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REPORT

Diverse communities of active Bacteria and Archaea along oxygen gradients in coral reef sediments

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Abstract Microbial communities inhabiting highly permeable sediments of Checker Reef in Kaneohe Bay, Hawaii, were characterized in relation to porewater geochemistry (O_2, NO_3^-, NO_2^-, NH_4^+, phosphate). The physiologically active part of the population, assessed by sequencing cDNA libraries of 16S rRNA amplicons, was very diverse, with an estimated ribotype richness ≥1,380 in anoxic sediment. Quantitative analysis of community structure by rRNA-targeted fluorescence in situ hybridization (FISH) indicated that the archaeal population (9–18%) was dominated by marine Crenarchaeota (5–9%). Planctomycetales were the most abundant group in the oxic and interfacial habitat (17–19%) but were a minority (<5%) in anoxic reef sediment, where γ-Proteobacteria were numerically dominant (18%). Another 9–14% of the microbial benthos belonged to β-Proteobacteria, predominantly within the order Nitrosomonadales, many cultured representatives of which are NH_4^+ oxidizers. The results of this study contribute to the phylogenetic characterization of benthic microbial communities that are important in organic matter degradation and nutrient recycling in coral reef ecosystems.

Keywords Coral reef sediment · DIN · Microbial community · Oxygen

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Introduction

Tropical coral reefs are among the most productive ecosystems and home to highly diverse marine biota, including a plethora of prokaryotic species. Bacterial and archaeal inhabitants of living coral have been investigated in the context of symbiotic relationships (Rohwer et al. 2002; Kellogg 2004; Wegley et al. 2004; Klaus et al. 2005) or coral disease and mortality (Breitbart et al. 2005; Kline et al. 2006; Ritchie 2006). Heterotrophic microbes in the underlying carbonate reef framework and of reef sediments are considered important in the remineralization of organic matter (OM) (Rasheed et al. 2003; Wild et al. 2005) and in the efficient recycling of nutrients that is crucial for sustaining high ecosystem productivity in extremely oligotrophic tropical seawater (D'Elia and Wiebe 1990; Capone et al. 1992; Tribble et al. 1994; Miyajima et al. 2001; Rasheed et al. 2002).

Many coral reef frameworks and their unconsolidated sediments are highly permeable structures, where waves, currents, and irrigating fauna can drive advective exchange between porewater and overlying seawater (Sansone et al. 1988; Tribble et al. 1992; Alongi et al. 1996; Haberstroh and Sansone 1999). For example, the porewaters of Checker Reef, a fully submersed patch reef in Kaneohe Bay, Hawaii, exhibited a deep oxycline, and their depth-integrated O_2 content increased with wave height (Falter and Sansone 2000a, b). Heterogeneity of physically and

biologically induced interstitial water flow generates short-term variability, microzonation, and steep local gradients in porewater geochemistry (Sansone et al. 1988; Alongi et al. 1996; Huettel et al. 1998; Precht et al. 2004). Efforts to characterize the benthic microbial communities are still in their infancy; a surface sediment of the Great Barrier Reef contained an estimated 30–140 operational taxonomic units (OTUs) in Bacteria, with maximum diversity at 3 cm depth (Hewson and Fuhrman 2006). The subsurface prokaryotic communities in geochemically distinct sediments of Moku o Loe, an inhabited reef island in Kaneohe Bay, Hawaii, showed a significant horizontal and vertical heterogeneity in denaturing gradient gel electrophoresis (DGGE) banding patterns and DNA-based clone library structure (Sørensen et al. 2007).

This study reports the first analysis of RNA in coral reef sediments, representing the currently active fraction of the benthic microbial population. RNA-based clone libraries were constructed to survey the identities of physiologically active Bacteria in Checker Reef sediments and to estimate their phylogenetic diversity. Fluorescence in situ hybridization (FISH) was applied to quantify the microbial community structure and its vertical heterogeneity. The communities in oxic, "interfacial" (suboxic, microoxic or temporarily oxic) and anoxic benthic reef habitats are compared in the context of the effects of O_2 on microbial community structure. Furthermore, putatively important microbial inhabitants are discussed with respect to their potential metabolic functions in the coral reef ecosystem.

Materials and methods

Study site, sample collection and preservation

Kaneohe Bay, Oahu, Hawaii (21°28′N, 157°48′W) harbors an extensive system of patch reefs at <2 m water depth that are covered with partially lithified carbonate sand and coral rubble (Tribble et al. 1990, 1992). These sediments are poorly sorted (size fractions from cobble to clay), with median grain sizes in the 5–10 mm range, and characterized by porosities of 0.3–0.5 and permeabilities of 10^{-8}–10^{-10} m^2 (Falter and Sansone 2000a; Hannides 2008). During most of the year, northeasterly trade winds drive surface waves across the reefs, with wave heights up to 0.5 m and frequencies predominantly between 0.10 and 0.18 s^{-1} (Tribble et al. 1992; Haberstroh and Sansone 1999; Falter and Sansone 2000a). In the sediment of Checker Reef, wave-driven exchange of porewater with the overlying water occurs within 2.1 d at 1 m depth (Tribble et al. 1992). Depending on wave action and sediment permeability, dissolved O_2 penetrates 15–50 cm into the sand, and concentrations of NO_3^- and NO_2^- peak at 15–25 cm depth (Haberstroh and Sansone 1999; Falter and Sansone 2000a, b).

On April 20, 2006, July 12, 2006 and May 21, 2007, porewater and sediment samples were collected from Checker Reef at 21°26.6′N, 157°47.6′W, using the well-point sampling method as previously applied to reef sands (Falter and Sansone 2000b; Sørensen et al. 2007). Briefly, a pointed stainless steel rod (1 m long, 1 cm diameter) and sleeve were driven into the sediment to the desired depth, at which the rod was removed, allowing porewater to seep into the sleeve. The top of the sleeve was sealed with a butyl rubber stopper fitted with a port, through which porewater was drawn into a 60 ml syringe. Samples were taken between 10 and 60 cm depth and from the overlying water. At each depth, the first 60 ml of porewater was discarded, before a second portion of 60 ml was retrieved for immediate measurement of O_2, and another 60 ml was stored in a polypropylene tube for transport, minimizing the headspace. The porewater volume withdrawn corresponded to that contained in a sediment sphere of radius 4.4–5.2 cm. Then the sleeve was driven 2–3 cm deeper into the reef to retrieve a small sediment sample, which was transferred from the bottom of the stake into a polypropylene tube. All samples were placed on ice during transport to the laboratory.

Within 3 h of sampling, porewater samples were syringe-filtered (pore size 0.2 μm), split into aliquots for NH_4^+, NO_3^-, NO_2^- and dissolved inorganic phosphate (DIP) analyses, and stored at −20°C. From each sediment sample, 2.0 g were placed in a 10 ml serum bottle, preserved with 3 ml of formaldehyde/acetic acid solution (2% each in filtered seawater), and stored at 4°C for later cell counts. For FISH, duplicate 0.5 cm^3 subsamples were put in 2 ml screw-cap tubes and incubated with 1.5 ml paraformaldehyde (3% in saline phosphate buffer, PBS) at 4°C for 4 h. After centrifugation (15,000 × g, 5 min, 4°C), the fixative was removed, and the sediment was washed three times with 1.5 ml PBS each, then preserved in 1 ml PBS/ethanol (50:50) and stored at −20°C. The remaining sediment volume was stored at −80°C for later extraction of nucleic acids and analysis of organic content.

Analyses of porewater solutes and OM content

Immediately upon sampling, porewater was added to a glass bottle until full, and its O_2 concentration was measured by an Orion 820 probe with a portable oxygen meter. Instrument readings were cross-calibrated with Winkler titrations (Winkler 1888) over a range of O_2 concentrations.

Concentrations of NH_4^+ in preserved porewater and seawater samples were determined spectrophotometrically by the phenol–hypochlorite method (Solórzano 1969).

Concentrations of NO_3^- and NO_2^- in preserved porewater and seawater samples were also measured spectrophotometrically (Bendschneider and Robinson 1952; Morris and Riley 1963). Where advisable (sulfidic smell or high NH_4^+ concentrations), zinc acetate was added to 1 mM to precipitate sulfide from NO_3^- samples before passing them over the catalytic column. DIP was analyzed by the molybdenum blue complexation method using ascorbic acid as the reductant (Murphy and Riley 1962; Koroleff 1976).

Sediment samples collected on July 12, 2006 were weighed into pre-combusted ceramic crucibles, dried under ventilation at room temperature for 3 days, and reweighed. The dry samples were then combusted at 550°C for 4 h and weighed again to determine their weight loss on ignition, considered equivalent to total organic matter (TOM) content (Santisteban et al. 2004).

Cell abundance

Microbial cells were extracted from the formaldehyde-preserved sediment samples by applying ultrasound in combination with acetic acid as suggested by Wild et al. (2006), with slight modifications. Briefly, samples were placed on ice and exposed to five pulses (5 s on, 10 s off) of ultrasound (22.5 kHz) emitted by the immersed tip of a probe (power output 7–8 W, Fisher Scientific Sonic Dismembrator 100). After settling of the sand grains, the supernatant was collected with a Pasteur pipette. The remaining sediment was shaken with 3 ml extractant (2% acetic acid in filtered seawater), let stand until sand grains settled out, and supernatant collected, with five repetitions. This sonication/washing procedure was repeated four more times, producing a combined supernatant of 105 ml cell suspension. The extract was stored at 4°C for less than 24 h until staining and enumeration.

Aliquots of cell suspension were stained with 4′–6′-diamidino-2-phenylindole (DAPI), concentrated on polycarbonate membrane filters (pore size 0.2 μm), and viewed under an epifluorescence microscope (Olympus BX51). Cell abundances were determined from 12 counting grids each on duplicate filters.

Whole-cell FISH

Fixed cells were extracted from the sediment as described for cell counts, but without sonication. Aliquots of the resulting cell suspension were concentrated on white polycarbonate membrane filters (0.2 μm pore size, Millipore), allowed to air dry, and stored in a petri dish at −20°C until hybridization. Filter sectors were incubated with Cy3-labeled oligonucleotide probes (Thermo Electron GmbH) and, if applicable, with equimolar amounts of unlabeled competitor oligonucleotide, for ~2 h in an equilibrated humidity chamber at 46°C (Manz et al. 1992; Snaidr et al. 1997). Information on the probes, their targets and optimal stringency applied is given in Table 1 and references therein. Excess reagent was removed with washing buffer at 48°C for 15 min. After rinsing with distilled water, the hybridized filter sectors were counterstained with DAPI, rinsed briefly with distilled water, air-dried in the dark, and mounted on glass slides in Citifluor™ AF87 (Citifluor Ltd.).

The slides were examined by epifluorescence microscopy under a magnification of 1,300×. On each of 1–3 filters, cells showing probe-conferred fluorescence and DAPI-stained cells in the same field of view were enumerated in 12–24 randomly chosen counting grids. In order

Table 1 Oligonucleotide probes applied in fluorescence in situ hybridization (FISH) analyses of carbonate sediments from Checker Reef

Target group	Probe	Stringency (% FA)	Reference
Neg. control	NON	35	Wallner et al. 1993
Archaea	Arch 915	20	Loy et al. 2002
Marine Crenarchaeota	GI-554	20	Manz et al. 1992
Bacteria (excl. anammox clade)	EUB 338 I–III	35	Daims et al. 1999
Planctomycetales (excl. anammox clade)	EUB 338 II	35	Daims et al. 1999
Anammox clade	Amx 368	15	Schmid et al. 2003
Phylum Bacteroidetes (excl. Flavobacteria)	CFB 719	30	Weller et al. 2000
Flavobacteria	CFB 563	20	Weller et al. 2000
β-Proteobacteria	BET 42a, unlabeled GAM 42a	35	Manz et al. 1992
γ-Proteobacteria	GAM 42a, unlabeled BET 42a	35	Manz et al. 1992
Phylum Nitrospirae	Ntspa 712	50	Daims et al. 2001
Nitrosomonadales	Nso 190	55	Mobarry et al. 1996

With exception of the competitors, all oligonucleotides were 5′-labeled with Cy3. Stringency refers to the concentration of formamide (FA) in the hybridization buffer

to compare the percentage of probe-detected cells to the percentage of false positives (NON 338), a *t*-test or a Behrens/Fisher test was applied, depending on the outcome of an F-test for possible differences in variance (Sachs 1997). All FISH data are given in % of DAPI-stained cells and represent the difference between the probe-related signal and the unspecific background signal. In comparisons of FISH data between different samples, the same statistical method was applied.

Extraction of RNA

From three sediment samples collected on July 12, 2006 at depths representing oxic, interfacial, and anoxic habitats, RNA was extracted by a combination of bead beating and phenol/chloroform treatment as described by Biddle et al. (2006). Briefly, samples were exposed to four iterations of increasingly harsh extraction by phenol/sodium dodecyl sulfate (SDS) and beating with 0.1 mm zirconia beads. After the combination of the aqueous extracts, RNA was transferred into chloroform, precipitated, washed with ethanol, and dissolved in nuclease-free water. Two-thirds of these extracts were immediately reverse transcribed into cDNA using the iScript™ system (Biorad) with random hexamer primers, whereas one-third of the RNA extract was retained for a negative control in downstream applications. Both RNA and cDNA were stored at −80°C until analysis.

Polymerase chain reaction (PCR)

PCRs of bacterial 16S rRNA, using cDNA templates, were performed on a Biorad MyiQ® thermocycler, with SYBR Green®-based real-time monitoring of the amplification. The PCR reactions contained 1 μM primers Bac 341f and Bac 521r (Sørensen et al. 2005), 3 mM Mg^{2+}, 0.4 mM of each dNTP, and 25 U ml^{-1} iTaq™ DNA polymerase (iQ SYBR Green® Supermix, Biorad). All reactions were subject to initial denaturation at 95°C for 3 min; then 30 cycles of 10 s at 95°C, 30 s at 58°C and 30 s at 72°C; followed by 3–5 min of final extension at 72°C. DNA of *Pseudomonas chlororaphis* ATCC 43928 was used for a positive control. The correct length and purity of the PCR products were verified by agarose gel electrophoresis. PCR products were stored at −20°C over night before further processing into a clone library.

Clone libraries

Clone libraries were established from amplificates of bacterial 16S rRNA in the three sediment extracts. These PCR products were ligated into the pCR®4TOPO vector, using the TOPO-TA Cloning kit (Invitrogen). The ligation products were then used to transform chemically competent *Escherichia coli* (OneShot®TOP10, Invitrogen). Transformants were grown over night at 37°C on LB agar/ampicillin (130 mg l^{-1}) plates. Plasmid preparation and sequencing were conducted by the University of Hawaii's Center for Genomics, Proteomics and Bioinformatics.

In order to determine the phylogenetic affiliation of the novel clones, their closest matches were sought in GenBank by BLASTN 2.2.18 (Altschul et al. 1997) and in RDP Release 9.60 by the Classifier (Wang et al. 2007) and Sequence Match tools. Novel sequences have been deposited into GenBank under accession numbers EU121687 – EU121844.

To estimate the community's ribotype richness from a sample of clones, a flat rank distribution approach (Lunn et al. 2004) was applied to the singleton-only libraries. For libraries consisting of singletons and doubletons, one could compute the $\hat{S}_{Chao1}$ estimator of species richness (Chao 1987), with corrective terms to account for undersampling bias (e.g., Kemp and Aller 2004; Chao 2005; Schloss and Handelsman 2005). This approach, however, is valid only where the singletons and doubletons represent the rare species of the community, i.e., the majority of phylotypes occur three or more times (Chao 1987, 2005), and therefore its application to the Checker Reef clone libraries was dismissed as misleading.

Results

Reef porewater geochemistry

The depth profiles of O_2, NO_3^-, NO_2^-, NH_4^+, and DIP concentrations in Checker Reef porewater samples are shown in Fig. 1. Dissolved O_2 was present down to 30–35 cm and 25–30 cm depth in April and July/May, respectively. Below this oxygenated layer, $[NH_4^+]$ and [DIP] became considerable, and a sulfidic smell was noticed. Porewater NO_3^- was depleted 0–5 cm deeper than O_2, and its maximum concentrations were recorded at 25–30 cm depth (Fig. 1).

For the following discussion, oxic habitats are defined by $[O_2] \geq 0.5\ [O_2]_{sat.}$, $[NH_4^+] < 3$ μM and the absence of dissolved sulfide. Sulfidic, O_2-free porewater characterized anoxic benthic habitats. Interfacial habitats are defined by $[O_2] < 0.5\ [O_2]_{sat.}$, peak $[NO_3^-]$, $[NH_4^+] < 10$ μM and the absence of olfactorily detectable sulfide from the reef porewater.

Organic material in the upper 60 cm of Checker Reef amounted to 3.2–4.0% (wet weight), without obvious variation over depth. The ratio of dissolved inorganic nitrogen (DIN) to DIP can be indicative of the quality of organic material degraded in the sediment. In Checker Reef porewaters, [DIN]:[DIP] ranged from 8 to 42 in July 2006 and

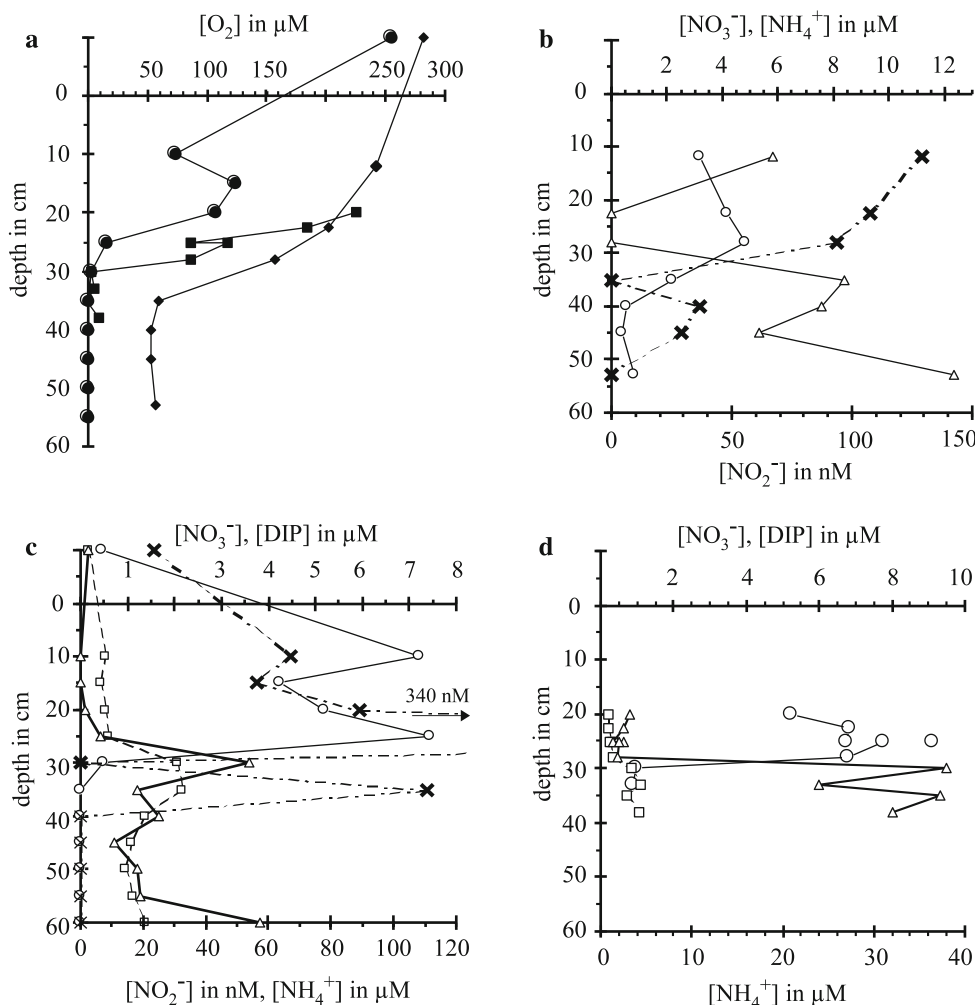

Fig. 1 Concentrations of O_2, NO_3^-, NO_2^-, NH_4^+ and dissolved inorganic phosphate (DIP) in interstitial waters collected from Checker Reef. (**a**) Depth profiles of [O_2] on April 20, 2006 (◆), July 12, 2006 (●), and May 21, 2007 (■); an apparent offset of 50–60 µM in April is likely due to air contamination during sampling. (**b**) Porewater concentrations of NO_3^- (○), NO_2^- (×), NH_4^+ (Δ) on April 20, 2006; DIP (□) was not measured. (**c**) July 12, 2006. (**d**) May 21, 2007; [NO_2^-] amounted to less than 1.5% of [NO_3^-]

from 24 to 52 in May 2007, without consistent trend with depth or relation to oxic, interfacial, and anoxic zones.

Microbial cell abundances are shown in Fig. 2. They were 10^8–10^9 (g sed)$^{-1}$, with larger numbers found in the top 10 cm and in the anoxic layer, while fewer cells occurred between 15 cm and 30 cm depth (Fig. 2). Microbial cell abundance was not significantly correlated with TOM or with any one of the porewater solutes analyzed.

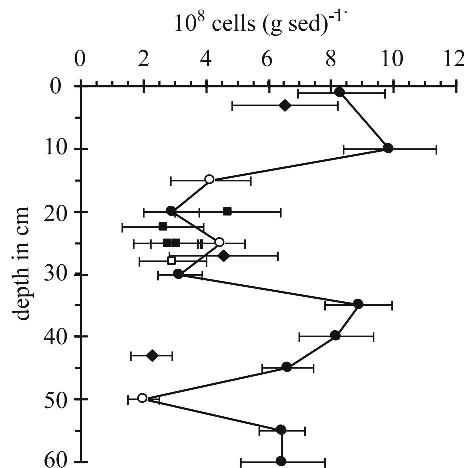

Fig. 2 Cell abundances in Checker Reef sediments on April 20, 2006: (◆); July 12, 2006: (●, ○); and May 21, 2007: (■, □). Error bars represent the standard deviation of 24 cell counts in the same sample. Open symbols mark where parallel samples were used for molecular analyses

Composition of the active microbial community

The physiologically active bacterial population of oxic, interfacial, and anoxic benthic habitats as defined above was surveyed by constructing clone libraries from environmental RNA extracts. Table 2 presents the resulting inventory for Checker Reef in comparison to DNA-based data from nearby Moku o Loe Reef (Sørensen et al. 2007). The bacterial ribotypes detected in Checker Reef were distributed over a broad range of phyla. Members of Chloroflexi, Acidobacteria, α-, γ-, and δ-Proteobacteria were well represented in these clone libraries; Bacteroidetes and unclassified neighbors of Cyanobacteria, while among the dominant groups in DNA-based Moku o Loe clone libraries, appeared to be minor contributors or absent from the RNA pool of Checker Reef (Table 2). Seven ribotypes

Table 2 Taxonomic affiliation of bacterial 16S rRNA phylotypes detected in cDNA from oxic, interfacial, and anoxic Checker Reef sediment

	% of all phylotypes within library					
	Checker Reef (RNA)			Moku o Loe Reef (DNA)		
	Oxic	Interfacial	Anoxic	Surface	35 cm depth	Anoxic
Cyanobacteria	2	6	0	19	0	0
Unclassified neighbors of Cyanobacteria	0	0	0	29	9	6
Chloroflexi	25	6	11	3	6	23
Bacteroidetes	0	2	0	16	4	8
Planctomycetes	4	6	2	10	13	6
α-Proteobacteria	6	14	5	6	3	6
β-Proteobacteria	0	2	0	0	7	0
γ-Proteobacteria	18	19	18	16	22	8
δ-Proteobacteria	4	11	18	0	12	21
Nitrospirae	0	2	5	0	3	4
Acidobacteria	33	22	5	0	13	4
Others	0	2	29	0	7	15
Unclassified	8	8	7			

Percentages refer to the total number of ribotypes found in the respective library (oxic: 51; interfacial: 63; anoxic: 44). Corresponding data based on DNA from Moku o Loe Reef sediments (Sørensen et al. 2007) are given for comparison. Both reef sites are located in Kaneohe Bay, about 0.7 nautical miles from each other. The total numbers of phylotypes reported in Sørensen's libraries were 31 (surface, oxic), 68 (35 cm depth) and 52 (75 cm depth, anoxic)

Table 3 Richness of operational taxonomic units (OTUs) in bacterial 16S rRNA clone libraries from Checker Reef sediment

Threshold identity	Library	Number of sequences occurring							Richness
		Once	Twice	Thrice	4 times	5 times	6 times	7 times	
≥99.5%	Oxic	49	2	0	0	0	0	0	51
	Interfacial	59	4	0	0	0	0	0	63
	Anoxic	44	0	0	0	0	0	0	44
	Combined	152	6	0	0	0	0	0	158
≥99.0%	Oxic	42	4	1	0	0	0	0	47
	Interfacial	54	3	1	1	0	0	0	59
	Anoxic	35	1	1	1	0	0	0	38
	Combined	129	9	3	2	0	0	0	143
≥95%	Oxic	33	5	1	0	0	0	1	39
	Interfacial	41	4	3	1	1	0	0	50
	Anoxic	24	5	0	1	0	1	0	31
	Combined	88	12	7	2	2	1	1	113

Sequence identity thresholds of 99.5, 99.0 and 95% were used as operational definitions of ribotype, species and genus level, respectively. Library sizes were 53 (oxic), 67 (interfacial), and 44 (anoxic) sequenced clones

(2, 3 and 2 from oxic, interfacial, and anoxic zones, respectively, all belonging to Chloroflexi or Acidobacteria) detected in Checker Reef sediment were closely related (95–98%) to phylotypes found at 35 cm or 75 cm depth in Moku o Loe Reef (Sørensen et al. 2007). Another three ribotypes from anoxic Checker Reef sediment, affiliated with δ-Proteobacteria, were closely related (92–94%) to an uncultured bacterium associated with *Montastrea franksi* in Bermuda (Rohwer et al. 2002).

For a quantitative description of diversity in the clone libraries, Table 3 presents the frequency distribution of sequenced clones that are unique (singletons), double, triple, or more clustered, when different threshold levels of sequence identity are applied. Sequences that show

≥99.5% identity, i.e., do not differ within the limits of analytical error, are termed ribotypes. Operational definitions of species and genus level are ≥99% (van Passel et al. 2006) and ≥95% sequence identity (Schloss and Handelsman 2004), respectively. At the ribotype level, the vast majority of sequences occurred only once, indicating that the diversity of the active bacterial community by far exceeded the size of the libraries. Applying a flat rank distribution approach (Lunn et al. 2004) to the singleton-only library from anoxic sediment yielded an estimate of ≥1380 different bacterial ribotypes in that habitat. The richness, i.e., number of different OTUs, among the sequenced clones was 86–88% and 70–74% of the library size at the species and genus level, respectively (Table 3). With the exception of one doubleton at the species level, overlaps between the three libraries occurred at no lower than genus level (Table 3).

Quantitative community structure

The quantitative structure of the microbial communities in oxic, interfacial, and anoxic Checker Reef sediments, as determined by FISH with group-specific, rRNA-targeted oligonucleotide probes (Table 1), is presented in Fig. 3. In July 2006, overall detection by the domain-specific probes was 58–66% of DAPI-stained cells, indicating similar levels of general physiological activity in all three communities. The relative abundance of bacteria did not change with depth ($p = 0.05$, t-test), whereas Archaea were more abundant in interfacial than in oxic and anoxic sediment ($p \leq 0.01$, Behrens/Fisher test, t-test). Two-thirds of the archaeal cells were identified as non-thermophilic marine Crenarchaeota. Note that only 50% of known sequences in this group are detected by the domain-specific probe Arch915, compared to 78%

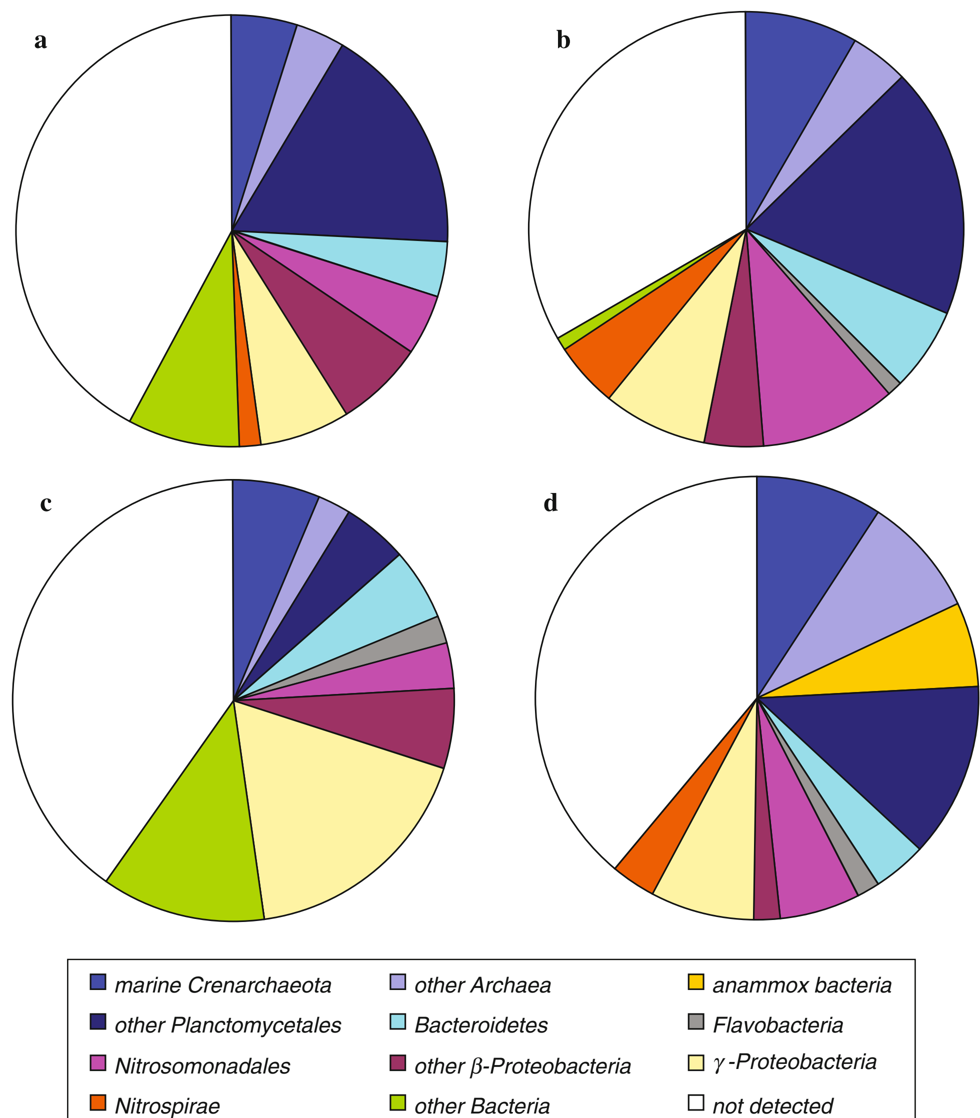

Fig. 3 Microbial community structure in Checker Reef sediments as determined by fluorescence in situ hybridization (FISH) analysis. (**a**) July 12, 2006: oxic sediment (15 cm depth); (**b**) July 12, 2006: interfacial sediment (25 cm depth); (**c**) July 12, 2006: anoxic sediment (50 cm depth); (**d**) May 21, 2007: interfacial sediment (28 cm depth). Note that anammox bacteria were only probed for in the May sample

coverage by the group-specific probe GI-554 (RDP-II database, version 8.1).

Planctomycetales were the most abundant bacterial group in the oxic and interfacial habitat, but comprised less than 5% of the community in anoxic sediment (Fig. 3). Their poor representation in the Checker Reef clone libraries (Table 2) can be explained by primer bias: only 47 of the currently 5,200 published 16S rRNA sequence fragments of planctomycetes contain the priming site complementary to Bac341f, the forward primer applied in the PCRs (RDP-II database, release 9.60). Another 9–14% of the benthic microbial population in Checker Reef belonged to the β-Proteobacteria, with a substantial share in the order Nitrosomonadales, especially in interfacial sediment (Fig. 3), where they were more abundant than in the oxic or anoxic habitat ($p < 0.01$, *t*-test). This apparently dominant group was severely underrepresented in the clone libraries (Table 2), without obvious blame to primer bias. Members of the γ-Proteobacteria were the most abundant group (18%) in anoxic sediment; they made up 7–8% of the inhabitants of oxic and interfacial sediment (Fig. 3). The relative abundance of γ-Proteobacteria was positively correlated with $[NH_4^+]$ in the porewater ($p < 0.05$, $n = 4$). Based on the 16S rRNA survey, γ-Proteobacteria in oxic and interfacial sediment were predominantly affiliated with the order Chromatiales, whereas Pseudomonadales abounded in the anoxic habitat. Bacteroidetes (excl. Flavobacteria) occurred at 4–6% of DAPI-stained cells in all three habitats, with slightly lower abundance in oxic than in interfacial sediment ($p < 0.05$, *t*-test). Even fewer Flavobacteria were detected. The phylum Nitrospirae was found mostly in the interfacial sediment, constituting 5% of the community there (Fig. 3). The relative abundance of Nitrospirae was positively correlated with $[NO_3^-]$ in the reef porewater ($p < 0.05$, $n = 4$).

In interfacial reef sediment collected in May 2007, overall detection by the domain-specific probes was 55% of DAPI-stained cells (Fig. 3d); note that the anammox clade is outside the phylogenetic coverage of these probes. Compared to the corresponding sample from July 2006 (Fig. 3b), the relative abundances of marine Crenarchaeota, Flavobacteria, and γ-Proteobacteria did not change ($p = 0.10$, *t*-test). Archaea were more abundant in May than in July ($p < 0.01$, *t*-test), while the relative abundances of all other groups probed for were higher in July than in May ($p < 0.05$, Behrens/Fisher test, *t*-test). Potential anammox bacteria constituted 6% of DAPI-stained cells in May 2007 (Fig. 3d) and they were not quantified in the July samples. Note that the probe Amx368 (Table 1) can also hybridize with the 16S rRNA of several microbes outside the anammox clade, mostly uncultured Planctomycetales and Archaea (SILVA database, Pruesse et al. 2007).

Discussion

Coral reef microbial benthos in oxic, interfacial, and anoxic habitats

Oxygen is involved in the mineralization of OM both as terminal electron acceptor of aerobic respiration and as oxidant for the reduced products of anaerobic respiration, such as NH_4^+ and sulfides. Metabolic pathways and the structure of the catalyzing microbial community are constrained by their redox environment. Porewaters of the highly permeable sediment of Checker Reef contained O_2 to a depth of $\sim$30 cm (Fig. 1) or more (Falter and Sansone 2000a). If O_2 consumption rates are as high as measured in various coral reef sediments (Sansone et al. 1990; Grenz et al. 2003; Rasheed et al. 2004; Wild et al. 2004a; Werner et al. 2006), then a concomitant high rate of O_2 transport is required. Oceanic swells and local waves in Kaneohe Bay drive interstitial flow in the upper 1 m of Checker Reef at vertical velocities of up to 216 cm d^{-1} (Tribble et al. 1992), providing an efficient mechanism of O_2 influx. The resulting deep oxic zone is a suitable habitat for a benthic population of obligately and facultatively aerobic Bacteria and Archaea.

Interfacial habitats are likely to favor a community of microaerophilic, aerotolerant, and facultatively aerobic species. Alternatively, a mosaic of oxic and anoxic microenvironments may be established, so that the microbial population in macroscopic samples of sediment will represent a blend of organisms belonging to the strictly aerobic and strictly anaerobic communities. Between the clone libraries of bacterial 16S rRNA from Checker Reef, with a combined size of 164 sequences, there were only one and three overlaps at species and genus level, respectively (Table 3). However, as these libraries are small compared to the estimated diversity of the natural communities (e.g., anoxic sediment: 44 clones, estimated richness $\geq$1,380), overlaps could have been missed.

The microbial community structure in oxic, interfacial, and anoxic benthic habitats of Checker Reef reflected the O_2-related ecophysiology of some detected groups. Planctomycetes, with the exception of anaerobic NH_4^+ oxidizing (anammox) bacteria, have been characterized as obligate or facultative aerobes (Fuerst 1995; Schlesner et al. 2004). Correspondingly, they were highly abundant in oxic and interfacial reef sediments, but much less so in the anoxic sediment (Fig. 3). Cultured representatives of Bacteroidetes appear exclusively anaerobic (Kirchman 2002), and in Checker Reef, members of this group were detected in oxic sediments at lower abundance than in the oxic/anoxic transition zone (Fig. 3). Members of the phylum Nitrospirae were almost absent from the anoxic habitat and reached significant abundance only in the interfacial

sediment of Checker Reef (Fig. 3). *Nitrospira* spp. are described as typically aerobic NO_2^- oxidizers (Daims et al. 2001; Koops and Pommerening-Röser 2001) and the dominant members of that guild in many systems (Revsbech et al. 2006). The apparent correlation between porewater [NO_3^-] and the abundance of Nitrospirae gives supportive evidence of their role as NO_2^- oxidizers in Checker Reef sediments. Further O_2-related differences in community structure were not resolved by the FISH analyses, as ecophysiology often varies at taxonomic levels lower than those targeted by the set of probes applied in this study. While the data from the clone libraries were phylogenetically more finely resolved, they lack information about abundance and suffer from considerable methodical bias (Table 2 vs. Fig. 3).

Potential metabolic roles of ribosome-rich reef microbes

The gross level of physiological activity of microbial cells is reflected in their ribosome content (Kemp et al. 1993; Kerkhof and Ward 1993), which in turn is a major limiting factor for occurrence in RNA-based clone libraries and for detection by rRNA-targeted FISH probes. The combined detection of Archaea and Bacteria in the FISH analyses of Checker Reef samples was 58–66% of DAPI-stained cells (Fig. 3) and appeared to be independent of the redox state of the porewater. For comparison, 13–84% of microbial cells were FISH-detectable in the few permeable sediments analyzed previously, all of them terrigenous silicate sands (Llobet-Brossa et al. 1998; Rusch et al. 2003; Ishii et al. 2004; Bühring et al. 2005). Aside from methodical limitations, these values may indicate that varying parts of the microbial population were temporarily inactive in response to the quickly changing porewater geochemistry of permeable sediments.

DNA-based molecular analyses manifest community structure by cell abundance and operon number, whereas RNA-based data reflect gene expression and growth rate. The census of sedimentary DNA pools (e.g., Sørensen et al. 2007) includes inactive and dead cells as well as extracellular DNA attached to the sand grains (Naviaux et al. 2005), whereas RNA-targeted methods probe the active fraction of the population. Between DNA- and RNA-based clone libraries established from the same sample of polluted soil (Nogales et al. 2001) or marine bacterioplankton (Moeseneder et al. 2005), fewer than 30 % of phylotypes were found in both libraries. The difference in outcome between DNA- and RNA-based surveys of coral reef bacterial benthos (Table 2) may be particularly pronounced due to rapid redox fluctuations that affect microbial activity and hence the RNA but not the DNA pool. In addition, there are reef-specific differences between Moku o Loe and Checker Reef, for example, in human impact and hydrographic regime. Both inventories provide valuable information on candidate groups or species to expect in the subsurface part of coral ecosystems.

Small suspended particles carried by interstitial water flows get trapped and efficiently degraded within the reef sediment (Rasheed et al. 2004; Wild et al. 2004a, b, 2005), presumably by heterotrophic Bacteria and Archaea. Benthic microorganisms occur at abundances of 10^8–10^9 cells g^{-1} (Fig. 2; Hansen et al. 1987; Wild et al. 2006; Sørensen et al. 2007), but the number of heterotrophically active cells remains unknown. Most phylogenetic groups detected in the RNA-based clone libraries and FISH analyses include heterotrophic members, and thus there is at least the potential for microbial OM degradation to be considerable. Checker Reef sediments were relatively rich in OM compared to sandy reef sediments elsewhere (Alongi et al. 1996, Rasheed et al. 2002, 2003, Schrimm et al. 2004). However, as TOM content was virtually invariant with depth, i.e., across a broad range of redox conditions and TOM influx rates, it is likely to represent refractory material, unavailable due to its chemical composition or to enclosure deep in the cavities and crevices of biogenic carbonate grains. Almost equally high TOM contents of 2.9–3.2% (wet weight) were measured in sediments from Hawaiian reefs at Kilo Nalu and Moku o Loe (data not shown). The porewater [DIN]:[DIP] ratio may indicate the source and state of degradation of the OM respired in the sediment, but is also influenced by denitrification and P binding onto mineral phases, especially $CaCO_3$ (Ogrinc and Faganeli 2006). The values for this ratio obtained in the present study, ranging between 8 and 52, envelope a previous estimate of 37 for Checker Reef porewater at 1 m depth (Tribble et al. 1990). Based on the data from Checker Reef (Haberstroh and Sansone 1999; Falter and Sansone 2000b), it has been argued that [DIN]:[DIP] in oxic reef porewaters is one-third to one-half of that in deeper porewaters (Atkinson and Falter 2003). Measurements from the present study cannot support this claim.

Chemoorganotrophs capable of hydrolyzing biopolymers, of fermenting or respiring organic compounds with NO_3^- or other electron acceptors are well represented in Planctomycetes (Fuerst 1995; Schlesner et al. 2004), Pseudomonadales, Oceanospirillales, β-Proteobacteria (excl. Nitrosomonadales), Bacteroidetes, and Flavobacteria (Shewan and McKeekin 1983; Cottrell and Kirchman 2000; Weller et al. 2000; Kirchman 2002). These prominent groups (Table 2, Fig. 3) could be involved in various steps and pathways of OM degradation in Checker Reef and Moku o Loe Reef sediments.

The benthic microbial communities of Checker Reef also hold the potential for chemoautotrophic metabolism, such as aerobic and anaerobic NH_4^+ oxidation. Nitrosomonadales,

with all cultured members characterized as chemolithotrophic or mixotrophic NH_4^+ oxidizers (Garrity et al. 2005), were the dominant fraction of β-Proteobacteria in Checker Reef. Marine Crenarchaeota, some of which carry genes for NH_4^+ oxidizing enzymes (Francis et al. 2007), constituted the major part of the archaeal benthos (Fig. 3). The significantly enhanced occurrence of both groups in interfacial sediment suggests that aerobic NH_4^+ oxidation may be an important bacterial and archaeal metabolism in habitats that experience the spatial or temporal proximity of both oxic and anoxic conditions. Bacteria with the putative capacity to oxidize NH_4^+ anaerobically with NO_2^- were present in the rRNA pool of interfacial reef sediment (Fig. 3d). More detailed investigations on these reef-dwelling anammox bacteria are underway in the authors' laboratory.

This ribotype survey and FISH quantification of physiologically active prokaryotes in permeable reef sediments suggest phylogenetically diverse benthic communities. Members of all detected groups have been found previously in temperate marine environments, but all Checker Reef ribotypes were novel. It remains an open question whether these reef microbes are endemic to reefs (i.e., specialized at the species level) or are cosmopolitan species forming assemblages that are reef-specialized at the community level. The microbial community structure in oxic, interfacial, and anoxic benthic habitats of Checker Reef largely reflected the O_2-related ecophysiology of most detected groups. Members of several abundant groups are likely contributors to the benthic decomposition of OM and regeneration of nutrients that are crucial for sustaining the high productivity of coral reefs in oligotrophic tropical marine environments.

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