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# Optimization of fluorescence *in situ* hybridization (FISH) for the identification of two polar coccoid green algae species

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**Abstract** Standard FISH protocols using fluorochrome-labeled oligonucleotide probes have been successfully applied for *in situ* detection. However, optimized protocols of FISH for specific eukaryotes in marine environments are often not developed. This study optimized the conditions of fluorescence *in situ* hybridization (FISH) by using two polar isolated microalgae. The modified conditions were as follows: (1) 10 mg · mL$^{-1}$ lysozyme solution pretreatment at 37°C for 30 min; (2) the hybridization buffer including 20% formamide; (3) the hybridization condition was 47°C for 6 h. The cells enumerated by FISH were compared with those enumerated by flow cytometry (FCM) and DAPI to confirm the cell loss and hybridization efficiency. The optimized protocol was also successfully applied to Arctic Ocean samples, which were found to be dominated by *Micromonas* sp. The modified protocol showed a high relative efficiency and could be successfully applied for the detection of specific microbial eukaryotes in environmental samples.

**Key words** Fluorescence *in situ* hybridization (FISH), *Chlorella vulgaris* strain Lw2008/02, *Micromonas* sp. strain CCMP2099.

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## 1 Introduction

Fluorescence *in situ* hybridization (FISH) is a powerful technique that allows the *in situ* detection of specific nucleic acid sequences (RNA and DNA). FISH employs fluorochrome-labeled RNA (DNA) oligonucleotide probes targeted at specific regions such as the 16S rDNA of prokaryotes or 18S rDNA of eukaryotes in environmental samples *in situ*. During the last decade, many attempts have been made to develop and optimize FISH protocols, especially for prokaryotes[1-3], and these methods have been applied to different specified populations and complex environmental

samples. For example, a specific FISH technique was used for localization and identification of soil microbial diversity[4]. By constructing catalyzed reporter deposition-fluorescence *in situ* hybridization, substantial increases in signal sensitivity have been available to access the inside of thecate dinoflagellate cells and bacterial cells in aquatic ecosystems[5]. In addition, rapid and effective FISH protocols have been developed to study *Bacillus* spores using *in situ* labeling within 1 h[2]. However, few optimized protocols for FISH have been developed and applied to quantify picoeukaryotes of interest in environmental samples.

Picoeukaryotes (<3 μm) represent component of major importance in terms of contribution to both biomass and primary productivity in various aquatic systems[6-7]. However, it is necessary to quantify the dominant phylogenetic groups of picoeukaryotes in the natural environment to understand their contribution to the microbial food web and biogeochemical cycles[8]. Due to their small size and simple morphology, oligonucleotide probes designed to target these specific taxa were particularly favorable. The FISH technique has proven to be useful for estimation of the abundance of picoeukaryotes in natural communities[8-9]. Previous studies have focused on estimation of the abundance of specified picoeukaryotes rather than modification of the FISH protocol itself. Both Biegala *et al*. (2002)[10] and Not *et al*. (2002)[11] described the TSA-FISH procedure and assessed natural samples successfully, but they did not consider the signal-to-background ratio or the hybridization efficiency. Biegala *et al*. (2003)[8] combined FCM with fluorescence microscopy to quantify environmental picoeukaryotes, but they emphasized sample enumeration rather than FISH efficiency. This lack of methods and studies for optimizing FISH for some pico-sized eukaryote populations has likely led to significant errors during investigations. Accordingly, targeting improved efficiency of the use of FISH for studies of such organisms is necessary.

Since FISH procedures have been described in previous studies[8,10-11], the present study focused on optimizing key hybridization steps and factors, especially signal intensity and the hybridized efficiency. An effective laboratory protocol that has the potential for application to the quantitative assessment of polar environmental picoeukaryotes was then developed. Specifically, we employed the optimized FISH procedures developed herein using rDNA-targeted probes for the detection of two lab coccoid green algae isolated from polar regions. The fluorescence intensity and hybridization efficiency primarily depend on sample processing and the FISH conditions applied, such as the permeabilizing reagent used, hybridization temperature and time, probe concentration, and formamide concentration[3, 11-13]; therefore, we optimized these key influencing factors. Both the fluorescence signal intensity and the FISH efficiency were taken into account in this study.

## 2 Material and methods

### *2.1 Cultures and strains*

*Chlorella vulgaris* strain Lw2008/02 was isolated from glacial water in the later

summer of 2006 in Kongsfjorden (79° N, 12° E)[14]. *Micromonas* sp. strain CCMP2099 is a tiny (1—2 μm) green alga with a pear-shaped naked cell body that swims with a single flagellum and is commonly isolated from Arctic waters (https://ccmp.bigelow.org/). The algae were cultured at the Key Laboratory for Polar Science of State Oceanic Administration(SOA) at the Polar Research Institute of China. Algal growth medium Bourelley[15] was used for *Chlorella vulgaris* strain Lw2008/02. The cultures were grown in 150 mL flasks at (6±0.5)°C under a 12∶12 h Light∶Dark regime. Light was provided by fluorescent bulbs at an irradiance of 5000 mol·$m^{-2}$·$s^{-1}$. Medium L1 (https://ccmp.bigelow.org/) was used for *Micromonas* sp. strain CCMP2099, and the culture was grown in 150 mL flasks at 4°C under a 12∶12 h Light∶Dark regime. Light was provided by fluorescent bulbs at an irradiance of 2000 mol·$m^{-2}$·$s^{-1}$.

### *2.2 Cell collection and fixation*

Exponential phase algal cells were collected from suspension cultures. To estimate cell losses due to leaching and hybridization, cells were counted before and after hybridization. Prior to hybridization, the concentrations of *Chlorella vulgaris* cells were determined by FCM (Flow Cytometry, Beckman Coulter Quanta, SC, USA) and DAPI staining, separately. The process of FCM counting was as follows: 1.5 mL sample cells were fixed with paraformaldehyde at a final concentration of 2% for 1 h at room temperature, after which the samples were diluted in PBS solution and mixed well. Data acquisition was conducted at a strict range of flow rates (100—500) for 1 to 3 min depending on the concentration of the target population. The flow speed was 4.67 μm·$min^{-1}$. The first trigger parameter was controlled at FL3 (670 long pass), while the second parameter was controlled at SS (side-angle scatter height).

After FCM, the remaining 1 mL of *Chlorella vulgaris* cells were concentrated by centrifugation (Eppendorf, Germany) at 9000×g for three min at room temperature. The cell pellet was then resuspended in 1 mL of phosphate-buffered saline (1× PBS solution at pH 7.4) and incubated for 5 min at room temperature. The cells were then concentrated by two successive centrifugation steps (9000×g for 3 min at room temperature). The concentrated cells were then added to 1 mL of MilliQ water, after which the samples were successively trapped onto 25 mm diameter, 0.2 μm-pore-size nucleopore black membrane filters (Whatman). Finally, the samples were stored at −20°C until the further processing.

### *2.3 Probe labeling*

The oligonucleotide probes, EUK1209R, EUK528 and PRAS02, coupled with Cy3 dye were used in this study (Table 1). The universal eukaryotic probes EUK1209R and EUK528 were adapted from Lim *et al.* (1993)[16] and Zhu *et al.* (2005)[17], respectively. PRAS02 is a specific probe that targets the order Mamiellales (Chlorophyta, Prasinophyceae), which was adapted from Biegala *et al.*

(2003)[8]. In the present study, EUK1209R and EUK528 targeted both eukaryotic algae, while PRAS02 was specific to *Micromonas* sp. strain CCMP2099. Pre-tests of these specified probes with the clone libraries of microbial eukaryotes isolated from the Arctic were conducted as well[18].

Table. 1 Oligonucleotide probes used in this study

| Probe | Specificity | Sequence (5′—3′) of probes | Target site | Tm(°C)[b] | Reference |
|---|---|---|---|---|---|
| EUK1209R | Eukaryote | GGGCATCACAGACCTG | 1195—1211 | 27.1 | Lim *et al*. (1993) |
| EUK528 | Eukaryote | CCGCGGTAATTCCAGCTC | 18S_nucl | 58 | Zhu *et al*. (2005) |
| PRAS02 | Mamiellales | CCCGTCCCGAGACCA ACG | 651—69 | 37 | Biegala *et al*. (2003) |

### 2.4 *Permeabilization of cells for optimization*

*Chlorella vulgaris* strain Lw2008/02 targeted by EUK1209R was used in the following tests. Three different enzymatic pre-treatment protocols were tested before hybridization: (a) the fixed cells were permeabilized using lysozyme [10 mg • $mL^{-1}$ in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0] for 30 min at 37°C; (b) the fixed cells were permeabilized as above for 60 min at 37°C; (c) the cells were fixed without permeabilization. In addition, the cell permeabilization conditions were evaluated by fluorescent microscopy.

### 2.5 *The procedure and optimized conditions for FISH*

Following dehydration by sequential 3 min washes in 50%, 80% and 96% ethanol, and then air dried. The samples were then stored at 4°C in the dark until further experiments were conducted.

For the fluorescent dye CY3, which is red under 550—570 nm, it was necessary to determine whether autofluorescence was removed before hybridization. To accomplish this, we compared samples to positive probe signals. To estimate the cell loss due to hybridization, the cells were counterstained with DAPI and compared to the results obtained following FISH. DAPI staining was conducted as follows: 10 μl 5 μg • $mL^{-1}$ DAPI (Sigma-Aldrich) was added to the samples, which were then incubated for 15 min in the dark at room temperature. The filters were then rinsed twice in 5 mL sterile $H_2O$ for 1 min and dried, after which they were observed by fluorescent microscopy[19].

During the hybridization reaction, the following hybridization protocols were employed: (a) to optimize the formamide concentration, 20%, 30% and 40% concentrations were employed[11]; (b) to optimize the signal intensity and hybridization efficiency, three hybridization times were employed, 2.5 h, 4 h and 6 h at 47°C. The procedure of hybridization as follows: 20 μL hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.35 mM sodium dodecyl sulfate (SDS), 0.1 mg • $mL^{-1}$ of poly(A), 0.2 mg • $mL^{-1}$ of bovine serum albumin (BSA) and 20%, 30% or 40%

formamide]was prewarmed at 37°C for 30 min, after which it was mixed with 5 μL blocking reagent. Next, 2 μL (1 μm) probe was added to the buffer, and hybridization was conducted.

Following hybridization, non-hybridized probes were washed by rinsing for 30 min in freshly prepared buffer[20 mM Tris-HCl, 6.3 mM EDTA, 0.35 mM SDS, 56 to 225 mM NaCl (depending on the formamide concentration)]that had been prewarmed at 37°C. The filters were then washed twice with excess MilliQ water and 96% ethanol for 3 min each. Finally, the filters were completely air dried in the darkness and then counterstained with DAPI.

### *2.6 Microscopic observation and data analyses*

Cells were all observed by fluorescent microscopy (Nikon Eclipse 80i connected to a system for picture a capture Digital Camera Dxm1200f) under the 1000× oil immersion objective. The autofluorescence of non-hybridized cells was observed under a 450—490 nm filter (green), which showed light green fluorescence if there was no autofluorescence and red fluorescence in the case of autofluorescence. Following hybridization, the samples were visualized under excitation/emission filters of 360/420 nm for DAPI (blue) and 550/570 nm for CY3 (red). For each sample, cells in 20 randomly selected microscopic fields were counted and the concentration was then determined as follows: cells • $mL^{-1}$ = (the average cells of each field/the volume of samples trapped onto filters) × (the area of the filters/the area of the field). All data were statistically analyzed by SPSS 17.0 (Analyze/CompareMeans/One-Way ANOVA). A multiple comparisons table containing the confidence intervals, the mean difference is significant at the 0.05 level.

### *2.7 Application to environmental samples*

Environmental samples were collected during the Third Chinese Scientific Expedition of the Arctic Ocean. The samples were treated with a final concentration of 2% paraformaldehyde as described above, after which they were passed through a 50 μm mesh prefilter and then trapped onto 25 mm diameter, 0.2 μm pore size nucleopore membrane filters (Whatman). The filters were then stored at −80°C until processing. The environmental sample B84-0 m, which was used in this study, was collected at 83°59.91′N, 144°16.50′W.

## 3 Results

*Chlorella vulgaris* strain Lw2008/02 targeting by EUK1209R was modified by altering the permeabilization, formamide concentration, hybridization time and temperature and amount of probes. Except that specification of three probes were set for two different coccoid green algae.

Before hybridization, the autofluorescence was examined by fluorescent micros-

copy. As shown in Fig. 1 (A. E. I.), the fluorescence of *Chlorella vulgaris* was successfully removed before each test. Permeabilization treatment conducted under three different conditions revealed that 10 mg • $mL^{-1}$ lysozyme for 30 min helped maintain the morphology of the *Chlorella* cells and caused a significant reduction in background signal (Fig. 1. B), while 60 min of lysozyme incubation caused slight cell dissolution (Fig. 1. C). Additionally, cells that were not subjected to lysozyme treatment showed low hybridization efficiency (Fig. 1. D), while 2 μL (1 μm) of lysozyme with 20 μL hybridization buffer produced the optimal results.

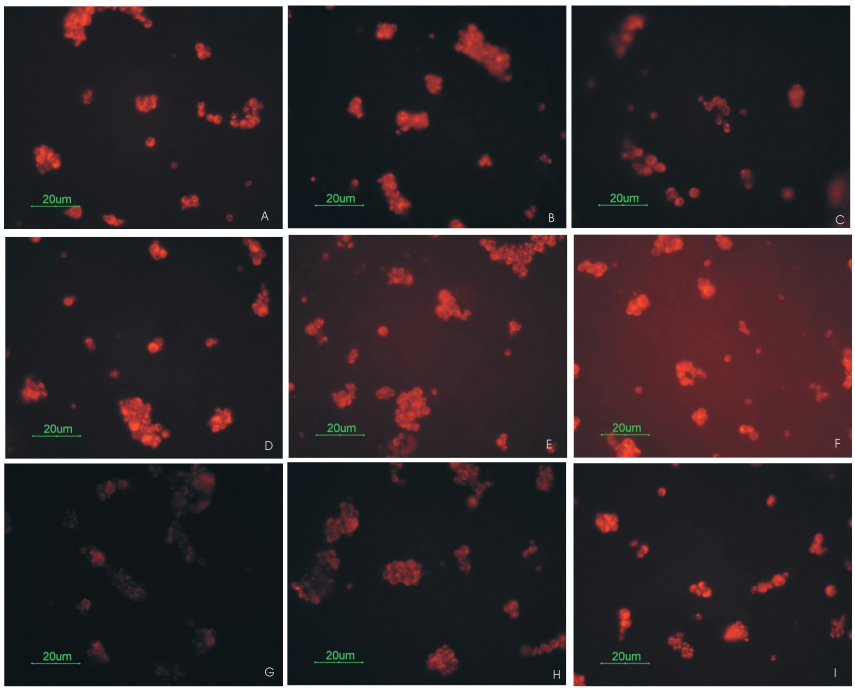


Fig. 1 FISH staining of *Chlorella vulgaris* (Lw2008/02) under different conditions including: (1) different permeabilization conditions; (2) different formamide concentration; (3) different hybridization times and temperatures. Before each condition was set, the autofluorescence was examined. (A) autofluorescence before permeabilization; (B) after enzyme treatment for 30 min; (C) after enzyme treatment 60min; (D) without enzyme treatment; (E) autofluorescence before the formamide concentration was set; (F) after 20% formamide treatment; (G) 30% formamide treatment; (H) 40% formamide treatment; (I) autofluorescence before hybridization time and temperature were set; (J) hybridization for 2.5 h at 47°C; (K) hybridization for 4 h at 47°C; (L) hybridization for 6 h at 47°C.

Three different concentrations of formamide (20%, 30% and 40%) were also evaluated. The results revealed that 20% formamide was ideal for specific hybridization and maintaining a low background signal, while 30% and 40% formamide resulted in a strong background signal (Fig. 1. F-H). Satisfactory signal intensity and hybridization efficiency were observed after incubation at 47°C for 6 h (Fig. 1. J-L).

Following hybridization, the hybridized cells were counterstained with DAPI. Microscopic analysis under the same visual field revealed that most of the cells were successfully hybridized (Fig. 2. A-B). To assess the cell loss and hybridization efficiency, FISH counts were compared with those obtained after FCM and DAPI staining(Fig. 3). The counts were found to differ significantly among groups ($df=3$, $p=0.002$). Specifically, FCM resulted in an average concentration of $2.186\times10^6$ cells $\cdot$ $mL^{-1}$, while DAPI before hybridization resulted in a concentration of $2.107\times10^6$ cells $\cdot$ $mL^{-1}$(B), which did not differ significantly from numbers ($P>0.05$) during filtering process. Following hybridization, DAPI (A) and FISH gave concentrations of $1.921\times10^6$ cells $\cdot$ $mL^{-1}$ and $1.903\times10^6$ cells $\cdot$ $mL^{-1}$, respectively (Fig. 3), which did not differ significantly ($P>0.05$).

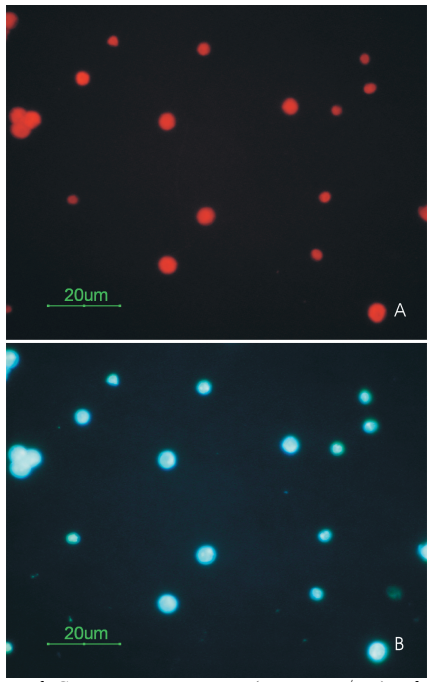


Fig. 2 Fluorescence microscopy of *Chlorella vulgaris* (Lw2008/02) after hybridization and subsequent counterstaining with DAPI. The picture shows the organisms under the same visual field (A&B). (A) The image under the CY3 filter; (B) The image under the DAPI filter.

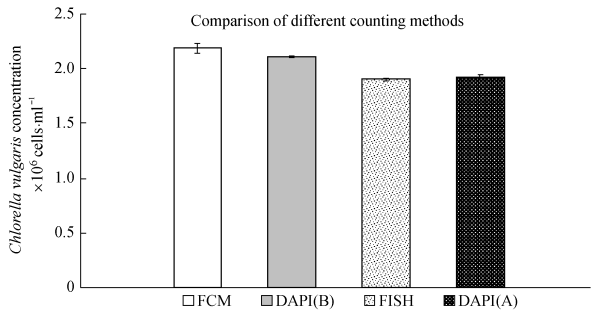


Fig. 3 The abundance of *Chlorella vulgaris* (Lw2008/02) (FCM, DAPI and FISH). DAPI (B): DAPI counts before hybridization; DAPI (A): DAPI counts after hybridization.

EUK1209R and EUK528 targeted both green algae strains evaluated in this study, *Chlorella vulgaris* strain Lw2008/02(Fig. 4. A) and *Micromonas* sp. strain CCMP2099 (Fig. 4. E). The specificity was compared based on relative signal intensity and hybridization efficiency under the same test conditions (Fig. 4. B. C. F. G). The results revealed that EUK1209R had higher fluorescence intensity than EUK528. Apparently, probe PRAS02 could not target *Chlorella* (Fig. 4. D), which confirmed its specificity for *Micromonas*. sp. (Fig. 4. H). As shown in Fig. 5, the FISH protocol developed here could be successfully applied to samples from the Arctic Ocean.

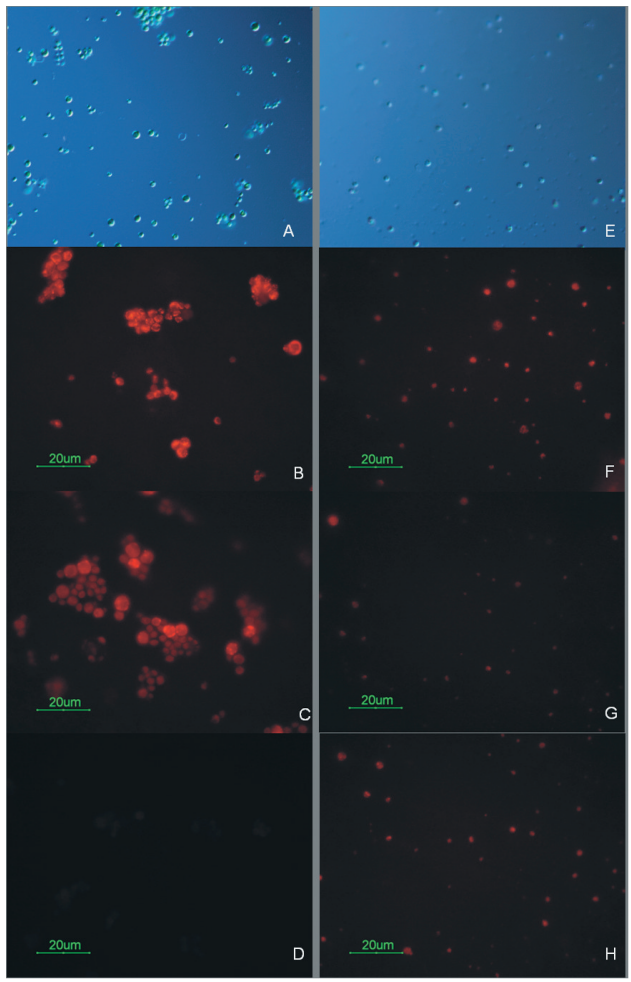


Fig. 4 Specification tests of Cy3-labelled probes used in this study. EUK1209R and EUK528 are universal probes that can target both eukaryotes evaluated herein, while PRAS02 is specific for *Micromonas* sp. (CCMP2099). (A) *Chlorella vulgaris* (Lw2008/02) under light microscopy; (B) *Chlorella vulgaris* (Lw2008/02) targeted by EUK1209R; (C) *Chlorella vulgaris* (Lw2008/02) targeted by EUK528; (D) *Chlorella vulgaris* (Lw2008/02) targeted by PRAS02; (E) *Micromonas* sp. (CCMP2099) under light microscopy; (F) *Micromonas* sp. (CCMP2099) targeted by EUK1209R; (G) *Micromonas* sp. (CCMP2099) targeted by EUK528; (H) *Micromonas* sp. (CCMP2099) targeted by PRAS02.

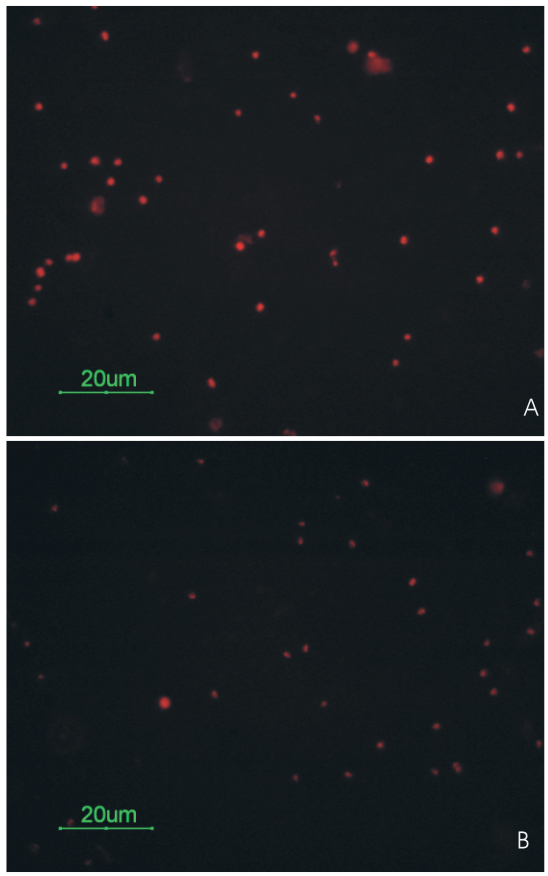


Fig. 5 Fluorescent micrographs for the natural community from the Arctic Ocean region, which is dominated by picoeukaryotes, primarily *Micromonas pusilla*. Cells were hybridized with EUK1209R and PRAS02. Cy3-labeled specific probes. (A) EUK1209R probes target picoeukaryotes. (B) PRAS02 probes target *Micromonas* sp..

The final optimized conditions determined based on the tests described above are shown in Table 2. Specifically, the optimum fluorescence intensity and hybridization efficiency was obtained by incubating the cells in 10 mg • $mL^{-1}$ lysozyme solution at 37°C for 30 min prior to hybridization, which was then conducted using 20% formamide in preheated hybridization buffer at 47°C for 6 h.

Table. 2 Optimal conditions for fluorescence *in situ* hybridization (FISH)

| Condition | |
|---|---|
| Permeabilization | 10 mg • $mL^{-1}$ lysozyme buffer at 37°C for 30 min |
| Probe amount | 2 μL(1 μm) in 20 μL hybridization buffer |
| Formamide concentration | 20% formamide |
| Hybridization time | Hybridization for 6 h |
| Hybridization temperature | Hybridization at 47°C |

## 4 Discussion

During most of the last two decades, natural fluorescence observed by epifluorescence microscopy or FCM and marker pigment analyses were the only quantitative techniques available for large-scale investigation of picoplankton diversity[20-21]. However, these methods usually lacked the resolution (especially epifluorescence microscopy and FCM) required for investigation at a fine taxonomic level. Recent advances within the field of biological oceanography and progress in quantitative (or semi-quantitative) molecular tools such as FISH have allowed more detailed and extensive phylogenetic surveys[8, 11, 22]. Indeed, FISH has proven to be a powerful molecular method for identification, visualization and quantification of organisms in microbial communities[23]. Despite the widespread use of FISH for more than a decade, the signal intensity of cells hybridized with the fluorophore-labeled probe sometimes below the detection limit. Low fluorescent responses in hybridized samples can occur as a result of a variety of factors, such as low ribosome content of cells, difficulty in permeating cell walls, and inaccessibility of target sites due to either secondary or tertiary structures of the rRNA (i. e., RNA-RNA interactions) or the effect of ribosomal proteins (i. e., RNA-protein interactions). The former two factors depend solely on the studied organism and can potentially be circumvented by changes in the experimental protocol, such as using a different fixative to better permeate the cell wall[24] or indirect labeling of the oligonucleotide to amplify the signal intensity per probe[25].

In this study, we optimized the FISH procedure for the identification of two polar coccoid green algae species (*Chlorella vulgar* and *Micromonas* sp.). Accordingly, we designed different experimental conditions in an attempt to develop an optimized protocol of FISH for analysis of these organisms. Different enzymes have often been used to increase probe permeability in general[26]. Since no universal or standard permeabilization procedure is available in the literature, in general, adapted protocols have had to be developed for specific organisms. Enzymatic permeabilization was easier for the wall-less flagellated *M. pusilla*. Pre-treatment of fixed biofilms with lysozyme produced significantly higher probe intensities[11]. Aditionally, autofluorescence can be diminished by ethanol washing, as described by Biegala *et al*. (2003)[8]. In the present study, 96% ethanol incubation appeared to reduce autofluorescence sufficiently.

The cells were targeted with the eukaryotic probes (EUK1209R and EUK528) and PRAS02. Hybridizations were then conducted using different hybridization mixture systems while targeting different species. In the present study, hybridization in 20% formamide in preheated hybridization buffer at 47°C for 6 h was found to the optimal condition. This method also worked well with the polar environmental samples.

The concentration of specific probes is also an important factor for FISH. Biegala *et al*. (2003)[8] assessed picoeukaryotes in the natural environment using 100 ng

probes in 20 μL hybridization buffer. Conversely, Junge *et al*. (2004)[12] used 250 ng probes in 65 μl hybridization buffer in a study of arctic water. However, in our study, it was suitable to mix 2 μL (1 μm) with 20 μL hybridization buffer.

To assess the cell loss and hybridization efficiency, we compared FISH counts with FCM and DAPI counts. No significant differences were observed before and after the samples were trapped onto filters, which confirmed the results of previous studies[8]. These findings suggested that several rounds of centrifugation at speeds as high as 9000×g did not induce significant cell loss.

It is well known that all cells should be stained with DAPI, but not all can be hybridized. Observation of the same microscopic visual field using the CY3 and DAPI filter revealed that most cells were hybridized successfully, and confirmed the high efficiency of the technique developed here.

Following hybridization, the FISH counts and DAPI (A) did not differ significantly ($P>0.05$). Conversely, the DAPI (B) and FISH counts differed significantly ($P<0.05$), indicating that cell loss may have occurred during the permeabilization and washing treatment. However, this discrepancy may also have been due to human error since the cells were counted manually by fluorescence microscopy, which has been shown to be subjective[8]. Otherwise, In the polar ecosystem, the concentration of picoeukaryotes can be 3 orders of magnitude lower than in lab cultures, the difference of cell losses could be narrowed[7].

In summary, an optimized FISH protocol that could be readily applied to identify and quantify taxa of interest such as *Micromons* sp. and *Chlorella* sp. in natural communities in polar regions was developed here. Further studies will be conducted to optimize TSA-FISH for comparison with standard FISH.

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