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Microscopic Overestimation of Heterotrophic Bacteria in Open Waters of China Seas

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Abstract Comparison of the abundances of heterotrophic bacteria in the East and South China Seas by stanctard epifluorescence microscopy and flow cytometry revealed that *Prochlorococcus* was miscounted as heterotrophic bacteria in DAPI stained samples. This could result in 5–31% overestimations of heterotrophic bacterial abundance in the study areas.

Key words: Epifluorescence microscopy, flow cytometry, heterotrophic bacteria, *Prochlorococcus*

In the late 80s, an extremely small (mean cell size of 0.6 μm) prokaryotic, oxy-phototrophic autotroph, *Prochlorococcus*, was discovered and proved to be widely present in the world ocean by the application of flow cytometry (FCM) [2]. In the central Pacific and Atlantic areas, up to more than 60% of autotrophic biomass is attributed by this tiny organism [1, 13]. However, the faint chlorophyll autofluorescence of *Prochlorococcus* prevents it to be efficiently observed by the standard epifluorescence microscopy approach [1]. On the contrary, *Prochlorococcus* could easily be miscounted as heterotrophic bacteria when samples are stained with DAPI for microscopic observation, as proved by flow and imaging cytometries [14]. Since most of the existing data of bacterial abundance were acquired by the epifluorescence microscopy method [6, 9, 15, 18], it is necessary to study this systematic error so that precise estimation of bacterial abundance and accurate evaluation on carbon budget of the microbial community can be achieved.

Although pico-sized phytoplankton was recognized to be the major fraction of total autotrophic biomass in the China sea areas many years ago [16], the presence of *Prochlorococcus* was not revealed until recently [7]. *Prochlorococcus* could hence have been practically counted as heterotrophic bacteria in the previous studies in these areas [15, 18]. However, there has been no information available on this error for these areas so far. We present here the results of a comparative study on the differences in quantification of heterotrophic bacteria by epifluorescence microscopy and FCM with field samples from the East and South China Seas.

Natural seawater samples were collected at Chinese JGOFS stations 204 (129.0°E, 30.0°N) and 418 (127.5°E, 28.7°N) in the East China Sea during February and March, 1997, and at stations 1 (114.0°E, 11.5°N), 18 (113.8°E, 9.0°N), 54 (111.6°E, 8.5°N), 48 (108.8°E, 7.0°N), and 28 (113.6°E, 6.4°N) in the South China Sea in November and December, 1997. Water samples were taken with 10-liter Niskin bottles. The sample pretreatment and preserving procedures for FCM analysis were the same as Jiao and Yang [7]. Samples were run on a FACSCalibur flow cytometer (Becton-Dickinson). FCM data were acquired and analyzed by CellQuest 2.2 (Becton Dickinson). Fluorescent beads (Fluorescence Scientific) of 0.474 μm were used as the internal reference. SYBR Green-I (Molecular Probes) was applied as the DNA stain for heterotrophic bacteria enumeration [10]. For microscopic analysis, samples were preserved either with 0.5% glutaraldehyde or with 2% formalin, and stored at 4°C for laboratory analysis. Aliquots of 3–10 ml seawater were filtered on to black Nuclepore filters (25 mm diameter, 0.2 μm pore size) and stained with 5 μg/ml 4', 6-diamidino-2-phenylindole (DAPI). Samples were then observed by Zeiss epifluorescence microscopy with UV-DAPI filter sets.

Flow cytometry analysis showed that the averaged depth integrated cell abundances of *Prochlorococcus* and heterotrophic bacteria were 3.5×10^4–5.1×10^4 and 2.2×10^5–2.7×10^5 cells/ml in the South China Sea, respectively. The vertical distribution patterns of the two were similar, both

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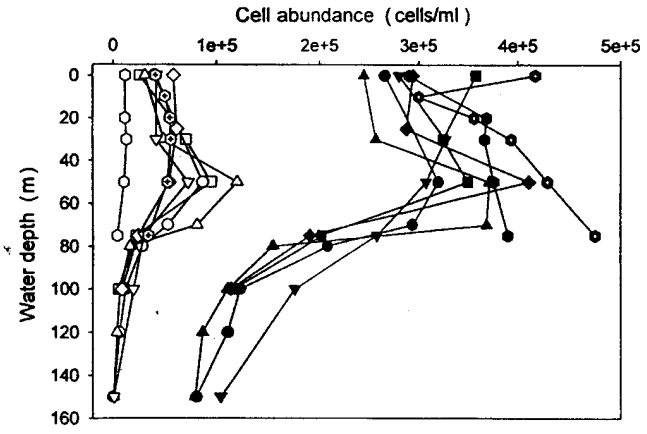

Fig. 1. Depth profiles of cell abundances of *Prochlorococcus* (open symbols) and heterotrophic bacteria (filled symbols).
The East China Sea (hexagon – Stn. 204, hexagon with cross – Stn. 408) and the South China Sea (circle – Stn. 1, square – Stn. 18, triangle – Stn. 28, diamond – Stn. 48, upside down triangle – Stn. 54).

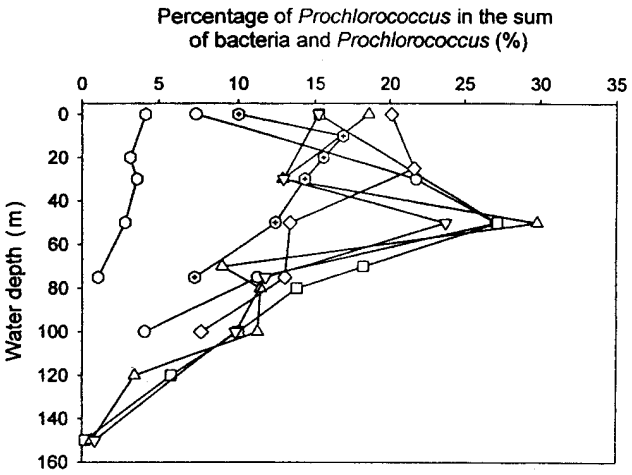

Fig. 3. Cell abundance percentage of *Prochlorococcus* in the sum of heterotrophic bacteria and *Prochlorococcus*.
The East and South China Seas indicate the potential overestimation of heterotrophic bacteria by epifluorescence microscopy (Symbols as in Fig. 1).

with maximum abundance layers occurring at around a 50-m depth. In the East China Sea, the averaged depth integrated cell abundances of *Prochlorococcus* ranged from 1.0×10^4 to 4.9×10^4 cells/ml, and those of heterotrophic bacteria from 2.6×10^5 to 4.0×10^5 cells/ml (Fig. 1). Obviously, *Prochlorococcus* was abundantly present in all of the sampling stations and was comparable in cell abundance to heterotrophic bacteria in most cases.

At all the sampling stations, cell abundance counts of heterotrophic bacteria by epifluorescence microscopy were distinctly different from those by FCM. The majority of the difference was distributed in the upper water column (less than 80 m) and showed a similar pattern to the vertical distribution pattern of *Prochlorococcus*. By coupling the corresponding data for each station, a significant correlation (r^2=0.7088) was found between the difference in bacterial cell abundance by the two methods and the cell abundance of *Prochlorococcus* (Fig. 2), suggesting that *Prochlorococcus* was responsible for the majority of the differences by the two methods. In fact, DAPI-stained *Prochlorococcus* cells could easily be included as bacterial cells under epifluorescence microscopy. Thus, in most cases, the presence of *Prochlorococcus* would cause an overestimation of the bacterial cell abundance in microscopy observations.

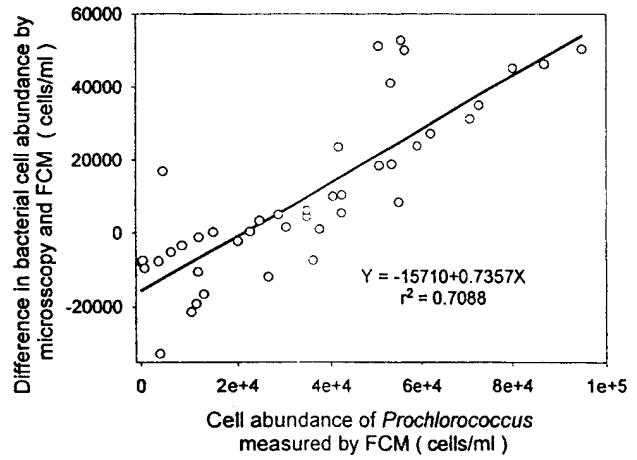

Fig. 2. Correlation between abundance of *Prochlorococcus* by FCM and the overestimation of abundance of heterotrophic bacteria by epifluorescence microscopy.

The percentage of *Prochlorococcus* cell abundance in the sum of *Prochlorococcus* and heterotrophic bacterial cell abundance ranged from 5% to 31%, with a high frequency level at 10–25% (Fig. 3). The lowest value occurred at Stn. 204 (129.0°E, 30.0°N) in the East China Sea and the highest at Stn. 28 (113.6°E, 6.4°N) in the South China Sea, indicating that the potential microscopic overestimation of heterotrophic bacteria was greater in the oligotrophic open ocean than in the mesotrophic areas. In vertical distribution, the percentage profiles peaked at the same depth as the maximum abundance layers of *Prochlorococcus*, i.e. the potential overestimation of heterotrophic bacterial abundance could be the largest at the depths where *Prochlorococcus* was most abundant.

The abundance of heterotrophic bacteria in the East China Sea [18] and the South China Sea [9] in previous studies were higher (up to 10^6 cells/ml) than that in the present study (at a level of 10^5 cells/ml). Except for other causes such as temporal and spatial differences, the systematic difference would most likely be due to the inclusion and exclusion of *Prochlorococcus*. Taking the converter of carbon biomass per cell as 53 fgC/cell for *Prochlorococcus* [1, 12] and 20 fgC/cell for heterotrophic bacteria [3-5, 8],

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this potential error would be 6.9–35.6% in terms of carbon biomass. This estimation is comparable to that of the studies in the Sargasso Sea (18–22%) [14].

Since the 1970s, picoplankton has been one of the major focuses in marine ecological studies. It has been recognized that picoautotrophs together with heterotrophic bacteria form the biggest compartment of the particulate organic carbon pool in the marine pelagic ecosystem, which is one of the key links in the biogeochemical cycling of biogenic elements. An accurate quantification of each group of these organisms is the prerequisite for a perspective understanding of the structure of marine living resources, and for the proper modeling of carbon budget in a marine ecosystem. The epifluorescence microscopy approach, by which most of the existing data on cyanobacteria and heterotrophic bacteria were acquired, has contributed much to our knowledge on marine picoplankton [11, 17], and it is still known to be the routine method for studying microorganisms in general laboratories all over the world. However, it is subject to major random errors for limited observations and uneven subsampling. Furthermore, the present study as well as a previous study [14] verified that the microscopy approach could introduce systematic errors in heterotrophic bacteria enumeration, when *Prochlorococcus* is present in the samples. With high sensitivity and thousands of acquisition events for every single sample, however, the FCM is the most powerful approach in quantitative analysis of microbial communities.

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