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Method for quantification of aerobic anoxygenic phototrophic bacteria

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Abstract **Accurate quantification of aerobic anoxygenic phototrophic bacteria (AAPB) is of crucial importance for estimation of the role of AAPB in the carbon cycling in marine ecosystems. The normally used method "epifluorescence microscope-infrared photography (EFM-IRP)" is, however, subject to positive errors introduced by mistaking cyanobacteria as AAPB due to the visibility of cyanobacteria under infrared photographic conditions for AAPB. This error could be up to 30% in the coast of the East China Sea. Such bias should be avoided by either subtracting cyanobacteira from the total infrared counts or using a flowcytometer equipped with specific detectors for discrimination between cyanobacteria and AAPB.**

Keywords: AAPB, EFM-IRP, quantification, cyanobacteria.

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Aerobic anoxygenic phototrophic bacteria (AAPB) are unique functional aerobic heterotrophic bacteria that can utilize sunlight for anoxygenic photosynthesis as needed in accordance with environmental conditions. They can turn photosynthesis on or off, and just use photosynthesis to supplement their normal bacterial diet of dissolved organic molecules. Kolber et al.'s article, discussing the contribution of AAPB to the carbon cycle in the ocean in *Science*[1], has brought a focus of great interest to the biological oceanography society. Obviously, AAPB is a part of bacterial photosynthesis unassessed heretofore[1)]. It has a big impact on the current marine primary production theory[2,3], and we should re-evaluate the carbon and energy cycle in the ocean based on oxygenic photosynthesis[4], for which we must first be sure that we use correct method for AAPB quantification.

1 Problems in AAPB enumeration

Currently, the most common method for enumeration of AAPB is epifluorescence microscope–infrared photography (EFM-IRP)[1]. In principle, the number of AAPB is determined based on the photosynthetic pigment——Bacteria Chlorophyll a (BChl a) that is incorporated into species-specific types of pigment-protein complexes, of which *in vivo* fluorescence peak is in the near-infrared region (>850 nm). The infrared fluorescence is supposed to be given only by AAPB cells. However, in practice, we found that this method would cause distinct errors due to interference by cyanobacteria.

2 Trouble shooting

(ⅰ) Laboratory experiments

(1) Materials. A cyanobacterium *Synechococcus* (CCMP1379) and an AAPB strain isolated from the surface water of the outer region of the Yangtze River estuary (location: 122.49° E, 28.99° N; salinity: 25.1; temperature: 27.6℃) were used in the experiment. The isolate was identified to be an AAPB strain according to the literature[5,6].

(2) EFM-IRP analysis. The diluted AAPB samples were stained with 5 μg/mL 4' 6-diamidino-2-phenylindole (DAPI) for 30 min in the dark, and then the cells were collected by filtration through 0.2 μm pore size black polycarbonate (PC) membranes (Whatman). Cells with Infrared fluorescent, diagnostic of the presence of Bchl a, were counted from nine microscopic fields using an epifluorescence microscope (Carl Zeiss Light Microscopy) with the following filter set (Chroma Technology Corp.): Excitation 350 to 550 nm, emission LP850 nm, beam splitter 650 nm, and viewed with an IR-sensitive charged-coupled device (CCD) camera (SPOT Diagnostic Instruments, Inc.) (Fig. 1).

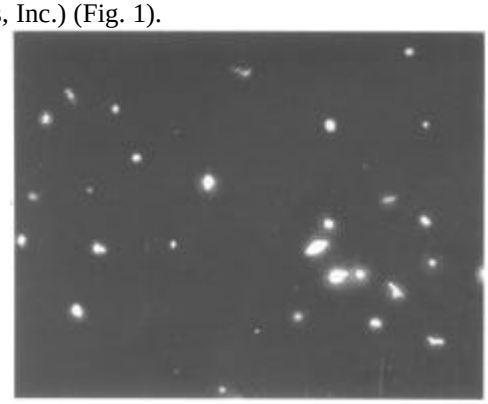

Fig. 1. Infrared fluorescence signals from AAPB.

The diluted *Synechococcus* samples were collected on 0.2 μm pore size black PC membranes. Cell counting was conducted twice under the following two microscopic settings respectively: ⅰ) the same as above filter set; ⅱ) excitation BP546 /12 nm, emission LP590 nm, beam splitter FT580 nm (Filter set 15, Carl Zeiss Light Microscopy), and viewed with CCD camera. The images captured by the CCD camera under the two conditions correspond to each other, verifying the visibility of cyanobacteria under the EFM-IRP conditions for AAPB counting (Fig. 2).

1) Referring to NAI NEWS ARCHIVE (http://nai.arc.nasa.gov/news_stories/news_print.cfm?ID=102): Photosynthesis: Take It or Leave It. By: Stephen Hart, October 17, 2001.

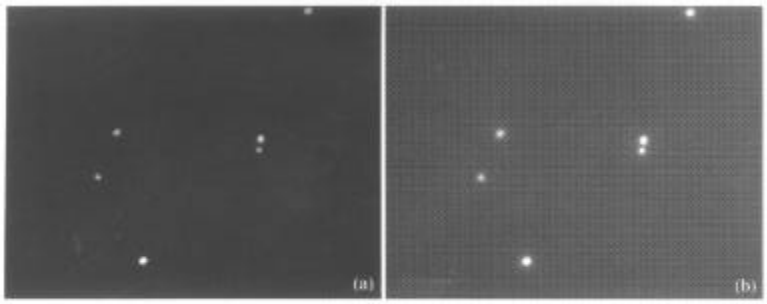

Fig. 2. Red (left) and infrared fluorescence (right) signals from *Synechococcus*.

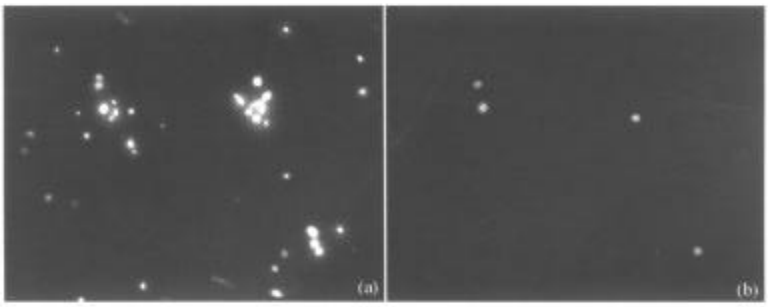

Fig. 3. Infrared (left) and red (right) fluorescence signals from *Synechococcus* and AAPB in the mixture of the two.

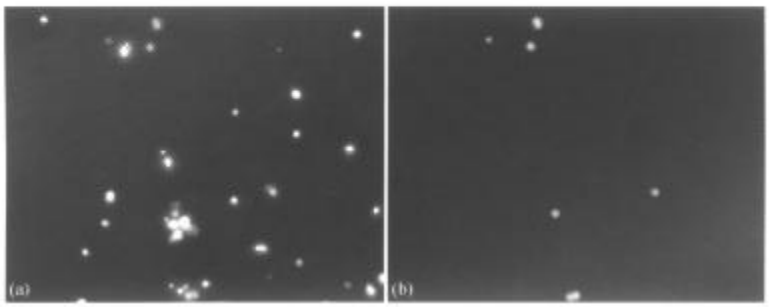

Fig. 4. Infrared (left) and red (right) fluorescence signals of a field seawater sample showing the AAPB false positives due to cyanobacteria.

The values obtained from nine microscopic fields were averaged and multiplied by appropriate factors to yield cell concentrations in the original samples. The abundance of AAPB and *Synechococcus* is 9.0×10^5 cells/mL and 2.1×10^5 cells /mL, respectively.

The mixture of AAPB and *Synechococcus* (equal volume) was collected on black PC membranes. Cells were counted by the same method as above (Fig. 3). The abundance of total cells is 5.6×10^5 cells/mL, which is about the average of the concentration of AAPB and *Synechococcus*. Errors in AAPB enumeration introduced by cyanobacteria in the EFM-IFP approach were thus confirmed.

(ⅱ) Field tests. Field samples were collected from the surface layer of the coastal water near Xiamen in July, 2003. Immediately after sampling, aliquot of 10 mL seawater was fixed for 15 min with 1% polyformaldehyde (PFA), and then was stained with DAPI before being filtered onto PC membranes. The membranes were then put on glass slides and stored at −20℃ in the dark for subsequent observation. With the same procedures as that in the laboratory, both red and infrared signals were captured by the CCD camera. Comparing the two sets of the red fluorescence and infrared images, one can easily see that *Synechococcus* cells also showed up in the infrared image (Fig. 4). It is thus shown that AAPB is overestimated by mistaking cyanobactrial cells by the EFM-IFP approach.

3 Rationales and error potentials

(ⅰ) Error rationales. Lab and field tests both verified that the presence of cyanobacteria would result in positive errors in AAPB enumeration by the EFM-IRP method due to the visibility of cyanobacteria under the infrared conditions. What is the rationale? Chlorophyl a, b, c, d, e contained in micro-algae all have no infrared fluorescence emission. However, the fluorescence spectrum of Photosynthetic System Ⅱ (PSⅡ) complex has a peak at 630—770 nm region and a vibrational subband in > 770 nm region; Light-Harvesting Compound Ⅱ (LHCⅡ) has a fluorescence peak in 660—900 nm and a vibrational small peak in > 725 nm region[7]. Additionally, the infrared fluorescence from cyanobacteria may be from the emission of heat energy as well. Namely, electrons transit from the ground state to the high energy state after being

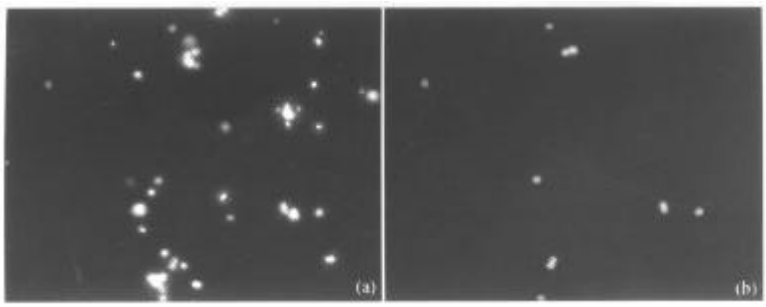

Fig. 5. Error engendered by cyanobacteria in the right photograph is about 28%.

excited, and excrescent energy will be released by fluorescence and heat energy as electrons return to the ground state[8]. The heat energy can generate infrared signals that can be captured by the CCD camera. Further study is needed to illustrate this point. Meanwhile, being 0.8—2.0 μm in diameter[9], *Synechococus* cells are overlapping with AAPB (length 1.0—2.5 μm; width 0.3—1.0 μm)[10] especially in infrared images.

(ⅱ) Error estimation. Seasonal investigations of AAPB and *Synechococcus* in the Yangtze River estuary of the East China Sea conducted from April 2002 to September 2003 showed that the two groups of microorganisms coexisted investigation-region-wide and year-round. Especially in summer, abundance of *Synechococcus* and AAPB is about $(2.0-3.0)\times10^4$ cells/mL and $(8.0-9.0)\times10^4$ cells/mL respectively in the Yangtze River estuary (Fig. 5), the positive error in AAPB due to the presence of *Synechococcus* is thus about 30% in summer. In other seasons, the errors are relatively small because of lower abundance of *Synechococcus*.

In addition to *Synechococcus*, another cyanobacterium *Prochlorococcus* is very abundant (10^5 cells/mL) in oceanic waters. It has been verified that *Prochlorococcus* could be miscounted as heterotrophic bacteria by microscopy[11,12] due to small diameter (0.6—0.8 μm)[13]. Normally, it is difficult for *Prochlorococcus* to be viewed by the CCD camera because fluorescence from *Prochlorococcus* is very faint and the fluorescence decays quickly. So, in practice, the presence of *Prochlorococcus* may not result in obvious positive errors in AAPB enumeration by the EFM-IRP method. Nevertheless, in principle, the potential positive errors introduced by *Prochlorococcus* exist.

4 Solutions

AAPB is distributed widely in the euphotic zone of global oceans[2]. Accurate quantification of AAPB is the first step for appropriate evaluation of the role of AAPB in carbon cycling in the ocean. In order to avoid obvious bias caused by the current enumeration EFM-IRP procedures, we put forward two solutions: ⅰ) Following the procedures described for infrared signal acquisition and red fluorescence (for cyanobacteria) acquisition to get the gross positive counts in the infrared image and *Synechococcus* counts in the red fluorescende image respectively, and then subtracting the latter from the former to get rid of cyanobacteria from AAPB. ⅱ) Applying Flow Cytometry (FCM) to AAPB analysis, i.e. adding an infrared fluorescence detector to the flowcytometer, exciting the samples with UV and visible dual lasers, and setting appropriate analytical conditions to discriminate the two groups by corresponding plots.

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References

1. Kolber, Z. S., Plumley, F. G., Lang, A. S. et al., Contribution of aerobic photoheterotrophic bacteria to the carbon cycle in the ocean, Science, 2001, 292: 2492—2495.
2. Beatty, J. T., On the natural selection and evolution of the aerobic phototrophic bacteria, Photosynthesis Research, 2002, 73: 109—114.
3. Jiao, N. Z., Sieracki, M. E., Zhang, Y. et al., Aerobic anoxygenic phototrophic bacteria and their roles in marine ecosystems, Chinese Science Bulletin, 2003, 48(6): 530—534.
4. Karl, D. M., Hidden in a sea of microbes, Nature, 2002, 415: 590—591.
5. Shiba, T, Simidu, U., Erythrobacter longus gen. nov., sp. Nov., an aerobic bacterium which contains bacteriochlorophylla, Int. J. Syst. Bacteriol., 1982, 32: 211—217.
6. Yurkov, V. V., Beatty, T., Isolation of aerobic anoxygenic photosynthetic bacteria from black smoker plume waters of the Juan de Fuca Ridge in the Pacific Ocean, Appl. Environ. Microbiol., 1998, 64: 337—341.
7. Zhang, S., He, J. F., He, F. T. et al., Fluorescence characterisctics of PS Ⅱ using ultrafast scanning image spectroscopy, Acta Photonica Sinica, 2001, 30(1): 6—10.
8. Ou, L., Yu, J. Y., Jin, X. G., Application Biochemistry, Beijing: Chemistry Industry Publication, 2001, 209—211.
9. Waterbury, J. B., Watson, S. W., Guillard, R. R. L. et al., Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium, Nature, 1979, 277: 293—294.
10. Yurkov, V. V., Beatty, T., Aerobic anoxygenic phototrophic bacteria, Microbiol. Mol. Biol. Rev., 1998, 62 (3): 695—724.
11. Jiao, N. Z., Yang, Y. H., Koshikawa, H. et al., Microscopic overestimation of heterotrophic bacteria in open waters of China Seas, J. Microbiol. Biotechnol., 2001, 11(5): 889—901.
12. Sieracki, M. E., Haugen, E. M., Cucci, T. L., Overestimation of heterotrophic bacteria in the Sargasso Sea: Direct evidence by flow and imaging cytometry, Deep-Sea Res., 1995, 42: 1399—1409.
13. Chisholm, S. W., Olson, R. J., Zettler, E. R. et al., A novel free-living prochlorophyte abundant in the oceanic euphotic zone, Nature, 1988, 334: 340—343.

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