Macroalgal blooms favor heterotrophic diazotrophic bacteria in nitrogen-rich and phosphorus-limited coastal surface waters in the Yellow Sea

Xiaoli Zhang, Yanjing Song, Dongyan Liu, John K. Keesing, Jun Gong

Abstract

Macroalgal blooms may lead to dramatic changes in physicochemical variables and biogeochemical cycling in affected waters. However, little is known about the effects of macroalgal blooms on marine bacteria, especially those functioning in nutrient cycles. We measured environmental factors and investigated bacterial diazotrophs in two niches, surface waters that were covered (CC) and non-covered (CF) with massive macroalgal canopies of Ulva prolifera, in the Yellow Sea in the summer of 2011 using real-time PCR and clone library analysis of nifH genes. We found that heterotrophic diazotrophs (Gammaproteobacteria) dominated the communities and were mostly represented by Vibrio-related phylotypes in both CC and CF. Desulfovibrio-related phylotypes were only detected in CC. There were significant differences in community composition in these two environments ($p < 0.001$) and a much higher abundance of nifH in CC ($4.55 \times 10^6$ copies l$^{-1}$) than in CF ($2.49 \times 10^6$ copies l$^{-1}$). The nifH copy number was inversely related to concentrations of ammonium and dissolved inorganic nitrogen and to the stoichiometric ratios of N:P and N:Si. This indicates that macroalgal blooms significantly affect diazotrophic abundance and community composition and that vibrios and Desulfovibrio-related heterotrophic diazotrophs adapt well to the (N-rich but P-limited) environment during blooming. Potential ecological and microbiological mechanisms behind this scenario are discussed.

1. Introduction

In recent decades, green tides, blooms of free-floating exotic or endemic green macroalgae in marine waters (Shimada et al., 2003; Charlier et al., 2007), are increasing along estuaries and coasts worldwide (Fletcher, 1996; Valiela et al., 1997; Largo et al., 2004). Nutrient loading, especially increased concentrations of dissolved inorganic nitrogen (DIN), is a dominant factor in macroalgal blooms because it controls the growth rate, productivity and onset of biomass collapse in macroalgae (Lapointe, 1997; Valiela et al., 1997; Teichberg et al., 2010). Opportunistic macroalgae can grow rapidly, forming high biomass abundance by assimilating and storing a large amount of N and resulting in low aquatic concentrations of this nutrient (Valiela et al., 1992, 1997; Menéndez et al., 2002).

Macroalgal canopies release large amounts of dissolved organic carbon (DOC), which fuel heterotrophic microbes, and increase biological oxygen demand and the frequency of anoxic events (D’Avanzo and Kremer, 1994; Alber and Valiela, 1994); furthermore, they may cause substantial changes in the carbon, sulfur, and phosphorus (P) cycles (Valiela et al., 1997; Viaroli et al., 2005).

A recent review has identified negative effects from macroalgal blooms on the abundance and diversity of a range of marine organisms (Lyons et al., 2014). There are only a few studies using 16S rRNA genes as markers to reveal bacterial communities during macroalgal blooms (Burke et al., 2011; Liu et al., 2011). However, it is not obvious how abundant macroalgal biomass and modified water chemistry will affect nitrogen (N) cycling microbes, which are major players in biological N$_2$ fixation, regeneration, and transformation, and which could also have chemical interactions with macroalgae (Goeree et al., 2010).

Diazotrophs (N$_2$-fixers) play an important role in ocean systems where inorganic nitrogen can represent a significant limiting factor.
for primary production (Falkowski et al., 1998). While large, colony-forming cyanobacterial _Trichodesmium_ has been considered a major contributor to N₂ fixation in the tropical and sub-tropical oligotrophic oceans (Zehr et al., 2000; Sanudo-Wilhelmy et al., 2001; Church et al., 2005; Tang et al., 2006; Langlais et al., 2008; Moisander et al., 2008), unicellular cyanobacteria and heterotrophic bacteria (e.g., _Proteobacteria, Firmicutes_) have also been recognized as important diazotrophs in marine waters (Riemann et al., 2010; Halm et al., 2012). Because N₂-fixing bacteria comprise a taxonomically diverse group, the functional gene _nifH_, which encodes one of the subunits of nitrogenase, has been widely used as molecular marker to identify the community composition of this functional group; furthermore, copy numbers of the _nifH_ gene can be quantified to indicate the genetic potential for diazotrophy in environments (Zehr et al., 1998; Church et al., 2008; Moisander et al., 2008). Four major phylogenetic clusters (I–IV) of the _nifH_ gene have been well recognized: phytootypes from Cluster I (subcluster 1A–1IV) were from Cyanobacteria and Alpha-, Gamma-, and Epsilonproteobacteria, Cluster II (subcluster 2A–2F) was from Archaea, Cluster III (subcluster 3A–3T) was from anaerobic bacteria such as _Chlostridium_, sulfate reducers, _Deltaproteobacteria_, and _Spirochaetes_, and Cluster IV (subcluster 4A–4F) was from non-functional archaeal _nifH_ homologues (Zehr et al., 2003).

Because biological N₂ fixation is an energy-demanding pathway, the DOC released from macroalgal canopies could power this process. There are also anoxic and suboxic micro-scale environments (e.g., interdigitated small parcels) in canopy-covered waters, and the mix of conditions changes from day to night. These low-oxygen niches and events may also favor the activity of diazotrophs during macroalgal blooms. It has been reported that the heterotrophic _Azotobacter_ on the surface of seaweed _Codium fragile_ could fix N₂ in situ (Head and Carpenter, 1975), in other environmental conditions, for instance, high concentrations of ammonium (NH₄⁺), and oxygen produced by photosynthesis, have long been known to inhibit diazotrophy (Head and Carpenter, 1975; Klugkist and Haaker, 1984; Howarth et al., 1988; Knapp, 2012). However, considerable N₂ fixation rates and abundant _nifH_ genes and transcripts have been recently detected in anoxic ammonium-rich waters in the central Baltic Sea (Farnelid et al., 2013). Therefore, the potential effects of macroalgal blooms on diazotrophs remain elusive.

We hypothesized that there was contrasting community composition and abundance in diazotrophs in canopy-covered and canopy-free surface waters in the Yellow Sea in China, where the world’s largest green tide occurred during the 2008 Olympic Games (Sun et al., 2008; Keesing et al., 2011). The seaweed species was _Ulva prolifera_ (Leliaert et al., 2009; Liu et al., 2010). To test this hypothesis, we measured environmental variables, including concentrations of macronutrients, and determined the copy numbers and phylogenetic affiliation of the _nifH_ gene in an _Ulva_ blooming area off Qingdao in 2011. We found the macronutrient structure appeared N-replete, but P and silicate (Si) were co-limiting. The _nifH_ gene copy numbers were found to be more abundant at the canopy-covered sites than the canopy-free sites, and the diazotrophic phytootypes were more represented by _Gammaproteobacteria Vibrio_ and _Deltaproteobacteria Desulfovibrio_ at the canopy-covered sites. We also discuss the following underlying mechanisms (e.g., DOC utilization, alleviation iron-limitation by bacteria and competition for P) to interpret these results.

2. Materials and methods

2.1. Study area and sample collection

Water samples were collected from the coast of Qingdao in the Yellow Sea during the summer of 2011 when _Ulva prolifera_ blooms occurred. The sampling area (approximately 35°N, 121°E) was approximately 30–80 km from the coast line of Qingdao, with water depths up to 30 m. The floating seaweed formed many distinct canopy patches measuring approximately 2 cm in thickness and several meters to kilometers in width. Surface water samples from five canopy-covered sites (CC1-5) located in the centers of the major algal patches (hereafter referred to as CC samples) were collected with a long syringe. Five replicates of canopy-free waters (CF1-5) between the major patches (hereafter referred to as CF samples) (Fig. 1) were also sampled. At each site, approximately two liters of surface seawater were collected with a sterilized polycarbonate bottle. No colony-forming cyanobacteria were observed using microscopic examination of the subsamples. The water samples were then filtered through a 20-μm-pore-sized mesh to exclude seaweed debris and microzooplankton, which were subsequently filtered through 0.2-μm-pore-sized poly-carbonate filters (47-mm diameter, Millipore, Germany) with a hand-operated vacuum pump. The filters were put into 2-ml pyrotubes and frozen in liquid nitrogen until DNA extraction. The filtered water samples were stored at −20 °C for nutrient measurements.

The water temperature, dissolved oxygen (DO), pH and salinity (measured using Practical Salinity Units) were measured at each site using an electronic probe (Hydrolab MS5, HACH, USA). The concentrations of NH₄⁺, nitrate (NO₃−), nitrite (NO₂−), dissolved silicate (Si) and soluble reactive phosphate (PO₄³⁻) were determined with a nutrient AutoAnalyzer (Seal, Germany).

2.2. DNA extraction and PCR amplification

The filters with microbial biomass were cut into pieces and the DNA was extracted using a FastDNA® Spin Kit for Soil (Mpbio, USA) based on the manufacturer’s protocol. The DNA concentration was quantified in triplicate with a spectrophotometer (Nanodrop ND 2000C, Thermo Scientific, USA). The _nifH_ gene, a commonly used molecular marker for nitrogenase, was targeted by PCR amplification of a 360–bp fragment based on specific primers Polf (5’-TGC GAY CCC AAR GCB GAC TC3’-3’) and PoIR (5’-ATS CCC ATC ATY TCR CCG GA3’-3’) (Poli et al., 2001). A PCR was performed in a Tprofessional Thermocycler (Biomera, Germany). The 25-μl PCR solution contained 1 μl of 1/10 dilution of DNA template, 400 nM of each primer, and 12 μl of PCR Master Mix, which contained DNA polymerase, buffers, salts and dNTP (Fermentas, USA). The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 40 s, and extension at 72 °C for 45 s, with a final elongation step at 72 °C for 7 min.

2.3. Cloning and sequencing

The PCR products of five replicated samples were pooled, purified with the Qiaquick PCR Purification Kit (Qiagen, Germany), ligated into the pTZ57 R/T vector (Fermentas), and then transferred into competent _Escherichia coli_ DH5α cells (Tiangen, China). Two clone libraries, one for water samples collected from canopy-covered sites (CC) and one for canopy-free sites (CF), were constructed. Positive recombinants were selected using X-Gal-IPTG LB indicator plates amended with ampicillin (100 mg/ml). The insertion was determined by PCR amplification with M13F and M13R primers. Approximately 280 clones in each library were randomly selected for restriction fragment length polymorphism (RFLP) analysis with the endonuclease _Haell_ (Fermentas) (Poli et al., 2001). Each RFLP type was defined as an operational taxonomic unit (OTU), and 1–3 representative clones of each OTU and 25 clones in total were sent for sequencing (Sangon, China).
based on fluorescent dye SYBR Green I. Each reaction was performed in a 25 μl solution containing 12.5 μl Maxima SYBR Green/ROX qPCR Master Mix 2× (Fermentas), 1 μl each primer (10 μM), and 1 μl 1/10 dilution of a DNA template. Standard curves were prepared from ten-fold serial dilutions ranging from 10⁷ to 10² copies of plasmids carrying the target gene fragment. Thermocycling was performed as follows: 2 min at 50 °C and 10 min at 95 °C, followed by a total of 40 cycles of 30 s at 95 °C, 40 s at 54 °C, 30 s at 72 °C, and 25 s at 80 °C. The specificity of the qPCR reaction was tested with a melting curve analysis (60–94 °C) in order to identify unspecific PCR products such as primer dimers or fragments with unexpected fragment lengths. The PCR product size was verified by agarose gel electrophoresis. The PCR amplification efficiencies were 87–103%, and the correlation coefficients (R²) for all of the assays were higher than 0.98.

2.6. Statistical analysis

To explore the effect of seaweed blooms on physicochemical factors, a student’s t-test was performed for water physicochemical variables (n = 5). Both Pearson’s (r) and Spearman’s correlation coefficients (ρ) were calculated to examine the links between the nifH gene abundance and environmental variables (n = 10). These analyses were performed using SPSS 13.0 for windows (SPSS, Chicago, USA).

The library coverages were calculated using the formula C = [1−(n1/N)] × 100, where n1 is the number of unique (frequency = 1) OTU detected in a library and N is the total number of clones in the same library (Mullins et al., 1995). The LIBSHUFF program (Singleton et al., 2001) provided a hypothesis test (Cramer-von Mises statistics) for comparing the libraries of the blooming samples (assigned to X) and the control (assigned to Y) to determine whether they were significantly different.

3. Results

3.1. Comparison of environmental factors between canopy-covered and canopy-free waters

Nutrients in both the canopy-covered and canopy-free surface waters appeared to be N-rich but poor in P and Si (Table 1). NH₄⁺ was the dominant nitrogen species, accounting for approximately 51% and 73% of the DIN in the CC and CF samples, respectively. Concentrations of SiO₄²⁻ were lower than 1.0 μM, whereas PO₄³⁻ represented the lowest macronutrients with less than 0.05 μM. This resulted in much higher stoichiometric ratios of N:P and N:Si. Comparison between the two types of water samples indicated that there were significantly lower concentrations of NH₄⁺ and DIN, and N:P and N:Si ratios in the CC (p < 0.05), whereas other environmental variables, nutrient levels, and stoichiometric ratios were not significantly different (p > 0.20) (Table 1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Canopy-covered (mean ± SE)</th>
<th>Canopy-free (mean ± SE)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>19.8 ± 1.15</td>
<td>18.30 ± 0.42</td>
<td>0.25</td>
</tr>
<tr>
<td>Salinity</td>
<td>32.5 ± 0.27</td>
<td>32.2 ± 0.02</td>
<td>0.35</td>
</tr>
<tr>
<td>DO (mg/l)</td>
<td>4.10 ± 0.22</td>
<td>3.98 ± 0.26</td>
<td>0.74</td>
</tr>
<tr>
<td>pH</td>
<td>8.28 ± 0.07</td>
<td>8.12 ± 0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>NH₃⁻ (μM)</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.76</td>
</tr>
<tr>
<td>NH₄⁺ (μM)</td>
<td>2.14 ± 0.15</td>
<td>5.33 ± 0.93</td>
<td>0.01</td>
</tr>
<tr>
<td>NH₃⁻ (μM)</td>
<td>1.94 ± 0.18</td>
<td>1.84 ± 0.28</td>
<td>0.77</td>
</tr>
<tr>
<td>DIN (μM)</td>
<td>4.19 ± 0.32</td>
<td>7.29 ± 1.05</td>
<td>0.02</td>
</tr>
<tr>
<td>PO₄³⁻ (μM)</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.00</td>
<td>0.20</td>
</tr>
<tr>
<td>SiO₄²⁻ (μM)</td>
<td>0.67 ± 0.05</td>
<td>0.58 ± 0.05</td>
<td>0.25</td>
</tr>
<tr>
<td>N:P</td>
<td>150.60 ± 37.09</td>
<td>296.64 ± 21.08</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N:Si</td>
<td>6.30 ± 0.26</td>
<td>12.86 ± 2.01</td>
<td>0.01</td>
</tr>
<tr>
<td>P:Si</td>
<td>24.38 ± 2.62</td>
<td>23.70 ± 5.57</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Significant differences (p < 0.05) in t-tests are highlighted in bold.
3.2. Community composition of bacterial diazotrophs

The coverages of 98.9% and 100.0% indicated that the majority of phylotypes in the two libraries were detected. Following RFLP screening of 562 (280 and 282 clones for CC and CF, respectively), a total of 12 unique OTUs were obtained, with 10 and 6 OTUs from the CC and CF samples, respectively.

All of the newly obtained \textit{nifH} gene sequences had similarity ranging from 64.3 to 95.5% and were phylogenetically placed into two major Clusters I and III (Fig. 2) based on the classification of \textit{nifH} genes by Zehr et al. (2003). The majority (91.1% of the total) of \textit{nifH} OTUs belonged to Cluster I and were primarily affiliated with sub-cluster 1I; the others fell into Cluster III (3E). The OTUs from the control were all classified in clusters I, whereas OTUs from the bloom presented in both Clusters I and III. According to the phylogenetic tree, the \textit{nifH} genes from this study were closely related to the representatives of \textit{Alpha-}, \textit{Gamma-}, \textit{Delta-} and \textit{Epsilonproteobacteria}; however, no cyanobacterial \textit{nifH} phylotypes were detected (Fig. 2).

Overall, \textit{Gammaproteobacteria}, in particular \textit{Vibrio}-related, \textit{nifH} OTUs dominated in both the CC and CF samples. Nevertheless, 8 clones from 2 \textit{Alphaproteobacteria} OTUs were exclusively detected from CF, with sequences that were 85.7% identical to \textit{Arcobacter} sp. [EF468430] and 93.4% identical to \textit{Azospirillum brasilienis} [X51500] \textit{nifH} sequences. Six OTUs (OTU3, OTU5, OTU8, OTU9, OTU10, OTU11) representing approximately 36% relative abundance were derived only from the CC library. All of these sequences were related to \textit{Vibrio}, \textit{Arcobacter}, \textit{Methylomonas}, and \textit{Desulfovibrio} \textit{nifH} sequences with 84.4–96.3% sequence identity (Fig. 2). Four OTUs (OTU1, OTU2, OTU4, and OTU7) accounting for 61.2% of the total abundance were found in the two libraries. These four OTUs exhibited sequence similarities ranging from 88.9 to 97.6% to the described species of the genera \textit{Vibrio}, \textit{Arcobacter}, and \textit{Klebsiella}.

Statistical comparison of homologous and heterologous coverage curves for these two libraries by LIBSHUFF indicated that the two libraries were significantly different, with \( p \) values of 0.0007 (XY comparison) and <0.0001 (YX comparison).

3.3. Copy numbers of the \textit{nifH} gene and correlations with environmental variables

The \textit{nifH} gene copy numbers quantified by qPCR revealed that the gene abundance ranged from \( 1.9 \times 10^6 \) to \( 3.4 \times 10^7 \) copies l\(^{-1}\) in the canopy-covered samples, and 55% to 66% for the canopy-free samples. The variation in this ratio was statistically significant (\( p \) < 0.0001). The \( nifH \) genes in the two libraries were significantly different, with \( p \) values of 0.0007 (XY comparison) and <0.0001 (YX comparison).
Significantly higher gene copies than the canopy-free samples (t-test, \( p = 0.002 \))

Table 2

<table>
<thead>
<tr>
<th>Canopy-covered</th>
<th>Copy number</th>
<th>Canopy-free</th>
<th>Copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>5.819</td>
<td>CF1</td>
<td>3.359</td>
</tr>
<tr>
<td>CC2</td>
<td>4.599</td>
<td>CF2</td>
<td>2.427</td>
</tr>
<tr>
<td>CC3</td>
<td>3.421</td>
<td>CF3</td>
<td>1.921</td>
</tr>
<tr>
<td>CC4</td>
<td>4.188</td>
<td>CF4</td>
<td>2.152</td>
</tr>
<tr>
<td>CC5</td>
<td>4.739</td>
<td>CF5</td>
<td>2.570</td>
</tr>
<tr>
<td><strong>Mean ± SE</strong></td>
<td><strong>4.553 ± 0.391</strong></td>
<td><strong>2.486 ± 0.245</strong></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Significant correlations between the \( nifH \) gene copy number and the stoichiometric ratios of N:P and N:Si, concentrations of ammonium (\( \text{NH}_4^+ \)), and dissolved inorganic nitrogen (DIN).

The qPCR detected considerable \( nifH \) copy numbers in both the CC and CF samples, indicating substantial N\(_2\) fixation potential in the N-rich coastal waters. This result contrasts with the often assumed N\(_2\) fixation ecology in aquatic environments, as high concentrations of \( \text{NH}_4^+ \) inhibit N\(_2\) fixation, and \( \text{NH}_4^+ \) uptake is energetically favorable compared to N\(_2\) fixation (Klugkist and Haaker, 1984; Zehr and Ward, 2002; Knapp, 2012). As rich \( \text{NH}_4^+ \) ambient waters could be easily accessible for macroalgae and phytoplankton, the N supply by diazotrophs via symbiosis seems unnecessary. However, \( nifH \) genes have been detected in nutrient-rich estuaries (e.g., Affourtit et al., 2001; Zehr et al., 2003; Jenkins et al., 2004), and substantial N\(_2\) fixation has been detected in N-replete waters (Knapp, 2012), suggesting that diazotrophs and their function could also be of importance when N is abundant in ambient waters (Farnelid et al., 2013).

Recent studies showed that heterotrophic N\(_2\)-fixers were more abundant in estuaries and coastal areas than in open oceans (Olson et al., 1999; Riemann et al., 2010; Farnelid et al., 2011). This notion is supported by our results indicating that all \( nifH \) phylotypes belong to heterotrophic diazotrophs (Fig. 2). Abundant Gammaproteobacteria diazotrophs have been frequently recorded in oligotrophic environments, such as the North (Fong et al., 2008) and South Pacific Oceans (Halm et al., 2012), the Sargasso Sea (Farnelid et al., 2011), and coral microbiomes (Lema et al., 2014). However, it is not common that Gammaproteobacteria phylotypes are dominant in diazotroph communities with N-rich samples as our study shows. A possible explanation is that the DOC released from macroalgae favors the growth of Gammaproteobacteria, which was previously suggested in that this bacterial group is often linked to the excretion of organic carbon from phototrophs (Zehr et al., 2007; Moisander et al., 2008). Many marine heterotrophic bacteria exhibit high levels of chemotaxis toward a variety of amino acids.
and sugars (Stocker and Seymour, 2012). Therefore, the DOC released by Ulva may attract heterotrophic N$_2$-fixers (e.g., vibrios) swimming towards canopy-covered waters, contributing to high abundance there.

It is interesting that the most abundant diazotrophs in our samples were related to Vibrio (Fig. 2). Many vibrios are known as both pathogens and diazotrophs in estuarine and coastal waters (Urdaci et al., 1988; Riemann et al., 2010). Nevertheless, vibrios and members of another Gammaproteobacteria genus Marinobacter can synthesize and secrete photosensitiser siderophores called vibrio-ferrin, which tightly binds Fe(III) and thereby increases its solubility (Yamamoto et al., 1994). Photolysis of the Fe(III)-vibrio-ferrin complex provides labile iron (Fe) to algal cells, presumably in exchange for DOM (Amin et al., 2009). As photosynthetic fixation of carbon and the utilization of nitrate require many Fe-containing enzymes, the rapid growth of macroalgae could be limited by Fe availability (Viaroli et al., 2005). Therefore, the dominance of vibrios in diazotrophic communities in this study could reflect the Fe-supply link between macroalgae and heterotrophic bacteria in the putatively Fe-limited waters.

In this study, the low concentrations of soluble reactive phosphate (0.02–0.04 µM) and the extremely high ratios of N : P (approximately 150–300:1) indicate severe P-limitation for macroalgae, phytoplankton and bacteria in the studied waters. Because bacteria have a high median half-saturation constant for orthophosphate uptake, they are very effective competitors with eukaryotic algae for orthophosphate (Ammerman, 2003). It has been reported that Vibrio cholerae biosynthesizes and stores very large amounts of inorganic polyphosphate for ATP biosynthesis when P is limiting in the environment (Ogawa et al., 2000; Jahid et al., 2006). Furthermore, the sulfate-reducing Desulfovibrio gigas can form P-rich granules inside cells under Fe limitation (Hensgens et al., 1996). The dissolved organic phosphorus released by macroalgae could be efficiently regenerated to inorganic phosphorus, taken up and stored by these bacteria, which enhances the capacity of vibrios and Desulfovibrio to survive environmental stresses in a low-phosphate and Fe-limited environment. This speculation is largely supported by our observation that vibrios and Desulfovibrio-related diazotrophic phylotypes were dominant in the studied waters. Therefore, both previous research and our evidence support the notion that Vibrio and Desulfovibrio are tolerant of low P availability or are better competitors for P in diazotrophic communities. Furthermore, strict anaerobes of Desulfovibrio were detected in Ulva canopy-covered but not in canopy-free waters, indicating the presence of anoxic micro-environments and decomposition at night time during macroalgal blooms, which could provide diverse niches to microorganisms, resulting in a different community composition of diazotrophs in CC samples.

The abundance of diazotrophs was inversely related to the N:P ratio in surface waters of the studied region of the Yellow Sea (Fig. 3). Although this finding is based on a small-scale dataset, it partly agrees with a recent resource-ratio model, which suggests that the ratios of N:Fe and N:P control the biogeography of diazotrophs in global surface oceans (Ward et al., 2013) and the phosphate controls the nifH gene copy numbers in oligotrophic North Pacific Ocean (Robidart et al., 2014).

In summary, we found that macroalgal blooms of Ulva prolifera caused dramatic decreases of ammonium concentrations and N:P ratios of canopy-covered surface waters in a coastal area of the Yellow Sea, where the DIN was rich, but the P and Si were limited. The blooms affected the community composition, and enhanced the abundance of N$_2$-fixing bacteria in these waters covered with accumulated macroalgal canopies. These changes in community composition and abundance of diazotrophs were related to the N:P ratio and could be attributed to a series of macroalgae–bacteria interactions. These include bacterial utilization of the DOM released from macroalgae and the Fe supply to macroalgae, and their competition for phosphorus. By investigating the diazotrophs in these very characteristic environments (N-rich, P-limited, and putatively DOC-rich and Fe-deficient), this study contributes to our understanding of the ecology of heterotrophic diazotroph niche adaptation and environmental controls, which is presently not well understood (Riemann et al., 2010). Nevertheless, the presence of an abundance of nifH genes does not necessarily mean that these microbes are actually active and functioning in such environments (Zehr et al., 2007); the gene expression and N$_2$–fixation rates need to be further investigated in order to assess the contribution of heterotrophic diazotrophs in nitrogen fixation during algal blooms in increasingly eutrophic estuarine and coastal waters.

Acknowledgments

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