INTRODUCTION

Nitrogen (N) is an essential requirement for life on Earth, but the largest reservoir — N₂ gas — is only directly accessible to N₂-fixing microbes known as diazotrophs. In marine systems, these diazotrophs are the only autochthonous source of new N, complementing allochthonous inputs from rivers and the atmosphere. In the past, N₂ fixation in marine environments has been associated with cyanobacteria such as *Trichodesmium*, but this focus has been expanding rapidly. N₂ fixation activity has recently become associated with a greater diversity of microbes including unicellular cyanobacteria (Zehr & Turner 2001, Montoya et al. 2004, Moisander et al. 2010, Zehr 2011) and heterotrophic bacteria (Rahav et al. 2013, Benavides et al. 2016, Jayakumar et al. 2017), and a greater diversity of locations than traditionally considered including colder waters (Großkopf & LaRoche 2012, Sipler et al. 2017), the aphotic water column (Benavides et al. 2016), oxygen minima zones (Fernandez et al. 2011, Jayakumar et al. 2017), and in deeper sulfidic waters yielded mRNA transcripts of *nifH*, even though NH₄⁺ was 1−5 µM. Multiple phylogenetic groups expressed *nifH*. Three uncultured groups of Cluster III type transcripts were detected, as well as 2 groups of Cluster I type sequences related to known sulfur oxidizers in the ε-proteobacteria and *Halorhodospira*. The depth range where N₂ fixation was found was also the depth range of chemoautotrophic production, as determined by a maximum in suspended organic nitrogen concentrations and from 16S rRNA at these depths, which was dominated by known chemoautotrophs *Sulfurimonas*, SUP05, and BS-GSO2. We suggest chemoautotrophy and competition with chemoautotrophs for ammonium as reasons for N₂ fixation in the presence of ammonium. Profiles of N₂ gas unequivocally show the importance of N loss in the suboxic zone of the Black Sea; however, our data suggest a role for N₂ fixation. These results suggest that N cycling is seldom unidirectional.

KEY WORDS: N₂ fixation · *nifH* · Black Sea · N cycling · Redox gradient
Potential N₂ fixation has been documented in many low oxygen systems, including all 3 of the largest oceanic oxygen-deficient zones (ODZs; O₂ < 10 nM), in the Equatorial North and South Pacific Ocean and the Arabian Sea (Fernandez et al. 2011, Jayakumar et al. 2012, Cheung et al. 2016). ODZs differ fundamentally from restricted basins because they lie over oxic rather than sulfidic waters. ODZs contain high concentrations of dissolved nitrate (NO₃⁻), though less than predicted from Redfield stoichiometry due to denitrification. The differences in Gibbs free energy of reactions between N₂ fixation (ΔG° = 87 kcal) and NO₃⁻ assimilation (ΔG° = 69 kcal) are small (Falkowski 1983). N₂ fixation in anoxic environments avoids the problem of oxygen permanently damaging the nitrogenase enzyme (Großkopf & LaRoche 2012, Bombard et al. 2016). Experiments with Crocosphaera indicate that much of the indirect energy costs of N₂ fixation are related to removal of oxygen from carbohydrate production followed by oxygen scrubbing by respiration of these carbohydrates, implying that N₂ fixation would be slightly more favorable than NO₃⁻ uptake under low oxygen conditions (Großkopf & LaRoche 2012). However, even in the euphotic zone, N₂ fixation can occur in the presence of NO₃⁻ if abundant phosphate is present (Knapp 2012). Techniques of single-cell bacteria for protecting nitrogenase from oxygen include photoautotrophs fixing N₂ at night and increasing respiration to consume O₂, and heterotrophs potentially producing extracellular organic polymers or attaching to particles to slow oxygen diffusion (Bombard et al. 2016). In contrast, N₂ fixation in the presence of NH₄⁺, such as can be found in restricted basins, is more surprising given the paradigm that N₂ fixation is an option of last resort because of its high energetic cost when compared to the use of NH₄⁺ in particular (Leigh & Dodsworth 2007). Nonetheless, evidence of N₂ fixation has been found in restricted basins such as the Baltic Sea (Farnelid et al. 2009, 2011), a deep-sea hypersaline basin in the Mediterranean Sea (Pachiadaki et al. 2014), and the meromictic Lake Cadango (Halm et al. 2009). More surprisingly, in the benthic sedimentary environment, N₂ fixation has been measured in the presence of >100 µM NH₄⁺ (Knapp 2012).

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Sulfide is common in restricted basins; in the redoxcline of the meromictic sulfide-rich Lake Cadacono, Switzerland, N₂ fixation by anaerobic consortia including the sulfur-oxidizing anoxygenic phototroph *Chlorobium* was documented (Halm et al. 2009). The Black Sea is also known for a very low-light-adapted population of *Chlorobium* (Manske et al. 2005), but the transcriptional activity and phylogeny of N₂-fixing microbes in the world’s largest anoxic basin has not been documented.

In the present study, we investigated N₂ fixation in the oxic, suboxic, and sulfidic zones of the northeast (NE) Black Sea using DNA, RNA, nutrients, and isotope ratio NO₃⁻ samples collected in May and October 2007.

**MATERIALS AND METHODS**

**Study site and sampling**

The Black Sea is a permanently anoxic basin with a well-defined redox gradient. Because of cyclonic gyre circulation (Poulain et al. 2005), isopycnal surfaces vary in depth within the basin and shoal in the central gyre regions (Murray et al. 1995). For this reason, most chemical gradients and features are found at different depths at different locations but commonly occur on the same density surfaces. To make data sets from different locations comparable, we plotted most of our data versus density, rather than depth.

In most of the Black Sea, the cold intermediate layer, with a characteristic core density of σθ ≈ 14.5, represents the lower boundary of water with direct contact with the surface. The oxycline and suboxic zone lie between the oxic cold intermediate layer and a sulfidic zone that stretches to the seafloor (maximum depth >2 km). Sampling was conducted in the NE Black Sea at a water depth of more than 1 km (single station: 44.35 ± 0.5°N, 37.7 ± 0.2°E). Care was taken to cross the Rim Current (Poulain et al. 2005), to minimize the influence of coastal waters. Samples were collected aboard the RV ‘AKBAHABT’ on 19–21 May and 3–5 October 2007. Water sampling was conducted with a CTD (SeaBird Electronics) rosette system, with 5 l Niskin bottles. Dissolved oxygen, nitrate, nitrite, ammonium, and hydrogen sulfide were measured using standard methods (Grashoff et al. 1999) within 24 h of sampling. Nitrate was reduced to nitrite using a cadmium column, and nitrite was measured using sulphanilamide and N[1-naphthyl]-ethylenediamine using a 2-channel Technicon Autoanalyzer II system. The detection for nitrite and nitrate was 0.02 μM. Ammonium was analyzed spectrophotometrically using the indophenole blue procedure. This technique has a detection limit near 0.3 μM. Oxygen and sulfide flasks were purged with argon prior to being filled to reduce oxygen contamination. Oxygen was measured using the Winkler method and sulfide was measured by iodometric titration. The detection limit for Winkler oxygen was between 1 and 2 μM. The detection limit for sulfide analyses was 0.3 μM (Stunzhas & Yakushev 2006). CTD and nutrient data for these cruises are publicly available from The Biological and Chemical Oceanography Data Management Office (BCO-DMO): www.bco-dmo.org/dataset-deployment/454619 for May and www.bco-dmo.org/dataset-deployment/454620 for October.

**Stable isotopes and geochemistry**

Suspended particulate organic matter (POM) samples were collected in 5 l acid-washed plastic bottles. Particulate material was filtered immediately onto precombusted 0.7 μm Whatman glass fiber filters, and dried in an oven at 60°C. Dried filters were subject to HCl fumes for <48 h in a vacuum dessicator without dessicant, before re-drying with dessicant. Nitrogen concentrations in the POM samples were measured in the Stable Isotope Laboratory, School of Oceanography, University of Washington, with a DeltaPlus Finnigan connected to a NC2500 CE Instruments Elemental Analyzer by a Finnigan MAT ConFlo II. No N was detected in blanks.

Frozen water samples from October 2007 were analyzed for both δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ using the denitrifying method, where denitrifying bacterium *Pseudomonas aureofaciens* transformed nitrate to N₂O, a gas measurable on the mass spectrometer (Casciotti et al. 2002). IAEA-N3, USGS 34 and USGS 35 were used as standards. Only samples with ≥0.7 μM nitrate were examined. Samples were analyzed at the University of Washington, Quaternary Research Center, on a DeltaPlus mass spectrometer with a Finnegan Precon system and GasBench. A blank, containing only bacteria and media, was analyzed for N₂O with every run and found to be negative. All samples were analyzed in duplicate. Error propagation included the standard deviation of duplicates samples as well as of the triplicate IAEA-N3 standards. Nitrite was not removed from the samples, but was below 0.06 μM.

δ¹⁸O-H₂O was analyzed on a mass spectrometer by Eric Steig’s laboratory at the University of Washington, using methods detailed in Steig et al. (2013).
Methane concentrations were obtained in October 2007 from 120 ml bottles with a 12 ml headspace. Samples were shaken and left to equilibrate before measuring on a gas chromatograph (LHM-80) with flame ionization detection. The methods are described in Yakushev et al. (2006).

**DNA and RNA analyses**

Samples for DNA and RNA extraction were collected directly from the Niskin bottles, using Millipore Sterivex filters in-line with a vacuum trap. RNA filtration was conducted first, then RNA was immediately fixed with RNAlater® (Ambion), sealed, refrigerated for approximately 1 h, and then frozen (−20°C). Care was taken to limit filtration to ≤30 min (typically 1–2 l). DNA filtration was subsequently conducted, and filters were sealed and frozen. Initial transport to Moscow (1 d) was conducted with cold packs and blocks of ice, after which samples were transferred to dry ice and shipped to the University of Washington. RNA extractions were conducted using a modified version of Poretsky et al. (2005; methods supplement). After thawing, the RNAlater® was centrifuged at maximum speed and the supernatant was discarded; the resultant pellet and excised filter paper were suspended with Buffer RLT of the Qiagen RNeasy Mini Kit, and bead-beating was conducted 4 times for 30 s each using 0.1 and 0.05 mm quartz-silica beads. After centrifugation (10 min at maximum speed), the supernatant was applied to a Qiagen RNeasy column and purified as per the manufacturer’s instructions. DNase digests were conducted with an on-column DNase I (Qiagen). cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen), using gene-specific primers (nifH623R; Steward et al. 2004), and including controls for DNA contamination in all cases (i.e. reagents without transcriptase). For DNA filters, extractions were performed using a combined freeze−thaw and enzymatic method, as per Fuchsman et al. (2011). RNA samples examined for nifH expression can be seen in Table 1.

cDNA and DNA amplification was conducted for nifH with an initial reaction using the primers nifH32F (5’-TGA GAC AGA TAG CTA TYT AYG GHA A-3’) and nifH623R (5’-GAT GTT CGC GCG GCA CGA ADT RNA TSA-3’) (Steward et al. 2004; 50°C annealing temperature, 34 cycles) and second amplification with nifH1 (5’-TGY GAY CCN AAR GCN GA-3’) and nifH2 (5’-ADN GCC ATC ATY TCN CC-3’) (Zehr & Turner 2001; 57°C annealing temperature, 34 cycles). For PCR reagents, 2X PCR Master Mix (Fermentas) was used. For nifH only, supplemental MgCl₂ was added for a final concentration of 6 mM. For terminal restriction fragment length polymorphisms (TRFLP), a tagged version of nifH1 (5’-[6-FAM]) was used. Similar to Farnelid et al. (2009), to test for nifH contamination, amplification without sample was conducted in duplicate for different stocks of nuclease-free H₂O. No bands were detected in this control via gel electrophoresis, but gel pieces were excised for the expected fragment size. DNA was purified using Qiagen gel purification spin columns, amplicons were first reconditioned to reduce heteroduplexes (Thompson et al. 2002), and cloning was conducted in duplicate using the StrataClone PCR Cloning Kit (Agilent Technologies).

Clones were sequenced using Sanger sequencing by the High-Throughput Genomics Unit (www.htseq.org). Using the Sequencher program (Gene Codes Corporation), vector and primer sequences were removed and chromatograms were hand-checked for error. Black Sea DNA and cDNA sequences were grouped separately at the 98% amino acid level and representative sequences from the interior of each cluster were used in phylogenetic trees. Cloned PCR controls yielded no nifH sequences. Phylogenetic trees were made and bootstrapped with RAxML (Stamatakis 2014). Sequence data from this study have GenBank accession numbers JN638619–JN638721 and KY069980–KY070119.

**Table 1.** nifH mRNA expression. Samples in which mRNA amplification was attempted are indicated for both May and October 2007. NA (not applicable) indicates no samples were available. nd (not detected) indicates that amplification was unsuccessful. Clusters I and III indicate the phylogenetic affiliation of amplified nifH. σθ: core density
16S rRNA and rDNA TRFLP analyses were performed for October 2007 samples using 27F-FAM and 1517R exactly as in Fuchsman et al. (2011). PCR products were cut with 4 restriction enzymes (HaeIII, Hpy1881, MspI, and MnlI) and analysis was performed on a MegaBACE 1000 apparatus in the Armbrust Lab at the University of Washington. Electrophoretic profiles were visualized with Dax software (Van Mierlo Software Consultancy). TRFLP profiles were normalized by total peak height. Most identified peaks were previously identified in Black Sea samples in Fuchsman et al. (2011, 2012). The Methylobacter phylotype was identified using in silico analysis of Black Sea sequences from Vetriani et al. (2003).

For nifH TRFLP, purified PCR products (QiaQuick columns; Qiagen) were separately digested for 2−6 h with 2 restriction enzymes (SetI and Hpy188III) and immediately ethanol precipitated according to the manufacturer’s instructions (Amersham Pharmacia Dynamics). Analysis was performed as above.

**RESULTS AND DISCUSSION**

**No evidence for oxic N$_2$ fixation**

The mixed-layer depth for both cruises (May and October) was 8−12 m. Oxygen concentrations were high in and right below the mixed layer, before decreasing with depth (Fig. 1). Nitrate concentrations increased below the euphotic zone to a maximum of 6–7 µM at $\sigma_0 = 15.5$ (120–130 m; Fig. 1).

In May 2007, conditions were particularly unfavorable for N$_2$ fixation in surface waters as surface nitrate...
was relatively high (0.6 µM) (Fig. 1). Though dissolved N:P ratios were typically <5 in surface waters in the central Black Sea (Fuchsmann et al. 2008), N:P ratios in May were also greater than Redfield with values >20 (data not shown). Diatoms (Chaetoceros curvisetus) were the dominant phytoplankton immediately prior to our May cruise (Silkin et al. 2014).

In October 2007, NO$_3^-$ was below detection in surface waters but NH$_4^+$ was detectable at low levels and N:P ratios were 8–10 in the top 50 m. Transcripts of Cluster I, II, and III type dinitrogen reductase (nifH) can be used as a proxy for N$_2$ fixation capacity (Bombar et al. 2016). Transcripts for nifH were not found in oxic waters in May and October 2007. We used in situ nitrate isotopes ($\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$) below the mixed layer, which integrate over long time periods, to ascertain the absence of appreciable N$_2$ fixation in surface waters. Nitrogen fixation in surface waters should produce isotopically light organic matter, which should sink and remineralize, affecting $\delta^{15}$N of nitrate (Knapp et al. 2008). Since nitrate assimilation and denitrification affect $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ equally (Granger et al. 2004, 2008), an enrichment in $\delta^{18}$O-NO$_3^-$ compared to $\delta^{15}$N-NO$_3^-$ could indicate N$_2$ fixation (Knapp et al. 2008). However, in the NE Black Sea in October 2007, $\delta^{15}$N-NO$_3^-$ (6–8‰) and $\delta^{18}$O-NO$_3^-$ (3–5‰) tracked together closely and mirrored the concentration profile (Fig. 2). No salinity $\delta^{18}$O-NO$_3^-$ correction (Knapp et al. 2008) was needed here since the total change in seawater $\delta^{18}$O for these depths ($\sigma_θ = 14.8$ to 15.9) was <0.4‰ (see Fig. A1 in the Appendix). After normalizing the profiles to values at $\sigma_θ = 15.5$, the deviation of $\delta^{18}$O versus the deviation of $\delta^{15}$N nitrate had a slope of 1.2 (Fig. 2). A slope of 1 is expected if nitrate assimilation and denitrification are the primary factors affecting nitrate (Granger et al. 2004, 2008). Three data points in the $\sigma_θ = 15.6$–15.8 range at the top of the suboxic zone had an enrichment in $\delta^{18}$O (Fig. 2), which we hypothesize was due to rapid cycling of remineralization and nitrite oxidation (Sigman et al. 2005, Frey et al. 2014). While an enrichment in $\delta^{18}$O-NO$_3^-$ compared to $\delta^{15}$N-NO$_3^-$ cannot prove the existence of N$_2$ fixation due to uncertainties related to this rapid cycling (Knapp et al. 2008), the lack of enrichment in oxic waters indicates that N$_2$ fixation was not an important source of N in oxic waters in October and in the preceding months.

Despite the lack of geochemical or mRNA evidence for appreciable N$_2$ fixation in the top 50 m of the water column in either May or October 2007, nifH DNA indicated N$_2$ fixation potential. This potential for N$_2$ fixation included detectable DNA from Cluster I and III diazotrophs in May (Figs. 3 & 4) but only...
Cluster III sequences in October 2007 (Fig. 3). All known conventional marine photosynthetic diazotrophs are in Cluster I, including colonial organisms such as *Trichodesmium* and single-celled genera such as *Crocosphaera*. Notably, no sequences associated with these common photosynthetic diazotrophs were found at this Black Sea station (Fig. 4). Some *nifH* DNA sequences from the top 50 m of the water column cluster with sequences found in the suboxic zone, including *Chlorobium* (Figs. 3 & 4). *Chlorobium phaeobacteroides* was originally cultured from the Black Sea and is known as an extremely low-
light-adapted green sulfur bacterium which needs sulfide to metabolize (Manske et al. 2005). Thus *Chlorobium* cannot be active in the oxic zone and must be brought there by mixing or active transport by zooplankton (Grossart et al. 2010). Other examples are represented by sequences in uncultured clusters (Figs. 3 & 5). The presence, in the top 50 m of the water column, of *nifH* DNA sequences do not indicate activity.
Suboxic zone geochemistry

A suboxic zone, where O₂ was less than 10 µM and H₂S was not detectable, was observed in both May and October 2007. The suboxic zone was relatively compressed in May due to both increased O₂ penetration from above and H₂S from below. The onset of sulfide varied from σθ = 16.06 (152 m) in May to σθ = 16.11 (144 m) in October. In the Black Sea, there is a constant flux of NH₄⁺ into the suboxic zone from the sulfidic zone, causing ammonium to be measurable at the bottom of the suboxic zone (Fig. 1; Fuchsman et al. 2008). For both cruises, nitrate had a maximum at the top of the suboxic zone and decreased with depth. NO₃⁻ was measurable at deeper isopycnsals than seen in the central Black Sea (Fig. 6). In May, NO₃⁻ reached σθ = 15.97 (145 m) where NH₄⁺ concentrations were 1.2 µM. In October, NO₃⁻ reached σθ = 16.17 (153 m) where ammonia concentrations were already 3.5 µM. Nitrite concentrations were always below 0.05 µM.

Suboxic N₂ fixation potential

DNA sequences found in the suboxic zone in this study (May σθ = 15.5, 16.1; October σθ = 15.8) include both Cluster I and Cluster III type nifH (Figs. 3 & 4; Chien & Zinder 1996). Many of these sequences were not closely related to characterized bacteria, the exception being a singleton similar to *Methylobacter tundripaludum*, a known methane oxidizer (Wartiainen et al. 2006), and 7 sequences that showed ≤1.8% amino acid divergence from the extremely low-light-adapted green sulfur bacterium *Chlorobium phaeobacteroides BS1*. This DNA profile is somewhat similar to RNA data from Lake Cadango (Halm et al. 2009), where *nifH* sequences from a meromictic lake were dominated by *Chlorobium* with some *Methylobacter* expression also detected (Figs. 3 & 4).

Suboxic nifH expression

Expression of *nifH* mRNA was investigated as a proxy for microbial N₂-fixing activity. No expression was found in the upper suboxic zone (σθ < 15.9) where nitrate was greater than 1 µM (Table 1). However, expression was detected via amplification for σθ = 15.9 and 16.1 in May, and for σθ = 16.1 and 16.3 in October. There was no operational taxonomic unit overlap between the 2 sets of RNA sequence data. All of the expressed sequences in May were Cluster III (Figs. 3 & 4). Cluster III includes many known sulfate reducers, including *Desulfovibrio* and *Desulfatibacillum*. Both mRNA and DNA from the Black Sea are well integrated with sequences from other low oxygen environments (Figs. 3 & 4). There were 3 mRNA clusters found in May. One mRNA cluster (JN638662) was closely related to sequences from the Eastern Tropical South Pacific as well as the suboxic San Pedro Basin and Chesapeake Bay bottom waters (Zehr et al. 2003, Hamersley et al. 2011, Bonnet et al. 2013). A second mRNA cluster (JN638658) was closely related to a sequence from San Pedro Basin (Hamersley et al. 2011). A third (JN638653) appeared similar to cultures from the Baltic Sea (Bentzon-Tilia et al. 2014).

*nifH* expression in October was only detected for Cluster I *nifH*, in contrast to the Cluster III in May. The closest characterized genera to the sequence JN638621 expressed in October were in the class ε-proteobacteria, and the closest genus to the sequence JN638632 was *Halorhodospira*, a phototrophic purple sulfur bacterium. The ε-proteobacteria cluster included a sequence group that was also found on the continental shelf in the Eastern Tropical South Pacific in the presence of sulfide (Loescher et al. 2014). Both of these cultured organisms are associated with oxidation of reduced S species, suggesting that S and N cycling in ODZ and anoxic basins may be more coupled than previously thought.

Depth profiles of *nifH* DNA TRFLP give more complete contours for the presence of N₂-fixing phytypes. Despite the difference in expression, the data indicate that these N₂-fixing organisms were present at both sampling dates and were primarily located in the suboxic–anoxic transition zone. Two phytypes were identified, one from each cluster, and neither were found shallower than σθ = 15.9. *Halorhodospira*-like *nifH* had a maximum at σθ = 15.95 in May and was more variable in October (Fig. 5). The JN638653-type *nifH* had a maximum around σθ = 16.0 in both May and October, but comprised a greater proportion of the community in October (Fig. 5). The ε-proteobacteria mRNA sequence was not identified in the *nifH* TRFLP, but TRFLP was not performed into the depths of the sulfidic zone, where its mRNA was found (σθ = 16.3). Other known *nifH* types were not resolvable with the given restriction enzymes.

Anoxic phototrophs and methanotrophs as N₂ fixers: further offshore?

Despite their abundance in the *nifH* DNA, photosynthetic green sulfur (*Chlorobium*) and methane-
oxidizing (Methylobacter) bacteria showed no evidence of N₂ fixation in the nifH expression data from the NE Black Sea (Figs. 3 & 4). *Chlorobium* did not appear to be active at all in the NE Black Sea. Both bacteria were present in 16S rDNA data sets, but when rRNA was amplified from the cDNA, Methylobacter-type 16S rRNA was present but *Chlorobium* was not (Fig. 6). The onset of sulfide at this station (144−154 m) was significantly deeper than seen in the Central Gyre. This supports previous work that has shown *Chlorobium* to be dormant in the periphery of the Black Sea where isopycnals containing sulfide are deeper than light can penetrate (Marschall et al. 2010).

While N₂ fixation by *Chlorobium* and Methylobacter was apparently repressed at the nearshore station reported here, where NO₃⁻ and NH₄⁺ profiles overlap (Fig. 6), nutrient profiles suggest potential N₂ fixation niches farther offshore. Unlike the station used in the present study, in the Western Gyre and the majority of the offshore region, NO₃⁻ and NH₄⁺ gradients exhibit greater separation and do not overlap (ca. 90 m; Fig. 6). In the Central Western Gyre, some of the suboxic zone was at least limited by fixed N, and, importantly for *Chlorobium*, sulfide was found nearer to the sunlit surface (Fuchsman et al. 2011). Regarding Methylobacter, methane concentrations were highest in the sulfide layer but were still measurable in the lower suboxic zone (Fig. 6). The Black Sea Methylobacter lives in the suboxic–anoxic transition zone, where methane appears to be consumed (Fig. 6). While NO₃⁻ and NH₄⁺ were present at these depths in the NE Black Sea, in the central Black Sea, Methylobacter lives in the depth range where N may be limiting (Fig. 6). Thus, though not active as N₂ fixers here, these 2 taxa still could be significant N₂ fixers in the offshore Black Sea. In 2001, N₂ fixation rates of up to 54 nmol kg⁻¹ d⁻¹ were measured in the suboxic zone of the offshore Black Sea (McCarthy et al. 2007), indicating N₂ fixation activity in this region.

Active suboxic diazotrophs: why fix N₂?

A growing body of evidence suggests that N₂ fixation occurs in the presence of high concentrations of fixed N in the environment, including marine phototrophic diazotrophs in the presence of NO₃⁻ (Voss et al. 2004, Holl & Montoya 2005); sedimentary heterotrophic N₂ fixers with NO₃⁻ or NH₄⁺ (Fulweiler et al. 2008, Knapp 2012); oxygen minimum zone N₂ fixers in the presence of NO₃⁻ (Fernandez et al. 2011, Jayakumar et al. 2012, Bonnet et al. 2013, Cheung et al. 2016); and anoxygenic photosynthetic and sulfate-reducing bacteria with and without NH₄⁺ (Halm et al. 2009). Here, we showed that expression

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**Fig. 5.** nifH terminal restriction fragment length polymorphism (TRFLP) profiles. Depth profiles of nifH DNA from TRFLP for 2 bacteria identified in *nifH* mRNA: (A) the Cluster I Halorhodospira-like clade and (B) the unknown Cluster III Black Sea sequence JN638653. Peak height is shown in relative fluorescence units (rfu). Arrows indicate the depth at which *nifH* mRNA for each organism was sequenced.
Fig. 6. Terminal restriction fragment length polymorphism (TRFLP) profiles of *Chlorobium* and *Methylobacter*. (A) Nitrate and ammonium concentrations and *Chlorobium* TRFLP peak height in relative fluorescence units (rfu) for the Western Gyre in 2005 (Fuchsman et al. 2011) and (C) in the northeast Black Sea in October 2007. (B) Methane concentrations (Fuchsman et al. 2011) and TRFLP peak height for methane-oxidizer *Methylobacter* for the Western Gyre. *Methylobacter* AY360488 was sequenced from the Black Sea from 1988 samples (Vetriani et al. 2003). (D) Methane concentrations and TRFLP peak height for *Methylobacter* for both 16S rRNA and 16S rDNA from the northeast Black Sea in October 2007. 16S rRNA from cDNA was not measured in the Western Gyre and was not detectable for *Chlorobium* in October 2007. Dashed lines indicate the suboxic zone.
of N₂ fixation genes was found in the presence of 1−5 µM NH₄⁺ in the transition zone of the Black Sea. It is unknown if expression continues below the depths sampled, where NH₄⁺ concentrations continue to increase to ~100 µM in bottom waters. While NO₃⁻ and NO₂⁻ require reduction before incorporation into biomass, NH₄⁺ does not, making fixation in the presence of the latter particularly puzzling, but not unprecedented. N₂ fixation has been measured in the presence of >100 µM NH₄⁺ in benthic environments and linked to sulfate reducers (Knapp 2012).

The N₂-fixing organisms reported here were found in a region of high chemoautotrophy associated with oxidation of reduced S. Chemoautotrophic activity in the transition zone in the Black Sea has been well documented (Yilmaz et al. 2006), and the suboxic–anoxic transition zone (σθ = 16.2–16.4) commonly exhibits a maximum in suspended particulate organic nitrogen (S-PON; Fig. 7) (Coban-Yildiz et al. 2006). The S-PON concentration maximum was only 0.45 µM at σθ = 16.0–16.1 in May 2007, but a notable S-PON concentration maximum of 0.7 µM was observed in October 2007 at σθ = 16.3 (Fig. 7). The

Fig. 7. Chemosynthesis maxima from May and October 2007. (A) Depth profile of 16S rRNA terminal restriction fragment length polymorphisms (TRFLP) from cDNA sampled in May 2007 for the 3 bacteria found to be chemosynthetic at the chemosynthesis maximum in Glaubitz et al. (2010). (B) Suspended particulate organic nitrogen (S-PON) concentrations for May 2007. (C) Depth profile of 16S rRNA TRFLP for the same 3 bacteria from cDNA sampled in October 2007. (D) S-PON concentrations for October 2007. Dashed lines indicate the boundaries of the suboxic zone. TRFLP peak height is shown in relative fluorescence units (rfu).
chemosynthesis maximum is known to be dominated by γ- and ε-proteobacteria (Grote et al. 2008), notably *Sulfurimonas*, SUP05, and BS-GSO2 bacteria (Glaubitz et al. 2010). *Sulfurimonas* has been linked to autotrophic denitrification in the Black and Baltic Seas (Brettar et al. 2006, Fuchsman et al. 2012). SUP05 is potentially an S-oxidizing nitrate reducer (Shah et al. 2017), and the role of BS-GSO2 is still unknown. From our mRNA samples, we detected expression of the 16S rRNA gene from *Sulfurimonas*, SUP05, and BS-GSO2 bacteria in both May and October 2007 (Fig. 7). In October, the 16S rRNA had a maximum at $\sigma_\theta = 16.3$, with the largest contribution due to the *Sulfurimonas* (Fig. 7). SUP05 and BS-GSO2 appeared to be more active in May than in October (Fig. 7). Autotrophic anammox bacteria were also present and active until $\sigma_\theta = 16.1$ in May and $\sigma_\theta = 16.3$ in October (Kirkpatrick et al. 2012). These shifts in the chemosynthesis maximum coincide with the shoaling of sulfide in May compared to October (Fig. 1).

We postulate that N₂ fixation may be a strategy to avoid competition for NH₄⁺ substrate at relatively low concentrations with rapid assimilatory (chemosynthesis) and dissimilatory (anammox) uptake by other microbes. Inhibition of N₂ fixation may also be relaxed by the availability of reducing equivalents in the sulfidic zone, minimizing redox drain for N₂ fixing cells (Leigh & Dodsworth 2007). Organisms in the presence of abundant sulfide may be using N₂ fixation as a way of dumping electrons and regulating the intracellular redox state (McKinlay & Harwood 1982). Extended sampling deeper into NH₄⁺-rich anoxic waters, quantification of transcripts, or other methods targeting diazotrophic activity could shed light on the range and/or NH₄⁺ thresholds for N₂ fixation in this environment.

In this context, it is interesting to note that on a thermodynamic basis, the reduction of N₂ with sulfide as electron donor may be Gibbs free energy neutral or even energy yielding at these depths. To test the effect that the availability of reductants (electron donors) may have on the thermodynamics of N₂ fixation in the presence of sulfide, we calculated the in situ free energy yield of N₂ fixation coupled to sulfide oxidation:

$$\text{N}_2 + \text{HS}^- + 4\text{H}_2\text{O} + \text{H}^+ \rightarrow 2\text{NH}_4^+ + \text{SO}_4^{2-} + \text{H}_2 \quad (1)$$

To calculate in situ free energy yields, we used:

$$\Delta G_f = \Delta G^o_f + RT\ln \frac{[\text{NH}_4^+][\text{SO}_4^{2-}][\text{H}_2]}{[N_2][\text{HS}^-][\text{H}^+]} \quad (2)$$

where $R$ is the ideal gas constant. A $\Delta G^o_f$ for Eq. (1) of 30.80 kJ mol⁻¹ was calculated based on $\Delta G^o_f$ of products and reactants from Amend & Shock (2001). We used values of $[\text{NH}_4^+]$, [HS⁻], pH and temperature measured in October (for density surfaces $\sigma_\theta = 16.1$ and $\sigma_\theta = 16.3$: $[\text{NH}_4^+] = 0.9$ and 4.5 µM; $[\text{HS}^-] = 0.3$ and 11.1 µM; pH of 7.89 and 7.91; and $T$ of 8.4°C and 8.5°C, respectively). We used dissolved concentrations of N₂ and sulfate from previous data (N₂ = 592 µM and sulfate = 17 mM; Fuchsman et al. 2008, Jørgensen et al. 2001, respectively) and assumed biological control of H₂ in the presence of sulfate (Hoehler et al. 1998), i.e. 1 nM or less. Activity coefficients were calculated using the Davies equation, using $I = 0.42$ for HS⁻. Activity coefficients for H₂ and N₂ were 1.2 (Amend & Shock 2001), and 0.128 for $\text{SO}_4^{2-}$ based on a salinity of 20 (Millero & Schreiber 1982). For $\sigma_\theta = 16.1$, $\Delta G = -2.4$ kJ mol⁻¹, and for $\sigma_\theta = 16.3$, $\Delta G = -3.2$ kJ mol⁻¹. Thus, *nifH* transcripts were detected at the same interfaces where the energetic cost of N₂ fixation in the presence of sulfide was eliminated, even becoming energetically favorable.

**CONCLUSIONS**

We have, for the first time, documented the activity of 5 distinct sequence types of Cluster III and Cluster I N₂-fixing bacteria in the lower suboxic and sulfidic layers of the Black Sea. This is inferred by the expression of *nifH* sequences. Many of these mRNA sequences were associated with S-cycling bacteria. The depth range of *nifH* transcription was in the zone of high chemoautotrophic activity and where NH₄⁺ was 1–5 µM. We suggest chemoautotrophy or competition with chemoautotrophs as a motivation for N₂ fixation in the presence of ammonium. In contrast, the lack of *nifH* mRNA and the stable isotopes of NO₃⁻ in oxic waters are consistent with no N₂ fixation in those waters during this time period. These data indicate where models of N cycling in this and, potentially, other anoxic basins may benefit from rate measurements of N₂ fixation to better understand the implications of this activity. Changes between May and October of the same year in the density levels of both the onset of oxygen and the onset of sulfide, and the activity of bacteria from 16S rRNA and *nifH* mRNA indicate that the Black Sea is not a stagnant system.

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**Appendix.** Additional data regarding oxygen isotope data and corrections

Fig. A1. δ¹⁸O-H₂O depth profile from the northeast Black Sea in May and October 2007; the dashed line represents data from the Western Gyre in 1995 (Rank et al. 1999). These δ¹⁸O-H₂O data were used to check for necessary corrections of δ¹⁸O-NO₃⁻ calculations

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