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

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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 (4.55×10^6 copies l^{-1}) than in CF (2.49×10^6 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 (Goecke et al., 2010).

Diazotrophs (N_2-fixers) play an important role in ocean systems where inorganic nitrogen can represent a significant limiting factor

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for primary production (Falkowski et al., 1998). While large, colony-forming cyanobacterial *Trichodesmium* has been considered a major contributor to N_2 fixation in the tropical and sub-tropical oligotrophic oceans (Zehr et al., 2000; Sañudo-Wilhelmy et al., 2001; Church et al., 2005; Tang et al., 2006; Langlois 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 N_2-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: phylotypes from Cluster I (subcluster 1A–1V) were from Cyanobacteria and *Alpha-*, *Beta-*, *Gamma-*, and *Epsilonproteobacteria*, Cluster II (subcluster 2A–2F) was from Archaea, Cluster III (subcluster 3A–3T) was from anaerobic bacteria such as *Clostridium*, sulfate reducers, *Deltaproteobacteria*, and *Spirochaetes*, and Cluster IV (subcluster 4A–4F) was from non-functional archaeal *nifH* homologues (Zehr et al., 2003).

Because biological N_2 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 N_2 *in situ* (Head and Carpenter, 1975). In other environmental conditions, for instance, high concentrations of ammonium (NH_4^+), 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 N_2 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 identified to be *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-limited. The *nifH* gene copy numbers were found to be more abundant at the canopy-covered sites than the canopy-free sites, and the diazotrophic phylotypes were more represented by *Gammaproteobacteria Vibrio* and *Deltaproteobacteria Desulfovibrio* at the canopy-covered sites. We also discuss the possible 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 microscopical 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 polycarbonate 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 NH_4^+, nitrate (NO_3^-), nitrite (NO_2^-), dissolved silicate (Si) and soluble reactive phosphate (PO_4^{3-}) were determined with a nutrient AutoAnalyser (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 CCS AAR GCB GAC TC-3′) and PolR (5′-ATS GCC ATC ATY TCR CCG GA-3′) (Poly et al., 2001). A PCR was performed in a Tprofessional Thermocycler (Biometra, 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 *HaeIII* (Fermentas) (Poly 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).