Distribution of free-living and particle-attached aerobic anoxygenic phototrophic bacteria in marine environments

Raphaël Lami1,2,7,*, Zuzana Čuperová3,4, Josephine Ras5,6, Philippe Lebaron1,2, Michal Koblížek3,4

1UPMC Univ Paris 06, UMR 7621, LOBB, Observatoire Océanologique, 66651 Banyuls-sur-Mer, France
2CNRS, UMR 7621, LOBB, Observatoire Océanologique, 66651 Banyuls-sur-Mer, France
3Institute of Physical Biology, University of South Bohemia, Zámek 136, 373 33 Nové Hrady, Czech Republic
4Institute of Microbiology CAS, Opatovický mlyn, 379 81 Třeboň, Czech Republic
5UPMC Univ Paris 06, UMR 7093, Lab. d’Océanographie de Villefranche-sur-Mer, 06230 Villefranche-sur-Mer, France
6CNRS, UMR 7093, LOV, 06230 Villefranche-sur-Mer, France
7Present address: College of Marine and Earth Studies, University of Delaware, Lewes, Delaware 19958, USA

ABSTRACT: Aerobic anoxygenic phototrophic (AAP) bacteria are bacteriochlorophyll a-containing prokaryotes which can use both light and organic compounds as energy sources. This functional group is ubiquitous in the euphotic zone of the oceans. Nevertheless, life strategies, distribution patterns and physiology of AAP bacteria remain largely unknown. We combined infrared fluorometry, microscopic counts and HPLC pigment analysis to characterize free-living and particle-attached AAP bacterial populations. Using a size-fractionation approach, we found that the size distribution of AAP bacteria and the fraction of particle-attached cells varied greatly among different marine environments. In the open sea environments (Atlantic Ocean, offshore Mediterranean Sea), the main portion of AAP bacterial fluorescence was in the <0.8 µm fraction, which indicates that the majority of AAP bacteria in these regions were free-living cells <0.8 µm. In these environments, only a few particle-attached AAP bacteria were found. In coastal Mediterranean waters, the fraction of larger cells increased together with a few particle-attached cells, but >50% of AAP bacteria were free living. In a coastal lagoon and in the deep chlorophyll a maximum at an offshore Mediterranean station, particle-attached AAP bacteria formed up to half of the AAP bacterial community. The results presented here suggest that AAP bacteria can take on either free-living or particle-attached lifestyles depending on environmental conditions.

KEY WORDS: AAP bacteria · Photoheterotrophy · Free-living bacteria · Particle-attached bacteria

INTRODUCTION

Recent studies have revealed that a large fraction of marine prokaryotes are not strictly heterotrophic or autotrophic, but adopt a variety of mixotrophic strategies (Béjà et al. 2000, Kolber et al. 2000, Zubkov & Tarran 2005). One group of these organisms, aerobic anoxygenic phototrophic (AAP) bacteria, performs photoheterotrophic metabolism. They require organic substrates for their metabolism and growth and they are able to supplement a fraction of their energy demands using light (Yurkov & Beatty 1998). AAP bacteria contain bacterial photosynthetic reaction centers with bacteriochlorophyll a (Bchl a) as the main light-harvesting pigment. The abundance of AAP bacteria varies greatly among different marine environments (Kolber...
et al. 2001, Cottrell et al. 2006, Mašín et al. 2006, Lami et al. 2007), but on average these bacteria account for about 2 to 4% of the total prokaryotes (Jiao et al. 2007).

Little is known about the distribution and physiology of marine AAP bacteria. Originally it was suggested that they would be abundant in oligotrophic environments, where the capacity to use light might represent a significant ecological advantage (Kolber et al. 2001). Later, AAP bacteria were also found in the highly eutrophic Baltic Sea (Koblížek et al. 2005), as well as in many freshwater environments (Mašín et al. 2006), which suggests that different AAP bacterial species can inhabit various environments and that their distribution is not determined solely by trophic status. Other factors such as light, temperature and nitrate availability were identified as potentially important factors controlling the abundance of AAP bacteria (Kolber et al. 2001, Mašín et al. 2006, Waidner & Kirchman 2007).

In the pioneering study by Kolber et al. (2000), the authors suggested that the majority of the AAP bacterial population in the tropical Pacific is composed of free-living cells <0.8 µm. In contrast, Allgaier et al. (2003) concluded that dinoflagellates could represent an ‘important ecological niche’ for AAP bacteria and speculated that these prokaryotes are directly attached to dinoflagellate cells. In addition, large fractions of particle-attached AAP bacteria were found in the Delaware estuary (Waidner & Kirchman 2007). Furthermore, analyses of the metagenomic data sets from the Global Ocean Sampling expedition indicated a significant fraction of particle-attached AAP bacteria in the open ocean (Yutin et al. 2007). These conflicting results raise the question of whether these microorganisms in the natural environment are mostly free-living or particle-attached, and how this changes under different conditions. To address these questions, we used size fractionation to examine free-living and particle-attached AAP bacteria in several contrasting marine environments.

### MATERIALS AND METHODS

**Sample collection.** Three Mediterranean sites (SOLA, POLA and MOLA) in the Gulf of Lion were repeatedly sampled in spring and early summer 2007 (Table 1). The observatory Stn SOLA is in the Bay of Banyuls-sur-Mer, Stn POLA is on the continental shelf (7.4 km offshore), and the offshore Stn MOLA is on the continental slope (37 km from the coast). Nine stations in the North Atlantic Ocean were sampled in April 2008 during the OC443 cruise aboard the RV ‘Oceanus’. The Sargasso Sea Stn 13.5 was sampled repeatedly during diel studies conducted on April 9 and 10, 2008; the other 8 stations were sampled only once. In the Mediterranean Sea and North Atlantic Ocean, samples were collected with General Oceanics 12 l Niskin bottles mounted on a rosette equipped with a SeaBird SBE911+ (SBE19 at the bay station) conductivity-temperature-depth (CTD) instrument. The coastal lagoon Lapalme was sampled on April 13 and May 1, 2007. Here, the samples were collected using acid-washed Nalgene PC bottles rinsed 3 times with sampled water.

**Fluorometry and particle size distributions.** Bchl a fluorescence was measured with an infrared (IR) kinetic fluorometer (Photon Systems Instruments) as described previously (Koblížek et al. 2007). To separate chlorophyll a (chl a) and Bchl a contribution in the IR fluorescence signal (>850 nm), the oxygenic phytoplankton was selectively blocked by adding 10⁻⁵ M (final concentration) of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron; PESTANAL, Fluka), which specifically binds photosystem II, but does not affect the bacterial reaction centers. Then, only the variable part of the kinetic IR signal originating from bacterial reaction centers was determined (Koblížek et al. 2005). This approach avoids potential interferences from other fluorescing compounds (not exhibiting variable fluorescence) or from electrical drifts within the detection system (occurring at different time scale).

### Table 1. Main sampling stations. Samples were collected in surface waters (3 m) at all stations, and every 20 m between 0 and 100 m at Stn MOLA. nd: no data

<table>
<thead>
<tr>
<th>Location</th>
<th>Position</th>
<th>Regime</th>
<th>Depth (m)</th>
<th>Sampling date</th>
<th>Temp. (°C)</th>
<th>Salinity (PSU)</th>
<th>Chl a (ng l⁻¹)</th>
<th>Bchl a (ng l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sargasso Sea</td>
<td>Stn 13.5</td>
<td>Open ocean</td>
<td>4875</td>
<td>9–10 Apr 08</td>
<td>21.2</td>
<td>nd</td>
<td>50</td>
<td>−1.5º</td>
</tr>
<tr>
<td>Mediterranean Sea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stn POLA</td>
<td>42° 28’N, 3° 15’E</td>
<td>Shelf</td>
<td>600</td>
<td>10 Apr 07, 23 May 07</td>
<td>14.4, 18.0</td>
<td>38.1, 37.6</td>
<td>590, 223</td>
<td>0.9, 1.8</td>
</tr>
<tr>
<td>Stn SOLA</td>
<td>42° 29’N, 3° 08’E</td>
<td>Coastal bay</td>
<td>27</td>
<td>21 May 07, 9 May 07</td>
<td>18.6, 16.5</td>
<td>37.8, 36.6</td>
<td>nd, 318</td>
<td>−4º, 3.9</td>
</tr>
<tr>
<td>Lapalme lagoon</td>
<td>42° 58’N, 3° 00’E</td>
<td>Coastal lagoon</td>
<td>1.2</td>
<td>13 Apr 07</td>
<td>11.0</td>
<td>28.0</td>
<td>800</td>
<td>22</td>
</tr>
</tbody>
</table>

*Estimated from the infrared fluorometry data
Particle size distributions were measured with the Multisizer 3 Coulter Counter (Beckmen Coulter). Only particles with equivalent spherical diameters of 6 µm and greater were considered and quantified using the total cumulative volume.

**AAP bacteria enumeration.** AAP bacteria were enumerated using previously described methods (Mašín et al. 2006). Briefly, bacterial cells were fixed using 2% paraformaldehyde (final concentration) and stored at 4°C. Later, the samples were collected onto 0.2 µm polycarbonate filters, dried, and stained with DAPI using a 3:1 mixture of Citifluor AF1 and Vectashield containing 1 µg ml–1 DAPI. First, the total DAPI-stained bacteria were recorded in the blue part of the spectrum (100 to 200 ms exposure). Then, IR emission (>850 nm) images were captured, showing both AAP bacteria and phytoplankton (from 15 to 35 s exposure). Finally, red chl a autofluorescence was recorded to identify chl a-containing organisms (0.5 to 1 s exposure). The acquired images were saved and semi-manually analyzed with the aid of AnalySiS software (Soft Imaging Systems) to distinguish between heterotrophic bacteria, picocyanobacteria and AAP bacteria for each sample. To obtain net AAP bacteria counts, the contribution of chl a-containing organisms to the IR image was subtracted (Schwalbach & Fuhrman 2005). For each individual sample, 8 to 12 fields of view were recorded and analyzed (total count of 400 to 600 DAPI cells sample–1).

**Pigment concentrations.** Pigment concentrations were determined using HPLC, following previously published protocols (Ras et al. 2008). Between 1 and 5 l seawater (depending on the trophic conditions) were filtered under low vacuum (<500 mbar) onto 2 stacked 25 mm GF/F filters which were frozen immediately at –80°C until analysis. The samples were extracted in 3 ml methanol (100%) for a minimum of 1 h, and sonicated. The clarified extracts were injected onto an Agilent Technologies 1100 series HPLC system equipped with a refrigerated autosampler and a column thermostat, according to a modified version of the method described by Ras et al. (2008). The sample extracts (100% methanol), premixed (1:1) with a buffer solution (28 mM tetrabutylammonium acetate), were injected onto a narrow reversed-phase C8 Zorbax Eclipse XDB column (3 × 150 mm; 3.5 µm particle size) which was heated to 60°C. Separation was achieved within 28 min with a gradient between a solution (A) of 28 mM tetrabutylammonium acetate: methanol (30:70; vol:vol) and a solution (B) of 100% methanol according to the following program: 0 min: 90% A, 10% B; 25 min: 5% A, 95% B; 28 min: 5% A, 95% B. The retention times and diode array absorbance spectra of each peak were used for identification purposes. Pigment concentrations were calculated from the peak areas with an internal standard correction (Vitamin E acetate, Sigma) and external calibration standards which were provided by DHU Water and Environment and Sigma. This method has proven to be satisfactory in terms of resolution, sensitivity, accuracy and precision, with the detection of about 25 separate phytoplankton pigments (Ras et al. 2008), and a lower limit of detection (3 times signal:noise ratio) for chl a and Bchl a of 0.1 ng l–1. The injection precision was 0.4%.

**RESULTS**

The objective of the present work was to examine free-living and particle-attached AAP bacteria in 5 contrasting marine environments: a Mediterranean coastal lagoon, Mediterranean coastal and offshore waters, North Atlantic Ocean (including the Gulf Stream) and the Sargasso Sea. AAP bacterial cells were frequently seen attached to particles in samples collected from coastal waters by IR microscopy: the individual AAP bacterial cells were typically attached together with other bacteria to irregular particles of organic matter as shown in Fig. 1. Monospecific aggregates of AAP bacterial cells or phytoplankton cells selectively colonized by AAP bacterial cells were extremely rare (data not shown). Unfortunately, direct microscopic observations are labor-intensive and proper quantification of particle-attached cells is extremely complicated. For this reason Bchl a fluorescence was used in this study as an easy and rapid way to detect AAP bacteria (Kolber et al. 2000, 2001, Kobližek et al. 2005, 2007).

**Evaluation of the filtration methods**

Size fractionations were conducted to determine the percentage of total Bchl a fluorescence associated with particles. For each sample, <0.8 µm and <2 µm Bchl a fluorescence signals were recorded with the original non-filtered water sample and the collected fractions, and the percentage of Bchl a signal in individual fractions was then calculated. Gravity filtration was used to minimize potential particle dislodging during the filtration processes (Ghiglione et al. 2007, Waidner & Kirchman 2007).

In preliminary experiments we found that as expected a 0.2 µm filtration always removed all the Bchl a signal. In a preliminary evaluation of our methods conducted at Stn POLA, we found that the majority (67%) of the fluorescence signal was in the 0.4 to 770 nm. The retention times and diode array absorption spectra of each peak were used for identification purposes.
0.8 μm fraction, with no signal below 0.4 μm (Fig. 2). Only small part of the total signal was found in the larger size classes (13% in 0.8 to 2.0 μm fraction, 7% in 2 to 5 μm and 12% in >5 μm fraction), which indicates that the majority of the AAP bacterial population was formed by small individual cells sized 0.4 to 0.8 μm (Fig. 2).

The disadvantage of this size-fractionation protocol was lengthy filtration leading to slow deterioration of the samples. To avoid potential artifacts, the procedure was simplified to a 2-step protocol separating only 3 size classes: small cells passing through 0.8 μm membrane filters, larger cells between 0.8 and 2 μm, and mostly particle-attached cells which do not pass through 2 μm membrane filters. Using this protocol, the standard error of triplicate determinations on the same sample was 2.8%. As the experimental error was small, in later experiments the samples were analyzed only once to speed up the fractionation protocol.

Collection of water samples onto GF/F filters is a very common approach in marine microbiology. For this reason, we decided to test (in addition to nucleopore filtration) the ability of GF/F filters to retain the Bchl.a fluorescence signal. The water samples collected at the coastal Stn SOLA in March and April 2007 were filtered through GF/F filters without vacuum. The filters retained 70% (in March) or 85% (April) of the Bchl.a signal, which equals 15 to 30% of the signal passing through the filter. At Stn POLA, GF/F retained only 42% of the Bchl.a signal (Fig. 2). The more dense MN GF-5 fiber glass filters (Macherey-Nagel) retained a larger fraction of the signal, but even these filters missed up to 30% of the Bchl.a signal in the oligotrophic regions (data not shown).

Mediterranean waters

The size distribution of the Bchl.a fluorescence signal was examined through March to May 2007 at the coastal bay Stn SOLA (Fig. 3). During this period, Bchl.a concentrations increased from 1.5 ng l⁻¹ in March to 3.5 ng l⁻¹ in May. The <0.8 μm fraction (tracing free-living AAP bacteria) was the largest one (50 to 55%, except April 16 to 23, during which it was 25 to 30%). The >2 μm fraction (tracing particle-attached AAP bacteria) ranged from 20 to 40%, while the 0.8 to 2 μm fraction varied between 10 and 40% (Fig. 3).

The spatial changes in the AAP bacterial size distribution were examined at 3 time-series stations in offshore waters of Banyuls-sur-Mer in April and May 2007 (Table 1). At these studied Mediterranean sites, the AAP bacterial abundance in surface waters (deter-
mined with IR epifluorescence microscopy) ranged from $4.4 \times 10^3$ to $65 \times 10^3$ cells ml$^{-1}$. Bchl a concentration measured by HPLC varied from 0.9 to 4.3 ng l$^{-1}$, and the Bchl a:chl a ratio was between 0.6 and 1%. At all these sites, the fraction of fluorescence in the $<0.8 \mu$m size fraction dominated the signal; however, its proportion varied. The highest fraction of the $<0.8 \mu$m signal was observed at the offshore Stn MOLA (up to 74% of the total signal in April), whereas at the shelf Stn POLA and the coastal site SOLA this fraction was smaller (55 to 65% of the total signal respectively) (Fig. 4). The 0.8 to 2 $\mu$m fraction was present in all samples and was 10 to 40% of the total signal depending on the station and sample. The opposite pattern was found for the large fraction (>2 $\mu$m), which mostly traces particle-attached AAP bacteria. Whereas at the offshore and shelf stations, this > 2 $\mu$m fraction was about 10% of the total signal, it accounted for about 30% of the total Bchl a fluorescence signal at the coastal site (Fig. 4).

The vertical size distribution of the Bchl a signal was also examined at the offshore Stn MOLA on May 23, 2007 (Fig. 5). Here, Bchl a fluorescence in the $<0.8 \mu$m fraction was high at all depths and was relatively constant (40 to 50% of the total Bchl a signal) (Fig. 5). A large fraction (50%) of the signal was recorded in the >2 $\mu$m fraction at the deep chlorophyll maximum (DCM). Interestingly, this pattern followed particulate matter volume determined using a Coulter counter. Particles and Bchl a concentrations measured by HPLC peaked at the DCM (up to 4.3 ng l$^{-1}$ of Bchl a and $70 \times 10^3$ $\mu$m$^3$ ml$^{-1}$ of particles at the DCM). AAP bacterial cell abundances varied from $6.6 \times 10^3$ to $34 \times 10^3$ cells ml$^{-1}$ and peaked at 60 m, just below the DCM (Fig. 5).

We analyzed samples from a coastal Mediterranean lagoon (Lapalme Lagoon, France, 28 PSU) as a representative of an environment with high concentrations of particles. This shallow lagoon is characterized by high turbidity, and high chl a (0.8 $\mu$g l$^{-1}$) and Bchl a (22 ng l$^{-1}$) concentrations (Table 1). In this lagoon, the percentage of total Bchl a fluorescence in the $<0.8 \mu$m fraction was 28%. The signal in the >2 $\mu$m fraction reached 30% (Fig. 4).

**North Atlantic Ocean**

We performed size-fractionation experiments in the northwest Atlantic in April 2008. In the Sargasso Sea, the Gulf Stream and in northern Atlantic waters, concentrations of Bchl a were between 0.8 and 1.6 ng l$^{-1}$. In the oligotrophic Sargasso Sea, 90 ± 2% (mean ± SE) of Bchl a passed through the 0.8 $\mu$m filter (calculated from 6 independent casts at Stn 13.5) and all Bchl a passed through the 2 $\mu$m filter (Fig. 6). In addition, 0.4 $\mu$m filtration usually removed >90% of the Bchl a signal, which indicates that in the Sargasso Sea the majority of AAP bacterial cells were between 0.4 and 0.8 $\mu$m in size, and particle-attached AAP bacteria were absent. The size-fractionation measurements were also performed along the transect from the Sargasso Sea (Stn 16, 33°N 65°W) to the coast (Woods Hole, MA). In 3 samples collected in the Sargasso Sea, the <0.8 $\mu$m fraction varied between 86 and 98% of the total, and >2 $\mu$m fraction was absent. In the Gulf Stream (38°N, 69.5°W) the <0.8 $\mu$m fraction decreased to 77%, whereas the >2 $\mu$m fraction was 9% of the total Bchl a signal. In the productive waters of the Mid-
dle Atlantic Bight, the small fraction further decreased to 50 to 68%, while the large >2 µm fraction increased to 22 to 30% of the total Bchl a signal (Fig. 6).

**DISCUSSION**

We investigated the distribution of particle-attached and free-living AAP bacteria by the detection of Bchl a fluorescence after size fractionation of seawater in various oceanographic contexts. Data on free-living versus particle-attached AAP bacterial populations in marine waters are scarce. In their pioneering paper, Kolber et al. (2000) reported that most AAP bacteria in the subtropical Pacific pass through the GF/F filter, which suggests that the community is composed mostly of single cells <0.7 µm. In contrast, large quantities of particle-attached AAP bacteria (31 to 94%) in estuarine waters were reported by Waidner & Kirchman (2007). Our data clearly show that the proportion...
of free-living and particle-attached AAP bacteria varies among the environments. In offshore and open-ocean environments, the majority of the AAP bacterial community was small (<0.8 µm) free-living cells, which agrees with the findings of Kolber et al. (2000). Interestingly, our estimate of size ranges for the Sargasso Sea community (0.4 to 0.8 µm) agrees with AAP bacterial cell sizes (0.4 to 1.0 µm) determined by Sieracki et al. (2006) in the same region using epifluorescence microscopy. Our observations are in contrast with metagenomic analyses conducted in the oligotrophic Sargasso Sea suggesting that the majority of AAP bacteria are in the 3 to 20 µm fraction (Yutin et al. 2007).

We found a measurable percentage of Bchl a fluorescence in the 0.8 to 2 µm fraction in all environments. This signal likely comes from large but free-living AAP bacteria. It has previously been shown both in the North Atlantic and South Pacific Oceans that these microorganisms are larger than average heterotrophic bacterioplankton cells (Sieracki et al. 2006, Lami et al. 2007). It has been suggested that large cells may be the most active members of the bacterioplankton community (Bird & Kalff 1993, Gasol et al. 1995).

A different pattern was observed in a coastal lagoon and bay, where up to one-third of the AAP bacterial community was particle-attached. This is consistent with the large fraction of particle-attached AAP bacteria reported by Waidner & Kirchman (2007) from Chesapeake and Delaware estuaries. Interestingly, this pattern follows the usual observations of particle-attached versus free-living prokaryotes in the sea, with the highest percentages of attached bacteria in environments with high concentrations of particles (Simon et al. 2002). Collectively, the data presented in the present study show that AAP bacteria, in comparison with regular heterotrophic prokaryotes, do not exhibit an enhanced tendency to attach to particles or to form clumps. However, as the AAP bacterial community is composed of phylogenetically diverse organisms (Yutin et al. 2007, Salka et al. 2008), some AAP bacterial species might have an enhanced tendency to attach to particles as a part of their lifestyle.

We observed high AAP bacterial abundances at the DCM, consistent with high Bchl a concentrations. This was previously found in other marine environments (Kolber et al. 2000, 2001, Sieracki et al. 2006). The slight decoupling between the peak of AAP bacterial cells and the peak of Bchl a may be due to regulation of Bchl a content per cell. The interpretation of this peak of photoheterotrophs is not completely clear. On the one hand, AAP bacteria and phytoplankton may require the same environmental conditions (light and nutrients) for their growth. Thus, they may occupy similar ecological niches and might strongly compete for similar resources. On the other hand, AAP bacteria may find at the DCM specific organic compounds provided by phytoplankton and required for their growth. Interestingly, we recorded a large fraction of AAP bacteria attached to particles at the DCM, twice as much as in surface and deep layers. Attachment of AAP bacterial populations also follows closely the distribution of particles in the water column as well as chl a concentrations. Thus, their attachment to particles probably also depends on the concentrations of particles, including phytoplankton. The attached AAP bacteria may use a large spectrum of molecules, including diverse dissolved organic matter provided by various algal groups.

For pigment analyses, planktonic samples are routinely collected onto glass fiber GF/F filters (Whatman). Kolber et al. (2000) reported that in the tropical Pacific most of the Bchl a signal passes through GF/F. In contrast, Goericke (2002) reported high retention of Bchl a on GF/F filters in his HPLC analyses. Based on our data, it seems that the use of GF/F filters for pigment analyses in open-ocean environments might lead to a significant underestimation of true Bchl a concentrations, as GF/F filters did not trap 30 to 60% of the total Bchl a signal in our experiments. This is consistent with earlier observations from the subtropical Atlantic, where 50 to 65% of the signal passed through GF/F filters (M. Kobližek unpubl. data). The GF/F filtration conducted at coastal Stn SOLA seemed to be more effective. However, in a previous study conducted in the Bay of Villfranche, France, the GF/F filters did not trap 30 to 50% of the signal (Kobližek et al. 2007).

The environmental variables that control AAP bacterial abundance and dynamics remain a matter of debate. Our data suggest that the characterization of ‘free-living’ versus ‘particle-attached’ is an important factor to explain the distribution and the ecology of AAP bacteria in marine environments. We have shown that AAP bacteria are mainly free living in the oceans. In addition, our data strongly suggest that in coastal bays and lagoons, large fractions of AAP bacterial cells have a particle-attached lifestyle. It has been shown that particle-attached bacteria can be the most active members of the prokaryotic community (Grossart et al. 2007). Thus, in coastal environments (lagoon, shelf) and at the DCM, some AAP bacteria may play significant roles in the mineralization of organic matter and particulate detritus degradation.

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LITERATURE CITED


