammonium inhibition of nitrate uptake for a diatom grown under low light conditions. *J. Exp. Mar. Biol. Ecol.* 228:151–65.

Zhou, J. J., Fernandez, E., Galvan, A. & Miller, A. J. 2000. A high affinity nitrate transport system from *Chlamydomonas* requires two gene products. *FEBS Lett.* 466:225–7.

Zingone, A., Percopo, I., Sims, P. A. & Sarno, D. 2005. Diversity in the genus *Skeletonema* (Bacillariophyceae). I. A reexamination of the type material of *S. costatum* with the description of *S. grevillei* sp. nov. *J. Phycol.* 41:140–50.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Time course of cell density and *NRT2* gene transcript level under three nitrogen conditions in *Skeletonema dohrnii*. Cells were grown under ammonium-sufficient (left column), nitrate-sufficient (middle column), or nitrogen limited (right column) conditions. The maximum biomass is indicated by a dashed line. ANOVA was used to test for significant differences in transcript levels among time points. *P* values are listed after the gene name in each panel. Error bars indicate ±1 SD of cell count in growth curves and represent ±1 SD of Q-RT-PCR replicate tubes in gene transcript levels.

Figure S2. Time course of cell density and *NRT2* gene transcript level under three nitrogen conditions in *Skeletonema menzelii*. Cells were grown under ammonium-sufficient (left column), nitrate-sufficient (middle column), or nitrogen limited (right column) conditions. The maximum biomass is indicated by a dashed line. ANOVA was used to test for significant differences in transcript levels among time points. *P* values are listed after the gene name in each panel. Identical letters above the time points indicate no significant difference between means of transcript levels (Fisher's least significant difference test). Different symbols represent two replicated experiments. Error bars indicate ±1 SD of cell count in growth curves and represent ±1 SD of Q-RT-PCR replicate tubes in gene transcript levels.

Figure S3. Time course of cell density and *NRT2* gene transcript level under three nitrogen conditions in *Skeletonema marinoi*. Cells were grown under ammonium-sufficient (left column), nitrate-sufficient (middle column), or nitrogen-limited (right column) conditions. The maximum biomass is indicated by a dashed line. ANOVA was used to test for significant differences in transcript levels among time points. *P* values are listed after the gene name in each panel. Different symbols represent two replicated experiments. Error bars indicate ±1 SD of cell count in growth curves and represent ±1 SD of Q-RT-PCR replicate tubes in gene transcript levels.

Table S1. Primers used in this study.

Table S2. *Skeletonema* species and the *NRT2* forms identified from the Marine Microbial Eukaryote Transcriptome Sequencing Project. The *NRT2* form identified is indicated by the number following the colon.

Table S3. *NRT2*-homologus sequences searched from the transcriptome database.