Cyanobacterial Diazotroph Distributions in the Western South Atlantic

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Inputs of new nitrogen by cyanobacterial diazotrophs are critical to ocean ecosystem structure and function. Relative to other ocean regions, there is a lack of data on the distribution of these microbes in the western South Atlantic. Here, the abundance of six diazotroph phylotypes: *Trichodesmium*, *Crocosphaera*, UCYN-A, *Richelia* associated with *Rhizosolenia* (Het-1) or *Hemiaulus* (Het-2), and *Calothrix* associated with *Chaetoceros* (Het-3) was measured by quantitative PCR (qPCR) of the *nifH* gene along a transect extending from the shelf-break to the open ocean along the Vitória-Trindade seamount chain (1200 km). Using *nifH* gene copies as a proxy for phylotype abundance, *Crocosphaera* signals were the most abundant, with a broad distribution throughout the study region. *Trichodesmium* signals were the second most abundant, with the greatest numbers confined to the warmer waters closer to the coast, and a significant positive correlation with temperature. The average signals for the host-associated diazotrophs (UCYN-A, Het-1, and Het-2) were consistently lower than for the other phylotypes. These findings expand measurements of cyanobacterial diazotroph distribution in the western South Atlantic, and provide a new resource to enhance modeling studies focused on patterns of nitrogen fixation in the global ocean.

Keywords: *nifH* gene abundance, *Crocosphaera*, UCYN-A, diatom diazotroph association, diazotroph, western South Atlantic, *Trichodesmium*

INTRODUCTION

Diazotrophic cyanobacteria are critical for driving inputs of new nitrogen (N) into the oligotrophic ocean and supporting carbon fixation (Gruber and Galloway, 2008; Zhang et al., 2019), particularly where marine primary production is limited by nitrogen (Moore et al., 2013). However, the composition and distribution of diazotrophs, as well as their contribution to dinitrogen (N_2) fixation, in the western South Atlantic remain largely unknown, and this presents a gap in global nitrogen fixation estimates and hampers related modeling efforts (Coles and Hood, 2007; Sohm et al., 2011; Snow et al., 2015; Landolfi et al., 2018; Tang and Cassar, 2019; Wang et al., 2019).

Much of what is known about marine nitrogen fixation is based on studies from the North and South Pacific (e.g., Church et al., 2009; Benavides et al., 2016; Zhang et al., 2019; Zehr and Capone, 2020), and the tropical and subtropical regions of the North Atlantic (*e.g.*, Orcutt et al., 2001; Capone et al., 2005; Montoya et al., 2019; Tang et al., 2019; Palter et al., 2020), with comparatively few observations in the South Atlantic (e.g., Fernández et al., 2010; Sohm et al., 2011; Snow et al., 2015; Wasmund et al., 2015). Visible surface "slicks" of *Trichodesmium* have been observed in the southwestern Atlantic Ocean (Carvalho et al., 2008; Detoni et al., 2016), which are usually associated with warm surface water of the poleward-flowing Brazil Current (BC), and its meanders at the shelf-break region. Nitrogen fixation is thought to be largely attributable to *Trichodesmium* in this region (Moore et al., 2014), yet nitrogen fixation by unicellular diazotrophic cyanobacteria *Crocosphaera watsonii*, host-associated groups like UCYN-A, and diatom-diazotroph associations (DDAs) in other areas of the global ocean can be equal to or greater than those of *Trichodesmium* (Church et al., 2005a; Foster et al., 2007; Church et al., 2009; Fernández et al., 2010; Wu et al., 2018; Zehr and Capone, 2020).
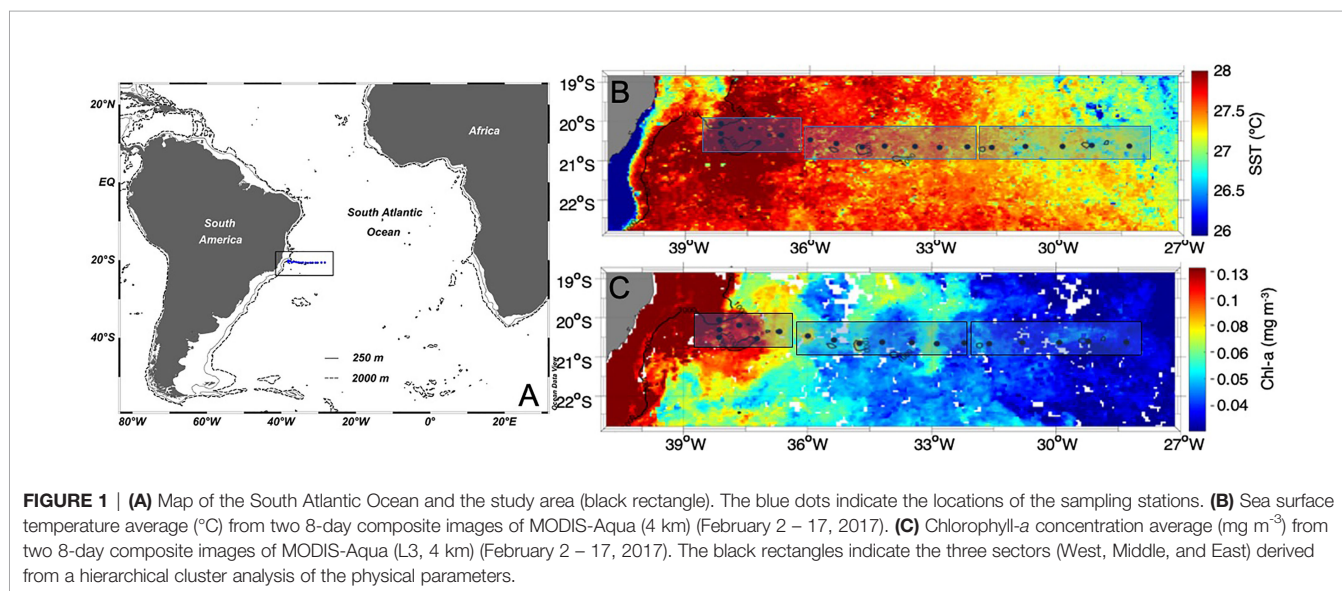
The distribution and abundance of diazotrophic cyanobacteria can be tracked with measurements of *nifH* gene copies for different phylotypes, among other approaches (e.g., Karlusich et al., 2021). Database compilations of diazotroph distributions (Luo et al., 2012; Tang and Cassar, 2019) provide critical resources for studying the factors which drive these organisms' distribution and for modeling nitrogen fixation. However, these observations are regionally skewed, and Tang and Cassar (2019) noted the limitations posed by the lack of observations in the South Atlantic. In the present study, we addressed this knowledge gap by examining diazotroph community structure in the western South Atlantic.

MATERIALS AND METHODS

Samples were obtained from 18 stations during a cruise aboard the R/V Alpha Crucis (February 2 – 17, 2017) along the Vitória-Trindade Ridge (21°S, 1200 km transect), extending from the continental slope to the western limb of the South Atlantic subtropical gyre (**Figure 1**). The first three stations were located in meridional positions, following the 1000 m isobath, and subsequent stations followed the deeper isobaths along approximately the same latitude (around 20°S).

Physical Parameters

Temperature, salinity, and fluorescence profiles were measured from the CTD downcast profiles (SeaBird CTD/Carrousel 911+system® with fluorescence sensor – *WetLabs ECO-AFL/FL*®) that were then used to compute the potential density of seawater and the mixed-layer depth (MLD). The MLD was calculated using the finite difference criteria method (Glover and Brewer, 1988, and Kara et al., 2000), with a temperature difference (dT) that best fit the station profiles (dT=0.1). Water column stability was estimated by the Brunt-Väisälä frequency (BV) ($\times 10^{-5}$ rad^2 s^{-2}), which considers the atmospheric gravity (m s^{-2}) and the potential density of seawater (kg m^{-3}). Sea surface temperature (SST) and chlorophyll a (Chl-a) concentration were derived from MODIS-Aqua satellite images (http://oceancolor.gsfc.nasa.gov). Sea Level Anomaly (SLA) products were obtained from Archiving, Validation, and Interpretation of Satellite Oceanographic Data (AVISO) (spatial resolution 1/8° × 1/8°). The water masses found in the study area were Tropical Water (TW) (Silveira et al., 2000), and West South Atlantic Central Water (WSACW) (Liu and Tanhua, 2019).

FIGURE 1 | (A) Map of the South Atlantic Ocean and the study area (black rectangle). The blue dots indicate the locations of the sampling stations. **(B)** Sea surface temperature average (°C) from two 8-day composite images of MODIS-Aqua (4 km) (February 2 – 17, 2017). **(C)** Chlorophyll-*a* concentration average (mg m^{-3}) from two 8-day composite images of MODIS-Aqua (L3, 4 km) (February 2 – 17, 2017). The black rectangles indicate the three sectors (West, Middle, and East) derived from a hierarchical cluster analysis of the physical parameters.

DNA Sampling, Extraction and qPCR Assays

Water samples were collected from the surface using a bucket, and sub-surface (10 m), DCM, and bDCM samples were collected with niskin bottles on the CTD rosette. Seawater (between 2.5 to 6 L) was filtered through polycarbonate filters (47 mm, 0.2 µm) (Millipore Sigma) using a peristaltic pump. Volumes were variable to keep the total filtration time to less than 2 h. Filters were flash frozen in liquid nitrogen and then stored at -80° C until extraction. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), with a minor modification to the lysis procedure. Lysis was performed by adding ~ 250 µL zirconium/silica beads (0.5 mm) to each sample with the lysis reagent, Buffer AP1. Extracted DNA was quantified spectrophotometrically using a Take3 micro-volume plate in a Synergy-H1 Hybrid Reader (Biotek).

The abundance of six nitrogen-fixing target phylotypes in the western South Atlantic were assayed by qPCR using primer and probe sequences for *nifH* (**Table S1**). This study targeted the unicellular diazotroph, *Crocosphaera watsonii* (also known as UCYN-B; Moisander et al., 2010); filamentous, *Trichodesmium* sp. (Church et al., 2005a); UCYN-A (Church et al., 2005a) and three diatom-diazotroph associations (DDAs), *Richelia* associated with *Rhizosolenia* (Het-1; Church et al., 2005b), *Richelia*-associated with *Hemiaulus* (Het-2; Foster et al., 2007), and *Calothrix*-associated with *Chaetoceros* (Het-3; Foster et al., 2007) (**Table S1**). All analyses were performed in triplicate in a CFX96 Touch Real-Time PCR detection system (Bio-Rad) in a total volume of 20 µL. The reaction mix contained 10 µL SsoAdvanced Universal Probes Supermix (Bio-Rad), 0.6 µM (final concentration) forward and reverse primers, 0.3 µM (final concentration) fluorogenic probe, and 6 µL DNA template. No template (water only) control samples were included in quadruplicate for all qPCR assays. Inhibition controls were included on all plates, where a 10^5 standard was added to one well of each sample to verify the quantitation cycle (Cq) values were the same as the 10^5 standard. When inhibition was detected, samples were re-run at a 1:10 dilution. Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15s followed by 60°C for 1 min. The *nifH* gene copy abundance for each phylotype was determined from the mean Cq value (n = 3), and standard curves (linear regression) generated for the appropriate primer and probe set. Each primer and probe set spanned 10^9 to 10^3 gene copies per reaction. The relationship between Cq values and gene copy number was linear from 10^3 to 10^9 target diazotrophs for all of the TaqMan primer and probe sets, and r^2 values for linear regressions were > 0.98. The standard curves were prepared from synthesized gBlocks gene fragments (IDT) of the target *nifH*. Amplification efficiencies were > 90% for all phylotype targets. Gene copy values were normalized by the sample volume to determine *nifH* copies L^{-1}.

Statistical Analyses

A hierarchical clustering analysis was applied (Euclidean distance and Ward method) (Legendre and Legendre, 1998) using surface physical variables (temperature, salinity, and SLA) to geographically sector the stations. Significant differences in *nifH* gene copy number were determined using the ANOVA with Tukey test (honestly significant difference – HSD) to compare all the averages across each depth. Pairwise Spearman rank correlations (Spearman's rho) were calculated between temperature and the *nifH* gene abundance for each phylotype.

RESULTS

Physical Parameters

The mixed layer depth (MLD) ranged from 20 to 66 m [average of 40 m (±10)], and the average BV above the MLD ranged from 0.1 to 2.7×10^{-5} rad^2 s^{-2}, indicating neutral stability of the water column (Pond and Pickard, 1983). Hierarchical clustering analysis divided the transect stations in this region of the western South Atlantic into three sectors, (West, Middle, and East) with temperature and salinity as follows: West [28.3°C (± 0.1), 37.5 (± 0.05), n = 6), Middle [27.6°C (± 0.2), 37.5 (± 0.09), n = 7], and East [27.3°C (± 0.2), 37.45 (± 0.05), n = 5]. Average temperature was significantly different between sectors (Tukey test (HSD), *p*-value < 0.02). Temperatures were highest in the West sector, likely associated with the influence of Brazil Current (BC) water (Silveira et al., 2000) (**Figure 2** and **Figure S1**).

Diazotroph Community Composition

The *nifH* gene copy number can be used as a proxy for the relative abundance of each phylotype, providing data on community composition. Recent studies have highlighted some of the potential caveats associated with this approach, including the lack of *nifH* genes in members of a genus previously considered to all be diazotrophic (Delmont, 2021), and the presence of "pseudo"-*nifH* genes in microbial genomes (primarily anaerobes) that do not carry the full complement of genes for nitrogen fixation (Mise et al., 2021). However, the approach applied here has been extensively validated (e.g. **Table S1**) and broadly applied for decades (Tang and Cassar, 2019; Karlusich et al., 2021), which makes its application valuable despite the potential caveats.

In the mixed layer (surface and 10 m samples), the diazotroph community in this dataset was dominated by *nifH* gene copies belonging to the unicellular diazotroph *Crocosphaera watsonii* (**Table 1**). In fact, comparing the entire transect average at each depth for each phylotype, the *Crocosphaera nifH* gene copies were significantly higher than the other phylotypes at each depth (*p*-value < 0.001) (**Table 1**). The abundance of *Trichodesmium nifH* gene copies varied from below the detection limit (BDL) to 209×10^3 *nifH* copies L^{-1} with the highest average abundances also observed in the mixed layer (**Table S2**; **Figure 3**). The average transect *nifH* gene copy number from host-associated diazotrophs (Het-1, Het-2, and UCYN-A) was consistently lower than that from the free-living diazotrophs in the mixed layer (**Table 1**). The UCYN-A *nifH* gene copies ranged from BDL to 63×10^3 *nifH* copies L^{-1} (**Table S2**). The Het-1 and Het-2 *nifH* gene copy

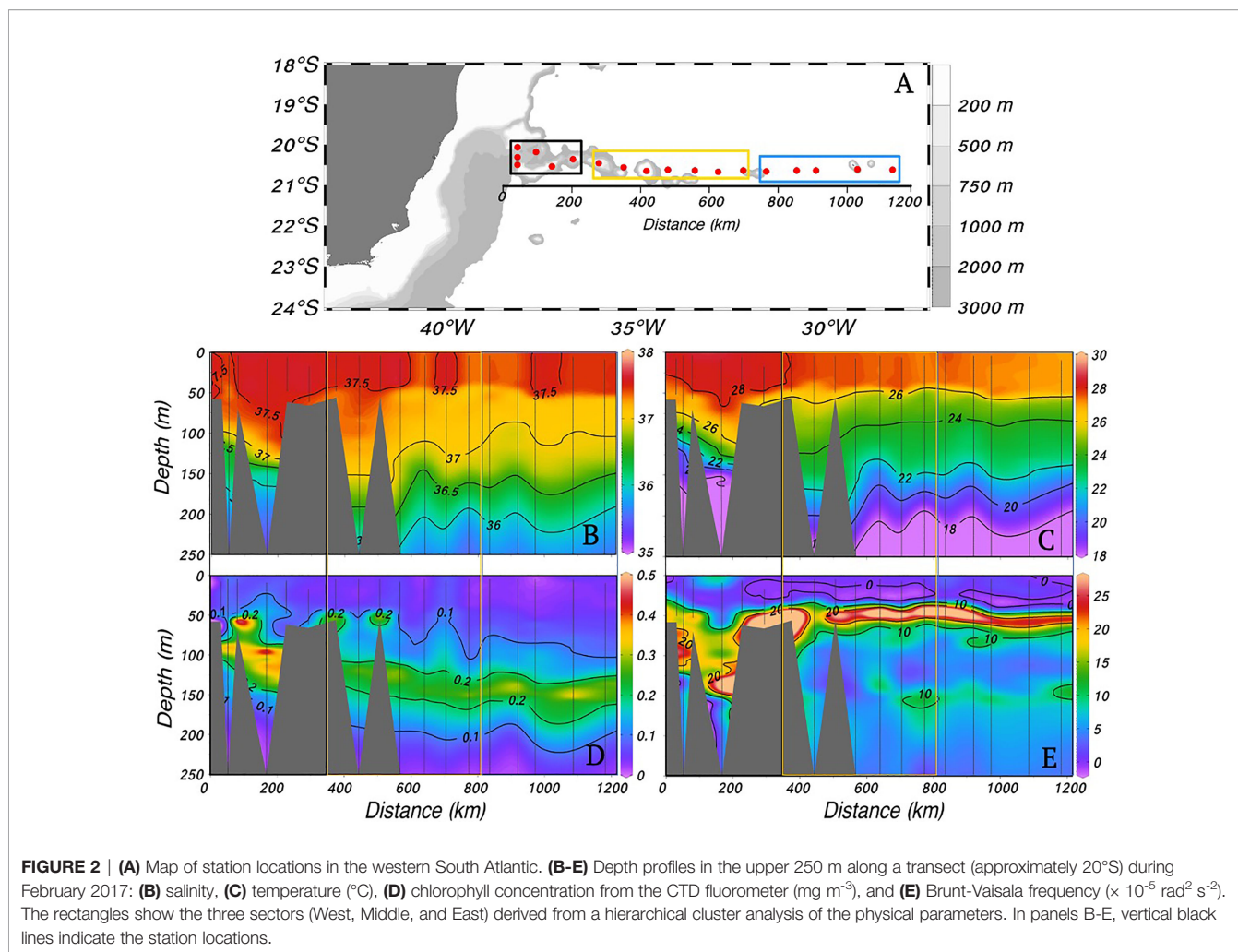**FIGURE 2 |** **(A)** Map of station locations in the western South Atlantic. **(B-E)** Depth profiles in the upper 250 m along a transect (approximately 20°S) during February 2017: **(B)** salinity, **(C)** temperature (°C), **(D)** chlorophyll concentration from the CTD fluorometer (mg m^{-3}), and **(E)** Brunt-Vaisala frequency (× 10^{-5} rad^2 s^{-2}). The rectangles show the three sectors (West, Middle, and East) derived from a hierarchical cluster analysis of the physical parameters. In panels B-E, vertical black lines indicate the station locations.

TABLE 1 | The nifH abundances (× 10^3 copies L-1 ± standard deviation) for each nitrogen-fixing phylotype (*Trichodesmium*, *Crocosphaera*, UCYN-A, Het-1 and Het-2).

		Trichodesmium	*Crocosphaera*	**UCYN-A**	**Het-1**	**Het-2**
WEST (n = 6)	Surf	63.3 (± 37)	70 (± 56.4)	0.02 (± 0.04)	0.13 (± 0.23)	1.72 (± 1)
	10m	69.3 (± 73)	78 (± 125)	BDL	0.02 (± 0.06)	0.8 (± 0.5)
	DCM	4.4 (± 7.3)	17.5 (± 18.5)	2.16 (± 2.5)	0.14 (± 0.2)	0.13 (± 0.1)
	bDCM	7.3 (± 9)	24.3 (± 18.6)	4.7 (± 6.7)	0.5 (± 0.8)	0.2 (± 0.2)
MIDDLE (n = 7)	Surf	4 (± 8)	128 (± 130)	4 (± 5.3)	0.43 (± 0.9)	0.8 (± 0.8)
	10m	4.4 (± 9.4)	121.8 (± 149)	2.2 (± 2.2)	0.5 (± 0.6)	0.6 (± 0.7)
	DCM	0.5 (± 0.8)	1 (± 0.4)	BDL	BDL	0.07 (± 0.1)
	bDCM	2 (± 5.4)	10.5 (± 18.3)	12.8 (± 24)	7 (± 16)	0.6 (± 0.8)
EAST (n = 5)	Surf	1.5 (± 2.5)	96 (± 28)	5.3 (± 3)	0.8 (± 1)	2.5 (± 3)
	10m	1 (± 1.2)	106 (± 49)	4.6 (± 2.5)	1.3 (± 1)	2.4 (± 3.5)
	DCM	BDL	0.7 (± 0.4)	BDL	0.6 (± 0.9)	0.6 (± 1.2)
	bDCM	BDL	0.6 (± 0.2)	6 (± 6.7)	9.5 (± 14.5)	0.9 (± 1.3)
TRANSECT (n = 18)	Surf	23 (± 36)[a]	100 (± 88)[b]	3 (± 4)	0.43 (± 0.8)[c]	1.6 (± 1.9)
	10m	25 (± 51)[a]	102 (± 116)[b]	2 (± 2.6)	0.6 (± 0.8)[c]	1.2 (± 2)
	DCM	1.3 (± 3.5)[a]	4.6 (± 10.5)[b]	0.5 (± 1.4)	0.2 (± 0.5)[c]	0.2 (± 0.6)
	bDCM	3 (± 6.5)	10.4 (± 17)[a]	5 (± 8.4)	6 (± 13)	0.6 (± 0.9)

Average values for each phylotype at each depth (surface (Surf), 10 m, DCM and bDCM) are reported for sectors (West, Middle, East) and the transect as a whole, combining all sectors (Transect). Significance (p > 0.001) is noted for the average transect nifH copy number for each phylotype at each depth. Het-3 was not detected in any sample along the 18 station transect.
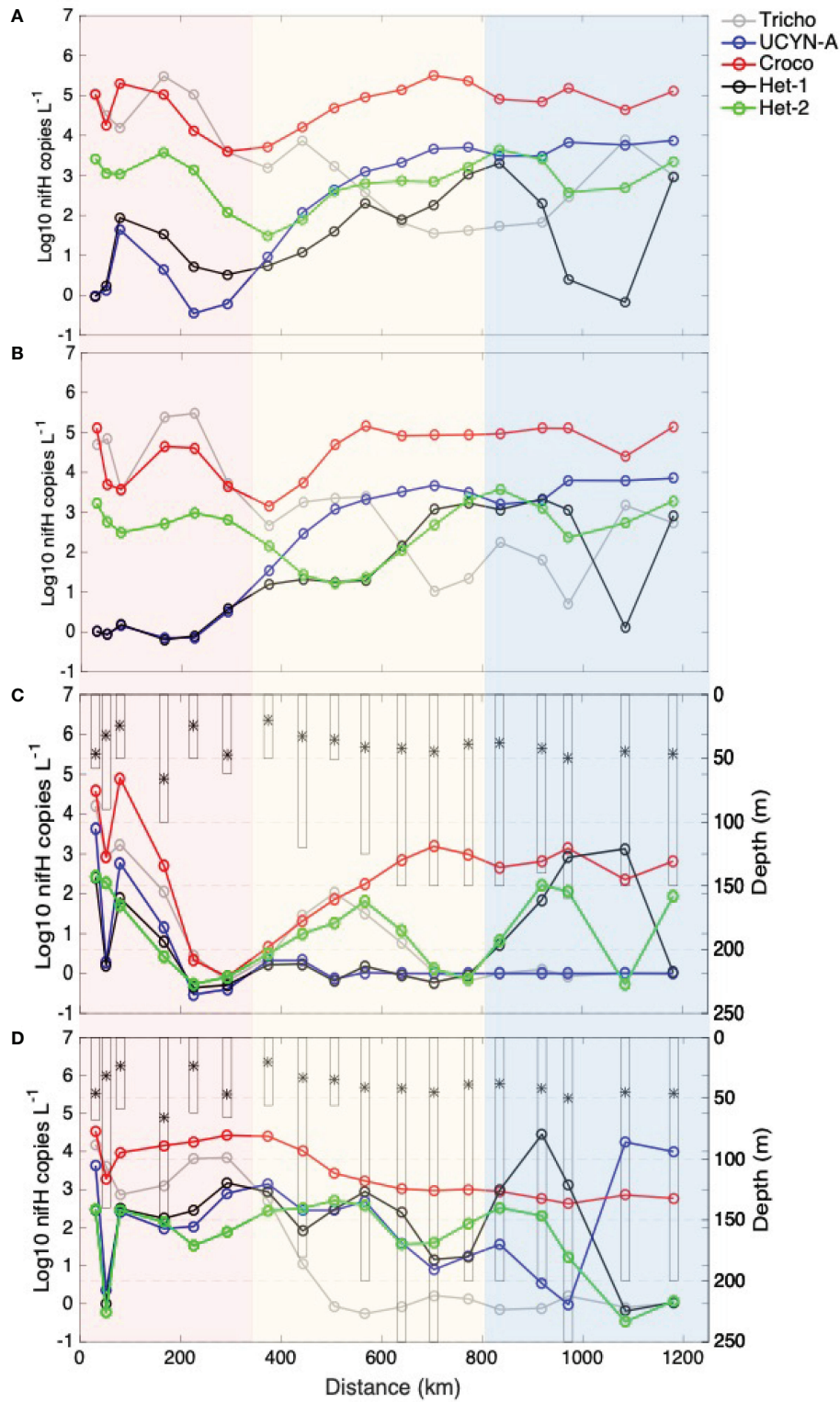
FIGURE 3 | Spatial distribution of *nifH* gene copies for each nitrogen-fixing phylotype [log10 (copies L⁻¹)]: *Trichodesmium* (Tricho) (grey), UCYN-A (blue), *Crocosphaera* (Croco) (red), Het-1 (black), and Het-2 (green), along the sampled transect through the three sectors: West (shaded in pink), Middle (shaded in yellow), and East (shaded in blue) derived from a hierarchical cluster analysis of the physical parameters. Data from each sample depth are plotted as follows: **(A)** surface, **(B)** 10 m, **(C)** DCM, where the DCM depths for each station are shown with bars **(D)** bDCM, where the bDCM depths for each station are shown with bars. The asterisk (*) denotes the mixed layer depth.

number in the dataset (all depths and stations) ranged from BDL to 42.6×10^3 *nifH* copies L^{-1} and BDL to 8.6×10^3 *nifH* copies L^{-1}, respectively (**Table S2**). Het-3 *nifH* genes were not detected in any sample. The average *nifH* gene copy number decreased with depth in all groups except Het-1 and Het-2 (**Table 1**; **Figure 3**). While the concentration of *nifH* genes for these two host-associated groups was low and variable overall, the average *nifH* gene copy number was highest in bDCM samples (**Table 1**; **Figure 3**). The *Trichodesmium* (Spearman's rho ≥ 0.74 p > 0.001), *Crocosphaera* (Spearman's rho ≥ 0.57 p > 0.001), and Het-2 (Spearman's rho ≥ 0.43 p > 0.001) phylotype abundances significantly positively co-varied with temperature.

DISCUSSION

Recent synthesis and modeling efforts (Tang and Cassar, 2019; Karlusich et al., 2021) have highlighted that diazotroph distribution and community structure are under-sampled in the South Atlantic. Herein, we addressed this knowledge gap with a western South Atlantic survey of *nifH* genes representing six diazotroph phylotypes. The transect extended 1200 km, roughly along 20°S, traversing three sectors (West, Middle and East). These sectors were distinguished by temperature, which was significantly higher in the West relative to the other sectors. This likely reflects the influence of tropical water associated with the BC. Temperature significantly positively co-varied with the *Trichodesmium*, *Crocosphaera* and Het-2 *nifH* gene copy abundances, and average *Trichodesmium* *nifH* gene copies were highest in the West sector. Water temperature is a well-known factor controlling the distribution of diazotrophs (Luo et al., 2014), and typically their distribution has been thought to be largely restricted to warm, stratified, high solar radiation regions in the tropics and subtropics.

The average *nifH* gene distribution along the transect was dominated by *Crocosphaera* signals, and the average mixed layer *nifH* gene copies (~100×10^3 *nifH* copies L^{-1}) were in the general range of *Crocosphaera* *nifH* gene abundance found in other regions such as the North Pacific (10^3 to 10^4 *nifH* copies L^{-1}; Church et al., 2005a) and the western tropical South Pacific (10^2 to 10^6 *nifH* copies L^{-1}; Stenegren et al., 2018). Recent imaging work by Karlusich et al. (2021) identified *Crocosphaera* sp. that were clustered together, or associated with large diatoms (e.g., *Climacodium*), in addition to solitary cells. This highlights the potential for disparate ecologies within the phylotype which may, in part, support the broad distribution observed here. The apparent dominance of the *Crocosphaera* phylotype in this dataset suggests that it is an important contributor to nitrogen fixation in this region. However, the activity of the phylotypes cannot be accurately extrapolated from these results.

The average mixed layer *Trichodesmium* *nifH* gene copies were the second most abundant on the transect (~20×10^3 *nifH* copies L^{-1}). This average is within the range observed in other systems such as the western tropical South Pacific (10^2 to 10^6 *nifH* gene copies L^{-1}; Stenegren et al., 2018). The distribution of *Trichodesmium* is known to be highly variable, ranging from concentrations so high they are visible in pigmented surface

slicks to non-detectable in this region (Lima et al., 2019). Here, the concentration of *Trichodesmium* *nifH* gene copies was significantly positively correlated with temperature, with a stronger correlation coefficient than that observed for *Crocosphaera*. *Crocosphaera* occupies a broader temperature range (15-30°C) than *Trichodesmium* (Fu et al., 2014), which might, in part, underpin the differences in the distribution of these two phylotypes in this study.

A recent study identified the widespread occurrence of a non-diazotrophic *Trichodesmium* species reconstructed from a global metagenomic survey (Delmont, 2021). By tracking *nifH* genes, this, and other studies that use this approach, may under-count *Trichodesmium* spp. Although the presence of the *nifH* gene for this phylotype assay suggests these *Trichodesmium* species have the ability to fix nitrogen, we can't exclude the possibility that some portion of the *Trichodesmium* population lacks the full complement of genes required for nitrogen fixation. Thus, the values reported here could be an underestimate of *Trichodesmium* abundance.

The host-associated diazotrophs (UCYN-A, Het-1, and Het-2) had lower average abundances of *nifH* genes than *Crocosphaera* or *Trichodesmium* in the mixed layer. Although UCYN-A is increasingly recognized as a widely-distributed and abundant diazotroph (Martínez-Pérez et al., 2016), the UCYN-A *nifH* gene copy number observed here was not the dominant signal. This pattern is consistent with a study by Stenegren et al. (2018) in the western tropical South Pacific, where UCYN-A1 and A2 ecotypes were low to non-detectable and the system was dominated by *Trichodesmium* and *Crocosphaera* signals. Also, Karlusich et al. (2021) found low abundances of the UCYN-A *nifH* gene in the South Atlantic Ocean, making up only 3-4% of the bacterioplankton population.

The low copy number, or lack of detection, of *nifH* genes from the other host-associated diazotrophs *Richelia* (Het-1 and Het-2) and *Calothrix* (Het-3) may be, in part, driven by the silica requirement and physiological ecology of their diatom hosts. These DDAs are often observed in the transitional waters of river plumes (Foster et al., 2009; Montoya et al., 2019), although they can be observed in other open ocean systems (Karl et al., 2012; Stenegren et al., 2018). Overall, the low abundances of Het-1 *nifH* gene copies (10^2-10^3 *nifH* gene copies L^{-1}) among samples over the transect were consistent with studies from the North Atlantic, which have generally found low abundances of Het-1 (~10^2 *nifH* copies L^{-1}) (Benavides et al., 2016), and are in the range of Het-1 *nifH* gene copies found in the western tropical South Pacific (10^1-10^5 *nifH* gene copies L^{-1}). We note that the lack of Het-3 *nifH* gene copies detected in this study is also consistent with the findings of previous work in the South Pacific and North Atlantic where Het-3 signals were low or non-detectable (Foster et al., 2007; Stenegren et al., 2018). In sum, the community composition of *nifH* phylotypes observed here is in the range of other systems, but requires more information across a broader swath of the South Atlantic to determine whether the patterns observed here are representative of this region.

A recent study by Tang and Cassar (2019) used a broad compilation of *nifH* phylotype data to model and then extrapolate phylotype distribution in the global ocean. They

predicted that the western South Atlantic would transition from *Trichodesmium* dominance to UCYN-A dominance at roughly 30°S, but acknowledged the lack of data for this region (Tang and Cassar, 2019). With the dominance of the *Crocosphera nifH* phylotype, the observations in this study do not support this model output, but are limited in number. Expanding on the data presented here with a larger set of observations and related chemical and physical data would be valuable for future synthesis and modeling efforts.

Here we observed mixed diazotrophic assemblages with the distribution of colonial, free-living, and host-associated forms along the transect. Tracing diazotroph abundance, and the factors that drive both abundance and community structure, are critical to building robust models of marine nitrogen and carbon cycling. This is only becoming more pressing in a changing ocean. The data presented herein provides valuable information on the composition and abundance of the diazotrophic community in an under-sampled region in the western South Atlantic. Further expanding studies such as this in the South Atlantic would help to validate models of diazotroph biogeography and their effects on ecosystem processes such as primary production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AMSD collected and processed samples. AMSD and AS analyzed the data. AMSD and PHRC contributed to the study implementation. STH and STD supported and advised the molecular methods. All authors discussed the results and contributed to the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.856643/full#supplementary-material

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