ORIGINAL ARTICLE

Bacteriochlorophyll and community structure of aerobic anoxygenic phototrophic bacteria in a particle-rich estuary

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Photoheterotrophic microbes use organic substrates and light energy to satisfy their demand for carbon and energy and seem to be well adapted to eutrophic estuarine and oligotrophic oceanic environments. One type of photoheterotroph, aerobic anoxygenic phototrophic (AAP) bacteria, is especially abundant in particle-rich, turbid estuaries. To explore questions regarding the controls of these photoheterotrophic bacteria, we examined their abundance by epifluorescence microscopy, concentrations of the light-harvesting pigment, bacteriochlorophyll *a* (BChl *a*) and the diversity of *pufM* and 16S ribosomal RNA (rRNA) genes in the Chesapeake Bay. Concentrations of BChl *a* varied substantially, much more so than AAP bacterial abundance, along the estuarine salinity gradient. The BChl *a* concentration was correlated with turbidity only when oceanic and estuarine waters were considered together. Concentrations of BChl *a* and BChl *a* quotas were higher in particle-associated than in free-living AAP bacterial communities and appear to reflect physiological adaptation, not different AAP bacterial communities; *pufM* genes did not differ between particle-associated and free-living communities. In contrast, particle-associated and free-living bacterial communities were significantly different, on the basis of the analysis of 16S rRNA genes. The BChl *a* quota of AAP bacteria was not correlated with turbidity, suggesting that pigment synthesis varies in direct response to particles, not light attenuation. The AAP bacteria seem to synthesize more BChl *a* when dissolved and particulate substrates are available than when only dissolved materials are accessible, which has implications for understanding the impact of substrates on the level of photoheterotrophy compared with heterotrophy in AAP bacteria.

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Introduction

Photoheterotrophic microbes satisfy their requirement for carbon and energy by consuming organic substrates and harvesting light energy. Photoheterotrophy is widespread among the main groups of microbes found in aquatic systems, including cyanobacteria, Proteobacteria and Bacteroidetes (Eiler, 2006) and is integral to microbial carbon and energy cycling in estuarine, coastal and oceanic waters. Measurements of light-stimulated bacterial production and amino-acid assimilation suggest that photoheterotrophy contributes directly to the flux of carbon (C) and energy in marine systems (Church

et al., 2004; Michelou *et al.*, 2007; Mary *et al.*, 2008). Nonetheless, there are large gaps in our understanding of how photoheterotrophic microbes adapt to light, dissolved organic carbon, particulate organic carbon and nutrients. Estuaries present valuable test cases for examining the relationship between photoheterotrophs and these environmental factors.

One group of photoheterotrophs, the aerobic anoxygenic phototrophic (AAP) bacteria, seems to be more abundant in estuarine waters than in other environments. The highest abundances of AAP bacteria occur in estuaries, such as Long Island Sound, Chesapeake Bay and Delaware Bay, where these bacteria can constitute more than 10% of total prokaryotic abundance (Schwalbach and Fuhrman, 2005; Waidner and Kirchman, 2007). Although ranging up to almost 25% of the total prokaryotic community in the oligotrophic South Pacific Ocean (Lami *et al.*, 2007), AAP bacterial abundance is typically less than 10% of the total community in

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oceanic environments. The AAP bacteria make up 1.5–15% of total prokaryotic abundance in the coastal Pacific Ocean off California (Schwalbach and Fuhrman, 2005), in tropical and polar waters (Schwalbach and Fuhrman, 2005; Cottrell and Kirchman, 2009), in the North Atlantic Ocean (Sieracki *et al.*, 2006) and in shelf waters of the mid-Atlantic bight and in the Pacific Ocean near Hawaii (Cottrell *et al.*, 2006). Abundances of AAP bacteria in the brackish Baltic Sea, which is much like an estuary with a large input of freshwater (Stepanauskas *et al.*, 2002), seem to be more similar to those found in the ocean, ranging from about 1–10% of the total prokaryotes (Salka *et al.*, 2008).

Bacteriochlorophyll *a* (BChl *a*) is the light-harvesting pigment and a component of the reaction center complex in AAP bacteria (Yurkov and Beatty, 1998; Kolber *et al.*, 2001). The concentration of BChl *a* in oligotrophic waters is low (~1%) compared with that of chlorophyll *a* (Goericke, 2002; Cottrell *et al.*, 2006; Sieracki *et al.*, 2006; Lami *et al.*, 2007). Nevertheless, the BChl *a* quota of AAP bacteria cells can be substantial and comparable to the divinylchlorophyll *a* quota of *Prochlorococcus* (Cottrell *et al.*, 2006). Such an investment by AAP bacteria in BChl *a* synthesis suggests that light harvesting is an important aspect of AAP bacterial metabolism. The BChl *a* quota of cultivated AAP bacteria varies as much as 10-fold in response to light availability (Koblížek *et al.*, 2003; Li *et al.*, 2006), similar to *Synechococcus* and *Prochlorococcus* (Mackey *et al.*, 2009). In addition, the BChl *a* quota of AAP bacteria is higher at night than during the day because BChl *a* synthesis is inhibited by light (Koblížek *et al.*, 2005, 2007). However, in oceanic waters the BChl *a* quota of AAP bacteria does not seem to vary systematically with depth in the water column (Cottrell *et al.*, 2006), suggesting that factors other than light alone may impact the pigment quota of AAP bacteria.

Particle attachment seems to be a key adaptation for the success of AAP bacteria. In Delaware Bay, 30–90% of AAP bacteria are associated with particles, whereas usually 20% or less of total prokaryotes are particle associated (Waidner and Kirchman, 2007). However, high percentages of particle-associated AAP bacteria may be specific to estuaries. In the Atlantic Ocean and Mediterranean Sea, 10–40% of AAP bacteria appeared to be associated with particles, whereas in a Mediterranean coastal lagoon about 50% of AAP bacteria were associated with particles (Lami *et al.*, 2009). Adaptations to the particle environment may involve responses to the availability of light controlled, in part, by light scattering and absorption by particles and to the availability of substrates that may be associated with detrital particles.

In this study, we assessed the concentration of BChl *a*, the abundance of AAP bacteria and the BChl *a* quota of AAP bacteria in the Chesapeake Bay to explore factors hypothesized to be integral to the

ecology of AAP bacteria. Particle-associated and free-living cells were examined to determine the relationship between these photoheterotrophs and concentrations of particulate detritus. Phylogenetic analyses of free-living and particle-associated AAP bacteria were used to explore the impact of physiological adaptation versus shifts in community structure on the BChl *a* quota of AAP bacteria.

Materials and methods

Sampling and oceanographic parameters

The main stem of Chesapeake Bay was sampled in June 2006. Seawater was collected from a depth of approximately 1 m using Niskin bottles (General Oceanics, Miami, FL, USA). Light attenuation was measured using a Secchi disc (Wildlife Supply Company, Yulee, FL, USA) and a Biospherical (San Diego, CA, USA) light meter. Salinity was determined using an SBE-9 conductivity meter (Sea-Bird Electronics, Bellevue, WA, USA). Free-living microbes were separated from the whole community by size-fractionation using vacuum filtration and 0.8- μ m pore-size polycarbonate (Millipore, Billerica, MA, USA) filters. Chlorophyll *a* (Chl *a*) was measured by fluorometry using 90% acetone extracts of particulate material collected on GF/F (Whatman, Piscataway, NJ, USA) filters (Parsons *et al.*, 1984).

Microscopic enumeration of AAP bacteria and total prokaryotes

Prokaryotes were enumerated by epifluorescence microscopy of paraformaldehyde-fixed samples that were filtered onto 0.2- μ m pore-size black polycarbonate filters. Total prokaryotes were enumerated after staining with a solution of 1 μ g ml⁻¹ 4',6-diamidino-2-phenylindole (Porter and Feig, 1980) in Tris buffer (pH 7.2) containing 0.5 M NaCl. The samples were analyzed using a computer-controlled microscope (Olympus Provis AX70, Central Valley, PA, USA) and image analysis software (ImagePro Plus, Media Cybernetics, Bethesda, MD, USA) as described previously (Cottrell and Kirchman, 2003). The average and s.d. value for 20 fields of view were estimated. The AAP bacteria were enumerated using an Intensified Retiga charge-couple device camera (Qimaging, Surry, BC Canada) and image analysis routines to enumerate cells that fluoresce blue when stained with 4',6-diamidino-2-phenylindole and have infra-red fluorescence, but not red or orange fluorescence (Cottrell *et al.*, 2008; Cottrell and Kirchman, 2009).

*BChl *a* concentration*

The concentration of BChl *a* was determined as previously described (Ras *et al.*, 2008). In brief, 200-ml of seawater was filtered onto GF/F (Whatman) filters that were then stored at -80 °C. The samples were extracted in 3 ml of 100% methanol for a

minimum of 30 min at -20°C and then briefly sonicated. After a further incubation for 30 min, at least, at -20°C , the extracts were clarified by vacuum filtration. The sample extracts, premixed with an equal amount of 28 mM tetrabutylammonium acetate, were then injected onto an Agilent Technologies (Foster City, CA, USA) 1100 series HPLC system fitted with a narrow reversed-phase C8 Zorbax Eclipse XDB column ($3 \times 150\text{ mm}$; $3.5\text{-}\mu\text{m}$ particle size), which was heated to 60°C . Separation was achieved within 28 min using a gradient composed of solution A containing 28 mM tetrabutylammonium acetate: methanol (30:70; vol:vol) and solution B containing 100% methanol according to the following program: 0 min: 90% A, 10% B; 22 min: 5% A, 95% B; 27 min: 5% A, 95% B. The BChl *a* chromatogram was recorded at 770 nm using an online diode array detector. The retention times and diode array absorption spectra of peaks were used for identification purposes. Pigment concentrations were calculated from the peak areas using an external BChl *a* standard (Sigma, St Louis, MO, USA) and an internal standard correction (vitamin E acetate, Sigma). The detection limit was of 1 ng l^{-1} for BChl *a* at a signal-to-noise ratio of 3 and a filtration volume of 0.2 l.

Diversity of *pufM* genes

Seawater samples were preserved for filter PCR (Kirchman *et al.*, 2001) in 2% paraformaldehyde at 4°C for 18 h. The preserved samples were then filtered in 10-ml aliquots onto 25-mm diameter, $0.2\text{-}\mu\text{m}$ pore-size polycarbonate filters, rinsed three times with deionized water and then stored at -20°C .

The diversity of *pufM* genes, which encode the M subunit of the photosynthetic reaction center complex, was examined using denaturing gradient gel electrophoresis (DGGE) of PCR amplicons using a modification of the two-step procedure described by Yutin *et al.* (2008). The first step was performed using the filter PCR approach in which the cells are added to the PCR reaction while on a polycarbonate filter (Kirchman *et al.*, 2001). In the first PCR reaction, the primers: *pufM*_uniF (5-GGNAAYYTNT WYTAYAAAYCCNTTYCA-3) and *pufM*_WAW (5-AY NGCRAACCACCANGCCCA-3) (Yutin *et al.*, 2005), were used in a reaction that included an initial 4-min denaturation step at 94°C , followed by a three-step cycle consisting of 1 min of denaturation at 94°C , 1 min of annealing at 50°C and 1 min of extension at 68°C . The cycle was repeated 10 times and the reaction concluded with a 10-min extension at 68°C . The reaction mixture contained 2.0 mM MgCl_2 , 0.25 mM dNTPs, $1\text{ }\mu\text{M}$ of *pufM*_uniF primer and $0.5\text{ }\mu\text{M}$ of *pufM*_WAW primer, $3\text{ }\mu\text{l}$ of a bovine serum albumin (stock concentration of 10 mg ml^{-1} ; Sigma-7030), 1/16th of a 25-mm polycarbonate filter containing the cells and one unit of BIO-X-ACT DNA polymerase (Bioline, Tauntun, MA, USA) in a total volume of $25\text{ }\mu\text{l}$.

The product of the first PCR reaction was used as the template in the second PCR step that used primers *pufM*_uniF and GC_WAW (5-CCGCCGCGC GGCGGGCGGGCGGGGGCACGGGGAYNGCRAACC ACCANGCCCA-3; Yutin *et al.*, 2008). The GC clamp in the GC_WAW primer increases the stability of denatured PCR products (Muyzer *et al.*, 1993). A volume of $2\text{ }\mu\text{l}$ of the first reaction mixture was added to the second PCR reaction that had the same reaction composition and cycling conditions as the first PCR mix, except that the reaction was cycled 30 times and the reaction volume was $40\text{ }\mu\text{l}$.

The PCR primers for the *pufM* gene were tested *in silico* to determine whether the *pufM* genes of anaerobic phototrophs, if present, would be amplified in our assays. The *in silico* test used the Amplify software (www.engels.genetics.wisc.edu/amplify) with sequences from strict and facultative anaerobes, including *Rhodobacter capsulatus*, *Rhodobacter blasticus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Thermochromatium tepidum*, *Rhodocyclus tenuis* and *Rhodomicrobium vannielii*.

Amplicons from filter PCR were analyzed by standard DGGE methods (Muyzer *et al.*, 1993) using an 8% acrylamide gel containing a 25–55% gradient of formamide and urea. Electrophoresis was performed at 60°C for 20 h at 100 V using a DCode DGGE apparatus (Bio-Rad, Hercules, CA, USA). After staining with ethidium bromide, images of the DGGE gels were recorded and analyzed using a Kodak (Rochester, NY, USA) Gel Logic 100 electrophoresis documentation and analysis system using the default setting for recognizing bands and determining band migration distances. Analysis of band distances was done using the Palaeontological Statistics (PAST) data analysis package (Hammer *et al.*, 2001). Select bands were sequenced using the Sanger method after being ligated to the TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) and cloned in *Escherichia coli* after the procedure recommended by the manufacturer. These sequence data have been submitted to the GenBank database under accession numbers GU079530–GU079579.

Diversity of 16S rRNA genes

The diversity of 16S ribosomal RNA (rRNA) genes was analyzed by filter PCR (Kirchman *et al.*, 2001) using the same samples analyzed for *pufM* genes. The DGGE analysis of 16S rRNA genes used PCR primers: GC358F and 517R (Muyzer *et al.*, 1993). The PCR cycling conditions included an initial 4-min denaturation step at 94°C , which was followed by a three-step cycle consisting of 1 min of denaturation at 94°C , 1 min of annealing at 50°C and 1 min of extension at 68°C . The cycle was repeated 35 times. The reaction concluded with a 10-min extension at 68°C . The reaction mixture contained 2.0 mM MgCl_2 , 0.25 mM dNTPs, $10\text{ }\mu\text{M}$ of each primer, $3\text{ }\mu\text{l}$ of a bovine serum albumin (stock concentration of 10 mg ml^{-1}) (Sigma-7030), 1/16th

of a 25-mm diameter polycarbonate filter containing the cells and 1 U of Bio-x-act DNA polymerase (Bioline) in a total volume of 25 μ l. The DGGE conditions, gel imaging and analysis were the same as those used for the *pufM* genes.

Statistical analysis

BChl *a* quotas and light attenuation were log-transformed for the correlation analysis.

Results

Environmental setting

Salinity decreased from 21 p.s.u. at the sampling site closest to the mouth of the bay to 5 p.s.u. at the site 240 km up the bay (Figure 1a). The light attenuation coefficient ranged from 0.72–0.96 m^{-1} at the sites less than 200 km from the mouth of the bay (Figure 1b). The turbidity was highest at the site furthest from the bay mouth where the light attenuation coefficient was 1.44 m^{-1} . The concentration of Chl *a* increased steadily from the lowest concentration of 6 $\mu\text{g l}^{-1}$ closest to the ocean to a maximum of 24 $\mu\text{g l}^{-1}$ at the site 200 km from the bay mouth. The concentration of Chl *a* was 11 $\mu\text{g l}^{-1}$ 240 km from the bay mouth. (Figure 1b).

Abundance of total prokaryotes and AAP bacteria

The abundance of AAP bacteria varied more than total prokaryotes along the 200 km estuarine transect. Direct counts of AAP bacterial cells varied about four-fold, from 0.2×10^6 – 0.8×10^6 cells per ml

(Figure 2a), whereas the total prokaryotic community varied less than two-fold, ranging from 6.5×10^6 – 1.0×10^7 cells per ml. Shifts in the contribution of AAP bacteria to the total prokaryotic community were greater than changes in absolute abundances. The relative abundance of AAP bacteria varied six-fold, from 2 to 12% of the total prokaryotic community within the estuary.

In contrast, the percentage of AAP bacteria found associated with particles was constant throughout the lower part of the estuary and variable in the waters more than 150 km from the estuary mouth. In the lower part of the estuary, on average $47 \pm 16\%$ of AAP bacteria were associated with particles, whereas in the upper region of the estuary, the percentage of particle-associated AAP bacteria was variable, ranging from 30 to 80% of the AAP bacterial community (Figure 2b).

Overall the percentage of total prokaryotic cells associated with particles averaged $34 \pm 8\%$, which was not significantly different from that of AAP bacteria (analysis of variance, $P > 0.05$; Figure 2b).

Bacteriochlorophyll *a*

The concentrations of BChl *a* varied much more than AAP bacterial abundances, but most of the

Figure 1 Salinity and light attenuation (a) and chlorophyll *a* (Chl *a*) concentration (b) on the Chesapeake Bay transect. The x-axis indicates distance from the mouth of the bay.

Figure 2 Abundance of total prokaryotes and the relative abundance of aerobic anoxygenic phototrophic (AAP) bacteria (a) in Chesapeake Bay determined by epifluorescence microscopy. The percentage of total prokaryotes and AAP bacteria that were associated with particles was determined by 0.8- μm pore-size fractionation (b). Representative error bars calculated by propagation of error are shown for two points only. The x-axis indicates distance from the mouth of the Chesapeake Bay.

Figure 3 Total concentration of bacterial chlorophyll *a* (BChl *a*) and percentage of BChl *a* associated with particles in Chesapeake Bay (a). The percentage of particle-associated BChl *a* was determined using 0.8- μ m pore-size fraction. Cellular BChl *a* quota of aerobic anoxygenic phototrophic (AAP) bacteria (b). Pigment content per cell was calculated by dividing the BChl *a* concentration by AAP bacterial abundance. The x-axis indicates distance from the mouth of the Chesapeake Bay.

variation was in the upper part of the estuary. In the lower part of the estuary, BChl *a* concentrations increased approximately 2.5-fold (the slope of the linear regression was 0.76 ± 0.14 and significantly different from zero, $P < 0.05$), from 50 ng l^{-1} near the mouth of the bay to 133 ng l^{-1} in the middle of the bay (Figure 3a). In contrast, 250 km from the bay mouth in the upper part of the bay BChl *a* concentration decreased more than 60-fold (slope = -1.6 ± 0.05 , $P < 0.05$) to levels below the detection limit of 1 ng l^{-1} .

The percentage of BChl *a* associated with particles followed essentially the same pattern as total BChl *a* (Spearman correlation: $r = 0.99$, $P < 0.05$). In waters more than 150 km from the mouth of the bay, the percentage of particle-associated BChl *a* decreased about 75-fold to $< 1\%$ of total BChl *a* 200 km from the bay mouth. However, the situation was different in the lower part of the bay where the percentage of particle-associated BChl *a* was high and constant, averaging $80 \pm 3\%$ (Figure 3a).

We examined the relationship between BChl *a* and turbidity (attenuation coefficient), including data from the northwest Atlantic Ocean (Sieracki *et al.*, 2006), coastal Atlantic Ocean (Cottrell *et al.*, 2006) and the South Pacific Ocean (Lami *et al.*, 2007) in our analysis (Figure 4). The correlation between the concentration of BChl *a* and

Figure 4 Relationship between turbidity and the concentration of bacterial chlorophyll *a* (BChl *a*) (a) and the cellular BChl *a* quota of aerobic anoxygenic phototrophic (AAP) bacteria (b) in the northwest Atlantic Ocean (Sieracki *et al.*, 2006), Chesapeake Bay, coastal Atlantic Ocean (Cottrell *et al.*, 2006) and South Pacific Ocean (Lami *et al.*, 2007).

turbidity was positive and statistically significant ($r = 0.63$; $n = 16$; $P < 0.05$; Figure 4a). However, when environments were examined individually different patterns emerged. In the Chesapeake Bay, the correlation between BChl *a* concentration and turbidity was negative and almost statistically significant ($r = -0.75$; $n = 7$; $P = 0.052$; Figure 4a). In contrast, there was no relationship between BChl *a* and turbidity in the oceanic data set that included the coastal North Atlantic Ocean and the South Pacific Ocean ($r = 0.07$; $n = 9$; $P = 0.33$; Figure 4a).

Bacteriochlorophyll a quota

The BChl *a* quota (BChl *a* per cell) of AAP bacteria varied substantially within the estuary (Figure 3b). The pattern was similar for total AAP bacteria and particle-associated AAP bacteria; BChl *a* quotas were low closest to and furthest away from the bay mouth and high in the middle of the bay. The peak in the middle of the bay was 0.28 fg per cell , which was four-fold higher than that near the mouth of the estuary and seven-fold higher than that in waters furthest from the bay mouth (Figure 3b).

The BChl *a* quota of particle-attached AAP bacteria was on average 1.8-fold higher than that of the total AAP bacterial community, although the difference was not statistically significant (*t*-test; $n=7$; $P=0.13$). There was a mid-bay peak in the BChl *a* quota for particle-associated AAP bacteria, similar to that of the total AAP bacterial community (Figure 2b). The BChl *a* quota of particle-associated AAP bacteria varied from 0.14 fg per cell near the mouth of the estuary to 0.55 fg per cell in the middle of the bay.

Unlike BChl *a* concentrations (Figure 4a), there was no significant correlation between the BChl *a* quota of AAP bacteria and turbidity for the data set that included the Chesapeake Bay, the coastal North Atlantic Ocean and the South Pacific Ocean ($r=0.31$; $P>0.05$; $n=16$; Figure 4b).

DGGE analysis of *pufM* gene diversity

The DGGE analysis revealed a modest level of *pufM* gene diversity within the estuary and obvious differences in the *pufM* genes at the 12 sites (Figure 5). The richness of the AAP bacterial community varied substantially between the sampling sites, evident in the two-fold variation in the number of DGGE bands of *pufM* genes (Table 1). The similarity between sampling sites varied substantially as well, with the Sorensen similarity index ranging from 0.27 to 1.0. Interestingly, sites that were completely similar based on *pufM* genes were

Figure 5 Dendrogram of *pufM* genes in the Chesapeake Bay. The cluster analysis used Sorensen similarity indices that were calculated on the basis of denaturing gradient gel electrophoresis (DGGE) band presence. The leaf labels are sampling locations that were numbered sequentially starting with the site furthest from the mouth of the bay. The scale bar represents 0.2-fold dissimilarity between DGGE banding patterns.

not adjacent to one another. For example, sites 2, 5, 7 and 9 were identical but differed from adjacent sites, such as sites 3, 4, 6 and 8, on the basis of DGGE of *pufM* (Figure 5). When band intensities were included in the analysis, pair-wise comparisons revealed significant differences between all of the communities (*t*-test; $P<0.05$) with one exception; sites 5 and 12 were not different from each other.

Sequence analysis of DGGE bands indicated that the *pufM* genes belonged to three subdivisions of the *Proteobacteria* (Table 2), including several clusters of *pufM* genes previously identified by Yutin *et al.* (2007). The *pufM* genes from site 1 belong to Group G, which includes *Alphaproteobacteria*, and Group I that includes *Betaproteobacteria*. The *pufM* sequences from site 5 belong to Groups E, I and J, which all include *Alphaproteobacteria*. Sequences from site 8 belonged to Groups E, G, I and sequences from site 12 belonged to those plus group K that includes *Gammaproteobacteria* (Table 2). Notably, *pufM* Group I includes a 40-kb DNA fragment from a metagenomic library from the nearby Delaware estuary (Waidner and Kirchman, 2005).

None of the *pufM* gene sequences obtained from the DGGE gels seemed to belong to anaerobic phototrophs based on sequence similarity. The *pufM* gene PCR primers used in this study are predicted to amplify the *pufM* genes of anaerobes on the basis of an *in silico* test. Five of the seven anaerobes that we tested, including *Rhodobacter capsulatus*, *Rhodobacter blasticus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum* and *Thermochromatium tepidum* are predicted to amplify, but were not found in our actual *pufM* analyses. *Rhodocyclus tenuis* and *Rhodomicrobium vannielii* failed the *in silico* PCR test.

Attached versus free-living communities

The DGGE analysis of *pufM* indicated that particle-associated AAP bacteria were similar to those found in the free-living community. The majority of DGGE bands were the same between the particle-

Table 1 Diversity of *pufM* and 16S rRNA genes inferred from DGGE banding patterns of PCR amplicons

	<i>PufM</i>				16S rRNA			
	Whole		<0.8 μ m		Whole		<0.8 μ m	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
No. of bands	7.38	0.92	7.13	1.46	16.17	2.04	18.33	1.51
Shannon index	1.81	0.14	1.79	0.16	2.53	0.18	2.69	0.11
Evenness	0.84	0.04	0.86	0.04	0.78	0.06	0.81	0.04

Abbreviations: DGGE, denaturing gradient gel electrophoresis; rRNA, ribosomal RNA.

Banding patterns generated from the whole community and cells in the <0.8 μ m bacterial size fraction.

Table 2 *pufM* genes seen in Chesapeake Bay identified based on the classification proposed by Yutin *et al.* (2007)

<i>pufM</i> group	Site	No. of clones	Closest BLAST hits	Accession	% Similarity
E	5, 8, 12	8	' <i>Thalassiohium</i> sp.' R2A62	AF394002	96–100
G	1, 8, 12	7	Clone 38B07	EU395537	98
			<i>Roseobacter</i> sp. Axw K-3b	EU196351	97
			Clone 36A06	EU395521	96
			Clone 1G02	EU191257	96–97
I	1, 5, 8, 12	26	<i>Roseovarius</i> sp. TM 1035	NC_007493	96
			Clone PUF_108_8_23	FJ619010	91–100
			Clone 38A10	EU395532	95–96
			Clone Clone-6	AB510455	94–96
			Gamma proteobacterium KT 71	AAOA01000002	96–100
			<i>Jannaschia</i> sp. CCS1	CP000264	91–92
			<i>Roseateles depolymerans</i>	AM773545	95
			<i>Loktanella vestfoldensis</i> SKA53	NZ_AAMS01000002	91–92
			<i>Acidiphilium rubrum</i>	AB005225	91–92
			Clone Clone-9	AB510458	96
J	5, 8, 12	4	Fosmid clone delriverfos06h03	AY912082	92
			<i>Methylobacterium</i> sp. 4–46	CP000830	87
			Clone 38C11	EU395540	87
			Clone 61G03	EU395567	96
K	12	4	Clone 61G01	EU395566	100
			Clone 2A04	EU191266	97
			Clone 2C03	EU191286	100

Abbreviation: BLAST, basic local alignment search tool.

Figure 6 Denaturing gradient gel electrophoresis (DGGE) gel of 16S ribosomal RNA (rRNA) genes amplified by PCR from environmental DNA extracted from the whole community (W) and the particle-associated fraction (P) at eight sites in Chesapeake Bay located 15–100 km from the mouth of the estuary.

associated and the free-living community. There was no significant difference in the *pufM* gene between the AAP bacteria in the whole community and the free-living size fraction, as revealed by DGGE (analysis of similarity; $P=0.7$). The average number of *pufM* DGGE bands was also similar between the two communities. The DGGE analysis of *pufM* genes revealed 7.4 ± 0.9 bands for the whole community and 7.1 ± 1.5 bands for the free-living size fraction (Table 1). In addition, there was no significant difference in the Shannon index of diversity for *pufM* genes in the whole community and in the free-living size fraction (t -test; $P>0.05$). The Shannon indices for the whole community and the free-living community were each 1.8 ± 0.14 and 1.79 ± 0.16 , respectively. Similarly, there was no significant

difference in evenness (t -test; $P>0.05$) for the whole community (Table 1).

In contrast, DGGE of 16S rRNA genes clearly resolved differences in the diversity of the entire bacterial community and of free-living bacteria (Figure 6). The composition of the whole communities at various sampling sites differed on the basis of DGGE banding patterns of 16S rRNA genes (analysis of similarity; $P<0.05$). Surprisingly, the number of 16S rRNA gene bands was slightly, but significantly, higher for the free-living size fraction than for the whole community (t -test; $P<0.05$; Table 1). Diversity of bacteria in the free-living fraction was also significantly higher, according to DGGE analysis of 16S rRNA genes (t -test; $P<0.05$). In contrast, the level of evenness was not significantly different between the two communities (t -test; $P>0.05$) and averaged about 0.8 for both (Table 1).

Discussion

The over arching goal of this study was to obtain a better understanding of organic matter cycling by photoheterotrophic microbes. We examined AAP bacteria because they are widespread and abundant in marine environments (Cottrell *et al.*, 2006; Sieracki *et al.*, 2006; Salka *et al.*, 2008), especially in estuaries such as the Chesapeake Bay (Schwalbach and Fuhrman, 2005; Waidner and Kirchman, 2007), where there is a large range of environmental conditions hypothesized to influence photoheterotrophy, including turbidity and concentrations of particulate material. Data on

particle-associated AAP bacteria seem to provide some clues regarding the success of these photoheterotrophs

in aquatic systems, but interpretation has not been straightforward. The association of AAP bacteria with particles could reflect several adaptations, ranging from those related to organic substrate utilization to others involved in light harvesting under the turbid conditions generated by particles. One of our objectives was to explore possible photoheterotrophic adaptations to light availability in turbid waters using BChl *a* and phylogenetic analysis of free-living and particle-associated AAP bacteria.

We suggest that differences in BChl *a* concentration reflect levels of AAP bacterial photoheterotrophy in estuarine and oceanic environments. Concentrations of BChl *a* in surface waters of the coastal Atlantic Ocean, the Gulf Stream (Cottrell *et al.*, 2006), the northwest Atlantic Ocean and the Sargasso Sea (Sieracki *et al.*, 2006) were less than 1 ng l^{-1} compared with $50\text{--}100 \text{ ng l}^{-1}$ in the Chesapeake Bay, suggesting that photoheterotrophy is 1–2 orders of magnitude greater in estuarine waters than in oceanic waters. However, it is not clear why BChl *a* concentrations are so much higher in estuarine than in oceanic environments. Differences in total microbial abundance are not large enough to explain the pigment concentrations. Photoadaptation such as that in phytoplankton (Cullen, 1982; Veldhuis and Kraay, 2004), which results in high pigment quotas in low-light environments such as estuaries, is one possible explanation.

We expected that because of photoadaptation, the BChl *a* quota of AAP bacteria would be higher in the estuary than in the ocean. Cultivated AAP bacteria modulate the BChl *a* quota as much as 10-fold in response to light availability (Koblížek *et al.*, 2003; Li *et al.*, 2006), similar to photoadaptation by phytoplankton. As a result of photoadaptation, phytoplankton C-to-chlorophyll *a* ratios are low in turbid estuaries, such as the Schelde (Lionard *et al.*, 2008), and high in clearer oceanic environments, such as the subtropical Atlantic Ocean (Veldhuis and Kraay, 2004). In contrast, the BChl *a* quota of AAP bacteria in Chesapeake Bay was not higher than that in the coastal North Atlantic Ocean and in the South Pacific Ocean. It seems that AAP bacteria do not photo-adapt, similar to phytoplankton, to low light availability in turbid estuarine waters.

Being associated with particles seems to give AAP bacteria a competitive advantage in estuaries, as proposed by Waidner and Kirchman (2007). Our results suggest that the benefit of high particle concentrations for AAP bacteria is related to a greater ability of particle-associated AAP bacteria to synthesize BChl *a*. The BChl *a* quota of particle-associated AAP bacteria was about two-fold higher than the BChl *a* quota of the total AAP bacterial community from the same water, although the

difference was not statistically significant due to large variability within both the particle-associated group and the total community. The strength of the relationship between BChl *a* quota and particle association should become clearer as additional data on BChl *a* concentrations become available. This, to the best of our knowledge, was the first study to examine BChl *a* concentrations in an estuary.

The high BChl *a* quota of AAP bacteria associated with particles was unexpected because detrital particles potentially contain labile organic substrates, which should lead to less phototrophy and lower BChl *a* quota. The BChl *a* quota of cultivated AAP bacteria is low when substrate concentrations are high (Rathgeber *et al.*, 2008). In natural communities, however, the impact of substrate availability on BChl *a* quota seems to differ from that seen in cultured strains, assuming that particle-associated AAP bacteria use particle-associated substrates. The negative correlation between BChl *a* quota and substrate availability in culture (Koblížek *et al.*, 2003) suggests that light harvesting provides energy in the absence of labile substrates. In contrast, our results suggest that AAP bacteria actually generate larger amounts of BChl *a* when they are associated with particles and have access to both dissolved and particulate substrates than when they are free-living and use only dissolved substrates.

Estuaries can have anoxic microhabitats that may harbor anaerobic anoxygenic bacteria that could have been counted by epifluorescence microscopy and contributed to the BChl *a* level we measured. However, these anaerobic counterparts of AAP bacteria do not seem to be abundant in oxic estuarine waters like the ones we examined (Crump *et al.*, 2007; Waidner and Kirchman, 2008). On the basis of metagenomic sequence analysis of photosynthesis genes and operons, *pufM*-bearing bacteria in the Delaware Bay lack the anaerobic versions of the *hemF* and *acsF* genes, but do possess the *acsF* gene, which is indicative of aerobic metabolism (Waidner and Kirchman, 2005). In addition, the PCR primers for *pufM* gene, which we used, should amplify the *pufM* genes of anaerobic AAP bacteria, including some that are facultative anaerobes, but these were not observed. Phylogenetic analysis of the *pufM* genes obtained in our study and in another estuarine study (Waidner and Kirchman, 2008) did not reveal sequences matching facultative anaerobes, such as *Rhodovulum* and *Rhodobium*.

A shift in community structure is another factor that could explain the BChl *a* quota of AAP bacteria in different regions of the Chesapeake Bay and in particle-associated AAP bacteria. However, the sequence analysis of *pufM* genes did not support the hypothesis that BChl *a* quota of AAP bacteria varies with community structure. The concentrations of BChl *a* were the same at sites within the estuary where the *pufM* gene sequences differed significantly. Furthermore, *pufM* genes and BChl *a* quota were not significantly correlated for the whole

community or for the free-living community (Mantel test, $P > 0.05$). Our results suggest that the high concentrations of BChl *a* in particle-associated AAP bacterial communities is the result of physiological adaptation, not of selection for AAP bacterial taxa with different *pufM* genes.

In contrast, DGGE analysis of 16S rRNA genes did reveal a significant difference between the whole community and the free-living community. Community structure data from estuaries and other marine systems also suggest that particle-associated and free-living bacterial communities differ (DeLong *et al.*, 1993; Crump *et al.*, 1999). In the Mackenzie River, the largest differences between particle-associated and free-living communities were observed in waters with the highest particulate organic material content (Garneau *et al.*, 2009). In the Columbia River, particle-associated bacterial communities are distinct from those found in the free-living communities (Crump *et al.*, 1999). Furthermore, the particle-associated communities differ between the river, estuary and coastal waters, whereas the free-living bacteria are similar (Crump *et al.*, 1999).

The affinity of AAP bacteria for particles seems to differ among estuaries and between estuaries and oligotrophic systems. In contrast to the Chesapeake Bay, where the relative abundance on particles of AAP bacteria and the rest of the prokaryotic community are equal, in the Delaware Bay the percentage of particle-associated AAP bacteria ranged from 30–90%, whereas only about 20% of the total prokaryotic community was associated with particles (Waidner and Kirchman, 2007). In contrast, the abundance of particle-associated AAP bacteria is higher in those estuaries than in more oligotrophic systems. In the Sargasso Sea and Mediterranean Sea, 60–90% of the AAP bacteria are free-living, based on *in vivo* BChl *a* fluorescence data (Lami *et al.*, 2009), much higher than what we observed in the Chesapeake Bay. Despite differences between the Delaware Bay and Chesapeake Bay, our results support the view that the percentage of particle-associated AAP bacteria is higher in estuaries than in offshore environments.

The image of photoheterotrophs using light energy to gain an advantage over strict heterotrophs is attractive but supportive data have remained largely elusive. The case of AAP bacteria, which are more abundant in coastal and estuarine environments than in oceanic environments, was one of the first signs that the role of photoheterotrophy is more complex than originally thought (Kolber *et al.*, 2000). The data presented here suggest that particle-associated AAP bacteria are able to synthesize larger amounts of BChl *a* to drive photoheterotrophy than free-living AAP bacteria. It now seems important to test the hypothesis that photoheterotrophy is limited by organic substrates, which would have implications for photoheterotrophy providing supplemental energy or alleviating C limitation.

Assessing the level of photoheterotrophy compared with heterotrophy in AAP bacteria and other photoheterotrophs under C-replete and C-limiting conditions will be essential for understanding the impact of photoheterotrophy on C cycling in aquatic systems.

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