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An In Situ Hybridization Protocol for Detection and Identification of Planktonic Bacteria

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Summary

The fraction of planktonic bacteria in oligo- and mesotrophic lakes that can be classified by in situ hybridization has been significantly increased with an optimized protocol. By the use of white polycarbonate filters, CY3-monolabeled oligonucleotide probes, and high quality fluorescence filter sets between 29 and 64% of the cells visualized by 4′,6-diamidino-2-phenylindole (DAPI) can be detected with a bacterial probe or group-specific probes by epifluorescence microscopy without further digital image enhancement.

Key words: In situ hybridization – Planktonic bacteria – Epifluorescence microscopy – Oligonucleotide probe – vRNA – CY3

There is a clear need for in situ identification of microorganisms in aquatic samples. The currently available techniques for analyzing structures and dynamics of aquatic microbial communities have severe limitations. The cultivation based techniques frequently suffer from low detection rates (*Ferguson* et al., 1984, *Jones,* 1977, *Kogure* et al., 1979, *Staley* and *Konopka*, 1985) and bad representation of true community structure (*Wagner* et al., 1993). Although improved techniques like the recently described dilution culture (*Schut* et al., 1993) might yield higher culturability the isolation of pure cultures remains slow and the subsequent identification cumbersome. Without cultivation total cell counts in water samples are today routinely determined by the combination of membrane filtration, staining with dyes specific for nucleic acids and epifluorescence microscopy (*Kepner* and *Pratt*, 1994). Only few (groups of) microorganisms can be directly identified in situ due to sufficient morphological detail or typical autofluorescence. Since the microscopic uniformity of microorganisms is in sharp contrast to their genetic diversity in situ hybridization with nucleic acid probes should facilitate their identification. The usefulness of the most common probe type, fluorescently labeled, rRNA-targeted oligonucleotides (*Amann* et al., 1990b, *DeLong* et al., 1989), has already been demonstrated for planktonic bacteria from eutrophic freshwater samples (*Hicks* et al., 1992, *Wagner* et al., 1993). In meso- and oligotrophic lakes, however, detection of planktonic bacteria with standard procedures (*Amann* et al., 1990b) was usually in the range of few percent or below (*Amann* et al., 1995). In the following we describe an improved protocol for the in situ identification of bacterioplankton in water samples.

Sequences and references for all oligonucleotide probes used in this study are given in Table 1. 5′-aminolinked oligonucleotides (MWG Biotech, Ebersberg, Germany) were labeled with the N-hydroxysuccinimidester of the carbocyanine dye CY3 (exitation, emission maxima in PBS buffer of 552 and 568 nm, respectively; Biological Detection Systems, Pittsburg, USA) using the standard protocol (*Amann* et al., 1990b).

Using a glass filter tower (GV025; Schleicher & Schuell, Dassel, Germany) cells are concentrated from lake water samples (1–5 ml) on white polycarbonate filters (diameter 25 mm, pore size 0.2 μm; type GTTP 2500; Millipore, Eschborn, Germany) which are placed on nitrocellulose support filters (25 mm; 0.45 μm; Sartorius, Göttingen, Germany) by applying a vacuum of < 500 mbar. They are subsequently fixed by overlaying the filter with 3 ml of a freshly prepared, PBS-buffered (pH 7.2) 4% paraformaldehyde (Sigma, Deisenhofen, Germany) solution for 30 min at room temperature. The fixative is removed by applying vacuum and the filter is subsequently overlayed with 3 ml each of PBS buffer and distilled water. Both are

Table 1. Oligonucleotide probes

Probe	Sequence (5'-3')	rRNA target pos.	Specificity	References
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338–355	Bacteria	*Amann* et al., 1990a
BET42a	GCCTTCCCACTTCGTTT	23S, 1027–1043	beta-subclass Proteobacteria	*Manz* et al., 1992
GAM42a	GCCTTCCCACATCGTTT	23S, 1027–1043	gamma-subclass Proteobacteria	*Manz* et al., 1992
NON338	ACTCCTACGGGAGGCAGC	–	negative control	*Wallner* et al., 1993

immediately removed by applying a vacuum. Alternatively, the water samples can be fixed before filtration by addition of one tenth volume of formalin (37% formaldehyde solution, Merck, Darmstadt, Germany) at the site of sampling. They should be further processed within a day as described above. Air-dried filters are ready for hybridization and can be stored at −20 °C or room temperature for several weeks without apparent changes. Each filter is cut in four sections which are placed on glass slides. Filter sections are overlayed with 20 µl of hybridization solution containing 0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 35% formamide, 0.01% SDS, and 50 ng of CY3-labeled oligonucleotide and incubated at 46 °C for 90 min in an equilibrated chamber. The filter is transferred to a prewarmed (48 °C) vial containing 50 ml of washing solution (70 mM NaCl, 20 mM Tris/HCl (pH 7.4), 5 mM EDTA, and 0.01% SDS) and incubated freely floating without shaking at 48 °C for 15 min. Due to firm adhesion to polycarbonate filters only approximately 10% of cells are lost during hybridization and washing. This was shown by comparative DAPI counting of parallel filters (N = 8; 89.5% +/− 6.2%). Filter sections are dried on Whatman 3M paper (Whatman Int. Ltd., Maidstone, UK), placed back on a glass slide, and overlayed with 50 µl of DAPI solution (1 mg/ml in 0.2 µm filtered, distilled water) for 8 min at room temperature in the dark. Subsequently, they are gently washed in 50 ml of 0.2 µm filtered, distilled water, dried on Whatman 3M paper, and mounted on glass slides with Citifluor AF1 (Citifluor Ltd., Canterbury, UK). Cells were viewed using an Axioplan epifluorescence microscope (Zeiss, Jena, Germany) equipped with a 50 W mercury high pressure bulb and specific filter sets (DAPI: Zeiss 01; CY3: Chroma HQ 41007, Chroma Tech. Corp., Brattleboro, VT, USA). Color photomicrographs were done on Kodak Ektachrome 1600 (Rochester, NY, USA). Exposure times were 4 s for DAPI and 15 s for CY3. Each microscopic field was first viewed with the CY3 filter set before switching to the DAPI filter set to avoid bleaching of CY3 during DAPI examination.

Using this protocol 29 to 64% of the cells visualized by DAPI in several oligo- and mesotrophic lakes could be detected with the CY3-labeled bacterial probe EUB338 (Table 2). Fluorescent signals were readily visible and could be documented with a relatively short exposure time of 15 s on a 1600 ASA diapositive film (Fig. 1). The high quality CY3 filter set, consisting of a 535/50 nm exciter, a 565 nm long pass dichroic mirror, and a 610/75 nm emission filter, visualizes probe-conferred CY3 in bright yellow from which red background signals originating from debris and algae can be readily distinguished (Fig. 1). Since yellow autofluorescence can not be ruled out in environmental samples it is, however, necessary to perform hybridizations with a negative control, e. g. probe NON338 which is complementary to probe EUB338 but not to rRNA. This negative control usually yielded values of 1% or less of the total cell counts except for the samples from Lago Cadagno which were exceptionally rich in autofluorescent bacteria and algae (Table 2). This constantly

Table 2. DAPI and in situ hybridization counts

Lake	Date of Sampling	Depth [m]	Trophic state[5]	P-conc.[6] [µgl^{-1}]	Total cell counts [$\times 10^6$ ml^{-1}]	Fraction [%] of total cells detected with probe			
						EUB338[7]	BET42a[7]	GAM42a[7]	NON338
Gossenköllesee[1]	May 1995	3	oligotrophic	3	0.4 (±0.01)	64 (±1)	19 (±10)	0 (±1)	n.d.
Piburger See[2]	April 1995	3	oligotrophic	8	2.0 (±0.3)	52 (±8)	11 (±4)	1 (±1)	n.d.
Herrensee[3]	July 1995	2	oligotrophic	3–12	1.6 (±0.2)	29 (±9)	6 (±2)	2 (±1)	0 (±0)
Großer Schwaigsee[3]	July 1995	2	mesotrophic	25–30	2.2 (±0.3)	46 (±9)	26 (±7)	0.3 (±1)	1 (±1)
Großer Ostersee[3]	July 1995	2	mesotrophic	20–25	2.6 (±0.2)	60 (±7)	13 (±2)	0.4 (±1)	1 (±1)
Lake Cadagno[4]	August	6	mesotrophic	20–30	3.2 (±0.7)	46 (±9)	21 (±4)	0 (±3)	7 (±3)
	1995	13	eutrophic	n.d.	4.2 (±0.7)	61 (±7)	17 (±3)	5 (±3)	5 (±1)

[1] Gossenköllesee is a high mountain lake in the Austrian Alps (2417 m a.m.s.l.)
[2] Piburger See is a small lake located in the Eastern Alps in Tyrol, Austria. The elevation is 913 m a.m.s.l.
[3] The Lakes belong to the Osterseen Group, which are located in Southern Bavaria, Germany et elevation 594–601 m a.m.s.l.
[4] Lake Cadagno is at 1923 m a.m.s.l. in the Piora Valley in the south of Switzerland
[5] According to *Vollenweider*, 1986
[6] Total phosphorus concentration as determined by the method of *Murphy* and *Riley*, 1962
[7] Numbers have been corrected by substracting NON338 counts

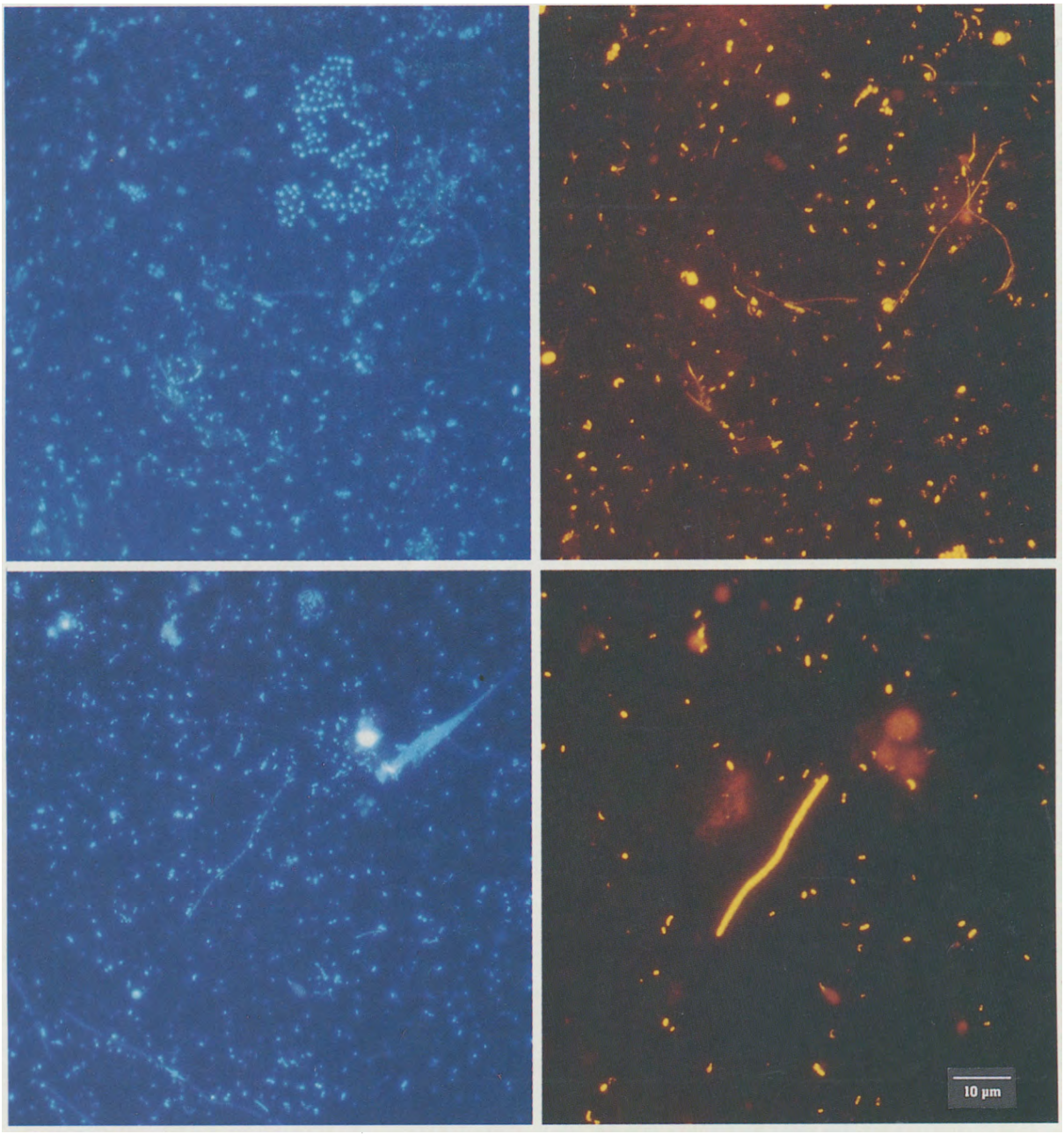

Fig. 1. In situ identification of bacteria in a water sample from Piburger See by a combination of hybridization with CY3-labeled, rRNA-targeted oligonucleotides and DAPI staining. Identical microscopic fields have been visualized with an epifluorescence microscope using filter sets specific for DAPI (left) and CY3 (right). Panel A. Hybridization with probe EUB338 specific for Bacteria. Panel B. Hybridization with probe BET42a specific for beta-subclass Proteobacteria. Bar, 10 µm (applies to all panels).

stratified mesotrophic alpine lake is subdivided in a sulphate-rich anoxic monimolimnion and an aerobic mixolimnion. The chemocline at a depth of 13 m was rich in autofluororescent Chromatiaceae.

In order to demonstrate the feasibility of further classification of the detected cells we applied oligonucleotide probes specific for beta- and gamma- subclass of Proteobacteria (*Manz* et al., 1992). Clearly separated cell populations were identified. Whereas between 6 and 26% of the DAPI stained cells hybridized with probe BET42a, usually less than 2% of the total cells were detected with probe GAM42a. The only exception was the sample taken from the chemocline of Lago Cadagno in which the abundant *Chromatiaceae* were correctly identified by probe GAM42a. This again demonstrates that in contrast to the results of cultivation-dependent community structure analyses, beta-subclass Proteobacteria are more abundant than gamma-subclass Proteobacteria in many aquatic samples (*Wagner* et al., 1993).

Explanations for the considerably increased detection rates with the new protocol in oligo- and mesotrophic lakes are the following: (i) the very photostable indocar-

bocyanine dye CY3 (*Southwick* et al., 1990) is significantly brighter than other fluorophores such as Texas Red, tetramethylrhodamine or fluorescein (*Wessendorf* et al., 1992). (ii) Due to its high hydrophilicity as compared to, e. g., the triphenylmethane dyes fluorescein and tetramethylrhodamine CY3 shows the lowest level of nonspecific binding (*Wessendorf* et al., 1992) further increasing its advantage in the most critical signal-to-noise ratio. (iii) With relative broad excitation and emission windows and consequently high fluorescence yields the CY3 filter set is optimized for the human eye with its ability for color discrimination and limited sensitivity. (iv) The dye Irgalan-Black commonly used to reduce the background of polycarbonate filters in total cell count determinations with DAPI or acridine orange (*Hobbie* et al., 1977) caused increased background on filters hybridized with CY3-labeled probes. Unstained white polycarbonate filters showed significantly less background fluorescence with the CY3 filter set when mounted in Citifluor. At the same time the autofluorescence with the standard Zeiss DAPI filter set was not at a level preventing the detection of even very small cells. Recently we observed that the DAPI background on white polycarbonate filters can be effectively reduced by using a modified filter set (Chroma # 3 1000, 360/40 nm exciter, 400 nm long pass dichroic mirror, 460/50 nm emission filter; Chroma Techn. Corp.).

In conclusion, the new protocol considerably broadens the applications of in situ hybridization with fluorescently labeled, rRNA-targeted oligonucleotide probes for a cultivation-independent in situ monitoring of microbial populations in aquatic samples. With its increased sensitivity this single cell identification protocol does not require the use of multiple probes with identical specificity (*Hicks* et al., 1992, *Lee* et al., 1993) which might be difficult to find for defined groups or species of bacteria. We anticipate that this protocol may also help to improve detections rates of microorganisms in other difficult samples such as sediments or soils.

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References

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., Stahl, D. A.: Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. *56,* 1919–1925 (1990a)

Amann, R. I., Krumholz, L., Stahl, D. A.: Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. *172,* 762–770 (1990b)

Amann, R. I., Ludwig, W., Schleifer, K.-H.: Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. *59,* 143–169 (1995)

DeLong, E. F., Wickham, G. S., Pace, N. R.: Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells. Science *243,* 1360–1363 (1989)

Ferguson, R. L., Buckley, E. N., Palumbo, A. V.: Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. *47,* 49–55 (1984)

Hicks, R., Amann, R. I., Stahl, D. A.: Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. *58,* 2158–2163 (1992)

Hobbie, J. E., Daley, R. J., Jasper, S.: Use of Nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. *33,* 1225–1228 (1977)

Jones, J. G.: The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. Freshwater Biology *7,* 67–91 (1977)

Kepner, R. L., Jr., Pratt, J. R.: Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol. Rev. *58,* 603–615 (1994)

Kogure, K., Simidu, U., Taga, N.: A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. *25,* 415–420 (1979)

Lee, S., Malone, C., Kemp, P. F.: Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Mar. Ecol. Prog. Ser. *101,* 193–201 (1993)

Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.-H.: Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. *15,* 593–600 (1992)

Murphy, J., Riley, J. P.: A modified solution method for the determination of phosphate in natural waters. Analyt. Chem. Acta *27,* 31–36 (1962)

Schut, F., DeVries, E. J., Gottschal, J. C., Robertson, B. R., Harder, W., Prins, R. A., Button, D. K.: Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. *59,* 2150–2160, (1993)

Southwick, P. L., Ernst, L. A., Tauriello, E. W., Parker, S. R., Mujumdar, R. B., Mujumdar, S. R., Clever, H. A., Waggoner, A. S.: Cyanine dye labeling reagents – carboxymethylindocyanine succinimidyl esters. Cytometry *11,* 418–430 (1990)

Staley, J. T., Konopka, A.: Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Ann. Rev. Microbiol. *39,* 321–346 (1985)

Vollenweider, R. A.: Scientific fundamentals of eutrophication of lakes and flowing waters with special reference to phosphorus and nitrogen. OECD/DAS/CSI *68.27,* 1–159 (1968)

Wagner, M., Amann, R., Lemmer, H., Schleifer, K. H.: Probing activated sludge with proteobacteria-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. *59,* 1520–1525 (1993)

Wallner, G., Amann, R., Beisker, W.: Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry *14,* 136–143 (1993)

Wessendorf, M. W., Brelje, T. C.: Which fluorophore is brightest? A comparison of the staining obtained using fluorescein, tetramethylrhodamine, lissamine rhodamine, Texas Red, and cyanine 3.18. Histochemistry *98,* 81–85 (1992)

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