An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments

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Abstract

In situ identification of prokaryotic cells in subsurface sediments is hampered by the low cellular rRNA contents of the target organisms. Fluorescence in situ hybridization with catalyzed reporter deposition (CARD–FISH) has the potential to overcome this limitation, and was therefore optimized for a 40 cm deep sediment core sampled from a tidal sandy flat of the German Wadden Sea. Treatment with methanol and H_2O_2 inactivated endogenous peroxidases and effectively reduced the background signal. Percentage of DAPI stained cells detected with the probe combination EUB(I-III), targeting nearly all the *Bacteria,* were comparable for CARD–FISH with a horseradish peroxidase (HRP)-labeled probe and FISH with a fluorescently monolabeled probe in the 2–3 cm depth interval (92% and 82%, respectively), but significantly higher with the HRP-labeled probe at 35–40 cm, the deepest layer sampled (63% with HRP vs. 26% with monolabeled probe). With CARD–FISH *Alphaproteobacteria* and the *Desulfobulbaceae* group of sulfate-reducing bacteria were detected only in the upper layers. In contrast, *Desulfosarcinales,* the *Bacteroidetes* group, *Planctomycetes, Betaproteobacteria,* and *Gammaproteobacteria* were found at all depths. Archaea were detectable with ARCH915-HRP after achromopeptidase treatment. Surprisingly, aggregates of Bacteria and Archaea were found, below 12 cm depth, that strongly resemble consortia involved in anoxic oxidation of methane that have previously been found in sediments near methane hydrate deposits. With the optimized CARD–FISH protocol, microbial populations could also be detected in deeper sediment horizons. Furthermore, the intensity of the CARD–FISH signals improved detection of rare organisms such as Archaea.
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1. Introduction

Comparative 16S rRNA sequence analysis and fluorescence in situ hybridization (FISH) are key methods for studying the diversity and composition of complex microbial communities [1]. Since the first application to marine sediments by Llobet-Brossa et al. [2], FISH has been frequently used to directly quantify specific microbial communities in sediments [3–7]. For example, in sediments from an intertidal mud flat in the German Wadden Sea, depth profiles of sulfate-reducing bacteria (SRB) showed a peak at 0.5–2 cm, corresponding with a near-surface peak in sulfate reduction rates, and local minima of potential electron donors such as acetate and lactate [4]. However, a recent review showed that, among environmental samples, detection frequencies (relative to total DAPI-stained cells) with the general bacterial probe EUB338 [8] were lowest for sediments – often less than 40% [9]. Populations of bacterial-sized Eukarya and Archaea in Wadden Sea sediments were

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small in this and a previous study [2] so most DAPI-stained cells are expected to be Bacteria. In addition, FISH detection generally decrease sharply with sediment depth [2,4,6]. Low cellular rRNA contents likely make many sediment microbes undetectable by FISH (e.g. [6]).

The sensitivity of FISH can be improved by combination with catalyzed reporter deposition (CARD–FISH; [10–12]). Probes for CARD–FISH are labeled with the enzyme horseradish peroxidase (HRP), which catalyzes the deposition of fluorochrome-labeled tyramides within the target cells. Recent publications have demonstrated increased detection for bacterial cells compared to standard methods [10,13]. Here we present an optimization of this method for marine sediments, using a 40 cm core from a sandy tidal flat sediment of the German Wadden Sea. Modifications included inactivation of endogenous peroxidases, achromopeptidase digestion to permeabilize proteinaceous cell walls; and higher hybridization and tyramide reaction temperatures to increase specificity and sensitivity.

Preliminary observations indicated that endogenous peroxidase activity (EPX) may produce background fluorescence in our samples. In shallow intertidal pools, where photosynthetic organisms produce high concentrations of oxygen, H_2O_2 is photochemically generated [21]. Permeable sandy sediments allow H_2O_2 to penetrate to deeper layers. Organisms living in such zones therefore may have active peroxidases or catalases to detoxify H_2O_2.

The standard cell permeabilization protocol uses lysozyme. This enzyme may not be effective for microorganisms with unusual cell wall structures, such as *Planctomycetes* and Archaea [14], which are considered important members of marine microbial communities (e.g. [7,15,16]). The *Planctomycetes* have strong proteinaceous cell walls [17,18], but gave bright hybridization signals in this study without any additional treatments. Archaeal cell walls are diverse: their components may include protein or glycoprotein surface layers (S-layers), pseudomurein, or methanochondroitin. The amino acids, sugars, and glycosidic bonds between sugar residues in these compounds often differ from those typical of bacteria [19]. Since most marine Archaea detected with 16S rRNA-based techniques belong to phylogenetic lineages with no cultured representatives as yet, their cell wall constituents remain unknown. Lysozyme has been reported to permeabilize the cell walls of *Methanosarcina mazei* [20], and protease might be effective for proteinaceous cell walls.

In previous studies, hybridization and washing temperatures of 35–37 °C were adopted in CARD–FISH to avoid thermo-inactivation of the HRP label [10–12,22,23]. The effect of this lower temperature on the hybridization stringency can be compensated for by addition of formamide. Higher hybridization and washing temperatures are nevertheless desirable, especially in highly complex samples such as marine sediments, to achieve high specificities. High hybridization temperatures also facilitate more rapid hybridization, since lowering the formamide concentration decreases the viscosity of the hybridization buffer [24]. We have therefore examined the effects of higher temperatures on hybridizations with HRP-labeled probes.

2. Materials and methods

2.1. Reference strains

The reference strains *Azospirillum brasilense* (DSM 1690), *Alcaligenes faecalis* (DSM30030), *Escherichia coli* (DSM 498), *Comamonas testosteroni* (DSM 50244), and *Pseudomonas pseudoalcaligenes* (LMG 1225) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Pirellula* sp. SL 1 was kindly provided by Dörte Gade from the Department of Microbiology, Max Planck Institute for Marine Microbiology.

2.2. Sample collection and preparation

A 9 cm-diameter sediment core was taken at low tide in September 2002 from the sand flat Janssand, in the backbarrier area of the Friesian Island Spiekeroog, NW Germany. Sample preparation was based on Llobet-Brossa et al. [2], with minor modifications. The core was sliced into the following layers: 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–18, 18–20, 20–22, 22–25, 25–30, 30–35, 35–40 cm (top to bottom). Each layer was split in two vertically, with consistent orientation, and mixed well with a sterilized spatula. The sediment color changed from gray to black between 1 and 3 cm depth. Below 5 cm, in particular around 20 cm, but also in the 25–30 cm layer, the sediments contained muddy black spots, which are caused by local enrichments of organic rich matter, e.g., sedimented microalgae [25].

Samples (0.5 cm^3) of the slices were fixed in 4% paraformaldehyde (1 part 24% paraformaldehyde and 5 parts filtered seawater) overnight at 4 °C. Fixed samples were washed three times with 1 × PBS, with centrifugation at 16,000g for 10 min between washes, and stored in PBS/ethanol (1:1) at −20 °C until further processing. Samples (100 μl) were then diluted with 900 μl of PBS/ethanol, and dispersed by sonication at minimum power for 20 s with a sonication probe MS73 (Sonopuls HD70; Bandelin, Berlin, Germany). The sonicated samples were mixed with PBS, 0.001% SDS and 0.1% agarose at 55 °C. Ten microliters of the sample suspensions were pipetted into the wells of Epoxy-printed 10-hole glass

slides (Carl Roth GmbH + Co., Karlsruhe, Germany) and dried at room temperature. The slides were dehydrated in ethanol (50% for 5 min, 80% for 1 min and 96% for 1 min), and then dried at room temperature.

2.3. FISH with Cy3-labeled oligonucleotide probes

Oligonucleotide probes labeled with Cy3 fluorochrome at the 5′ end were purchased from ThermoHybaid (Interactiva Division, Ulm, Germany). Hybridizations and microscopic counting of hybridized and DAPI (4′,6-diamidino-2-phenylindole)-stained cells were performed as previously described [2,10,26]. For each probe and sample, at least 700 DAPI-stained objects were counted.

2.4. Sample pretreatments for CARD–FISH

For bacterial cell wall permeabilization, samples were treated with lysozyme solution as previously described [10]. Spotted slides were covered with lysozyme solution (10 mg/ml of lysozyme [Bio Chemika, Cat. No. 62970], 50 mM EDTA and 0.1 M Tris–HCl), and incubated in a humid chamber at 37 °C for 1 h, washed with MilliQ water for 1 min, dehydrated in ethanol as described above, and stored at −20 °C until further processing. In order to permeabilize archaeal cell walls, the preparations were subsequently treated with achromopeptidase as previously described [13]. For inactivation of EPX, the slides were covered with 0.15% H_2O_2 in methanol for 30 min at room temperature, washed with 96% ethanol, and dried.

2.5. CARD–FISH

Samples were processed as previously described [10,13] with the following modifications. Each sample on the slide was covered with 10 μl of hybridization buffer [10] including 2% (wt/vol) blocking reagent (Roche Molecular Biochemicals, Mannheim, Germany). Stringencies were regulated for each probe by adjusting the formamide concentration (Table 1). Slides were hybridized in a humid chamber for at least 2 h at 35 °C, then washed in 50 ml of prewarmed washing buffer [10] at 37 °C for 30 min. Prior to tyramide signal amplification, the samples on the slide were equilibrated with PBS amended with 0.05% Triton X-100 for 15 min at room temperature. After carefully removing excess liquid by soaking up with blotting paper, slides were covered with the substrate mix [13], consisting of 1 × PBS, 0.1% blocking reagent, 0.0015% H_2O_2, and 1/500 parts of fluorescein-labeled tyramide, and incubated for 30 min at 37 °C. During the course of this study, a more effective amplification protocol (enhancer method), with a substrate mix containing 2 M NaCl and 10 μg/ml p-iodophenylboronic acid, was developed in our lab [27]. We used this protocol for detection of Archaea. After the signal amplification, slides were washed with PBS amended with 0.05% Triton X-100 for 15 min at room temperature and with sterile MilliQ water for 1 min. They were dehydrated in an ethanol series and finally dried. DAPI staining and microscopic observations were done as described above.

To eliminate the faint fluorescein signals of non-target strains with BET42a and GAM42a, we used a Cy3-labeled competitor instead of non-labeled competitor for the hybridizations. A mixing ratio of 3:1 Cy3-labeled competitor to HRP-labeled probe completely suppressed the background green fluorescence, probably due to Cy3 quenching of fluorescein emission.

For double staining with probes ARCH915 or EelMS932 and DSS658, two sequential hybridizations were performed. To minimize signal loss after the first hybridization, slides were washed with PBS/Triton-X

Table 1
HRP labeled oligonucleotide probes used in this study

Probe name	Specificity	Sequences (5′–3′)	FA[a] (%)	T_h[b] (°C)	Reference
EUB(I-III)	Bacteria	Mixture of following three probes	55	35	[51]
EUB338	Most Bacteria	GCT GCC TCC CGT AGG AGT			[8]
EUB338P	*Planctomycetes*	GCA GCC ACC CGT AGG TGT			[51]
EUB338V	*Verrucomicrobium* spp.	GCT GCC ACC CGT AGG TGT			[51]
NON338	Antisense of EUB338	ACT CCT ACG GGA GGC AGC	55	35	[52]
ALF968	*Alphaproteobacteria*	GGT AAG GTT CTG CGC GTT	60	35	[53]
BET42a	*Betaproteobacteria*	GCC TTC CCA CTT CGT TT	55	35	[53]
GAM42a	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	55	35	[53]
CF319	*Bacteroidetes*	TGG TCC GTG TCT GAG TAC	55	35	[54]
PLA886	*Planctomycetes*	GCC TTG CGA CCA TAC TCC C	55	35	[28]
DSR651	*Desulfobulbaceae*	CCC CCT CCA GTA CTC AAG	55	35	[55]
DSS658	*Desulfosarcinales*	TCC ACT TCC CTC TCC CAT	70	35	[55]
ARCH915	Archaea	GTG CTC CCC CGC CAA TTC CT	35	46	[56]
ANME1-350	ANME1	AGT TTT CGC GCC TGA TGC	60	35	[30]
EelMS932	ANME2	AGC TCC ACC CGT TGT AGT	20	46	[30]

[a] Formamide concentration in the hybridization buffer.
[b] Hybridization temperature.

for 1 min at RT, then with water for 1 min, and dehydrated in an ethanol series. Alexa$_{488}$- and Alexa$_{546}$-labeled tyramides were used for the first and second signal amplification, respectively.

2.6. Test for endogenous peroxidase activity

To check for EPX, the substrate mix for signal amplification was overlaid on the sample which had not been hybridized with an oligonucleotide probe and incubated at 37 °C for 30 min. Cells with fluorescent signals were counted microscopically.

3. Results

3.1. Total cell counts

Uneven distribution of sediment samples on glass slides can affect the accuracy of cell counts. Addition of SDS improved the spreading behavior of samples. In addition DAPI counts increased with SDS concentration, reaching a maximum at 0.001% SDS. Although 0.01% SDS resulted in similar counts and a more even distribution, some cells had diffuse DAPI signals at this concentration, probably due to the onset of cell lysis (not shown). Therefore, 0.001% SDS was used in all sample preparations. The vertical profile of total cell counts showed a maximum at 2–3 cm, then generally decreased with depth (Fig. 1(a)). Sediment samples with numerous black spots, especially in the 18–20 cm layer and a part of 25–30 cm horizon, showed higher cell counts than the other deep samples, which had fewer such inclusions.

In order to evaluate both methodological and biological variation in cell counts with the CARD–FISH protocol, three subsamples of each parallel sample from the 0–3 cm sediment layers were hybridized with HRP-labeled EUB(I-III) probe. The average DAPI counts of the parallel samples were 30.5 and 44.1 × 10^8 cells cm^{-3}, and the standard deviations were 1.34 and 2.62 × 10^8 cells cm^{-3}, respectively. The average detection frequency of EUB(I-III) positive cells of the parallel samples were 83.8% and 80.4% of DAPI counts, and the standard deviations were 0.78% and 2.59%, respectively. Variations within subsamples were small in all cases. Relatively large difference between the DAPI counts of the parallel samples suggests some biological variability. Biological variation may be caused by uneven distribution of black spots, especially in deeper sediment layers.

Counts with the FISH and CARD–FISH protocols were compared using HRP- and Cy3-labeled EUB(I-III) probes. The counts with both methods were highest at 2–3 cm depth, and then decreased with depth. The detection frequencies (relative to total DAPI-stained cells) were comparable in the upper layers of the sediment
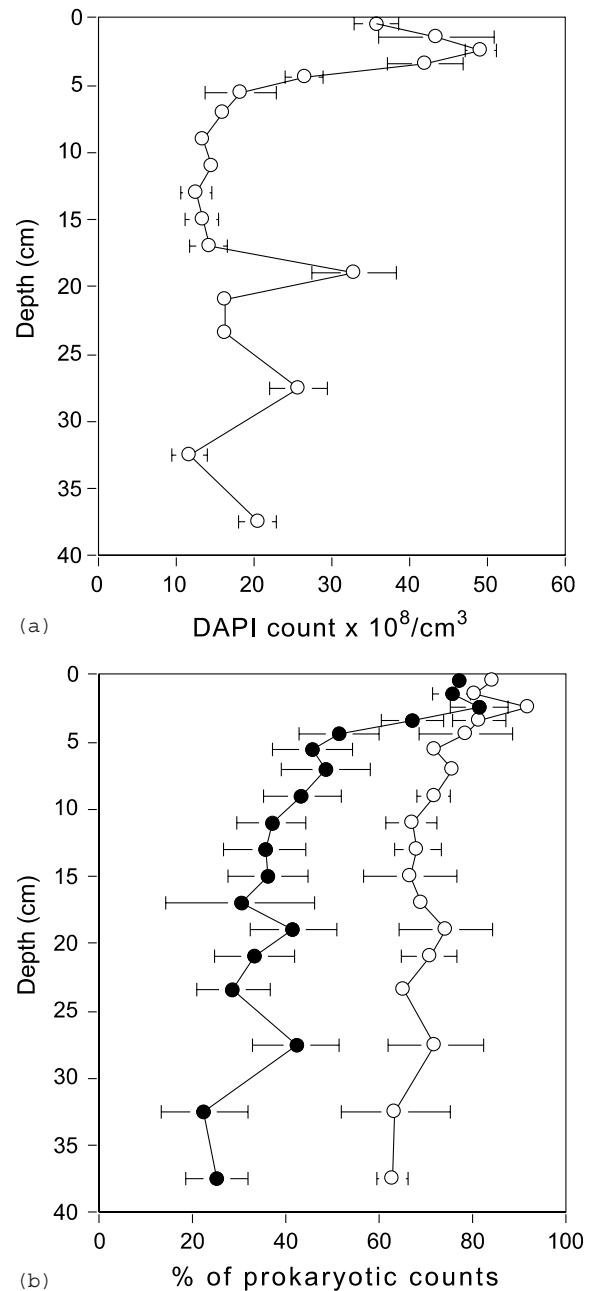

Fig. 1. Vertical profiles of (a) DAPI counts and (b) cells hybridizing with Cy3-labeled (solid symbols) or HRP-labeled (open symbols) EUB(I-III) probes as a percentage of DAPI-stained cells. DAPI-stained cells were counted at least two times per sample. Values for each probe are averages two parallel samples. Error bars indicate standard deviations.

ment (92.1 ± 1.3 vs. 81.5 ± 6.3% at 2–3 cm depth for CARD–FISH and FISH, respectively), but detection frequencies were more than two times higher with CARD–FISH in the deepest layer (62.9 ± 3.1 vs. 25.5 ± 6.7% at 35–40 cm depth, Fig. 1(b)). The observed signal intensities were not homogeneous from cell to cell at a given depth even with CARD–FISH. The general trend of decreasing detection with depth was less steep with CARD–FISH than with FISH.

3.2. Inactivation of endogenous peroxidase activity

Approximately 4% of DAPI-stained cells had visible green fluorescence signals after the CARD procedure without HRP-labeled probe (data not shown). The signal intensities decreased with depth. These signals are considered to have originated mainly from EPX. To inactivate this background fluorescence, we examined treatments such as long fixation (48 h) with 4% and 12% paraformaldehyde; high temperature (95 °C); and treatment with urea, HCl, 1% diethyl pyrocarbonate (DEPC) in PBS, or methanol with 0%, 1% and 3% H_2O_2 (21). All treatments other than the methanol treatment resulted in decreased DAPI counts, insufficient inactivation, or high background signals. Methanol treatments with different amounts of H_2O_2 showed effective inactivation, although high concentrations of H_2O_2 (>1%) resulted in lower total cell counts (Table 2). Therefore, methanol without H_2O_2 was initially used for EPX inactivation. However, this was not sufficient for the enhancer protocol (data not shown). Pretreatment with 0.15% H_2O_2 in methanol successfully inactivated the EPX without any loss of DAPI counts (Table 2). At the same time, increasing the concentration of blocking reagent in the hybridization buffer to 2%, and additional washing at 37 °C for 5 s in PBS with 0.05% Triton X-100 after the signal amplification step [27], substantially reduced the background fluorescence.

3.3. Increased detection of bacterial groups

Bright hybridization signals with the probe for *Planctomycetes* (PLA886) were observed with both a pure strain (*Pirellula* sp. SL 1; Fig. 2(a)), and in sediments without any pretreatment, although signal intensities with the pure strain were not homogeneous. PLA886-targeted cells in the sediment samples were morphologically uniform. They were coccoid and generally showed the circular FISH signals around a central DAPI spot reported previously for natural populations of PLA886-targeted cells [28]. This type of staining was not seen in pure cultures of *Pirellula* sp., where the DAPI and FISH
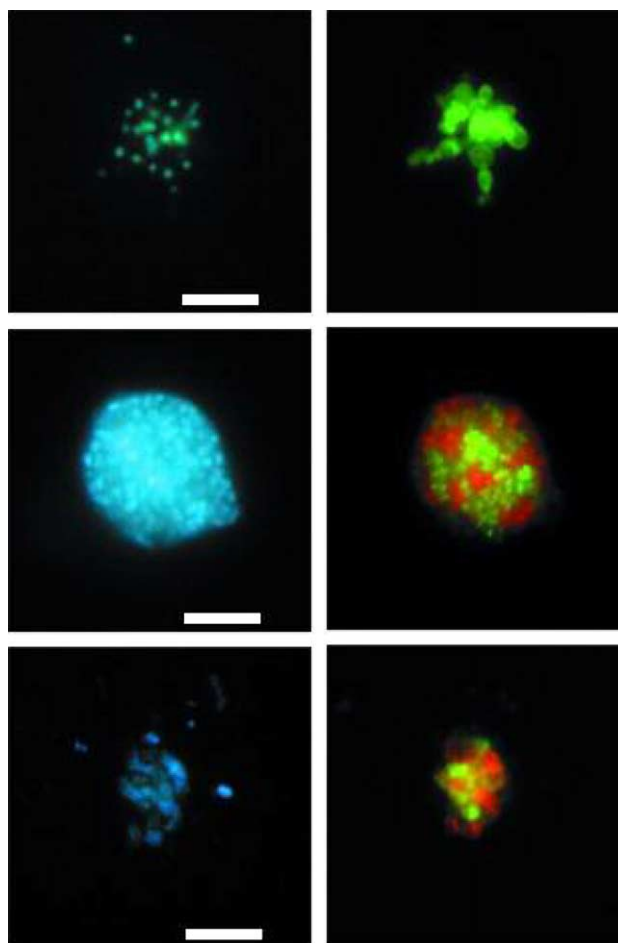

Fig. 2. Photomicrographs of CARD–FISH stained microorganisms. Left panels depict DAPI staining (blue) and right panels depict probe signals (green and red). (a) *Pirellula* sp. SL 1 hybridized with PLA886-HRP; (b) sediment (14–16 cm depth) hybridized with ARCH915 (green) and DSS658 (red); (c) sediment (14–16 cm depth) hybridized with EelMS932 (green) and DSS658 (red). Scale bar, 5 μm.

Table 2
Effect of methanol treatments on DAPI and EPX counts

Treatments	% DAPI counts after treatment [SD (n = 2)]	% EPX cell/DAPI [SD (n = 2)]
None	100	6.17 (0.7)
Methanol	98.7 (2.9)	0.1(0.07)
Methanol/0.15% H_2O_2[a]	103.5 (4.8)	ND[b]
Methanol/1% H_2O_2	88.7 (3.6)	0.07 (0.05)
Methanol/3% H_2O_2	55.5 (5.0)	0.03 (0.02)

[a] This treatment was combined with 2% blocking reagent in the hybridization buffer and hot washing (see text).

[b] ND – no EPX cells were observed in 20 microscopic fields.

signals are sometimes separated from each other and not circular. Probe PLA886 also hybridized with some diatoms. As already indicated before [28] this probe binds to many Eukaryotes. It perfectly matches with 185 of 210 *Bacillariophyta* 18S rRNA sequences in the database. However, significant morphological differences allowed for the discrimination of true *Planctomycetes* and eukaryotic algae [5]. High abundance of *Planctomycetes* was observed over the whole depth of the core, with a maximum of 9.6×10^7 cells cm^{-3} at 3–4 cm depth (Fig. 3(a)). Likewise, *Bacteroidetes, Betaproteobacteria, Gammaproteobacteria* and *Desulfosarcina* spp.-related cells (*Desulfosarcinales*) were observed throughout the whole depth. Abundances of *Bacteroidetes* ranged from 7.8×10^7 to 5.8×10^8 cells cm^{-3}, with a maximum at 2–3 cm depth (Fig. 3(b)). *Betaproteobacteria* showed a maximum in the surface layer (0 to 1 cm depth), with abundances ranging from 2.9×10^7 to 2.2×10^8 cells cm^{-3} (Fig. 3(c)). *Gammaproteobacteria* were generally
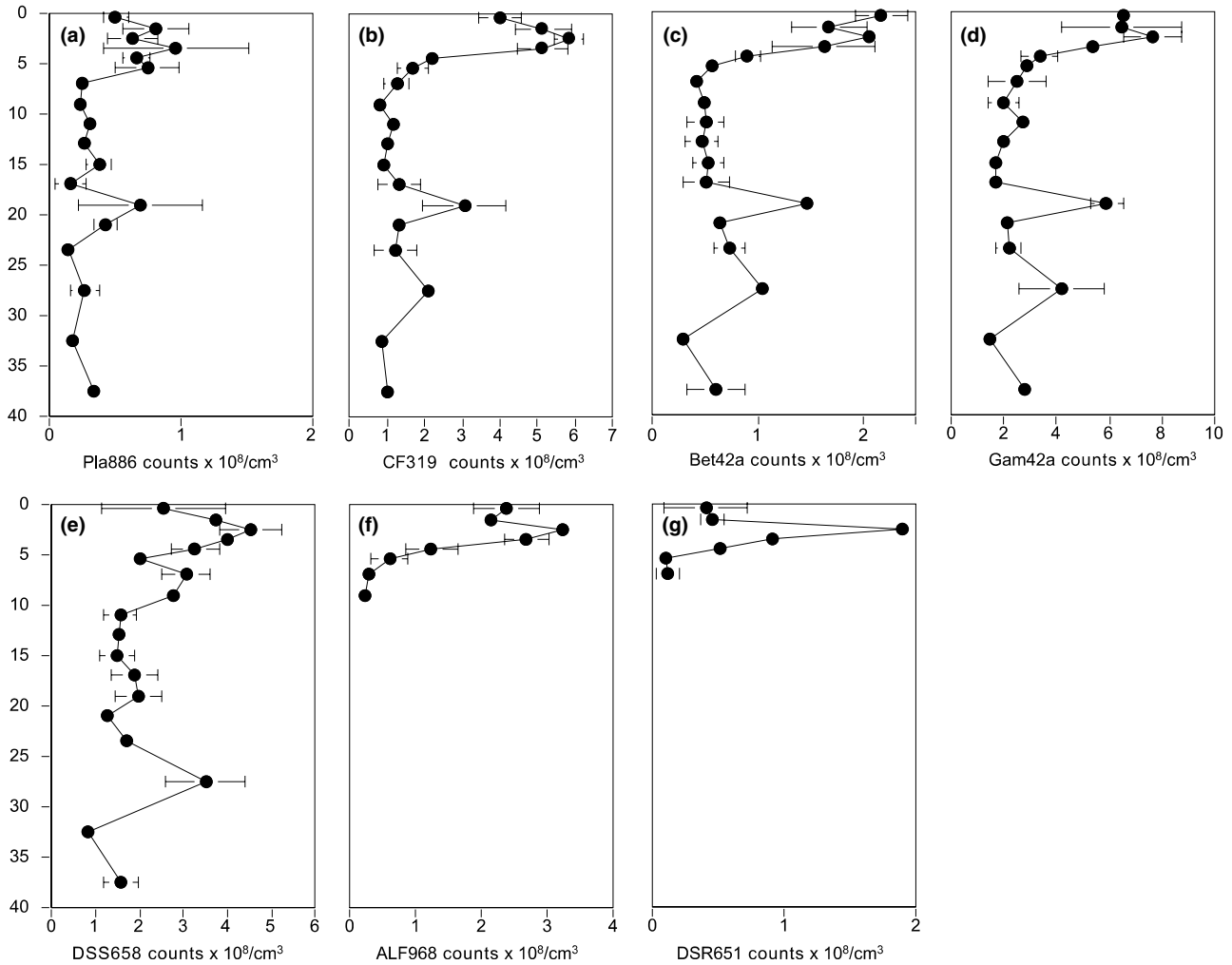
Fig. 3. Vertical profiles of counts with group-specific probes: (a) PLA886; (b) CF319; (c) BET42a; (d) GAM42a; (e) DSS658; (f) ALF968; (g) DSR651. Values are average counts of two parallel samples. Error bars indicate the standard deviations.

the most abundant group, ranging from 1.4 to 7.6×10^8 cells cm^{-3} (Fig. 3(d)). Interestingly, in the layer dense in black spots (18–20 cm depth), distinct peaks of cell numbers for all targeted populations except the *Desulfosarcinales* were observed. *Desulfosarcinales* ranged from 8.3×10^7 to 4.6×10^8 cells cm^{-3} (Fig. 3(e)). This group had the lowest relative abundance at 18–20 cm depth, comprising 6.0% of DAPI counts. In contrast, *Alphaproteobacteria, Desulfobulbaceae* were found only in the upper layer, with a maximum at 2–3 cm (3.2 and 1.9×10^8 cells cm^{-3}, respectively; Fig. 3(f) and (g)). The signal intensities of *Bacteroidetes, Betaproteobacteria* and *Desulfosarcinales* decreased with depth in spite of the use of HRP-labeled probes. On the other hand, most of cells hybridizing with the *Planctomycetes* and *Gammaproteobacteria* probes had similar signal intensities over the whole depth, suggesting relatively high rRNA contents even in deeper layers of the sediments. Bacteria such as *Desulfosarcinales, Planctomycetes* and *Gammaproteobacteria* were sometimes observed in large single-

group aggregates, which may cause methodological variation in the counting. The sum of all tested groups accounted for 30–52% of DAPI counts. The general Archaeal probe ARCH915 was also tested. At this point, even with enzyme pretreatments, we did not observe signals bright enough for reliable identification of Archaea.

3.4. Thermo-stability of HRP-labeled probes

To examine the thermo-stability of probe-conjugated HRP, hybridization/washing at higher temperatures were tested. Surprisingly, the signal intensities after hybridization at 55 °C for 2 h and washing at 57 °C for 30 min were similar to those after hybridization at 35 °C for 2 h and washing at 37 °C for 30 min. Evidently hybridization at elevated temperatures does not inactivate probe-conjugated HRP, and conventional hybridization/washing temperature (46/48 °C) can be used. This thermotolerance also allowed the tyramide amplifi-

cation to be performed at high temperature. This might result in more efficient tyramide catalysis, explaining the increase in the EUB(I-III) detection frequencies (relative to total DAPI-stained cells) for the 30–35 cm depth sample from $71.2 \pm 0.45\%$ with a 37 °C incubation to $80.2 \pm 4.2\%$ with a 48 °C incubation. Signal amplification at more elevated temperature (55 °C) resulted in high background signals (data not shown), probably due to excess production of active tyramides or spontaneous reaction of tyramides with H_2O_2. We used this improved method for the detection of Archaea.

3.5. Detection of archaea

After the lysozyme and achromopeptidase treatments clearer hybridization signals from both single and aggregated cells were observed with ARCH915, although counts remained low with less than 1% of DAPI-stained cell counts. Interestingly, conspicuous microbial aggregates occurred in samples below 12 cm depth. Double hybridizations with probes ARCH915 and DSS658 confirmed the association of two different populations (Fig. 2(b)). HRP-labeled probes, ANME1-350 and EelMS932, were used to differentiate between ANME-1 and ANME-2 Archaea, which are distinctive phylogenetic groups considered to be involved in anaerobic oxidation of methane in methane seep sediments [30–32]. Only EelMS932-hybridized cells were observed (Fig. 2(c)). The aggregates were observed at most depths below 12 cm, except for the layers rich in black spots (18–20 cm depth). The concentration of aggregates was very low; e.g., there was about one aggregate per 5×10^4 DAPI counts in the 14–16 cm depth sample.

4. Discussion

With the optimized CARD–FISH method described here, benthic microbial populations – including Archaea – could be detected in both surface and deep sediment layers, even at low abundance. The optimization is mainly based on the inactivation of EPX with methanol treatment, improved cell wall permeability, and the use of higher temperatures during the hybridization and signal amplification steps.

Schallenberg et al. [38] found that silt and clay particles in sediment may mask cells and cause underestimation of direct counts if sample dilution is insufficient. This effect decreased with decreasing silt and clay content [38]. The sediments around the sampling site in the present study were primarily sand (e.g. [25,39]). The total prokaryotic cell numbers were relatively high, ranging between 12 and 49×10^8 cells cm^{-3}, which is in the higher range of counts reported for sandy sediments, including the Wadden Sea [2–4,40]. Therefore, we do

not believe our samples were significantly undercounted. The relatively high total counts in this study might be partly due to the improvement of cell detachment from sediment particles with SDS.

A combination of methanol treatment, inclusion of 2% blocking reagent in hybridization buffer and washing at 37 °C after the amplification step effectively suppressed background fluorescence. Although the abundance of the archaeal population was below the usual detection limit in sediments, which is about 1%, the effective suppression of background fluorescence enabled us to detect rare signals with archaeal probes.

Planctomycetes have attracted many researchers because of their unique phylogenetic position [42], metabolic activities [43,44] and morphology [29]. The tough proteinaceous cell walls of some species can maintain their original shape even after a 10% SDS treatment at 100 °C [17,18]. The clear CARD–FISH signals from both the pure strain and the samples, without any pretreatment, suggest that their cell walls allow penetration of HRP-labeled probes. The heterogeneity of FISH signals with the pure strain (Fig. 2(a)) suggests that further improvements in the protocol are possible. However, also the cell compartmentalization of *Planctomycetes* [29] may cause this variation.

With the CARD–FISH, vertical profiles of bacterial groups were clearly shown. A previous study suggested substantial populations of *Planctomycetes* multiplying in the anaerobic zone of sediments [5]. Our detection of *Planctomycetes* in much deeper zone supports this finding. A more systematic study is necessary to gain further insights into the diversity and function of marine *Planctomycetes*. Likewise, *Bacteroidetes*, *Betaproteobacteria* and *Gammaproteobacteria* were observed throughout the core. Although these functionally diverse groups have often been found in 16S rRNA gene clone libraries from marine sediments [16,45–48], and detected by FISH [2,4,5,7], their abundance in the deeper parts of sandy sediments has not been quantified previously. *Desulfosarcinales* were also abundantly identified throughout the sandy core. In contrast, *Desulfobulbaceae* were found only in the upper layer, with a maximum at 2–3 cm (Fig. 3(g)). The distributions of these SRB are described in detail in elsewhere [49].

The thermo-tolerance of the HRP probes shown in this study may be explained by the modification of the HRP during the conjugation with oligonucleotide probes. In the synthesis of HRP-labeled oligonucleotide probes, positively charged primary amino groups of HRP react with *N*-succinimidil *S*-acetylthioacetate [35]. This might neutralize the surface charge of HRP, resulting in a more stable tertiary structure. Actually this conjugation is similar to the chemical thermo-stabilization of HRP which is accomplished by neutralizing the positive charge of its free amino groups with succinimide ester [33,34].

Hybridizations with the HRP-labeled probes in this study were performed at 46 °C, the same temperature used in standard protocols for fluorescently-labeled probes [36]. Together with CARD amplification at high-temperature and application of the enhancer, this is currently the most sensitive FISH method. Archaeal cell could only be clearly visualized in subsurface sediment by this sensitive method and a special enzymatic cell permeabilization, suggesting low rRNA contents and dense cell walls. It came as a surprise, when, subsequently, aggregates consisting of ANME-2 Archaea and *Desulfosarcinales* could be detected which were morphologically highly similar to consortia involved in the anaerobic oxidation of methane [30,32,37]. In sandy tidal flats, the presence of consortia mediating the anoxic oxidation of methane might be explained by methane production in sulfate depleted organic rich areas, i.e., black spots [25]. Higher cell counts in the black spots have been observed in this study as well as a previous study [41]. Giani et al. [50] showed that mineralization of high organic loads in sandy marine sediments resulted in methane emission after sulfate was depleted. The methane produced may diffuse to more sulfate-rich sediment layers, thereby fueling the conspicuous DSS/ANME-2 consortia found. The low abundance of *Desulfosarcinales* in the layer dense in black spots (18–20 cm depth) is also in line with sulfate depletion. Microsensor studies to determine local concentrations of methane, sulfate and sulfide are planned. Another potential source of methane would be advective transport from deeper layers, which can, at this time, not be ruled out.

This study shows that by proper pretreatment of the samples and with an improved CARD–FISH protocol, even low-frequency microbial groups can be quantified in complex samples such as marine sediments. This method should also be useful for the investigation of prokaryotic populations in other environments.

Acknowledgement

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