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Colonization in the Photic Zone and Subsequent Changes during Sinking Determine Bacterial Community Composition in Marine Snow

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Due to sampling difficulties, little is known about microbial communities associated with sinking marine snow in the twilight zone. A drifting sediment trap was equipped with a viscous cryogel and deployed to collect intact marine snow from depths of 100 and 400 m off Cape Blanc (Mauritania). Marine snow aggregates were fixed and washed *in situ* to prevent changes in microbial community composition and to enable subsequent analysis using catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). The attached microbial communities collected at 100 m were similar to the free-living community at the depth of the fluorescence maximum (20 m) but different from those at other depths (150, 400, 550, and 700 m). Therefore, the attached microbial community seemed to be "inherited" from that at the fluorescence maximum. The attached microbial community structure at 400 m differed from that of the attached community at 100 m and from that of any free-living community at the tested depths, except that collected near the sediment at 700 m. The differences between the particle-associated communities at 400 m and 100 m appeared to be due to internal changes in the attached microbial community rather than *de novo* colonization, detachment, or grazing during the sinking of marine snow. The new sampling method presented here will facilitate future investigations into the mechanisms that shape the bacterial community within sinking marine snow, leading to better understanding of the mechanisms which regulate biogeochemical cycling of settling organic matter.

The formation of macroscopic organic aggregates such as marine snow is an important process for the removal of photosynthetically fixed carbon from the surface ocean to the deep sea (1). The export of organic matter is the main driver for the oceanic sequestering of atmospheric carbon dioxide on long time scales (2).

Settling marine snow aggregates harbor diverse microbial communities that play an important role in the degradation of the organic compounds and release of dissolved nutrients and organic matter to the water column (3–5). Therefore, the attached microbial communities may play an important role in the regulation of the magnitude and efficiency of the biological carbon pump. However, little is known about bacterial community composition and dynamics within marine snow collected below the depths reached by scuba divers. This is because the fragile nature of marine snow makes the collection of individual, intact marine snow particles nearly impossible.

Previous investigations have relied on size fractionation of water samples to observe and compare clustering of free-living bacteria to that of bacteria associated with particles (5–8). These studies observed that the composition of the microbial communities associated with particles differed from that of free-living communities in the surrounding water column. However, other studies have found similarities between the free-living and attached microbial communities (9–11), potentially indicating methodological pitfalls in the separation of free-living and attached fractions of the microbial communities by fractionated filtration. Further, fractionated filtration does not select for sinking particles but includes both suspended and sinking particles above a certain size.

Until now, most studies on attached microbial communities from depths below scuba range were done not directly on aggregates collected *in situ* but through artificial aggregate formation using roller tanks (12–14), through size fractions collected by filtration of water samples to differentiate between free-living and aggregate-associated bacteria (6, 15), or by investigations of bulk material collected using sediment traps (16). Though these methods provide very good model aggregates, the scientific community still needs a method to sample individual, intact, *in situ*-formed marine snow aggregates at any depth throughout the water column.

This study presents a new method using gel sediment traps to fix, wash, and collect intact sinking marine snow at any depth *in situ*. This method was used to analyze the bacterial community of individually collected marine snow from 100- and 400-m depths. The microbial communities were analyzed using catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and confocal laser scanning microscopy (CLSM). Additionally, a biovolume approach was used to estimate the abundances of different bacterial groups within the aggregates.

MATERIALS AND METHODS

Sampling. The free-living and aggregate-associated bacterial communities were sampled during RV *Maria S. Merian* cruise 18-1 (MSM 18-1) in the area off Cape Blanc (Mauritania) (Fig. 1). The free-living bacterial

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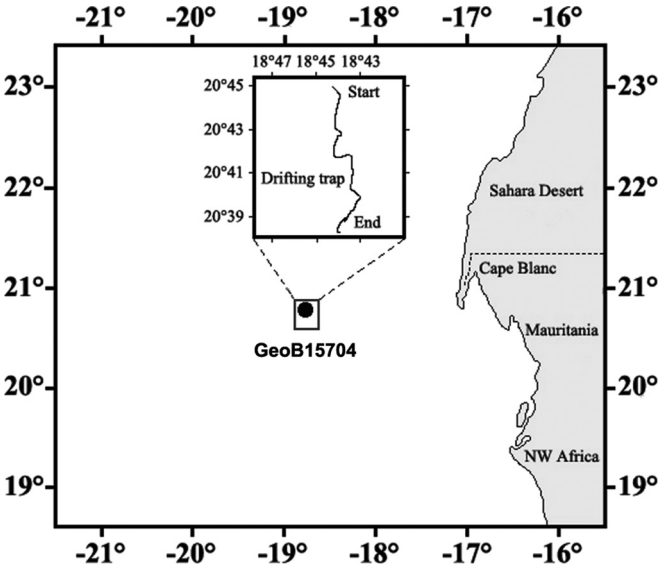

FIG 1 Map of the study area off Cape Blanc, Mauritania. Water samples were collected at depths of 20, 150, 400, 550, and 700 m at station GeoB15704 to quantify the free-living microbial community. Additionally, a surface-tethered freely drifting sediment trap was deployed at station GeoB15704 to collect intact, settling marine snow at 100 and 400 m. The inset shows the track of the surface-tethered drifting sediment trap.

communities were investigated from water samples collected with a CTD Rosette sampler at depths of 20, 150, 400, 550, and 700 m on 1 May 2011 at station GeoB15704 (20°35′N, 18°03′W), which was at the continental slope and had a total water depth of ~780 m. At each depth, one liter of water was fixed with formaldehyde (1% final concentration) for 1 to 3 h at room temperature. Thereafter, the samples were size fractionated, and only the size fraction between 3 and 0.22 μm was used for the investigations of the free-living bacteria. Triplicate filtrations of 0.05 to 0.2 liters of seawater were made for each depth and stored frozen at −20°C.

Intact marine snow was collected with a free-drifting sediment trap at depths of 100 and 400 m (Fig. 2). At each collection depth, two 1-m-long trap cylinders with an inner diameter of 10.4 cm were equipped with ~200 ml of a viscous cryogel (Tissue-Tek OCT [optimum cutting temperature] compound cryogel; Sakura Finetek, Alphen aan den Rijn, Netherlands) to intercept and preserve settling particles without destroying their original size and structure. Previous studies did not show any differences between fresh samples of microbial communities and those embedded in Tissue-Tek cryogel (19). Additionally, visual inspections of laboratory-formed aggregates did not show any changes in their shape and volume after they had been allowed to settle into the gel. The shape and sizes of the aggregates were also preserved after freezing and thawing of the gels. The cryogel was placed in a tight-fitting collection jar at the bottom of each trap cylinder (Fig. 2). The two trap cylinders were attached to the trap array gyroscopically to ensure a vertical position of the cylinders during the entire deployment. This prevented the aggregates coming into contact with the cylinder walls while they settled through the trap cylinders. Twelve small buoyancy balls placed at the surface line of the trap array served as wave breakers to reduce the hydrodynamic effects on the sedi-

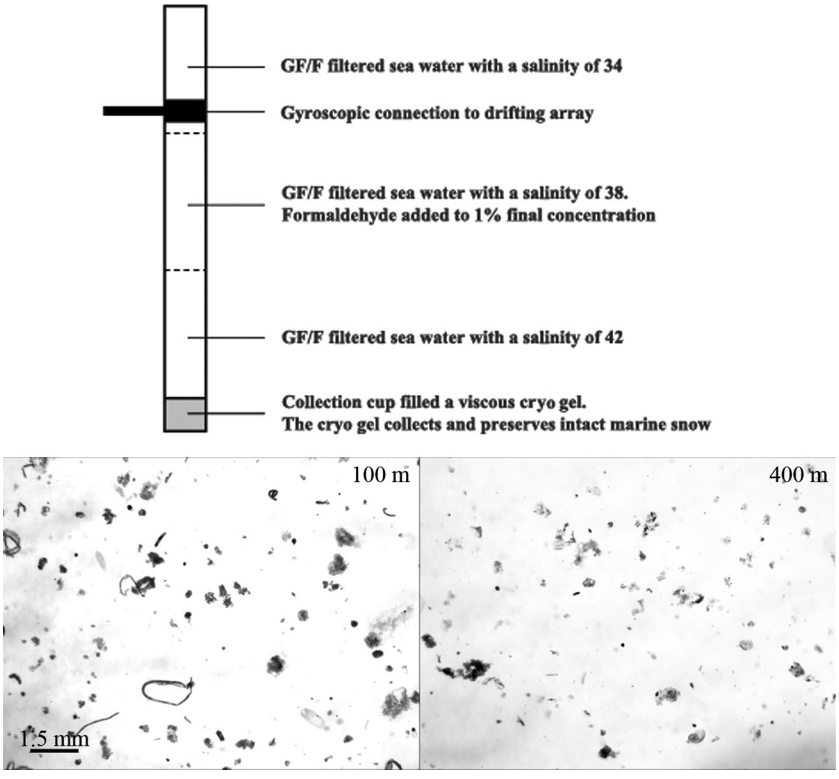

FIG 2 (Top) Drawing of one of the collection cylinders deployed on the sediment trap. The cylinder was filled with the different densities of GF/F-filtered seawater. The lower layer was denser than the layers above. This created three distinct density layers. By adding formaldehyde to the middle layer, we were able to fix the microbial community within marine snow and other aggregates sinking through this layer. The bottom density layer did not contain any formaldehyde and served to wash the fixed marine snow and other aggregates to avoid overfixation of the attached microbes, which prevents the use of fluorescence *in situ* hybridization. A collection cup filled with a viscous cryogel was placed at the very bottom of the sediment trap cylinder. The viscous gel collected the fixed and washed marine snow and other aggregates and preserved their size and structure. (Bottom) Images of the aggregates collected in the cryogel at 100 m (left) and 400 m (right).

TABLE 1 List of oligonucleotides used in this study

Oligonucleotide	Target organism(s)	Sequence (5′→3′)	*E. coli* position	FA (%)[a]	Reference
EUB 338[b]	Most *Bacteria*	GCTGCCTCCCGTAGGAGT	338–355	35	55
EUB II[b]	*Planctomycetales*	GCAGCCACCCGTAGGTGT	338–355	35	56
EUB III[b]	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	338–355	35	56
NON338	Control	ACTCCTACGGGAGGCAGC	338–355	35	57
ARCH915	*Archaea*	GTGCTCCCCCGCCAATTCCT	915–934	35	58
CREN554	*Thaumarchaeota*	TTAGGCCCAATAATCMTCCT	554–573	0	59
EURY806	*Euryarchaeota* marine group II	CACAGCGTTTACACCTAG	806–823	0	60
SAR11 441	SAR11 clade	AAAAAATACAGTCATTTCCTCCCCCGAC	441–463	25	61
ROS537	*Roseobacter* clade	CAACGCTAACCCCCTCC	537–553	35	62
GAM42a[c]	*Gammaproteobacteria*	GCCTCCCCACATCGTTT	1027–1043	35	63
ALT1413	*Alteromonas*/*Colwellia*	TTTGCATCCCACTCCCAT	1413–1430	40	64
PSA184	*Pseudoalteromonas*	CCCCTTTGGTCCGTAGAC	184–201	30	64
SAR324 1412	SAR324 clade	GCCCCTGTCAACTCCCAT	1412–1429	35	65
CF319a	*Bacteroidetes*	TGGTCCGTGTCTCAGTAC	319–336	35	66
PLA46	*Planctomycetes*	GACTTGCATGCCTAATCC	46–63	30	67
SYN405	*Synechococcus*	AGAGGCCTTCATCCCTCA	405–422	30	68
PRO405	*Prochlorococcus*	AGAGGCCTTCGTCCCTCA	405–422	30	68

[a] FA, formamide concentration of the hybridization buffer.
[b] Used in a mix.
[c] Used in a mix with unlabeled BET42a as competitor.

ment traps. The sediment trap was deployed on 30 April 2011 at 20°45′N, 18°44′W and recovered after 3 days at 20°38′N, 18°43′W (Fig. 1). Upon trap recovery, the gel-filled collection jars from 100- and 400-m depths were removed from the trap and stored at −20°C until further processing (Fig. 2).

During identification of bacterial groups with CARD-FISH, it is important to fix the bacteria in 1% formaldehyde (final concentration). However, overfixation can prevent staining with the CARD-FISH probe; therefore, the fixed material needs to be washed. To enable both fixing and washing of the bacterial community within the settling aggregates as they sank through the trap cylinders, three density layers, each ~30 cm high, were made in the cylinders above the gel collection jar. This was done by adding different amounts of sea salt to Whatman glass microfiber filter (GF/F)-sieved seawater collected from the surface with the ship's seawater pump (Fig. 2). The bottom layer had a salinity of ~42. The middle layer had a salinity of ~38 and a 1% final concentration of formaldehyde, while the top layer (salinity, ~34) had only a slightly higher density than the surrounding seawater. No formaldehyde was added to the top and bottom layers, and hence, the latter functioned as the washing layer (Fig. 2). Due to the changes in water density for the different layers, the sinking velocities of the aggregates were slowed when they entered the denser water layers in the traps. Simple estimations using Navier-Stokes drag equation (20) suggested that the residence time of a 1-mm aggregate within each density layer in the trap was ~4 to 5 h for both depths. This residence time is sufficient for both fixation and washing. Inspection of the three-layer density gradient in the sediment cylinders after trap retrieval showed that each of the three layers had remained intact during the deployment, suggesting that both fixing and washing of the settling aggregates were successful.

Aggregate isolation, filtration, and volume determination. The water in the sediment cylinders was siphoned out after 12 h sedimentation, and the gel collection jars were removed from the trap cylinders. Around 30 diatom-dominated marine snow aggregates of similar sizes (0.5 to 1 mm in diameter) were isolated from the gel trap deployments at both 100 and 400 m. The individual aggregates were photographed from above and the side before they were removed from the gels. This was done with a Canon EOS 550D digital camera equipped with a Sigma 105-mm macrozoom objective. The total volume of each aggregate was calculated assuming an ellipsoid shape, and the equivalent spherical diameters (ESD) were then calculated from the volumes. Thereafter, 20 aggregates from each collection depth were individually washed in 1× phosphate-buffered saline (PBS) buffer solution overnight and then broken apart by shaking before they were filtered individually onto 0.22-μm-pore-size polycarbonate filters (Millipore, Billerica, MA, USA) to be used for CARD-FISH analyses.

CARD-FISH. Quantification of the free-living bacterial and archaeal community was done using CARD-FISH according to Thiele et al. (21) with specific oligonucleotide probes (Table 1). Triplicate filters from water depths of 20, 150, 400, 550, and 700 m were stained with 4′,6-diamidino-2-phenylindole (DAPI) subsequently to the CARD-FISH procedure, and the DAPI and CARD-FISH counts were done with a Zeiss AxioImager.Z2 microscope (Carl Zeiss AG, Jena, Germany) with an automated stage. Image acquisition was done using the software package AxioVision, release 7.6 (Carl Zeiss AG, Jena, Germany), comprising an automated focusing routine, an automated sample area definition using the macro MPISYS, and a manual image quality assessment (22–24). The software took pictures of the DAPI channel (350 nm) and the FISH channel (488 nm) at different fields of view along a track on the sample. These picture pairs were used for the quantification of microbial cells. Only samples with a minimum of 15 picture pairs were taken into account and evaluated using the software ACMEtool 0.76 (www.technobiology.ch).

Analyses of the bacterial communities within marine snow were done using CARD-FISH as described above. Filters containing individual aggregates were cut into one half and two quarters and subjected to CARD-FISH. The half-filters were used for hybridization with a specific probe, while one quarter of the filter was hybridized with the EUB I-III probe mix (Table 1). For each probe, three aggregates were used, and counts were determined relative to the EUB I-III counts from the same filter.

Confocal laser scanning microscopy. Single aggregates were analyzed using a confocal laser scanning microscope (CLSM) (Zeiss LSM 780; Carl Zeiss AG, Jena, Germany) and the software ZEN2011 (Carl Zeiss AG, Jena, Germany). Three aggregates were analyzed with every probe by selecting two random fragments from each aggregate for the scanning, using single scans of ~30 to 40 s per focal plane. This resulted in a total of 6 z-stacks per probe, which were used to determine biovolumes of the specific bacterial clade hybridized with this probe, and a total of 38 (100 m) and 39 (400 m) z-stacks of EUB I-III-stained cells, which were used to determine total cell numbers.

Specific biovolume calculation. Due to the three-dimensional structure of the aggregate, direct cell counting is difficult. Autofluorescence of the aggregate matrix often quenches CARD-FISH signals and hence hinders cell detection and a precise enumeration of cells. Furthermore, cells

in close proximity to or overlying one another cannot be distinguished, thus leading to underestimations of total cell numbers. Therefore, the use of biovolume estimations to calculate cell numbers on aggregates circumvented these difficulties and obtained higher precision than tests using manual cell counting. PHLIP (Phobia laser scanning microscopy image processor, v4.0.) was used to obtain biovolumes for each channel of the z-stacks (25) in Matlab R2010b (Mathworks Inc., Natick, MA, USA) using manual threshold settings optimized for each sample.

Biovolume measurements and total bacterial cell count calculation. A single representative picture was made from each z-stack for each probe to calculate the total cell counts per sample. To do this, the average biovolume of single CARD-FISH-stained bacterial cells was measured from each of the extracted representative single pictures using the YABBA software (22). The total cell counts were calculated by dividing the total cell biovolume with the average biovolume of a single cell. The total cell numbers for each specific probe were divided by the total EUB I-III cell numbers to estimate the abundance of each specific probe relative to the total bacterial abundance. Different intensities of the CARD-FISH signals could lead to errors in the calculation of cell abundance. However, these errors were circumvented by the use of a CLSM. The excitation of the fluorescent dye by a laser with a specific wavelength resulted in CARD-FISH signals with rather uniform intensities, while the confocal technique of the CLSM reduced the problems of halo formation. Hence, these error sources had only minor influences on the calculation of cell counts.

In addition to the probe channel, one channel with a broad detection range was scanned to measure the autofluorescence of each analyzed aggregate fragment for the calculation of its total volume. Total bacterial abundance per volume aggregate was obtained by dividing the total cell numbers by the volume of the analyzed aggregate fragment. However, the filtration of the fragmented aggregates caused the fragments to lose their pore water and collapse on the filters. Therefore, the measured volumes of the investigated fragments must be considered much smaller than they would be in their hydrated state, since marine snow components typically have porosities of ~99% (26, 27) with the pore space mainly consisting of exchangeable pore water (89 to 98%) and transparent exopolymer particles (28). Therefore, an underestimation of the aggregate volume by excluding the pore water and allowing the aggregate to collapse on the filter is likely. Hence, the bacterial cell numbers per aggregate volume might be overestimated due to the loss of aggregate pore space after filtration, whereby the measured aggregate volumes potentially were <100 times smaller than the *in situ* volumes of the aggregates. Thus, the measured volumes of the investigated aggregate fragments were corrected by multiplying with a factor of 100 to estimate the minimum total bacterial abundances per aggregate volume.

RESULTS

***In situ* sampling and facilitation of marine snow aggregates.** Several particle types, including diatom aggregates and fecal pellets, were captured and preserved in their original form and structure within the gel at both trap depths (Fig. 2). Only individual diatom dominated aggregates with similar sizes were picked from the gel traps. The average ESD was 0.70 ± 0.22 mm ($n = 31$) at 100 m and 0.63 ± 0.19 mm ($n = 31$) at 400 m, and there were no significant differences between the two depths (Student's t test; $P > 0.05$).

Bacterial abundances on aggregates. The total corrected bacterial abundances per volume aggregate were not significantly different ($P = 0.053$; Mann-Whitney rank sum test) between the aggregates collected at the two depths. However, a tendency toward a higher abundance per volume aggregate and more variability in *in situ*-collected marine snow at 400 m in comparison to that at 100 m was observed. Cell numbers ranged from 1.4×10^8 cells cm^{-3} to 6.3×10^9 cells cm^{-3}, with a median of 3.8×10^8 cells cm^{-3} at a depth of 100 m, compared to a range of 4.2×10^6 cells cm^{-3} to 1.5×10^{10} cells cm^{-3} with a median of 8.9×10^9 cells cm^{-3} at 400 m (Table 2). The abundance of free-living bacteria in the bulk water was 3.4×10^5 at 150 m and 1.0×10^5 cells ml^{-1} at 400 m (Table 2).

The cyanobacterial genus *Synechococcus* accounted for the majority of the microorganisms within the collected aggregates at both depths, with a relative abundance of 40.4% ± 12.2% at 100 m and 49.2% ± 36.8% at 400 m (Table 2; Fig. 3). Cells of *Synechococcus* were also present in the surrounding water column, but with a much lower relative abundance (2.3% ± 1.3% at 150 m and 5% ± 1.3% at 400 m) (Table 2). The relative abundance of *Bacteroidetes* within the aggregates was significantly higher at 100 m (42.4% ± 28.2%) than at 400 m (6.6% ± 3.3%), while the abundance of *Bacteroidetes* in the free-living community was only 3.7% ± 0.6% at 150 m and 2.8% ± 0.2% at 400 m (Table 2). The relative abundance of *Gammaproteobacteria* within the aggregates was 13.7% ± 10.5% at 150 m and 10.6% ± 3.8% at 400 m and these organisms comprised only ~3% of the free-living community at both 150 m and 400 m (Table 2). *Alteromonas* accounted for the majority of *Gammaproteobacteria* within the aggregates collected at 100 m, while it contributed significantly less to the *Gammaproteobacteria* ($P = 0.022$; Student's t test) at 400 m (Table 2; Fig. 3). At both depths, the free-living *Alteromonas* strains had a relative abundance of ~2%. *Pseudoalteromonas* organisms showed a stable relative abundance of ~2 to 3% in the free-living fraction, while the value was ~4 to 5% within aggregates at both depths (Table 2). *Roseobacter* organisms had a relative abundance of ~1% at both depths for the free-living fraction, while within aggregates they comprised 9.7% ± 11.8% at 100 m and 7.6% ± 10.2% at 400 m (Table 2; Fig. 3). The relative abundance of *Planctomycetes* within the aggregates was 2.5% at 100 m but was significantly lower, <1%, at 400 m (Student's t test; $P = 0.02$) (Table 2; Fig. 3). The free-living *Planctomycetes* fraction had a stable relative abundance of ~1.5% at both depths (Table 2). *Thaumarchaeota*, *Euryarchaea* marine group II, and the SAR324 clade were too scarce to account for significant fractions of the bacterial community within the aggregates but were observed in the free-living fraction (Table 2).

Qualitative comparisons of the vertical distribution of attached and free-living bacteria. To test for similarities between the attached and free-living bacterial communities at different depths, a cluster analysis on the relative abundances of free-living bacteria found at water depths of 20, 150, 400, 550, and 700 m and the relative abundances of attached bacteria from the 100- and 400-m depths was performed. Only the bacterial groups that were found within the aggregates were included when the relative abundances of the free-living bacteria were calculated (Table 3). Since *Alteromonas* and *Pseudoalteromonas* seemed to comprise the majority of the *Gammaproteobacteria*, the cluster analysis was done with *Alteromonas* and *Pseudoalteromonas* and without *Gammaproteobacteria* (Fig. 4). The distances for the dendrogram were calculated using Spearman's rank based on the relative abundances. The dendrogram clustered the attached microbial community from 100 m together with the free-living community observed at 20 m (Fig. 4).The microbial community attached to the aggregates collected at 400 m did not cluster directly together with the attached community from 100 m or with any of the free-living samples, except the free-living community from 700 m. When only bacterial groups present within the aggregates were considered, the free-living communities at 400 and 550 m were similar

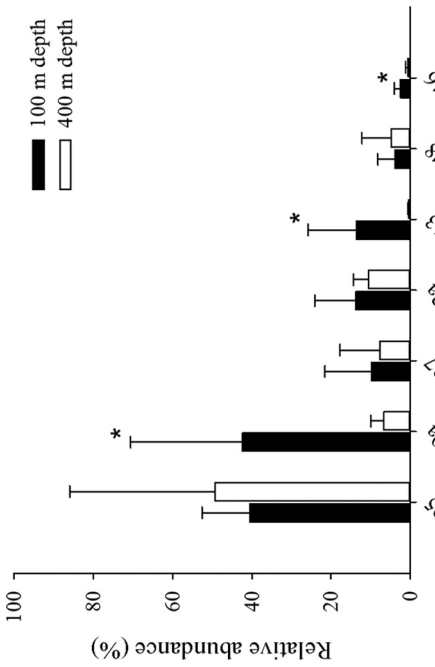

FIG 3 Comparison of relative abundances of the attached bacterial community found within marine snow aggregates collected at 100 m and 400 m. Abundances of *Synechococcus* (SYN405), *Bacteroidetes* (CF319a), *Roseobacter* (ROS537), *Gammaproteobacteria* (GAM42a), *Alteromonas* (ALT1413), *Pseudoalteromonas* (PSA184), and *Planctomycetes* (PLA46) cells are shown. The asterisks indicate the clades which were significantly different between 100 and 400 m.

and to some extent were also similar to the community found at 150 m. The community observed for the free-living bacteria at 700 m did not cluster with bacteria at any of the other depths sampled (Fig. 4).

DISCUSSION

The use of drifting sediment traps equipped with gel allows settling aggregates to be washed and fixed in formaldehyde *in situ* while also preserving their three-dimensional shape, structure, and size. The gel trap enables studies on truly aggregate-attached microbial communities from depths where macroaggregates are scarce and easily missed when small water volumes are sampled. This experimental approach therefore offers a major advantage for studies of microbial communities within settling aggregates compared to the use of size-fractionated filtrations. These result in the destruction of aggregates and can lead to uncertainty as to the origin of microorganisms collected in the different size fractions.

The results gained from the gel trap method agree well with previous determinations of total bacterial abundance per volume aggregate (for example, see references 29, 30, and 31) and showed that bacteria were highly enriched within aggregates in comparison to a similar volume of surrounding water. This resulted in enrichment factors of >1,000-fold, as observed previously (31, 32). Despite the large enrichment factors, the relative proportion of attached to free-living bacteria was only 0.3% at 100 m and 2.4% at 400 m. This seems typical for such depths in oceanic environments (26, 33, 34) and is a result of the relative scarcity of marine snow at greater depths (35–37) (for example, during the present study, *in situ* particle camera deployments showed total aggregate volumes of 3.7 and 0.4 cm^3 per m^3 water at 100 and 400 m, respectively). The ability of free-drifting traps to collect and preserve settling aggregates is therefore an advantage when studying particle-associated microbes at depths where macroaggregates are at low concentrations and have very patchy distributions.

By collecting particles from different depths, insight into the

TABLE 2 Microbial numbers and relative abundances for aggregate-attached and free-living organisms[a]

Organism(s)	100 m					400 m				
	Attached		Free-living (150 m[b])			Attached		Free-living		
	No. (10^7 cells cm^{-3})	Abundance (% of total cells)	No. (10^3 cells ml^{-1})	Abundance (% of total cells)	EF (10^3)	No. (10^7 cells cm^{-3})	Abundance (% of total cells)	No. (10^3 cells ml^{-1})	Abundance (% of total cells)	EF (10^3)
Total bacteria	38		340		1.1	890		100		89
Roseobacter	3.7	9.7 ± 11.8	6.5	1.5 ± 0.4	5.9	71	7.6 ± 10.2	1.5	1 ± 0.3	474.7
Bacteroidetes	16	42.4 ± 28.2	16	3.7 ± 0.6	10	62	6.6 ± 3.3	4.1	2.8 ± 0.2	152
Gammaproteobacteria	5.2	13.7 ± 10.5	13	2.9 ± 0.1	4.1	98	10.6 ± 3.8	4.9	3.3 ± 0.4	199.8
Alteromonas	5.2	13.6 ± 12.2	7	1.6 ± 0.6	7.6	8.9	0.3 ± 0.3	3.2	2.2 ± 0.6	27.8
Pseudoalteromonas	1.4	3.8 ± 4.4	11	2.6 ± 2	1.3	43	4.8 ± 7.4	2.8	1.9 ± 0.9	152.6
Planctomycetes	0.95	2.5 ± 1.6	6	1.4 ± 0.7	1.6	8.9	0.6 ± 0.6	2.3	1.6 ± 0.4	38.7
Synechococcus	15	40.4 ± 12.2	9.7	2.3 ± 1.3	15.7	440	49.2 ± 36.8	7.4	5 ± 1.3	589.3
SAR11			63	14.5 ± 6.5				25	16.6 ± 7.4	
Thaumarchaeota			90	20.9 ± 4.7				32	21.4 ± 2.5	

[a] The microbial numbers are given as cells per cm^3 aggregate (attached) or cells per ml seawater (free living). The relative abundance is calculated against the EUB probe and presented as an average of counts within three aggregates (attached) or three filter counts (free living). Values are averages and standard deviations. The enrichment factor (EF) is presented as the percent of microbes attached per cm^3 aggregate relative to microbes found per ml seawater.
[b] No samples for free-living microbial communities were collected at 100 m, and therefore the 150-m samples were used.

TABLE 3 Relative abundances in percentages of attached and free-living microbes[a]

Cell type	Depth (m)	Abundance (% of total cells)					
		Roseobacter	*Bacteroidetes*	*Alteromonas*	*Pseudoalteromonas*	*Planctomycetes*	*Synechococcus*
Attached	100	8.6	37.7	12.1	3.4	2.2	35.9
	400	11.0	9.6	0.4	7.0	0.9	71.2
Free-living	20	15.7	66.2	4.4	4.0	3.3	6.5
	150	11.6	28.4	12.5	19.7	10.6	17.3
	400	6.8	19.1	15.2	13.2	10.8	34.9
	550	9.5	18.5	17.2	13.4	13.2	28.2
	700	9.7	24.0	8.7	16.2	10.8	30.6

[a] Only microbial groups observed within the aggregates were used to calculate the relative abundances by dividing the microbial abundance for each group by the total number of microbes in all groups. The relative abundances were used to compare the attached microbial community structure with the free-living bacteria at different depths using a dendrogram (Fig. 4).

origin of the particle-associated microbial communities could be gained. Cluster analysis showed similarities between the free-living microbial community sampled at 20 m and that attached to aggregates collected at 100 m (Fig. 4). This suggests that the microbial community within the aggregates collected at 100 m was, to a large extent, determined by bacterial colonization of particles in the photic zone, e.g., 20 m. Hence, the bacterial communities within settling aggregates were "inherited" from the chlorophyll maximum (16, 38). Interestingly, the aggregate-attached community collected at 400 m was different from the free-living communities at any depth down to 700 m and from that attached at 100 m (Fig. 4). This suggests that little *de novo* colonization occurred at greater depths and that the majority of aggregate colonization most likely took place in the surface ocean (i.e., 20 m), where a high abundance of microbes and suspended and slowly sinking particles persisted. It was surprising that the attached community at 400 m was similar to the free-living community at 700 m. Considering that the station sampled for free-living communities was only 780 m deep and was on the continental slope, it is possible that the surface sediment, which must be assumed to be formed by settling aggregates, was resuspended and caused the high similarity to the attached community at 400 m.

Further evidence supporting the idea that the particle-associated microbial community is recruited at the surface is provided by the high relative abundance of *Synechococcus* in the aggregates. The photoautotrophic *Synechococcus* organisms made up the majority of the attached microbes at both 100 m and 400 m, even though it is not beneficial for this organism to live at these dark depths. The high numbers of *Synechococcus* within the aggregates could be explained by active chemotaxis toward the aggregates while they were forming in the euphotic zone (39). Such chemotactic behavior may be triggered by nitrogenous compounds (40), which are often highly enriched within aggregates compared to the surrounding water (41).The high enrichment of attached *Synechococcus* at 400 m suggests that the cells were trapped within the large, rapidly sinking aggregates and thus transported to the deeper, darker region of the ocean.

Synechococcus made up the majority of the attached microbial communities at 100 and 400 m; however, there were substantial shifts in other parts of the attached microbial communities. It is of course possible that the aggregates collected at 400 m had not settled vertically from 100 m but been transported via lateral advection. Still, the facts that the aggregates at both depths were dominated by diatoms and that *Synechococcus* accounted for the majority of the bacteria within them support a common origin. Therefore, the differences observed in the attached microbial community at 400 m compared to the attached community at 100 m can be considered due to ecological successions over time, as-

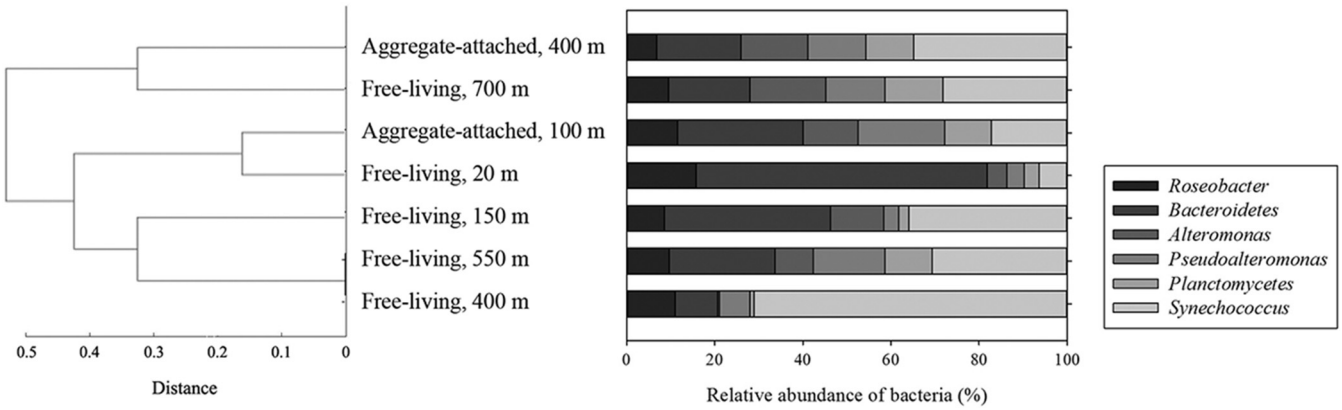

FIG 4 (Left) Dendrogram showing hierarchical cluster analysis using the relative abundances of only the microbial groups observed within marine snow. The dendrogram shows the distance between the attached microbial community at 100 and 400 m and the free-living community at 20, 150, 400, 550, and 700 m. Spearman's rank was used to calculate the distances. (Right) Relative abundances, in percentage of attached and free-living microbes, are shown for the groups observed within the aggregates (Table 3).

suming that the deeper aggregates were older than those from shallower depths. The investigated aggregates from the two depths were of similar sizes and types, and it is therefore likely that their sinking velocities were similar, too (42). Typically, 0.7-mm aggregates sink with velocities between 50 and 100 m day^{-1} off Cape Blanc (36); hence, the aggregates collected at 400 m would have been 3 to 6 days older than those collected at 100 m. Ecological successions of the particle-associated community might be determined by an initial outcome of attachment and detachment (43) followed by growth of some bacterial groups within the aggregates and also by colonization of the aggregates by microbes during their descent. Additionally, grazing pressure on the attached microbes will also shape the microbial community structure within the aggregates.

Interestingly, there was a tendency toward higher abundance in both the total bacterial numbers and all the individual bacterial groups within the aggregates collected at 400 m compared to those collected at 100 m. Though not significantly different, this trend might suggest a high bacterial growth rate and/or high colonization rate on the aggregates while they settled from 100 to 400 m. To test if colonization was the dominant mechanism leading to increased abundance, the total water volume "filtered" by an aggregate as it settles from a depth of 100 m to 400 m can be estimated. The average aggregate diameter at 100 m was 0.7 mm, which means that an aggregate will sweep an area of 3.9×10^{-3} cm^2 clear of particles while it sinks. Thus, a settling distance of 300 m in a vertical direction will result in 115.5 ml of water being "filtered." The abundances of free-living bacteria found at 400 m for the aggregate-attached dominant groups *Roseobacter*, *Gammaproteobacteria*, and *Synechococcus* (Table 2; Fig. 3) suggest that the aggregate potentially encountered 1.7×10^5, 5.7×10^5, and 8.5×10^5 bacterial cells during its descent, respectively. This suggests that if colonization was the only mechanism responsible for increased aggregate-associated microbial cell numbers at 400 m compared to 100 m, every *Synechococcus* and *Roseobacter* cell encountered during the descent would need to attach and remain within the aggregates, while one in three *Gammaproteobacteria* cells encountered would need to attach and remain attached to the aggregates. Previous studies of mechanisms of attachment/detachment by marine snow-associated bacteria have shown that the bacteria have a residence time on the aggregates of only approximately 3 h (43). Therefore, colonization alone cannot explain the attached bacterial abundance at 400 m being higher than that observed at 100 m.

Generally, the structure of pelagic microbial communities are controlled by growth and top down control mechanisms, such as grazing (44) and viral lysis (45). Of course, for attached microbial communities, attachment and detachment play an additional role (46). Since colonization seems to have had little influence on the regulation of the microbial community structure in this study and since viral lysis usually leads to a decrease of one species (45) and not several clades (Fig. 3), growth and/or grazing might have been the major regulating mechanisms. Assuming aggregate settling velocities between 50 and 100 m day^{-1}, it would take between 3 and 6 days to settle vertically from 100 to 400 m. When these values are compared to typical bacterial generation times between 0.4 and 2 day^{-1} for attached bacteria (e.g., see references 13 and 47), it seems realistic that growth could have sustained the relatively high bacterial abundance observed within the aggregates collected at 400 m. This suggests that the grazing impact on the microbial community must have been rather low.

In this study, the main process determining the attached microbial community structure was the colonization in the upper photic part of the water column during aggregate formation (16). This "initial" community structure was then altered during the 4 to 8 days it took the aggregates to sink from the surface ocean to 400 m. These alterations seemed to occur primarily through growth rather than continuous colonization. This contradicts the general belief that colonization is the main mechanism determining the attached microbial community at greater depths (43, 48). It is, however, possible that other regulating factors, such as quorum sensing (49), viral infection (50), hydrostatic pressure changes (51–53), and temperature changes (54), had important influences on the outcome of the internal competition between the microbial groups attached to the aggregates, and it will be interesting to use the present method to address those questions.

The data set presented here encourages us to further develop the method of *in situ* fixation, washing, and collection of individual intact marine particles using drifting sediment traps deployed at various depths. This study focused on CARD-FISH analysis within individual aggregates from just two depths, but the method enables collection and preservation of aggregates from any depth and offers a way to truly separate microorganisms attached to sinking aggregates from the free-living communities. This allows much more detailed studies of the aggregate-attached communities in relation to size, type, and age of the aggregates and will thus provide a better understanding of turnover rates through the water column, which will help toward better understanding and predictions of oceanic biogeochemistry.

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