Enumeration of Microalgae in Different Cell Growth Phases with Sandwich Hybridization Integrated with Nuclease Protection Assay

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Received 27 April 2017; revised 24 July 2017

Sandwich hybridization integrated with nuclease protection assay (NPA-SH), which takes advantage of high copy number of rRNA molecules in cells, has been developed to quantify microalgae. Seven microalgae in different cell growth phases were analysed by NPA-SH to find how the amounts of RNA had influence on enumeration of microalgae with NPA-SH. It was found that the calibration curves of NPA-SH from samples in different growth phases would influence the quantification results. Although the cell densities enumerated with the calibration curves from stable samples were higher than those by microscopy in the logarithmic growth phase, when rRNA greatly accumulated, it was very significant to detect samples from natural marine environments with NPA-SH. The results from this method can reflect the growth potentiality of microalgae, which will present the evidence for pre-warning of harmful algal blooms.

[Keywords: cell growth phases; rRNA index; NPA-SH]

Introduction

Among about 4,000 marine planktonic microalgae described so far all over the world, there are about 300 species those can trigger the harmful algal blooms (HABs), among which around 97 species are toxic. Thus it is basic for HABs study to identify and quantify microalgae species. Conventional methods using the light or electron microscopes for their identification and enumeration have been indispensable tools; however, because the classification criteria are mainly some morphological characteristics, such as general form, cell size and shape, flagellum numbers and position, they are obscure in varied environments, labour-intensive and tedious, making it difficult to quantify large number of routine samples in long-term monitoring and high-throughput sampling projects. In addition, the variable shapes and sizes of these microalgae in different environmental conditions or in their growth phases make it difficult to identify and count exactly, especially when several morphologically similar microalgae coexist in samples. Consequently, some improved monitoring, rapid detection and enumeration methods of microalgae are crucial because monitoring methods based on microscopy are time-consuming and costly if a large number of samples need to be processed.

In recent years, other techniques including flow cytometry, chemotaxonomy, spectrofluorometry, oligonucleotide probes, real-time PCR and immunoassays detection have been introduced into this field, playing increasing vital roles in routine monitoring programs. Those methods based on molecular probe techniques such as whole cell fluorescent in situ hybridization (FISH), sandwich hybridization assays (SHA), PCR-based assays and antibody probes, offering the advantage of high specificity, sensitivity and convenience of automation, have the potential to identify and quantify microalgae quickly for routine application of monitoring phytoplankton in field surveys.

Among those molecular probe techniques, the rRNA-targeted SHA established by Scholin7 was used to quantify several microalgae (e.g. Pseudo-nitzschia pungens, Heterosigma akashiwo, Fibrocapsa japonica and Alexandrium fundyense), showing good potential in analysing microalgae. Then an improvement on the basis of SHA was developed by integrating a nuclease protection assay to elevate the stringency of hybridization. This method, sandwich hybridization integrated with nuclease protection assay (NPA-SH), makes use of the ability of S1 nuclease, degrading single stranded nucleic acids to yield 5’ phosphoryl mono or oligonucleotides and leaving double stranded...
DNA, RNA or DNA/RNA hybrids intact, to convert rRNA easily degraded to stable DNA probes stoichiometrically. Thus it not only takes advantage of high copy numbers of rRNA but also overcomes unstable defect of SHA and enhances specificity, which offers better performance in specificity and repeatability. Twelve red tide species, which cause frequently HABs in many coastal waters of China, have been detected by NPA-SH. Results showed that this method have good reliability, specificity and accuracy in analyzing the samples from cultured, mixed and field samples. NPA-SH has taken advantage of high copy number of rRNA molecules with the assumption that the rRNA amounts keep constant in each cell; however, it is not the case. The rRNA content in each cell is dependent on the nutrient conditions, physiological status and growth phases. Especially in different cell growth phases, the amounts of RNA change, which probably give influence to enumeration with this method. In this study, seven microalgae were analyzed by NPA-SH in different cell growth phases, from which it could be found the variability of RNA amount in unit cell from different growth phases, and how this variability had influence on enumeration of microalgae with NPA-SH.

Materials and Methods

Seven microalgae, Chaetoceros curvisetus, Skeletonema marinoi, Symbiodinium sp., Akashiwo sanguinea, Prorocentrum minimum, Alexandrium catenella and Scrippsiella trochoidea, were collected from China sea (Table 1). A unicellular culture was established by isolating species with micropipettes. The strains were cultured axenically in f/2 medium at temperature between 22°C and 25°C on a light-dark cycle of 12:12 with a photon flux density of 60 µmol m⁻² s⁻¹. The cells of microalgae were daily removed from cultures and counted by microscopy for three independent replicates. Three replicates of each dilution were analyzed. Absorbance of NPA-SH was plotted against the cell number from microscopy to establish individual calibration curve. The correlation between the absorbance and the number of cells was calculated, and the linear regression equations were obtained to calculate those cell numbers.

When the cells of microalgae were counted by microscopy every day, the same number of microalgae cells was collected again and analysed by NPA-SH (Table 3). The cell numbers were calculated by the linear regression equations from NPA-SH for those microalgae.

After seven microalgae were analyzed by NPA-SH, a ratio \( R \), named as rRNA index, was calculated for every species, which expressed the variation trend of rRNA content in a microalga cell at different cell growth stages. The equation was \( R = \lg \left( \frac{OD}{N} \right) \), where OD was the absorbance determined at 450 nm with reference at 630 nm with NPA-SH, and N was the cell number enumerated by microscopy.

Results

The cells of seven microalgae were serially diluted and analysed at the same time with both NPA-SH and microscopy. The NPA-SH absorbance at 450 nm referenced against 630 nm was plotted against the microscopic enumeration of cell numbers (Figure 1). Seven microalgae were analysed by NPA-SH, and rRNA index was calculated for every specie. The cell growth curves and rRNA indexes for these species were plotted (Figure 2). It could be found that different microalgae had different growth phases, but they had approximately same characteristics. rRNA indexes were variable at different stages of cell growth. In the initial growth stage, the cell densities of microalgae rose gradually, and the R indexes
increased during this period, showing that the rRNA content in a cell had a remarkable accumulation at this time. At the logarithmic phase, the cell densities of these microalgae increased rapidly and got to the maximum, but the increasing of R indexes was not as fast as that in the earlier stage, and their fluctuation ranges were quite small. In general, the maximum cell density appeared following $R$ index. At the stationary phase, the cell densities fluctuated near the maximum, and the $R$ indexes remained stable and began to decline. In the declined phases, the cell densities began to fall, and the $R$ indexes also decreased gradually, which showed that rRNA was degrading and the cells were dying.

**Discussion**

Through converting degraded rRNA in cells to stable DNA probes stoichiometrically, NPA-SH can be used to quantify cell numbers; however, rRNA content in cells is not constant, which depends on the physiological or nutrient status of cells. Because the calibration curves of NPA-SH are produced by cells, which growth stage cells come from will probably influence the quantification results. rRNA amounts change during different cell growth stage; especially in the logarithmic growth phase, rRNA amount in a cell increase tremendously. The possible reason is that cells will step into a period of fast division, and it is necessary to synthesize a large amount of protein to meet growth needs. Because rRNA plays a vital role in synthesizing protein, its content in a cell has to increase to provide material basis for cells growth and division.

In this study, the calibration curves of NPA-SH came from the samples in the stationary phase. Thus, the cell densities enumerated by NPA-SH were higher than those by microscopy in the logarithmic growth phase, when rRNA greatly accumulated. While in the declined growth phase, the cell densities enumerated by NPA-SH were lower than those by microscopy, when rRNA greatly degraded and cells were dying. In the previous experiments, the detection results of not only cultured samples but also field ones by NPA-SH were both consistent with those of microscopy observations. The reason may be that the cells collected in laboratory cultures are at the stationary phase, and the microalgae cells in natural marine environments are also in the stable period, which lead...
to the roughly identical results with these two methods. In natural marine environments, microalgae are commonly in the stable phase. Only when HABs begin to develop and break out, the microalgae are in the logarithmic growth phase. Therefore, it is acceptable to calculate the cell numbers in natural samples with the calibration curves from cells in the stable phase. Even if the field samples come from the swift proliferation period of HABs, the cell concentrations calculated by NPA-SH are higher than those by microscopy, which is significant because public health officials and wildlife biologists require up-to-date trends indicating where potentially toxic species are and whether their population is rising or falling or is exceeding some threshold value that warrants attention. Furthermore, ultraprecise enumeration is probably not necessary in the context of providing an early warning of increasing microalgae, so that even a twofold error in estimating cell numbers is of little concern. Thus, although

Fig. 1 — Calibration curves of absorbance at 450 nm referred at 630 nm against the microscopic enumeration cell numbers for seven kinds of microalgae.
the cell concentrations calculated by NPA-SH are higher than those by microscopy during the red tides break out, it reflects the proliferation potentialities and up-to-date trends of microalgae. Therefore, the variability of rRNA contents will not affect monitoring these microalgae with NPA-SH. The results from this method will reflect the growth potentialities of microalgae and present the evidence for HABs pre-warning, which is a promising technique in identification and quantification of microalgae in routine monitoring programs.

Fig. 2 — Growth curves from microscopy and rRNA indexes ($R$) from NPA-SH for seven HABs species

Conclusions
When NPA-SH is used to enumerate the cell numbers of microalgae, it should be attention that the physiological or nutrient status of cells make the effect on the quantification results. When microalgae are in swift proliferation period, the cell concentrations quantified by NPA-SH are higher than those by microscopy; however, to HABs pre-warning and public health, the NPA-SH is still a promising technique in routine monitoring programs.
Acknowledgements

This work was supported by the Public Science and Technology Research Funds Projects of Ocean (201205031) and Shandong Provincial Natural Science Foundation, China (ZR2014DM007).

References