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Original Research

Distribution of Microbial-Selected Populations in Lake North Mamry by Fluorescent *in situ* Hybridization

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Abstract

Fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has become one of the major techniques in environmental microbiology, allowing rapid and reliable definition of prokaryotes and quantification of population sizes. The aim was to demonstrate the applicability of the FISH method to study bacterioplankton composition in North Mamry Lake, and to follow the dynamics of two populations of common bacteria. We analyzed the phylogenetic composition of free-living bacterioplankton assemblage using oligonucleotide probes specific for *Bacteria* as well as for β-*Proteobacteria* and *Cytophaga-Flavobacterium* groups. Up to 53% of bacteria detected with DAPI could be detected via FISH by applying the universal bacterial probe for domain *Bacteria* (Eub338). Percentage of *Cytophaga-Flavobacterium* cluster did not exceed 20%. Members of the β –*Proteobacteria* appeared to be the most abundant group.

Keywords: rRNA-targeted oligonucleotide probes, fluorescent in situ hybridization, bacterial community composition

Introduction

Complex microbial communities carry out various environmental processes which are essential to maintaining ecosystems. The detection, identification and isolation of a wide range of microorganisms from the environment are integral parts of microbial ecology studies aimed at the role of prokaryotes in ecosystem functioning. Conventional cultivation of microorganisms is selective and is biased towards the growth of specific bacteria, resulting in an incomplete picture of the real community composition [42, 13]. Most of the cells visualized by microscopy are viable, but they could not form visible colonies on plates [29]. Usually less then one percent can be isolated on artificial media, very few microorganisms from the aquatic community have been described and the remaining 90% could only be found using cultivation-independent molecular tools [3]. Since the mid 1980s various approaches based on molecular biological methods have been used to identify microorganisms in natural samples [44]. There are several reviews [4, 19, 43] of the application of molecular techniques in various ecosystems. The whole-cell hybridization technique with fluorescently rRNA-targeted oligonucleotide probes (FISH) [10, 15, 2] has been widely used to directly identify specific bacteria at the single-cell level within complex samples. Traditionally FISH has been performed with DNA oligonucleotide probes. Such probes are usually 15-25 nucleotides long attached to a fluorochrome at the 5'end and target ribosomal RNA-small subunit rRNA (16S rRNA) or large subunit rRNA (23S rRNA). rRNA is an ideal target because it is present in all living cells in high copy numbers, 10^5 per metaboli-

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cally active bacterial cell [2], and it is relatively stable, permitting identification of individual cells. Only targeted cells, which contain the signature sequence on the rRNA, are stained. So far, several hundred rRNA-targeted specific oligonucleotide probes have been described with an online database [23]. The most frequently used dyes are green fluorescein, red tetrametylrhodamine and the indocarbocyanine dyes Cy3. The signal of hybridized cells can be monitored either by epifluorescence microscopy, confocal microscopy [25] or flow cytometry [2]. FISH allows us to address various ecological issues

i) to bypass cultivation problems,

ii) to identify and to enumerate prokaryote sub-populations in natural systems and analyze their spatial distribution and

iii) to obtain together with biomass determination structural insight in mixed-population communities [42, 3, 1, 31, 35].

However, in some cases a direct microscopic visualization of bacteria by FISH with monolabeled probes remains difficult. In some cases a relatively large fraction (<50%) of cells cannot be visualized after hybridization due to the small bacteria size, small ribosome content or low cell permeability [38]. This appears to be a serious limitation of the method. During the past decade the FISH technique has been continuously improved to increase the sensitivity of the method. Preincubation with chloramphenicol [30] using PNA probes [46], polyribonucleotide probes [33] and helper probes [14], and hybridization with more than one fluorescently labeled oligonucleotide probe [11] have made the method relatively rapid and widely applied for identification and quantification of bacteria in mixed microbial communities [6, 43]. Recently CARD-FISH protocol, the novel catalyzed reporter deposition, was developed for microbial community analysis [34] resulting in increased sensivity without reducing specifity. This approach permits the detection of small bacteria with low ribosome content. It is based on hybridization with HRP-labeled oligonucleotide probes and subsequent tyramide signal amplification [7, 39]. Using the improved CARD-FISH technique, Sekar and coworkers [40] successfully permeabilized the cell walls of *Actinobacteria*. This bacterial lineage in freshwater samples range from 32 to >55% of all DAPI-stained cells and was a numerically important *Bacteria* of picoplankton.

Here we analyzed the distributions of microbial selected populations in Lake North Mamry (Great Masurian Lakes) by FISH providing a first estimate of bacterial community composition within this freshwater system.

Materials and Methods

North Mamry Lake is a typical postglacial, dimictic mesotrophic lake with marked summer and winter stratification. Background information on the main limnological characteristics is found in other publications [22.9]. Duplicate samples from surface water (0.5 m depth) were sampled between April 2002 and February 2003 at approximately three-month intervals in a sterile screw-topped flask (SCHOTT, Germany). Samples for community analysis (5 to 10 ml) were fixed with freshly buffered prepared paraformaldehyde (PFA, pH-7.4) to a final concentration of 2% [vol/vol] and stored for several hours at 4°C. Then the samples were filtered through white polycarbonate filters (type GTTP; pore size, 0.2µm diameter, 47 mm; Millipore, Eschborn, Germany), rinsed twice with 5 ml of sterile water, dried at room temperature and stored at -20°C. Additionally, samples for determining bacterial abundance were fixed with formalin (1% final concentration [wt/vol]) and stored at 4°C until processed further. Total bacterial numbers were determined after filtration of sub samples (2 ml) through black membrane filters (type GTTP; pore size, 0.2µm; diameter 25 mm; Millipore, Eschborn, Germany) and staining with 4.6-diamidino-2-phenylindole (DAPI), final concentration 0.1µg ml^{-1} [37]. Whole-cell in situ hybridization of sections from the polycarbonate filters were performed with the oligonucleotide probes Eub338 [2], Non 338 [44], Bet 42a [24], unlabeled Gam42a as a competitor for Bet42a probe [24], and Cf319a [25] as described previously by Pernthaler et al. [32] The probe sequences, hybridization conditions and references are given in Table 1. Oligonucleotides labeled with the cyanine dye Cy3 were synthesized by Interactiva (Ulm, Germany). After FISH the filters were air dried and mounted on glass slides in a previously described mix amended with DAPI, final

Table 1. Oligonucleotide probes used in this study.

Probe	Specifity	Sequence (5' – 3') of probe	Target site[a] (rRNA position)	%FA[b] in situ	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S (338-335)	35	2
NON388	control	ACTCCTACGGGAGGCAGC	16S (338-335)	35	44
CF319a	Cytophaga-Flavobacterium cluster	TGGTCCGTGTCTCAGTAC	16S (319-336)	35	24
BET42a	Beta sublass of *Proteobacteria*	GCCTTCCCACTTCGTTT	23S (1027-1043)	35	23
GAM42a	gamma-competitor	GCCTTCCCACATCGTTT	23S (1027-1043)	35	23

[a]16S rRNA *Escherichia coli* numbering [b]Percentage of formamide (FA) in in situ hybridization bufer

concentration 1µg ml^{-1} [33]. Bacterial cells on the filter sections were observed with an Axioplan epifluorescence microscope (Zeiss, Jena, Germany) equipped with filter sets for DAPI [Zeiss 01], Cy3 [chroma HQ 41007; Chroma Tech, Corp.] and a 100x Plan Apochromat objective. The fractions of FISH-stained bacteria in at least 1.000 DAPI-stained cells per sample were quantified.

Statistic

Duplicates samples from the lake were taken in order to determine variability of DAPI counts. Probe-specific cell counts are presented as a percentage of cells visualized by DAPI, and the mean abundances and standard deviations were calculated. For the comparison of percentage of hybridized cells at different sampling dates, analysis of variance on ranks by Duncan's post-hoc comparisons was applied using Statystica 7.0.

Results and Discussion

Bacterial in situ identification appears to describe supplementary features of microbial communities. The group-specific rRNA-targeted oligonucleotide probes have proved to be a sufficient tool for characterizing bacterial community composition in various aquatic environments [3]. Our results from FISH with fluorescently monolabeled (5'-Cy3) oligonucleotide probes indicated that in the surface layer on average 53.0% (±2.27) to 42.7% (± 9.67) of all DAPI stained cells hybridized with Eub338 probe during spring thermal mixing (April) and summer stagnation (September) (Fig. 1). In fall and winter samples the percentage rates were even lower and accounted on average for 41.2 to 31.0% of bacterial count stain cells. A similar range of values has been reported from different environments [17, 26, 8, 12, 21]. The numbers of cells hybridizing the Domain *Bacteria* showed a seasonal pattern. The visualization of resident bacteria hybridizing with Eub338 and other probes is shown in Fig. 2. The lower percentage of Eub in winter could be linked to environmental factors (i.e. temperature). In FISH, visualization with the oligonucleotide probes is dependent on the quantity of ribosomes in the cell. The relationship between cell activity and temperature is a likely cause of the observed *Bacteria* results.The percentage of Eub338 peaked in spring and summer and perhaps input of organic matter stimulated bacterial growth and activity. The generally low percentage of Eub338 could be caused by the high percentages of *Actinobacteria* that are more difficult to be hybridized by monolabeled FISH. Actinobacterial abundances in the studied lake as detected by CARD-FISH, a more sensitive technique for microbial community analysis [39, 40], range from 15.4 to 29% (Skowrońska et al. in preparation). In our investigation β-*Proteobacteria* (probe Bet42a) represented a large fraction of bacterioplankton in the examined lake. The highest density was observed in spring thermal mixing mean, 17.4% (± 1.7) or 1.6 x 10^6 cells in ml^{-1}. The ratio of β-*Proteobacteria* ranged from 6 to 19% DAPI stained cells, with distinct maxima in spring. This supports other findings on the spread occurrence of this group in the plankton of lakes, rivers and reservoirs [1, 26, 17, 20]. There was a statistically significant difference with detection of beta proteobacteria in spring samples ($p<0.004$) compared to the other study period (Fig. 1). Pernthaler et al. [31] observed the highest biomass of this group in high mountain lakes after ice break, suggesting that they are the first, compared to other groups, to respond to thermal mixing and the allochtonous input of nutrients. A few years later Glöckner et al. [17] studied this lake and hypothesized that β-*Proteobacteria* were able to rapidly utilize the main annual input of organic carbon. Bouvier and Del Giorgio [8] observed a highly significant relationship between this group of bacteria and the DOC concentration, suggesting that the distribution of this lineage may be linked to DOC. Members of *Cytophaga-Flavobacteria* (CF) phylotypes (Cf319a probe) are usually found in all marine and freshwater samples [16, 8]. Most bacteria belonging to this group are chemoorganotrophic to be proficient in degrading various biopolymers such as cellulose and chitin [24]. The abundance of these bacteria in free-living assemblages is due to release from detritus-

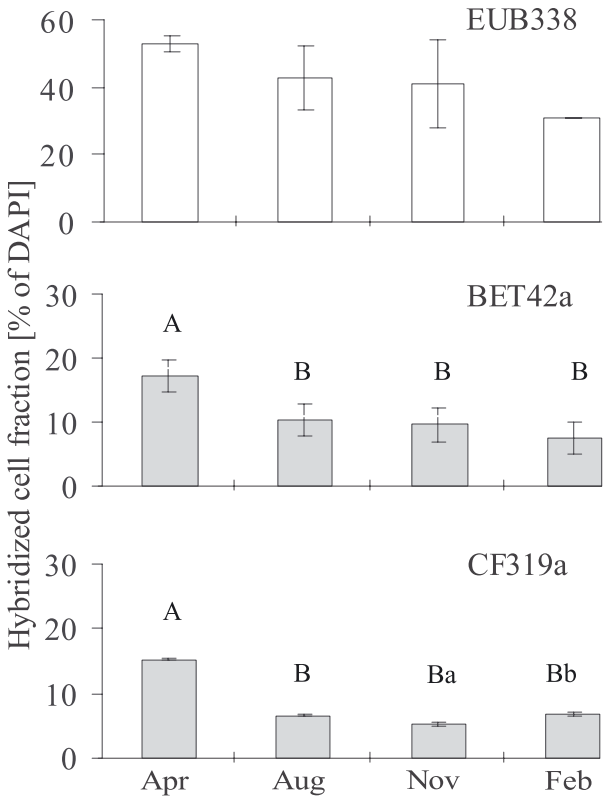

Fig. 1. Taxonomic composition of the bacterial communities as analyzed by FISH. Values are expressed as percentage of hybridized cell counts of total counts of DAPI-stained cells. Error bars indicate the range of duplicates. Values with different letters differ at: A, B – $p<0.01$, a, b – $p<0.05$.

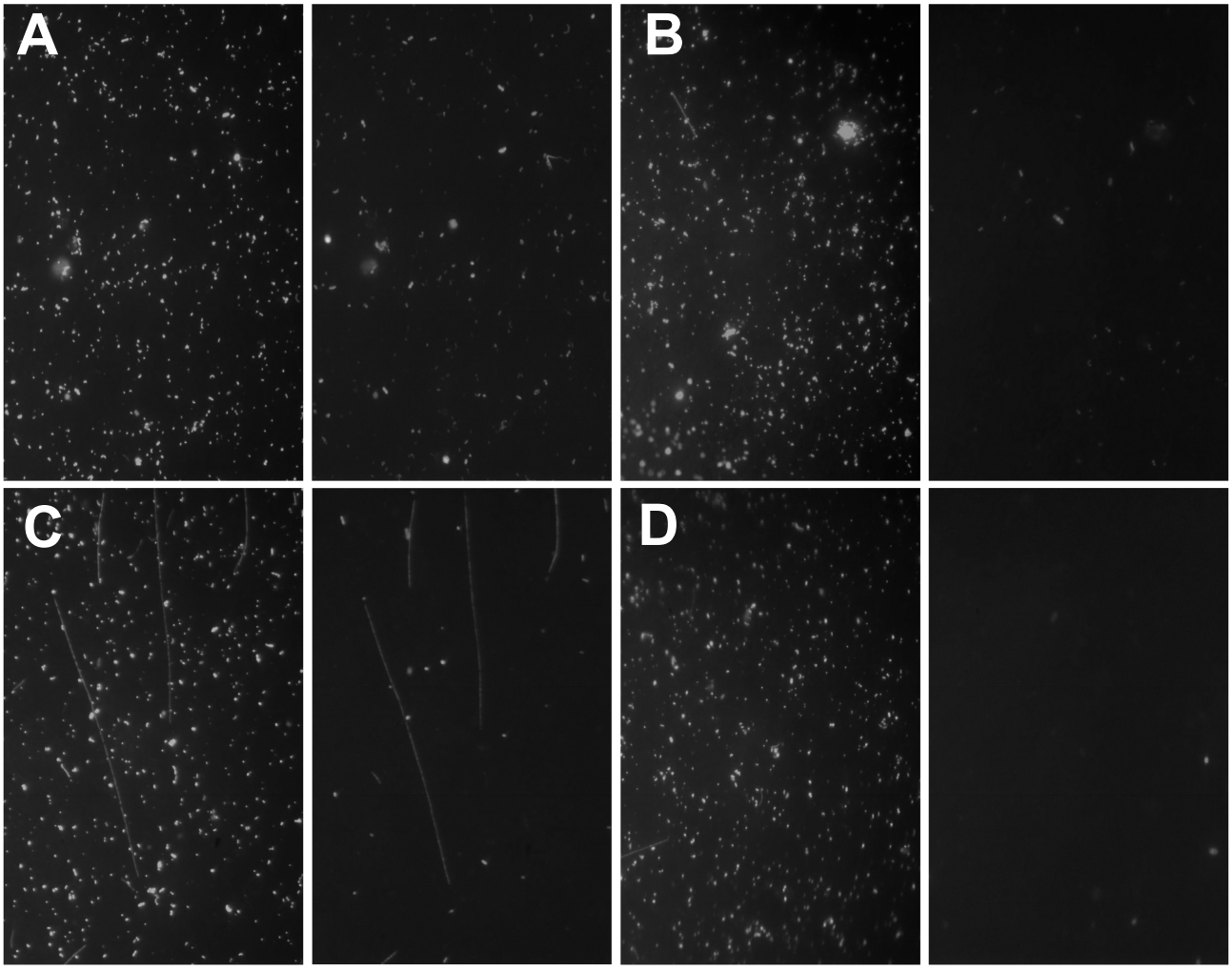

Fig. 2. Typical morphotypes of bacteria hybridizing with Cy3-labeled oligonucleotide probes (epifluorescence micrographs), (Left) UV excitation DAPI staining, (Right) hybridized cells with probe Eub338 (A, B), with probe Bet42a (C, D), with probe Cf319a (E, F), and with probe Non338 (G, H).

associated communities. In our study the highest percentage rates and abundances of CF cluster were recorded during spring thermal mixing mean, 15.2% (± 0.13) or 8 x 10^5 ml^{-1} ($p<0.0006$). In other examined periods (fall and winter) a gradual decrease was noted from 5.22 (± 0.07) up to 7.0% (± 0.21). Other authors have presented similar results; Pinhassi and Hagstrom [36] observed relatively high amounts of members of the CF group in spring in the North Baltic Sea and reported a seasonal succession for this group. In turn, Pernthaler et al. [31] reported the largest fraction of filamentous morphotypes of CF in a high mountain lake accounting for more than 50% of the biomass after ice break. Klammer et al. [21] and Eilers at al. [13] also found in late spring peaks in the amount of members of the CF group, which may respond to phytoplankton blooms. Some authors also believe that CF bacteria together with β-*Proteobacteria* share the ability to degrade complex organic macromolecules. Our results presented here agree with the known distribution patterns of the composition of microbial assemblages in lake water.

Conclusions

The objective of this study was to demonstrate the applicability of the FISH method to studies of bacterioplankton community composition. We were able to provide for the first time information on distributions of selected microbial populations based on a culture-independent technique in North Mamry Lake. The in situ identification of the dominant members of bacteria and their temporal succession is a necessary first step to unveil their ecological function in the examined system. Using the different oligonucleotide rRNA probes we obtained the relative abundance of different bacterial groups regardless of their culturability. FISH techniques offer the first insights at the full bacterial composition that is lost by other techniques like molecular fingerprinting or reverse line blotting. Needless to say, studies combining modern molecular and traditional cultivation-based techniques should give new insights into the ecology of lakes.

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References

1. ALFREIDER A., PERNTHALER J., AMANN R., SATTLER B., GLOCKNER F. O., WILLE A., PSENNER R. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. Appl. Environ. Microbiol. **62**, 2138, **1996**.
2. AMANN R. I., BINDER B. J., OLSON R.J., CHISHOLM S. W., DEVEREUX R., STAHL D. A., Combination of 16S-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56**, 1919, **1990**.
3. AMANN R., LUDWIG W., SCHLEIFER K. H., Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**, 143, **1995**.
4. AMANN R., GLÕCKNER F. O., NEEF A., Modern methods in subsurface microbiology-in situ identification of microorganisms with nucleic acid probes. FEMS Microbiol. Rev. **20**, 191, **1997**.
5. AMANN R., LUDWIG W., Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol. Rev. **24**, 555, **2000**.
6. AMANN R., FUCHS B. M., BEHRENS S., The identification of microorganisms by fluorescence in situ hybridization. Current Opinion in Biotechnology **12**, 231, **2001**.
7. BOBROW M. N., HARRIS T. D., SHAUGHNESSY K. J., LITT G. J., Catalyzed reporter deposition, a novel method of signal amplification. J. Immunol. Methods **125**, 279, **1989**.
8. BOUVIER T. C., DEL GIORGIO P. A. Compositional changes in free-living bacterial communities along salinity gradient in two temperature estuaries. Limnol. Oceanogr. **47**, 453, **2002**.
9. CHROST R. J., SIUDA W. Microbial production, utilization, and enzymatic degradation of organic mater in upper tropogenic water layer in the pelagial zone of lakes along the eutrophication gradient. Limnol. Oceanogr. **50,** 4, **2005**.
10. DELONG E. F., WICKHMAN G. S., PACE N. R. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**, 1360, **1989**.
11. DELONG E. F., TAYLOR T. T., MARSH T. L., PRESTON C. M. Visualization and enumeration of marine planktonic *Archaea* and *Bacteria* by using polirybonucleotide probes and fluorescent in situ hybridization. Appl. Environ. Microbiol. **65**, 5554, **1999**.
12. DEL GIORGIO P.A., BOUVIER T. C. Linking the physiologic and phylogenetic successions in free-living bacterial communities along an estuarine salinity gradient. Limnol. Oceanogr. **47,** 471, **2002**.
13. EILERS H., PERNTHALER J., GLÖCKNER F. O., AMANN R. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66**, 3044, **2000**.
14. FUCHS B. M., GLÖCKNER F. O., WÜLF J., AMANN R. Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. **66**, 3603, **2000**.
15. GIOVANONNI S. J., BRITSCHGI T. B., MOYER C.L., FIELD K. G., Genetic diversity in Sargasso Sea bacterioplankton. Nature **345**, 60, **1990**.
16. GLÖCKNER F-O., AMANN R., ALFREIDER A.,.PERNTHALER J., PSENNER R., TREBESIUS R., SCHLEIFER K.-H. An in situ hybridization protocol for detection and identification of planktonic bacteria. System. Appl. Microbiol. **19**, 403, **1996**.
17. GLÖCKNER F-O., FUCHS B. M., AMANN R. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. **65**, 3721, **1999**.
18. GLÖCKNER F-O., ZAICHIKOV E, BELKOVA N., DENISSOWA L., PRENTHALER J., PERNTHALER A., AMANN R. Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. Appl. Environ. Microbiol. **66**, 5053, **2000**.
19. HEAD I. M., SAUNDERS J. R., PICKUP R. W. Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial Ecol. **35**, 1, **1998**.
20. KIRCHMAN D. L., DITTEL A. I., FINDLAY S. E. G., FISHER D. Changes in bacterial activity and community structure in response to dissolved organic matter in the Hudson River. Microb. Ecol. **35**, 243, **2004.**
21. KLAMMER S., POSH T., SONNTAG B., GRIEBLER CH., MINDL B., PSENNER R. Dynamics of bacterial abundance, biomass, activity, and community composition in the oligotrophic Traunsee and the Traun River Austria. Water, Air and Soil Pollution **2**, 137, **2002**.
22. KUFEL L. Uncoupling of chlorophyll and nutrients in lakes-possible reasons, expected consequences. Hydrobiologia **443**, 59, **2001**.
23. LOY A., HORN M., WAGNER M. probeBase: an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Res. **31** (1), 514, **2003**.
24. MANZ W., AMANN R., LUDWIG W., WAGNER M., SCHLEIFER K. H., Phylogenetic oligonucleotide probes fort the major subclasses of Proteobacteria: Problems and solutions. Syst. Appl. Microbiol. **15**, 593, **1992**.
25. MANZ W., AMANN R., VANCANNEYT M., SCHLEIFER K-H. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. Microbiology **140**, 2849, **1996**.
26. METHE B. A., HIORNS W. D., ZEHR J. P. Contrast between marine and freshwater bacterial community composition: analyses of communities in Lake George and six other Adirondack lakes. Limnol. Oceanogr. **43**, 368, **1998.**

27. MOTER A., GÖBEL U. B., Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J. Microbiol. Methods **41**, 85, **2000**.
28. NEEF A., Anwendynng der in situ –Einzelzell-identifizierung von bakterien zur populationsanalyse in komplexen mikrobiellen biozonosen. Ph. D. Thesis. Technische Universitat Munchen, Munich, Germany, **1997**.
29. OLSEN R. A. BAKKEN L.R. Viability of soil bacteria, optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Microb. Ecol. **13**, 59, **1987**.
30. OUVERNEY C. C., FUHRMAN J. A. Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramfenicol. Appl. Environ. Microbiol **63**, 235, **1997**.
31. PERNTHALER J., GLÖCKNER F-O., UNTERHOLZNER S., ALFREIDER A., PSENNER R., AMANN R. Seasonal community and population dynamics of pelagic Bacteria and Archaea in a high mountain lake. Appl. Environ. Microbiol. **64**, 4299, **1998**.
32. PERNTHALER J., GLÕCKNER F-O., SCHONHUBER W., AMANN R. Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes. Methods in Microbiology **30**, 207, **2001**.
33. PERNTHALER A., PRESTOM C. M., PERNTHALER J., DELONG E. F., AMANN R. A comparison of fluorescently labeled oligonucleotide and polynucleotide probes for detection of pelagic marine bacteria and Arachaea. Appl. Environ. Microbiol. **68**, 661, **2002**.
34. PERNTHALER A., PERNTHALER J., AMANN R. Fluorescence in situ hybridization and catalyzed reporter deposition fort he identification of marine bacteria. Appl. Environ. Microbiol. **68**, 3094, **2002**.
35. PERNTHALER J., ZOLLNER E., WARNECKE F., JURGENS K. Bloom of filamentous bacteria in a mesotrophic lake identity and potential controlling mechanism. Appl. Environ. Microbiol. **70**, 6272, **2005**.
36. PINHASSI J., HAGSTRÖM A. Seasonal succession in marine bacterioplankton. Aquat. Microb. Ecol. **24,** 245, **2000**.
37. PORTER K. G., FEIG Y. S., The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**, 943, **1980**.
38. SEKAR R., PERNTHALER A., PERNTHALER J., WARNECKE F., POSH T., AMANN R. An improved protocol for quantification of freshwater *Actinobacteria* by fluorescence in situ hybridization. Appl. Environ. Microbiol **69**, 2928, **2003.**
39. SCHÖNHUBER W., FUCHS B., JURETSCHKO S., AMANN R. Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl. Environ. Microbiol **63**, 3268, **1997**.
40. SEKAR R., PERNTHALER A., PERNTHALER J., WARNECKE F., POSH T., AMANN R. An improved protocol for quantification of freshwater *Actinobacteria* by fluorescence in situ hybridization. Appl. Environ. Microbiol **69**, 2928, **2003**.
41. SKOWROŃSKA A., Application of molecular methods to investigate biodiversity of bacterioplankton in lake water. Doctoral thesis, Biblioteka Główna UW-M w Olsztynie p. 82, **2004**. [In Polish]
42. WAGNER M., AMANN R., LEMMER H., SCHLEIFER K.-H. Probing activated sludge with oligonucleotides specific for Proteobacteria: inadequacy of culture-dependent methods for describing community structure. Appl. Environ. Microbiol. **59**, 1520, **1993**.
43. WAGNER M., HORN M., DAIMS H., Fluorescence in situ hybridization for the identification and characterization of prokaryotes. Current Opinion in Microbiology **6**, 302, **2003**.
44. 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, **1993**.
45. WARD D. M., WELLER R., BATESON M. M., 16S rRNA sequences reveals numerous uncultured microorganisms in a natural community. Nature (London) **345**, 63, **1990**.
46. WORDEN CHISHOLM s. W., BINDER B. J. In situ hybridization of *Prochlorococcus* and *Synechococcus* spp. Marine cyanobacteria with rRNA-targeted peptide nucleic acid probes. Appl. Environ. Microbiol. **66**, 284, **2000**.