Molecular identification of picoplankton populations in contrasting waters of the Arabian Sea

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ABSTRACT: The composition of picoplankton in the southern oligotrophic, northern mesotrophic waters and deep oxygen minimum zone (OMZ) of the Arabian Sea was determined by 16S ribosomal RNA gene cloning and fluorescence *in situ* hybridisation (FISH). It was hypothesised that the composition of the heterotrophic picoplankton would be different in these contrasting waters. To reduce the total diversity, cells were sorted by flow cytometry according to their scatter and DNA content before PCR amplification. The 16S rRNA clone libraries resulting from flow-sorted populations were different and often dominated by a small number of clades. Libraries from the *Prochlorococcus*-dominated southerly waters were dominated by sequences related to uncultured clusters of SAR11, SAR86 and Actinobacteria (HGC I). From surface waters of the *Synechococcus*-dominated northern part of the Arabian Sea, mostly sequences related to the uncultured gammaproteobacterial group 'Svalbard' and HGC I were retrieved. The clone libraries from the OMZ were also dominated by sequences falling in the clades SAR11 and SAR406, but included sequences related to those of sulfate-reducing (*Desulfosarcina*, *Desulfofrigus*) and sulfide-oxidising bacteria (endosymbionts of *Riftia* and *Calyptogena*). With a recently developed more sensitive FISH protocol approximately 60% of all DAPI stained cells could be identified by general probes as Bacteria, Cren- or Euryarchaeota in both provinces of the Arabian Sea; 40% remained undetected. On this level and on that of the major phylogenetic groups like Alpha- and Gammaproteobacteria only minor differences were detected by FISH. However, the composition of heterotrophic picoplankton clearly differed for the proteobacterial subgroups SAR86, SAR11 and SAR116. These were more abundant in the oligotrophic waters throughout the water column than in the mesotrophic surface waters and the OMZ. This supports our original hypothesis that the contrasting waters in the Arabian Sea harbor different heterotrophic picoplankton communities. In the future, FISH with a larger set of probes for more narrow phylogenetic groups will enable us to quantify these differences in more detail.

KEY WORDS: Phylogenetic composition · Arabian Sea · Oxygen minimum zone · OMZ · Fluorescence *in situ* hybridisation · Catalyzed reporter deposition (CARD)-FISH · Oligonucleotide probes · Flow cytometry

INTRODUCTION

The Arabian Sea is enclosed in the east and north by the Indian subcontinent and in the west by the Arabian Peninsula and the Somalian coast. Strong winds during the monsoon periods, especially during the southwest monsoon, lead to upwelling and concomitantly result in high productivity (e.g. Barber et al. 2001, Smith 2001). In contrast to other oceanic provinces, where a regular exchange with oxygenated water occurs, an extensive oxygen minimum zone (OMZ) develops in the deeper water column of the Arabian Sea (Sarma 2002). Typically suboxic conditions prevail throughout the whole year in a stable water layer between 200 and 1000 m depths with oxygen concentrations that often falls below 5 µM (Morrison et al. 1999).

Numerous studies have shown the strong coupling between the physical conditions and the biological

processes in the Arabian Sea (e.g. Brock & McClain 1992, Barber et al. 2001, Shalapyonok et al. 2001). For example during the southwest monsoon between June and September the open waters of the northern Arabian Sea basin are dominated by *Synechococcus* and eukaryotic picophytoplankton, indicating mesotrophic conditions, whereas the southern waters are dominated by *Prochlorococcus* (Burkill et al. 1993, Campbell et al. 1998, Tarran et al. 1999). Even though the phytoplankton composition and productivity changes seasonally with the monsoon cycle (Campbell et al. 1998, Tarran et al. 1999), the productivity remains virtually the same throughout the year (Pomroy & Joint 1999, Ducklow et al. 2001). Little is known about the diversity of the heterotrophic picoplankton and how dominant groups vary within the basin. Riemann et al. (1999) found dominant phylotypes such as members of the SAR11 clade or Deltaproteobacteria by DGGE in the surface water layers, and an increase of diversity with depth. The presence of Crenarchaeal membrane lipids was detected by Damste et al. (2002) in water layers below 500 m. However, a study of the heterotrophic picoplankton composition and dominant phylogenetic groups has yet to be reported.

Currently the method of choice for examining microbial diversity is the so-called full cycle rRNA approach (Olsen et al. 1986, Amann 1995). This approach is culture independent and therefore has the potential to find uncultured and new microbial species in the marine environment (Giovannoni et al. 1990, Fuhrman et al. 1993, Massana et al. 1997). It starts with the isolation of DNA, followed by a PCR amplification of the 16S rRNA genes, cloning and sequencing. The sequences are subjected to a comparative sequence analysis, in which the phylogenetic relationships of the respective organisms can be reconstructed. Probes specific for the different microbial groups present in the clone libraries are designed. Finally the *in situ* abundance of microbial groups is determined by fluorescence *in situ* hybridisation (FISH) in the original sample. In a modification of the general approach, we tried in the present study to reduce the rather large diversity to the most abundant groups by flow cytometric cell sorting (Venter et al. 2004). Approximately 3 to 4 subpopulations of picoplankton can be categorized based on light scatter and DNA content (Gasol et al. 1999, Gregori et al. 2001, Lebaron et al. 2001, Zubkov et al. 2001b). Here $>10^4$ sorted cells of each subpopulation were directly used for 16S rRNA clone library construction (Wallner et al. 1997, Zubkov et al. 2003). The most frequently retrieved sequences were used as guidance for selection and development of oligonucleotide probes for FISH analysis.

Here we report data on heterotrophic picoplankton diversity and composition in contrasting pelagic regions of the Arabian Sea. Our hypothesis was that there should be pronounced differences in the picoplankton composition of the surface mixed layer of the southern *Prochlorococcus*-dominated waters, the surface mixed layer of the northern *Synechococcus*-dominated waters and the oxygen depleted OMZ waters of the Arabian Sea.

MATERIALS AND METHODS

Sampling. Seawater samples were collected with a rosette of 20 l Niskin bottles mounted on a conductivity-temperature-density (CTD) profiler during cruise CD132 on board the RRS 'Charles Darwin', on a transect from the equator to 21° N primarily along the 67° E meridian across the Arabian Sea in September 2001 (Fig. 1). Oxygen measurements in surface waters were typically about 200 μM but northwards from 7° N, concentrations at depths >100 m within the OMZ fell below 20 μM. This is typically equivalent to <10% of oxygen saturation. Subsamples for picoplankton analyses were fixed with 1% (v/v) paraformaldehyde (PFA) in 1.8 ml aliquots for maximum 24 h at 4°C. Then, cells were enumerated on board the ship and the rest of the material was frozen at –20°C for subsequent flow cytometric sorting and molecular analyses ashore. The samples used in this study were all from depth profiles of Stns A (3.8° N, 67.0° E), B (19.0° N, 67.0° E) and C (20.9° N, 63,7° E) (Fig. 1).
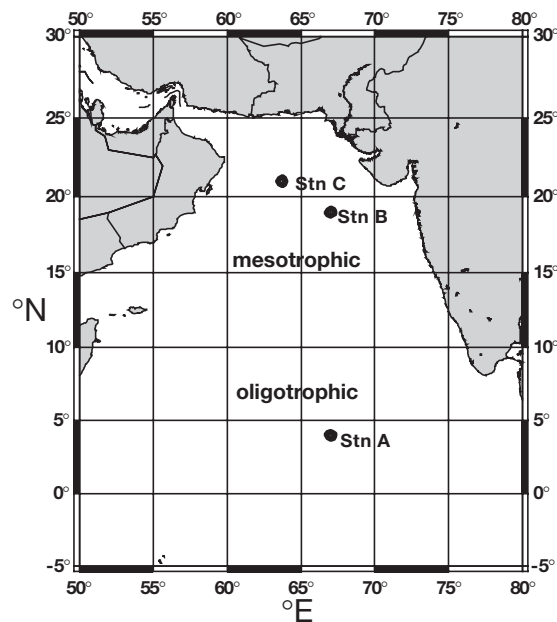

Fig. 1. Map showing the sampling locations for the present study

Flow cytometry. The abundance of *Synechococcus* and *Prochlorococcus* cyanobacteria was determined on board in fresh unfixed samples using a FACSort flow cytometer (BD Biosciences) as described previously (Zubkov et al. 1998). Total picoplankton were enumerated after PFA fixation and staining with SYBR Green (1:10 000 dilution of stock; Sigma) using the FACSort flow cytometer as described previously (Marie et al. 1997). Flow sorting was done on a FACS Star Plus (BD Biosciences). Cells were excited by an Argon ion laser tuned to 488 nm with an output power of 500 mW. A photomultiplier tube (PMT) and a broader obscuration bar replaced the forward scatter diode. Forward scatter (FSC) was analyzed through a 488 ± 10 nm bandpass filter typically at a PMT voltage of 350 V and logarithmic amplification mode. Green fluorescence (FL1) of SYBR Green-stained cells was measured through a 530 ± 20 nm bandpass filter typically at a PMT voltage of 750 V and logarithmic amplification mode (Fuchs et al. 2000). Online analysis was carried out on a bivariate dotplot diagram. The dotplot diagram was also used to define sorting windows (gates) for subsequent flow cytometric sorting. A 0.2 µm prefiltered 0.1 % NaCl (w/v) solution was used as a sheath fluid for sorting. Sheath fluid was pressurized at 12 psi (82 730 Pa). A drop drive was typically set at 25 150 Hz; drop delay was set around 12 drops depending on the daily performance of the instrument and the highest purity sort mode 'Normal-C' at 3 deflected drops was selected. The performance was evaluated by sorting a known number of beads onto microscopic slides and enumeration under an epifluorescence microscope. At least 2×10^4 cells were sorted for molecular analysis of identified flow cytometric bacterial populations (Fig. 2) and 1×10^4 cells were sorted from the population identified as *Synechococcus* based on flow cytometric characteristics. The latter population was used as a control of sorting purity.

PCR, cloning, sequencing. All fluidic lines of the instrument were cleaned with a 1 % hypochloric acid solution and rinsed with excessive amount of sterilized water prior to sorting for PCR amplification. Three representative samples were flow sorted for 16S rRNA gene amplification and cloning: a sample collected at 50 m represented the surface mixed layer of the *Prochlorococcus*-dominated oligotrophic part of the Arabian Sea (Stn A); a pooled sample of 2 subsamples, collected at 25 and 49 m, respectively, represented the surface mixed layer of the mesotrophic *Synechococcus*-dominated part (Stn B); and a sample collected from the OMZ at 248 m on Stn B. Sorted cell fractions (approximately 50 µl) were concentrated on small sterilized pieces of polycarbonate filter (GTTP, 0.2 µm pore size, Millipore) and directly subjected to PCR amplification (Zubkov et al. 2001a). Approximately

500 µl sheath fluid was used as a negative control. The primer pairs GM3 (5′-AGAGTTTGATCMTGGC-3′) (Muyzer et al. 1995) and GM4 (5′-TACCTTGTTAC-GACTT-3′) (Kane et al. 1993) were used for amplification of the bacterial 16S rRNA genes, respectively. For PCR, 10 µl of 10x Taq-polymerase buffer (10 mM Mg^{2+}; TaKaRa Bio), dNTPs (2.5 mM each), bovine serum albumin (3 mg ml^{-1}), 0.5 µl of Taq polymerase (5 U ml^{-1}, TaKaRa Bio), and 1 µl of primers (15 µM) were mixed and adjusted to a final volume of 100 µl with ultrapure, sterile water. The PCR was run on a Mastercycler (Eppendorf) at the following cycling conditions: 1 cycle at 94°C for 5 min followed by 1 min at 70°C and 35 cycles at 94°C for 1 min, 48°C for 2 min, 72°C for 3 min and a final step at 72°C for 10 min. The PCR products were checked by agarose (1 % w/v) gel electrophoresis. PCR products were purified with the QIAquick PCR purification kit (Qiagen). The purified products were ligated using the pGEM-T-Easy (Promega) and the TOPO TA cloning kit (Invitrogen) and cloned into high efficiency competent cells of *Escherichia coli* (JM109) as described by the manufacturer. The transformed cells were plated on Luria-Bertani (LB) agar plates containing ampicillin (100 µg ml^{-1}), isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 80 µg ml^{-1}) and incubated at 37°C overnight. Colonies of plasmid-bearing clones were transferred to liquid LB medium containing ampicillin (100 µg ml^{-1}). The clones were screened for correctly sized inserts by a direct agarose gel electrophoresis of lysed clones. Plasmids of positive clones were isolated using the QIAprep plasmid extraction kit (Qiagen). The quality of the plasmid was checked by agarose gel electrophoresis. For the sequencing reaction, 1 µl of plasmid DNA (50 to 100 ng µl^{-1}) was used. The plasmid inserts were sequenced on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) with the primers GM1 (Muyzer et al. 1993), M13F and M13R (Messing 1983). Nearly full length 16S rRNA sequences were deposited in GenBank under the accession numbers AY907718 to AY907823.

Catalyzed reporter deposition (CARD)-FISH. Filters for counts of CARD-FISH-stained cells were embedded in low-gelling point agarose (concentration 0.2 %, MetaPhor, BioWhittaker Molecular Applications) and dried at 37°C for 10 min. Embedded cells were permeabilized by subsequent treatments with lysozyme (10 mg ml^{-1}, Fluka) in 0.05 M EDTA, 0.1 M Tris-HCl, pH 8.0) for 60 min at 37°C and achromopeptidase (120 U in 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0) for 30 min at 37°C. For detection of Archaea, embedded cells were permeabilised with 0.1 M HCl for 1 min. After washing in sterile water and dehydration with 96 % (v/v) ethanol for 1 min, the preparations were air-

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Table 1. Probes used in the present study. FA: formamide (v/v)

Probe	Target organisms	*Escherichia coli* position[a]	Sequence (5'→3')	FA[b] (%)	NaCl[c] (mM)	Source
CREN554	Crenarchaeota	554–573	TTAGGCCCAATAATCMTCCT	20	145	Massana et al. (1997)
EURY806	Euryarchaea	806–823	CACAGCGTTTACACCTAG	20	145	Teira et al. (2004)
EUBI-III	Bacteria	338–355	GCWGCCWCCCGTAGGWGT	55	13	Amann et al. (1990), Daims et al. (1999)
NON338	Control	338–355	ACTCCTACGGGAGGCAGC	55	13	Wallner et al. (1993)
GAM42a[d]	Gammaproteobacteria	1027–1043[e]	GCCTTCCCACATCGTTT	55	13	(Manz et al. 1992)
ALF968	Alphaproteobacteria	968–986	GGTAAGGTTCTGCGCGTT	55	13	(Neef 1997)
ROS537	*Roseobacter* clade	537–553	CAACGCTAACCCCCTCC	55	13	Eilers et al. (2001)
SAR86-1245	SAR86-clade	1245–1262	TTAGCGTCCGTCTGTAT	55	13	Zubkov et al. (2001b)
CF319a	Bacteroidetes	319–336	TGGTCCGTGTCTCAGTAC	55	13	Manz et al. (1996)
HGC69a	Actinobacteria	1901–1918[e]	TATAGTTACCACCGCCGT	40	37	Roller et al. (1994)
SAR11-486	SAR11-clade	486–503	GGACCTTCTTATTCGGGT	60	9	Present study
SAR116-1-447	Cluster 1 of SAR116-clade	447–464	GCTACCGTCATCATCTTC	65	7	Present study
SAR116-2-436	Cluster 2 of SAR116-clade	436–452	CATCTTCACCAGTGAAAG	45	26	Present study
SAR406-97	SAR406-clade	97–114	CACCCGTTCGCCAGTTTA	65	7	Present study

[a] Probe target position on *E. coli* 16S rRNA according to Brosius et al. (1981)
[b] Formamide concentration in CARD-FISH hybridisation buffer
[c] NaCl concentration in washing buffer
[d] Including an unlabeled competitor probe BET42a (5'-GCCTTCCCACTTCGTTT-3'), see Manz et al. (1992) for details
[e] Probe target position on *E. coli* 23S rRNA according to Brosius et al. (1981)

dried and stored at −20°C until further processing. FISH on these pre-treated filter sections were performed using 5′-horseradish peroxidase (HRP)-labeled oligonucleotide probes, as described previously (Pernthaler et al. 2002). The hybridisation buffer contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 10% dextran sulfate (w/v), 1% blocking reagent (Boehringer), 0.05% Triton X-100 (v/v) and varying amounts of formamide, depending on the probe used (Table 1). The blocking reagent was prepared in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5). Hybridisation buffer and probe working solution (50 ng µl^{-1}) were mixed 300:1. Filter sections (10 to 15) were put in a 0.5 ml reaction vial and placed in the buffer. The reaction vial was incubated in a hybridisation oven at 35°C on a rotation shaker for a minimum of 2 h. Next, the filter sections were transferred to 50 ml of pre-warmed washing buffer and incubated at 37°C for 10 min. The washing buffer contained varying amounts of NaCl (depending on the probe, Table 1), 5 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 0.01% (w/v) sodium dodecyl sulfate. After the washing step, filters hybridised with HRP-labeled probes were transferred to 15 ml of 1× phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.6) amended with 0.05% Triton X-100 for 25 min at room temperature to equilibrate the probe-delivered HRP. Then, the filters were transferred onto blotting paper to remove excess buffer and subsequently incubated in the substrate mix. Tyramide signal amplification was performed using custom fluorescein-labeled tyramides dissolved in dimethyl formamide containing 20 mg ml^{-1} 4-iodophenyl boronic acid (Pernthaler et al. 2004). One part of the tyramide solution was added to 300 parts of amplification buffer (1× PBS, 2 M NaCl, 0.1% blocking reagent, 10% Dextran sulfate, 0.0015% H_2O_2). The amplification reaction was run at 37°C for 15 min in the dark. The filters were then washed with 1× PBS amended with 0.05% Triton X-100 for 15 min at room temperature followed by thorough washing with sterile water and then with 96% ethanol for 1 min. Counterstaining of CARD-FISH preparations with 4,6-diamidino-2-phenylindole (DAPI; 1 µg ml^{-1}), mounting, and microscopic evaluations were performed as described previously (Glöckner et al. 1996). The fraction of FISH-stained bacteria in at least 1000 DAPI-stained cells per sample was quantified. Prior to evaluation the slides could be stored at −20°C up to several days without loss of fluorescence intensity.

Treeing, probe design, probe testing. All sequences were analyzed with the ARB program

package (Ludwig et al. 2004). Sequences were imported into the dataset present at the Ribosomal Database Project (RDP) in January 2004 (~95 000 rRNA sequences; http://rdp.cme.msu.edu). Sequences were aligned to the next relative in the dataset. Trees were calculated with the maximum likelihood, maximum parsimony and neighbor joining method, both with and without a 50 % similarity filter. Consensus trees were calculated for each group of interest and used as basis for choosing the target organisms for probe design. Probe design was done with the ARB tool PROBE_DESIGN. The new probes were first tested on original material at low stringency, and those detecting cells were further optimized using the Clone-FISH method (Schramm et al. 2002). Optimal hybridisation conditions were determined by hybridising IPTG induced *E. coli* clones, carrying inserts that either fully matched the probe or had mismatches in the probe target region. Optimized probes were then used for quantification of the populations *in situ*. Probes used in this study are listed in Table 1.

RESULTS

Flow cytometric analysis

Flow cytometric analyses of the picoplankton communities were carried out using light scatter versus DNA fluorescence dotplots. In both oceanic provinces the flow cytometric signatures of the surface mixed layer were similar (Fig. 2). Three main populations could be distinguished: 1 population with low DNA content (LNA; Fig. 2A,C) and 2 populations with high DNA content which showed low (HNA I) and high scatter properties (HNA II; Fig. 2A,C). HNA II scatter signals were higher in mesotrophic compared to oligotrophic surface waters indicating a larger average cell size. Samples from the OMZ at 248 m depth showed only 2 major populations, 1 with a low (LNA, Fig. 2E) and 1 with a high cellular DNA content (HNA; Fig. 2E).

Flow cytometric sorting for the construction of 16S rRNA clone libraries

To evaluate efficiency and purity of flow cytometric sorting, autofluorescent *Synechococcus* cells were sorted based on their specific phycoerythrin fluorescence and subsequently subjected to 16S rRNA gene amplification and cloning (Syn; Fig. 2B,D). Of the clones sequenced, 13 out of 14 and 14 of 16 were related to *Synechococcus* group A (similarity > 99 %) (Zubkov et al. 2003) in the mesotrophic and oligo-
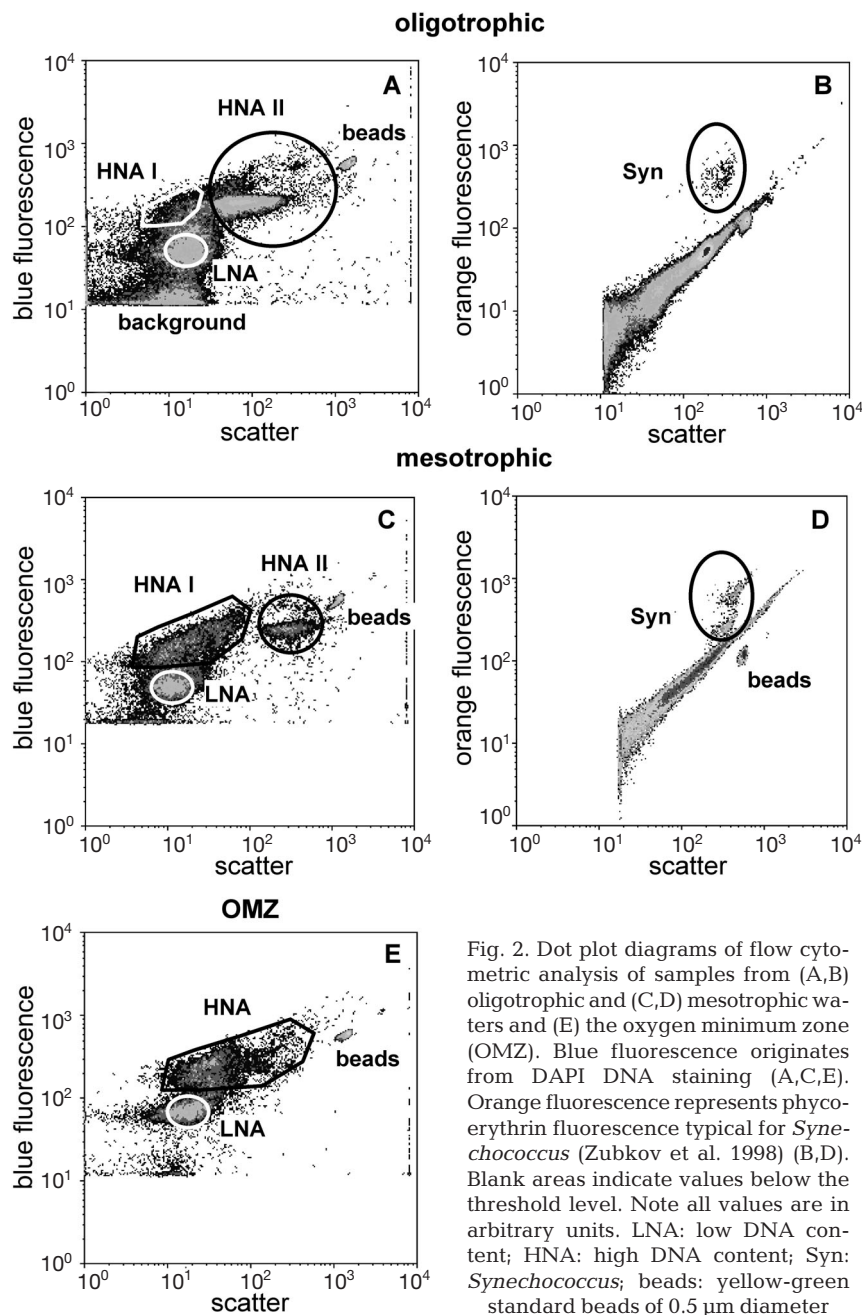

Fig. 2. Dot plot diagrams of flow cytometric analysis of samples from (A,B) oligotrophic and (C,D) mesotrophic waters and (E) the oxygen minimum zone (OMZ). Blue fluorescence originates from DAPI DNA staining (A,C,E). Orange fluorescence represents phycoerythrin fluorescence typical for *Synechococcus* (Zubkov et al. 1998) (B,D). Blank areas indicate values below the threshold level. Note all values are in arbitrary units. LNA: low DNA content; HNA: high DNA content; Syn: *Synechococcus*; beads: yellow-green standard beads of 0.5 μm diameter

Table 2. Affiliation of the clone sequences retrieved from sorted bacterioplankton cells. Closest relatives were determined with the ARB program package. The names of the clusters are defined arbitrarily if no cultured representative was available

Cluster	No. of sequences Full	No. of sequences Partial	Next relative	Accession no.	Similarity (%)
Alphaproteobacteria					
SAR116	2	2	Uncultured alpha-proteobacterium clone NAC11-16	AF245641	92
SAR11	4	35	Uncultured alpha-proteobacterium clone SAR407	U75253	99
	4	1	Uncultured alpha-proteobacterium (MB-C2_128)	AY093481	98
Olavius loisae symbiont		1	*Olavius loisae* symbiont	AF104474	90
Paracoccus		1	Uncultured alpha-proteobacterium	AJ567557	93
Roseobacter	2		Uncultured bacterium	M63810	94
Oceanospirillum	2		Uncultured alpha-proteobacterium	AF353236	86
Uncultured alpha	1		Uncultured bacterium clone Artic95B-2	AF355036	91
Betaproteobacteria					
Methylophilus	1		*Methylophilus methylotrophus*	L15475	99
Gammaproteobacteria					
Alteromonas		3	*Alteromonas* sp. ANSW2-2	AF427478	99
Uncultured gamma	4		Uncultured gamma-proteobacterium KI89C	AB022713	92
Gamma symbionts (*Bathymodiolus*)	2	7	Uncultured bacterium	M99445	98
Gamma symbionts (*Riftia*)		1	Aggregate clone 47	L10949	95
Cycloclasticus	1	1	*Cycloclasticus pugetii*	L34955	90
SAR86	5	8	Uncultured bacterium EBAC	AF268217	93
SAR86	1	1	Uncultured bacterium CHAB-I-7	AJ240911	97
SAR86	2		Uncultured bacterium ZD0433	AJ400356	95
Svalbard cluster	8	10	Uncultured bacterium ZD0408c	AJ400349	90
Chloroplast	1		Unidentified prasinophyte chloroplast OM5	U70715	98
Deltaproteobacteria					
Bdellovibrio	2		Clone OM27	U70713	86
Desulfofrigus		3	Uncultured bacterium clone Eel-BE1B3	AF354151	91
Desulfosarcina	1	1	Uncultured bacterium clone Sva0081	AJ240975	98
Cytophagales					
Capnocytophaga		1	*Capnocytophaga granulosa*	X97248	92
CFB 1	1		Uncultured bacterium clone JTB250	AB015264	87
CFB 2	4		Uncultured bacterium clone Arctic97A-17	AF354617	86
CFB 3	1		Uncultured bacterium clone C1B008	AF419690	98
Microscilla	1		Uncultured bacterium clone JTB250	AB015264	86
Flexibacter 1	5	2	Uncultured bacterium NAC60-3	AF245645	96
Flexibacter 2	1		Uncultured bacterium ATAM173a_2	AF359539	95
Polaribacter	2		Uncultured bacterium ZD0403c	AJ400347	95
Saprospira	2		*Saprospira grandis*	M58795	88
Actinobacteria					
Marine cluster 1	11	19	Uncultured bacterium SAR432	AF110142	96
Marine cluster 2		3	Uncultured bacterium	AJ347031	96
Nitrospina	2	2	*Nitrospina gracilis*	L35503	92
Planctomycetales					
Pirellula	1	1	Uncultured *Pirellula* clone 6O13	AF029077	92
Cluster 1		3	Uncultured planctomycete clone 0319-7F4	AF234144	83
Cluster 2	1		Uncultured planctomycete clone agg27	L10943	95
Cyanobacteria					
Prochlorococcus HLII	7		*Prochlorococcus marinus* subsp. *pastoris* strain TATL2 (HLII)	AF001476	99
Synechococcus group A	27		Uncultured *Synechococcus* clone NAC1-5	AF245618	99
SAR324 cluster	6		Uncultured bacterium clone SAR276	U65915	99
SAR406 cluster					
SAR406	9	24	Uncultured bacterium clone SAR406	U34043	83
	1		Uncultured bacterium clone OCS146	AF001653	97
Verrucomicrobiales					
	1	1	Uncultured bacterium	AY028219	94
	1		Uncultured bacterium CHAB-II-49	AJ240909	88
Chloroflexaceae	1	2	Uncultured sponge symbiont	AF186417	85
OP11		1	Isolated bacterium ko116	AJ224539	83

trophic waters, respectively. The 16S rRNA genes were obtained from all populations using >2 × 10⁴ sorted cells. Between 14 and 49 clones were sequenced from each library. In total 327 clones were subjected to comparative sequence analyses (Table 2). Putative chimeras (Hugenholtz & Huber 2003) and sequences derived from potential contaminations were excluded from further analysis (n = 19).

Comparative 16S rRNA sequence analysis

In the oligotrophic waters most of the clones (19/21) sequenced from the LNA clone library belonged to the SAR11 clade within the Alphaproteobacteria. Other sequences were affiliated to an uncultured cluster CFB 3 of the Bacteroidetes (1/21), and to an uncultured cluster of gammaproteobacterial symbionts of the mussel *Bathymodiolus* (1/21). The oligotrophic HNA I clone library was codominated by the cluster HGC 1 of marine Actinobacteria (16/39) and by SAR86 related Gammaproteobacteria (12/39). Other sequences comprised affiliates to an uncultured cluster 1 related to *Flexibacter* species (*Flexibacter* 1; 4/39), the SAR406 cluster of Firmicutes (4/39), the SAR116 of Alphaproteobacteria (2/39) and an uncultured cluster related to the *Roseobacter* clade (SAR83; 1/39). Members of the HNA II population were mainly related to a cluster of high-light adapted *Prochlorococcus* (HLII; 7/23), and to the SAR406 cluster (4/23). One clone each was related to *Capnocytophaga*, *Methylophilus*, and *Oceanospirillum*, to an uncultured cluster of Alphaproteobacteria, to an uncultured cluster 2 of Planctomycetes, SAR116, *Roseobacter* and to an uncultured cluster of Verrucomicrobiaceae. In the mesotrophic surface water sample investigated, the LNA library was dominated by 2 distinct clone groups: an uncultured cluster I of marine Actinobacteria (HGC I; 14/49) and an uncultured cluster of Gammaproteobacteria (Svalbard-cluster; 16/49). Other sequences were related with SAR406 (6/49), SAR86 (3/49), the *Flexibacter* cluster 1 (2/49), *Polaribacter* (2/49), an uncultured cluster 1 and 2 of the Cytophagales (CFB 1; 1/49; CFB 2; 2/49), SAR11 (2/49), and *Oceanospirillum* (1/49). Cloning from the HNA I population was repeatedly difficult. Only 7 clones could be retrieved in total, which were affiliated to uncultured members of the Chloroflexaceae (2/7), the SAR86 cluster (2/7) the Svalbard-cluster (2/7) and the SAR406 cluster (1/7). Clones affiliated to the cluster SAR324 were the most abundant in HNA II sorted cells (6/29). A high diversity was found in the other 23 clones sequenced from that subpopulation: 3 sequences related to *Alteromonas*, 2 sequences each from the clades Nitrospina, *Pirellula*, SAR406, CFB cluster 2, *Saprospira*, *Bdellovibrio*, and 1

sequence each from the *Flexibacter* cluster 2, *Cycloclasticus*, *Paracoccus*, SAR116, Verrucomicrobiaceae, Chloroflexaceae, *Microscilla*, and a chloroplast. In the OMZ, only 2 picoplankton populations were observed and sorted for cloning (Fig. 2E, Table 3). The LNA fraction was dominated by SAR11 sequences (23/31). Other sequences were related to a Planctomycetes cluster 1 (3/31), SAR406 (2/31), an uncultured cluster of gammaproteobacterial symbionts of *Bathymodiolus* (1/31), an uncultured cluster II of marine Actinobacteria (HGC 2; 1/31), and the OP11 phylum (1/31). The HNA population was dominated by members of the SAR406 cluster (15/34) and bacteria related to uncultured symbiotic Gammaproteobacteria of *Bathymodiolus* (gamma symbionts *Bathymodiolus*; 7/34). Other groups detected in the sorted HNA fraction were *Desulfofrigus* (3/34), Nitrospina (2/34), *Desulfosarcina* (1/34), an uncultured cluster of symbiotic Gammaproteobacteria of *Riftia* (gamma symbionts *Riftia*; 1/34) relatives to *Cycloclasticus* (1/34), HGC cluster II (1/34), a group of uncultured symbiotic Alphaproteobacteria of *Olavius loisae* (1/34), *Paracoccus* (1/34), and a *Verrucomicrobium* related sequence.

Probe design and testing

Probes were designed for the clusters SAR11, SAR406, SAR116, a cluster of Gammaproteobacteria related to *Bathymodiolus* symbionts and 2 marine clusters of Actinobacteria. All newly designed probes were tested at low stringency on original sample material. However only probes specific for SAR11, SAR116 and SAR406 detected cells above background and were therefore further optimized by Clone-FISH (Schramm et al. 2002). Probe SAR11-486 showed specific signals after hybridisation in a buffer containing 60% (v/v) formamide. Two probes specific for the SAR116 cluster, SAR116-1-447 and SAR116-2-436, were used at 65% (v/v) and 45% (v/v) formamide in the hybridisation buffer, respectively. SAR406-97, a probe specific for most of the known members of the SAR406 clade, was used at a formamide concentration of 65%.

CARD-FISH on total picoplankton samples

Between 32 and 66% of all DAPI-stained cells could be detected by probes specific for Bacteria and Archaea (Fig. 3A). The fraction of cells detected with the bacterial probe EUBI-III ranged between 38 and 40% in oligotrophic waters (Fig. 3A) and was clearly lower in mesotrophic waters (21 to 28%). Bacterial counts in the OMZ were between 23% (300 m, Stn C) and 38% (150 m, Stn B). The abundance of Archaea

Table 3. Number of sequences retrieved from the different sorted picoplankton fractions. Names of the clades are defined in Table 2. Names of the clones are given in brackets behind the sorting gates. LNA: low DNA content; HNA: high DNA content; Syn: *Synechococcus*

	Stn A: 50 m		Stn B: surface		Stn B: OMZ
	LNA (A312xxx)		**LNA (A712xxx)**		**LNA (A722xxx)**
19	SAR11	16	Svalbard cluster	23	SAR11
1	CFB cluster 3	14	HGC marine cluster 1	3	Planctomycetes cluster 1
1	Gamma symbionts (*Bathymodiolus*)	6	SAR406	2	SAR406
		3	SAR86	1	OP11
		2	CFB cluster 2	1	Gamma symbionts (*Bathymodiolus*)
		2	*Flexibacter* cluster 1	1	HGC marine cluster 2
		2	*Polaribacter*		
		2	SAR11		
		1	*Oceanospirillum*		
		1	CFB cluster 1		
	HNA I (A313xxx)		**HNA I (A713xxx)**		**HNA (A723xxx)**
16	HGC marine cluster 1	2	Svalbard cluster	15	SAR406
12	SAR86	2	Uncultured *Chloroflexus*	7	Gamma symbionts (*Bathymodiolus*)
4	SAR406	2	SAR86	3	*Desulfofrigus*
4	*Flexibacter* cluster 1	1	SAR406	2	HGC marine cluster 2
2	SAR116			2	Nitrospina
1	*Roseobacter* (SAR83)			1	Uncultured *Verrucomicrobium*
				1	Gamma symbionts (Riftia)
				1	*Desulfosarcina*
				1	*Olavius loisae* symbiont
				1	*Cycloclasticus*
	HNA II (A314xxx)		**HNA II (A714xxx)**		
7	*Prochlorococcus* HLII	6	SAR324		
4	Uncultured gamma	3	*Alteromonas*		
4	SAR406	2	Nitrospina		
1	SAR116	2	SAR406		
1	*Capnocytophaga*	2	*Pirellula*		
1	*Roseobacter* (SAR83)	2	CFB cluster 2		
1	Uncultured *Verrucomicrobium*	2	*Saprospira*		
1	*Oceanospirillum*	2	Uncultured *Bdellovibrio*		
1	*Methylophilus*	1	SAR116		
1	Uncultured alpha	1	Uncultured *Verrucomicrobium*		
1	Planctomycetes cluster 2	1	Uncultured *Chloroflexus*		
		1	*Microscilla*		
		1	*Flexibacter* cluster 2		
		1	*Cycloclasticus*		
		1	*Paracoccus*		
		1	Chloroplast		

was determined by 2 probes specific Euryarchaeota (Eury806) and Crenarchaeota (Cren554). Both probes together yielded between 14% (Stn A, 54 m) and 28% (Stn B, 150 m) probe-positive cells in the samples examined (Fig. 3A). The Euryarchaeota dominated the archaeal fraction at Stn A (9 to 13%), the mesotrophic surface waters (11 to 19%) and the OMZ (10 to 22%). In oligotrophic waters, the abundance of Crenarchaeota was between 5% (54 m) and 8% (71 m), and ranged between 4% (300 m) and 8% (120 m) in the OMZ. In turn, 40% to sometimes 60% of the picoplankton cells could not be identified by FISH, even though an optimized protocol of CARD-FISH was used (Pernthaler et al. 2002). The Alphaproteobacteria

were monitored with the probe Alf968 (Fig. 3A,B). The percentage of this group was between 11 and 18% of all DAPI stained cells. The relative abundance of detected Alphaproteobacteria in the mesotrophic waters tended to increase with depth (11, 14, and 18%), but remained similar in the oligotrophic waters (12 to 15%). They were less abundant in the OMZ (3 to 7%). Probes specific to the 3 subgroups of Alphaproteobacteria were applied: SAR11-486, specific for the SAR11 clade, SAR116-1- 447 and SAR116-2-436, together targeting the SAR116 clade, and Ros537, targeting the *Roseobacter* clade including the SAR83 cluster (Fig. 3B). Members of the SAR11 clade comprised 1 to 6% in the oligotrophic waters. Few SAR11
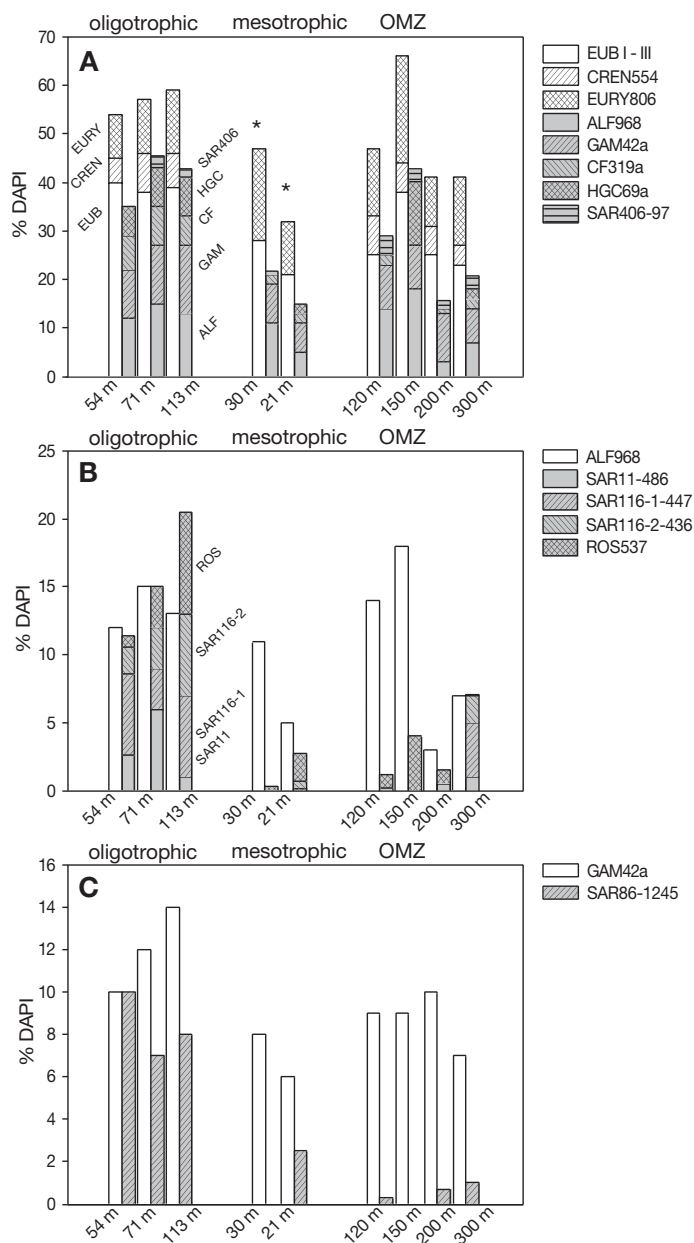
Fig. 3. Abundances of the different groups of picoplankton determined by FISH. *: CREN554 hybridizations not done; oligotrophic: samples from Stn A from mixed surface waters (54 m), the pycnocline (71 m) and below the pycnocline (113 m); mesotrophic: surface mixed water samples from Stn B (30 m) and Stn C (21 m); OMZ: samples from Stn B (120 and 150 m) and Stn C (200 and 300 m). (A) Hybridizations with general group-specific probes; (B,C) hybridizations with probes specific for subgroups of (B) Alphaproteobacteria and (C) Gammaproteobacteria; probes are listed in Table 1

present in the OMZ (1 to 6%). *Roseobacter* counts were similarly low in the 3 water masses studied. The percentage of the Gammaproteobacteria was slightly higher in oligotrophic waters (10 to 14%) compared to mesotrophic waters (6 to 9%) and the OMZ (7 to 10%) (Fig. 3A,C). Within the Gammaproteobacteria, the SAR86 clade accounted for more than half of the Gammaproteobacteria in oligotrophic waters (7 to 10%) but were virtually absent in mesotrophic waters and OMZ (<3%) (Fig. 3C). Counts of members of the Cytophagales were higher in oligotrophic waters (6 to 8%) than in mesotrophic waters (<2%) (Fig. 3A). The probe HGC69a specific for Actinobacteria gave significant counts in oligotrophic waters (6 to 8%), whereas the Actinobacteria were <2% in mesotrophic waters (Fig. 3A) but high (13% at 150 m) in the top part of the OMZ. Members of the uncultured SAR406 cluster could not be detected in oligotrophic waters and were <1% in mesotrophic waters (Fig. 3A). Their relative abundances ranged between 2 and 4% in the OMZ.

DISCUSSION

Picoplankton composition

On the level of photoautotrophic picoplankton the contrasting waters of the Arabian Sea are characterized by a pronounced switch between *Prochlorococcus* and *Synechococcus*, which are dominant in the oligotrophic southern and the mesotrophic northern waters, respectively (e.g. Campbell et al. 1998, Tarran et al. 1999, Zubkov et al. 2003). The cloning and FISH data obtained in this study support the hypothesis that differences also exist in the heterotrophic picoplankton composition.

This is, for example, evident for SAR11. Sequences related to this clade of Alphaproteobacteria dominated the LNA fractions of the oligotrophic *Prochlorococcus*-dominated southern province. This is in line with the small cell size and low DNA content reported for SAR11 by Rappe et al. (2002). SAR11 sequences also dominated the clone library of the sorted small LNA cells from the OMZ. Both findings are in agreement with those of Riemann et al. (1999) conducted in the same area, who found SAR11 sequences in surface waters and below 200 m water depth by DGGE analysis. The cloning results were confirmed by the newly designed and tested probe SAR11-441. SAR11 cells could be detected at the *Prochlorococcus*-dominated Stn A with relative abundances of 6%, but was low at the OMZ (1% and below). The corresponding absolute cell numbers of 2.6×10^7 l^{-1} were in the range found below the photic zone in samples from the Western Atlantic by Morris et al. (2002). Interestingly,

cells could be detected in the OMZ (<1%) and none in the surface mesotrophic waters. Members of the SAR116 clade reached relatively high percentages of 6 to 12% in the oligotrophic waters. They were not detected in the mesotrophic surface waters but were

SAR11 was neither detected by sequencing nor FISH in the mesotrophic *Synechococcus*-dominated surface waters. *Pelagibacter ubique*, recently isolated as the first cultured member of the clade SAR11 (Rappe et al. 2002), grows preferentially under oligotrophic conditions. Why *Pelagibacter*-related bacteria are present in the OMZ has still to be further investigated and perhaps has something to do with the history of the OMZ water formation. Alternatively, it could be due to seasonality of these organisms, which was observed in Atlantic waters (Malmstrom et al. 2004).

Another still uncultured alphaproteobacterial group, the SAR116 clade, was first described by Mullins et al. (1995) for the Sargasso Sea. Members of this cluster were later detected in both Pacific and Atlantic coastal waters (Rappe et al. 1997, Suzuki et al. 2001). With the 2 newly designed probes for the SAR116 cluster they could be detected in substantial amounts (up to 12%) in all depths at the oligotrophic Stn A. It seems that SAR116 is not restricted only to oligotrophic conditions. They were also found in the HNA sorted clone library from mesotrophic surface waters, but could not be detected by FISH. Interestingly, SAR116 cells were encountered in the same water sample from 300 m depth in the OMZ, where SAR11 cells were found. The co-occurrence of both groups in this particular water sample and the absence of these 2 groups in other samples from the OMZ suggest a patchy distribution in an otherwise homogeneous water body. To investigate such a fine-scale distribution of picoplankton groups, the water column has to be examined at higher resolution. Patchiness might also be the reason why, based on FISH data, Actinobacteria peaked in the OMZ at 150 m at Stn B (13%) and were below 2% abundance at other stations in the OMZ. In the LNA clone library from mesotrophic waters most clones were affiliated with an uncultured cluster of marine Actinobacteria (HGC 1) (Crump et al. 1999, Rappe et al. 2000, Kelly & Chistoserdov 2001). HGC 1 could also be found in the sorted HNA I fraction in the oligotrophic stations. Unfortunately, probe development was not successful for that group of Gram-positive Bacteria. No FISH signals could be retrieved with any of the newly designed probes. This could be due to insufficient probe target sites accessibility (Behrens et al. 2003). It is also possible that the diversity within the HGC I is high and the picoplankton groups targeted with the developed probes were only present in low abundance.

Sequences affiliated to the clade SAR406 were found in almost all of the clone libraries. Consequently, a specific probe for this group was designed, tested and applied. SAR406 is a deep branching group within the Firmicutes and was first detected in the Atlantic and the Pacific Oceans (Gordon & Giovannoni 1996). By dot blot hybridisation Gordon & Giovannoni (1996)

detected up to 3% of SAR406 at Bermuda and up to 1% off the Oregon coast at depths below the chlorophyll maximum. They were able to show a strong correlation between low chlorophyll concentration and high abundance of SAR406 in surface water samples. Similarly, in samples from the Arabian Sea, SAR406 could be detected by FISH only in samples below the chlorophyll maximum (2 to 4%).

Members of the *Roseobacter* clade were only found in the clone libraries of the sorted large cell fractions (HNA I and HNA II) of the picoplankton in oligotrophic waters. FISH confirmed the large cell size. Previously, members of this clade were shown to be linked to phytoplankton blooms in mesotrophic oceanic regions (Gonzalez et al. 2000, Zubkov et al. 2001a), but they were also found in oligotrophic regions (Fuhrman et al. 1993). Our FISH counts varied from below 1 to 8% and showed no clear relationship with a trophic regime. *Roseobacter* cells were present even at the low oxygen concentrations in the OMZ.

The hypothesis of significant differences in picoplankton composition is also supported by data on the gammaproteobacterial SAR86 cluster. This clade seems to flourish only in oligotrophic *Prochlorococcus*-dominated waters. Although they were detected in low numbers by FISH in the mesotrophic and oxygen-depleted waters, SAR86 were one of the most abundant clone types in the HNA I sorted population at Stn A and had high counts were obtained by FISH at the same station.

The clone libraries obtained from the OMZ contained some sequences absent in the fully oxygenated waters. One cluster comprised sequences related to those of symbiotic bacteria from *Calyptogena* and *Riftia*, oxidising reduced sulfur compounds. Test hybridisations with specific probes for these clusters failed. An explanation might be a low *in situ* abundance. Populations below 1% of all DAPI-stained picoplankton cells are difficult to detect by FISH. Interestingly, sequences related to the above mentioned groups were also retrieved from a water sample taken in the suboxic zone of the Namibian upwelling region (e.g. clone ZA2329c, accession number AF382101, B. M. Fuchs et al. unpubl.). Another surprise was the retrieval of clones related to sulfate reducing bacteria of the genera *Desulfosarcina* and *Desulfofrigus*. Similar sequences have been retrieved before in OMZ waters below 200 m (Riemann et al. 1999). It has been previously reported that sulfate reducing bacteria can survive under suboxic conditions (Santegoeds et al. 1998, Minz et al. 1999). We therefore speculate that at certain times and certain locations, specific bacterial populations might catalyze sulfate reduction and sulfide oxidation in the water column of the Arabian Sea.

Methodological aspects

Flow cytometric sorting was effective in subdividing total picoplankton diversity. The sorting of orange fluorescent cells resulted almost exclusively in sequences related to *Synechococcus* group A. *Prochlorococcus* cells are typically larger than an average picoplankton cell. This explains why they were retrieved in the large HNA II population of the oligotrophic station, Stn A. Clone libraries of sorted heterotrophic picoplankton cells were often dominated by a single clade. FISH confirmed for example that SAR11, the most frequent clone type in the LNA sorted fraction, was indeed present in high numbers in oligotrophic waters. In other cases like the SAR116 the high numbers detected by FISH in the oligotrophic Stn A were not mirrored by high clone frequencies in any of the clone libraries from that region. We conclude that even after enrichment of subpopulations by flow cytometry, a PCR bias might have occurred (Wintzingerode et al. 1997, Curtis et al. 2002).

Our inability to detect all of the picoplankton cells by CARD-FISH might be mainly due to an overfixation of the cells. The possibility cannot be excluded that some of the samples were shortly defrosted during transportation or storage and therefore the fixation process of the cells continued. Overfixation makes cells impermeable for HRP-labeled oligonucleotide probes even after a vigorous permeabilization treatment. Tests with other samples, which were allowed to defrost for 24 h, showed a decline in hybridisation efficiency of about 20 to 30 % (B. M. Fuchs unpubl.). It is therefore of paramount importance to ensure a continuous transportation at –20°C to maintain the quality of the samples.

CONCLUSIONS AND OUTLOOK

Recent studies indicate that the diversity of marine picoplankton is high but not infinite (e.g. Venter et al. 2004). Hagström et al. (2002) estimated a maximum number of approximately 1200 bacterial species present in the world's oceans. The same study speculates that there might be a maximum of 50 dominant species present in the bacterioplankton community. In order to obtain a better understanding of seasonal and regional differences in picoplankton composition it is necessary to move from a qualitative assessment of species presence to the exact quantification of specific cell number and biomass of defined microbial taxa. In the present study we started to quantify subgroups of the heterotrophic picoplankton in contrasting waters of the Arabian Sea. We found evidence that, although the same proteobacterial subgroups are present (e.g. SAR11), they show pronounced differences in relative

and absolute abundance. In the future, automated FISH counting will facilitate the study of fine-scale distributions of picoplankton groups at high resolution (Pernthaler et al. 2003, Sekar et al. 2004).

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