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2 High levels of heterogeneity in diazotroph diversity and activity within a putative hotspot for
3 marine nitrogen fixation

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22

23 Abstract

24 Australia's tropical waters represent predicted "hotspots" for nitrogen (N₂) fixation based on
25 empirical and modelled data. However, the identity, activity and ecology of diazotrophs
26 within this region are virtually unknown. By coupling DNA and cDNA sequencing of
27 nitrogenase genes (*nifH*) with size fractionated N₂ fixation rate measurements, we elucidated
28 diazotroph dynamics across the shelf region of the Arafura and Timor Seas (ATS) and
29 oceanic Coral Sea during Austral spring and winter. During spring, *Trichodesmium*
30 dominated ATS assemblages, comprising 60% of *nifH* DNA sequences, while *Candidatus*
31 *Atelocyanobacterium thalassa* (UCYN-A) comprised 42% in the Coral Sea. In contrast,
32 during winter the relative abundance of heterotrophic unicellular diazotrophs
33 (δ -proteobacteria and γ -24774A11) increased in both regions, concomitant with a marked
34 decline in UCYN-A sequences, whereby this clade effectively disappeared in the Coral Sea.
35 Conservative estimates of N₂ fixation rates ranged from < 1 to 91 nmol L⁻¹ d⁻¹, and size
36 fractionation indicated that unicellular organisms dominated N₂ fixation during both spring
37 and winter, but average unicellular rates were up to 10-fold higher in winter than spring.
38 Relative abundances of UCYN-A1 and γ -24774A11 *nifH* transcripts negatively correlated to
39 silicate and phosphate, suggesting an affinity for oligotrophy. Our results indicate that
40 Australia's tropical waters are indeed hotspots for N₂ fixation, and that regional
41 physicochemical characteristics drive differential contributions of cyanobacterial and
42 heterotrophic phylotypes to N₂ fixation.

43

44 Keywords: *nifH* amplicon sequencing / seasonal nitrogen fixation / tropical ocean /
45 heterotrophic diazotrophs

46 Subject category: Microbial ecology and functional diversity of natural habitats

47 Running title: Diazotroph dynamics in the tropical Australian ocean

48

49 INTRODUCTION

50 Biological nitrogen (N₂) fixation is a fundamental process within the ocean, helping to
51 alleviate nitrogen limitation, thereby supporting primary production and the sequestration of
52 carbon to the deep sea (Sohm et al. 2011; Karl et al. 2012). N₂ fixation is mediated by a
53 diverse range of microorganisms (Zehr et al., 2003), including the photoautotrophic
54 cyanobacterium *Trichodesmium*, as well as unicellular cyanobacteria, diatom-associated
55 cyanobacteria and heterotrophic bacteria, all of which are distributed across tropical and
56 subtropical latitudes (Capone et al., 1997, 2005; Montoya et al., 2004; Moisander et al., 2008,
57 2010, 2014; Foster et al., 2009).

58 High rates of marine N₂ fixation have been observed in Australia's tropical waters (Montoya
59 et al., 2004) and N₂ fixation rate models predict rates sometimes exceeding 100 μmol m⁻² d⁻¹
60 in this region (Luo et al., 2014). Additionally, ecosystem models predict cyanobacterial
61 diazotrophs will be abundant in northern Australian waters (Monteiro et al., 2010). Indeed,
62 *Trichodesmium* has long been recognised as an important member of the phytoplankton in
63 this region (Hallegraeff and Jeffrey, 1984; Burford et al., 1995, 2009), and unicellular
64 diazotrophs are assumed to be highly active here as well (Montoya et al., 2004). However,
65 compared to the South Pacific Ocean, where unicellular diazotrophs including *Candidatus*
66 *Atelocyanobacterium thalassa* (UCYN-A), *Crocospaera watsonii*, and the γ-proteobacterial
67 clade γ-24774A11 are known to be abundant (Moisander et al., 2010, 2014), we currently
68 lack any detailed understanding of patterns in the diversity, activity and ecology of
69 diazotrophs within tropical Australian waters.

70 We surveyed two distinct oceanographic provinces in northern Australia, which play
71 important roles in global climate and ocean circulation. These include the semi-enclosed
72 Arafura and Timor shelf sea regions (ATS), which form part of the Indian Pacific Warm Pool

73 (Alongi et al., 2011), and the open ocean Coral Sea, where the South Pacific western
74 boundary current originates (Qu and Lindstrom, 2002). The ATS is considered autotrophic
75 (McKinnon et al., 2011) and highly productive, particularly during the tropical dry season
76 (Austral winter) (Alongi et al., 2011), despite relatively low surface nitrogen concentrations
77 ($< 2 \mu\text{M}$ nitrate) and no deep-water reservoir of nutrients (Lyne and Hayes, 2005). Annual
78 primary production in the Coral Sea is relatively low and nitrogen limitation is predicted
79 (Condie and Dunn, 2006), with the upper 100 m of the water column being highly
80 oligotrophic throughout the year (Lyne and Hayes, 2005).

81 N_2 fixation has been found to be a significant biogeochemical feature of this important region
82 of the ocean (Montoya et al., 2004; Luo et al., 2014), but we observed substantial
83 physicochemical variability, manifest in differential temperature and salinity signatures and
84 nutrient availability, between the ATS and Coral Sea, which may influence the relative
85 importance of diazotroph activity, particularly given the highly dynamic nature of diazotroph
86 communities (Robidart et al., 2014). By combining *nifH* sequencing and size fractionated
87 $^{15}\text{N}_2$ rate measurements, we assessed spatial and temporal patterns in the diversity and
88 activity of N_2 fixing bacteria across this region, with the aim of characterising the dynamics
89 of diazotrophy within this putative global N_2 fixation hotspot.

90

91

92 MATERIALS AND METHODS

93 Sample Collection

94 Sampling was performed during two voyages aboard the *R/V Southern Surveyor*, consisting
95 of a 2500 km transect from Darwin to Cairns conducted in the Austral spring (October 2012;

96 ss2012_t07; Figure 1A), and a 5000 km transect from Broome to Brisbane during the Austral
97 winter (July-August; ss2013_t03; Figure 1B). October marks the beginning of the tropical
98 wet season, while the July-August period corresponds to the middle of the dry season.

99 Seawater was sampled daily at dawn during both transects for diazotroph diversity (DNA)
100 and gene expression (cDNA) analyses and N₂ fixation rate measurements, as well as mid-
101 afternoon for analysis of diazotroph diversity (DNA) only, resulting in stations separated by
102 100 - 300 km. Samples were collected from the surface at all stations, and the chlorophyll
103 maximum (cmax) when a fluorescence peak was discernible in the water column (see
104 Supplementary Information).

105

106 N₂ fixation rates

107 Net ¹⁵N₂ assimilation (Montoya et al., 1996) was measured to obtain estimates of N₂ fixation
108 by the whole community (WC) and < 10 μm unicellular size fraction (USF) of diazotrophs as
109 previously described (Church et al. 2009; see Supplementary Information). Experiments
110 conducted with surface and cmax samples were incubated at *in situ* temperature and light
111 levels for 24 h and terminated by filtration (see Supplementary Information). Assimilation
112 rates were calculated as previously described (Montoya et al. 1996), based on a theoretical
113 enrichment of ca. 8 atom% ¹⁵N₂, and are considered conservative estimates of N₂ fixation due
114 to the known incomplete dissolution of the ¹⁵N₂ gas bubble (Mohr et al., 2010).

115

116 Nucleic acid collection and extraction

117 At each station, 4-8 L of seawater was filtered through 0.22 μm Sterivex filter units (EMD
118 Millipore, Billerica, MA, USA). Filters were immediately frozen in liquid nitrogen and stored

119 at -80°C. Community DNA was extracted using the PowerWater DNA Extraction Kit
120 (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions,
121 including an additional 10 min heating step with solution PW1 to ensure complete cell lysis.
122 DNA yield was quantified using a Broad Range DNA Qubit™ Assay (Invitrogen, Carlsbad,
123 CA, USA) with a Qubit™ 2.0 Fluorometer.

124 RNA samples were collected from all N₂ fixation incubation stations. Seawater (1-2 L) was
125 filtered through a 0.22 µm Durapore membrane filter (Millipore) within 15 minutes of
126 collection, after which RNAlater solution (300 µl; Ambion, Austin, TX, USA) was added and
127 filters were frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted as
128 previously described (Frias-Lopez et al. 2008; Stewart et al. 2010; see Supplementary
129 Information).

130

131 *nifH* PCR amplification and amplicon pyrosequencing

132 A nested PCR protocol was used to amplify an ~359 bp region of the nitrogenase gene, using
133 the degenerate primers: *nifH3*, *nifH4*, *nifH1* and *nifH2* (Zani et al., 2000; Zehr and Turner,
134 2001). Equal volumes of DNA or cDNA were used as template (2 µl) in the first stage of the
135 reaction, and 1 µl of PCR product was used as template in the second stage, using previously
136 described reaction conditions (Messer et al. 2015; see Supplementary Information).

137 The *nifH* amplicons were sequenced using the 454 FLX Titanium pyrosequencing platform
138 (Roche, Molecular Research LP, USA) following an additional 10 PCR cycles with custom
139 barcoded *nifH1* and *nifH2* primers under the same PCR reaction conditions (Dowd et al.
140 2008; Farnelid et al. 2011, 2013; Messer et al. 2015; Supplementary Information). Raw
141 sequences were quality filtered, whereby sequences with a quality score < 25 and reads < 200

142 bp long were removed, and clustered into operational taxonomic units (OTUs) at 95%
143 sequence identity (Penton et al., 2013) using UCLUST (Edgar, 2010) and rarefied to the
144 lowest number of sequences per sample (872 sequences) in QIIME (Caporaso et al., 2010).
145 To assign putative taxonomy, representative sequences from *nifH* OTUs were aligned to the
146 closest sequence in a custom *nifH* database (updated in April 2014) (Zehr et al., 2003; Heller
147 et al., 2014) and placed in a phylogenetic tree using the maximum parsimony tool in ARB
148 (Westram et al., 2011). Translated *nifH* sequences were compared to the Ribosomal Database
149 Project's *nifH* protein database using FrameBot from the Fungene pipeline (Fish et al., 2013;
150 Wang et al., 2013).

151

152 Statistical analyses

153 Rarefied sequence data were square-root transformed and a resemblance matrix generated
154 using Bray-Curtis similarity. Environmental parameters (Supplementary Table 1) were
155 normalised and a resemblance matrix generated using Euclidean distance (Clarke and
156 Warwick, 2001). Statistical analyses, including analysis of similarities (ANOSIM; Clarke
157 1993), distance based linear modelling (DistLM) and distance-based redundancy analysis
158 (dbRDA) (Legendre and Anderson, 1999; McArdle and Anderson, 2001), were performed in
159 the PRIMER + PERMANOVA software package (v6; Clarke & Warwick 2001).

160 In order to identify associations (linear regression, p) between N_2 fixation rates, expressed
161 *nifH* OTUs and environmental parameters, we calculated the maximal information coefficient
162 (MIC) between all variable ($n = 255$) pairs from all samples ($n = 28$) using the MINE
163 statistics package (Reshef et al., 2011). Strongly co-linear variables ($p > 0.9$ or > -0.9) were
164 removed from the analyses. After correction for multiple testing (Benjamini and Hochberg,
165 1995), statistically significant co-occurrence relationships ($P < 0.01$; $MIC > 0.473$) between

166 pairs of variables were input into Cytoscape v 2.8 (Smoot et al., 2011) and used to generate
167 network diagrams for visualisation.

168

169

170 RESULTS

171 Physicochemical characteristics of the ATS and Coral Sea

172 During both spring and winter, ATS waters were warmer, exhibited lower salinities and had
173 higher nutrient concentrations and phytoplankton abundances than Coral Sea waters (Figure
174 1, Supplementary Table 1). Mean sea surface temperature (SST) in the ATS was 27.4 °C in
175 spring and 26.4 °C in winter, compared with 26.5 °C and 24.3 °C in the Coral Sea. During
176 both sampling periods, salinity increased from 33.5 to > 35 PSU at a longitude of
177 approximately 143 °E, reflecting the transition from the shelf region (ATS) to the open ocean
178 (Coral Sea) (Figure 1). Mean nitrate and phosphate concentrations were greatest during the
179 spring, being 3.5 and 3.8 times greater in the ATS than the Coral Sea respectively, with this
180 discrepancy falling to 2.6 and 1.8 times in the winter. N:P ratios were always < 16:1
181 indicating an excess of phosphate compared to nitrate and ammonium, particularly in surface
182 waters (Supplementary Table 1). We also observed intra-region nutrient differences, for
183 example silicate concentrations were highest in the eastern region of the ATS in spring (5.97
184 $\mu\text{mol L}^{-1}$; SS7), and the western region in winter (5.74 $\mu\text{mol L}^{-1}$; WS1; Figure 1). Pigment
185 analyses indicated that phytoplankton communities within the ATS were dominated by
186 microphytoplankton such as diatoms, whereas pico- and nanophytoplankton were relatively
187 more abundant in the Coral Sea (Supplementary Table 1).

188

189 N₂ fixation rates

190 N₂ fixation rates within the WC and USF ranged from < 1 to 91 nmol L⁻¹ d⁻¹, and at times
191 displayed substantial variability between the ATS and Coral Sea and between seasons (Figure
192 2). During the Austral spring, mean WC N₂ fixation rates (± sd.) were 23 ± 32 nmol L⁻¹ d⁻¹
193 compared to 6 ± 7 nmol L⁻¹ d⁻¹ in the USF. However, it is notable that the majority of N₂
194 fixation occurred within the < 10 μm size class at three out of four ATS sites (Figure 2A).
195 Within ATS waters there was a peak in N₂ fixation at site SS5 where WC rates reached 71 ±
196 10 nmol L⁻¹ d⁻¹, significantly greater than the USF rates of 16 ± 3 nmol L⁻¹ d⁻¹ (One-way
197 ANOVA, Tukey HSD, P < 0.05). This was the only ATS site sampled for N₂ fixation rate
198 measurements where a cmax was observed, and rates within it were comparatively low at ≤ 4
199 nmol L⁻¹ d⁻¹ for both size classes (Figure 2A).

200 Spring N₂ fixation rates in the Coral Sea were lower than in the ATS, with mean rates of 13 ±
201 5 (WC) and 7 ± 6 nmol L⁻¹ d⁻¹ (USF). However, within this region the contribution made by
202 unicellular organisms was higher, being ~50% of the total. Rates in Coral Sea surface waters
203 reached a maximum of 18 ± 2 (WC) and 14 ± 13 (USF) nmol L⁻¹ d⁻¹ at SS16 (Figure 2A). N₂
204 fixation rates in the cmax were at times higher than in the surface waters, reaching up to 24 ±
205 0.6 (WC) and 18 ± 5 (USF) nmol L⁻¹ d⁻¹ (Figure 2A).

206 A marked increase in N₂ fixation was observed in both regions during the Austral winter
207 (Figure 2B). Within ATS surface waters, WC N₂ fixation increased almost three-fold in
208 winter, with a mean of 60 ± 15 nmol L⁻¹ d⁻¹ across the six sites. Furthermore, the majority of
209 this activity was attributable to the USF (One-way ANOVA, Tukey HSD, P > 0.05), such that
210 mean USF rates increased almost ten-fold in winter to 57 ± 23 nmol L⁻¹ d⁻¹. Maximum rates
211 recorded in the ATS surface waters were similar to the spring (Figure 2). Only one winter

212 ATS site had a discernible c_{max} and here, in contrast to the spring, N_2 fixation was also
213 relatively high (Figure 2B).

214 Mean winter N_2 fixation rates in the Coral Sea were four and seven-times higher than during
215 the spring in the WC ($56 \pm 32 \text{ nmol L}^{-1} \text{ d}^{-1}$) and USF ($47 \pm 28 \text{ nmol L}^{-1} \text{ d}^{-1}$) respectively.
216 While mean N_2 fixation rates were again lower in the Coral Sea than the ATS, the maximum
217 rates recorded in Coral Sea surface waters were the highest observed, reaching 91 ± 7 (WC)
218 and $71 \pm 18 \text{ nmol L}^{-1} \text{ d}^{-1}$ (USF) at station WS16, towards the southern end of the transect
219 (Figure 2B). During the winter, four Coral Sea sites had discernible c_{max} , where N_2 fixation
220 rates were also relatively high (Figure 2B).

221

222 Diazotroph population dynamics in ATS and Coral Sea waters

223 A total of 174 *nifH* OTUs were resolved from our samples. Phylogenetic analysis revealed
224 the presence of photoautotrophic, photoheterotrophic and heterotrophic diazotrophs during
225 both transects, and these clustered with environmental *nifH* sequences originating from the
226 Pacific and Atlantic Oceans and the South China Sea (Supplementary Figure 1).

227 Three *nifH* OTUs were dominant across the data-set, comprising 67% of total *nifH* DNA
228 sequences retrieved. These included OTU6956, which was 100% identical to *Trichodesmium*
229 *erythraeum* (IMS 101; hereafter *Trichodesmium*) in *nifH* amino acid (aa) composition,
230 OTU6352, which was 100% identical in aa composition to UCYN-A and clustered with the
231 UCYN-A1 ecotype (Supplementary Figure 1), and OTU4713, which shared 91% similarity in
232 aa composition to the γ -proteobacteria *Pseudomonas stutzeri*, and clustered within the
233 γ -24774A11 clade (Moisander et al., 2008, 2014). Despite the ubiquity of these dominant
234 OTUs, significant partitioning of diazotroph population structure was observed between the

235 ATS and Coral Sea (Figure 3; ANOSIM, Global R: 0.471, $P < 0.001$) and between the
236 Austral spring and winter sampling (ANOSIM, Global R: 0.399, $P < 0.001$). No significant
237 differences were observed between surface and cmax diazotroph communities.

238 During the Austral spring, *Trichodesmium* comprised 60% of *nifH* sequences in the ATS, and
239 at some sites reached over 80% of sequences in both the surface and cmax (SS3 and SS5
240 respectively; Figure 3A, B). γ -24774A11 was also detected throughout the ATS during the
241 spring, where it represented 14% of total sequences, and reached a maximum abundance of
242 over 40% of the diazotroph population at SS8 and SS6 (surface and cmax respectively;
243 Figure 3A, Supplementary Figure 2).

244 In contrast, the Coral Sea was dominated by the unicellular cyanobacterium UCYN-A during
245 the spring. UCYN-A1 was conspicuously absent from the ATS samples, but comprised 42%
246 of the total *nifH* sequences in the Coral Sea, with a maximum abundance of 77% at SS16
247 (Figure 3A). Like the ATS, γ -24774A11 was also a significant feature of Coral Sea
248 springtime diazotroph populations, where it constituted 21% of sequences, and reached up to
249 34 and 46% of diazotrophs at the surface and cmax at SS13 and SS14 respectively (Figure
250 3A; Supplementary Figure 2).

251 During the Austral winter, *Trichodesmium* still generally dominated throughout the ATS,
252 representing 35% of total *nifH* sequences, but there was an increase in the relative number of
253 heterotrophic diazotrophs compared to the spring (Figure 3). The putative heterotrophic δ -
254 proteobacterial OTUs 359, 7075, and 811, which shared between 96 and 99% aa identity to
255 *Desulfuromonas acetoxidans*, collectively comprised 19% of total sequences, and up to 55%
256 of the diazotroph population in ATS surface waters (Figure 3C; Supplementary Figure 2).
257 Notably these OTUs only accounted for 7% of diazotrophs in the ATS during the spring
258 (Supplementary Figure 2).

259 Sequences associated with heterotrophic diazotrophs also increased during the winter in the
260 Coral Sea, relative to the spring (Figure 3C, D). However, here they were primarily
261 associated with γ -24774A11, which represented 34% of wintertime Coral Sea *nifH*
262 sequences, reaching a maximum of 64% of diazotrophs at the surface, and 43% at the cmax
263 (Figure 3C, D). In contrast to the spring sampling where they dominated, UCYN-A
264 sequences only comprised 3% of Coral Sea diazotrophs during the winter and remained
265 absent from the ATS (Supplementary Figure 2).

266

267 Patterns in *nifH* expression

268 Consistent with DNA profiles, *Trichodesmium*, UCYN-A1 and γ -24774A11 dominated *nifH*
269 transcripts across the data-set (Figure 4), and significant differences were observed between
270 transcription profiles from the ATS and Coral Sea (ANOSIM, Global R: 0.595, P < 0.01). In
271 ATS surface waters, *Trichodesmium* (OTU6956) and γ -24774A11 (OTU4713) dominated
272 *nifH* transcripts during the spring. Specifically, *Trichodesmium* comprised up to 86% of
273 transcripts at sites where high rates of N₂ fixation attributable to the > 10 μ m size class were
274 recorded (SS5, Figure 4A). γ -24774A11 represented up to 52% of transcripts during the
275 spring transect (SS8; Figure 4A), even though unicellular N₂ fixation was recorded at
276 relatively low levels (< 1 nmol L⁻¹ d⁻¹; Figure 2A).

277 Within Coral Sea surface waters in spring, *nifH* transcripts mainly consisted of
278 *Trichodesmium*, UCYN-A and γ -24774A11 (Figure 4A). While *Trichodesmium* transcripts
279 decreased throughout the Coral Sea, UCYN-A1 transcripts increased from 4 to 39%, towards
280 southern latitudes where the peak in Coral Sea springtime N₂ fixation was observed (Figure
281 4A; Figure 2A). Transcripts associated with γ -24774A11 were most abundant in cmax

282 samples, where they represented up to 71% of expressed *nifH* genes (Figure 4B), although
283 c_{max} N₂ fixation rates were relatively low (< 1 nmol L⁻¹ d⁻¹; Figure 2A).

284 Similar to the spring sampling, *Trichodesmium* transcripts were often highly abundant in
285 ATS surface waters during the winter, accounting for 98% of transcripts at station WS3
286 (Figure 4C). Two additional OTUs, OTU1924 and OTU798, also constituted a significant
287 fraction (up to 76 and 67%) of transcripts in the ATS, however these OTUs shared only 20
288 and 25% aa identity respectively with available *nifH* sequences, with closest matches to
289 members of the Firmicutes. Interestingly, transcripts associated with γ -24774A11 and
290 UCYN-A were not detected in the ATS during the winter (Figure 4C) despite high unicellular
291 N₂ fixation rates (Figure 2B).

292 In contrast to the spring, where cyanobacterial transcripts were dominant, *nifH* expression in
293 Coral Sea surface waters was more variable during the winter. For example, both γ -
294 24774A11 and OTU7453, which shared 98% aa identity with the cyanobacterium
295 *Mastigocladus laminosus*, contributed up to ~56% of *nifH* transcripts at stations where
296 unicellular N₂ fixation rates were high (WS15 and WS16 respectively; Figure 4C; Figure 2B).
297 Notably, no transcripts associated with UCYN-A ecotypes were detected in Coral Sea surface
298 waters during the winter, but all three ecotypes were present in transcripts from the c_{max} ,
299 along with *Trichodesmium*, γ -24774A11, and some additional cyanobacterial diazotrophs
300 (Figure 4C, D).

301

302 Potential drivers of diazotroph populations and activity

303 Analysis of the diazotroph populations using both *nifH* DNA (Figure 5A) and cDNA (Figure
304 5B) data indicated clear separation between ATS and Coral Sea regions. DistLM identified

305 SST and salinity as significant explanatory variables ($P < 0.05$), reflecting the increase in
306 salinity and decrease in temperature associated with the Coral Sea region (Figure 1, Figure
307 5A, B; Supplementary Table 2). Dissolved silicate and phosphate were also significant ($P <$
308 0.05) explanatory variables in both the DNA and cDNA DistLM analysis, as were the
309 photoprotective and photosynthetic carotenoids alloxanthin and fucoxanthin ($P < 0.05$), all of
310 which exhibited higher relative concentrations in the ATS compared with the Coral Sea.
311 Interestingly, dissolved oxygen and divinyl chlorophyll *a* were significant ($P < 0.05$)
312 explanatory variables within the *nifH* DNA model but not within the *nifH* cDNA model
313 (Figure 5A, B). However for both models, $> 50\%$ of the total variation between diazotroph
314 populations remained unexplained.

315 Network analysis revealed that N_2 fixation by the USF exhibited a significant, but only
316 moderately strong, negative correlation to ammonium concentration ($p = -0.43$; MIC strength
317 $= 0.66$; $P < 0.01$), and was also negatively correlated to the relative abundance of UCYN-A2
318 transcripts ($p = -0.33$; MIC strength $= 0.71$; $P < 0.01$), possibly reflecting the relatively low
319 abundance of this ecotype (Supplementary Figure 2). N_2 fixation was not significantly
320 correlated to any other environmental parameters or *nifH* OTUs.

321 A number of *nifH* OTUs were negatively correlated with phosphate and silicate (Figure 6A,
322 B), including two of the most abundant diazotrophs, OTU4713 from the γ -24774A11 clade,
323 and OTU6352 from the UCYN-A1 ecotype. These γ -24774A11 and UCYN-A1 OTUs were
324 also positively correlated to salinity, to each other, and a range of other *nifH* OTUS including
325 the UCYN-A2 (OTU83) and UCYN-A3 (OTU2020), and other γ -24774A11 OTUs
326 (OTU2710 and OTU454; Figure 6C, D; Supplementary Figure 1). No significant correlations
327 were observed between *nifH* transcripts and the concentration of dissolved inorganic nitrogen
328 species.

329

330

331 DISCUSSION

332 Identifying the factors that influence the composition and activity of diazotrophs is key to
333 understanding the relative importance of N₂ fixation on local and global scales (Zehr and
334 Kudela, 2011; Robidart et al., 2014). N₂ fixed by diverse diazotrophic taxa may have
335 different fates within the marine environment (Glibert & Bronk 1994, Mulholland 2007,
336 Foster et al. 2011, Karl et al. 2012, Benavides et al. 2013) and therefore characterisation of
337 the composition of active N₂ fixing assemblages, combined with size fractionated N₂ fixation
338 rates, is necessary to determine the differential contribution of newly fixed N to pelagic
339 ecosystems. Here we report changes in the biogeographical distribution and activity of
340 diazotrophs across a broad tropical region, which has been identified as a potential global
341 “hotspot” for marine N₂ fixation (Montoya et al., 2004; Monteiro et al., 2010; Luo et al.,
342 2014).

343 Previously, Montoya et al. (2004) reported rates of USF N₂ fixation up to 480 nmol L⁻¹ d⁻¹ in
344 ATS waters (25 m below surface) during the Austral spring. Herein, we sampled during both
345 spring and winter, at similar latitudes (within 0 - 1 °S) and longitudes (within 0.3 – 2 °E), yet
346 observed maximum USF rates that were substantially less than those reported by Montoya et
347 al. (2004). Recently, Raes et al. (2014) reported mean WC rates of ~36 nmol L⁻¹ d⁻¹ within
348 the westerly region of the ATS during the Austral spring. While not directly comparable due
349 to methodological differences (Wilson et al., 2012), these values are in line with those we
350 measured in the same region during the Austral winter, suggesting relatively high rates are
351 maintained here across seasons. Based on methodological comparisons, there appears to be
352 no clear trend in the level of N₂ fixation rate underestimation using the Montoya et al. (1996)

353 method, due to multiple factors influencing the dissolution of the $^{15}\text{N}_2$ bubble (Mohr et al.,
354 2010; Großkopf et al., 2012). However, comparisons made in the North Pacific and Atlantic
355 Oceans indicate that this method could lead to N_2 fixation underestimates of 50 % (Wilson et
356 al. 2012; Benavides et al. 2013), or greater depending on the composition of the diazotroph
357 community (Großkopf et al., 2012).

358 Compared with near surface waters of similar latitudes, including the tropical western South
359 Pacific ($< 1 \text{ nmol L}^{-1} \text{ d}^{-1}$; Moisaner et al. 2010), western equatorial Pacific ($< 40 \text{ nmol L}^{-1} \text{ d}^{-1}$;
360 $\text{Bonnet et al. 2009}$), eastern tropical South Pacific (ca. $\leq 1 \text{ nmol L}^{-1} \text{ d}^{-1}$; Dekaezemacker et
361 al. 2013), and the tropical South Pacific Gyre ($\leq 3 \text{ nmol L}^{-1} \text{ d}^{-1}$; Raimbault & Garcia 2008),
362 the maximum conservative rates of N_2 fixation reported here during the Austral winter (91
363 $\text{nmol L}^{-1} \text{ d}^{-1}$) are relatively high. Indeed, our observations, along with those previously
364 reported (Montoya et al., 2004; Raes et al., 2014) support the proposition that the tropical
365 waters of northern Australia are a “hotspot” of diazotroph activity within the Southern
366 Hemisphere. However, our data also demonstrate significant temporal and spatial variability
367 in N_2 fixation in this region, which we propose is driven by the highly dynamic and
368 heterogeneous nature of the resident diazotroph populations.

369 Across our data-set, the majority of N_2 fixation activity was observed within the USF, and
370 USF N_2 fixation rates were greater in winter than spring, with mean rates ten-times higher in
371 the ATS and seven-times greater in the Coral Sea. While it must be noted that *Trichodesmium*
372 is known to release some of the N_2 it fixes as dissolved organic nitrogen (Glibert and Bronk,
373 1994), and therefore a proportion of ^{15}N - N_2 fixed could have been transferred to the USF
374 during the incubation, these increased USF rates occurred in parallel to an increase in the
375 relative abundance of δ - and γ -proteobacterial *nifH* sequences and γ -24774A11 *nifH*

376 transcripts. This highlights the potential importance of heterotrophic diazotrophs to
377 biogeochemical cycling during the winter across these two quite different regions.

378 This study provides the first detailed characterisation of active diazotroph populations
379 throughout northern Australia. It must be noted that amplicon sequencing approaches can
380 only reconcile relative abundances, and therefore do not allow for the absolute quantification
381 of colonial versus single-celled diazotrophs, and that it is difficult to directly equate
382 diazotroph communities to N₂ fixation activity. However, we identified a range of
383 photoautotrophic, photoheterotrophic and heterotrophic bacteria which share high similarities
384 in *nifH* sequences to those recovered from similar oceanic environments (e.g. Langlois et al.
385 2005; Bombar et al. 2013; Moisander et al. 2010; Thompson et al. 2014; Moisander et al.
386 2014).

387 Despite its shelf sea nature, ATS diazotroph communities typically resembled those of
388 similar latitudes, such as the tropical Atlantic Ocean, where *Trichodesmium* dominates
389 (Langlois et al., 2005; Foster et al., 2009; Goebel et al., 2010). However, heterotrophic
390 groups were also a feature of ATS communities, and we observed a shift from
391 γ -proteobacterial to δ -proteobacterial phylotypes between spring and winter. Conversely,
392 Coral Sea communities contained a greater diversity of cyanobacterial phylotypes, including
393 ecotypes of UCYN-A alongside a lower frequency of *Trichodesmium* sequences, as well as
394 heterotrophic diazotrophs. The composition of Coral Sea diazotroph populations appears
395 similar to those found within the wider tropical and subtropical South Pacific (Moisander et
396 al., 2010, 2014; Halm et al., 2012). However, a seasonal shift in the composition of Coral Sea
397 populations was also observed, such that the relative abundance of UCYN-A ecotypes
398 decreased while γ -24774A11 increased between spring and winter respectively. Previously,
399 Moisander et al. (2014) reported the distribution of γ -24774A11 to be ubiquitous and

400 relatively homogeneous in South Pacific surface waters during the Austral autumn. By
401 examining spatial and temporal patterns in diazotroph community dynamics, we show that
402 the distribution and relative abundance of γ -24774A11 is variable and that the significance of
403 this group may increase during the Austral winter.

404 Surprisingly, no significant differences between surface and cmax diazotroph communities
405 were observed in the present study, with *Trichodesmium*, UCYN-A1 and γ -24774A11
406 sequences all detected down to 120 m below surface. Previously, *Trichodesmium* and
407 unicellular diazotrophs have been shown to have differential depth distributions in the
408 western South Pacific Ocean, with *Trichodesmium* and γ -24774A11 most abundant in upper
409 euphotic zone waters and UCYN-A more abundant deeper within the water column
410 (Moisander et al., 2010, 2014). We found that all three of these groups were relatively
411 abundant in both surface and cmax waters depending on the sampling region and season. We
412 also detected *nifH* expression by *Trichodesmium* 90 m below surface, and UCYN-A1 and
413 γ -24774A11 100 m below surface, indicating that all three groups were active at depth too.
414 This suggests that the physicochemical variables identified to be potential drivers of
415 diazotroph distribution in the present study, were most relevant over horizontal rather than
416 vertical scales, which could be due to similarities between surface and cmax physicochemical
417 signatures (Supplementary Table 1).

418 The differences in diazotrophic taxa and N₂ fixation activity observed between the ATS and
419 Coral Sea during both seasons are likely attributable to the observed physicochemical
420 conditions, including higher SST, lower salinities and higher nutrient concentrations in the
421 ATS compared with the Coral Sea, features which are characteristic of the study regions
422 (Condie and Dunn, 2006; Alongi et al., 2011; Ceccarelli, 2011). In particular, SST was
423 identified as a strong indicator of the dominant diazotrophic taxa in our study regions. We

424 observed that *Trichodesmium* dominated communities in the warmer waters of the ATS,
425 displaying maximum relative abundances when SST was > 27 °C, while UCYN-A dominated
426 communities in the cooler Coral Sea waters, although maximum relative abundances of
427 UCYN-A1 transcripts occurred when SST was ~26 °C during the spring. Previously, UCYN-
428 A have been shown to occur at specific temperature optima between 24-26 °C in the western
429 South Pacific (Moisander et al., 2010, 2014). In contrast, during the cooler winter sampling,
430 when SST in the Coral Sea was closer to 24 °C, we observed an increase in relative
431 abundances of γ -24774A11, while the relative abundance of UCYN-A decreased
432 substantially. Maximum relative abundances of γ -24774A11 occurred where SST was ~25
433 °C, and maximum γ -24774A11 *nifH* expression occurred at 25.8 °C, suggesting a
434 temperature optima around 25-26 °C for this group. Recently, the occurrence of the γ -
435 24774A11 clade has been found to be positively, non-linearly correlated with temperature,
436 with maximum abundances associated with surface waters > 26 °C (Moisander et al., 2014).
437 Overall, our data are consistent with the observed distributions of these organisms across a
438 range of oceanic provinces (Capone et al., 1997; Mazard et al., 2004; Langlois et al., 2008;
439 Moisander et al., 2010), and supports previous findings that temperature is an important
440 determinant of diazotroph spatiotemporal dynamics.

441 Dissolved silicate and phosphate concentrations, and the concentration of the pigments
442 alloxanthin and fucoxanthin, were also identified as significant discriminating factors
443 explaining some of the heterogeneity between ATS and Coral Sea diazotroph populations.
444 Whether these correlations indicate a direct or indirect effect on diazotroph abundance and
445 consequently N₂ fixation is unclear. Despite high silicate concentrations and pigment
446 indications of diatom dominated phytoplankton communities in the ATS, only one OTU
447 associated with the heterocystous cyanobacterial symbiont of diatoms, *Richelia*, was
448 observed in our sequence data, and this represented a total of only 6 *nifH* sequences (data not

449 shown). The significant negative correlation observed between silicate and UCYN-A1 and γ -
450 24774A11 transcripts, and UCYN-A1 and fucoxanthin, could be indicative of shifting
451 phytoplankton communities between the ATS and Coral Sea, given that UCYN-A is known
452 to live in association with a prymnesiophyte host (Thompson et al., 2012; Hagino et al., 2013;
453 Krupke et al., 2013). Currently, the lifestyle (e.g. free-living, particle attached, or symbiont)
454 of γ -24774A11 remains unknown (Langlois et al., 2015), however it has been speculated that
455 it may depend upon phytoplankton produced dissolved organic carbon (Moisander et al.,
456 2012, 2014). While strongly co-linear variables were removed from our analyses, silicate was
457 inversely correlated to salinity and positively correlated to temperature, so therefore it could
458 also be indicative of a water mass tracer rather than a biological causation.

459 Conversely, phosphate availability is known to directly influence N₂ fixation and *nifH*
460 expression in natural populations of diazotrophs (Sañudo-Wilhelmy et al., 2001; Rees et al.,
461 2006; Turk-Kubo et al., 2012), as well as the oceanic distribution of diazotrophs in general
462 (Sohm et al., 2011). In the present study, phosphate concentrations were relatively high (e.g.
463 0.27 and 0.26 $\mu\text{mol L}^{-1}$) in the ATS where *Trichodesmium* and δ -proteobacterial dominated
464 communities were observed. In contrast, in the Coral Sea, where UCYN-A1 and γ -24774A11
465 dominated communities, phosphate concentrations were comparatively low (e.g. 0.09 and
466 0.07 $\mu\text{mol L}^{-1}$). This implies a potential role of phosphate limitation in the shift in diazotroph
467 community composition. UCYN-A1 and γ -24774A11 both appear to be broadly distributed
468 throughout low nutrient marine waters (Thompson et al., 2014; Langlois et al., 2015), and it
469 has been hypothesised that these taxa thrive in oligotrophic conditions (Church et al., 2008;
470 Krupke et al., 2014). In line with our findings in the ATS and Coral Sea, Moisander et al.
471 (2014) recently demonstrated that γ -24774A11 was significantly negatively correlated to
472 soluble reactive phosphorous in the western South Pacific. Taken together, our findings

473 suggest that UCYN-A and γ -24774A11 increase in relative abundance and activity when
474 oligotrophic conditions prevail.

475 While no correlations were observed between the different diazotrophic groups and oxidised
476 forms of N, USF N₂ fixation rates exhibited a moderate negative correlation to ammonium
477 concentration, although this is unlikely to be due to an inhibitory effect, because
478 concentrations observed here were below those expected to inhibit N₂ fixation (Knapp, 2012).
479 It has been suggested that tropical Australian waters are constantly nitrogen limited (Condie
480 and Dunn, 2006) and across all sites and depths measured, N:P ratios indicated an excess of
481 phosphate relative to nitrate and ammonium ($N:P \leq 6$). This may confer a competitive
482 advantage to diazotrophic taxa (Moutin et al., 2008; Knapp, 2012), and suggests that N₂
483 fixation could play an important role in helping to alleviate nitrogen limitation within this
484 region.

485 However, it is notable that much of the variability between ATS and Coral Sea *nifH* profiles
486 remained unexplained in our analysis, and despite combined analyses and stringent
487 standardisation across samples, some rare OTUs in the DNA appeared highly active in the
488 cDNA, potentially indicating a disconnect between relative organismal abundance and N₂
489 fixation activity at some locations. Therefore, other factors may have had a significant
490 influence on diazotroph distribution and activity, for instance, dissolved iron (dFe)
491 availability is known to limit marine N₂ fixation (Sohm et al., 2011) and dFe additions can
492 stimulate diazotrophic activity (Moisander et al., 2012; Turk-Kubo et al., 2012). In the
493 western South Pacific ocean, Moisander et al. (2012) demonstrated that the abundances of
494 unicellular diazotrophs, including UCYN-A and γ -24774A11, increased substantially in
495 response to dFe amendments, but also exhibited signs of iron and phosphorous co-limitation.
496 While we did not measure dFe throughout the ATS and Coral Sea during the present study,

497 previous evidence suggests that concentrations of particulate Fe are relatively high in the
498 Timor region of the ATS (Waite et al., 1995), but dFe concentrations may be relatively low
499 (Sohm et al., 2008), and *Trichodesmium* colonies have been shown to experience Fe
500 limitation in this region (Kustka et al., 2003). While this indicates that dFe availability could
501 have influenced diazotroph distributions during our study, additional work exploring dFe
502 dynamics, and the potential influence on diazotroph diversity and activity in the ATS and
503 Coral Sea, is required to further understand the mechanisms structuring diazotroph
504 populations and N₂ fixation throughout these regions.

505 Despite the relatively low concentrations of dissolved inorganic nitrogen, primary production
506 peaks in both the ATS and Coral Sea during the Austral winter (Lyne and Hayes, 2005;
507 Brewer et al., 2007). It was during this season we observed a substantial increase in both
508 unicellular N₂ fixation and the relative abundance of heterotrophic *nifH* sequences. Primary
509 production estimates from the winter sampling are detailed elsewhere (Robinson et al. In
510 Prep.), however using this data we calculated that wintertime USF in the ATS and Coral Sea
511 could supply up to 46 and 42 % respectively of the N required to sustain biomass assimilation
512 from measured primary production rates (assuming Redfield ratios for particulate matter).
513 Thus we propose that primary production here may be enhanced by the influx of newly fixed
514 N mediated by heterotrophic diazotrophs. While it has been argued that the abundances of
515 heterotrophic N₂ fixing bacteria in oceanic environments are not considered sufficient to
516 account for measured rates of N₂ fixation (Turk-Kubo et al., 2013), a recent large-scale
517 analysis of the abundance and distribution of marine γ -proteobacterial *nifH*, indicates that
518 this phylotype could play a significant role in oceanic N₂ fixation (Langlois et al., 2015). It
519 must be noted that the fate of N fixed by marine heterotrophic diazotrophs has not yet been
520 determined, and as such direct evidence of production supported by heterotrophic N₂ fixation
521 is lacking. However, our limited data suggests that heterotrophic diazotrophs are an important

522 component of N₂ fixing populations throughout tropical northern Australia, and that they may
523 contribute to relatively high USF rates of N₂ fixation. Given the apparent importance of
524 heterotrophic diazotrophs in our study region, future research determining the ultimate fate of
525 N fixed by these heterotrophs will be valuable for estimating the extent to which these
526 populations ultimately influence pools of bioavailable N and support primary production.

527 Our study has shown that the composition and activity of diazotrophs in Australia's tropical
528 waters are highly variable across shelf and open ocean environments. Overall, our rate
529 measurements confirm that diazotroph activity is an important process here, but our
530 molecular and statistical analyses suggests that the distinct physicochemical characteristics of
531 these waters drive heterogeneity in populations of photoautotrophic, photoheterotrophic and
532 heterotrophic N₂ fixing bacteria. This heterogeneity in turn leads to substantial changes in
533 rates of N₂ fixation and the subsequent addition of newly fixed N to the ocean. As such,
534 spatial (even within relatively localized boundaries) and seasonal shifts in diazotroph
535 diversity and activity must be considered in future regional and global marine N cycle
536 budgets and modelling efforts. This can only be achieved by further attempts to more
537 precisely map marine diazotrophic processes with increased spatiotemporal resolution.

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548 Conflict of Interest Statement

549 The authors declare no conflict of interest.

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795 | Figure Legends

| Figure 1. Physical and chemical characteristics of surface waters sampled within the ATS (stations SS1-8; WS1-12) and Coral Sea (stations SS9-17; WS13-18) during the (A) spring and (B) winter. Note the different scales within and between A and B.

800 | Figure 2. Mean N₂ fixation rates (\pm s.e., n = 3) performed by the whole community (WC) and < 10 μ m unicellular size fraction (USF) at the surface and chlorophyll maxima during the spring (SS; A) and winter (WS; B) transects.

| Figure 3. Relative abundance of *nifH* OTUs recovered from community DNA (% sequences) at the surface and chlorophyll maxima during the spring (SS; A & B) and winter (WS; C & D) transects. In parentheses are the closest cultured representatives that share \geq 90% amino
805 | acid identity with the detected sequences.

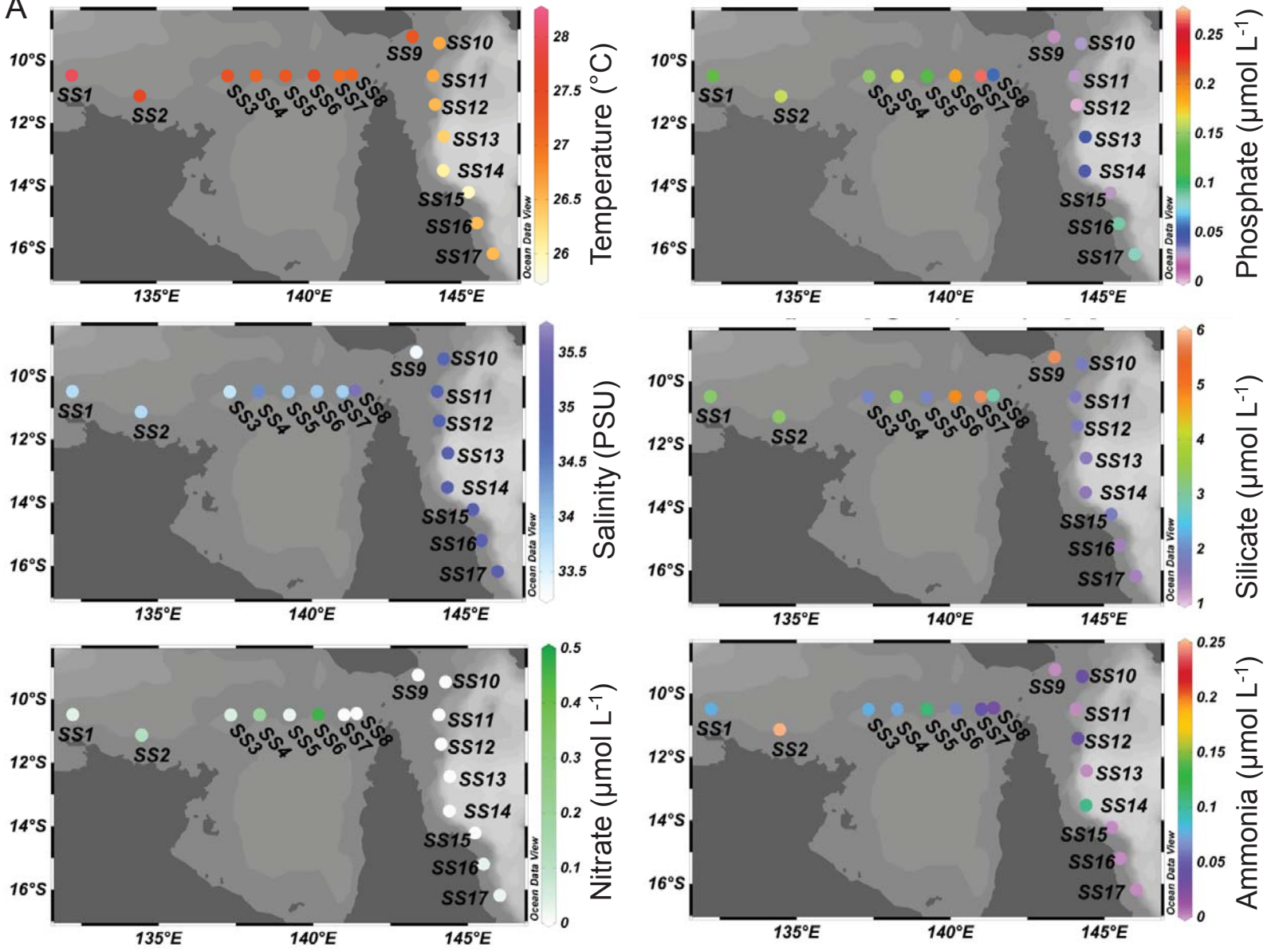
| Figure 4. Relative abundance of *nifH* transcripts detected in surface and chlorophyll maxima waters for spring (A & B respectively) and winter (C & D respectively) voyages.

810 | Figure 5. Distance based redundancy analysis constrained by the significant (P < 0.05) explanatory environmental variables for the observed variation in *nifH* composition within (A) DNA and (B) cDNA profiles in spring (S) and winter (W).

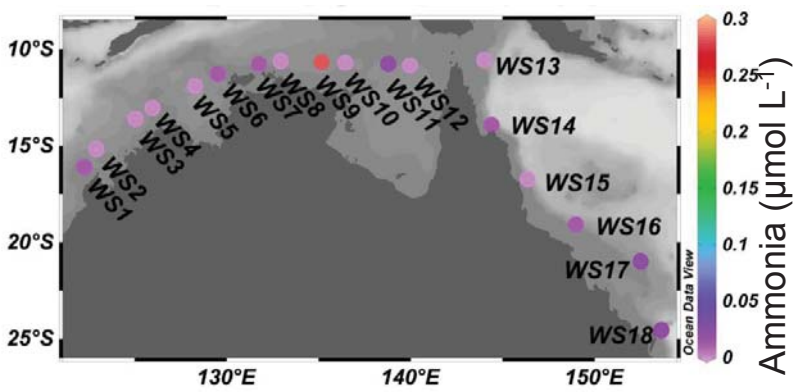
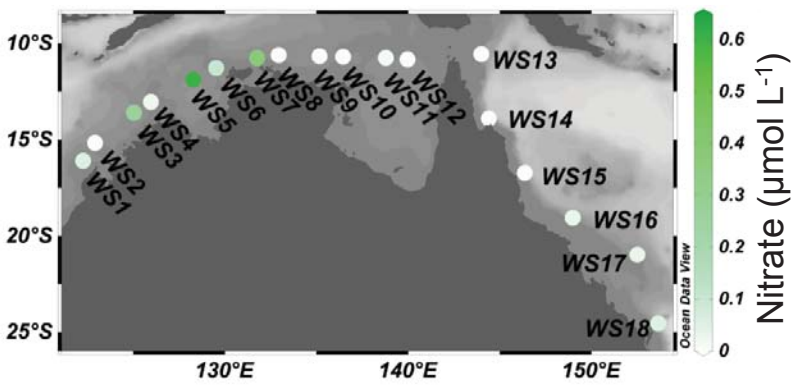
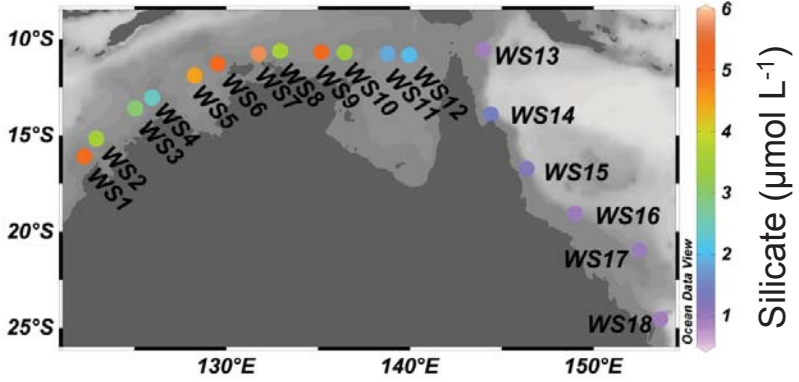
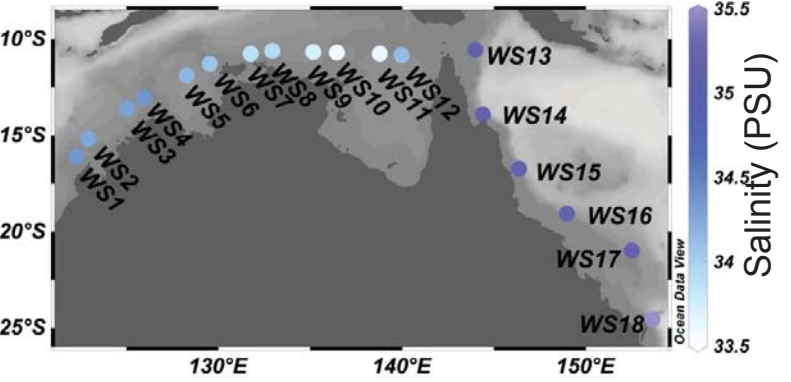
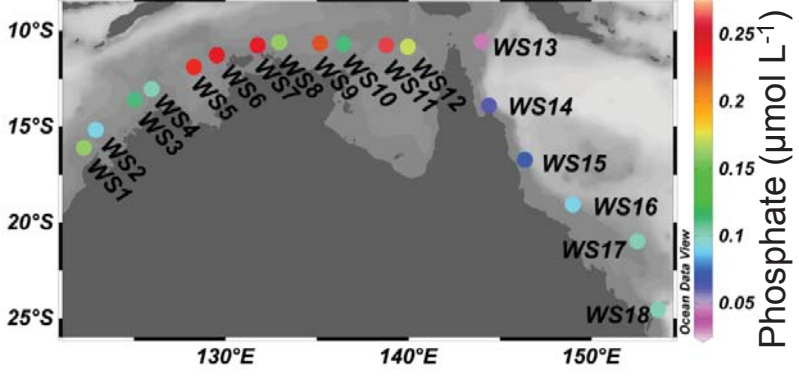
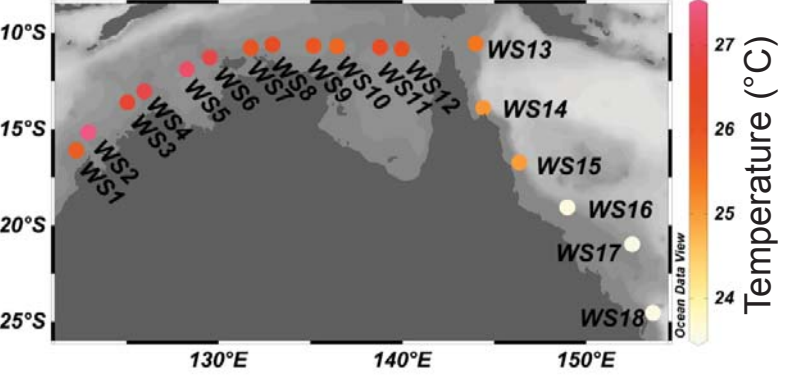
| Figure 6. Network analysis demonstrating significant (P < 0.01) associations only, between (A) phosphate concentrations and (B) silicate concentrations, and other environmental variables as well as the relative abundance of expressed *nifH* OTUs. In addition, significant (P < 0.01) associations between (C) OTU4713 of the γ -24774A11 clade and (D) OTU6352
815 | *Candidatus* Atelocyanobacterium thalassa (UCYN-A1), environmental data and the relative abundance of other expressed *nifH* OTUs are also shown. Circular nodes = *nifH* cDNA OTUs, the relative abundance is demonstrated by the relative size of the node; diamond

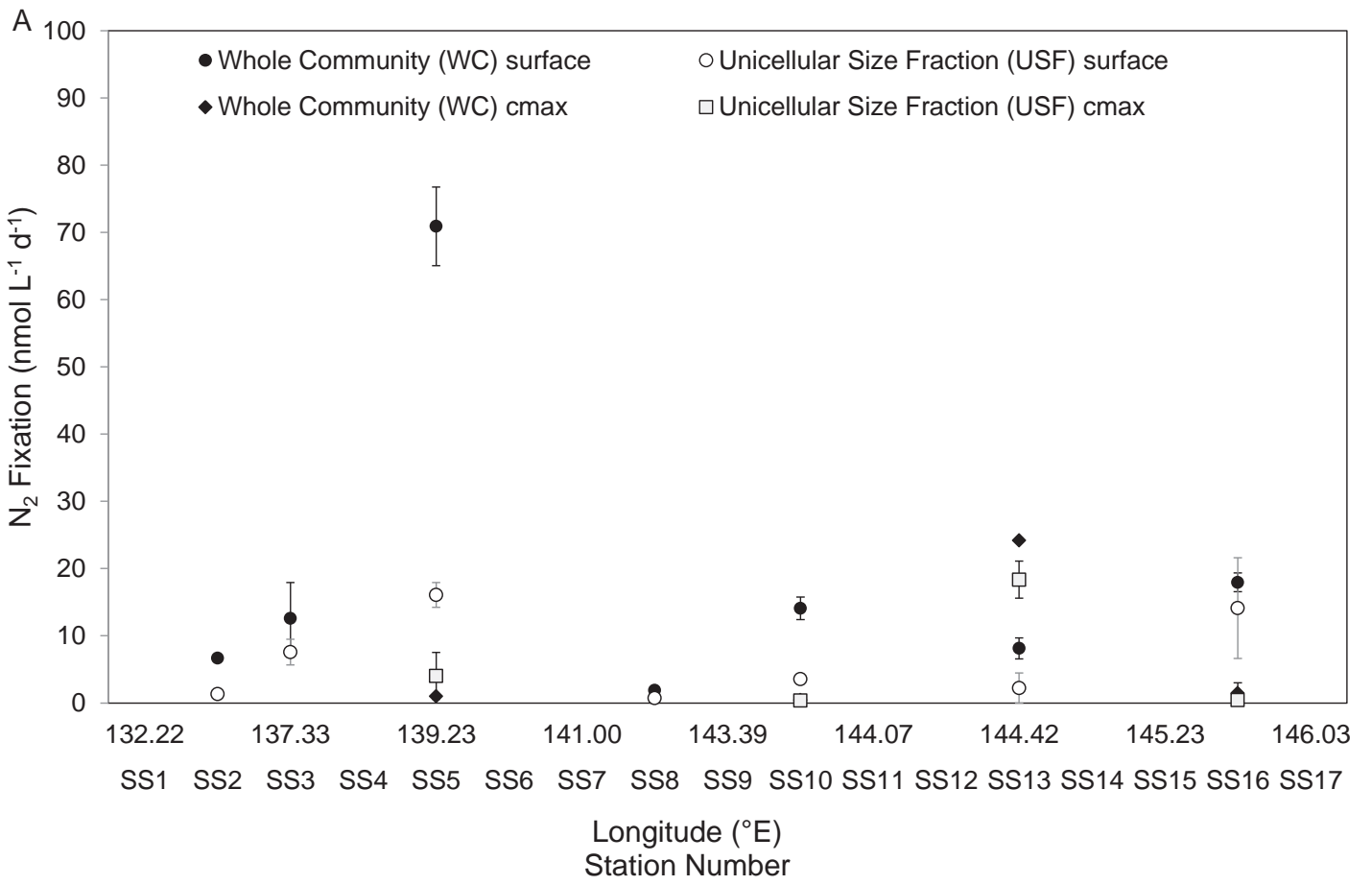
nodes = physical variables; square nodes = dissolved inorganic nutrients; hexagon nodes = photosynthetic pigments. Positive linear regressions between nodes are denoted by solid lines, negative linear regressions are dashed. Line colour represents the strength of the test statistic, MIC: high $> 0.8 < 1$ = blue; medium $> 0.6 < 0.8$ = green; low $> 0.47 < 0.6$ = black.

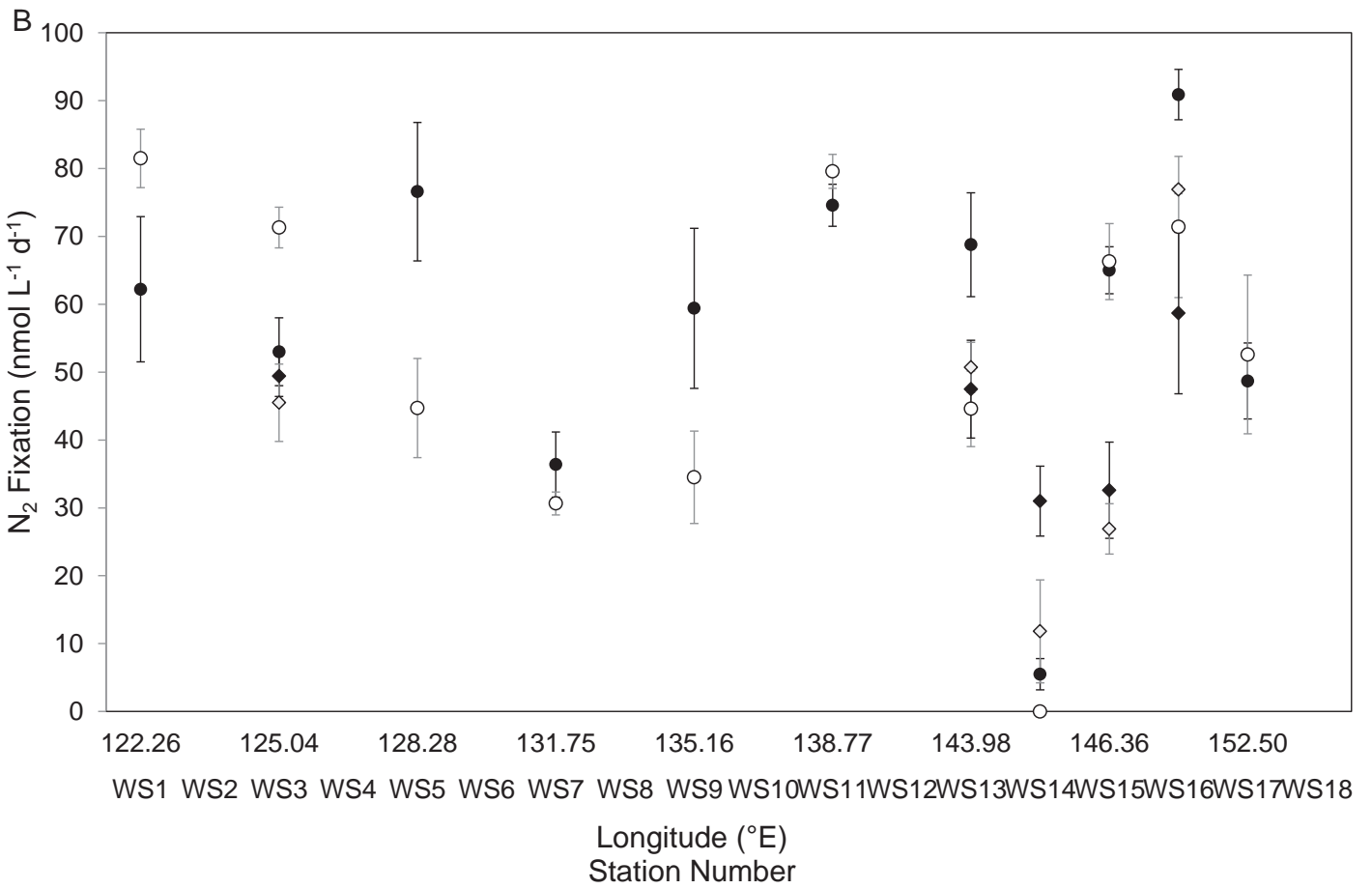
A



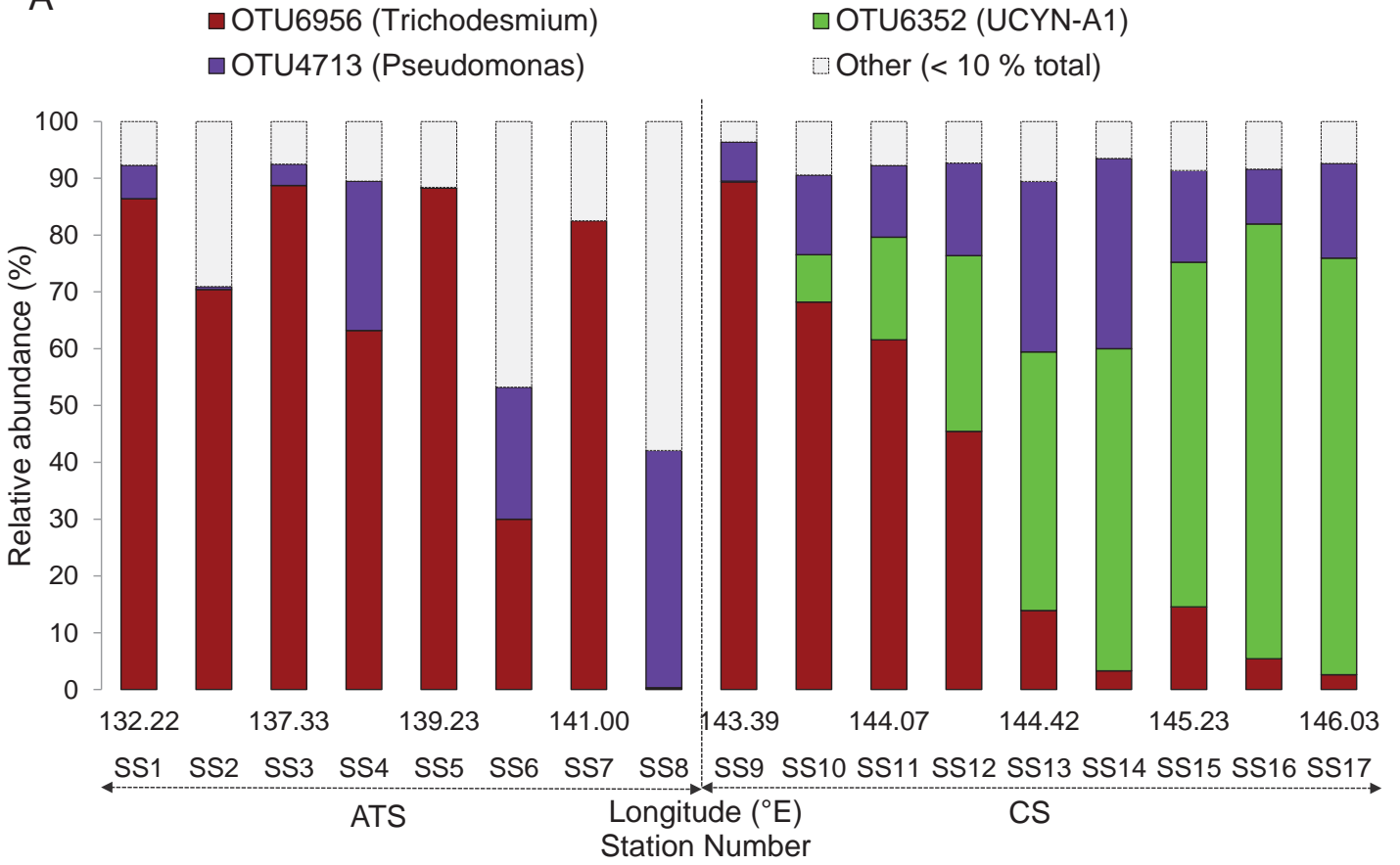
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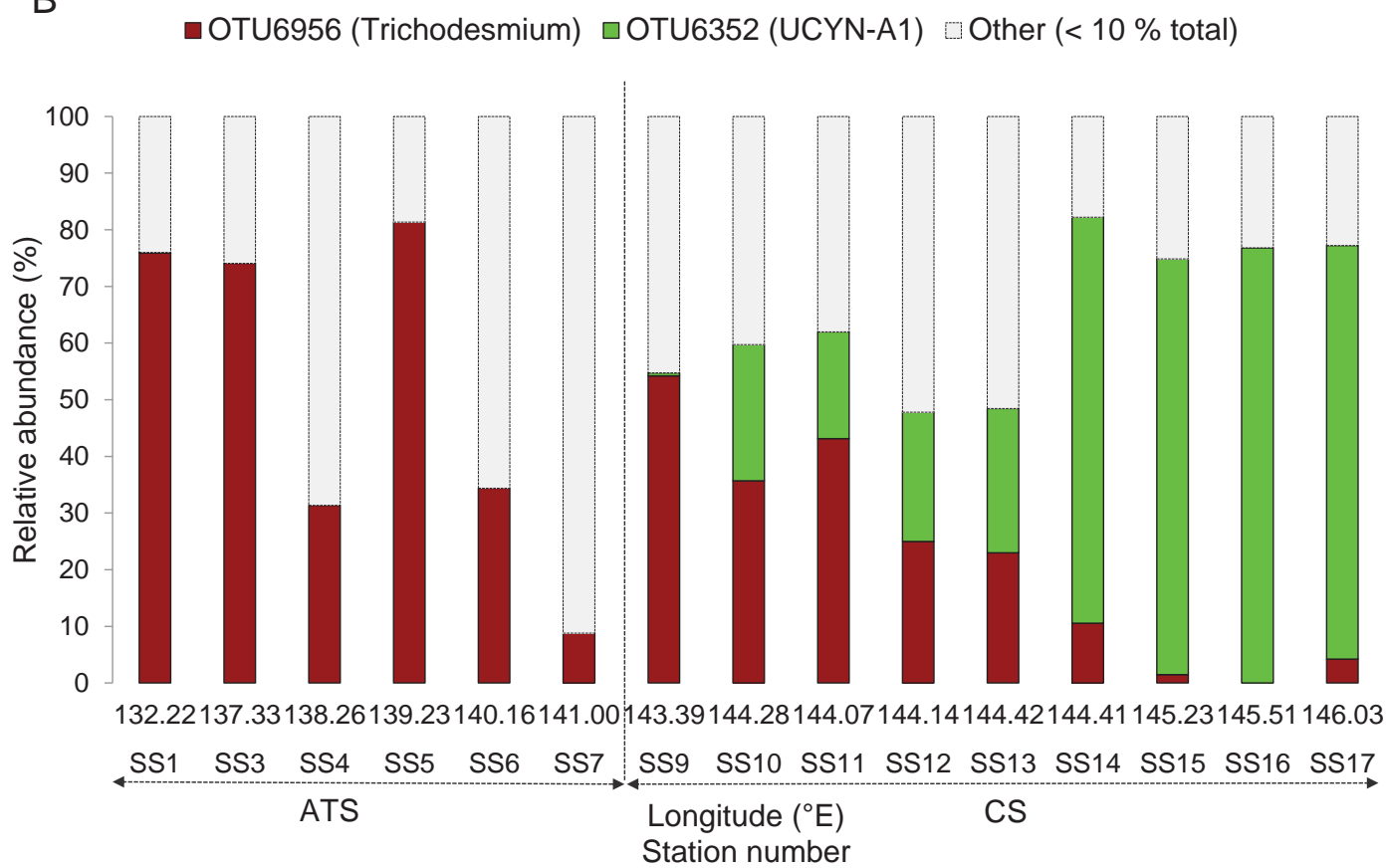




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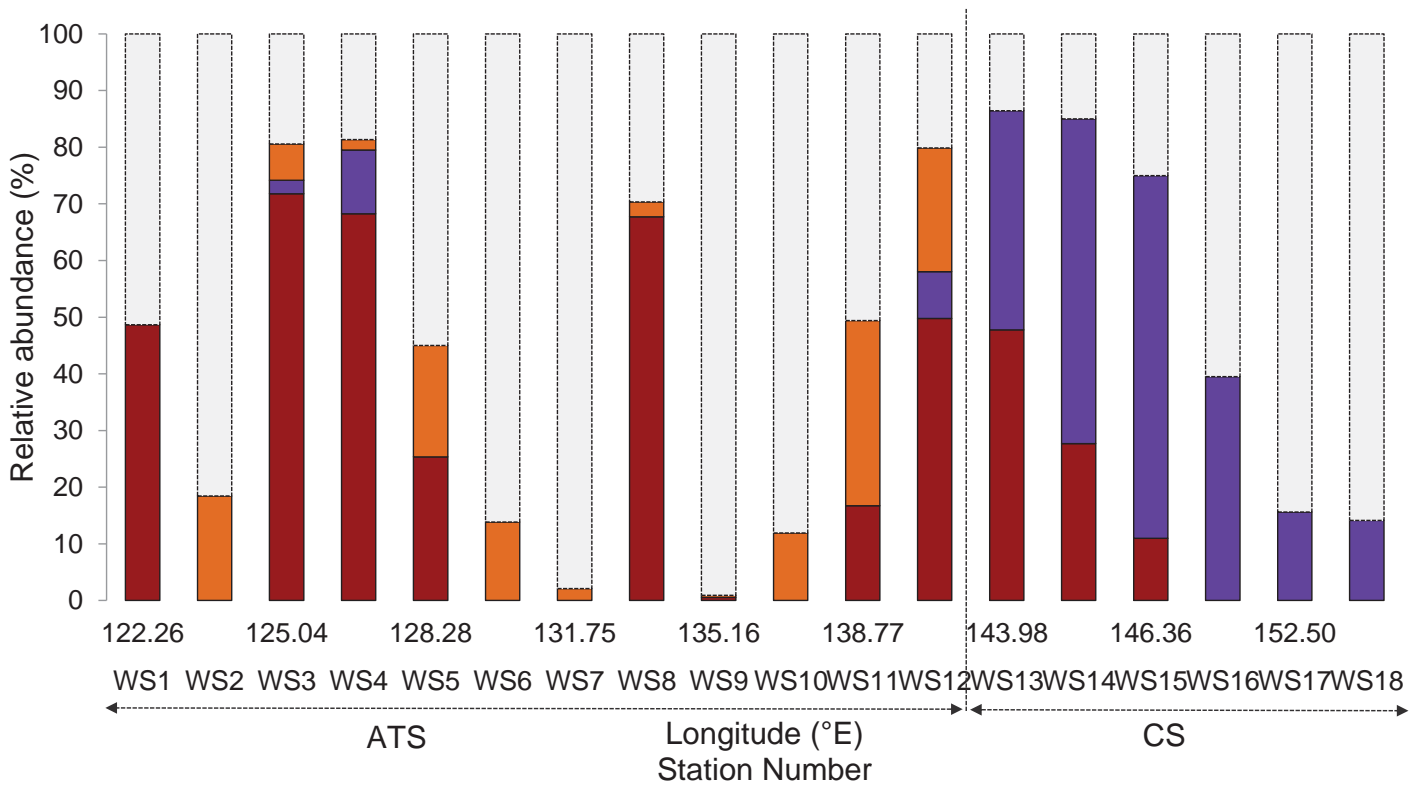


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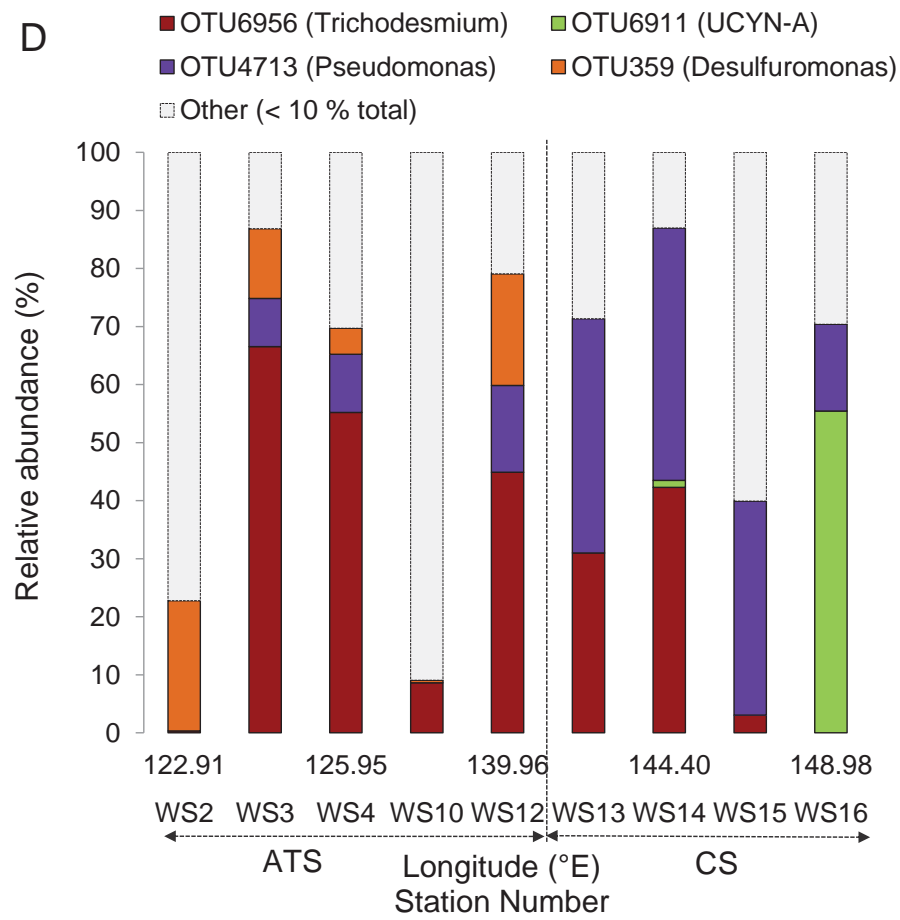


C

■ OTU6956 (*Trichodesmium*) ■ OTU4713 (*Pseudomonas*)
■ OTU359 (*Desulfuromonas*) □ Other (< 10 % total)

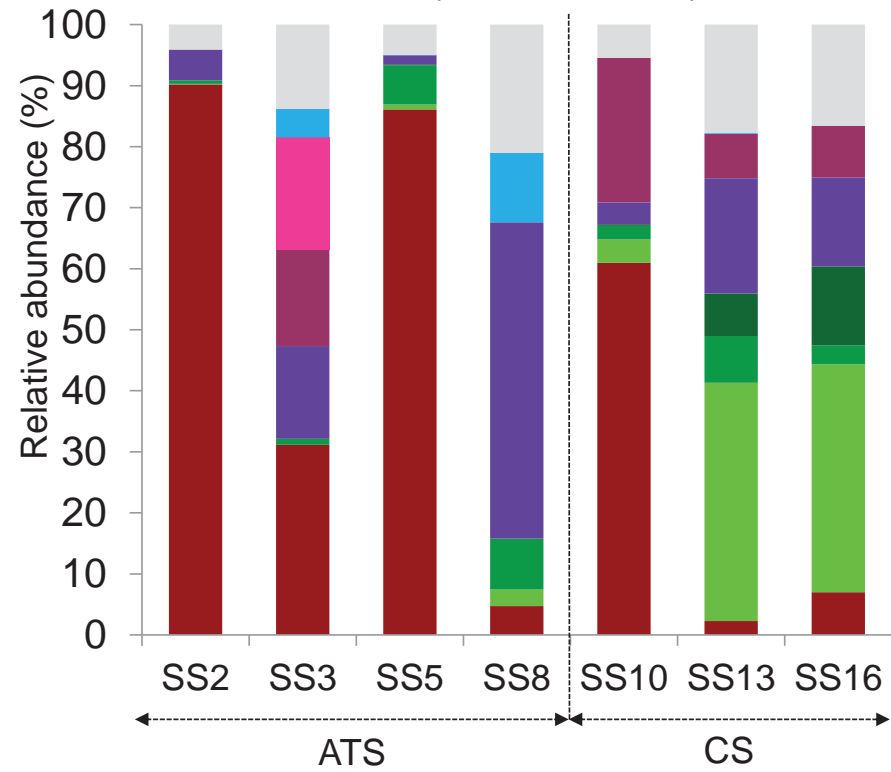


D



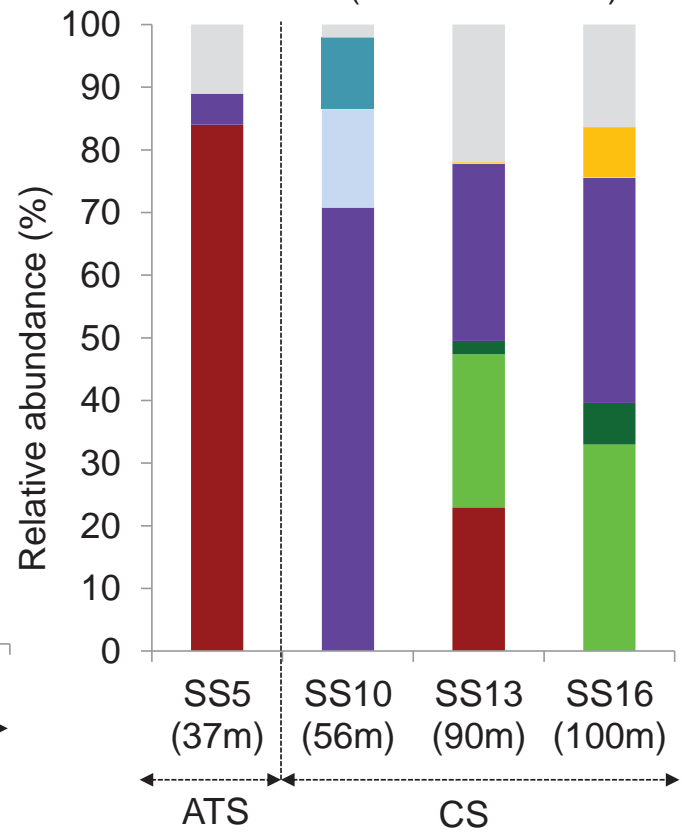
A

- Other (< 2% total)
- OTU7453 (Mastigocladus)
- OTU7434 (Crocospaera)
- OTU6863 (Pseudomonas)
- OTU4713 (Pseudomonas)
- OTU2020 (UCYN-A3)
- OTU83 (UCYN-A2)
- OTU6352 (UCYN-A1)
- OTU6956 (Trichodesmium)

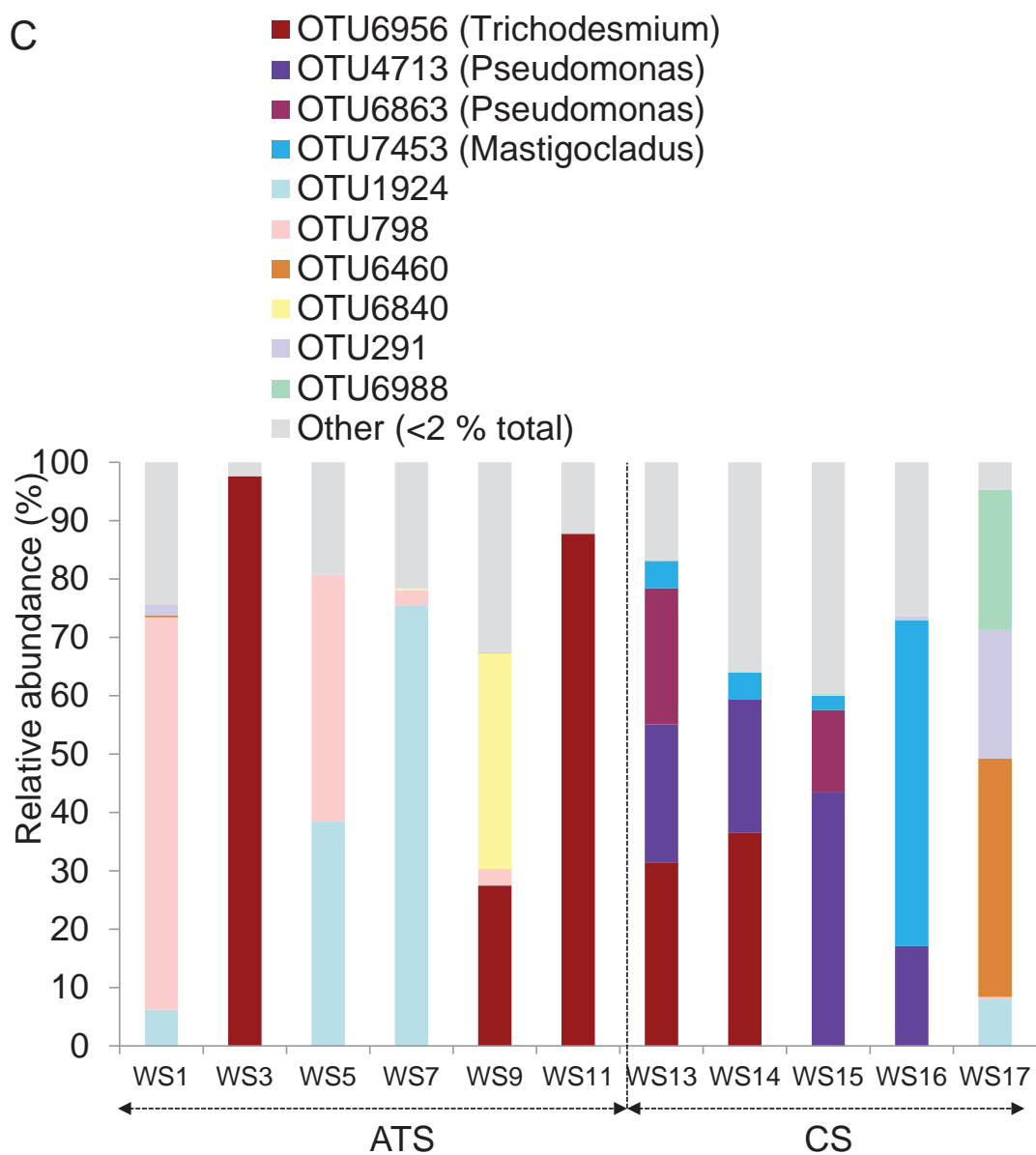


B

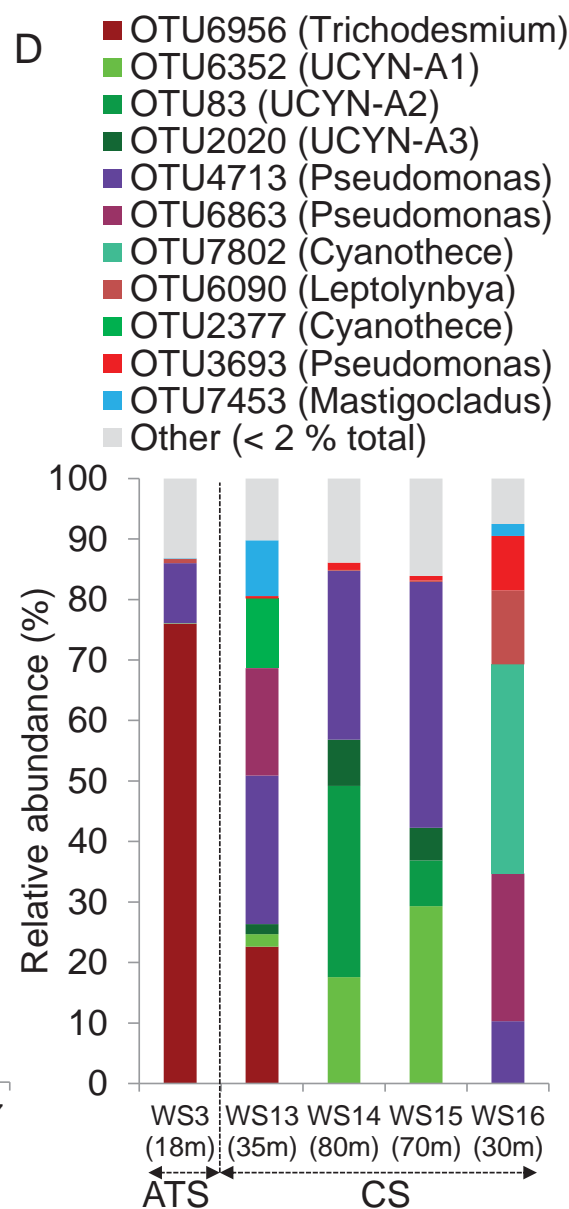
- Other (< 2 % total)
- OTU6460
- OTU4014
- OTU7728
- OTU4713 (Pseudomonas)
- OTU2020 (UCYN-A3)
- OTU6352 (UCYN-A1)
- OTU6956 (Trichodesmium)



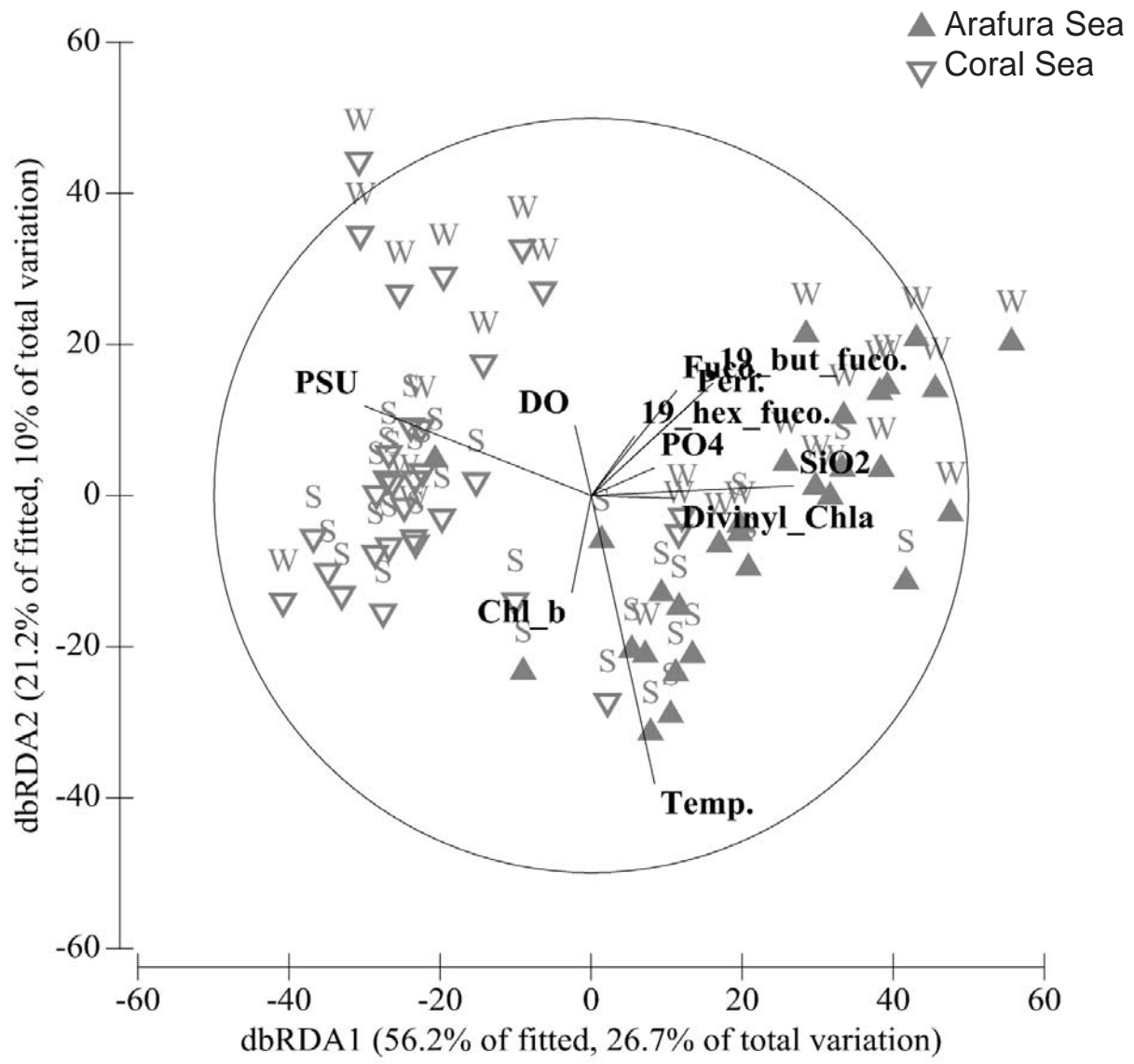
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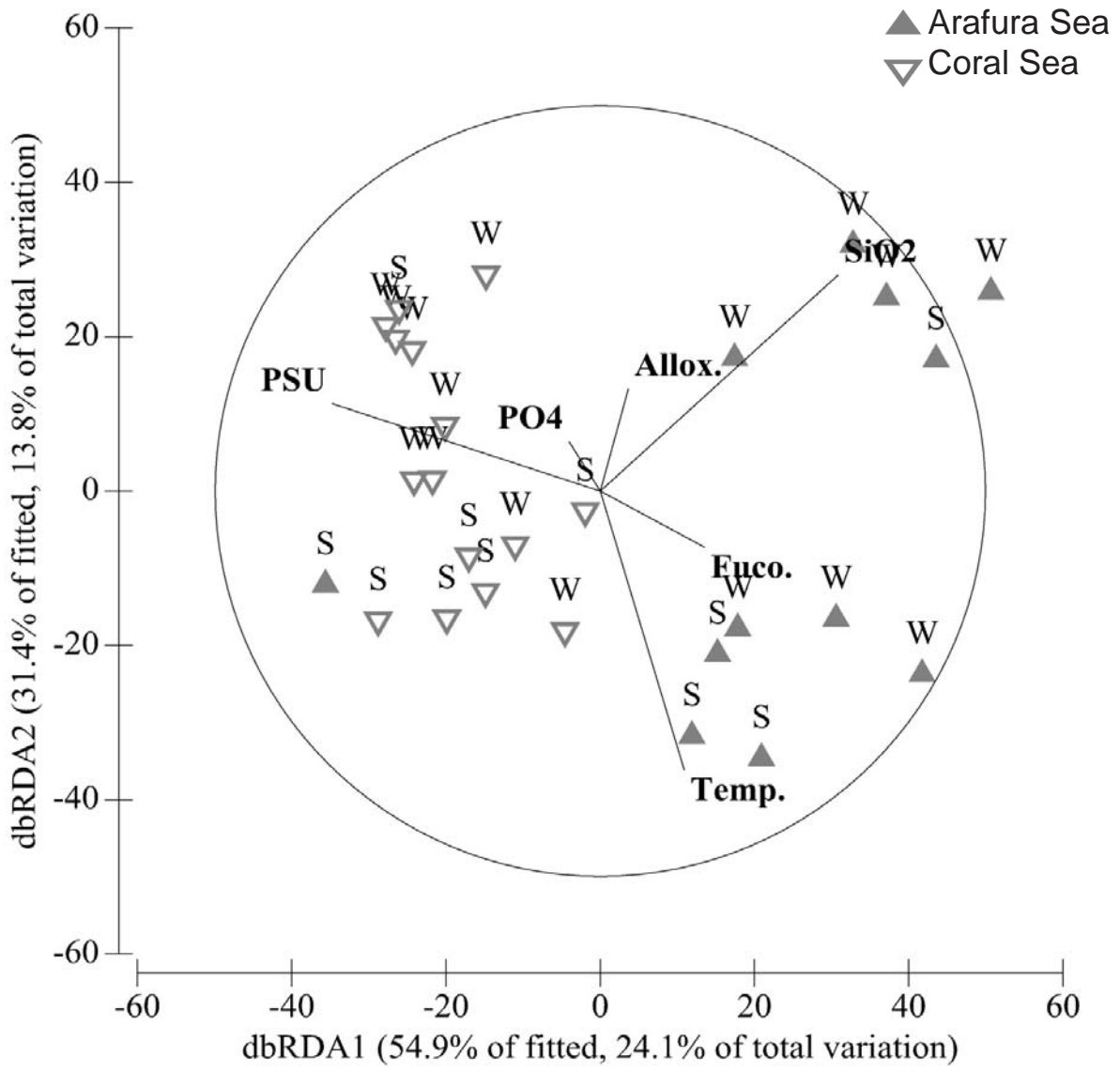
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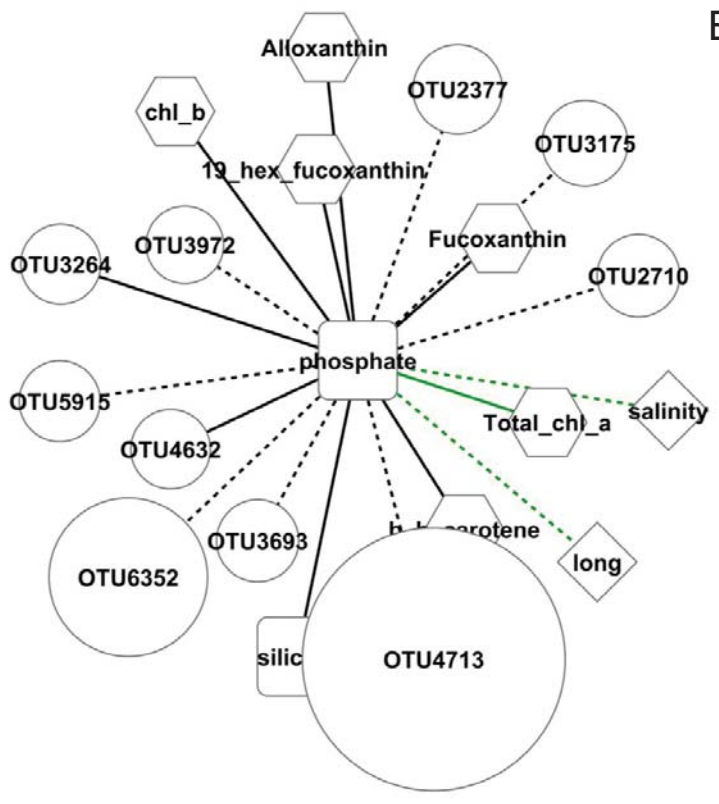
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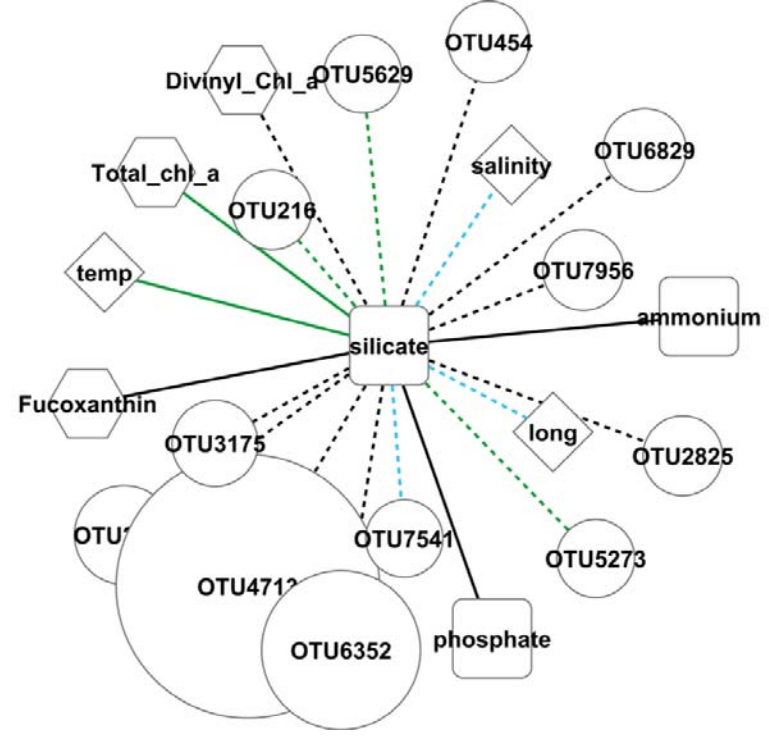
B



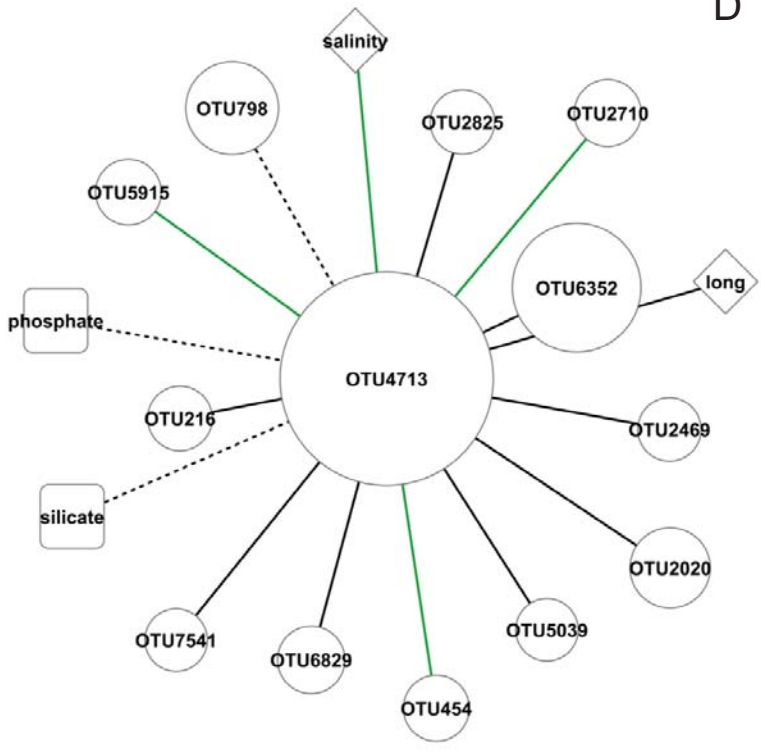
A



B



C



D

