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Diel variations in frequency of dividing cells and abundance of aerobic anoxygenic phototrophic bacteria in a coral reef system of the South China Sea

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ABSTRACT: With the ability of photoheterotrophic metabolism, aerobic anoxygenic phototrophic (AAP) bacteria are an important bacterial group in the marine microbial community. We investigated the diel variations of AAP bacteria in the coral reef water of the South China Sea, and report, for the first time, the frequency of dividing cells (FDC) of AAP bacteria. Our results showed that AAP bacterial abundance ranged from $1.54 \times 10^4 \pm 3.21 \times 10^2$ to $3.59 \times 10^4 \pm 3.04 \times 10^3$ cells ml^{-1}. The diel pattern of AAP bacterial abundance had 2 peak values, one at night around 21:00 h and the other in the daytime at around 9:00 h. Tidal current and light were the primary factors controlling the diel variations of AAP bacteria in this area during our study period. FDC values in AAP and total bacteria were both higher during the night than during the day. The FDC values in AAP bacteria (3.53 ± 1.09 to 9.43 ± 0.80%) were significantly higher than those in total bacteria (1.06 ± 0.06 to 3.14 ± 1.38%), indicating higher growth rate of AAP bacteria than total bacteria in the study area.

KEY WORDS: Aerobic anoxygenic phototrophic (AAP) bacteria · Diel variation · Frequency of dividing cells · FDC · Abundance

INTRODUCTION

Aerobic anoxygenic phototrophic (AAP) bacteria are a functional group of microorganisms that can utilize light energy and play a particular role in carbon cycling in the ocean (Kolber et al. 2000, 2001). These bacteria form 0.5 to 16% of the total bacteria in the euphotic zone of various marine environments (Kolber et al. 2001, Cottrell et al. 2006, Jiao et al. 2007, Zhang & Jiao 2007). They depend on aerobic respiration based on organic substrates for growth, but also derive a significant portion of their energy requirements from light through bacteriochlorophyll *a* (Bchl *a*)-containing reaction centers (Rathgeber et al. 2004).

Previous research on diel changes of AAP bacteria in marine environments focused on Bchl *a* concentration (Koblizek et al. 2005, 2007) or photochemistry signals (Kolber et al. 2000). Clear and large diurnal changes in Bchl *a* contents were reported in the Baltic Sea (Koblizek et al. 2005) and the euphotic zone of the Atlantic Ocean (Koblizek et al. 2007). A distinct decline of pigment occurring during daylight hours was ascribed to the complete inhibition of Bchl *a* synthesis by light, in combination with a concurrent turnover of the cells (Koblizek et al. 2005). However, research carried out in the surface water of the Pacific indicated the absence of a diel cycle in bacterial photosynthesis, implying a lack of photoinhibition and no diel coherence in cell division (at least as indicated by photochemistry; Kolber et al. 2000). There are few data concerning the growth rate of AAP bacteria in the field. Koblizek et al. (2007) reported the AAP bacterial growth rate in the Atlantic Ocean, assessed from the diel cycle rate of Bchl *a* concentration. In the present study, we investigated diel variations of abundance and frequency of dividing cells (FDC) of AAP bacteria

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in a coral reef system near Yongxing Island (112.32° E, 16.83° N) in the South China Sea. Time series observation was conducted from 21 to 25 February 2006 in the coral reef system near Yongxing Island. Combining the Time-series observation based InfraRed Epifluorescence Microscopy protocol (Jiao et al. 2006) and the FDC method for bacterial growth rate estimation (Hagstrom et al. 1979), provided us with an alternative way to assess AAP bacterial growth rate. This work was aimed at examining the differences between AAP and total bacteria in diel variation as well as their controlling mechanisms.

MATERIALS AND METHODS

Water sampling. The reef rim surrounding Yongxing Island supports a coral community dominated by *Acropora* spp. (Li et al. 2004). Our study site, located about 250 m from the coast, was a shallow water environment with a depth of 3 to 5 m. Diurnal samples were collected from the surface water at 3 h intervals. Briefly, subsamples for AAP and total bacterial abundance analysis were collected using 100 ml brown polypropylene bottles. Immediately after sampling, triplicate 20 ml seawater samples were fixed with paraformaldehyde (1% final concentration, 20 min) and stained with 4′6-diamidino-2-phenylindole (DAPI; 5 mg ml^{-1} final concentration) and then filtered onto 25 mm diameter, 0.2 µm pore-size black polycarbonate (PC) membranes (Whatman) for microscopic observation. They were stored at –20°C before analysis. Triplicate 2 ml subsamples were fixed with glutaraldehyde (0.5% final concentration, 20 min) and stored at –80°C for analysis of autotrophic picoplankton abundance by flow cytometry. For chl *a* concentration, triplicate 200 ml water samples were filtered directly onto 47 mm diameter GF/F membranes (Whatman) and stored at –20°C before analysis. Surface water temperature was monitored continuously using a Yellow Springs Instrument (YSI) meter (YSI6600), and the precision of the YSI probes was 0.01°C for temperature. Light intensity (solar radiation intensity) was measured using a radiometer (Delta OHM HD2302). The data of tidal height came from the observation station on Xisha Island.

Determination of abundance of AAP and total bacteria. Abundance of AAP bacteria was determined using an advanced 'Time-series observation based cyanobacteria calibrated InfraRed Epifluorescence Microscopy (TIREM)' protocol, which was described by Jiao et al. (2006). Infrared fluorescence from Bchl *a* was the diagnostic signal of AAP bacteria. Cells were viewed with an infrared-sensitive charge-coupled device (CCD) camera (DP30 Diagnostic Instruments) on an epifluorescence microscope with a 100 W mercury lamp (Olympus Light Microscopy BX61). DAPI-stained images (DAPI-images), cyanobacterial images (Cyano-images), and infrared images (IR-images) were acquired for each microscopic view field using a 100× oil immersion objective. All images were captured using automatic exposure with a gain limit of 8. Time series images of DAPI-, Cyano-, and IR-images were obtained for 10 to 15 min at intervals of 60 s from the start of the exposure. Image analysis was conducted to obtain dynamic curves of IR-, Cyano-, and DAPI-counts from each microscopic view field. The accurate estimation of AAP bacteria was calculated from the formula: AAP counts = (plateau count of infrared positive cells)—(plateau count of cyanobacterial cells) (Jiao et al. 2006). Thirty microscopic fields of each sample were viewed, and the means were multiplied by appropriate factors ($A_1/A_2/V$, where A_1 is the area of a filter, A_2 is the area of 1 microscopic view field, and V is the volume of water filtered) to yield AAP bacterial concentrations in the original samples. Abundance of total bacteria was obtained by directly counting DAPI-images acquired in the TIREM procedure and multiplication by the same factors as AAP bacteria.

Determination of FDC. FDC in total bacterial cells was counted according to Hagstrom et al. (1979) and Fukuda et al. (2006). A cell was counted as dividing if a clear invagination of the cell wall could be seen, but not a clear separatory space between daughter cells. DAPI images captured in the TIREM procedure were used for observation of dividing cells for total bacteria. Fifteen microscopic fields (average 130 cells $field^{-1}$) and a minimum of 30 dividing bacterial cells were counted for each sample. For determination of the FDC of AAP bacteria, a combination of the TIREM approach (Jiao et al. 2006) and the method for identifying dividing cells (Hagstrom et al. 1979) was used. Firstly, we picked out the AAP bacteria cells by comparing Cyano-images, IR-images, and DAPI-images captured in the TIREM procedure and subtracting the Cyano-positive cells from the IR-images. To do this, images needed to be processed in Image-Pro Plus (IPP) software (Media Cybernetic, version 6.0). The IR-images were stained with red color using the tint function in IPP, and Cyano-images were stained with green color. IR- and Cyano- images from the same microscopic field were merged together to form a new image using the merge function in IPP. The cells that retained red color in the merged image and could be found in the same position in the corresponding DAPI-image were AAP bacteria. The local zoom function in IPP was applied to enlarge these cells in order to clearly show their features. We then identified the dividing cells in the AAP cells using the same standard as that for total

bacteria described at the beginning of this section. Thirty microscopic fields of each sample were counted. The FDC of both AAP and total bacterial cells were calculated using the following formula: FDC (%) = abundance of dividing cells × 100/abundance of AAP (or total bacteria).

Determination of chl *a*, nutrient concentration, and autotrophic picoplankton abundance. Chl *a* concentration was determined fluorometrically (Parsons et al. 1984) with a Turner Designs Model 10 fluorometer (Sigma). Dissolved inorganic phosphate (DIP; PO_4^{3-}) and inorganic nitrogen (DIN; NO_3^-, NO_2^-) were measured using the standard molybdenum blue procedure and the standard pink azo dye method (Pai et al. 1990a,b), respectively. The precision of the methods was about 4% for DIP and 1% for DIN. Generally, surface nutrients in the oligotrophic ocean are found at the nano-mole level and, in our study area, surface DIN (<0.3 µM) was below the detection limit. Abundances of picoeukaryotes, *Synechococcus*, and *Prochlorococcus* were determined by flow cytometry (Jiao et al. 2002) on an Epics Altra II (Beckman Coulter) flow cytometer, equipped with a 306C-5 argon laser (Coherent).

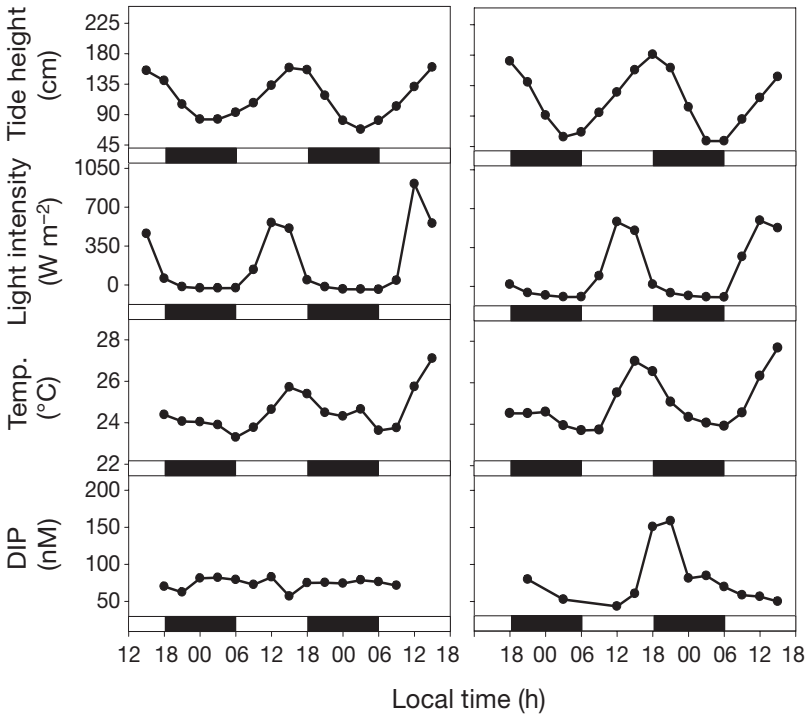

Fig. 1. Diel variations in tidal height, light intensity, water temperature, and concentration of dissolved inorganic phosphorus (DIP) in the surface water of the sampling site. Diurnal observations were made from 21 to 23 (left) and 23 to 25 (right) February 2006. Horizontal black (white) bars show night (day)

RESULTS

Environmental parameters

The tide was irregularly diurnal, with the highest water level at about 18:00 h and the lowest at around 0:00–03:00 h (Fig. 1). The light intensity was strongest from 12:00 to 15:00 h in this area (Fig. 1). During our observation period, the diel changes of water temperature ranged from 23.30 to 27.68°C, with the highest value at around 15:00 h and the lowest at around 06:00 h (Fig. 1). Diel changes in the concentration of DIP in the water were not as clear as those in tide and temperature. In the diurnal observation during 21 to 23 February, the changes in DIP concentration were not pronounced, and the values fluctuated around 74.08 nM. However, during 23 to 25 February, a rapid increase in DIP concentration occurred at night (around 18:00 to 21:00 h), reaching values twice as high as the lowest value in the daytime (Fig. 1).

Diel changes in AAP and total bacterial abundance

Abundance of AAP bacteria showed obvious diel changes, with values ranging from (±SD) $1.54 \times 10^4 \pm 3.21 \times 10^2$ to $3.59 \times 10^4 \pm 3.04 \times 10^3$ cells ml^{-1}. The highest value occurred at around 21:00 h and dropped to the lowest value at around 00:00 h of the next day, and then the value rose until 09:00 h. After that, the value dropped again at around 12:00 h and then rose until 21:00 h (Fig. 2a). The diel change in AAP bacterial abundance during 23 to 25 February was more obvious than that during 21 to 23 February, especially for the changes during the day (Fig. 2a).

The values of total bacterial abundance (±SD) ranged from $5.32 \times 10^5 \pm 1.07 \times 10^5$ to $1.80 \times 10^6 \pm 1.88 \times 10^5$ cells ml^{-1}. The diel pattern of total bacterial abundance was similar to that of AAP bacteria, but the changes during the day were 3 h behind those of AAP bacteria (Fig. 2b). The highest value of total bacterial abundance in the night occurred at around 21:00 h and lowest value at 00:00 to 03:00 h, while in the day, the highest values occurred at 12:00 h and the lowest values occurred during 15:00 to 18:00 h (Fig. 2b).

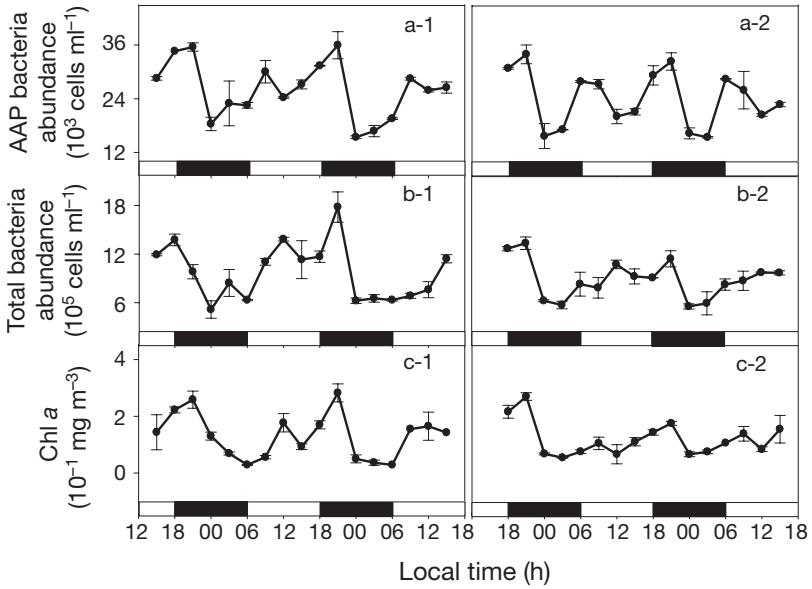

Fig. 2. Diel variations in (a) aerobic anoxygenic phototrophic (AAP) bacterial abundance, (b) total bacterial abundance, and (c) chl *a* concentration in the surface water of the sampling site. Diurnal observations were made from 21 to 23 (left, 1) and 23 to 25 (right, 2) February 2006. Horizontal black (white) bars show night (day). Error bars indicate the SD of triplicate measurements

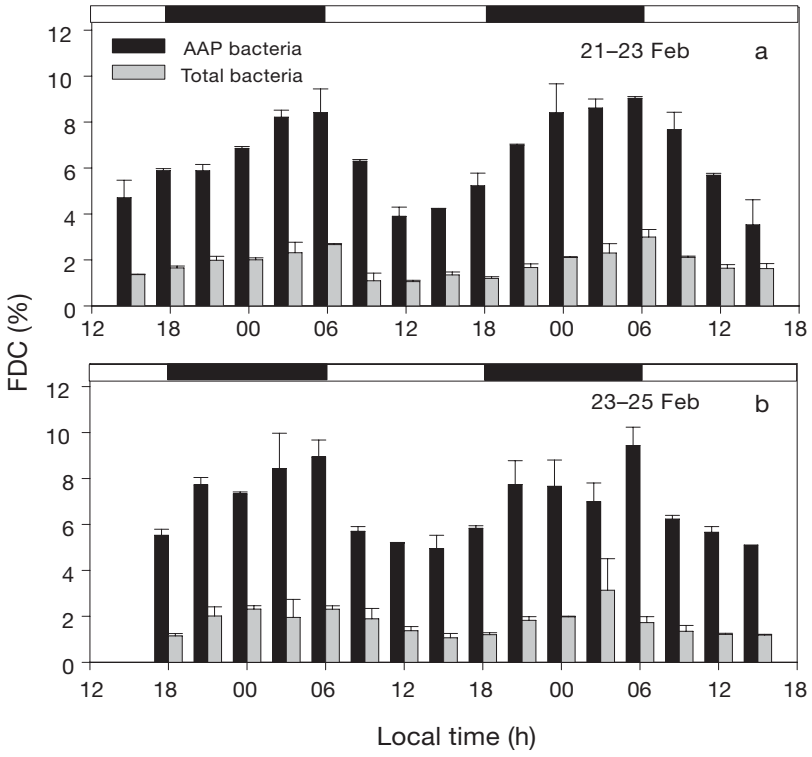

Fig. 3. Diel changes in the frequency of dividing cells (FDC) of aerobic anoxygenic phototrophic (AAP) bacteria (black columns) and total bacteria (grey columns) during (a) 21 to 23 and (b) 23 to 25 February 2006. Horizontal black (white) bars show night (day). Error bars indicate the SD of triplicate measurements

Diel changes in chl *a* concentration and autotrophic picoplankton abundance

In the diurnal observation of 21 to 23 February, the concentration of chl *a* showed an obvious diel change, basically similar to that of total bacterial abundance (Fig. 2c). The highest concentrations of chl *a* were measured at 18:00 to 21:00 h, and the lowest values were at midnight or just before dawn. Also, during the day there was an increase from 06:00 to 12:00 h and a decrease after 12:00 h. In contrast, in the diurnal observation of 23 to 25 February, chl *a* concentrations were lower than the first observation, especially during the whole day from 24 to 25 February (Fig. 2c). The autotrophic picoplankton abundance (Appendix 1, Fig. A1) showed a similar diel pattern to that of chl *a* contents in the diurnal observation of 21 to 23 February, while in the observation of 23 to 25 February, the diel changes were less pronounced (Fig. A1).

Diel changes in the FDC of AAP and total bacteria

Diel patterns of FDC were similar for both AAP and total bacteria: higher values during the night and lower values during the day. The peak values in the diurnal cycles were at 03:00 to 06:00 h and then decreased until 12:00 to 15:00 h (Fig. 3). Thereafter, the FDC values began to increase until the next peak value at night. The range in FDC of AAP bacteria (±SD) during our observation period was from 3.53 ± 1.09 to 9.43 ± 0.80%, while the FDC of total bacteria (±SD) ranged from 1.06 ± 0.06 to 3.14 ± 1.38%. The FDC of AAP bacteria was about 2.17 to 5.75 times that of the total bacteria (Fig. 3).

DISCUSSION

Diel variation in AAP abundance and possible regulation parameters

During our study period, a distinct diel pattern of AAP bacterial abundance was observed in this coral reef system, that is, abundance of AAP bacteria showed 2 peaks during a day-night cycle (Fig. 2a).

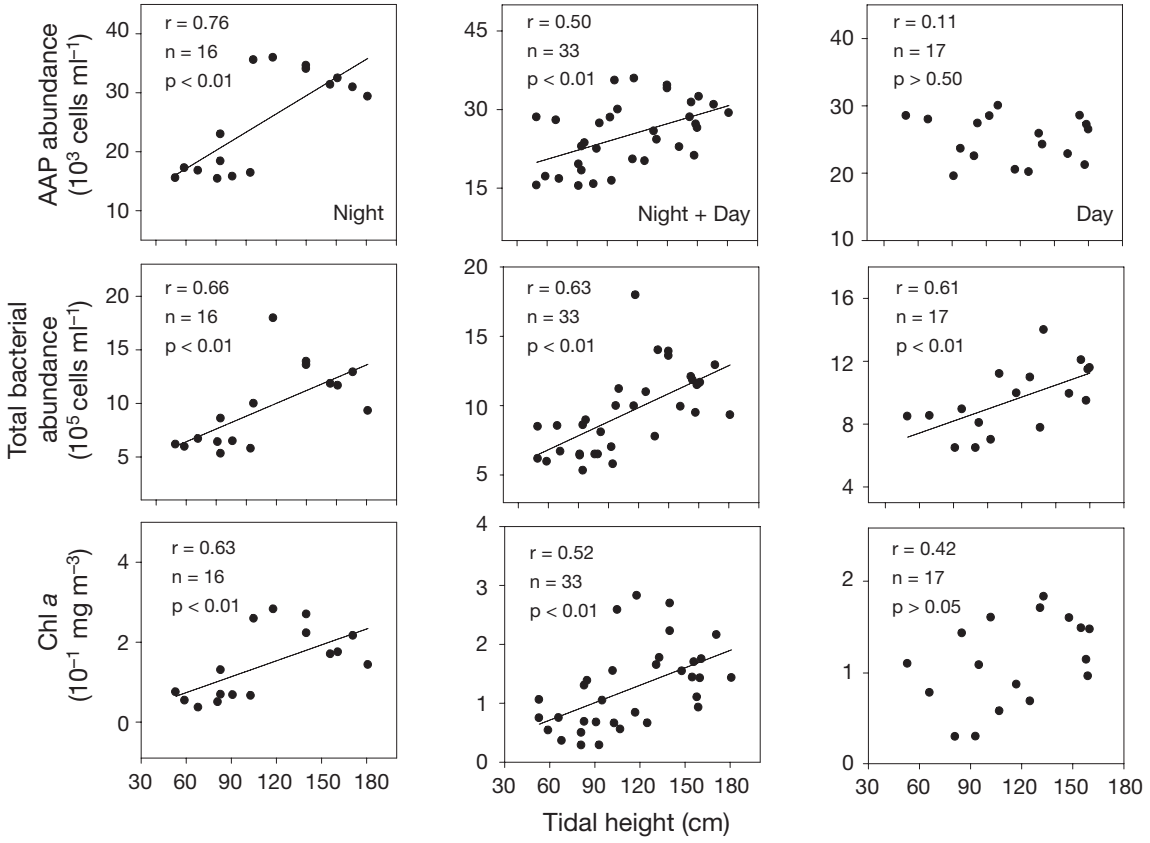

Fig. 4. Correlations between changes in aerobic anoxygenic phototrophic (AAP) bacterial abundance, total bacterial abundance, or chl *a* concentration and tidal height during night time (left), daytime (right), and the whole day (middle)

This pattern might be due to this particular ecosystem, which is shallow, with high productivity and activity of coral, and is affected by tidal cycle, light intensity, temperature, nutrients, and other factors.

Of the environmental factors we measured, variations in tidal height were most similar to those of AAP bacterial abundance, especially at night (Figs. 1 & 2). The highest and lowest values of AAP bacterial abundance at night happened at the times nearest to the highest and lowest water levels, indicating close relationships between AAP bacterial abundance and tidal currents (Fig. 4). Meanwhile, the changes of total bacterial abundance and chl *a* concentration at night were also close to those of tidal height (Fig. 4). In such a shallow coral reef ecosystem, the potential interactions between tidal current and organisms are described as follows: (1) During the flood tide, a large number of heterogenous organisms from the surrounding water (Sorokin 1994), coral surface mucus (Huettel et al. 2006), and sediments enter into the water covering the coral reef. Meanwhile, growth of local organisms during the flood tide could also be a reason for increasing biomass. (2) Increased biomass of small organisms stimulates the activity of consumers, which in turn limits further increase of pico-sized organisms. (3) During the ebb tide, many organisms are carried to the surrounding water (Sorokin 1994), stick to the coral mucus (Huettel et al. 2006), or settle to the bottom. Our results could provide some evidence of such processes. Here, the abundance of *Prochlorococcus* (Appendix 1, Fig. A1), which is much more abundant in the oligotrophic environment (Partensky et al. 1999) and is normally negatively related to other picoplankton in biomass distribution (Zhang et al. 2008), increased greatly along with the other autotrophic picoplankton with the flood tide during 21 to 23 February, indicating an intrusion of non-local organisms from the peripheral oligotrophic water sources.

During the day, the close correlation between AAP bacterial abundance and tidal height was broken (Fig. 4), suggesting that changes in AAP bacterial abundance might be controlled by factors other than just tidal current. High abundance from 06:00 to 09:00 h might result from cell division at 03:00 to 06:00 h, when FDC values were the highest during the diurnal changes (Fig. 3). Cell division leading to the high abundance in the morning has also been revealed in previous research (Kuipers et al. 2000). The decrease

of AAP abundance during 12:00 to 15:00 h might be a result of a combination of slow growth (Fig. 3) and light inhibition. Our research area was a shallow coral reef system, and solar radiation was most intense at around 12:00 to 15:00 h (Fig. 1), when the growth of bacteria could be inhibited and photo-induced cell lysis could occur (Müller-Niklas et al. 1995, Pakulski et al. 1998). In addition, viral lysis of bacteria mainly occurs around noon to afternoon (Winter et al. 2004), which might be another factor leading to a drop in AAP abundance at noon.

Diel variations of FDC of AAP bacteria versus total bacteria

In the present study, we observed for the first time the dividing cells of AAP bacteria using epifluorescence microscopy, which is a convenient and direct method for assessing bacterial growth rates (Hagstrom et al. 1979, Newell & Christian 1981, Fukuda et al. 2006). Observations of dividing cells have been conducted for many organisms, such as heterotrophic microprotozoa (Sherr & Sherr 1983), autotrophic picoplankton (Affronti & Marshall 1994), and heterotrophic bacteria (Hagstrom et al. 1979, Newell & Christian 1981, Fukuda et al. 2006), but no application to AAP bacteria has been reported to date.

The FDC of AAP and total bacteria showed clear diel patterns, with higher values during the night and lower values during the day (Fig. 3). Similar diel patterns have been reported for FDC of heterotrophic bacteria by Kuipers et al. (2000). Also, FDC values of picophytoplankton were higher in the early evening in natural environments (Campbell & Carpenter 1986). These similarities indicated that the pattern of higher division rate during night might be prevalent in marine microorganisms. Cell division accomplished mainly in darkness may avoid exposure of DNA to potentially harmful UV radiation during cell division (Kuipers et al. 2000).

Growth rates of AAP versus total bacteria implied by FDC

Our results revealed significantly higher FDC of AAP bacteria than of total bacteria (paired *t*-test, $p < 0.001$; Fig. 3). According to the positive relationships between FDC and growth rates as reviewed by Newell et al. (1986), higher FDC of AAP bacteria indicates higher growth rates. Based on the FDC data, we estimated the growth rate (μ) of AAP and total bacteria using the formula proposed by Newell & Christian (1981): $\ln \mu = 0.299\mathrm{FDC} - 4.961$. The results showed that the growth rate (±SD) of AAP bacteria in this area was 0.50 ± 0.16 to 2.86 ± 0.68 d^{-1} (Appendix 1, Fig. A2), and the average generation time (ln 2/μ) was about 15 h. The growth rate (±SD) of total bacteria was 0.23 ± 0.00 to 0.45 ± 0.18 d^{-1} (Appendix 1, Fig. A2), with an average generation time of about 60 h. The average growth rate of AAP was about 4 times of that of total bacteria). Similar higher growth rates of AAP bacteria than total bacteria were also reported in the productive North Atlantic (Koblizek et al. 2007). Given that the cell sizes of AAP bacteria are usually larger than those of other bacteria (Lami et al. 2007, Zhang et al. 2008, AAP bacteria may play a more important role than suggested by their abundance in carbon cycling in the ocean.

Although the estimation of bacterial growth rate through the FDC approach is rough, it provides a way to distinguish AAP production from total bacteria, which is impossible for traditional methods such as ^{3}H-adenine incorporation (Christian et al. 1982). However, we must point out that conclusions from the FDC approach should be viewed with caution given potentially unbalanced growth of different types of bacteria in the calibration experiments (Newell et al. 1986), diverse positive relationships between growth rates and FDC (linear or non-linear; Newell & Christian 1981, Newell et al. 1986), and other factors. Further studies are needed for accurate assessment of AAP bacterial growth rate.

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Appendix 1. Diel variations in the abundance of autotrophic picoplankton and in the growth rate of AAP and total bacteria

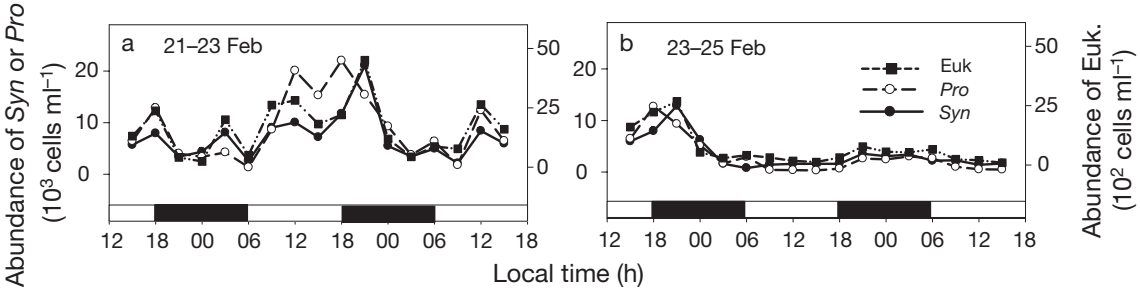

Fig. A1. Diel variations in the abundance of autotrophic picoplankton (*Syn*: *Synechococcus*; *Pro*: *Prochlorococcus*; Euk: eukaryotes) in the surface water of the sampling site. Diurnal observations were made from (a) 21 to 23 and (b) 23 to 25 February 2006. Horizontal black (white) bars show night (day)

Appendix 1 (continued)

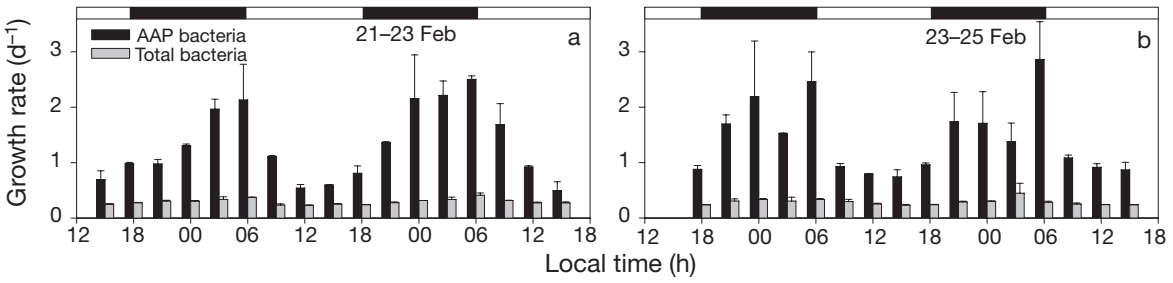

Fig. A2. Diel changes in growth rates of aerobic anoxygenic phototrophic (AAP) bacteria (black columns) and total bacteria (grey columns). Diurnal observations were made from (a) 21 to 23 and (b) 23 to 25 February 2006. Horizontal black (white) bars show night (day). Error bars indicate the SD of triplicate calculations

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