Temporal dynamics of *Prochlorococcus* cells with the potential for nitrate assimilation in the subtropical Atlantic and Pacific oceans

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Running Title: Temporal dynamics of *Prochlorococcus narB*

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22 **ABSTRACT**

23 Utilization of nitrate as a nitrogen source is broadly conserved among marine
24 phytoplankton, yet many strains of *Prochlorococcus* lack this trait. Among cultured strains,
25 nitrate assimilation has only been observed within two clades of *Prochlorococcus*: the high-light
26 adapted HLII clade and the low-light adapted LLI clade. To better understand the frequency and
27 dynamics of nitrate assimilation potential among wild *Prochlorococcus,* we measured seasonal
28 changes in the abundance of cells containing the nitrate reductase gene (*narB)* in the subtropical
29 North Atlantic and North Pacific oceans. At the Atlantic station, the proportion of HLII cells
30 containing *narB* varied with season, with the highest frequency observed in stratified waters
31 during the late summer, when inorganic nitrogen concentrations were lowest. The Pacific station,
32 with more persistent stratification and lower N:P ratios, supported a perennially stable
33 subpopulation of HLII cells containing *narB*. Approximately 20-50% of HLII cells possessed
34 *narB* under stratified conditions at both sites. Since HLII cells dominate the total
35 *Prochlorococcus* population in both ecosystems, nitrate potentially supports a significant fraction
36 of the *Prochlorococcus* biomass in these waters. The abundance of LLI cells containing *narB*
37 was positively correlated with nitrite concentrations at the Atlantic station. These data suggest
38 that *Prochlorococcus* may contribute to the formation of primary nitrite maxima through
39 incomplete nitrate reduction and highlight the potential for interactions between *Prochlorococcus*
40 and sympatric nitrifying microorganisms. Further examination of these relationships will help
41 clarify the selection pressures shaping nitrate utilization potential in low-light and high-light
42 adapted *Prochlorococcus*.

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44 **INTRODUCTION**

45 The cyanobacterium *Prochlorococcus* is often the numerically dominant phototroph in the
46 tropical and subtropical oceans where it contributes substantially to net primary production
47 (Flombaum et al. 2013). This genus encompasses several phylogenetic clades consisting of cells
48 that are high-light adapted (clades HLI – HLVI) or low-light adapted (clades LLI – LLVI)
49 (Huang et al. 2012; Biller et al. 2014). As such, several *Prochlorococcus* clades are associated
50 with sets of physiological properties (Moore and Chisholm 1999; Ahlgren et al. 2006), reflecting
51 adaptations that apparently influence the distributions of these clades along environmental
52 gradients (Bouman et al. 2006; Johnson et al. 2006; West and Scanlan 1999). For example, cells
53 belonging to the high-light adapted HLII clade (eMIT9312 ecotype) have a relatively high
54 optimal temperature for growth and dominate *Prochlorococcus* populations in warmer latitudes
55 (Johnson et al. 2006). Cells belonging to the low-light adapted LLI clade (eNATL2A ecotype)
56 are relatively tolerant of light shock, and unlike cells belonging to other low-light adapted clades,
57 can persist during vertical mixing events that expose cells to higher photon fluxes at the surface
58 (Malmstrom et al. 2010).

59 The closest relatives of *Prochlorococcus* are the marine *Synechococcus* (Rocap et al.
60 2002; Scanlan et al. 2009). Although similar in many respects, *Prochlorococcus* differs from
61 *Synechococcus* in several ways, including having smaller and more streamlined genomes
62 (Partensky and Garczarek 2010) and using divinyl chlorophylls *a* and *b* for harvesting light
63 energy (Goericke and Repeta 1992). Most cultured strains of *Synechococcus* are capable of
64 nitrate assimilation (Ahlgren and Rocap 2006), whereas initial *Prochlorococcus* isolates lacked
65 this ability (Moore et al. 2002; García-Fernández et al. 2004; Kettler et al. 2007). As a result,
66 modeling efforts at that time used the absence of nitrate assimilation by *Prochlorococcus* as a
67 defining feature of this phytoplankton group (Follows et al. 2007). That said, this feature of

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68 *Prochlorococcus* remained an enigma given that nitrogen is often the proximal limiting nutrient
69 controlling phytoplankton growth in marine ecosystems (Tyrrell 1999), and most cyanobacteria
70 have pathways for utilizing nitrate as a source of nitrogen (Ohashi et al. 2011).

71 More recently, field studies have revealed nitrate uptake by wild populations of
72 *Prochlorococcus* in the North Atlantic Subtropical Gyre (Casey et al. 2007) as well as the
73 presence of nitrate assimilation genes in uncultivated *Prochlorococcus* genomes (Martiny et al.
74 2009*b*). We then identified and isolated strains capable of nitrate assimilation and have shown
75 that axenic cultures of *Prochlorococcus* are capable of growth using nitrate as the sole nitrogen
76 source (Berube et al. 2015). Comparative genomics of these isolates indicated the possibility of
77 multiple gains and losses of nitrate utilization genes during the divergence of *Prochlorococcus*
78 from *Synechococcus* (Berube et al. 2015); even so, the capacity for nitrate assimilation appears to
79 be more closely tied to ribotype phylogeny than functions associated with phosphorus
80 assimilation (Martiny et al. 2009*a*; Berube et al. 2015). It is now clear that within this single
81 genus, there are cells with distinct differences in their ability to access nitrate in the oceans.

82 Although nitrate is one of the more abundant forms of nitrogen available to marine
83 phytoplankton (Gruber 2008), nitrate utilization is tightly regulated by cyanobacteria (Ohashi et
84 al. 2011). Cyanobacteria discern the nitrogen status of the cell by sensing changes in the cellular
85 level of 2-oxoglutarate which serves as an acceptor molecule for newly assimilated nitrogen. 2-
86 oxoglutarate accumulates in cells under nitrogen limitation and activates the NtcA transcriptional
87 regulator which is responsible for inducing the expression of multiple nitrogen assimilation genes
88 (Lopatovskaya et al. 2011). Like other cyanobacteria, *Prochlorococcus* responds to nitrogen
89 starvation by activating a putative NtcA regulon (Tolonen et al. 2006). Cyanobacteria generally
90 prefer ammonium as a nitrogen source and quickly inhibit the expression of nitrate assimilation
91 genes in the presence of sufficient concentrations of ammonium (Ohashi et al. 2011). This

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92 preference is often explained by the difference in oxidation state of these compounds (Ohashi et
93 al. 2011). Yet, recent studies of marine *Synecococcus* suggest that a limiting step in the nitrate
94 assimilation pathway, rather than the availability of reducing power, might be partly responsible
95 for this preference (Collier et al. 2012).

96 What selection pressures control the distribution of nitrate assimilation genes in wild
97 *Prochlorococcus* populations? Metagenomic data from surface waters have indicated that
98 *Prochlorococcus* genes conferring the ability to use nitrate are relatively more abundant in the
99 Caribbean Sea and Indian Ocean (Martiny et al. 2009*b*) – areas with lower than average nitrate
100 concentrations. Yet, how nitrate utilization potential is distributed within wild *Prochlorococcus*
101 populations, and how this distribution is related to temporal and vertical variations in the
102 availability of nitrogen, remain open questions. To address this issue, we looked at seasonal
103 changes in the distribution and abundance of cells containing the gene for nitrate reductase
104 (*narB*) in wild *Prochlorococcus* populations in two open ocean ecosystems: the North Atlantic
105 Subtropical Gyre (Sargasso Sea, Bermuda Atlantic Time-series Study, BATS) and the North
106 Pacific Subtropical Gyre (Station ALOHA, Hawai'i Ocean Time-series, HOT) (Karl and Lukas
107 1996; Steinberg et al. 2001). *Prochlorococcus* are abundant at both sites (Campbell et al. 1994;
108 DuRand et al. 2001) and numerically dominated by the high-light adapted HLII clade (eMIT9312
109 ecotype); when averaged over the entire year during our study period, the HLII clade constitutes
110 approximately 60% and 75% of the total *Prochlorococcus* population at BATS and HOT,
111 respectively, as derived from the work of Malmstrom et al. (Malmstrom et al. 2010).

112 While each of these long standing time-series stations is located in oligotrophic, open
113 ocean waters, they differ in the finer details of their physics and nutrient dynamics. The Atlantic
114 site displays substantial seasonal variations in temperature and nutrient concentrations because it
115 is subject to stronger convective mixing during the winter and early spring; these events disrupt

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116 stratification in the euphotic zone and transport nutrient rich water to the surface (Steinberg et al.
117 2001). In contrast, the Pacific site is characterized by more consistent stratification of the water
118 column and higher concentrations of inorganic phosphorus (Wu et al. 2000; Cavender-Bares et al.
119 2001; Steinberg et al. 2001). Indeed, inorganic N:P ratios are often well below the Redfield ratio
120 of 16:1 at the Pacific site, suggesting that cells here are typically limited by nitrogen availability
121 (Wu et al. 2000).

122 Studies comparing these two sites have been instrumental in understanding how
123 environmental factors influence the population dynamics of *Prochlorococcus* clades and the
124 genes they carry (Malmstrom et al. 2010; Coleman and Chisholm 2010). Here we expand on this
125 comparative approach by examining the presence of the *Prochlorococcus* nitrate reductase gene
126 (*narB*) as a proxy for nitrate assimilation potential by *Prochlorococcus* at these sites.

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127 **MATERIALS AND METHODS**

128 **Gene target for nitrate assimilation potential**. Nitrate reductase (NarB) is the first
129 enzyme in the nitrate assimilation pathway and its gene (*narB*) is a useful marker for inferring the
130 potential for this trait in wild phytoplankton populations (Paerl et al. 2011; Paerl et al. 2012).
131 Analysis of metagenomic data from the Global Ocean Sampling (GOS) expedition revealed two
132 variants of *Prochlorococcus narB* (Martiny et al. 2009*b*) – one with a GC content of ~30% and
133 adjacent sequences most closely related to HLII *Prochlorococcus* genomes, and the other with a
134 GC content of ~40% and adjacent sequences most closely related to LLI *Prochlorococcus*
135 genomes. These two variants will hereafter be referred to as 'HLII *narB'* and 'LLI *narB'*. To
136 date, nitrate assimilation genes have only been observed in cultured isolates of *Prochlorococcus*
137 belonging to the HLII and LLI clades (Berube et al. 2015). Based on genomic and metagenomic
138 data, these genes are typically colocalized in a single region of the chromosome in these clades
139 (Martiny et al. 2009*b*; Berube et al. 2015), suggesting that *Prochlorococcus* with the potential for
140 nitrate assimilation usually contain a single copy of *narB*. Thus, gene copy number is assumed to
141 generally represent *narB*-containing cell abundance. More recently, a metagenomics study of
142 anoxic marine zones uncovered evidence of nitrate assimilation genes within the genomes of low-
143 light adapted *Prochlorococcus* possibly belonging to the LLV or LLVI clades (Astorga-Eló et al.
144 2015). While we continue to search for these genes in other clades, our analyses here are
145 restricted to the HLII and LLI clades. Fortunately, these two clades represent the most abundant
146 high- and low-light adapted *Prochlorococcus* groups at our study sites (Malmstrom et al. 2010),
147 and thus, among *Prochlorococcus*, they likely have the strongest biogeochemical imprint on
148 these systems.

149 ***Primer design***. The design of quantitative polymerase chain reaction (qPCR) primers for
150 the detection of cells containing HLII *narB* and LLI *narB* (Table 1) was based on an alignment of

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151 *Prochlorococcus narB* sequences derived from cultures (Berube et al. 2015) and several ocean
152 metagenome databases in which *narB* gene sequences could be linked to *Prochlorococcus* DNA
153 (Venter et al. 2004; Rusch et al. 2007; DeLong et al. 2006; Martiny et al. 2009*b*). Sequences were
154 also identified in HOT and BATS metagenomes (Coleman and Chisholm 2010) based on
155 similarity to the *narB* sequences observed in *Prochlorococcus* genomes. Since the number of LLI
156 clade sequences are eclipsed by the relatively high number of HLII clade sequences in most
157 surface water metagenomes, additional *narB* sequences were obtained from *narB* clone libraries
158 prepared from DNA obtained at depths of 75m and 125m on the HOT179 cruise at Station
159 ALOHA. These clone libraries were constructed using degenerate primers narB34F and
160 narB2099R (Table 1) which target both variants of the nearly full length *Prochlorococcus narB*.
161 The *narB* amplicons were cloned into pCR4 (Life Technologies, Grand Island, NY, USA) and
162 sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core. Partial putative *narB*
163 sequences have been deposited in GenBank (accession numbers KM411385-KM411429).
164 *Prochlorococcus* and *Synechococcus narB* nucleotide sequences were aligned by codon
165 using transAlign (Bininda-Emonds 2005) and manually curated. The DISTMAT tool in the
166 EMBOSS software suite (Rice et al. 2000) was used to evaluate sequence similarity. Sequences
167 within the HLII *narB* group were 96.3 ± 4.5 percent identical and sequences within the LLI *narB*
168 group were 95.3 ± 2.2 percent identical. Sequences between the HLII and LLI *narB* groups were
169 62.3 ± 3.2 percent identical. Using this alignment, qPCR primers were designed against the
170 *Prochlorococcus* HLII and LLI *narB* consensus sequences using NCBI Primer-BLAST (Ye et al.
171 2012). Degenerate nucleotides at variable positions were introduced as long as they did not
172 significantly impact specificity or amplification efficiency. Based on the aligned sequence data,
173 these primers are expected to capture at least 80% of the diversity of each *narB* variant (the
174 proportion of *Prochlorococcus narB* sequences with exact matches to our primer sequences).

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175 Primer specificity was further examined with the in-silico polymerase chain reaction (isPCR)
176 algorithm (Kuhn et al. 2013) using the NCBI RefSeq Release 71 database in order to assess the
177 possibility that these primers could amplify sequences other than *Prochlorococcus narB*. No
178 other potential targets were observed in this database when using the default isPCR parameters
179 (4000 bp maximum amplicon size, a minimum of 15 perfect matches at the 3' end of each
180 primer, and a minimum length of 15 bp where there must be 2 matches for each mismatch).
181 Relaxing the stringency to a minimum of 5 perfect matches at the 3' end of each primer only
182 identified weak hits to terrestrial animals, soil bacteria, and *Halomonas* sp. GFAJ-1 (an
183 extremophile isolated from a hypersaline, alkaline lake). None of these are predicted to result in
184 PCR amplicons given the high proportions of mismatches across the length of the primers and
185 none of these organisms are found at significant concentrations in the oligotrophic open ocean.
186 No *Synechococcus* targets were observed, which is likely a reflection of the high degree of
187 divergence in GC content between *Synechococcus* and the HLII and LLI clades of
188 *Prochlorococcus* (Scanlan et al. 2009). Thus, our primers are expected to be specific for each
189 variant of *Prochlorococcus narB*.

190 ***qPCR standards***. Cells of *Prochlorococcus* SB (Shimada et al. 1995; Berube et al. 2015)
191 and *Prochlorococcus* MIT0917, both containing a single copy of *narB*, were used as standards in
192 qPCR assays to measure the abundance of cells containing the HLII and LLI variants of *narB*
193 respectively. These standards were processed in the same manner as field samples as previously
194 described (Ahlgren et al. 2006; Zinser et al. 2007; Malmstrom et al. 2010). While a single
195 *Prochlorococcus* strain was observed to contain duplicate copies of *narB*, genomic and
196 metagenomic evidence suggests that this is a rare occurrence and that most *narB*-containing
197 *Prochlorococcus* contain a single copy of this gene (Berube et al. 2015). The MIT0917 strain is a
198 derivative of the P0903-H212 enrichment culture (Berube et al. 2015) and has been deemed

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199 unialgal based on observations of a single *Prochlorococcus* population using flow cytometry and
200 by the presence of a single 16S-23S rRNA internal transcribed spacer (ITS) sequence as
201 determined by direct sequencing of its ITS PCR amplicon. The ITS sequence for MIT0917 has
202 been deposited in GenBank under accession number KM281884.

203 Each strain was maintained at 24°C in Pro99 medium (Moore et al. 2007) containing 800
204 μmol L^{-1} of nitrate as the sole nitrogen source. SB was grown at a constant illumination of ~30
205 μmol quanta m^{-2} s^{-1} and MIT0917 was grown at a constant illumination of ~10 μmol quanta m^{-2}
206 s^{-1}. Cells were enumerated using an Influx Cell Sorter (BD Biosciences, San Jose CA, USA) as
207 previously described (Olson et al. 1985; Cavender-Bares et al. 1999).

208 ***qPCR assay conditions***. Reaction parameters for qPCR were optimized on a set of
209 plasmid clones containing full-length *Prochlorococcus narB* sequences, genomic DNA from
210 *Prochlorococcus* strains MIT0604, SB, and MIT0917, and environmental DNA obtained at
211 depths of 75 and 125m on the HOT179 cruise. Plasmids containing the *narB* sequence from
212 *Synechococcus* WH8102 and *Synechococcus* WH7803 were used as negative controls; these
213 strains belong to the 5.1A and 5.1B groups of *Synechococcus* subcluster 5.1, the dominant
214 subcluster of *Synechococcus* inhabiting oligotrophic open ocean waters. All assays were
215 performed on a LightCycler 480 System (Roche Applied Science, Indianapolis, IN, USA).
216 Reaction conditions were optimized across gradients of temperature and primer concentrations.
217 Standards and samples were processed and analyzed as described previously (Ahlgren et al.
218 2006; Zinser et al. 2007; Malmstrom et al. 2010) in order to facilitate comparison with ITS qPCR
219 measurements of *Prochlorococcus* ecotype abundance (Malmstrom et al. 2010). Assays were
220 performed in 15 µl reaction volumes with 6 µl template and the following final concentrations of
221 reaction components: 1x QuantiTect SYBR Green PCR Mix (Qiagen, Germantown, MD, USA)
222 and 0.5 μmol L^{-1} of each forward and reverse primer. Reactions were pre-incubated at 95°C for

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223 15 min to activate the polymerase and then cycled at 95°C for 15 s, 60°C for 15 s, and 72°C for
224 30 s. The HLII *narB* assay (using primers ProHLIInarB-qPCR-F and ProHLIInarB-qPCR-R)
225 used 45 amplification cycles and the LLI *narB* assay (using primers ProLLInarB-qPCR-F and
226 ProLLInarB-qPCR-R) used 50 amplification cycles. The HLII *narB* and LLI *narB* primer sets did
227 not amplify the *Synechococcus narB* negative controls. PCR amplification efficiencies and limits
228 of quantification (defined as the minimum number of cells per reaction that remained within the
229 linear portion of standard curves) were determined using serial dilutions of *Prochlorococcus* cells
230 that were processed as qPCR standards as previously described (Ahlgren et al. 2006; Zinser et al.
231 2007; Malmstrom et al. 2010). The amplification efficiency for the HLII *narB* assay was 102%
232 and 95% respectively for strains SB and MIT0604, with a limit of quantification of 7 cells per
233 reaction (equivalent to 8 cells ml^{-1} seawater). The amplification efficiency for the LLI *narB* assay
234 was 89% and 92% respectively for MIT0917 and MIT0915, with a limit of quantification of 29
235 cells per reaction (equivalent to 32 cells ml^{-1} seawater). The MIT0915 strain is a derivative of the
236 P0902-H212 enrichment culture (Berube et al. 2015). Melting curve analysis was used to assess
237 specificity in each reaction as previously described (Malmstrom et al. 2010).
238 ***Validation of the qPCR assay***. The qPCR assay was validated on a set of qPCR samples
239 obtained on the HOT186 and BATS216 cruises for which there also exists metagenome sequence
240 data from depths of 25, 75, and 110m for HOT186 and depths of 50 and 100m for BATS216
241 (Coleman and Chisholm 2010). These metagenome sequence libraries enabled us to use an
242 independent method to examine the frequency of *narB* in the total *Prochlorococcus* population at
243 each site and evaluate the specificity of our qPCR assay (Table 2). For the metagenome data sets,
244 sequence reads were mapped to consensus HLII and LLI *narB* sequences using BLASTN with a
245 minimum bit score of 40 and a minimum read coverage of 20%. Hits were then screened against
246 the NCBI nt database using BLASTN to ensure they did not have better matches to taxa other

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247 than *Prochlorococcus* or to genes other than *narB*. The counts of single copy core

248 *Prochlorococcus* genes (Coleman and Chisholm 2010) and *Prochlorococcus narB* genes in each

249 metagenome library were normalized to gene length to account for the probability of detecting

250 the respective gene. The frequency of each *narB* variant in the *Prochlorococcus* population was

251 determined by dividing the normalized occurrence of *Prochlorococcus narB* genes by the

252 average normalized occurrence of single copy core *Prochlorococcus* genes (Table 2). For the

253 qPCR assay, the frequency of each *narB* variant in the total *Prochlorococcus* population was

254 determined by dividing the abundance of cells containing each *narB* variant by the total

255 abundance of *Prochlorococcus* measured by flow cytometry (Table 2). In the event that qPCR

256 samples were not obtained at the same depth as the metagenome library, the closest depth was

257 also evaluated. Each method resulted in similar measurements of *narB* gene frequency, thus

258 indicating that our qPCR assay is specific for each variant of *Prochlorococcus narB* and that it

259 detects most of the HLII *narB* and LLI *narB* sequences at our study sites (Table 2).

260 ***Analysis and interpretation of Prochlorococcus narB abundance data***. The abundance

261 of *Prochlorococcus* cells containing HLII *narB* and LLI *narB* genes was determined at the

262 Atlantic and Pacific sites over 2 full seasonal cycles (October 2005 – December 2007), using the

263 identical field samples examined by Malmstrom et al. (Malmstrom et al. 2010). In addition, the

264 abundance of cells belonging to the eMIT9312 and eNATL2A ecotypes of *Prochlorococcus* for

265 the HOT186 cruise were determined for the first time using the methods described by Malmstrom

266 et al. (2010). *Prochlorococcus* cell abundance data based on qPCR have been deposited with the

267 Biological and Chemical Oceanography Data Management Office (BCO-DMO).

268 Correspondence between the abundance of HLII cells (eMIT9312 ecotype) measured by

269 qPCR and total *Prochlorococcus* cells measured by flow cytometry indicates that ITS primers

270 detect the majority of HLII cells at both sites (Zinser et al. 2006; Malmstrom et al. 2010). Thus, it

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271 was possible to estimate the proportion of HLII cells with the potential for nitrate assimilation by
272 normalizing the abundance of cells containing the HLII *narB* gene to the abundance of
273 *Prochlorococcus* cells belonging to the HLII clade (Malmstrom et al. 2010). Proportions were
274 only determined if the absolute concentrations of both HLII cells and cells containing HLII *narB*
275 (assuming single copies of the ITS sequence and *narB* gene per cell, respectively) were greater
276 than 50 cells ml^{-1} in order to avoid potentially aberrant ratios.

277 In contrast, we could not normalize the abundance of cells containing the LLI *narB* gene
278 to the abundance of *Prochlorococcus* cells belonging to the LLI clade (eNATL2A ecotype)
279 because our ITS primers are not able to capture all of the low-light adapted *Prochlorococcus*. The
280 sum of ITS qPCR measurements for 5 *Prochlorococcus* ecotypes tends to underestimate total
281 *Prochlorococcus* abundance in deep waters where low-light adapted cells comprise a significant
282 fraction of the total population (Zinser et al. 2006; Ahlgren et al. 2006; Malmstrom et al. 2010).
283 To confirm the suspicion that ITS primers may be missing LLI cells, we tested the ability of LLI
284 clade ITS qPCR primers (NATL3f and NATL2r) (Ahlgren et al. 2006; Malmstrom et al. 2010) to
285 detect MIT0917, which has an ITS sequence that falls within the LLI clade. Our ITS qPCR
286 primers could not detect MIT0917. While we have tried to redesign the LLI clade ITS qPCR
287 primer set using an extensive data set of sequences from ITS sequence clones (Martiny et al.
288 2009*a*), we have not been successful. Thus, in the context of this study, we could only determine
289 the absolute abundance of LLI cells containing the *narB* gene (assuming a single copy of *narB*
290 per cell) and not their frequency of occurrence within the total LLI population.

291 ***Environmental data and statistical analyses***. *Prochlorococcus* ecotype abundance, mixed
292 layer depths, and photosynthetically active radiation (PAR) estimates were obtained from
293 Malmstrom et al. (Malmstrom et al. 2010). Chemiluminescent based measurements of low-level
294 nitrogen (nitrate + nitrite) concentrations from the HOT program and colorimetric based

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295 measurements of nitrite and nitrate + nitrite concentrations from the BATS program were
296 obtained from the Ocean Data View website (http://odv.awi.de/en/data/ocean/). Nitrite
297 concentration data were not collected by the HOT program during the period of our study. Nitrate
298 + nitrite concentrations were interpolated using weighted-average gridding and then mapped onto
299 our sampling depths using 2D estimation in Ocean Data View 4.6.2. All data were log (x + 1)
300 transformed before statistical analysis. Spearman rank correlation coefficients were calculated in
301 R using the stats package to assess the relationship between the abundance of cells containing
302 HLII *narB* or LLI *narB* and the abundance of *Prochlorococcus* cells belonging to the HLII and
303 LLI clades. Partial Spearman correlation coefficients were calculated in R using the ppcor
304 package to determine the relationship between the abundance of *narB*-containing
305 *Prochlorococcus* and nitrite or total inorganic nitrogen (nitrate + nitrate) concentrations while
306 controlling for the influence of light.

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307 **RESULTS AND DISCUSSION**

308 ***Seasonal changes in narB occurrence in high-light adapted Prochlorococcus cells.*** The
309 concentrations of both total *Prochlorococcus* (measured by flow cytometry) and those belonging
310 to the HLII clade (measured by qPCR) are typically higher at the Pacific site compared to the
311 Atlantic site (Malmstrom et al. 2010) (Fig. 1a), as were the abundances of cells in this clade that
312 contained *narB* (Fig. 1b). Total HLII clade cells and cells that contained the HLII *narB* gene were
313 co-localized as indicated by a strong positive correlation between their abundances (Spearman
314 correlation; Pacific, $R = 0.89$, $p < 0.01$; Atlantic, $R = 0.95$, $p < 0.01$). Twenty to 50% of HLII
315 clade cells were estimated to possess *narB* throughout much of the year at the Pacific site, but the
316 same was true only during the late summer and autumn at the Atlantic site (Fig. 1c). During the
317 winter months at the Atlantic site, the proportion of HLII cells containing *narB* drops to less than
318 10% of the total HLII population (Fig. 1c).

319 We examined these trends in the context of nutrient supply using nitrate + nitrite
320 concentrations (Fig. 1d) as a proxy for the availability of inorganic nitrogen (ammonium
321 concentrations were not measured by the HOT and BATS programs during the period of our
322 study). While we expect nitrogen limiting conditions to be more prevalent when inorganic
323 nitrogen concentrations are at their lowest, assessing the limiting nutrient for phytoplankton
324 growth is complicated by a number of factors: the availability of measurements for other
325 potentially limiting nutrients (e.g. phosphorus or iron) which are often below limits of detection,
326 knowledge of the bioavailability of different chemical forms of nutrients, and consideration of the
327 flexible elemental stoichiometry of marine microorganisms (Mulholland and Lomas 2008).
328 Further, nutrient fluxes rather than concentrations are likely more important determinants of
329 nutrient limitation. That said, evidence suggests that *Prochlorococcus* is limited by nitrogen at
330 the Pacific site (Van Mooy and Devol 2008), where phosphate concentrations are often more than

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331 an order of magnitude greater than at the Atlantic site (Wu et al. 2000; Cavender-Bares et al.
332 2001). In contrast, both nitrogen and phosphorus are known to limit primary production at the
333 Atlantic site (Moore et al. 2013), where seasonal dynamics contribute to alternating states of
334 phosphorus limitation in the winter and nitrogen limitation in the summer (Wu et al. 2000).
335 Cyanobacteria experiencing nitrogen limitation typically induce the expression of
336 alternative nitrogen assimilation pathways; thus we expect that cells with the genomic potential
337 for the assimilation of nitrate will be at a selective advantage under nitrogen limiting conditions.
338 Our data are generally consistent with this hypothesis. At the Atlantic site, the abundance of HLII
339 cells containing *narB* increased with decreasing concentrations of inorganic nitrogen (nitrate +
340 nitrite) when the effect of light availability was taken into account (Spearman partial correlation
341 coefficient, $R = -0.55$, $p < 0.01$), but was independent of nitrite alone (Spearman partial
342 correlation coefficient, $R = -0.02$, $p = 0.71$). Thus, as intense nutrient cycling is expected to drive
343 the system towards nitrogen limitation when the water column is stratified (Wu et al. 2000), we
344 observe an increase in the proportion of HLII cells with the potential for nitrate assimilation (Fig.
345 1c). A weaker, but also significant negative correlation between inorganic nitrogen
346 concentrations and the abundance of HLII cells containing *narB* was observed at the Pacific site
347 (Spearman partial correlation coefficient, $R = -0.26$, $p < 0.01$), which has low inorganic nitrogen
348 concentrations throughout the year. Overall, these data are consistent with more general patterns
349 observed in metagenomic data sets which have shown greater occurrence of the *Prochlorococcus*
350 *narB* gene among sequences obtained from low nitrogen waters (Martiny et al. 2009*b*).
351 While most *narB*-containing HLII cells were found in the nitrogen depleted surface layers
352 at both sites, we also observed several instances of HLII populations with relatively high
353 proportions of *narB*-containing cells in the lower reaches of the euphotic zone (Fig. 1c). Short-
354 lived (<10 days) vertical transport events supplying nitrate to the base of the euphotic zone at the

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355 Pacific site have been previously observed (Johnson et al. 2010). Given that *Prochlorococcus*
356 cells are primarily nitrogen limited at the Pacific site (Van Mooy and Devol 2008), these
357 injections of nitrate into the euphotic zone may result in higher relative growth rates of *narB*-
358 containing cells at the base of the euphotic zone. While the HOT program does not measure
359 ammonium concentrations because they are below the 50 nM detection limit of their colorimetric
360 assay (Chiswell et al. 1990), it is expected that extremely low ammonium concentrations would
361 result in the expression of nitrate assimilation genes in cyanobacteria (Ohashi et al. 2011). Thus,
362 it is possible that low ammonium availability coupled with intermittent nitrate injections might
363 contribute to the episodic enrichment of *narB* genotypes in HLII populations under these higher
364 nitrate conditions. Greater temporal and spatial sampling resolution, facilitated by automated
365 instrumentation (Ottesen et al. 2011), as well as a better understanding of ammonium
366 concentrations and fluxes, could help resolve the causality of these features.
367 Selection for genes conferring traits that facilitate access to additional pools of a particular
368 limiting nutrient has been observed previously in *Prochlorococcus.* For example, phosphorus
369 assimilation genes such as alkaline phosphatase and phosphonate transporters are more abundant
370 in phosphorus limited environments relative to phosphorus replete environments (Rusch et al.
371 2007; Coleman and Chisholm 2010; Feingersch et al. 2012). Similarly, *Prochlorococcus* cyanate
372 transporter genes are highly abundant in stratified and nitrogen depleted waters, but not in mixed
373 water columns, of the Red Sea (Kamennaya et al. 2008; Kamennaya and Post 2013). Given that
374 the capacity for nitrate assimilation appears more closely tied to ribotype than many phosphorus
375 acquisition functions (Martiny et al. 2009*a*; Berube et al. 2015), our observations could be
376 explained by covariation of nitrate assimilation potential and other genes under selection. We
377 consider it more likely, however, that nitrogen limitation drives the selection of high-light

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378 adapted *Prochlorococcus* cells capable of nitrate assimilation and potentially other nitrogen

379 assimilation pathways.

380 ***Distribution patterns of low-light adapted Prochlorococcus with the potential for nitrate***

381 ***assimilation.*** Cells belonging to all low-light adapted clades of *Prochlorococcus* reach their

382 maximum abundances deeper in the water column (Malmstrom et al. 2010) where fewer photons

383 penetrate and nutrients are typically at higher concentrations (Letelier et al. 2004; West and

384 Scanlan 1999). Accordingly, low-light adapted *Prochlorococcus* are usually at negligible

385 concentrations in the surface mixed layer. The LLI clade (eNATL2A ecotype) however, which is

386 the focus of our analyses here, stands out among low-light adapted cells in that they tolerate

387 intermittent periods of excess light intensity during deep mixing events that transport cells to the

388 well lit surface (Zinser et al. 2007; Malmstrom et al. 2010). At the same time, the LLI cells are

389 distinct from high-light adapted *Prochlorococcus* with regard to their gene content,

390 photophysiology, and phylogenetic similarity to other low-light adapted clades (Kettler et al.

391 2007; Malmstrom et al. 2010).

392 Although we could not normalize the abundance of cells containing LLI *narB* to total LLI

393 cell abundance, as we were able to do for the HLII cells (see methods), we can report absolute

394 counts of cells containing LLI *narB* (Fig. 2,3). This provides information on where this subgroup

395 of LLI cells are located in the water column, and how their distribution and abundance changes

396 with season. The spatial distribution of cells containing LLI *narB* overlaps with that of total LLI

397 cells (Spearman correlation; Pacific, $R = 0.97$, $p < 0.01$; Atlantic, $R = 0.94$, $p < 0.01$) (Fig. 2a,b).

398 Cells containing LLI *narB* are almost fully restricted to intermediate depths of the euphotic zone

399 at both sites, except during periods of deep mixing when their range expands into the surface

400 waters, albeit in low numbers. This is consistent with our understanding of the photophysiology

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401 of members of this clade – e.g. their tolerance of light stress, relative to other low-light adapted

402 *Prochlorococcus*, when mixed into surface waters.

403 We observed that cells containing the LLI *narB* gene are found in greatest abundance just

404 above or within the top layer of the nitracline (Fig. 2b,c), and in close proximity to the primary

405 nitrite maximum at the Atlantic site (Fig. 3). Because these are low-light adapted cells, however,

406 we know that they are relatively more abundant in deeper waters because of their adaptation to

407 low light intensities. Controlling for the inverse relationship between light and inorganic nitrogen

408 in the water column, there is no significant relationship between the abundance of cells

409 containing LLI *narB* and nitrate + nitrite concentration at either site (Spearman partial correlation

410 coefficient; Pacific, $R = 0.03$, $p = 0.70$; Atlantic, $R = 0.00$, $p = 0.95$). At the Atlantic site

411 however, there is a positive correlation between the abundance of cells containing LLI *narB* and

412 nitrite concentration when light is taken into account (Spearman partial correlation coefficient;

413 Atlantic, $R = 0.54$, $p < 0.01$). Although independent nitrite measurements are not available from

414 the Pacific site, historical data indicate that maximal nitrite concentrations occur at a mean depth

415 of 126 m at the Pacific site (Dore and Karl 1996) – close to depths where cells containing LLI

416 *narB* are most abundant.

417 What can the proximity of the LLI *narB* populations to the nitracline and the primary

418 nitrite maximum suggest about the selection pressures determining the distribution and

419 abundance of *narB* in low-light adapted cells? The primary nitrite maximum is a common feature

420 of both the Pacific and the Atlantic sites (Dore and Karl 1996; Lomas and Lipschultz 2006). This

421 peak in nitrite concentration may result from either the production of nitrite by ammonia

422 oxidizing microorganisms (Dore and Karl 1996; Newell et al. 2013) or by the incomplete

423 reduction of nitrate by phytoplankton (Dore and Karl 1996; Lomas and Lipschultz 2006). At

424 present, it is unclear which process is the primary driver of the formation of the nitrite maximum,

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425 and it may be that these processes are subject to diel and seasonal variability (Mackey et al.
426 2011). But, given that LLI clade cells and ammonia oxidizing microorganisms potentially coexist
427 within the primary nitrite maximum, competition for ammonium may be intense enough to favor
428 the selection of LLI cells capable of assimilating nitrate. Further, *Prochlorococcus* could
429 contribute to the formation of the primary nitrite maximum through incomplete nitrate reduction
430 and excretion of nitrite.

431 ***Implications for nitrate assimilation by Prochlorococcus in subtropical gyres***. We have
432 shown that high-light adapted *Prochlorococcus* cells belonging to the HLII clade (eMIT9312
433 ecotype) – the most abundant group of *Prochlorococcus* in the oceans by an order of magnitude –
434 are more likely to possess the *narB* gene in the surface of highly stratified waters characterized
435 by nitrogen depletion (i.e. during the summer in the Sargasso Sea and throughout much of the
436 year in the North Pacific Subtropical Gyre). This suggests that *narB*-containing HLII
437 *Prochlorococcus* have a greater selective advantage under low nitrogen conditions and contrasts
438 with a hypothesis we put forth some years ago, derived from a global simulation model, which
439 posited that *Prochlorococcus* cells would be more likely to lose *narB* under similar conditions
440 (Bragg et al. 2010). In light of our current study, this model could possibly be improved by
441 parameterizing different concentrations of ammonium under which nitrate assimilation genes are
442 expressed for different phytoplankton groups. Evaluating the costs and benefits of maintaining
443 the capacity for nitrate assimilation as well as considering the potential role of frequency
444 dependent selection (Bragg et al. 2010; Cordero and Polz 2014) could also aid in the refinement
445 of this model.

446 Potential biogeochemical and ecological interactions between *Prochlorococcus* and
447 nitrifying microorganisms should also be examined in light of our evidence that *narB*-carrying
448 *Prochlorococcus* are abundant under potentially nitrogen limiting conditions. Nitrification occurs

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449 throughout the euphotic zone and is a significant source of nitrate for marine phytoplankton
450 (Yool et al. 2007; Clark et al. 2008). Given the high substrate affinities exhibited by oligotrophic
451 ammonia oxidizing microorganisms (Martens-Habbena et al. 2009; Newell et al. 2013),
452 *Prochlorococcus* could face intense competition for ammonium. Under these conditions,
453 *Prochlorococcus* cells that are capable of nitrate assimilation may be at a selective advantage
454 because they can utilize the end products of both ammonia oxidation (nitrite) and nitrite oxidation
455 (nitrate). A greater understanding of *Prochlorococcus'* growth kinetics on these nitrogen sources,
456 as well as knowledge of the co-occurrence patterns of *narB*-containing *Prochlorococcus* and
457 nitrifiers, will be needed to elucidate these potential interactions.

458 Further, our understanding of the occurrence of *narB* in other high-light adapted
459 *Prochlorococcus* clades is limited given that cells belonging to the HLII clade are over-
460 represented in culture collections (Biller et al. 2014; Biller et al. 2015) and also dominate regions
461 from which most metagenomic data sets have been collected (Rusch et al. 2007). There is
462 evidence, however, that cells belonging to the HLIII and HLIV clades do not contain the genes
463 necessary for nitrate assimilation due to the high iron requirement of this pathway and the
464 observed dominance of these clades in iron limited and hence high nitrogen environments (Rusch
465 et al. 2010; Malmstrom et al. 2013). Our understanding of the distribution of nitrate assimilation
466 potential among low-light adapted *Prochlorococcus* is also influenced by the limitations of our
467 culture collection (Biller et al. 2015), but we are optimistic that targeted isolations or single cell
468 genome analysis will uncover more *narB*-containing low-light adapted cells. Single cell
469 sequencing designed to explore the evolutionary history of the *narB* gene among diverse
470 *Prochlorococcus* ribotypes will be particularly powerful in resolving how genes conferring the
471 potential for nitrate assimilation may co-vary with genetic markers for other ecologically relevant
472 attributes as it is clear that the distribution of specific traits among cells within the

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473 *Prochlorococcus* 'federation' (Biller et al. 2015) is the result of a complex interplay between
474 many different selective pressures in the marine environment.

475 Finally, given *Prochlorococcus*' numerical dominance and its potential for efficient
476 nutrient uptake facilitated by its small size (Chisholm 1992), it likely contributes to setting the
477 lower bounds of inorganic nitrogen concentrations in the surface waters of these systems. We
478 note that *Prochlorococcus* cells containing *narB* were particularly abundant in the water column
479 of the Atlantic site when Casey et al. first observed nitrate uptake by wild *Prochlorococcus* in the
480 autumn of 2005 (Casey et al. 2007). Thus, it is possible that the *Prochlorococcus* genotypes
481 detected in our study could be assimilating nitrate in the wild. The fact that at least 50% of
482 *Prochlorococcus* lack the ability to utilize nitrate in these environments, however, suggests that
483 carrying nitrate assimilation genes imposes a fitness cost to some cells, perhaps associated with
484 maintaining these genes in the genome or regulating their expression.

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716 **Table 1**. Oligonucleotide primers used in this study.

Name	Sequence	Reference
ITS-F	5'-CCGAAGTCGTTACTYYAACCC-3'	(Rodrigue et al. 2009)
ITS-R	5'-TCATCGCCTCTGTGTGCC-3'	(Rodrigue et al. 2009)
NATL3f	5'-ACCTAGCTTCTTGTCATCTTTTTAT-3'	(Ahlgren et al. 2006)
NATL2r	5'-CATGAGATGCTTTATTCTTTCTAATC-3'	(Ahlgren et al. 2006)
narB34F	5'-TGCCCWTATTGYGGTGTWGGHTG-3'	This study
narB2099R	5'-ATBGGRCATGWYTKYTCRTGC-3'	This study
ProHLIInarB-qPCR-F	5'-AGGCAAGAGGGTACAGCAGCAG-3'	This study
ProHLIInarB-qPCR-R	5'-GCRTTTGGTTGGCCAGTYAAGG-3'	This study
ProLLInarB-qPCR-F	5'-TGMGACACCTAATGGTCGAGCCC-3'	This study
ProLLInarB-qPCR-R	5'-TGCCATTGTCCDAGGTAACGYC-3'	This study

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718 **Table 2**. Validation of the *narB* qPCR assay by comparison with metagenomic sequence data.
719 Using qPCR, we determined the proportion of *Prochlorococcus* cells containing HLII *narB* or
720 LLI *narB* relative to the total concentration of *Prochlorococcus* measured by flow cytometry.
721 Using metagenomic data (Coleman and Chisholm 2010), the proportion of total *Prochlorococcus*
722 containing HLII *narB* or LLI *narB* was determined by comparing the occurrence of
723 *Prochlorococcus narB* genes with the average occurrence of single copy core *Prochlorococcus*
724 genes [sensu (Coleman and Chisholm 2010)]. When qPCR samples were not available for the
725 same depth as the metagenome library, qPCR measurements of *narB* were obtained for the
726 nearest depth. Note that qPCR and metagenome samples were not necessarily obtained on the
727 same hydrocast. Agreement between these two methods indicates that the qPCR assay is
728 detecting the majority of cells containing HLII *narB* or LLI *narB*.

	% *Prochlorococcus* containing *narB* estimated by qPCR or bioinformatics approaches			
	HL *narB*		**LL *narB***	
Depth (m)	**qPCR** *narB* qPCR count per total cell count	**Metagenomic** *narB* hits per average core gene hit	**qPCR** *narB* qPCR count per total cell count	**Metagenomic** *narB* hits per average core gene hit
Pacific Site (Hawai'i Ocean Time-series; HOT cruise 186; October 18-24, 2006)				
25	28.4%	28.9%	0.0%	0.5%
75	10.7%	15.8%	0.0%	0.0%
100	3.4%	n.a.	4.1%	n.a.
110	n.a.	3.5%	n.a.	5.7%
Atlantic Site (Bermuda Atlantic Ocean Time-series Study; BATS cruise 216; October 10-14, 2006)				
40	21.4%	n.a.	0.1%	n.a.
50	n.a.	9.5%	n.a.	0
60	0.0%	n.a.	2.1%	n.a.
100	0.7%	2.5%	7.6%	7.4%

729 n.a. samples not available for this depth.

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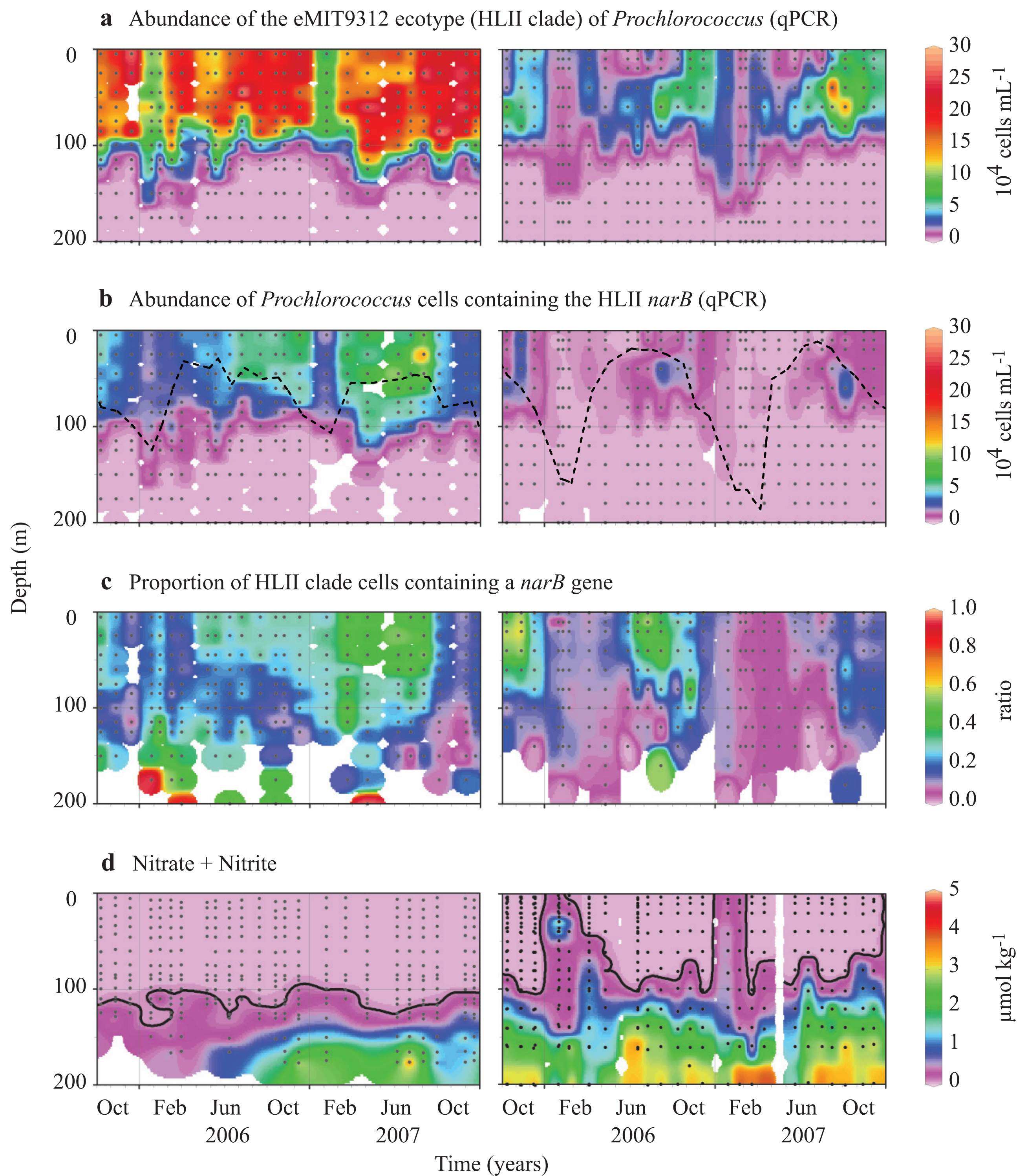

Fig. 1. Distribution and abundance of total HLII *Prochlorococcus* cells and HLII cells containing *narB* in the Pacific and Atlantic Oceans. (**a**) the abundance of HLII clade cells (eMIT9312 ecotype) determined by qPCR (Malmstrom et al. 2010), (**b**) the abundance of HLII clade cells containing *narB* determined by qPCR, (**c**) the fraction of HLII clade cells containing *narB* determined by normalizing the abundance of *narB*-containing HLII clade cells to total HLII clade cells, (**d**) nitrate + nitrite concentrations obtained from the HOT and BATS programs (Karl and Lukas 1996; Steinberg et al. 2001). Dashed lines represent the depth of the mixed layer (Malmstrom et al. 2010) and solid lines represent the nitracline depth defined as the depth at which nitrate + nitrite equals 0.1 µmol kg^{-1} (Le Borgne et al. 2002). Plots were created using Ocean Data View 4.6.2 and data were interpolated using weighted-average gridding.

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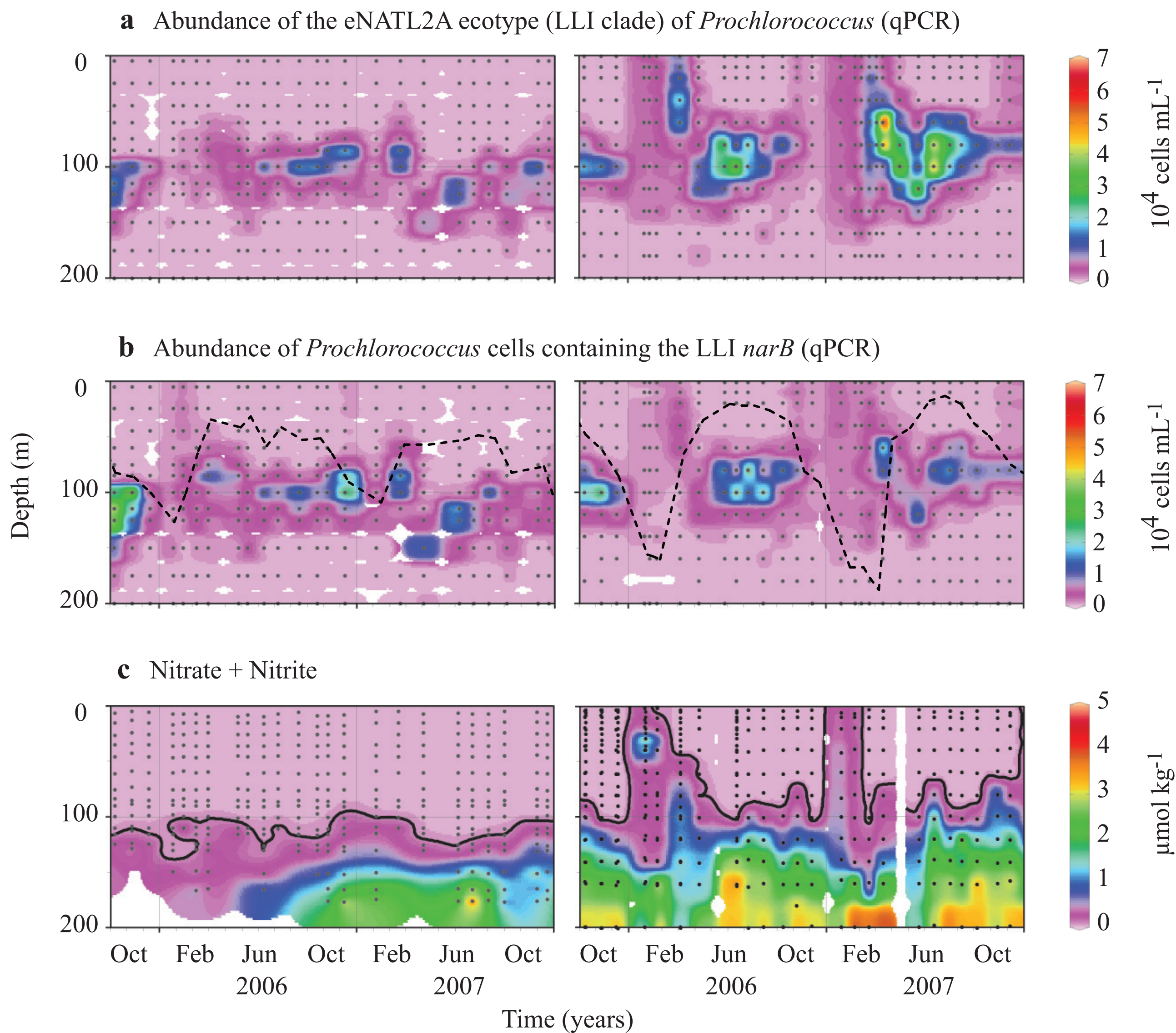

Fig. 2. Distribution and abundance of total LLI *Prochlorococcus* cells and LLI cells containing *narB* in the Pacific and Atlantic Oceans. (**a**) the abundance of LLI clade cells (eNATL2A ecotype) determined by qPCR (Malmstrom et al. 2010), (**b**) the abundance of LLI clade cells containing *narB* determined by qPCR, (**c**) nitrate + nitrite concentrations obtained from HOT and BATS. Dashed lines represent the depth of the mixed layer (Malmstrom et al. 2010) and solid lines represent the nitracline depth defined as the depth at which nitrate + nitrite equals 0.1 μmol kg^{-1} (Le Borgne et al. 2002). Plots were created using Ocean Data View 4.6.2 with data interpolated using weighted-average gridding.

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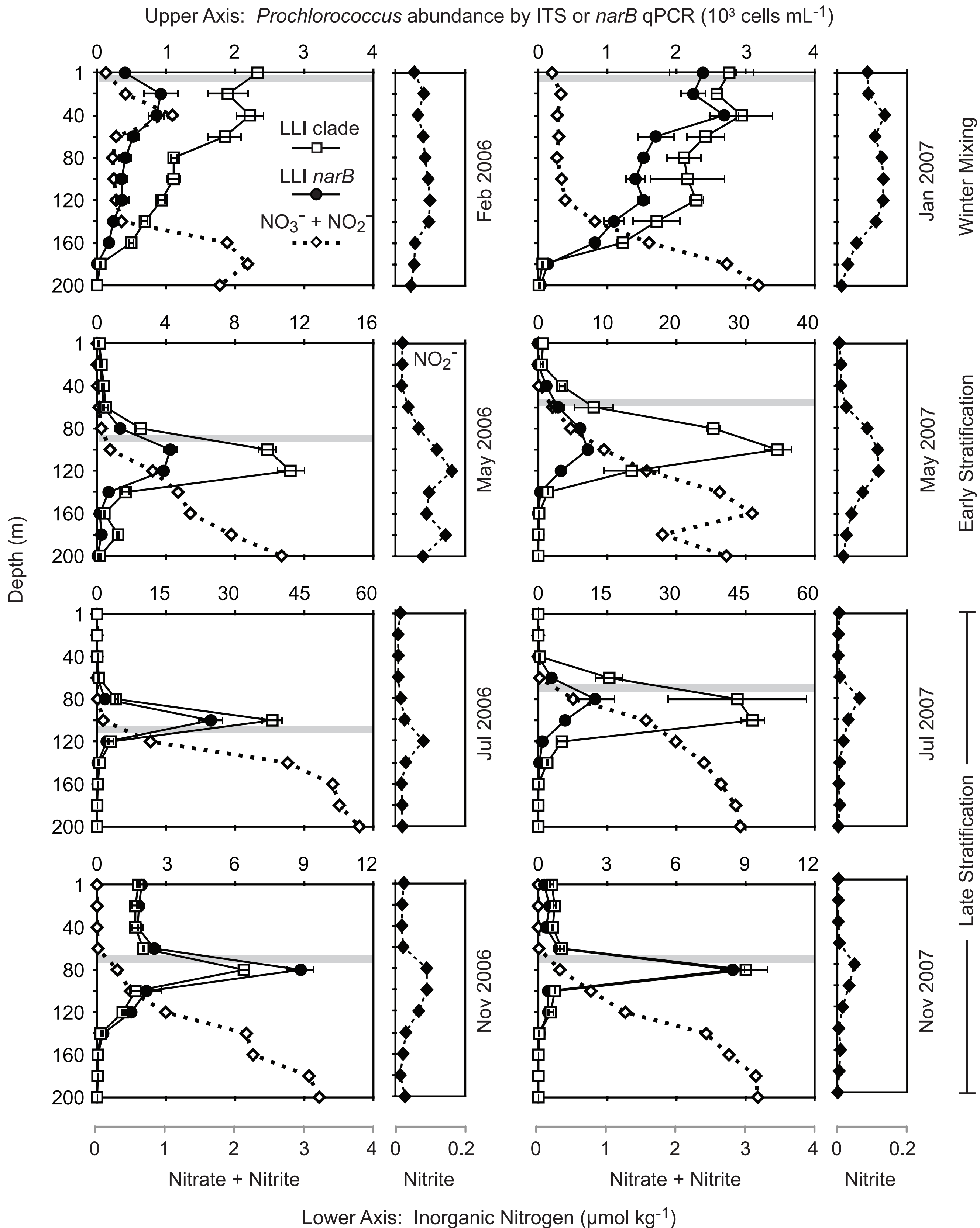

Fig. 3. Distribution of total LLI *Prochlorococcus* cells and LLI cells containing *narB* relative to the nitracline and primary nitrite maximum at the Atlantic site (BATS Station). The abundances of LLI clade cells (ITS qPCR) and *narB*-containing LLI clade cells (*narB* qPCR) are plotted for 8 representative depth profiles. Inorganic nitrogen (nitrate + nitrite) and nitrite concentrations were interpolated in Ocean Data View 4.6.2 in order to match depths of the qPCR samples. Gray bars represent the nitracline depth defined as the depth at which nitrate + nitrite equals 0.1 μmol kg^{-1} (Le Borgne et al. 2002). Within each water column, the greatest abundance of cells containing LLI *narB* are found immediately above or within the top layer of the nitracline and in close proximity to the primary nitrite maximum under stratified conditions.

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