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In Situ Diazotroph Population Dynamics Under Different Resource Ratios in the North Pacific Subtropical Gyre

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Major advances in understanding the diversity, distribution, and activity of marine N2-fixing microorganisms (diazotrophs) have been made in the past decades, however, large gaps in knowledge remain about the environmental controls on growth and mortality rates. In order to measure diazotroph net growth rates and microzooplankton grazing rates on diazotrophs, nutrient perturbation experiments and dilution grazing experiments were conducted using free-floating in situ incubation arrays in the vicinity of Station ALOHA in March 2016. Net growth rates for targeted diazotroph taxa as well as Prochlorococcus, Synechococcus and photosynthetic picocrykaryotes were determined under high (H) and low (L) nitrate:phosphate (NP) ratio conditions at four depths in the photic zone (25, 45, 75, and 100 m) using quantitative PCR and flow cytometry. Changes in the prokaryote community composition in response to HNP and LNP treatments were characterized using 16S rRNA variable region tag sequencing. Microzooplankton grazing rates on diazotrophs were measured using a modified dilution technique at two depths in the photic zone (15 and 125 m). Net growth rates for most of the targeted diazotrophs after 48 h were not stimulated as expected by LNP conditions, rather enhanced growth rates were often measured in HNP treatments. Interestingly, net growth rates of the uncultivated prymnesiophyte symbiont UCYN-A1 were stimulated in HNP treatments at 75 and 100 m, suggesting that N used for growth was acquired through continuing to fix N2 in the presence of nitrate. Net growth rates for UCYN-A1, UCYN-C, Crocosphaera sp. (UCYN-B) and the diatom symbiont Richelia (associated with Rhizosolenia) were uniformly high at 45 m (up to 1.6 ± 0.5 d−1), implying that all were growing optimally at the onset of the experiment at that depth. Differences in microzooplankton grazing rates on UCYN-A1 and UCYN-C in 15 m waters indicate that the grazer assemblage preyed preferentially on UCYN-A1. Deeper in the water column (125 m), both diazotrophs were grazed at substantial rates, suggesting grazing pressure may increase with depth in the photic zone. Constraining in situ diazotroph growth and mortality rates are important steps for improving parameterization for diazotrophs in global ecosystem models.

Keywords: diazotroph, growth rates, mortality rates, in situ incubations, dilution technique, nifH, qPCR
INTRODUCTION

Our knowledge of the patterns of marine microbial biodiversity and functional activities has rapidly expanded (Sunagawa et al., 2015; Carradec et al., 2018) with the application of ‘omic’-based techniques. Spatial patterns, however, are ultimately a function of growth and mortality rates of individual taxa, which are processes that are difficult to measure in natural populations. This is especially true for microorganisms responsible for biological nitrogen (N$_2$) fixation (diazotrophs) in the marine environment because, despite their importance, they are present at low abundances and many lineages do not have cultivated representatives. Thus, it is not well understood how shifts in the availability of critical nutrients drive changes in the growth (and mortality) of natural populations of diazotrophs.

Marine diazotrophs are diverse, comprised of both phototrophic and heterotrophic Bacteria and Archaea. They vary greatly in morphology and physiology and are expected to have different optimal growth conditions and mortality factors. The filamentous colonial diazotroph *Trichodesmium* and the heterocyst-forming symbionts of diatoms (diatom-diazotroph associations, or DDAs) have long been recognized as important N$_2$-fixers. These groups are distributed globally through tropical and subtropical regions, and are a major source of new N in temperate, oligotrophic regions (Villareal, 1999; Church et al., 2005a,b, 2009; Sohm et al., 2011b). Multiple lineages of unicellular N$_2$-fixing cyanobacteria are also globally distributed (Moisander et al., 2010; Luo et al., 2012) and are a quantitatively important source of new N (Montoya et al., 2004). This is especially true in the oligotrophic North Pacific subtropical gyre (NPSG) (Dore et al., 2002; Church et al., 2009).

The free-living cyanobacterial diazotroph *Crocosphaera* (UCYN-B) occasionally “blooms” in the NPSG, but arguably the more important unicellular diazotroph in this region is the uncultivated cyanobacteria group A (UCYN-A), which are present year-round in the NPSG. They reach peak abundances in the spring-early summer months and are comprised of diverse sublineages (Thompson et al., 2014; Farnelid et al., 2016; Turk-Kubo et al., 2017). Two of these sublineages, UCYN-A1 and UCYN-A2 are known to live in symbiosis with a prymnesiophyte algae (Thompson et al., 2012; Thompson et al., 2014). Both UCYN-A1 and UCYN-A2 have greatly reduced genomes, having lost important cyanobacterial metabolic capabilities including oxygenic photosynthesis and carbon fixation (Tripp et al., 2010; Bombar et al., 2014). These symbiotic associations are capable of high cellular rates of N$_2$ fixation (Martinez-Perez et al., 2016), presumably supported by the transfer of carbon from the host in exchange for reduced N from UCYN-A (Thompson et al., 2012).

Other unicellular cyanobacteria that have been reported include the unicellular cyanobacterial group C (UCYN-C; Foster et al., 2007), which includes some cultivated isolates (Tanuchi et al., 2012), however, little is known about their distribution or importance in the NPSG. *Trichodesmium* and several DDA lineages are also found in the NPSG (Letelier and Karl, 1996; Church et al., 2005a,b, 2009; Sohm et al., 2011b). *Richelia* spp. associated with *Rhizosolenia* (Het-1) and *Hemiaulus* (Het-2), are found in the NPSG and the latter has been identified as the diazotroph that contributes to vertical export following summer blooms (Karl et al., 2012). Diverse non-cyanobacterial diazotrophs have been reported in the NPSG (Bombar et al., 2013; Gradoville et al., 2017), but their distributions and activities, as well as their quantitative significance to biological N$_2$ fixation, are not well understood (Turk-Kubo et al., 2014; Bombar et al., 2016; Moisander et al., 2017).

Controls on the N$_2$ fixation activity and distribution patterns of diazotrophs involve the interplay between the availability of nutrients needed for growth and activity and their ability to compete for these (often limiting) nutrients. Diazotrophic phytoplankton have a competitive advantage over non-diazotrophic phytoplankton when N is limiting, due to their ability to access gaseous N$_2$. However, there are various costs associated with fixing N$_2$, including a high cellular demand for Fe and the energy required to protect against inhibition of the oxygen-sensitive nitrogenase enzyme in aerobic environments, which are thought to result in a competitive disadvantage over non-diazotrophic phytoplankton when N is available. Under non-Fe limited conditions, diazotrophic phytoplankton are thought to be outcompeted by non-diazotrophic phytoplankton for the limited P at high N:P ratios (Schade et al., 2005). Thus the known biogeography of diazotrophs, at the basin scale, can be best predicted by models that consider the supply ratios of Fe:N and P:N (Ward et al., 2013).

It has been widely assumed that marine N$_2$ fixation is inhibited by the presence of nitrate, however, recent discoveries of diazotrophs and measurements of N$_2$ fixation rates in nitrate-replete marine environments such as coastal systems (Mulholland et al., 2012; Thompson et al., 2014), regions with seasonal upwelling (Sohm et al., 2011a; Moreira-Coello et al., 2017), mesopelagic waters (Hammersley et al., 2011; Benavides et al., 2018), the Bering (Shiozaki et al., 2017) and Chukchi Seas (Shiozaki et al., 2018) in the Arctic, the Great Belt (Bentzon-Tilia et al., 2015) and oxygen minimum zones (Fernandez et al., 2011), suggests that the influence of DIN availability on diazotroph biogeography and N$_2$ fixation is not well-understood. The assumption that DIN inhibits N$_2$ fixation is based on several lines of evidence including the competitive disadvantage faced by diazotrophs due to the energetic tradeoff between N$_2$ fixation verses nitrate assimilation (Falkowski, 1997), early observations of *Trichodesmium* only in N-depleted surface waters of the oligotrophic ocean (Capone et al., 1997), and direct measurements of nitrate and/or ammonia inhibition of N$_2$ fixation in culture experiments (e.g., Mulholland and Capone, 1999; Holl and Montoya, 2005). However, there is increasing evidence that the presence of DIN alone may not exclude diazotroph activity and N$_2$ fixation. In culture, evidence is emerging that short-term exposure to nitrate may not inhibit growth or N$_2$ fixation in the unicellular diazotroph *Crocosphaera* (Dkea bezemacker and Bonnet, 2011) and that N$_2$ fixation in *Trichodesmium* is not inhibited at nitrate concentrations typically found in surface waters (Knapp, 2012). Furthermore, the ratio of available N to P may play a critical role, as reduced cellular N$_2$ fixation rates at high nitrate concentrations can be offset by increased abundances when P is available in *Trichodesmium* (Knapp et al., 2012). Additionally, continuing to invest in N$_2$
fixation in the presence of DIN has been hypothesized as a
unconventional strategy to balance redox states (Zehr and
Bombar, 2015; Bombar et al., 2016 and references therein).

Cyanobacterial diazotroph net growth rates are a balance
between taxa-specific requirements for growth (temperature,
light, nutrients), and the ability to compete for limiting
nutrients and avoid mortality (grazing and viral lysis). Each of
these terms is challenging to evaluate in natural populations,
thus predictive models rely heavily on culture-derived information,
which is heavily biased toward Trichodesmium, to parameterize
the N₂-fixing functional group (Dutkiewicz et al., 2009). Few
direct measurements of diazotroph net growth rates have been
made in natural populations. Net growth rates of UCYN-A1,
Crocosphaera, and the gammaproteobacteria γ-24774A11 have
been reported to be stimulated by P, Fe+P, and to a lesser extent
carbon additions in the South Pacific Ocean (Miosander et al.,
2011). However, increases in diazotroph abundances (growth) or
the transcription of the nifH gene (a proxy for N₂-fixing activity)
are not always observed upon addition of P (Zehr et al., 2007;
Turk-Kubo et al., 2012; Krupe et al., 2015; Barcelos Ramos
et al., 2017). Turk-Kubo et al. (2015) observed fluctuations in
net growth in mesocosms sampled for 23 days, underscoring that
this is a dynamic process in which the top-down controls such as
grazing and viral lysis are likely to be important factors.

Very little is known about diazotroph mortality in the marine
environment. Grazing and viral lysis are assumed to be important
factors affecting net growth rates and standing stocks, yet
both are difficult to quantify. There have been a number of
studies documenting the grazing of different copepod species
on Trichodesmium (O’Neil et al., 1996; O’Neil, 1998), UCYN-A
(Scavotto et al., 2015; Conroy et al., 2017), UCYN-B (Conroy
et al., 2017), UCYN-C (Hunt et al., 2016) and Richelia associated
with both Rhizosolenia (Het-1) and Hemialus (Het-2) (Hunt
et al., 2016; Conroy et al., 2017), through direct observation or
detection in full-gut copepods. Collectively, these studies indicate
that direct transfer of diazotroph derived N to microzooplankton
(>200 µm) is an important factor in marine food webs.

There are few direct observations of microzooplankton
(<200 µm) grazing on diazotrophs, despite their prominent role
as primary consumers in the oligotrophic open ocean (Schmoker
et al., 2013). A recent study measured microzooplankton grazing
rates on Crocosphaera during a summertime Crocosphaera bloom
in the NPSG and concluded that grazing and viral lysis were likely
controlling their abundances, rather than bottom-up factors
(Wilson et al., 2017). Wilson et al. (2017) used a modification
of the dilution technique developed by Landry and Hassett
(1982) and flow cytometry to measure growth rates. This
technique has been widely used to measure microzooplankton
grazing rates on pico- and nanoplankton communities (0.2–2 µm
and 2–20 µm size classes, respectively) in the ocean, and the
underlying assumptions and limitations have been discussed in
detail elsewhere (Landry et al., 1995; Schmoker et al.,
2013). Growth and mortality rates of the entire phytoplankton
community can be calculated using changes in chl a during the
incubation, however, this approach can also be used to measure
these parameters on individual taxa such as Prochlorococcus and
Synechococcus (Chen et al., 2009), as well as Crocosphaera (Wilson
et al., 2017).

In order to measure how shifts in the available nutrients
influence the growth of natural diazotroph populations,
free-floating in situ incubation arrays, originally described in
Böttjer et al. (2017), were used to investigate net growth rates of
phototrophic picochloroplaston and diazotrophic taxa as a function
of depth and N:P ratio. In addition, microzooplankton grazing
rates on diazotrophs were measured using a modified dilution
method (Landry et al., 1995), at two depths in the photic zone
(15 and 125 m) using a combination of in situ floating arrays and
deckboard incubations. Diazotroph net growth rates were often
stimulated by HNP conditions, which was an unexpected result,
and the depth at which HNP conditions were favorable was
taxa-dependent. Microzooplankton grazing pressure on the two
most abundant diazotrophs, UCYN-A1 and UCYN-C, increased
with depth, which was consistent with the pattern observed for
the total phytoplankton community.

**MATERIALS AND METHODS**

**Experimental Incubations**

Five experiments were conducted as part of this study. Changes in
diazotroph growth rates in response to shifts in available nutrient
reserves were measured in growth experiment 1 (G1). Two
experiments, grazing dilution experiment 1 (GR1) and 2
(GR2), were conducted to measure microzooplankton grazing
rates on specific diazotrophic taxa, using a modified dilution
technique amended with N, P and Fe (Landry et al., 1995). In
parallel to the GR1 and GR2 experiments, smaller scale dilution
experiments (GR1-N and GR2-N) amended only with P and Fe
were conducted, based on the assumption that the addition of N
substrates may inhibit diazotroph growth.

All experiments were conducted in the core of an anticyclonic
eddy situated to the east of Station ALOHA in the NPSG between
March 24 and 28, 2016 from aboard the R/V Kilo Moana
(cruise no. KM1605). Seawater from the appropriate depths was
collected for all experiments using a rosette of Niskin bottles
coupled to a SeaBird CTD detector. Handling of water for
each experiment type differed, and is detailed in sections below,
however, all experiments were conducted in small (2.3 or 4 L)
bottles, which have been recently shown to bias against large
(20–200 µm) diazotrophic taxa (Follett et al., 2018). Experiments
G1 and GR2 were incubated at fixed depths as part of a free-
drifting array approach based on Böttjer et al. (2017), that
makes it possible to incubate in situ temperatures and irradiances.
GR1, GR1-N, and GR2-N were incubated in simulated in situ
light and temperature conditions in an on-deck incubator. Dates,
experimental details and environmental parameters specific to
each experiment are detailed in Table 1.

**Diazotroph Net Growth Rate Incubations (G1)**

Seawater collected from 25, 45, 75, and 100 m was dispensed
directly from the Niskin bottles into 4 L polycarbonate bottles,
taking measures to randomize the filling of the bottles. The
polycarbonate bottles were acid-washed with trace metal clean
TABLE 1 | Growth (G1) and grazing rate (GR1, GR2) experiment details.

| Date         | Lat       | Long             | Experiment type                  | % dilution levels | Incubation method | Depth(s) (m) | Incubation period (h) | MLD (m) | DCM (m) | Nitrocline (m) |
|--------------|-----------|------------------|----------------------------------|-------------------|-------------------|--------------|-----------------------|----------|----------|---------------|
| G1           | March 25–27, 2016 | 22°23.799′ N    | Grazing (4 pt. dilution)         | 20,40,60,80,100   | In situ array     | 25, 48, 75, 100 | 24                     | 53       | 140      | 125           |
| G2           | March 27–28, 2016 | 22°33.276′ N    | Grazing (-N; 3 pt. dilution)     | na                | Deckboard         | 20,60,100    | 24                     | 53       | 140      | 125           |
| GR2-N        | March 27–28, 2016 | 22°33.276′ N    | Grazing (4 pt. dilution)         | 20,40,80,100      | Deckboard         | 60,100       | 24                     | 53       | 140      | 125           |

MLD, mix layer depth; DCM, deep chlorophyll maximum; na, not applicable.

HCl prior to each experiment, however, these incubations are not considered trace metal clean. No pre-filtration to remove grazers was used, so measured growth rates determined from changes in cell abundances over the length of the incubation incorporate cell death, and are therefore net growth rates.

Replicate bottles from each depth were amended with either NaNO₃ (high N:P, HNP) or KH₂PO₄ (low N:P, LNP) for final concentrations of 2 and 0.5 µM, respectively. All incubation bottles, including the controls (C), received FeCl₃ at a final concentration of 10 nM. Fe was added to ensure that the response of the diazotrophic community was to changes in the N:P ratio under non-Fe limiting conditions, as Fe is generally not considered to limit primary production at Station ALOHA (Boyle et al., 2005). Water was collected around 2 a.m. and the 4 L bottles were kept in the dark until the array was deployed before dawn. T₀ samples were immediately subsampled for phototrophic picoplankton cell counts using flow cytometry, nutrients, chl a and DNA. The G1 array was incubated in situ for 48 h, with bottles floating at the depths from which the water was originally sampled. The array deployment and recovery occurred at dawn, to minimize manipulating the communities in daylight. All bottles were kept in the dark until they were subsampled.

Incubations to Measure Microzooplankton Grazing Rates on Diazotrophs (GR1 and GR2)

For the dilution experiments GR1 and GR2, seawater from the appropriate depth was transferred into acid-washed 23 L polycarbonate carboys, which were then gently combined into a 50 L carboy to ensure homogeneity between treatments. Filtered seawater (FSW) for diluent was prepared by filtering experimental seawater through a Pall 0.2 µm Acropak 1550 Capsule Filter with Supor Membrane (Pall Corp, Port Washington, NY, United States), and was stored at room temperature in the dark while the experiment was setup. FSW was combined with whole sea water (WSW) in 2.3 L incubation bottles at ratios described below for each experiment and in Table 1. The bottles were amended with N, P and Fe at final concentrations of 2.0 µM NO₃⁻, 0.2 µM NH₄⁺, 0.5 µM PO₄³⁻, and 0.1 µM Fe. For each experiment, a control set of triplicate bottles containing 100% WSW were not nutrient-amended in order to determine net growth rates during the incubation period. T₀ samples were taken from the 50 L carboy and immediately subsampled for chl a and DNA. All dilution experiments were initiated at night, to minimize photoadaptation or light shock.

GR1 consisted of five dilution levels, 20, 40, 60, 80, and 100% WSW. In parallel with GR1, a smaller-scale dilution experiment (GR1-N) was conducted that consisted of three dilution levels (20, 60, and 100% WSW) in triplicate 1.2 L bottles. Triplicate 1.2 L bottles containing 100% WSW were not amended with nutrients as noted above. The remaining bottles were amended with only P and Fe at final concentrations of 0.5 µM PO₄³⁻, and 0.1 µM Fe. All bottles (GR1 and GR1-N) were incubated for 24 h in simulated in situ light and temperature conditions in an on-deck incubator with a continuous flow of surface seawater to simulate conditions at 15 m. At T₂₄, all bottles were subsampled for chl a and DNA.

GR2 consisted of four dilution levels at each depth, 20, 40, 80, and 100% WSW. Incubation bottles were deployed on the in situ incubation array at 15 and 125 m and incubated for 24 h. As with GR1, a smaller-scale dilution experiment (GR2-N) was conducted in parallel with GR2, but only with 15 m water. GR2-N consisted of two dilution levels (60 and 100% WSW) in triplicate 1.2 L bottles. Nutrient amendments and WSW control bottles were consistent with GR2-N described above. The GR2-N bottles were incubated as described above for GR1-N. At T₂₄, all bottles were subsampled for chl a and DNA.

Quantifying Diazotroph Abundances Using Quantitative PCR and Calculating Net Growth and Mortality Rates

Samples for DNA extraction were gently filtered using peristaltic pumps through Sterivex filters (Millipore) for growth rate experiments or 0.2 µm pore-size 25 mm diameter Supor filters (Pall Corp, Port Washington, NY, United States) in sterile swinnex holders (Millipore) for grazing experiments. Sterivex filters were immediately sealed, and Supor filters were transferred into 2.0 mL bead beating tubes filled with 0.1 and...
0.5 mm sterile glass beads. Filters were flash frozen in liquid nitrogen and stored at −80°C until extraction. Just prior to DNA extraction, the sterilax cartridges were broken and the filters were transferred into the bead beating tubes described above.

DNA was extracted using a protocol chosen for high quality recovery of DNA from algal cells. Details of this protocol are described in Moisander et al. (2008). Briefly, cells were disrupted using freeze-thaw, bead beating, and proteinase K digestion steps prior to a Qiacube® (Qiagen) automated on-column DNA extraction and clean-up protocol using DNase kit (Qiagen) components. Concentration and quality of purified DNA were measured using a NanoDrop (Thermo Scientific, Waltham, MA, United States). Extracted DNA was stored at −20°C until use.

Abundances of nine diazotrophic taxa were determined using quantitative PCR (qPCR) assays based on Taqman® chemistry. Primers and probes targeting the following diazotrophic taxa were used: unicellular cyanobacterial group A1 (UCYN-A1; Church et al., 2005a), unicellular cyanobacterial group A2/A3 (UCYN-A2/A3; Thompson et al., 2014), Crocosphaera (UCYN-B: Moisander et al., 2010), Cyanothecelike organisms (UCYN-C; Foster et al., 2007), Trichodesmium spp. (Church et al., 2005a), Richelia associated with Rhizosolenia (Het-1; Church et al., 2005b), Richelia associated with Hemiaulus (Het-2; Foster et al., 2007), Calothrix associated with Chaetoceros (Foster et al., 2007), and a gammaproteobacterial group (γ-24774A11; Moisander et al., 2008). All nine taxa were quantified in G1, but only the most abundant taxa present in the water at the time of the experimental work (UCYN-A1 and UCYN-C) were quantified in the grazing rate experiments.

Details of qPCR standard generation, plate design, thermocycling parameters, inhibition tests, determination of the limit of detection (LOD) and quantification (LOQ), as well as abundance calculations are described in Goebel et al. (2010). The LOD and LOQ for G1 and GR1 were 13 and 100 nifH copies L\(^{-1}\), respectively. The LOD and LOQ for GR2 were between 33-50 and 267-400 nifH copies L\(^{-1}\), respectively.

Net growth rates from G1 were calculated as in Turk-Kubo et al. (2015) using the equation \( k = 2.303 \left( \frac{\log N_t}{(t_2-t_1)} \right) \) where \( N_t \) = abundance at time \( t \) seconds, and assuming that there is a single nifH copy per cell. It should be noted that there is evidence of multiple genome copies in natural populations of Trichodesmium (Sargent et al., 2016), and little is known about genome copy numbers in natural populations of other diazotrophic taxa. Therefore, growth rates based on qPCR may be impacted if the number of genome copies is variable across time in a given population. However, based on the strong linear relationship between cell counts L\(^{-1}\) and nifH copies L\(^{-1}\) across variable sampling times reported by Sargent et al. (2016), the number of genome copies appears to be consistent, at least for Trichodesmium.

Net growth rates in G1 were determined for UCYN-A1, UCYN-B, UCYN-C in all depths and treatments, but only in surface waters for Het-1 (25 and 45 m) and Het-2 (25 m), due to low abundances for these latter taxa lower in the water column. UCYN-A2/A3, Trichodesmium, Het-3 and γ-24774A11 were often detectable, but abundances were too low for establishing reliable rates (detected, not quantified; DNQs). Comparisons between treatments and the controls were made using one way analysis of variance, followed by testing independent variables with a pairwise t-test in R (R Development Core Team, 2012).

Mortality rates (m) on individual diazotrophic taxa in GR1, GR1-N, GR2, and GR2-N were calculated using the slope of Model I linear regressions of qPCR-based diazotroph apparent growth rates in each nutrient-amended dilution vs. the dilution level, using the established convention that a negative slope indicated positive grazing pressure (Landry and Hassett, 1982). Nutrient enriched growth rates (\( \mu_t \)) of each prey were determined using the y-intercept of the regression of nutrient-amended treatments, which represents a theoretical scenario where all grazing pressure is removed from the incubations. Intrinsic (unenriched) growth rates (\( \mu_u \)) were calculated from the nutrient-enriched intrinsic growth rates, corrected for differences in growth rates between amended and unamended treatments of 100% WSW (Landry et al., 1995). Mortality rates are reported only when the linear regressions were significant (\( p \leq 0.05 \)), otherwise they are reported as non-significant (ns).

**Phototrophic Picoplankton Community Composition and Net Growth Rates in G1**

Subsamples (2 mL) for the enumeration of Prochlorococcus, Synechococcus and photosynthetic picoeukaryotes (PPEs) were collected from G1 at times \( T_0 \) and \( T_48 \) and immediately fixed with electron microscopy grade gluteraldehyde (Electron Microscopy Sciences, Hatfield, PA) for a final gluteraldehyde concentration of 0.25% v/v. Samples were fixed in the dark for 15 min, then flash frozen in liquid nitrogen and stored at −80°C until processing. Cells were enumerated using a BD Influx cell sorter with a 488 nm Sapphire laser (Coherent, Santa Clara, CA, United States). Large particles were removed from samples prior to counting using a CellTrics® filter with 30 µm mesh (Partec, Swedesboro, NJ, United States). Synechococcus cells were identified based on their phycoerythrin signal (orange fluorescence), and non-phycoerythrin containing cells (Prochlorococcus and PPEs) were identified using chl a fluorescence (red fluorescence) and forward scatter (FSC; a proxy for cell size). Data was triggered on the FSC channel, events were counted for 10 min, and data was processed using FlowJo v10.0.7 (Tree Star, Inc., Ashland, OR, United States). Net growth rates (G1) were calculated from abundances as described above.

**Measuring Shifts in Microbial Community Composition During G1 Using 16S rRNA Gene Tag Sequencing**

Bacterial community composition was characterized using the V3/V4 hypervariable region of the 16S-rRNA gene. Universal primers targeting Bacteria, Bakt_341F and Bakt_805R (Herlemann et al., 2011) were modified with common sequence linkers (Moonsamy et al., 2013) to facilitate library preparation.
RESULTS

Initial Environmental Conditions and Microbial Community Composition

All three experiments, G1, GR1, and GR2, were conducted during spring 2016 in the NPSG with water collected at the center of an anticyclonic eddy characterized by enhanced chlorophyll located 60 km north of Molokai (22.5° N and 156.5° W). The eddy appeared to be decreasing in strength at the time of sampling, with low and variable currents at the center. The mixed layer depth and depth of the deep chlorophyll maximum (DCM) were apparent at 87 and 140 m, respectively (Table 1). Nitrate + nitrite concentrations were very low in the top 100 m of the water column at 2.8 ± 3.2 nM, while PO4³⁻ concentrations were 0.11 ± 0.01 µM. Prochlorococcus abundances at G1 T0 were 7.2 × 10⁴ ± 6.9 × 10³ cells mL⁻¹ at 25 m and increased slightly to 9.6 × 10⁴ ± 5.2 × 10³ cells mL⁻¹ at 100 m; Synechococcus and photosynthetic picoeukaryote (PPE) abundances were consistent throughout the top 100 m at 7.5 × 10² ± 4.8 × 10¹ cells mL⁻¹ and 1.5 × 10⁵ ± 4.7 × 10⁴ cells mL⁻¹, respectively. The most abundant diazotrophs were unicellular cyanobacterial phytootypes UCYN-A1 and UCYN-C, which had maximum abundances of 4.7 × 10⁴ and 1.2 × 10⁴ nifH copies L⁻¹, respectively. All other targeted diazotrophs were detected in the water column, but at much lower abundances than UCYN-A1 and UCYN-C (Figure 1), including the large (20–200 µm) diazotrophic taxa (Trichodesmium and DDAs), which is consistent with known annual patterns in their abundances (Church et al., 2009). However, it cannot be ruled out that these abundances are underestimated given a recent report that small bottle incubations may bias against the larger diazotrophs, presumably due to heterogeneity in these populations resulting from buoyancy and vertical migration (Follett et al., 2018).

Net Growth Rates in Photosynthetic Microbial Populations Exhibited Taxa- and Depth-Specific Responses to Different Nutrient Ratios

The responses of the major photosynthetic populations, Prochlorococcus, Synechococcus, and PPEs to HNP and LNP conditions during G1 were characterized using net growth rates derived from FCM-based cell counts (Figures 2A–D) as well as changes in chl a concentrations (as a proxy for photosynthetic biomass; Figures 2E–H). Over the course of the experiment, chl a content increased in all treatments and at all depths except at 100 m, where T₁₀ and T₄₈ chl a concentrations were similar (Figures 2E–H). Significant (p ≤ 0.01) increases in chl a content with respect to control concentrations were measured only in the HNP treatment at 25 m (an approximately 2-fold increase; Figure 2E).

Increases in chl a concentrations in the 25 m HNP treatment may be due to a significant (p ≤ 0.05) stimulation of biomass from the PPE population compared to T₄₈ controls (Figure 2A). This was the only depth and treatment where the PPE population had higher net growth rates than the controls, indicating that the PPE population was P-limited in surface waters. Synechococcus growth rates were highest in the LNP treatment at 25 m, implying that these populations were P-limited at the initiation of the experiment, despite ambient PO₄³⁻ concentration of 0.11 ± 0.01 µM in the top 100 m of the water column (Figure 2A). In contrast, Prochlorococcus growth rates were...
FIGURE 1 | Diazotroph abundances ([log(x+1)] nifH copies L$^{-1}$) throughout the water column at the beginning of growth rate (G1) and dilution grazing rate (GR1 and GR2) experiments. (A) UCYN-A1, (B) UCYN-C, (C) UCYN-A3, (D) UCYN-B, (E) Trichodesmium, (F) Het-1, (G) Het-2, (H) Het-3, (I) γ-24774A11. Only the most abundant diazotrophs, UCYN-A1 and UCYN-C, were measured for GR1 and GR2. Error bars are replicate incubations.

Diazotroph Net Growth Rates Exhibit Taxa- and Depth-Specific Responses to Different Nutrient Ratios (G1)

Despite relatively stable abundances throughout the top 100 m (Figure 1), individual diazotrophic taxa exhibited depth-dependent responses to changes in nutrient ratio (Figure 3 and Supplementary Table S1). In surface waters (25 m), all of the diazotrophs had negative growth rates in the control treatment (Figures 3A,E,I,M,O). In 25 m HNP treatments, UCYN-B and UCYN-C had positive and enhanced growth rates (with respect to the control treatment) of 0.25 ± 0.07 d$^{-1}$ (Figure 3E) and 0.27 ± 0.10 d$^{-1}$ (Figure 3I), respectively; other groups had negative or close to zero growth rates. The only diazotroph that had enhanced growth rates in the LNP treatment at this depth was Het-2 at 0.24 ± 0.10 d$^{-1}$ (Figure 3O).

Surprisingly, the most abundant diazotrophs, UCYN-A1, UCYN-B, UCYN-C and Het-1 had high net growth rates across all controls and treatments at 45 m (Figures 3B,F,J,N), despite all having peak abundances at 25 m (Figure 1). UCYN-A1 and UCYN-B growth rates were uniformly high and insensitive to nutrient additions (1.32 ± 0.02 d$^{-1}$ and 1.39 ± 0.08 d$^{-1}$, respectively; Figures 3B,F), suggesting that these taxa were growing optimally at this depth at the start of the experiment, and growth was not inhibited or enhanced by experimental treatments. In contrast, both UCYN-C and Het-1 growth rates were slightly enhanced in LNP treatments (Figures 3J,N). UCYN-C growth rates in the control (2.11 ± 0.24 d$^{-1}$) were less than those measured in LNP treatments (2.71 ± 0.26 d$^{-1}$), and significantly ($p \leq 0.05$) stimulated only in HNP treatments at 45 m (Figure 2B).
the same was true for Het-1 (control 1.42 ± 0.17 d⁻¹ vs. LNP 1.74 ± 0.11 d⁻¹). These results suggest UCYN-C and Het-1 have a competitive advantage in the N-limited conditions at this depth.

Deeper in the water column (75 and 100 m incubations), net growth rates could only be determined for the unicellular taxa. There was no significant difference in net growth rates between control and LNP/HNP treatments at these depths for UCYN-B and UCYN-C (Figures 3G,H,K,L). However, UCYN-A1 net growth rates were significantly stimulated (p ≤ 0.05), with respect to control incubations, under HNP conditions at 75 m (0.63 ± 0.05 d⁻¹; Figure 3C) and 100 m (0.70 ± 0.08 d⁻¹; Figure 3D).

**Shifts in Prokaryote Microbial Community Composition During G1**

16S rRNA gene variable region amplicon tag sequencing was used to determine whether there were significant changes in microbial community composition. *Prochlorococcus* (21.8–51.3% of sequences per sample) and SAR11 (10.0–16.6% of sequences per sample) dominated the prokaryote community throughout
the experiment (Supplementary Table S2). To samples showed that there was a relatively constant microbial community within the top 100 m (Figure 4A), consistent with the presence of a deep mixed layer at the time of the experiment. Furthermore, there were no large shifts in relative abundances of these taxa in response to HNP and LNP treatments, including...
Prochlorococcus at 45 m, indicating that although cell numbers increased, the relative proportion of Prochlorococcus to other microbes (mainly SAR11) remained stable. Even at the finest taxonomic resolution, Prochlorococcus oligotypes (Eren et al., 2013) were also consistent across depths and treatments (data not shown).

There were minor changes in community composition between T₀ and T₄₈ at 75 and 100 m depths, but not between experimental treatments (Figure 4B). Ordination analysis using the Jaccard ecological index indicated that community shifts were driven by increases in relative abundances of the following four taxa in all treatments: Rhodobacterales (denovo1), Alteromonadales (denovo4), Oceanospirillales (denovo9), and Flavobacteriales (denovo89) (Figure 4A). These taxa are frequently found in associations and on particles and are known to respond quickly to incubation conditions (Fontanez et al., 2015). It is important to note that treatments often did not cluster together, indicating that changes in nutrient ratios did not drive any consistent patterns; T₄₈ samples clustered according to depth (Figure 4B). Furthermore, there was not a correlation between relative abundances and diazotroph net growth rates in samples where a diazotroph had differential responses to HNP/LNP treatments, e.g., UCYN-A1 at 75 or 100 m.

**Microzooplankton Grazing Pressure on UCYN-A1 and UCYN-C Was Highest Near the Deep Chlorophyll Maximum**

During GR2, grazing rates on the bulk phytoplankton community, based on changes in chl a, indicated that there was no detectable grazing pressure in surface waters (Figure 5A), while grazing pressure was significant near the DCM (125 m; Figure 5B). The measured mortality rate (m) on the bulk phytoplankton community at 125 m was 0.60 d⁻¹, which is higher than the mean rate for this region (0.29 d⁻¹) reported in Schmoker et al. (2013). Enriched (μ₁n) and intrinsic (μ₀) growth rates at 15 m were 0.66 and 0.24 d⁻¹, respectively, indicating that the addition of nutrients stimulated phytoplankton growth rates in surface waters. The community seemed less responsive to added nutrients at 125 m, with μ₁n and μ₀ of 0.59 and 0.62 d⁻¹, respectively.

Patterns of grazing mortality specifically for UCYN-A1 and UCYN-C were consistent with the depth-dependent patterns observed for the phytoplankton community. In GR1, no significant grazing pressure was measured at 15 m for either diazotroph taxa, however, variable mortality rates on UCYN-A1, and UCYN-C were measured during GR2 (Figure 6). In GR2, grazing rates on UCYN-A1 were lower at the surface (15 m; 0.24 d⁻¹) than deeper in the photic zone (125 m; 1.00 d⁻¹; Figure 6). UCYN-C did not seem to have significant grazing pressure at 15 m, while at 125 m, mortality rates were high (1.75 d⁻¹). Enrichment with nutrients did appear to stimulate UCYN-A1 growth rates at 15 m, with enriched (μ₁n) and intrinsic (μ₀) growth rates of 0.16 and −0.13 d⁻¹, respectively. However, this was not the case for samples collected deeper in the water column for either taxa. Grazing rates for other diazotrophs were not measured due to their low abundances in T₀ samples. Together, the chl a-based phytoplankton mortality rates and qPCR-based diazotroph mortality rates indicated that grazing pressure was less intense near the surface than deeper in the water column.

Grazing dilution incubations with water from 15 m were conducted during GR1 and GR2, using a nutrient addition that did not include additional fixed N (GR1-N and GR2-N), as the presence of N has been shown to inhibit diazotroph growth in cultures (e.g., Holl and Montoya, 2005). Mortality rates on UCYN-A1 in GR2-N and GR1-N were higher than in the typical modified dilution rate experiments at 1.25 and 0.69 d⁻¹, respectively (Figure 6). In these experiments, enriched growth rates for UCYN-A1 were positive with μ₀ ranging between 0.28 and 0.82 d⁻¹. No significant mortality rates were measured for UCYN-C in these experiments. These findings are inconsistent with growth rates measured in G1 where growth rates at 25 m were negative in response to P additions (LNP treatment).

**DISCUSSION**

**Diazotrophs Are Capable of High in Situ Growth Rates**

Early culture-based studies suggested that diazotrophs should have slower growth rates than their non-diazotrophic
TABLE 2 | Compilation of diazotroph growth rates measured in situ, along with selected culture-based measurement.

| Diazotroph                | Maximum net growth rate d⁻¹ | Method | Location               | Experimental Conditions or in situ | Reference                          |
|---------------------------|-----------------------------|--------|------------------------|-----------------------------------|-----------------------------------|
| UCYN-A²                   | 0.4                         | ¹³C    | Station ALOHA          | In situ                           | Thompson et al., 2012             |
| UCYN-A²                   | 0.5                         | ¹³C    | North Atlantic         | In situ                           | Krupke et al., 2015               |
| UCYN-A²                   | 0.8                         | cell size, modeled | North Pacific   | In situ                           | Goebel et al., 2008               |
| UCYN-A (small)³           | 0.6³                        | ¹³C    | North Atlantic         | In situ                           | Martinez-Perez et al., 2016       |
| UCYN-A (large)³           | 1.1³                        | ¹³C    | North Atlantic         | In situ                           | Martinez-Perez et al., 2016       |
| UCYN-A1                   | 1.3                         | qPCR   | NPSG                   | HNP treatment; 45 m               | This study                        |
| UCYN-A1                   | 1.3                         | qPCR   | NPSG                   | LNP treatment; 45 m               | This study                        |
| UCYN-A1                   | 1.3                         | qPCR   | NPSG                   | C treatment; 45 m                 | This study                        |
| UCYN-A1                   | 0.2                         | qPCR   | South Pacific          | P treatment                       | Turk-Kubo et al., 2015            |
| UCYN-A1                   | 0.7                         | qPCR   | Southwest Pacific      | P treatment                       | Turk-Kubo et al., 2015            |
| UCYN-A2                   | 1.7                         | qPCR   | Southwest Pacific      | P treatment                       | Turk-Kubo et al., 2015            |
| UCYN-B                    | 1.5                         | qPCR   | NPSG                   | LNP treatment; 45 m               | This study                        |
| UCYN-B                    | 1.3                         | qPCR   | NPSG                   | HNP treatment; 45 m               | This study                        |
| UCYN-B                    | 1.3                         | qPCR   | NPSG                   | C treatment; 45 m                 | This study                        |
| UCYN-B                    | 0.6                         | qPCR   | South Pacific          | Fe and P-Fe treatments            | Moisander et al., 2011            |
| UCYN-B                    | 1.4                         | qPCR   | Southwest Pacific      | P treatment                       | Turk-Kubo et al., 2015            |
| UCYN-B                    | 0.6                         | FCM    | NPSG                   | In situ                           | Wilson et al., 2017               |
| UCYN-C                    | 2.7                         | qPCR   | NPSG                   | LNP treatment; 45 m               | This study                        |
| UCYN-C                    | 2.4                         | qPCR   | NPSG                   | HNP treatment; 45 m               | This study                        |
| UCYN-C                    | 2.1                         | qPCR   | NPSG                   | C treatment; 45 m                 | This study                        |
| UCYN-C                    | 2.2                         | qPCR   | Southwest Pacific      | P treatment                       | Turk-Kubo et al., 2015            |
| Richelia in Rhizosolenia (Het-1) | 1.3                         | qPCR   | Southwest Pacific      | P treatment                       | Turk-Kubo et al., 2015            |
| Richelia in Rhizosolenia (Het-1) | 1.7                         | qPCR   | NPSG                   | LNP conditions; 45 m              | This study                        |
| Richelia in Rhizosolenia (Het-1) | 1.7                         | qPCR   | NPSG                   | HNP treatment; 45 m               | This study                        |
| Richelia in Rhizosolenia (Het-1) | 1.4                         | qPCR   | NPSG                   | C treatment; 45 m                 | This study                        |
| Richelia in Hemiaulus (Het-2) | 2.2                         | qPCR   | Southwest Pacific      | P treatment                       | Turk-Kubo et al., 2015            |
| Richelia in Hemiaulus (Het-2) | 0.2                         | qPCR   | NPSG                   | LNP treatment; 25 m               | This study                        |
| Richelia in Hemiaulus (Het-2) | 0.6                         | ¹⁵N    | North Pacific          | In situ                           | Foster et al., 2011               |
| Calothrix in Chaetoceros (Het-3) | 1.1                         | qPCR   | Southwest Pacific      | P treatment                       | Turk-Kubo et al., 2015            |
| Trichodesmium             | 0.1                         | ¹³C    | North Atlantic         | In situ                           | Martinez-Perez et al., 2016       |

Selected growth rates for cultivated isolates:

- Cyanothece, sp ATCC 51142: 1.92 d⁻¹ (Thompson et al., 2012; Martinez-Perez et al., 2016).
- UCYN-C TW3: 0.84 d⁻¹ (Taniuchi et al., 2012).
- Cyanothece, sp ATCC 51142: 2.4 d⁻¹ (Reddy et al., 1993).
- Crocosphaera watsonii WH8501: 0.48 d⁻¹ (Goebel et al., 2008).
- Trichodesmium erythraeum IMS101: 0.51 d⁻¹ (Goebl et al., 2008).
- Richelia in Rhizosolenia: 0.67 d⁻¹ (Villareal, 1989; Villareal, 1990).

Maximum rates from each study are reported and negative rates (when reported) are not included. NPSG, North Pacific Subtropical Gyre; ¹³C, measuring the incorporation of ¹³CO₂ using nanoSIMS; qPCR, quantitative PCR; FCM, flow cytometry. ³⁵C⁴Assumed by the authors of this study to be UCYN-A1 based on size and sample location. ³⁵C⁵Assumed by the authors of this study to be UCYN-A3 based on size and sample location. ³⁵C⁶Estimated from Figure 3 in Martinez-Perez et al. (2016).

...The maximum net growth rate measured for UCYN-A1 in this study, 1.3 d⁻¹, is higher than reported in the North Atlantic (0.19 d⁻¹; Moisander et al., 2011) and in the South Pacific (0.73 d⁻¹; Turk-Kubo et al., 2015) (Table 2). These previously reported net growth rates were from experimental manipulations of surface waters amended with P (or P + Fe, in the case of Moisander et al. [2011]), conditions which are generally thought to favor diazotrophs. Interestingly, net growth rates of UCYN-A1 were uniformly high in samples from 45 m, independent of treatment (1.3 d⁻¹), even though peak abundances of UCYN-A1 were measured at 25 m (Figure 1).
Maximum net growth rates for UCYN-B (1.5 d\(^{-1}\)), UCYN-C (2.7 d\(^{-1}\)), Het-1 (1.7 d\(^{-1}\)) and Het-2 (0.2 d\(^{-1}\)) were comparable to rates measured in a P-perturbation experiment in the New Caledonia lagoon (Turk-Kubo et al., 2015). As with UCYN-A1, maximum net growth rates were measured exclusively in the samples collected at 45 m, and were high across all treatments. This was not observed in the growth rates for Prochlorococcus, Synechococcus or the PPEs (Figure 2). These findings imply that all diazotrophic taxa were either growing at optimal maximum rates at the start of the incubation, and maintained these rates throughout the 48 h incubation, or that the combination of light, temperature and Fe was optimal for all taxa at this depth (10 nM Fe was added to all incubation bottles, including controls). These results are surprising given that peak abundances for all diazotrophs were measured at 25 m depth, as well as the physiological differences among diazotrophs and the evidence that there is niche partitioning throughout the water column (Moisander et al., 2010). Results from the grazing rate experiments clearly show that grazing pressure increases with depth, which may explain why peak abundances were found at 25 m, but the highest net growth rates were found at 45 m. Preliminary growth rate experiments conducted in July 2015 yielded very similar results, with net growth rates of the most abundant diazotrophs being high in control, HNP and LNP treatments at a single depth (see Supplementary Figure S1). Further research, and perhaps different tools for measuring growth rates in natural populations, are needed to determine the environmental factors behind this observation.

There have been relatively few measurements of in situ diazotroph growth rates, but when reported, they are often from nutrient perturbation experiments, where nutrient limitation may have been alleviated (Table 2). Due to this, along with differences in experimental design and measurement of growth rates, it is difficult to compare rates across studies, and between in situ and culture-based studies. However, results obtained in this and other recent studies together indicate that diazotrophs are capable of high net growth rates in situ over relatively short time periods, even in the presence of competition and mortality (grazing/viral lysis), which underscores the gaps in our understanding of their physiology and cellular requirements. In global ecological models, diazotroph growth rates are typically parameterized using \( \frac{1}{2} \) the maximum growth rate of a comparably sized non-diazotroph, to compensate for the energetic costs of fixing N\(_2\) (Dutkiewicz et al., 2009). Direct comparisons between
large diazotrophs (*Trichodesmium*, DDAs) and their non-diazotrophic counterparts cannot be drawn from this study, but growth rates measured for the smaller, non-symbiotic diazotrophs (*Crocosphaera* and UCYN-C) are comparable to, and in many cases higher, than rates measured for *Synechococcus* (Figure 2).

**Diazotrophs Can Grow in Nitrate-Replete Conditions**

Diazotrophs are predicted to be unable to coexist with non-diazotrophs at high N:P ratios under equilibrium conditions when Fe is not limiting (Schade et al., 2005). This is based on resource ratio theory (Dutkiewicz et al., 2009 and references therein), which uses basic physiological requirements for a specific phytoplankton taxa to define the minimum concentration of a given nutrient needed for survival. Thus, under the conditions in the HNP treatments of this study, non-diazotrophs would be expected to outcompete diazotrophs for available P (0.11 ± 0.01 µM at the beginning of the experiment). However, findings from this study indicate that diazotrophic taxa can compete for nutrients on relatively short temporal scales (48 h) under conditions thought to favor "velocity adapted" organisms capable of responding quickly to pulses of nutrients, such as diatoms (Sommer, 1984).

These findings may be explained in part due to growth possibly being uncoupled from the energetically demanding process of N₂ fixation in HNP conditions, as some taxa can assimilate nitrate directly from the environment for growth (Agawin et al., 2007; Großkopf and LaRoche, 2012), or live in symbiosis with eukaryotic phytoplankton (diatoms, haptophytes) presumed to assimilate nitrate. The accessibility of a fixed N source has been shown to be a requirement for the initiation of N₂ fixation in free-living soil diazotrophs, given the upfront investment a cell must make to generate N-containing cellular components (Norman and Friesen, 2017), thus the ability to acquire and utilize diverse sources of DIN and DON may also be a critical strategy for marine diazotrophs.

Further research is needed to verify the N source(s) used for growth by each diazotroph taxa under these conditions, and whether N₂ fixation continues in nitrate-replete conditions. We cannot speculate about whether growth in these experiments was supported by increased N₂ fixation or nitrate. However, there is growing evidence that diazotrophic taxa can compete for nutrients on relatively short temporal scales (48 h) under conditions thought to favor "velocity adapted" organisms capable of responding quickly to pulses of nutrients, such as diatoms (Sommer, 1984).

UCYN-A1 lacks the genetic ability to directly assimilate nitrate (Tripp et al., 2010), so the observation that UCYN-A1 grows in the presence of nitrate deeper in the water column implies that when nitrate is available to the host, the symbiont either continues to fix N₂ or obtains reduced N from its host to support growth. Although there have been several recent studies that have measured the response of UCYN-A1 to nutrient amendments (Moisander et al., 2011; Turk-Kubo et al., 2012; Krupke et al., 2015), it has been uncommon to include N species among the suite of nutrients added, since N₂ fixation is understood to be either P or Fe limited (or P/Fe co-limited) in oligotrophic waters (Mills et al., 2004; Moore et al., 2009). However, two recent studies conducted in the Subtropical North Atlantic did include N addition as a treatment. Langlois et al. (2012) reported increased UCYN-A *nifH*-based abundances (ca. an order of magnitude) after the addition of ammonium nitrate to surface waters in one of the three experiments conducted in this region, indicating that the association was able to grow in N-replete conditions. However, despite greatly increased abundances of UCYN-A, no stimulation of bulk N₂ fixation rates were measured in their N amended treatment. Without data on cell-specific N₂ fixation rates it is unclear whether UCYN-A stopped fixing N₂ in this incubation. However, Krupke et al. (2015) reported no reduction in cell-specific N₂ fixation rates or *nifH* transcript production after the addition of 2 µM ammonium nitrate to UCYN-A populations in the North Atlantic suggesting that UCYN-A continues to fix N₂ even when reduced N is available to the association.

It has been speculated that UCYN-A1 may reach peak abundances in oligotrophic waters with elevated nutrient conditions coinciding with entrainment of nutrient-rich waters from vertical mixing in the South Pacific Ocean (Moisander et al., 2010). At Station ALOHA, UCYN-A1 abundances peak in spring months when light intensity increases and the mixed layer begins to shoal, entraining nutrient-rich waters into the sunlit surface waters. However, it is unclear which component(s) of the deep nutrient pool are responsible for leading to peak UCYN-A abundances. Furthermore, Henke et al. (2018) found that UCYN-A1 peak abundances coincided with slightly elevated *in situ* nitrate concentrations in a time series conducted in the New Caledonia Lagoon. Results from this study may provide some insight into these observations, given that UCYN-A1 growth rates responded positively to the introduction of nitrate deeper in the water column. It is unclear why this was not the case for the samples collected at shallower depths, where more light energy was available for the prymnesiophyte host, and where the PPE population did have increased growth rates in the HNP treatment (Figure 2A), but active grazing on UCYN-A1 in surface waters may have depressed net growth rates. These findings underscore the likely tradeoff between conditions optimal for photosynthesis, N₂ fixation and availability of nutrients, and provide valuable insight to this enigmatic symbiosis which may help explain its niche preferences for deeper waters (closer to the nutricline than other diazotrophs), and occurrences in colder, sometimes N-replete environments.
that Crocosphaera in HNP conditions for UCYN-B are not unexpected given UCYN-C was greatest near the DCM, and it is possible that than N was limiting growth in the HNP treatments deeper at the surface (25 m), implying that light or a nutrient other than N was limiting growth in the HNP treatments deeper in the water column. Microzooplankton grazing pressure on UCYN-C was greatest near the DCM, and it is possible that the same may be true for UCYN-B. Positive growth rates in HNP conditions for UCYN-B are not unexpected given that Crocosphaera growth and N₂ fixation are insensitive to nitrate concentrations in culture (Großkopf and LaRoche, 2012). The same is assumed to be true for UCYN-C in the NPSG, as Cyanothecae isolates belonging to this group can grow on nitrate (Agwin et al., 2007). However, the qPCR assay used in this study does target a cluster that contains uncultivated marine sequence types, as well as the endosymbiont of the freshwater diatom Rhopalodia gibba. Therefore, without knowing with better certainty the source organism in the NPSG, the ability of the organisms targeted with the UCYN-C assay to assimilate nitrate remains speculative.

Stimulated growth rates in LNP treatments, conditions expected to favor diazotrophs due to N-limitation, were only measured for Het-2 at 25 m and UCYN-C and Het-1 at 45 m (Figures 3,4,5,N). Notably, Het-2 was found only at the surface and did not have positive net growth rates in HNP treatments, unlike Het-1 (45 m). Both Het-1 and Het-2 lack the genetic capability to assimilate nitrate (Hilton et al., 2013; Hilton, 2014). The observation that Het-2 does not increase in abundance when its host is in an environment with replete nitrate availability, implies a different ecological strategy than the Het-1 symbiosis. Results from these incubations suggest that the Het-2 symbiosis has a competitive advantage in surface waters under LNP conditions in the NPSG.

Microzooplankton Actively Graze on Diazotrophs

Very little is known about mortality processes for diazotrophs in the oligotrophic marine environment. To the best of our knowledge, this is the first report of the measurement of in situ microzooplankton grazing on UCYN-A1 and UCYN-C. Interestingly, these data suggest that the rate of microzooplankton grazing differed depending on the location in the water column and the grazed diazotrophic taxa (Figure 6). UCYN-A1 experienced significant grazing pressure at the surface (GR2; m = 0.20 d⁻¹) that exceeded measured growth rates. UCYN-C was not grazed at the surface, yet UCYN-A1 and UCYN-C were present at similar abundances at this depth (Figure 1). These findings imply that the grazer assembly preyed preferentially on UCYN-A1 in surface waters. Different mortality rates measured for co-occurring photosynthetic populations (Prochlorococcus, Synechococcus, and PPEs) have been previously reported (Chen et al., 2009), and this situation appears to also be true for diazotrophs. Numerous factors contribute to preferential feeding of microzooplankton including prey size, cell surface properties, viral infection, nutrient composition, and the release of dissolved substances (Jürgens and Massana, 2008; Chrzanowski and Foster, 2014). However, cell size is generally a dominant factor determining the suitability of prey to microzooplankton consumers. UCYN-A1 lives in association with a prymnesiophyte, while UCYN-C occurs as free-living solitary cells. Thus, we speculate that differences in the size of the host-symbiont association of UCYN-A1 and the solitary cells of UCYN-C may explain the preferential grazing by the consumer assemblages on UCYN-A1 in surface waters. Size dependent grazing relationships would implicate microzooplanktonic protists (20–200 µm in size) as their consumers. This size category is dominated by ciliates, dinoflagellates and a variety of rhizarian taxa at Station ALOHA (Pasulka et al., 2013; Hu et al., unpublished data from the present study).

Interestingly, deeper in the water column, both diazotrophs were grazed at substantial rates (Figure 6), and there was general agreement between depth trends in the chl a-based grazing rates, which provide bulk microzooplankton grazing rates on the phytoplankton community, and qPCR-based grazing rates on diazotrophic taxa (Table 2 and Figure 4). The mortality rate for UCYN-C at 125 m was the highest measured (1.75 d⁻¹), and greater than both intrinsic and enriched growth rates for this cyanobacterial group. Additionally, in contrast to results from surface waters, the grazing rate on UCYN-A1 (1.00 d⁻¹) was lower than the grazing rate for UCYN-C at this depth, and was also lower than its intrinsic growth rate (1.36 d⁻¹), indicating that grazing pressure was not controlling UCYN-A1 abundances deeper in the water column. It is not clear if these depth-related differences in grazing pressure reflected differences in the feeding activities of the grazer community between the two depths, differences in the taxonomic composition of the consumer community, or both. General depression of grazing activity near the ocean surface might be inferred from the lower mortality rates observed for chl a-based measurements and the results for UCYN-C, but mortality of UCYN-A1 in surface waters was substantial in most cases. Moreover, previous work at station ALOHA (Pasulka et al., 2013) and molecular diversity studies conducted during the present study (Hu et al., unpublished; Connell et al., unpublished) have documented substantive changes in absolute and relative abundances of the various consumer taxa between surface waters and the lower euphotic zone (i.e., commensurate with the depths sampling in this study). Therefore, taxonomic composition and feeding behavior may both contribute to the depth-related differences in cyanobacterial mortality rates observed in this study.

More measurements are needed at finer depth resolution throughout the photic zone, and in different seasons (typically characterized by different dominant diazotrophic taxa) to better constrain microzooplankton grazing pressure on diazotroph populations, and to identify their consumers. However, our experiments verify that it is possible to use the dilution method.
technique to investigate mortality of diazotrophic taxa, when their abundances are high enough to remain above detection limits when diluted.

CONCLUSION

Diazotroph growth and mortality rates are critical variables for global ecosystem models. The high energetic cost associated with the ability to fix N\textsubscript{2} is typically offset in model parameterizations by assuming lower growth rates of diazotrophs relative to non-diazotrophic counterparts, as well as higher requirements for intracellular N:P and Fe:P ratios (e.g., Monteiro et al., 2010, 2011). Collectively, these assumptions are based on the tradeoffs of an organism which is never N-limited and the high energetic cost of N\textsubscript{2} fixation. However, these assumptions are based primarily on Trichodesmium data and observations, and are validated by very few in situ measurements, particularly for unicellular diazotrophs. Furthermore, estimates of grazing on diazotrophs have been based on a simple interaction between predator and prey based on size (Monteiro et al., 2010) and sometimes palatability (Dutkiewicz et al., 2009). For these reasons, direct measurements of growth rates and microzooplankton grazing rates on diazotrophs are valuable, especially considering that for many of the important diazotroph groups, we are unable to define these terms using culture-based experiments.

The in situ growth rate experiment (G1) yielded several surprising results. In general, in situ net growth rates measured were high, often comparable to or greater than culture-based measurements (when comparisons can be made). LNP treatments, which were anticipated to provide diazotrophs with a competitive advantage, were not always the favorable conditions for growth at a given depth. Net growth rates for most diazotroph taxa appeared to be stimulated in the HNP treatments. More research is needed to determine whether the N needed for growth under these conditions was supported by assimilation of nitrate, or N\textsubscript{2} fixation, acknowledging that the answer to this question is likely taxa-specific. However, these results suggest that the availability of DIN may be favorable on short time scales for natural populations of diazotrophs.

This is the first study to directly measure microzooplankton grazing rates on UCYN-A1 and UCYN-C. Future studies should focus on identifying the potential consumers of these minute diazotrophs, as well as using dilution grazing experiments to constrain mortality at higher spatial and temporal resolution. However, together with previously reported observations of copepod grazing on diazotrophs (O’Neil et al., 1996; O’Neil, 1998; Hunt et al., 2016; Conroy et al., 2017), these findings provide further indication that diazotroph derived N is, in some cases, directly transferred to higher trophic levels through grazing.

In summary, the high in situ net growth rates reported here, the competitive ability of some taxa in N-replete conditions, and measurement of depth-dependent microzooplankton grazing on diazotrophs, provide new insight into their ecological roles, and indicate that current parameterizations for this functional group in global ecosystem models may need reevaluation.

AUTHOR CONTRIBUTIONS

KT-K, HF, and JZ designed the growth rate experiment. PC, DC, KT-K, and JZ designed the grazing experiments. MH, KT-K, PC, and DC executed the experiments at sea. KT-K processed all samples for molecular and flow cytometry analysis, with help from MH. PC and DC processed chlorophyll samples from the grazing experiments. KT-K analyzed the data sets, with input from PC and DC on grazing rate experimental data. KT-K wrote the manuscript with input from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01616/full#supplementary-material

FIGURE S1 | Results from growth rate experiments conducted in the vicinity of Station ALOHA in July 2015. Experiments were conducted and samples were processed as described for G1, but with water from a single depth (25 m) incubated in deck board incubators with in situ simulated light and temperature. The incubation was conducted during a Crocosphaera bloom described in detail in Wilson et al., 2017, but other diazotroph taxa were present (A). Differential responses in net growth rates to HNP and LNP conditions were not seen for any of the phototrophic phytoplankton groups (B). Diazotroph growth rates for the dominant diazotrophs (C–H) were uniformly high across all treatments and the controls, similar to the results from G1 at 45 m.

TABLE S1 | Growth rate data used in Figure 5.

TABLE S2 | OTU table, which includes taxonomy strings for each OTU for 16S rRNA data from G1.

TABLE S3 | Growth rate data used in Figure 5.

TABLE S4 | Growth rate data used in Figure 5.
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