Vol. 41: 163–169, 2005 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published November 25

Quantification and distinction of aplastidic and plastidic marine nanoplankton by fluorescence *in situ* hybridization

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ABSTRACT: We developed a protocol for the microscopic detection of nanoplankton by fluorescence *in situ* hybridization (FISH) with 18S rDNA targeting oligonucleotide probes in combination with tyramide signal amplification (TSA). The use of tyramides labeled with a UV-excitable fluorochrome allowed simultaneous identification and classification of the hybridized organisms according to their trophic modes. The protocol was initially validated on pure cultures and was subsequently compared with a standard technique for the enumeration of protists in a time series from North Sea waters. Cell counts with the new protocol were significantly higher for both aplastidic and plastidic nanoplankton, mainly due to superior detection of cells between 2 and 5 µm. FISH-TSA with specific probes for Pedinellales and a group of novel stramenopiles revealed that these lineages of bacterivores were not abundant in coastal North Sea surface waters at the time of investigation. In combination with specific 18S rRNA targeted oligonucleotide probes the new protocol may provide a valuable tool for a simultaneous rapid analysis of the identity and trophic mode of nanoplankton in environmental samples.

KEY WORDS: Aplastidic nanoplankton · Plastidic nonoplankton · Picoeukaryotes · Fluorescence · *In situ* hybridization · Tyramide signal amplification

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INTRODUCTION

Flagellates are commonly the most abundant group of protists in nanoplanktonic communities and hence play a pivotal role in marine and freshwater microbial food webs (Fenchel 1982, Sanders et al. 1989), yet it is difficult to quantitatively investigate aquatic flagellates at an appropriate taxonomic resolution. In ecological studies, flagellates and other protists are frequently quantified by epifluorescence microscopy (Caron 1983, Brandt & Sleigh 2000). However, due to small cell size and sample treatment (e.g. fixation and filtration), hardly any discriminative morphological features of these fragile organisms remain visible in such preparations. Thus, only bulk parameters such as the total abundance of aplastidic (heterotrophic, HNAN) or plastidic (mixo- and autotrophic, PNAN) nanoplankton (2 to 20 µm) are usually determined. This prohibits a more detailed analysis of the role and fate of different key protists in microbial food webs.

The use of molecular markers such as 16S rRNA genes has led to the discovery of numerous new prokaryotic lineages (Giovannoni et al. 1990, Fuhrman et al. 1992, Rappé et al. 1999). Cultivation-independent analyses of the composition of marine prokaryotic communities have been successfully performed by fluorescence *in situ* hybridization with rRNA-targeted oligonucleotide probes (FISH) (Eilers et al. 2001, Morris et al. 2002). Increasing numbers of eukaryotic 18S rRNA gene sequences from marine pelagic habitats have revealed that the large diversity of protists has likewise escaped detection by classical methods (Díez et al. 2001, López-García et al. 2001, Moon-van der Staay et al. 2001, Moreira & López-García 2002,

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Massana et al. 2002). Many of the new lineages are very divergent from cultivated members, and thus it is not possible to infer their biology, e.g. if they feature photosynthetic plastids. Hence, to unveil the ecological role of these organisms in marine food webs, it is essential to simultaneously determine their identity, their type of trophic metabolism, and their *in situ* abundances (Moreira & López-García 2002).

So far, only a few studies have used FISH with 18S rRNA-targeted oligonucleotide probes to identify and enumerate HNAN in marine samples (Lim et al. 1993, 1999, Caron et al. 1999, Massana et al. 2002). In order to routinely apply FISH to protists in ecological studies some difficulties must be overcome. The often weak signal intensities of hybridized marine nano- and picoeukaryotes from purely phototrophic lineages can be significantly enhanced by FISH with horseradish peroxidase (HRP)-labeled oligonucleotides and tyramide signal amplification (TSA) (Not et al. 2002). In the presence of hydrogen peroxide, HRP reacts with the phenolic group of the fluorescently labeled tyramide. The resulting, highly active tyramide radical covalently binds to other electron-rich compartments, e.g. proteins, in the immediate vicinity of the HRP molecule. Because the ribosome-bound enzyme is able to react with copious tyramide molecules, the fluorescence accumulates in the cell and thus enhances the signal manifold (Bobrow et al. 1991).

However, the masking of the chlorophyll *a* autofluorescence in auto- and mixotrophic organisms by commonly used fluorescent dyes remains a problem in assemblages of aplastidic and plastidic nanoplankton and impedes their differentiation.

Here we present a protocol for the rapid quantification of nanoplankton populations by FISH-TSA, also known as CARD-FISH (Pernthaler et al. 2004). With a eukaryotic probe and a UV-excitable fluorochrome ($Alexa_{350}$ it allowed a simultaneous discrimination of aplastidic and plastidic forms. We validated this new method in samples from the coastal North Sea by comparison with a standard technique for the enumeration of protists by epifluorescence microscopy (Sherr et al. 1993). Additionally, we applied 2 group-specific oligonucleotide probes targeting potentially bacterivorous stramenopile flagellates to quantify their *in situ* abundance in coastal North Sea plankton.

MATERIALS AND METHODS

Development of FISH-TSA protocol. Protistan cultures of different trophic modes were obtained from the Culture Collection of Algae and Protozoa (CCAP, UK) and the Center for Culture of Marine Phytoplankton (CCMP, Maine, USA). As reference organisms for heterotrophic flagellates we used *Paraphysomonas imperforata* (CCAP 935/13), *Developayella elegans* (CCAP 1917/1), *Pteridomonas danica* (CCAP 1969/1) and *Cafeteria roenbergensis* (CCAP 1900/1). Autotrophic reference organisms were *Dictyocha speculum* (CCMP 1381) and *Heterosigma akashiwa* (CCMP 452). A representative of a mixotrophic flagellate was *Ochromonas* sp. (CCMP 584).

Protists of different trophic modes were mixed and fixed with particle-free formaldehyde (final concentration 1.8% v/v) at 4°C for a minimum of 1 h and filtered onto white polycarbonate membranes (ATTP, 0.8 µm pore size, 25 mm diameter; Millipore). Filters were hybridized with the HRP-labeled general eukaryotic probe EUK516 (Amann et al. 1990) or the probe NON338. The probe NON338 was originally designed as a negative control probe for bacteria (Wallner et al. 1993), but a BLAST search (Altschul et al. 1997) revealed that it does not target at any known protistan 18S rRNA sequence either. Hence, NON338 was used as a negative control probe for protists in this study.

The hybridization buffer (2 ml) contained 0.9 M NaCl, 0.02 M Tris-HCl of pH 8.0, 20% v/v formamide, 20% v/v blocking reagent (Boehringer: 10% w/v in maleic acid buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]), 0.1% v/v sodium dodecyl sulfate (SDS), and deionized particle-free water (dH_2O). An aliquot was mixed with the oligonucleotide probe (1 ng μl^{-1}) and filters were covered with this mixture. The filters were hybridized in a hybridization chamber containing the remaining hybridization buffer for 2 h at 35°C. Filters were subsequently washed with washing buffer (dH_2O with 0.225 M NaCl, 0.02 M Tris-HCl, 5 mM EDTA, and 0.1% SDS) for 30 min at 37°C. The filters were briefly rinsed twice in dH_2O and equilibrated in TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.05% v/v Tween 20; Bio-Rad Laboratories) for 15 min at room temperature (RT). Moist filters were placed on a glass plate and covered with 50 µl of the tyramide solution (1× phosphate-buffered saline [PBS], 0.0015% v/v H_2O_2, 0.1% v/v blocking reagent and 2% v/v $Alexa_{350}$ tyramide, obtained from Molecular Probes) for 30 min at RT in the dark. Next, filters were briefly rinsed in TNT buffer at RT, transferred to fresh TNT buffer, and washed for 15 min at 55°C. Filters were rinsed again twice in dH_2O, air-dried in the dark, and embedded in a 1:5 mixture of the mounting fluids Vecta Shield (Vecta Laboratories) and Citifluor AF1 (Citifluor) on microscopic slides. Cells were visualized by epifluorescence microscopy at 1000× magnification (Zeiss Axiophot 2 microscope, Carl Zeiss). The blue hybridization signals were detected at UV-excitation (Zeiss Filter Set 01, Carl Zeiss). The autofluorescence of phototrophic cells was detected as dark red signals at UV-excitation, and was visible even more prominently as bright

orange signal at green excitation (Chroma HQ 41007 filter set, Chroma Technical Corporation).

Application of FISH-TSA protocol to environmental samples and comparison with standard enumeration method. North Sea surface water was collected over 10 d during August and September 2002 near the island of Helgoland (German Bight). Samples were fixed as described above. For the determination of total nanoplankton abundances (2 to 20 µm cell size) subsamples of 8 to 10 ml were concentrated on Irgalan Black-stained polycarbonate membranes (0.8 µm pore size, 25 mm diameter) and double-stained with 4′-6 diamidino-2-phenylindole (DAPI, 1 µg ml^{-1} staining time 3 min) and fluorescein isothiocyanate (FITC, 33 µg ml^{-1} staining time 6 min; modified from Sherr et al. 1993). Filters were embedded on microscopic slides and examined by epifluorescence microscopy. Eukaryotic cells were identified by their DAPI-stained nucleus (UV excitation and Zeiss filter set 01) and FITC-stained body outline (blue light excitation and Zeiss Filter Set 09). HNAN and PNAN were distinguished by the possession or lack of plastid autofluorescence as observed predominantly under blue light (orange-red emission), but also under UV excitation (dark red emission); 50 microscopic fields per sample were evaluated. For the FISH procedure, subsamples were processed as described above. We applied 2-sided Wilcoxon-tests for paired samples to test for differences between the 2 methods with respect to total counts of HNAN and PNAN and the relative proportions of PNAN (SPSS10.0, SPSS).

Design and application of group-specific oligonucleotide probes. The oligonucleotide probe PED1646 (5′-TTA CCT TCG ATC GGT AGG-3′, Position 1646, numbered according to the respective nucleotide position in the 18S rRNA of *Saccharomyces cerevisiae*) targeting the lineage Pedinellales/Rhizochromulinales was designed using the software package ARB (Ludwig et al. 2004). The probe accessibility data of Behrens et al. (2003) were used for the selection of the probe target site on the 18S rRNA and the determination of its base position in *S. cerevisiae*. Specificity evaluation against ARB and GenBank database (Altschul et al. 1997) revealed that the probe PED1646 has at least 3 mismatches with non-target sequences. 5′-HRP-labeled probe PED1646 was purchased from ThermoHybaid. Stringent hybridization conditions were established for cultures of *Pteridomonas danica* (CCAP 1969/1, target organism) and *Dictyocha speculum* (CCMP 1381, non-target organism) by concentration series of formamide in the hybridization buffer and subsequent microscopic inspection. An optimal formamide concentration, i.e. the concentration at which all target cells fluoresce brightly while non-target cells are not detectable, was determined as 40% (v/v) formamide in the hybridization buffer. For further details on probe design see, e.g., Pernthaler et al. 2004).

FISH-TSA with the HRP-labeled probes PED1646 and NS4, the latter targeting a subgroup of novel stramenopiles (Massana et al. 2002), was performed as described above using the same set of coastal North Sea samples.

RESULTS AND DISCUSSION

FISH-TSA of protists with $Alexa_{350}$

HNAN and PNAN could be clearly distinguished in all cultures (e.g. Fig. 1a,b) and environmental samples (Fig. 1c–h) by our protocol. Hybridization of protists with the HRP-labeled general eukaryotic probe EUK516 resulted in bright blue signals at UV excitation (Fig. 1a,c,d,g). No positive hybridization signals were detected with the probe NON338. HNAN exhibited blue fluorescence only (e.g. Fig. 1a, cell on right), but no or only a very faint background signal at green excitation (e.g. Fig. 1b, cell on right). PNAN appeared partly blue and red at UV-excitation (e.g. Fig. 1a, cell on left) and exhibited a bright orange fluorescence of the cellular plastids when excited by green light (e.g. Fig. 1b, cell on left). HNAN and PNAN from North Sea samples with cell sizes between 2 and 12 µm are shown in Fig. 1c–h.

The trophic mode of eukaryotic protists is usually distinguished by the possession or lack of plastid autofluorescence (Sieburth et al. 1978, Caron 1983). In our protocol, autofluorescence in phototrophic protists is not lost during the hybridization procedure (Fig. 1). The loss of the autofluorescence during FISH as reported by Lim et al. (1996) might be due to an ethanol treatment to dehydrate the cells. We found that this step is not required for FISH of planktonic protists. However, we observed a shift in the excitation optimum of the autofluorescence to longer wavelengths. Usually, chlorophyll *a* emits orange-red light when excited by blue light. After hybridization we detected autofluorescence as a bright orange signal at green excitation, which might be a consequence of pigment degradation. This shift in excitation and emission wavelengths did not reduce the detectability of phototrophic organisms, as reflected by statistically indistinguishable relative proportions of PNAN in environmental samples when counted by FISH or DAPI/FITC staining (Table 1; average = 83 vs. 82% of total counts, respectively; $p > 0.05$). This also demonstrated that the new protocol was not biased against cells of a certain trophic mode, i.e. the difference between aplastidic and plastidic cells were detected as well as with the standard method.

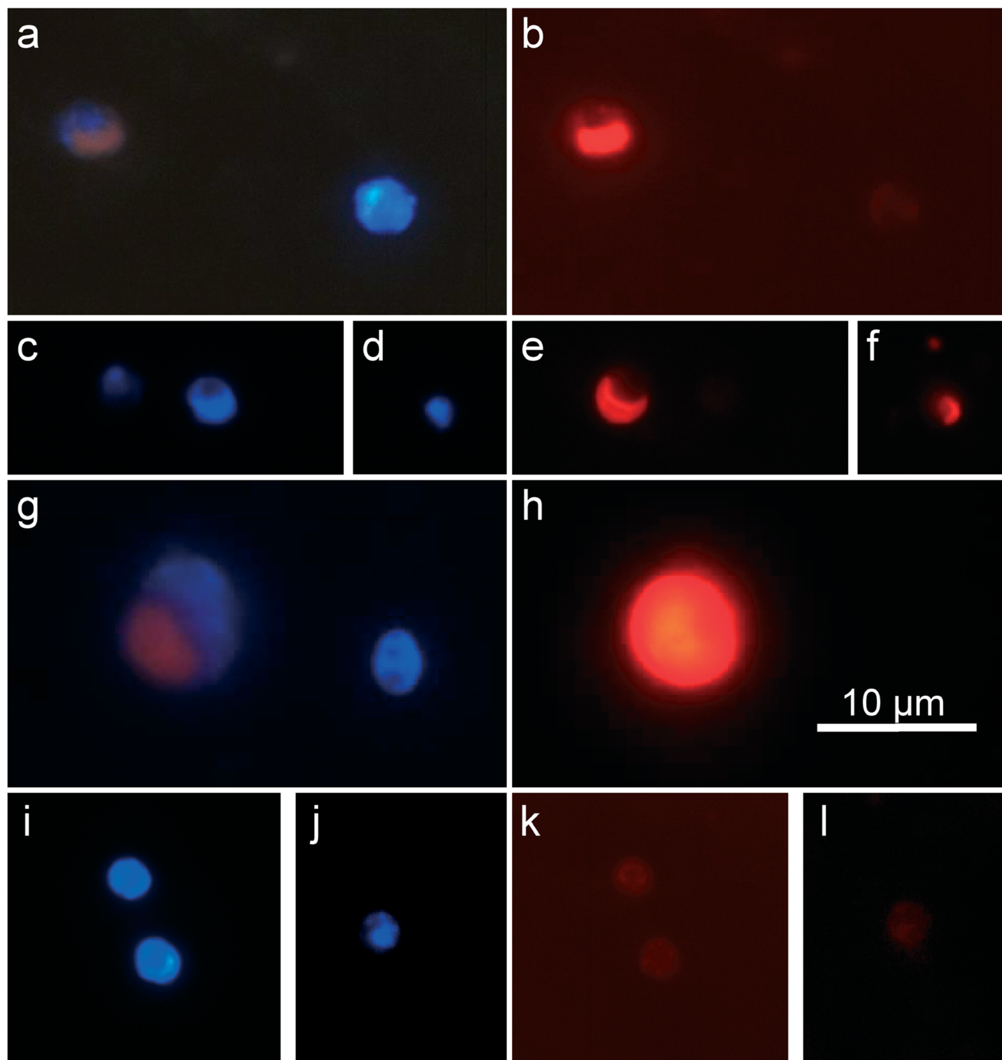

Fig. 1. Epifluorescence microscopy true-color images of nanoplankton from cultures and environmental samples. On left: blue hybridization signals at UV-excitation, with red chlorophyll *a* autofluorescence sometimes visible; On right: orange autofluorescence of chlorophyll *a* of phototrophic organisms at green excitation, with no or only faint background signal from purely heterotrophic cells. Scale bar is valid for all images. Hybridization with Probe EUK516. (a,b) Mixed culture of *Ochromonas* sp. (mixotrophic) and *Paraphysomonas imperforata* (heterotrophic). (c–h) Nanoplankton from North Sea samples. (i–l) Group-specific hybridizations. (i,k) *Pteridomonas danica* (heterotrophic) hybridized with probe PED1646. (j,l) Small eukaryote from North Sea sample (heterotrophic) hybridized with Probe NS4

The sulfoindocyanine dye Cy3 is one of the brightest fluorochromes commercially available, and therefore has been used as a fluorescent label for FISH of marine bacteria or protists (Eilers et al. 2001, Pernthaler et al. 2001, Massana et al. 2002). Unfortunately, the emission of Cy3 and of other dyes with an emission wavelength between 450 and 600 nm overlaps with plastid autofluorescence. Therefore discrimination between hybridized and phototrophic or between hybridized heterotrophic and phototrophic eukaryotic cells is not feasible. The design of oligonucleotide probes that are specific for a functional subgroup, e.g. bacterivorous flagellates, may be impossible for many lineages. Because flagellates are of polyphyletic origin, i.e. belong to many different and separate phylogenetic categories, one cannot design a general probe targeting all flagellates. Moreover, aplastidic and plastidic forms co-occur in closely related species (Cavalier-Smith 2000), e.g. within the ecological important group of marine stramenopiles (Brandt & Sleigh 2000, Massana et al. 2002), which makes it impossible to design a phylogenetically broader probe for a functional guild. Theoretically, species-specific oligonucleotide probes would circumvent these problems (Rice et al. 1997, Caron et al. 1999). However, individual flagellate species may occur at very low *in situ* abundances (Lim

Table 1. Relative proportion of phototrophic nanoplankton as percentage of total nanoplankton abundances determined by DAPI/FITC and EUK516 counts

Date (2002)	DAPI/FITC	EUK516
Aug 19	82	83
Aug 21	89	86
Aug 23	74	85
Aug 26	73	77
Aug 28	69	84
Aug 30	89	91
Sep 02	90	89
Sep 04	78	64
Sep 05	89	81
Sep 06	83	89

et al. 1999). Therefore, such highly specific probes may be an inadequate tool for a rapid analysis of flagellate community composition (Caron et al. 2004).

The UV-excitable fluorochrome $Alexa_{350}$ is the dye with an emission wavelength that is farthest away from that of the photopigment autofluorescence. Thus, a simultaneous detection of the fluorescent staining of hybridized nanoplankton cells and a classification into aplastidic and plastidic forms is possible. Currently the $Alexa_{350}$-labeled tyramides are commercially available only in a rather expensive kit. Therefore, custom synthesis might be a cheaper alternative (Hopman et al. 1998, Pernthaler et al. 2004).

Comparison with standard enumeration method

The abundances of total nanoplankton (2 to 20 µm) in our samples ranged between 1.9×10^3 to 6.3×10^3 cells ml^{-1} and 3.4×10^3 to 8.4×10^3 cells ml^{-1} with the standard staining technique and the new protocol, respectively. HNAN accounted for 3.8×10^2 to 9.2×10^2 cells ml^{-1} with the standard technique and for 6.3×10^2 to 1.3×10^3 with the new protocol (Fig. 2a). PNAN abundances were 1.4×10^3 to 5.6×10^3 cells ml^{-1} and 2.2×10^3 to 7.1×10^3 cells ml^{-1} with the standard and new protocol, respectively (Fig. 2b). These cell counts accorded well with abundances given for similar coastal habitats in the North Sea (Fenchel 1982, Geider 1988, Nielsen & Richardson 1989, Brandt & Sleigh 2000), the Mediterranean (Massana et al. 2002) and the NW Atlantic (Lim et al. 1996, Sanders et al. 2000).

Quantitative comparison between FISH-TSA and DAPI/FITC staining revealed that cells were not lost due to the hybridization procedure. On the contrary, the detection rate of nanoplankton with the new protocol was higher than the counts obtained with DAPI/FITC-stained cells in most samples (Fig. 2). Statistical analysis showed that these differences were significant for both, HNAN ($p = 0.022$) and PNAN ($p = 0.013$). The higher abundances could be mainly attributable to a population of small aplastidic or plastidic cells of approximately 2 to 3 µm in diameter (Fig. 1d,f). In fact, even a population of cells <2 µm could be detected by FISH, which accounted for 7.16×10^2 to 2.53×10^3 cells ml^{-1}. However, such cells were not included in the quantitative comparison because they could not be unambiguously identified by the DAPI/FITC method (i.e. distinguished from bacteria).

Picoeukaryotes, i.e. cells with a size of ≤2 µm (Moreira & López-García 2002), are an important fraction of the plankton community in the open ocean (Díez et al. 2001). Such small eukaryotes can be easily overlooked, or they can be mistaken for bacteria when stained with general dyes such as DAPI for DNA and FITC for proteins. Lim et al. (1996) also reported higher total nanoplankton abundances with biotinylated EUK-probes due to the better detection of cells attached to particles and of small sizes. FITC has the advantage of staining the whole cell and flagella, if pre-

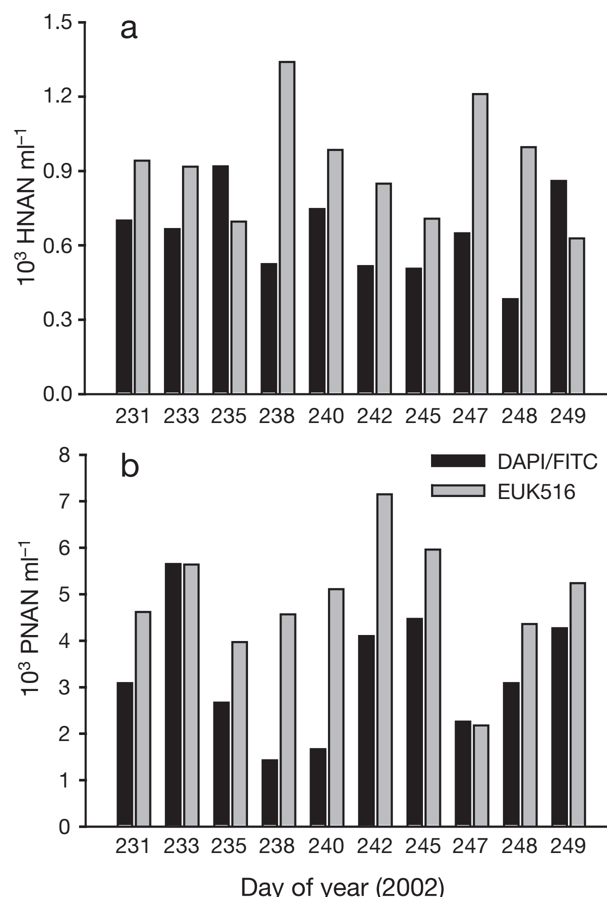

Fig. 2. Cell counts of (a) aplastidic (heterotrophic, HNAN) and (b) plastidic (mixo- and autotrophic, PNAN) nanoplankton (2 to 20 µm) in North Sea samples using 2 different methods, DAPI/FITC and eukaryotic probe EUK516

sent, thus permitting a better discrimination of picoeukaryotes from prokaryotes than by DAPI-staining only. However, FITC might also mask the weak autofluorescence signals of cells with low chlorophyll *a* content, and complicate the classification of the trophic mode. FISH-TSA with the fluorochrome $Alexa_{350}$ may help to eliminate these problems because (1) oligonucleotide probes can be selected that are specific for the phylogenetic lineages of interest (i.e. the eukaryotic probe will not detect bacteria) and (2) the fluorochrome is not excited by blue or green light (i.e. will not mask even low amounts of chlorophyll autofluorescence).

Although the abundances obtained with the new protocol were significantly higher than with the standard technique, they were still in the same order of magnitude. This difference alone would not justify the effort and higher costs of FISH with tyramide signal amplification. However, our approach might help to open the 'black box' of the community composition of planktonic pico- and nanoeukaryotes, and to distinguish between individual phylogenetic lineages of predators, mixotrophs or primary producers in the marine water column.

Probe design and detection of targeted protists in environmental samples

An important task in aquatic microbial ecology is to identify the phylogenetic groups of nanoflagellates that control bacterial populations in the ocean. Pedinellids such as *Pteridomonas* spp. and *Actinomonas* spp. are bacterivorous flagellates that are commonly enriched in marine samples (Vørs 1992). However, little is known about their *in situ* abundance and their potential impact within marine microbial food webs. Therefore, we designed an oligonucleotide probe that is specific for the lineages Pedinellales/Rhizochromulinales, a subgroup of the Dictyophyceae within the Heterokonta (stramenopiles). A taxonomic re-evaluation and phylogenetic classification of this group is given by Sekiguchi et al. (2003).

The probe PED1646 was successfully tested on selected cultures (Fig. 1i,k). However, pedinellids and rhizochromulinids were not detected in coastal North Sea surface waters during the period August and September 2002. Members of a lineage of uncultured, bacterivorous novel stramenopiles as detected by the probe NS4 (Massana et al. 2002) were occasionally found in these samples (Fig. 1j,l). These organisms constituted <1 % of the total aplastidic nanoplankton, i.e. <10 cells ml^{-1}, on average. The lack of autofluorescence in these cells supports experimentally derived conclusions that the NS4 organisms were purely heterotrophic (Massana et al. 2002).

CONCLUSIONS

FISH-TSA with 18S rRNA targeted oligonucleotide probes is sufficiently sensitive to quantitatively detect phylogenetic lineages of small aplastidic and plastidic protists in environmental samples. The UV-excitable fluorescent dye $Alexa_{350}$ does not overlap with the autofluorescence of plastids, and therefore allows a simultaneous identification of nanoplankton cells with specific FISH probes and a classification according to the cell's trophic mode (heterotrophic versus phototrophic). Moreover, by combining this FISH staining approach with uptake experiments of fluorescently labeled bacterial prey (Massana et al. 2002), the bacterivory of different mixotrophic protists might be studied. Our protocol could thus be a valuable direct method for the identification and quantification of potential key players in marine microbial food webs with defined ecological functions.

Acknowledgements. We thank G. Gerdts for hosting us in his laboratory, M. Schattenhofer for technical assistance, and A. Purdy for critical reading of the manuscript. This work was supported by the European Union (EVK3-2001-00194 BASICS) and the Max Planck Society.

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Editorial responsibility: Karel Šimek,
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Submitted: May 6, 2005; Accepted: August 3, 2005
Proofs received from author(s): October 25, 2005