Salinity as a key control on the diazotrophic community composition in the Southern Baltic Sea

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Abstract. Over the next decade, the Baltic Sea is predicted to undergo severe changes including decreased salinity due to altered precipitation related to climate changes. This will likely impact the distribution and community composition of Baltic Sea dinitrogen (N_2) fixing microbes, of which especially heterocystous cyanobacteria are adapted to low salinities and may expand to waters with currently higher salinity, including the Danish Strait and Kattegat, while other high-salinity adapted N_2 fixers might decrease in abundance.

In order to explore the impact of salinity on the distribution and activity of different diazotrophic clades, we followed the natural salinity gradient from the Eastern Gotland and Bornholm Basins through the Arkona Basin to the Kiel Bight and combined N_2 fixation rate measurements with a molecular analysis of the diazotrophic community using the key functional marker gene for N_2 fixation *nifH*, as well as the key functional marker genes *anf* and *vnf,* encoding for the two alternative nitrogenases.

We detected N_2 fixation rates between 0.7 and 6 nmol N L^{-1} d^{-1}, and the diazotrophic community was dominated by the cyanobacterium related to *Nodularia spumigena* and the small unicellular, cosmopolitan cyanobacterium UCYN-A. *Nodularia* was present in gene abundances between 8.07 x 10^5 and 1.6 x 10^7 copies L^{-1} in waters with salinities of 10 and below, while UCYN-A reached gene abundances of up to 4.5 x 10^7 copies L^{-1} in waters with salinity above 10. Besides those two cyanobacterial diazotrophs, we found several clades of proteobacterial N_2 fixers and alternative nitrogenase genes associated with *Rhodopseudomonas palustris*, a purple non-sulfur bacterium. Based on principal component analysis (PCA), salinity was identified as the primary parameter describing the diazotrophic distribution, while pH and temperature did not have a significant influence on the diazotrophic distribution. While this statistical analysis will need to be explored in direct experiments, it gives an indication for a future development of diazotrophy in a freshening Baltic Sea with UCYN-A retracting to more saline North Sea waters and heterocystous cyanobacteria expanding as salinity decreases.

1 Introduction

30   The Baltic Sea (Fig. 1) is a marginal, brackish sea characterized by a natural salinity gradient increasing from the North-East to the South-West. The Baltic Sea covers an area of 415000 km^2 with a permanent halocline in the Baltic Sea proper, preventing vertical mixing, oxygen (O_2)-depleted waters in the deeper basins and coastal systems, accompanied with the occasional accumulation of hydrogen sulfide (H_2S) and ammonium (NH_4^+) below the chemocline (Conley et al., 2002; Hietanen et al., 2012; Lennartz et al., 2014). It is further challenged by a high land-derived influx of phosphorus leading to a

35   substantial internal surface water and sedimentary phosphorus load (Gustafsson et al., 2017; Stigebrandt and Andersson, 2020). Prior studies have shown that the Baltic Sea experienced a 10-fold increase in O_2-depleted areas over the last 115 years, covering an area of 12000 – 70000 km^2 making it one of the ocean areas most severely affected by deoxygenation (Diaz and Rosenberg, 2008; Reusch et al., 2018). Between 1871 and 2013, the Baltic Sea showed an increase in temperature of 0.1^oC decade^{-1} exceeding the average global trend of about 0.06^oC decade^{-1} (Reusch et al., 2018; Rutgersson et al., 2014)

40   and decreased salinity (1-2 kg^{-1}) (Liblik and Lips, 2019). Nitrogen (N) deposition rates are now among the highest in marine areas (Reusch et al., 2018). The resulting eutrophication severely affects sensitive coastal areas resulting in high pelagic production, frequent events of anoxia, and decreased biodiversity (Breitburg et al., 2018; Carstensen et al., 2014; Maar et al., 2016; Reusch et al., 2018; Rutgersson et al., 2014). In this context, the Baltic Sea has been described as a "time machine" for how future oceans will respond to climate change (Reusch et al., 2018), making it an ideal environment to investigate

45   biological fixation of dinitrogen gas (N_2 fixation) in response to such changes.

N_2 fixation is the primary external source of new N to life in the ocean and plays a crucial role for primary production, and thus carbon dioxide (CO_2) uptake from the atmosphere (Capone and Carpenter, 1982; Gruber, 2005). Specialized organisms, called diazotrophs, carry out this highly energy-demanding process. The diversity of diazotrophs is not easy to describe, as those organisms spread across the microbial tree of life, and are found in both the bacterial and archaeal domains

50   (Zehr et al., 1998). N_2 fixation is catalyzed by an enzyme complex, the nitrogenase, which exists in three different subtypes: the molybdenum (Mo)-iron (Fe) type referred to as Nif, the vanadium (V)-Fe type called Vnf, and a Fe-Fe type called Anf (Chisnell et al., 1988; Robson et al., 1986). The different metal cofactors are essential in the context of the redox sensitivity of the three nitrogenases, respectively. Mo is preferentially available in the presences of at least traces of O_2, contrary to V and Fe, which are more soluble and thus available under anoxic conditions (Bennett and Canfield, 2020; Bertine and

55   Turekian, 1973; Crusius et al., 1996; Dixon and Kahn, 2004; Morford and Emerson, 1999). The differential availability of those trace metals along redox gradients might have played a role in the evolutionary development of those nitrogenase types (Anbar, 2008; Mus et al., 2019) and their distribution might change in a future ocean impacted by acidification, deoxygenation (Keeling et al., 2010; Löscher et al., 2014; Schmidtko et al., 2017; Stramma et al., 2008) and desalination (Olofsson et al., 2020). To date, most studies focused mainly on Nif-type nitrogenases in the marine environment, while Vnf

60   and Anf nitrogenases were often overlooked. Molecular detection of the *nifH* gene does not fully recover the diversity of *vnfH* and *anfH,* possibly resulting in an underestimation of alternative nitrogenases in the environment (Affourtit et al., 2001;

Farnelid et al., 2009, 2013; Man-Aharonovich et al., 2007; Steward et al., 2004; Zehr et al., 1995). Nonetheless, the few available studies focusing on alternative nitrogenases identified them to be abundant and diverse and suggested to play a role for N_2 fixation in various ecosystems, including the upper water column of the ocean, O_2-depleted waters, and Baltic Sea sediments (Bellenger et al., 2011, 2014; Betancourt et al., 2008; Christiansen and Löscher, 2019; Farnelid et al., 2009; Loveless et al., 1999; McRose et al., 2017; Tan et al., 2009; Zehr et al., 2003; Zhang et al., 2016).

In the Baltic Sea, N_2 fixation is considered to take place in sunlit surface waters mainly and carried out by three genera of heterocystous cyanobacteria, *Aphanizomenon sp, N. spumigena* and *Anabaena spp.* (now referred to as *Dolichosperum spp.* (Klawonn et al., 2016; Wacklin et al., 2009)), all of them containing the classic Nif-nitrogenase (Janson et al., 1994; Leppanen et al., 1988)*,* and contributed massively to N_2 fixation with rates of up to $115 – 295$ nmol N L^{-1} d^{-1} (Larsson et al., 2001). At the same time, cyanobacteria consume only a fraction of the N they fix with estimates in the range of $33.33 – 85.72$ nmol N L^{-1} d^{-1} (Janson et al., 1994; Larsson et al., 2001; Wasmund, 1997). Using an EA-IRMS-based approach, Klawonn et al., 2016 measured N_2 fixation rates up to 300 ± 216 nmol N L^{-1} d^{-1} and based on cell-specific measurement of *Aphanizomenon spp.* and *N. spumigena*, revealed that they release up to 35% of fixed N, translating into a substantial fraction of fixed N leaking out, available for other primary producers (Ploug et al., 2010, 2011). In addition, low rates of N_2 fixation (0.44 nmol N L^{-1} d^{-1}) have been described in anoxic waters of the Baltic Sea (Farnelid et al., 2013). These rates were accompanied by a highly diverse Nif-containing heterotrophic diazotroph community at and below the chemocline in anoxic NH_4^+-rich waters (Farnelid et al., 2013). Heterotrophic diazotrophs were closely related to those in other O_2-depleted marine systems regions including the eastern tropical South (ETSP) and North Pacific (ETNP), the Arabian Sea (AS), and an anoxic basin in the Californian Bight (Fernandez et al., 2011; Hamersley et al., 2011; Jayakumar et al., 2012, 2017; Löscher et al., 2014). Non-cyanobacterial diazotrophs have been found to fix N_2 actively. However, it is still inconclusive what regulates and controls their N_2 fixation activity.

Over the last three decades, the Baltic Sea has experienced a trend of freshening in the range of 1-2 kg^{-1} in surface waters (Liblik and Lips, 2019) or between 0.4 to 1.2 (Saraiva et al., 2019). Earlier studies suggested that salinity impacts the diazotrophic community composition and the activity of nitrogenases in pure cultures of *Azotobacter* sp. marine microbial mats and estuaries (Dicker and Smith, 1981; Marino et al., 2006; Severin et al., 2012). Surface salinity in the Baltic Sea ranges between 0 and 10 (Fig. 1) making it one of the major drivers for microbial composition and distribution in the Baltic Sea (Dupont et al., 2014; Olofsson et al., 2020; Wulff et al., 2018). Typically, *Aphanizomenon sp.* dominates less saline waters (e.g., Bothnian Sea) and grow best with salinities around 5 (Lehtimäki et al., 1997). *Aphanizomen sp.,* however, has also been frequently reported to grow at salinities reaching up to 5-8 such as the northern Baltic Proper (Olofsson et al., 2020). *N. spumigena* prefers the higher saline waters in southern part (e.g. the Southern Baltic Proper) with an optimum growth in salinities of 8-10 (Lehtimäki et al., 1997; Rakko and Seppälä, 2014). Additionally, the small, unicellular cyanobacterial symbiont UCYN-A has been detected in the Baltic Sea (Bentzon-Tilia et al., 2015). This cosmopolitan diazotroph has previously been shown to be abundant throughout most marine systems (Tang et al., 2019; Zehr et al., 2016), and to substantially contribute to N_2 fixation rates (Martínez-Pérez, C., Mohr, W., Löscher, 2016; Mills et al., 2020).

However, the factors controlling the biogeography of UCYN-A is poorly understood. Though, latest study suggested salinity having a positive correlation to UCYN-A biogeography (Li et al., 2021). These observations indicate potential changes in the cyanobacterial composition in future freshening events (Olofsson et al., 2020; Wasmund et al., 2011). To now explore the impact and importance of salinity for diazotrophy in the Baltic Sea and in order to complement existing datasets on N_2 fixation from high-productivity seasons, we carried out a survey including chemical profiling, and rate measurements in the low productive fall season. The focus of this study is a molecular genetic assessment of the diazotrophic community diversity and genetic activity including heterocyst and unicellular cyanobacteria, heterotroph diazotrophs and diazotrophs carrying alternative nitrogenase genes. In addition, we compared our data to available and to be able to predict the future distribution of Baltic Sea diazotrophs and of N_2 fixation rates.

2 Material and Methods

2.1 Seawater sampling

Samples were collected during the cruise AL528 using the German research vessel RV Alkor from 17.09.2019 to 28.09.2019 along a transect through the major basins of the Baltic Sea (see Fig. 1 for a cruise plan and table S2 for overview of all stations). Specifically, samples were collected at a station in the Kiel fjord (KB06), one in the Arkona basin (H21), four stations in the Bornholm Basin (BB08, BB15, BB23, BB31) and the Eastern Gotland Basin (GB84, GB90a, GB107, GB108), respectively, at water depths between 3 to 115 meters (m) using a 10 L Niskin bottle rosette equipped with a conductivity- temperature- depth (CTD) sensor. Water for dissolved inorganic nitrogen (NO_x (Nitrate (NO_3^-)+ Nitrite (NO_2^-)) was collected from the Niskin bottles, filtered through an acid-washed 0.8 µm cellulose acetate filter (Sartorius™), using a syringe and stored in 20 ml scintillation vials at -20°C until analysis. Samples for PO_4^{3-} analysis were filtered through a 200 µm mesh to avoid contamination with large zooplankton. Samples were filled in 20 ml scintillation vials and stored at -80°C until analysis. NO_x and PO_4^{3-} samples were analyzed on a SKALAR SAN^{plus} analyzer (Thermo Fischer, Waltham, US) according to Grasshoff (1999) with a detection limit of 0.05 µmol N L^{-1} for NO_x and 0.03 µmol P L^{-1} for PO_4^{3-}. Nutrient samples were taken in triplicates, handled with nitrile gloves and all equipment was rinsed with MilliQ water in between stations to avoid contamination. Samples for dissolved inorganic carbon (DIC) were collected by filling triplicates of 12 mL exetainers bubble-free at stations KB06, H31, H21 and BB15. Samples were fixed with 20µl of a saturated mercury chloride solution and stored at room temperature until analysis. DIC measurements were carried out using a 2 mM $NaHCO_3$ solution as standard and as described previously (Hall and Aller, 1992). From all stations and depths 0.5 to 1 L of seawater were collected from Niskin bottles and were immediately filtered onto a 0.22 µm pore size membrane filters (Millipore, Bilaterica, USA; exact seawater volumes were constantly recorded), and filters were stored at -80˚C until DNA extraction and further analysis.

2.3 $^{15}N_2$ and ^{13}C seawater incubations

N_2 and C fixation rates were determined using stable isotope labelling at KB06, H21, BB15, BB08 and GB107, at water depths between 3 and 41 m. We used the bubble addition method (Montoya et al., 1996) to ensure comparability to previous studies from the Baltic Sea. Triplicate water samples were filled from Niskin bottles into 2.4 L glass bottles until it was top-filled (Schott-Duran, Wertheim, Germany). To each incubation bottle, 1 mL $^{15}N_2$ gas ($^{15}N_2$ 98%+ Lot#I-16727, Cambridge Isotope Laboratories, Inc., USA) and 1 mL $H^{13}CO_3$ (1g 50 mL^{-1}, Sigma-Aldrich, Saint Louis Missouri US) was added through an air-tight septum and bottles were inverted to ensure mixing with final calculated concentrations of 0.8 atom % $^{15}N_2$ and 10 µg mL^{-1} $H^{13}CO_3$ (approximately 3.8 atom %). After an incubation time of 24 h, samples were filtered onto pre-combusted GF/F filters (GE Healthcare Life Sciences, Whatman, USA). A parallel set of triplicate samples was collected from each sampling depth for determining the natural abundance of N and C isotopes in the particulate organic material. Filters were stored at -20°C until further analysis. Filters were then acidified and dried in an oven at 65 °C. Dried filters were analysed on an Elemental Analyzer Flash EA 1112 series (Thermo Fisher), coupled to an isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Fisher).

2.2 Molecular methods

For nucleic acid extraction, 0.22 µm pore sized Millipore membrane filters (Millipore, Bilaterica, USA) were flash-frozen in liquid N and subsequently crushed. DNA was purified with the MasterPure Complete DNA and RNA purification Kit (Lucigen, Wisconsin, US) according to the manufacturer's protocol, with the minor modification of using 1 mL of lysis buffer to completely cover the filter pieces. RNA was purified using Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). The remaining DNA was removed with the gDNA wipeout mix, and a cDNA library was constructed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) with the supplied RT primer set. The nucleic acid concentration and quality were checked spectrophotometrically on a MySpec spectrofluorometer (VWR, Darmstadt, Germany).

A total of 15 DNA samples were amplified for *nifH* using nested PCR with primers and PCR conditions as described in Zani et al. (2000). *anf/vnfD* were amplified using a nested PCR with primers and conditions as described in Bellenger et al. (2014) and McRose et al. (2017). Amplicons were TOPO TA-cloned (Topo TA cloning Kit for sequencing, Thermo Fisher Scientific, Waltham, US) and Sanger-sequenced. Sequencing was carried out at the Institute of Clinical Molecular Biology in Kiel, Germany. Samples for *nifH* and *anf/vnfD* amplification were the same as the ones where N_2 rate measurements were carried out (KB06, H21, BB15, BB08 and GB107 between 3 and 41 meters, see table S1).

Sequences were quality checked and trimmed with BioEdit (Hall, 1999). *nifH* sequences were trimmed to 321 bp and *vnf/anfD* to 591 bp. This resulted in 182 *nifH* and 69 *vnf/anfD* amplicon sequences. Sequences were BLASTX-searched against the NCBI database. A reference library was created, and sequences with lower than 80% identity were discarded. Sequences were aligned in MEGAX (Kumar et al., 2018), and a maximum likelihood tree was constructed using 1000

bootstraps. To explore the overall microbial diversity, 23 samples from Kiel Fjord (station KB06, depth 5 and 10 m)), Arkona Basin (station H2, depth 4, 12, 42 m and station H31, depth 4 and 12 m), Bornholm Basin (Station BB23, depth 4, 48, 85 m, station BB15, depth 7, 41, 65 m and station BB08, 5 and 10 m) and East Gotland Basin (Station GB84, depth 9, 29, 110 m, station GB107, depth 8, 40 and 112 m and station GB108, depth 10 and 115 m) were sent to amplicon sequencing of the 16s rDNA V1V2 region using Illumina HiSeq technology. Depths were chosen based on the water column chemistry (halocline, oxycline, oxygen minimum zones and chlorophyll peaks). Sequencing was carried out by the Institute of Clinical Molecular Biology in Kiel, Germany. Raw reads were trimmed, quality filtered in dada2 (v1.18) (Callahan et al., 2016), and taxonomically annotated using SILVA database (v138) (Quast et al., 2013). Visualization of the cyanobacterial distribution was accessed with *phyloseq* (1.34.0) (McMurdie and Holmes, 2013).

Quantitative real-time PCRs were carried out targeting *nifH* clade-specifically for *Nodularia*, UCYN-A and gamma-proteobacterial diazotrophs (Gamma AO, Gamma PO) as described previously (e.g., Boström et al., 2007; Langlois et al., 2008; Loescher et al., 2014). As standards, serial dilutions of plasmids (10^7 to 10^1 copies) containing the target *nifH* genes were used. Samples, standards, and non-template controls were run in duplicates on a Biorad qPCR machine (Biorad, Hercules, USA), and reactions were considered uncontaminated if no amplification could be detected in non-template controls after 40 qPCR cycles. To further ensure that no DNA contamination was present in our cDNA, we ran additional qPCR reactions of RNA samples. No amplification was observed. Sequences were submitted to GenBank with accession numbers MZ063808-MZ063873 and MZ063874-MZ064057 and 16S rDNA with accession number SAMN20309768-SAMN20309783.

2.4 Statistical Methods

Principle component analyses (PCA) on qPCR and environmental data were performed in R using the *vegan* package (Oksanen et al., 2020; R Foundation for Statistical Computing, 2017). The *simper* function (from *vegan* package) was used to identify parameters with the highest impact on diazotroph abundance. A principal component analysis (PCA) was subsequently performed using *prcomp* and visualized with the *factoextra* package (Kassambara and Mundt, 2020). To maximize the amount of explained variance of the dataset, the figure included both component one versus two (Fig. 8A), and one versus three (Fig. 8B).

3 Results and Discussion

3.1 Hydrochemistry

During the cruise, surface waters above 40 m water depth exhibited the typical Baltic Sea salinity gradient with salinities decreasing from South-West to North-East (10 – 24 in the Kiel and Arkona Basin and 7-9 in the Bornholm and Eastern Gotland Basins), accompanied by lower temperatures of 13-14°C in the Bornholm and Eastern Gotland Basin compared to

slightly higher temperatures of 15-16°C in the Kiel Fjord and Arkona Basin (Fig. 2). A thermocline was observed at 45 m in the Bornholm Basin and 65 m in the Eastern Gotland Basin. The coastal station we sampled in the Arkona basin showed a

190   similar stratification pattern (Fig. 2) with a salinity of 10 on the surface and 17 below 30 m water depth. This stratification pattern is typical for the region and time of the year and could also be observed from annual data from the International Council for the Exploration of the Sea (ICES Copenhagen, 2020) (Fig. 1, B). Surface waters were well-oxygenated with O_2 concentrations above 156.31 µmol L^{-1} in the top 40 m of the water column. Low O_2 water masses with concentrations below 15 µmol L^{-1} were only observed in the Bornholm Basin in waters deeper than 65 m (Fig. 2).

195   Compared to the South-western part, the DIC concentrations were lower in the upper water column of the Eastern Gotland and Bornholm Basin with concentrations of around 1.6 mmol L^{-1} and increased towards the south-western part of the cruise track. An opposite trend was visible in nutrient distribution with NO_x (nitrite + nitrate) and phosphate (PO_4^{3-}) decreasing from the North-Eastern part of the cruise track towards the South-Western part with surface water concentrations of 9.04 µmol L^{-1} for NO_x and 2.98 µmol L^{-1} for PO_4^{3-} in surface waters of the Eastern Gotland Basin and 0.27 µmol L^{-1} for

200   NO_x and 0.18 µmol L^{-1} for PO_4^{3-} in the Kiel Fjord (Fig. 2). The observed nutrient concentrations were in the range of the Helsinki commission (HELCOM) dataset containing data from 2015 to 2020 and the HELCOM core indicator report (Helcom, 2018, Fig S2). The detected somewhat higher nutrient concentrations in the Bornholm and Eastern Gotland Basins could result from a decaying phytoplankton bloom releasing nutrients including ammonia, decreased microbial activity or increased eutrophication. High chlorophyll concentrations in the Bornholm basin (3 µM Chl a in August and 2 µM Chl a in

205   September), derived from the HELCOM dataset (from same areas as this cruise), indeed give evidence of a decaying phytoplankton bloom in the Bornholm Basin in September 2019 (Fig. S3). These values are in range with the HELCOM indicator report with an average of 4 µM Chl a from June to September, and a previous study presenting a range between 2 and 6 µM Chl a (Helcom, 2018; Suikkanen et al., 2010).

   Our dataset indicates an N:P ratio [µmol L^{-1}:µmol L^{-1}] well below the Redfield ratio of 16:1 in surface waters along the

210   cruise track, with excess P classically supposed to promote N_2 fixation. However, NO_x was still available to the phytoplankton and microbial community as supported by a positive intercept of the trendline, which might be considered unfavourable for N_2 fixation (Fig. 3). Remaining NO_x concentrations in the surface waters above 40 m thus speak for a limitation of primary production by either micronutrients, temperature, or light availability and less for a limitation by N or PO_4^{3-} (Arrigo, 2005; Saito et al., 2008). Nitrogen repletion is supported by POC: PON ratios of 3.6 to 4.94 in the Kiel Fjord

215   and Arkona Basin and 2.92 to 6.15 in the Bornholm and Eastern Gotland Basins (Fig. 3), which are close to the Redfield ratio (Redfield, 1934). Altogether, nutrient concentrations do not strongly support a niche for N_2 fixation.

3.2 Nitrogen fixation

While no clear niche for N_2 fixation could be identified, we still detected N_2 fixation rates in our samples from water depths between 3 and 41 m (Fig. 4). N_2 fixation rates were between 0 to 6 ± 4.73 nmol N L^{-1} d^{-1} in the Arkona Basin, 0.7 ± 0.97 to

220   27.3 ± 38.21 nmol N L^{-1} d^{-1} in Bornholm Basin and 0.67 ± 0.51 to 2 ± 0.4 nmol N L^{-1} d^{-1} in the Eastern Gotland Basin (Fig. 4). In Kiel Fjord, N_2 fixation rates were below the detection limit (0.03 nmol N L^{-1} d^{-1}) of our method. One outlier was observed in the Bornholm Basin at a water depth of 5 m, with an N_2 fixation rate of 27.3 nmol N L^{-1} d^{-1}. This high rate was only detected in one out of our three replicates and was not included in our further analysis. Without this outlier, a conservative estimate of N_2 fixation in the Bornholm Basin would be between 0.7 ± 0.97 and 5.27 ± 1.37 nmol N L^{-1} d^{-1}.

225   Though these rates are at the lower end of previous observations from the Baltic Sea (Table S3), they are comparable to a previous study from the same region, but not the same season, with N_2 fixation rates of 7.6 ± 1.76 nmol N L^{-1} d^{-1} from July and August (Farnelid et al., 2013). N_2 fixation activity is supported by the $\delta^{15}N$ -PON in our control samples which was in the range of control samples was found to be 0.3 to 2 ‰ in samples from surface waters above 40 m from the Eastern Gotland and Bornholm Basins (Fig. 4). Values between -2 to +2 ‰ are considered indicative for N_2 fixation (Dähnke and

230   Thamdrup, 2013; Delwiche and Steyn, 1970).

      Seasonal variations might explain the observed lower N_2 fixation rates if compared to previous studies. N_2 fixation rates are generally described sensitive to low temperatures (Brauer et al., 2013; Church et al., 2009; Englund and Meyerson, 1974; Moisander et al., 2010; Stal, 2009) with the energetic costs of N_2 fixation sharply rising at temperatures below 21 °C (Brauer et al., 2013). During our cruise, surface temperatures were between 12 °C and 16 °C and would most likely play a role in the

235   low rates observed. Yet, growth of *N. spumigena* has been demonstrated at 4 °C suggesting a relevance during wintertime in the Baltic Sea (Olofsson et al., 2019). Additionally, lower light intensities during fall and winter could directly influence the abundance of cyanobacterial diazotrophs in the Baltic Sea (Dera and Woźniak, 2010; Staal et al., 2002).

      N_2 fixation rates were accompanied by low C fixation rates compared to rates in the micromolar range as typically detected in the Baltic Sea (e.g., (Klawonn et al., 2016))(Fig. 4), which were, however, increasing over depth, with an average

240   C fixation of 29.16 ± 10.89 nmol C L^{-1} d^{-1} in Kiel Fjord, 42.62 ± 7.86 nmol C L^{-1} d^{-1} the Arkona Basin, 40.52 ± 13.16 nmol C L^{-1} d^{-1} in Bornholm Basin and 58.52 ± 15.42 nmol C L^{-1} d^{-1} in Eastern Gotland Basin. Assuming Redfield stoichiometry, N_2 fixation sustained between 8.8 and 108% with an average of 43% of C fixation during our cruise. These rates are comparable with the contribution of N_2 fixation to C fixation in other oceanic regions, including the Atlantic (20-25%) and Pacific Ocean (1-4%), the Mediterranean Sea (8%), and the Bay of Bengal (<1%) (Church et al., 2009; Dore et al., 2002; Fonseca-Batista et

245   al., 2019; Rahav et al., 2013; Saxena et al., 2020; Tang et al., 2019) (Table 1). The high contribution to primary production indicates that N_2 fixation may promote or extend primary production into the fall season if only at low rates. Other sources, however, might include land or riverine input.

3.3 Diazotrophic community composition

      We assessed the diazotrophic diversity based on *nifH* and *vnf/anfD* amplicons (Fig. 5 and 6). Sequences belonging to *N.*

250   *spumigena* (19%) and UCYN-A (26%) were dominant in the sequence pool. While *Nodularia* clades are classically abundant in the Baltic Sea waters (Boström et al., 2007; Farnelid et al., 2013; Klawonn et al., 2016; Larsson et al., 2001),

only one previous report of UCYN-A from the Baltic Sea exists, where it had been detected in waters of the Danish Strait and the Great Belt (Bentzon-Tilia et al., 2015). Moreover, we detected *Pseudanabaena*-like sequences representing 8 % of the sequence pool consistent with previous studies (e.g. Acinas et al., 2009; Farnelid et al., 2013; Klawonn et al., 2016; Stal et al., 2003). Only 1.5 % of the sequences belonged to *Aphanizomenon sp.,* a diazotroph known to dominate N_2 fixation early in the season with salinity at 5-6 (Klawonn et al., 2016; Svedén et al., 2015). The low number found in this study might be seasonally related.

Notably, based on our *nifH* sequence analysis, we did not identify, *Dolichospermum spp.*, one of the common N_2 fixing cyanobacteria for the Baltic Sea (Bentzon-Tilia et al., 2015; Boström et al., 2007; Farnelid et al., 2013). However, sequences related to *A. lemmermannii* were recovered from our 16S rDNA amplicon sequencing (Fig. S1). This discrepancy might speak for a bias against their specific *nifH* genes by our approach, or the clades we found in the 16S rDNA dataset might lack a *nifH* gene.

We detected *Nodularia*-like sequences (primer/probe constructed against *N. spumigena* and environmental *Nodularia* clusters, see Boström et al., 2007), in abundances of 3.37×10^5 to 4.06×10^7 copies L^{-1} at 3-5 m water depth and 8.64×10^4 – 4.46×10^6 copies L^{-1} at water depths of 20-41 m. *Nodularia*-specific *nifH* transcript abundance decreased over depth from 4.20×10^5 (5 m) to 3.5×10^2 (20 m) transcripts L^{-1} (Fig. 7). *Nodularia* followed the temperature-salinity gradient with the highest abundance (up to 1.6×10^7 copies L^{-1}) in waters with salinity at 10 and below (the Bornholm and Eastern Gotland basin) and lowest abundances (up 1.46×10^6 copies L^{-1}) in waters with a salinity above 10 (Kiel Fjord and the Arkona basin). *Nodularia*-specific *nifH* gene and transcript abundances were in the same range as previously described (10^6 gene copies L^{-1} and 10^5 transcripts L^{-1} (Farnelid et al., 2013)). UCYN-A was identified based on both *nifH* and 16S rDNA sequences and quantified using cluster-specific qPCR. UCYN-A was present in abundances of 3.73×10^3 – 9.97×10^7 copies L^{-1}, similar to the one available previous study where the abundance of *nifH* specific for UCYN-A peaked in September (approximately 10^6 copies L^{-1}) (Bentzon-Tilia et al., 2015) (Fig. 7). The same study showed *nifH* expression (roughly 10^3 transcript L^{-1}) of UCYN-A in the Baltic Sea surface waters (1 m). These transcript abundances are comparable with those obtained from our study with transcript abundances between 2.98×10^2 and 3.73×10^2 transcripts L^{-1} at 5 m water depth and up to 3.71×10^5 transcripts L^{-1} at a water depth of 20 m (Fig. 7). Notably, the above-mentioned study took place in the Danish Strait, and thus in more saline waters (salinity of 13-17).

Contrary to *Nodularia,* UCYN-A reached highest abundances (up to 4.52×10^7 copies L^{-1}) in higher saline waters impacted by the North Sea (the Kiel Fjord and Arkona Basin) and the lowest abundance (up to 6.29×10^5 copies L^{-1}) in low saline waters (Fig. 7, Supplementary Figure S1). UCYN-A is increasingly found throughout the oceans, often playing an important role in N_2 fixation (e.g. Martínez-Pérez, C., Mohr, W., Löscher, 2016; Tang and Cassar, 2019). Over the recent years, UCYN-A has been shown to have a crucial part in N_2 fixation and has been identified all over the globe, from polar to tropical regions and tolerating between 4ºC and 30ºC (Gradoville et al., 2020; Harding et al., 2018; Martínez-Pérez, C., Mohr, W., Löscher, 2016; Mills et al., 2020; Short and Zehr, 2007; Tang and Cassar, 2019). Despite UCYN-A exposing

highest abundances in Baltic Sea waters with higher salinity, we found UCYN-A present and active across the salinity gradient.

Besides those cyanobacterial diazotrophs, we detected several clades of proteobacteria related to *Rhodopseudomonas palustris* (4%)*, Pelobacter sp* (9%) and gamma proteobacteria of the Gamma-AO clade (3%, Langlois et al., 2008). We quantified gamma-proteobacterial *nifH* in abundances between 1.78×10^4 -1.82×10^5 copies L^{-1} (Gamma AO) and their transcripts in abundances of 2.89×10^3 - 3.91×10^3 transcripts L^{-1}. Despite lack of clones clustering with Gamma PO, the group could be detected in abundances between 8.21×10^2 -1.37×10^5 copies L^{-1} and their transcripts in abundances between 3.04×10^3 - 8.05×10^3 transcripts L^{-1}, indicating a potential contribution to N$_2$ fixation of those clades (Fig. 7). Diazotrophic proteobacteria are common in the ocean, however, their role for N$_2$ fixation is not fully understood (Benavides et al., 2018; Chen et al., 2019; Turk-Kubo et al., 2014). Diazotrophs related to Cluster III were dominated by *Desulfovibrio*-like (1.5%), *Opitutaceae*-like (6%) and *Clostridium*-like sequences (1.5%). However, both *nifH* gene and transcript abundances were below the detection limit of our qPCR. Altogether, the gene and transcript abundances of cyanobacterial diazotrophs were three to four orders of magnitude higher than those of non-cyanobacterial clusters suggesting that heterotrophic microbes may have only played a minor role for N$_2$ fixation in Baltic Sea surface waters during our cruise.

In the pool of alternative nitrogenase sequences of the *anfD* type, we identified the purple non-sulfur bacterium *Rhodopseudomonas palustris*. We could not recover any *vnfD* sequences, which might be an adaptation to low vanadium concentrations in the Baltic Sea (Bauer et al., 2017). The role of alternative nitrogenases in the environment is poorly understood, partly due to a lack of data and detection systems. Our data shows however the presence of alternative nitrogenases in the Baltic Sea surface water, which may sustain N$_2$ fixation under certain conditions, for example in anaerobic micro-niches (Bertagnolli and Stewart, 2018; Farnelid et al., 2019; Paerl and Prufert, 1987; Pelve et al., 2017), including sinking particles (Chakraborty et al., 2021; Pedersen et al., 2018).

3.4 Factors impacting the diazotrophic distribution

To identify parameters impacting on the distribution of diazotrophs during our cruise, we carried out a *simper* analysis. Interestingly, pH and temperature were less important for describing the diazotrophic distribution. The PCA suggested that depth and salinity explained the distribution of both the diazotrophic community and N$_2$ fixation best (Fig. 8). Moreover, it revealed a correlation of N$_2$ fixation and the *Nodularia* abundance and less of a correlation between N$_2$ fixation and UCYN-A or other diazotroph clusters (Fig. 8). A decreasing salinity would thus not only promote the distribution of *Nodularia*-like diazotrophs, and decrease UCYN-A abundances, but also promote N$_2$ fixation rates in such a future scenario. Intuitively, it is of little surprise that our data (Fig. 8) and previous studies indicate salinity to be a key control for cyanobacterial distribution and N$_2$ fixation in the Baltic Sea (Dupont et al., 2014; Olofsson et al., 2020; Rakko and Seppälä, 2014). A recent study also suggest a positive correlation between UCYN-A and salinity (Li et al., 2021). While a study based on a compiled dataset (1979-2017) indicated that salinity does not affect the biovolumes of the filamentous *N. spumigena* but rather species-interactions (Karlberg and Wulff, 2013; Olofsson et al., 2020). Our study cannot evaluate such interactions and their impact

of N_2 fixation; however, our data do point towards *Nodularia* expanding into low salinity waters in the future thus complementing UCYN-A and possibly increasing N_2 fixation rates in those waters.

320   Our analysis did not show temperature and pH to be major descriptors of N_2 fixation during this cruise, consistent with previous findings with elevated CO_2 concentrations not causing any response in N_2 fixation in the Baltic Sea (Olofsson et al., 2019; Paul et al., 2016; Wulff et al., 2018). Moreover, a very recent study showed that ocean acidification has an impact on the diazotroph community composition and ecology and can decrease bulk N_2 fixation rates in the subtropical Atlantic Ocean (Singh et al., 2021). Most likely, the contradicting results are due to other factors obscuring the stimulation by N_2 fixation

325   (Karlberg and Wulff, 2013; Wannicke et al., 2018). Besides pH, temperature has also been suggested as a control on diazotrophy, either directly or indirectly, by impacting on O_2 solubility (Stal, 2009).

Besides, increasing nutrient loads in the Baltic Sea might further stimulate primary production leading to increased anoxia and PO_4^{3-} release from sediments (Ingall and Jahnke, 1997), which will, in turn, fuel N_2 fixation and primary production (Canfield, 2006; Saraiva et al., 2019). Filamentous cyanobacteria benefit from excess PO_4^{3-} (Olofsson et al.,

330   2016), thus, excess PO_4^{3-} resulting from progressive deoxygenation might stimulate blooms of *Nodularia* (Degerholm et al., 2006; Schoffelen et al., 2018).

In case of a potential future freshening of the upper water column (Liblik and Lips, 2019) in combination with increased PO_4^{3-} availability both through land-derived influx and through phosphorus mobilization via bottom-water anoxia (Gustafsson et al., 2017; Ingall and Jahnke, 1997; Stigebrandt and Andersson, 2020; Vahtera et al., 2007), N_2 fixation by e.g.

335   *Nodularia* will likely increase in the region covered by our cruise. This could lead to a scenario with increased bloom formations and expansion of such large heterocystous cyanobacteria across the Baltic Sea, as previously observed (Kahru et al., 1994; Kahru and Elmgren, 2014). An increased organic matter load would possibly lead to enhanced respiration by heterotrophic microbes promoting the further expansion of O_2 depleted waters. This could lead to increased denitrification resulting in N-loss and emission of N_2O, fuelling global warming, an increase in euxinic events as already observed in

340   coastal Baltic waters (Breitburg et al., 2018; Carstensen et al., 2014; Lennartz et al., 2014). Further, blooms of *Nodularia* could lead to an increased load of Baltic Sea waters with the toxin Nodularin (Sivonen et al., 1989), which can harm Baltic Sea biota and poison animals (Main et al., 1977; Nehring, 1993) and ultimately humans by impacting e.g. fishery industry (Karjalainen et al., 2007). Latest studies, however, shows that projection of increased sea level rise counteracts freshening events in the Baltic Sea making it uncertain whether salinity increases or decreases in the future (Meier et al., 2021, 2022).

345   Regardless, potential changes in salinity might impact the diazotrophic community as described above. The uncertainty in changing salinity makes it unclear in which extent diazotrophs might be impacted.

4 Conclusion

During our cruise, we explored the diazotrophic community composition and N_2 fixation rates along the natural salinity gradient in the South Baltic Sea in the fall season. N_2 fixation rates were detectable but low for the Baltic Sea and sustained

350 by a diazotrophic community dominated by the heterocystous cyanobacterium *Nodularia* and the small unicellular cyanobacterium UCYN-A. The two different types of cyanobacteria occupied two different niches defined by salinity ranges, respectively, with *Nodularia* dominating in lower saline waters (8-9) and UCYN-A in high-salinity waters (>10). Statistical analysis revealed that N_2 fixation is quantitatively mainly driven by *Nodularia* clades and that both, *Nodularia* abundances and N_2 fixation rates, are best explained by salinity. In the context of a predicted freshening of the Baltic Sea, the

355 habitat of *Nodularia*-like heterocystous cyanobacteria would extend towards the South-Western part of the Baltic Sea, possibly replacing the community of UCYN-A and increasing N_2 fixation in those waters. Enhanced N_2 fixation might facilitate primary productivity, and organic matter export to waters below the euphotic zone and could thus have severe impacts on the Baltic Sea biogeochemistry including increased respiration, O_2 and N loss.

Author contribution

360 C. Reeder and C. Löscher designed the experiments and C. Reeder carried out the experimental work. J. Javidpour and I. Stoltenberg designed the sampling strategy during the expedition and contributed essential datasets. C. Reeder and C. Löscher prepared the manuscript with contributions from all the co-authors.

Competing interest

The authors declare that they have no conflict of interest.

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745   **Figures**
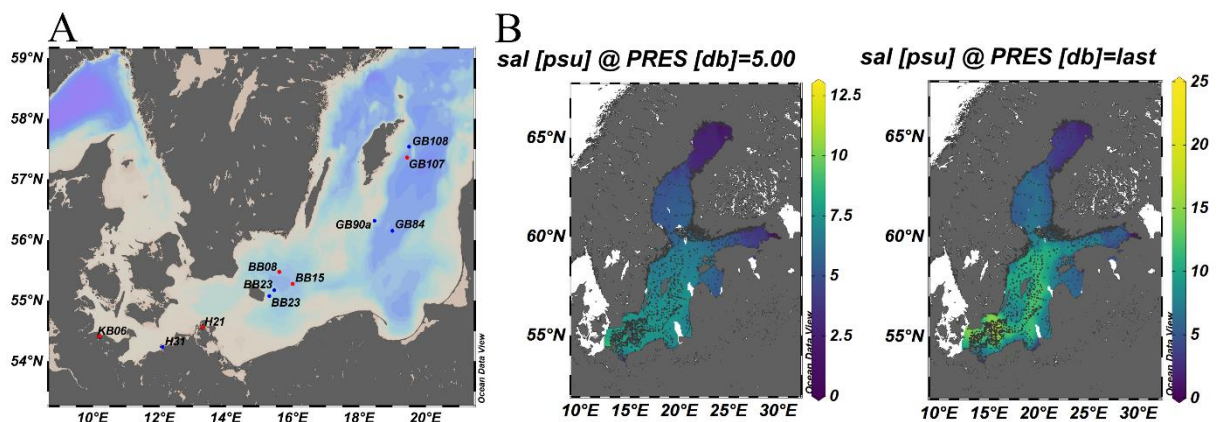
Figure 1. Overview of cruise track and stations from this study (A) Distribution of O₂, salinity (sal) in surface water (5 m) and the deepest sample at the station (last) in the Baltic Sea. Stations from where N₂ fixation were obtained are coloured red. Data for figure B were obtained through ICES oceanography spanning from 2019-08-01 to 2019-10-30 (ICES Copenhagen, 2020).

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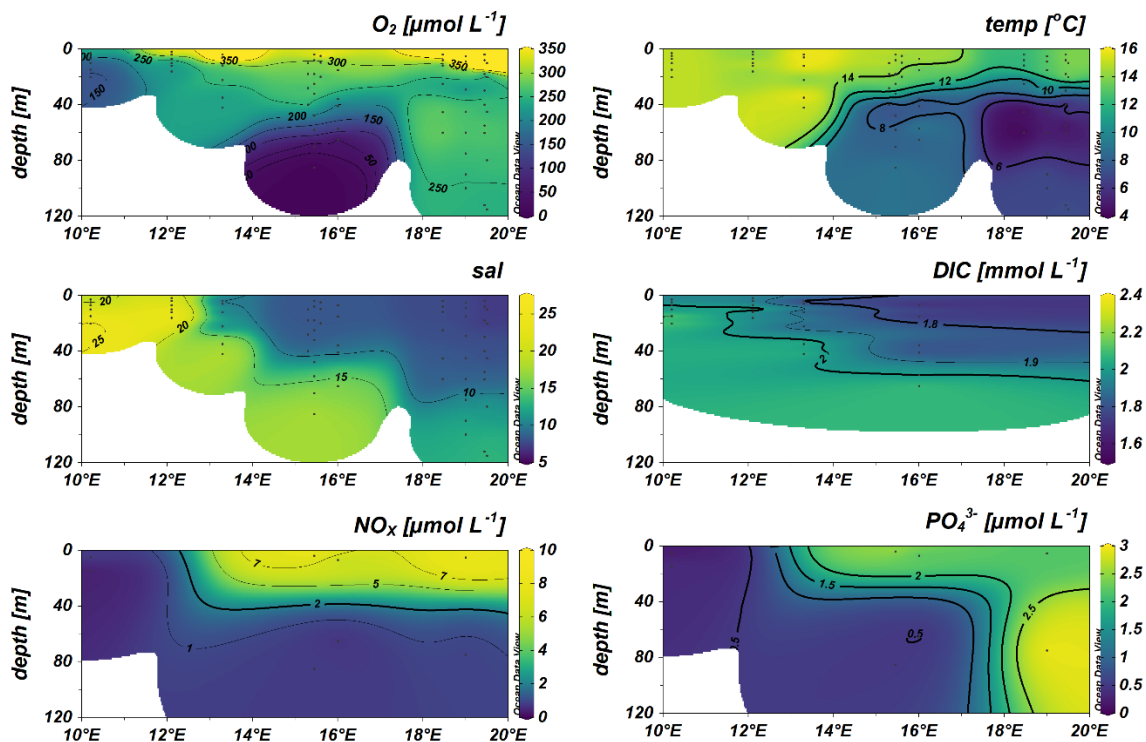
Figure 2 Distribution of surface water O₂ (μmol L⁻¹), temperature (temp) (°C), salinity (sal), dissolved inorganic carbon (DIC) (mmol L⁻¹) and NO_X (nitrate + nitrite) and PO₄³⁻ in μmol L⁻¹.

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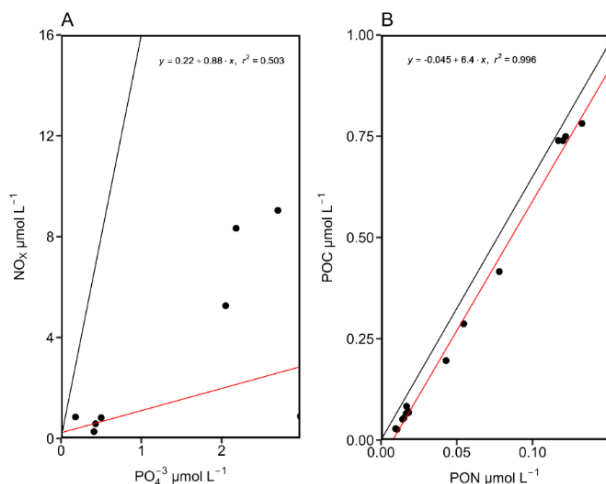
Figure 3 N:P (A) and C:N (B) ratios from station KB06, BB23, BB15, BB08 and GB107. The black lines depict the N:P (16:1) and C:N (106:16) ratios after Redfield. The red lines indicate the trendlines of our data. An N:P ratio below the canonical Redfield ratio of 16:1 suggests a niche for N_2 fixation However, the positive intercept speaks for DIN still being available, which might be considered as unfavorable for N_2 fixation (A). A C:N ratio below Redfield might suggest a slight deficiency in N (B), this might result from degrading organic matter in the POM pool.
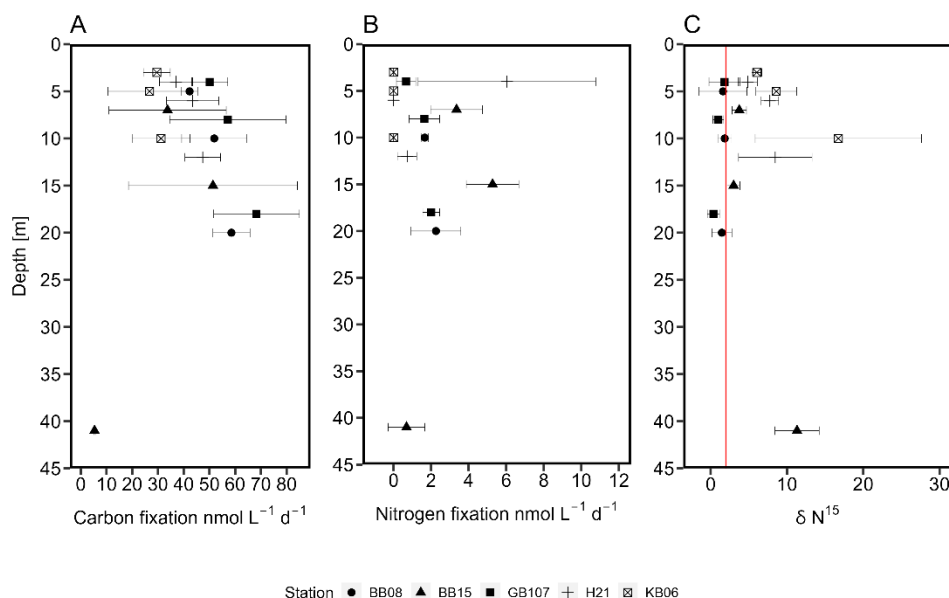
Figure 4 C (A) and N_2 (B) fixation rates [nmol L^{-1} d^{-1}]. Similar in all stations (five stations covering three depths), C fixation increased over depth, followed by a decrease at a water depth of 41 m. Higher rates were observed in the Bornholm and Eastern Gotland Basins compared to the Kiel and Arkona Basins. N_2 fixation rates increased slightly over depth, followed by a decrease at 41 m water depth in the Bornholm Basin. (C) $\delta^{15}N$ [‰] from station KB06, H21, BB08, BB15 and GB107. The red line indicates a $\delta^{15}N$ of 2 ‰. Values below 2‰ speak for previous or current N_2 fixation (Dähnke and Thamdrup, 2013).
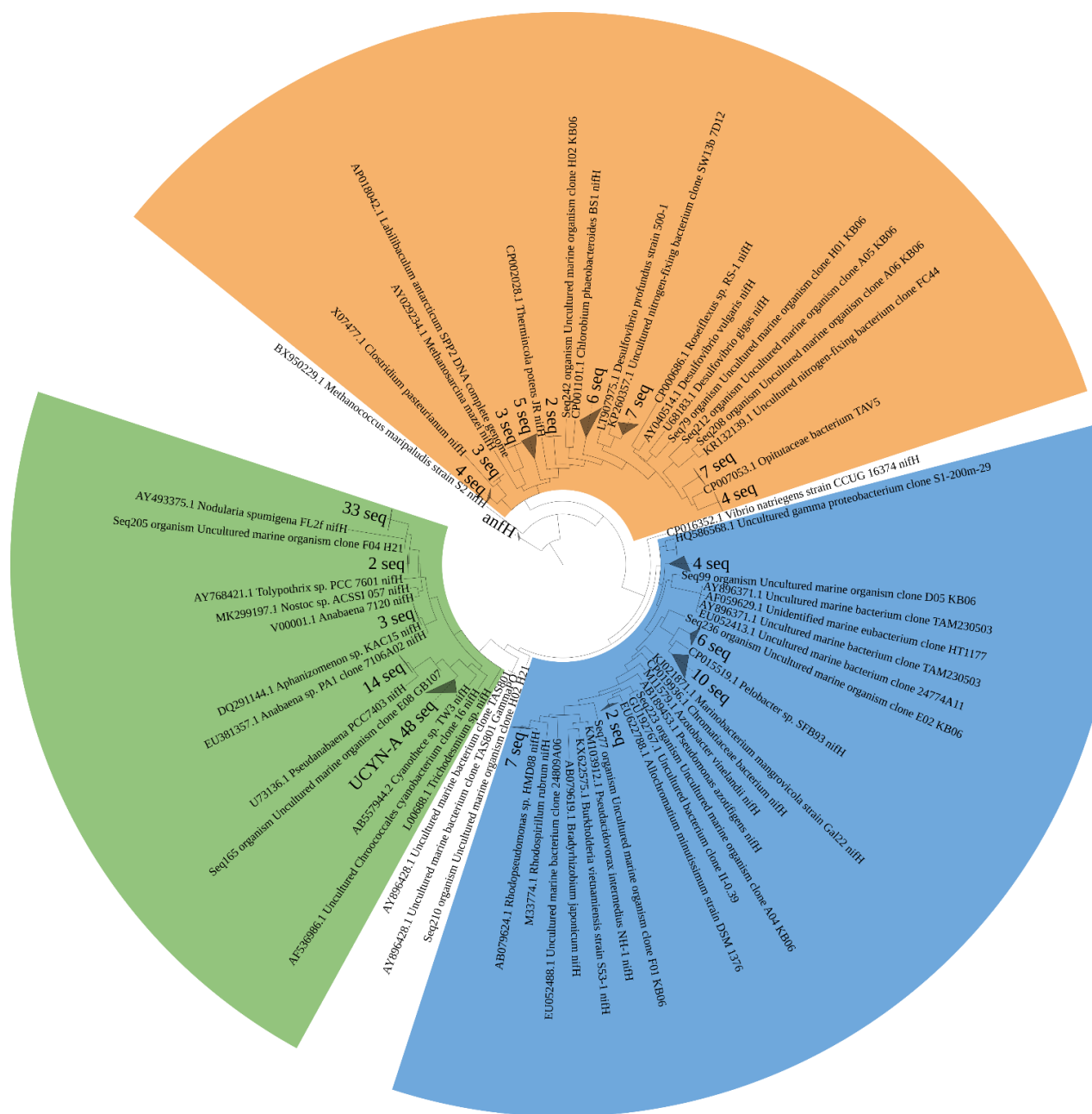
Figure 5 Maximum likelihood tree of *nifH* amplicons (321 bp) identified by Sanger sequencing. Clusters identified are denoted as grey triangles while identified individual sequences are denoted as 'Seq + NCBI submission ID'. Cluster I cyanobacteria are shown in green, Cluster I proteobacteria are shown in blue, and Cluster III diazotrophs are shown in orange.

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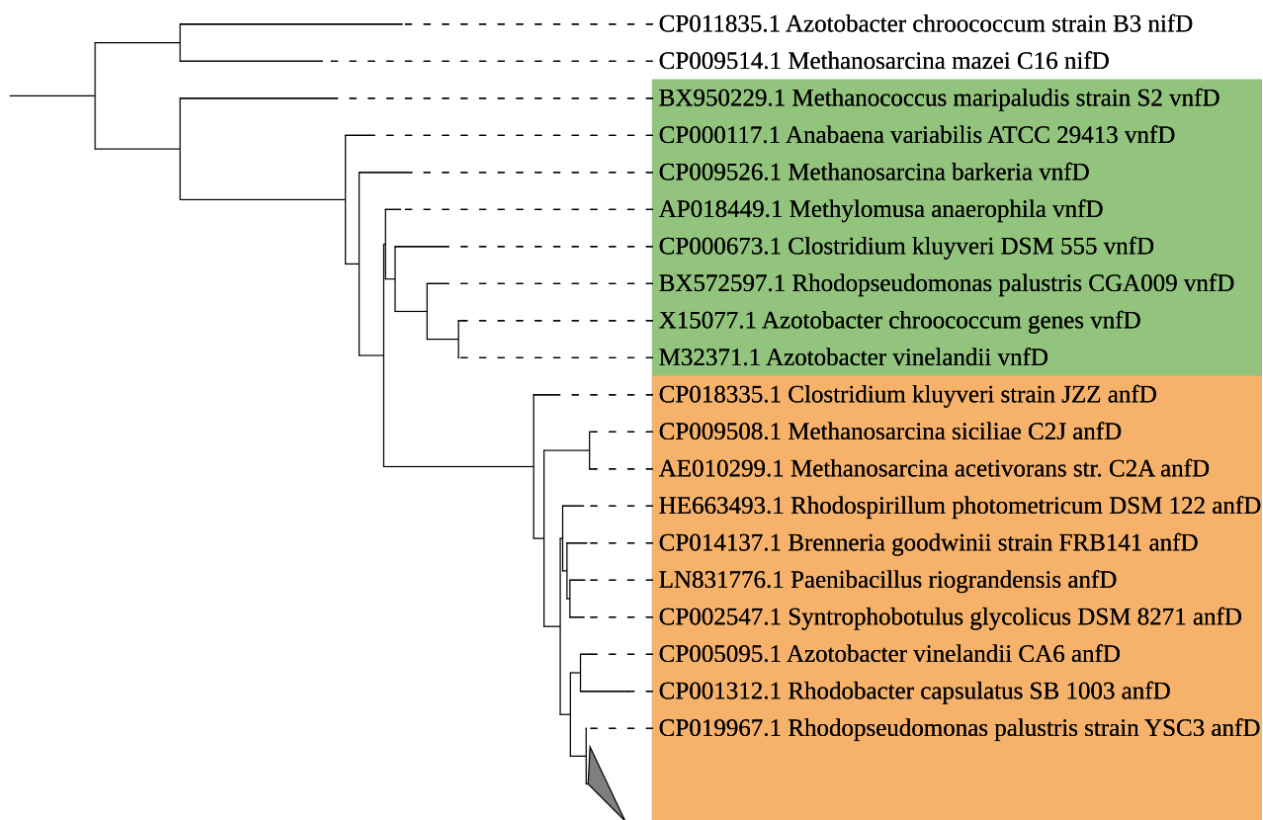
Figure 6 Maximum likelihood tree of *vnf/anfD* amplicons (591 bp) identified by Sanger sequencing. All recovered sequences clustered with *Rhodopseudomonas palustris* (grey triangle). *anfD* sequences are represented in brown, *vnfD* sequences are represented in green. A *nifD* Sequence has been chosen as the outgroup.

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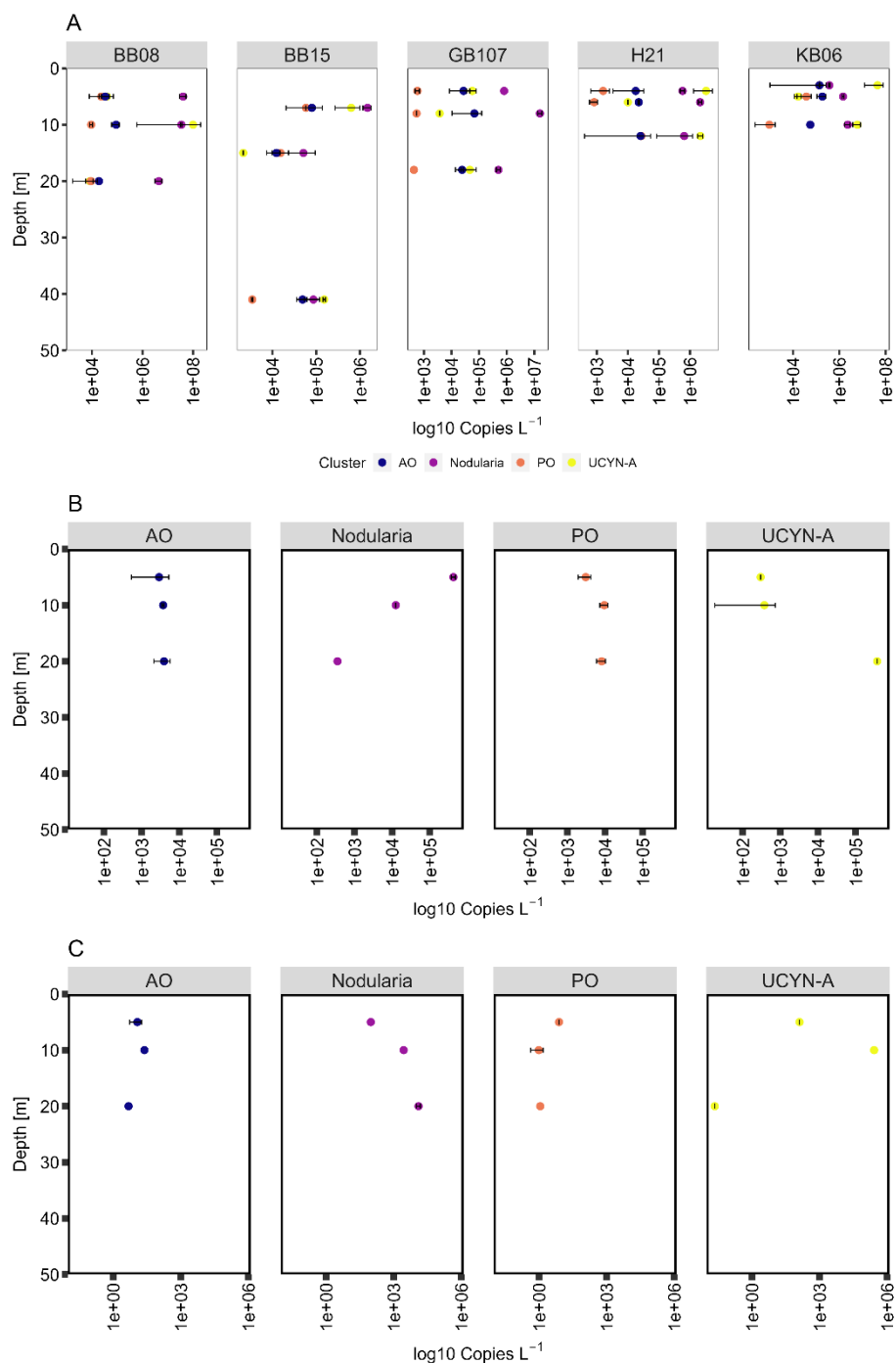
Figure 7 Cluster-specific nifH (A) gene, (B) transcript abundances and (C) transcript per gene copy of Nodularia, UCYN-A and Gamma-PO/AO. In general, both cyanobacterial groups are decreasing in nifH gene abundance over depth at all stations. Transcript abundances of Nodularia-nifH were higher in surface waters (5 m), contrary to UCYN-A, which showed an opposite trend. Gamma-PO/AO gene and transcripts abundance generally increased over depth.

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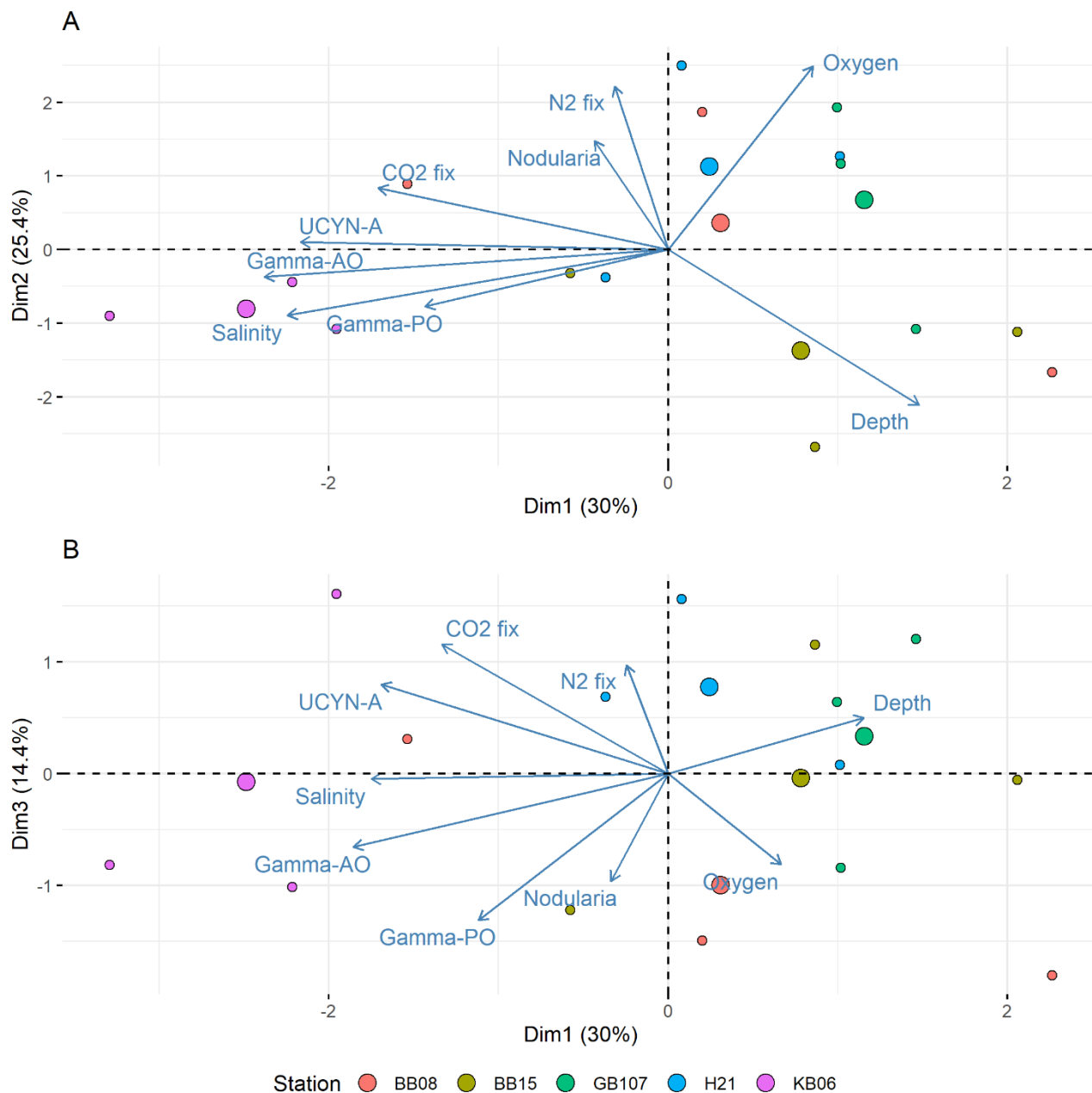
Figure 8 Principal component analysis (PCA) of components one and two (A), one and three (B). Positive correlations were identified between salinity and UCYN-A, while *Nodularia* and salinity are negatively correlated. Moreover, N$_2$ fixation correlated positively with *Nodularia*. Stations are color-coded. Each circle denoted one sampling point, bigger circles indicate the center for each station.

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Table 1 Contribution of N$_2$ fixation to primary production (PP) in different ocean basins. Values represent mean values for the respective regions.

Location	PP Contribution (%)	Source
North east Atlantic	25	(Fonseca-Batista et al., 2019; Tang et al., 2019)
West Northeast Atlantic	20	
Mediterranean Sea	8	(Church et al., 2009; Dore et al., 2002; Fonseca-Batista et al., 2019; Rahav et al., 2013; Saxena et al., 2020)
North Pacific Ocean	1-4	
Bay of Bengal	<1	
Baltic Sea	8-25	This study

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