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Seasonal variation in marine-snow-associated and ambient-water prokaryotic communities in the northern Adriatic Sea

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ABSTRACT: The structure and activity of prokaryotic communities were determined in marine snow and in the ambient water of the northern Adriatic Sea in different seasons (autumn, spring and summer). The seasonal variation in the composition of marine-snow-associated and ambient-water bacterial communities was assessed by T-RFLP (Terminal Restriction Fragment Length Polymorphism) on the 16S rRNA gene (16S rDNA) and 16S rRNA transcript (16S rRNA) level. On the 16S rDNA level, the bacterial community composition of the marine snow and ambient water was similar in summer and autumn, but not in spring. In contrast, on the 16S rRNA level, indicative of the active bacterial community, the marine-snow-associated bacterial community was different from that of the ambient-water, and different from the bacterial community on the 16S rDNA level, except in autumn. To phylogenetically characterize the bacterial and archaeal community composition associated with marine snow and the ambient water, clone libraries of 16S rDNA and 16S rRNA were constructed from 2 contrasting seasons. Phylogenetic profiling revealed a higher similarity among bacterial communities in summer compared to late autumn. Certain bacterial and archaeal groups were exclusively associated with summer or autumn marine snow, suggesting that marine-snow-associated prokaryotic communities are subjected to successional changes similar to ambient-water communities. Moreover, the presence of bacterial groups enriched in marine snow including *Vibrionales* and sulphate-reducing bacteria is consistent with niche partitioning and metabolic adaptations of the particle-associated microbiota.

KEY WORDS: Marine snow · Free-living bacteria · Marine snow-associated bacteria · *Archaea* · T-RFLP · 16S rRNA · 16S rDNA · Northern Adriatic Sea

INTRODUCTION

Macroscopic organic particles known as marine snow are a common feature in coastal and open-ocean waters. These particles are mainly formed by aggregation of dissolved exopolymeric substances, mostly polysaccharides of primarily photosynthetic origin (Alldredge & Silver 1988, Simon et al. 2002). In their mucous matrix, marine snow may entrap minerals, zooplankton faecal pellets, abandoned larvacean houses and dead, senescent and active phytoplankton cells (Alldredge & Gotschalk 1990, Simon et al. 2002). Regardless of its composition, marine snow is densely colonised by a diverse prokaryotic community (Kaltenböck & Herndl 1992, Müller-Niklas et al. 1994, Rath et al. 1998, Turk et al. 2010), that plays a

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crucial role in the solubilisation and remineralisation of marine-snow-bound particulate organic matter (Smith et al. 1992, Ploug et al. 1999) as well as in the formation and transformation of marine snow (Heissenberger et al. 1996).

The complex marine-snow-forming matrix provides microhabitats that considerably differ physically and chemically from the surrounding seawater (Alldredge & Cohen 1987, Ploug et al. 1997). Thus, the bacterial community occupying these microenvironments often differs significantly from that in the ambient water (DeLong et al. 1993, Rath et al. 1998, Riemann et al. 2000, Moeseneder et al. 2001a, Grossart et al. 2006, Kellogg & Deming 2009), while the per-cell productivity and prokaryotic growth rates depend on the nutritive quality of the marine snow and consequently on its origin and age (Alldredge & Gotschalk 1990).

In the first stage of its development, marine snow enriched in labile organic matter is colonised by 'opportunitrophs': chemotactic, motile heterotrophic bacteria from the surrounding seawater rapidly responding to sudden nutrient pulses and attracted by the strong chemical gradients in the marine snow's surrounding microsphere, as shown in laboratory studies (Grossart et al. 2003, Stocker et al. 2008). The attached prokaryotic community is later subjected to successional changes in the environment, since the marine-snow-forming matrix becomes more and more recalcitrant due to intensive microbial degradation (Müller-Niklas et al. 1994, Turk et al. 2010). However, marine snow is linked to the ambient water by a dynamic exchange of cells, which provides functionally and taxonomically diverse microbes that colonise the particle in different successional stages (Grossart et al. 2003). Although the relationship between the marine-snow-associated and free-living bacterial community has been explored in different marine environments (DeLong et al. 1993, Rath et al. 1998, Moeseneder et al. 2001b, Kellogg & Deming 2009, Eloe et al. 2011), most of the studies have investigated particle-associated communities at a single time point only, whereas successional changes of the marine-snow-attached prokaryotic communities have rarely been assessed.

In the northern Adriatic Sea, the contrasting conditions in different seasons lead to the formation of marine snow of different sizes and shapes (Stachowitsch et al. 1990, Precali et al. 2005), making this regional sea an ideal site to study the seasonal dynamics of marine-snow-associated prokaryotic assemblages. Stable, low-turbulence, summer conditions in the northern Adriatic Sea enable the aggregation of exopolymers into bigger mucous macroaggregates, also described as gel-like aggregates (GEA) reaching up to a few meters in length (Müller-Niklas et al. 1994). In addition to GEA, small marine-snow particles are formed under the turbulent autumn and winter conditions, when increased freshwater input and sediment re-suspension provide nutrients fuelling phytoplankton production (Mozeti et al. 2012). Despite this great variety in size and shape of marine snow, and consequently microhabitats for prokaryotes, the majority of studies has focussed on large GEA (Müller-Niklas et al. 1994, Azam et al. 1999, Herndl et al. 1999, Najdek et al. 2002, Kovac et al. 2004, Degobbis et al. 2005, Flander-Putrle & Malej 2008, Faganeli et al. 2009, Turk et al. 2010). Using fluorescence *in situ* hybridization (FISH), Danovaro et al. (2009) detected a high number of potentially pathogenic microorganisms (*Escherichia coli*, *Vibrio* sp.) and reported an almost 2-times higher richness in the GEA-associated bacterial community (based on Automated Ribosomal Intergenic Spacer Analysis [ARISA] fingerprints) than in the surrounding seawater. While the succession of marine snow from the initial phase of development until its mature stage, GEA, and the associated microbial activity in the northern Adriatic have been previously described (Kaltenböck & Herndl 1992, Müller-Niklas et al. 1994, Flander-Putrle & Malej 2008), the marine-snow-associated prokaryotic community in different forms of marine snow occurring in this area has not been thoroughly assessed. In fact, only one phylogenetic 16S rDNA sequencing-based analysis of the GEA-associated bacterial community has been performed in the northern Adriatic Sea so far, revealing a surprisingly high proportion of *Deltaproteobacteria* (Rath et al. 1998).

In addition to the lack of phylogenetic information, the effects of the marine-snow-associated prokaryotic community and its activity on the ambient water prokaryotes (i.e. the free-living community) are largely unknown. It has been shown that marine-snow-attached bacteria exhibit a higher per-cell exoenzymatic activity than free-living bacteria (Karner & Herndl 1992, Smith et al. 1992, Del Negro et al. 2005, Zoppini et al. 2005), interpreted as a loose hydrolysis-uptake coupling in attached bacteria in contrast to the tight hydrolysis-uptake coupling in free-living bacteria (Hoppe et al. 2002). The consequence of the loose hydrolysis-uptake coupling in attached bacteria is that a certain fraction of the cleavage products generated by the exoenzymatic activity is released into the ambient water (Baltar et al. 2009). This leakage of dissolved organic sub-

stances from the marine snow supports the growth of the heterotrophic prokaryotic community in the intimate vicinity of the particle (Kiørboe & Jackson 2001).

In this study, we investigated the composition of marine-snow-associated and ambient-water prokaryotic communities in 3 different seasons. Knowing that the bacterial community composition changes seasonally in the temperate coastal water column (Fuhrman et al. 2006, Sintes et al. 2013), the question addressed here is whether the marine-snow-attached bacterial community exhibits similar seasonal changes or whether marine snow represents a more stable environment than the ambient water. Based on 16S rDNA and 16S rRNA clone libraries, FISH and terminal-restriction fragment length polymorphism (T-RFLP), the phylogenetic composition and the differences between marine-snow-associated and ambient-water bacterial and archaeal assemblages were assessed in contrasting seasons.

MATERIALS AND METHODS

Study site and sampling

Sampling was carried out 2.3 km off Piran (Gulf of Trieste, northern Adriatic Sea; Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a073p211_supp.pdf) in 3 different seasons: late autumn (10 December 2009; the sampling date was considered as autumn, since the temperature throughout the water column was comparable to those of October and November), spring (23 April and 7 June 2010), and summer (15 July, 12 and 26 August 2010). The depth of highest marine-snow abundance (between 7 and 13 m depth) was first determined onboard by a submersible camera, and later confirmed by a SCUBA diver who selectively collected marine snow using sterile syringes (60 ml). Syringes were filled with as many particles as possible while minimizing the inclusion of ambient water (Kaltenböck & Herndl 1992, Müller-Niklas et al. 1994, Rath et al. 1998). Ambient water was collected at the same depth as marine snow using Niskin bottles attached to a CTD profiler. Marine snow and ambient water were stored in a cooling box in the dark at 8 to 15°C until arrival at the laboratory (within 1 h). The inorganic nutrient concentrations (PO_4^{3+}, NO_2^-, NO_3^- and NH_4^+) were measured in unfiltered seawater following standard colorimetric methods (Grasshoff et al. 1999).

Catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)

Samples were fixed with paraformaldehyde (PFA, 2% final conc.) and kept at 4°C in the dark for 8 to 18 h, then filtered onto a 0.2 µm polycarbonate filter, dried and stored at −20°C. The CARD-FISH protocol was carried out as described by Teira et al. (2004) using the probe mix Eub338 (mix of Eub338-I, -II and -III) to target *Bacteria* (Amann et al. 1990, Daims et al. 1999), a mix of Cren537 (Teira et al. 2004) and Cren554 (Massana et al. 1997) probes to target most of the marine *Thaumarchaeota* (Brochier-Armanet et al. 2008) and the Eury806 probe for *Euryarchaeota* (Teira et al. 2004). Negative control counts were performed with HRP-Non338 probe (Wallner et al. 1993), always accounting for <1% of total DAPI-stained cells. Samples were processed in triplicate by counting cells in 10 to 20 microscopic fields, enumerating at least 500 DAPI-stained cells filter^{-1}. The total prokaryotic abundance (PA) was calculated based on the enumerated DAPI-stained cells.

Prokaryotic heterotrophic production (PHP)

Prokaryotic heterotrophic production was estimated from the ^{3}H-leucine incorporation rate, employing the centrifugation method (Smith & Azam 1992). PHP (in µg C l^{-1} h^{-1}) was calculated according to Kirchman et al. (1985).

Nucleic acid extraction

Samples of 500 ml of ambient water and 60 ml of marine snow were filtered onto 0.2 µm pore-size filters to collect the total prokaryotic community and the marine-snow-associated community, respectively. All filters were flash-frozen in liquid nitrogen and stored at −80°C until DNA/RNA extraction. Extraction of DNA/RNA was done using phenol/chloroform and beadbeating based on protocols described previously (Moeseneder et al. 2001b, Paul 2004) with slight modifications. The detailed protocol is provided in the Supplement at www.int-res.com/articles/suppl/a073p211_supp.pdf.

RNA was recovered from subsamples of DNA/RNA extract using Deoxyribonuclease I, amplification grade (Invitrogen) according to manufacturer's instructions. RNA samples were checked for DNA contamination by PCR using 16S rDNA bacterial primers (conditions described below). If DNA was still pres-

ent, the samples were subjected to an additional DNase digestion. cDNA was synthesized from RNA extract using Superscript III First-Strand Synthesis supermix (Invitrogen) following the manufacturer's instructions.

T-RFLP fingerprinting

For T-RFLP fingerprinting, the bacterial 16S rRNA gene and transcripts were amplified using the *Bacteria*-specific 6-carboxyfluorescein (FAM)-labelled primer 27F and the VIC-labelled universal primer 1492R (Lane 1991). A total of 2 µl of DNA or 4 µl of cDNA were added per 50 µl reaction, consisting of 0.5 µM of primers, 2U of *Taq* polymerase, 1× *Taq* buffer, 160 µM of each dNTP, 2 mM of $MgCl_2$ and 0.4 mg ml^{-1} of BSA. Samples were amplified by an initial denaturation step at 95°C (for 4 min), followed by 30 to 35 cycles of 95°C (for 40 s), 55°C (for 30 s), and 72°C (for 1.5 to 2 min). Cycling was completed by a final extension at 72°C for 7 min. PCR products of the expected size were cleaned using AgaroseGel Extract Mini Kit (5Prime) or PCRExtract Mini Kit (5Prime) following the manufacturer's protocol. Cleaned PCR products were digested with *Hha*I restriction enzyme at 37°C for 12 h, followed by inactivation at 65°C for 20 min. Aliquots of the digested PCR product were mixed with Hi-Di formamide and internal lane standard 1200 LIZ (Applied Biosystems) and denatured at 95°C for 3 min. Separation and detection of fluorescently labelled fragments were done with a 3130xL Genetic Analyzer (Applied Biosystems).

T-RFLP electropherograms were analyzed with GelComparII software (Applied Maths). Due to inefficient amplification of archaeal 16S rRNA genes and transcripts with fluorescently labelled primers, only bacterial communities were screened by T-RFLP.

Cloning and sequencing

Bacterial 16S rDNA and 16S rRNA, and archaeal 16S rDNA from marine snow and ambient water collected on 10 December 2009 and 26 August 2010 were amplified in PCR reactions under the same conditions as described above, with a modified annealing time of 40 s and an elongation time of 2 min. Primer pairs 27F/1492R and 21F/958R (DeLong 1992) were used to amplify bacterial and archaeal genes, respectively. PCR reactions (2 to 3) of the same sample were pooled and cleaned with PCRExtract Mini Kit (5Prime). PCR products (2 to 4 µl) were cloned into the pCRII TOPO cloning vector (Invitrogen) according to the manufacturer's protocol. A total of 2 µl of cloning reaction was used for transformation of OneShot TOP10 chemically competent cells (Invitrogen). Up to 192 white colonies were picked per sample. Sequencing was carried out by Macrogen Europe using the 27F and 21F primer for *Bacteria* and *Archaea*, respectively.

Sequence analysis

Low quality sequence fragments were trimmed with DNA Baser software v.3.0. The presence of chimeras was checked with Mallard v.1.02 and Pintail v.1.0. bioinformatics toolkits. The sequences were blasted against the SILVA database (Release 106) (Pruesse et al. 2007). To estimate the efficiency of the cloning effort, rarefaction analysis was done using MOTHUR (Schloss et al. 2009). Operational taxonomic units (OTUs) were defined as the group of sequences differing <3% in the 16S rDNA or rRNA sequence using the decrease redundancy online tool (ExPASy). A representative sequence of each OTU was used to construct a neighbour-joining phylogenetic tree. Based on the phylogenetic tree and the sequence abundance per OTU, UniFrac similarity among all samples was calculated using the UniFrac online tool (Lozupone et al. 2006). The UniFrac environment distance matrix was used to construct similarity dendrograms using the UPGMA clustering method with Primer software (Primer-E).

Sequences obtained in this study were deposited in GenBank under the accession numbers KF185114 to KF186162.

Statistical analyses

Because our data exhibited a non-normal distribution (Shapiro test) and/or contained few individuals per sample, we used non-parametric tests. The Mann-Whitney test was used to examine the differences between total and marine-snow-associated bacterial and archaeal abundance, bulk and cell-specific heterotrophic production and the number of OTUs obtained by T-RFLP for the whole sampling period. Within each fraction, the same parameters (except the OTU number) were compared between sampling dates using the non-parametric Friedman test. Univariate analyses were performed using R software. The presence/absence matrix representing the distribution of different T-RFLP fragments obtained with the forward and reverse primer on both

16S rDNA and rRNA levels was used to calculate the Jaccard similarity matrix with Primer software. Ordination of the similarities was completed by non-Metric Multidimensional Scaling (nMDS) analysis (Kruskal 1964a,b), where samples with higher similarity are plotted closer together in a multi-dimensional space. ANOSIM analysis was performed using R software to test the significance of season, bacterial life mode (marine-snow-associated vs. total communities) and DNA vs. RNA levels on the clustering of bacterial communities. Bacterial communities within the 16S rDNA level were also compared with ANOSIM, according to season and their life modes.

RESULTS

Seasonal variation in environmental parameters and particle characteristics

The water column was well-mixed in December and highly stratified in summer, with the maximum temperature difference between the surface and the bottom layer (28.5 and 15.3°C, respectively) occurring in July. Nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) concentrations were highest in December (Table S1 in the Supplement at www.int-res.com/articles/suppl/a073p211_supp.pdf) and steadily decreased in spring (when phytoplankton blooms are usually detected; Mozeti et al. 2012). In June, NO_3^- and NO_2^-concentrations were 4- to 14-fold lower than in December. Ammonium concentration did not follow any particular trend and mostly ranged between 0.3 and 0.5 µM (Table S1).

The highest marine-snow concentration was observed between 10 and 13 m depth, depending on the season. Size and shape of the marine snow were highly variable: the particles collected in December, April, July and early August were whitish, small particles (from 1 to 5 mm diam.) also known as 'flocs', while particles collected in June were 5 to 10 cm long comet-shaped macroaggregates, called 'stringers' (Stachowitsch et al. 1990). In late August, even larger marine snow developed, mostly as irregularly shaped 10 to 20 cm long 'ribbons' (Precali et al. 2005).

Prokaryotic abundance, growth rate and cell-specific heterotrophic production

PA was significantly higher in the marine snow than in the ambient water throughout the sampling period (unilateral Mann-Whitney test, $p < 0.001$). In marine snow, PA increased from 0.4×10^6 to 7.3×10^6 cells ml^{-1}, and in ambient water from 0.6×10^6 to 3.2×10^6 cells ml^{-1} from late autumn into late summer (Fig. 1B). A similar trend was observed for PHP (Fig. 1A), which was significantly higher (unilateral Mann-Whitney test, $p = 0.015$) in marine snow (66.8 to 190 pmol leu l^{-1} h^{-1}) than in ambient water (52 to 182 pmol leu l^{-1} h^{-1}). The difference in PHP was the highest in June and late August, when stringers and long ribbon- shaped marine snow were present in the water column (Fig. 1A).

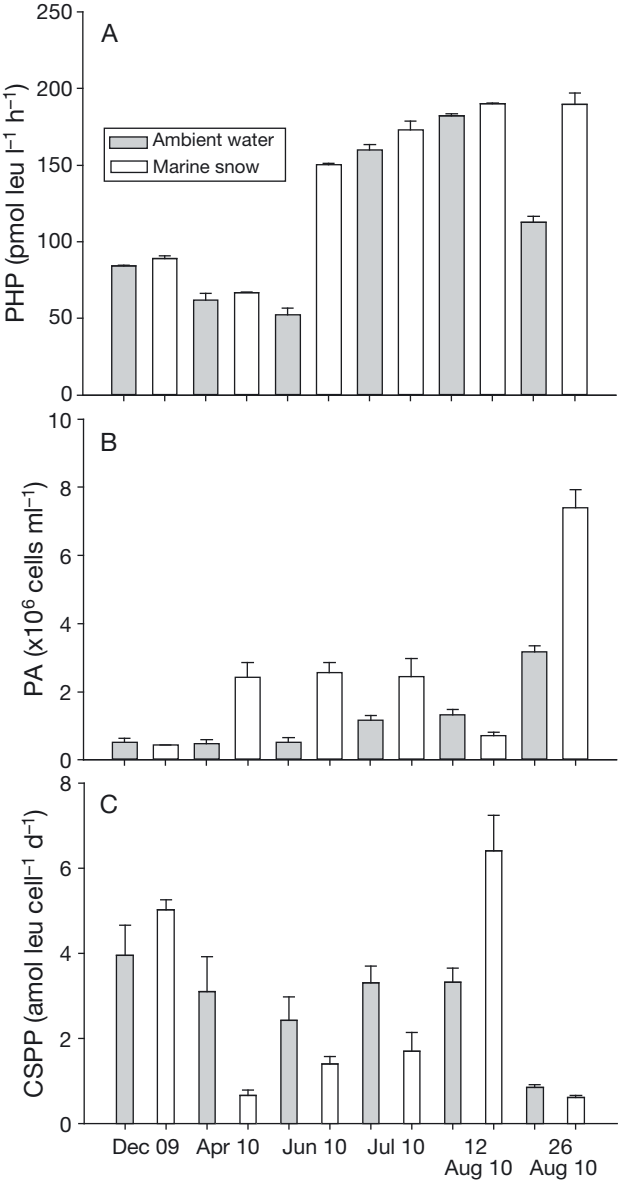

Fig. 1. (A) Prokaryotic heterotrophic production (PHP), (B) prokaryotic abundance (PA) and (C) prokaryotic cell-specific production (CSPP) in marine snow and ambient water. Error bars are SD

Cell-specific prokaryotic heterotrophic production (CSPP) measured in marine snow displayed large variation among different seasons (0.6 ± 0.05 to 6.4 ± 0.9 amol leu cell^{-1} d^{-1}) and was frequently lower than in ambient water (2.4 ± 0.6 to 4.0 ± 0.7 amol leu cell^{-1} d^{-1}), except in December and mid-August, when the highest CSPP in marine snow (5.0 ± 0.2 and 6.4 ± 0.9 amol leu cell^{-1} d^{-1}, respectively) of all the sampling dates was recorded (Fig. 1C). CSPP in ambient water was not significantly different between seasons (Friedman test, $p > 0.05$), but decreased considerably in late August (0.9 ± 0.06 amol leu cell^{-1} d^{-1}) when high PA coincided with low PHP (Fig. 1C).

Bacteria and *Archaea* in ambient water and marine snow as determined by CARD-FISH

CARD-FISH analyses of ambient water and marine snow revealed that *Bacteria* dominated the prokaryotic community throughout the sampling period (Fig. S2 in the Supplement at www.int-res.com/articles/suppl/a073p211_supp.pdf). The contribution of *Bacteria* to total PA was significantly higher in the ambient water than in the marine snow (unilateral Mann-Whitney test, $p = 0.001$) and showed no significant variation between seasons (Friedman test, $p = 0.06$). In ambient water, *Bacteria* outnumbered *Archaea* 1.2- to 7.6-fold and accounted for 64 to 78% of DAPI-stained cells. Within *Archaea*, *Thaumarchaeota* (22 ± 11% of DAPI-stained cells) dominated over *Euryarchaeota* (13 ± 8% of DAPI-stained cells) in the ambient water (Fig. S2).

In marine snow, bacterial abundance varied between 46 and 68% of DAPI-stained cells. In contrast to the archaeal contribution in ambient water, *Euryarchaeota* were more abundant than *Thaumarchaeota* in marine snow, accounting for 36 ± 7% of DAPI-stained cells, while *Thaumarchaeota* contributed only 28 ± 10% of DAPI-stained cells (Fig. S2). From autumn towards the summer, the abundance of both archaeal groups increased in marine snow, but decreased in the ambient water, resulting in a significantly higher archaeal contribution in marine snow than in the ambient water (unilateral Mann-Whitney test, $p < 0.001$).

Bacterial community composition in ambient water and marine snow determined by T-RFLP

T-RFLP analysis of the bacterial community revealed in total 144 OTUs from the FAM-labelled forward primer and 83 OTUs from the VIC-labelled reverse primer. Bacterial richness (i.e. the number of 16S rDNA OTUs detected sample^{-1}) was not significantly different between the ambient water and marine snow (Mann-Whitney test, $p = 0.06$; Table S2 in the Supplement at www.int-res.com/articles/suppl/a073p211_supp.pdf). In late August, however, the large marine snow exhibited a higher bacterial richness than the ambient water. The abundance of 16S rRNA OTUs (the potentially active bacterial community) followed the same seasonal trend as the total bacterial community obtained on the16S rDNA level (Table S2). However, the richness of the 16S rRNA based bacterial community was 1.5 to 6 times lower than that of the corresponding bacterial community on the 16S rDNA level, except in April, when the richness of the potentially active bacterial community was higher than that of the total community in marine snow (Table S2).

Bacterial communities (based on both the DNA and RNA level) significantly clustered according to season (ANOSIM, $R = 0.19$, $p = 0.03$), while bacterial life mode (ambient-water vs. marine-snow-associated) did not significantly shape bacterial community structure ($R = 0.08$, $p = 0.06$).

The nMDS analysis (final stress of 0.16, Monte Carlo test of stress significance, $p = 0.01$; see Table S3 in the Supplement at www.int-res.com/articles/suppl/a073p211_supp.pdf for more details) identified 5 clusters at the 35% similarity level, 1 cluster comprising bacterial communities from December, one containing spring communities and 3 comprising mainly summer samples (Fig. 2). The bacterial communities on the 16S rDNA and the 16S rRNA level appeared to

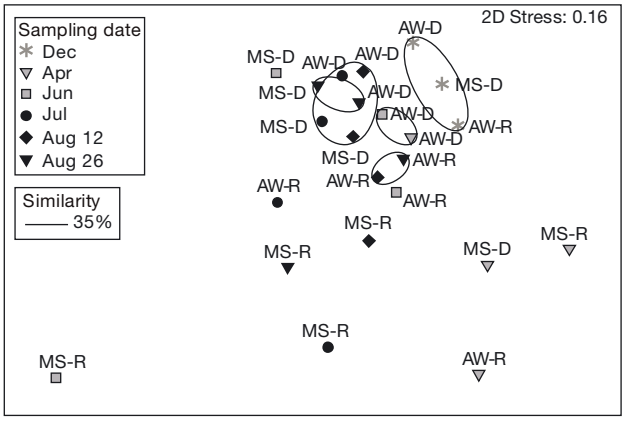

Fig. 2. Non-metric Multidimensional Scaling (nMDS) ordination of similarity in bacterial communities assessed by terminal restriction fragment length polymorphism (T-RFLP). Symbols represent different sampling dates. MS: marine snow, AW: ambient water, D: 16S rDNA, R: 16S rRNA

be clearly separated along the nMDS axis (Fig. 2), as also indicated by ANOSIM analysis (R = 0.29, p = 0.001).

Phylogenetic composition of marine-snow-associated and ambient-water prokaryotic communities

In total, 9 clone libraries were constructed from ambient water and marine snow in December and August (Table 1). The rarefaction curves indicated that we undersampled bacterial diversity, while the sequencing effort was sufficient to cover archaeal diversity (Fig. S3 in the Supplement at www.int-res.com/articles/suppl/a073p211_supp.pdf). The highest bacterial diversity (Margalef, Da = 17) was observed in ambient water in December as compared to the marine-snow-associated community (Da = 14; Table 1). In agreement with the results obtained by T-RFLP, bacterial diversity of the ambient water collected in August (Da = 9.8) was lower than that of the marine-snow-associated bacterial community collected on the same date (Da = 15; Table 1).

Four bacterial groups (*Bacteroidetes, Cyanobacteria, Alphaproteobacteria* and *Gammaproteobacteria*) were found to be highly abundant (i.e. more than 20% of all clones in at least one of the samples; Fig. 3). *Bacteroidetes* were highly abundant in August (29 and 21% of clones obtained from the ambient water and marine snow, respectively), and less abundant in December (8 and 14% in ambient water and marine snow, respectively). Within *Bacteroidetes, Flavobacteria* were the most abundant (16S rDNA) and most active (16S rRNA) group in both seasons (Fig. 4A). Few members of *Bacteroidia* and *Sphingobacteria* were detected in the ambient water in December, and few *Cytophaga* and *Sphingobacteria* in August. *Cyanobacteria* were also highly abundant in August in marine snow and the ambient water (26 and 46% of the total number of clones, respectively). In December, *Cyanobacteria* dominated in marine snow (35%

Table 1. Diversity indices obtained for the different bacterial (Bac) and archaeal (Arch) clone libraries. Dec: December 2009, Aug: August 2010, AW: ambient water, MS: marine snow, rDNA: 16S rDNA genes, rRNA: 16S rRNA, na: not applicable

Sample	Number of clones (N)	Number of OTUs (S)	Margalef diversity (Da)
16S rDNA Dec MS_Bac	130	69	14
16S rDNA Dec AW_Bac	180	91	17
16S rRNA Dec AW_Bac	87	46	10
16S rDNA Aug MS_Bac	178	77	15
16S rDNA Aug AW_Bac	168	51	9.8
16S rRNA Aug AW_Bac	158	51	9.9
16S rDNA Dec MS_Arch	53	15	3.5
16S rDNA Dec AW_Arch	60	6	1.2
16S rDNA Aug AW_Arch	35	1	na

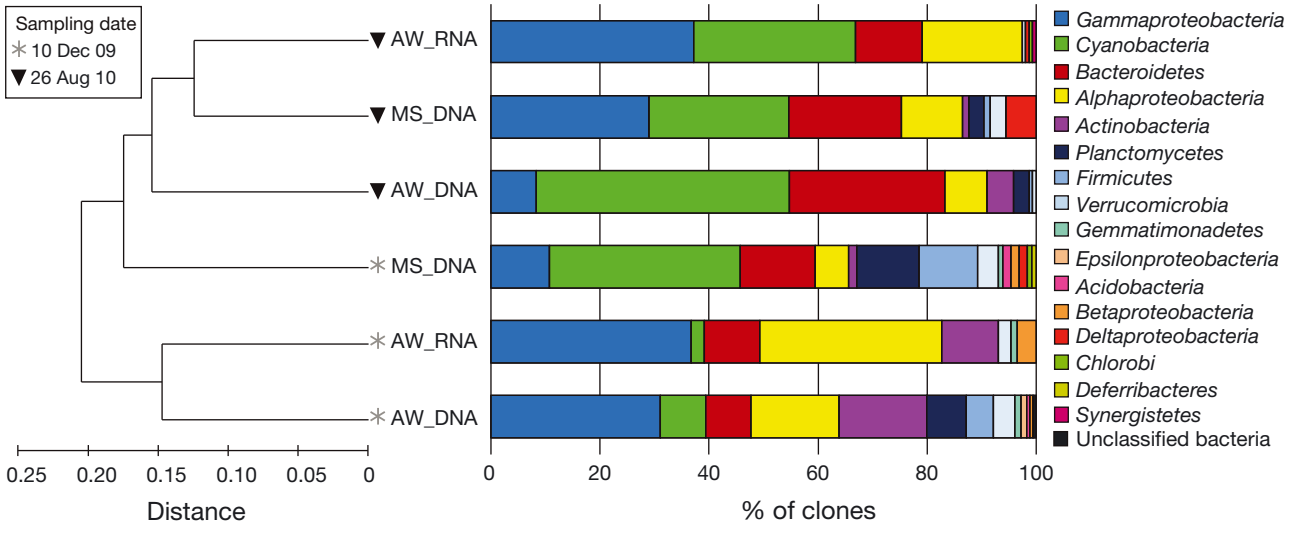

Fig. 3. Bacterial community composition in marine snow (MS) and ambient water (AW) in 2 contrasting months (August and December) based on cloning and sequencing. UPGMA dendrogram constructed based on UniFrac environment distance matrix (Lozupone et al. 2006) followed by the proportion of clones affiliated to each phylogenetic group compared to the total number of clones obtained for each individual sample. Distances between samples are given in UniFrac units (0 = composition identical, 1 = composition completely different). DNA: 16S rDNA, RNA: 16S rRNA

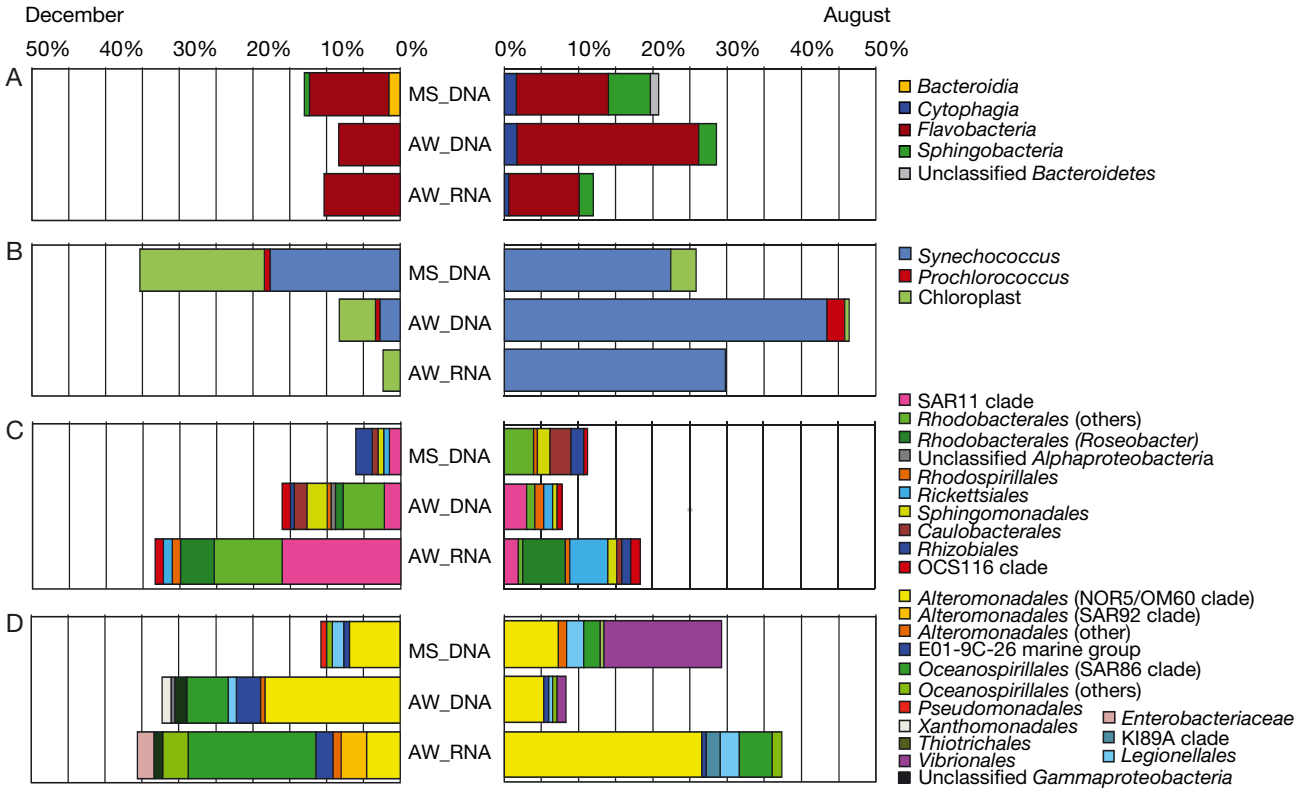

Fig. 4. Phylogenetic classification of the 4 most abundant (>20% of clones) bacterial groups of the northern Adriatic Sea obtained in December and August. (A) *Bacteroidetes*, (B) *Cyanobacteria*, (C) *Alphaproteobacteria*, (D) *Gammaproteobacteria.* The length of differently colour-coded bars indicates the proportion of clones affiliated to each specific phylogenetic group compared to the total number of clones in each corresponding clone library. Dec: December 2009, Aug: August 2010, AW: ambient water, MS: marine snow, rDNA: 16S rRNA genes, rRNA: 16S rRNA

of all clones), but were less abundant and less active in ambient water, while they were highly active (30% of all clones) in August (Fig. 4B).

Alphaproteobacteria accounted for <20% of all clones in the 16S rDNA libraries, but were the most abundant group (33% of all clones) in the 16S rRNA library in December (Fig. 3). Within *Alphaproteobacteria*, members of the SAR11 clade were absent from the large mucous aggregates in August (Fig. 4C) and not very abundant in other clone libraries (2 to 3%), with the exception of the 16S rRNA library of the ambient water in December (16% of all clones). Members of the *Roseobacter* clade could not be detected in marine snow and were detected in low abundance in the ambient water only in December (1% of all clones), but were disproportionally active in December as well as in August (5 and 6% of all 16S rRNA clones, respectively; Fig. 4C). Other *Rhodobacterales*, however, were found in association with large mucous aggregates in August (4% of all clones).

The distribution pattern of gammaproteobacterial clones was different between August and December samples (Fig. 3). In December, their abundance was highest in the ambient water (31% of all clones) while they represented only 10% of the clones in marine snow. The opposite was true in August (8 and 30% of all clones in the ambient water and in large mucous aggregates, respectively). *Gammaproteobacteria* dominated both 16S rRNA ambient water libraries (37% of all clones in December and August). Within *Gammaproteobacteria, Alteromonadales* from the NOR5 clade dominated almost all libraries (5–18 and 5–27% of all clones in rDNA and rRNA libraries, respectively), except the one from the large mucous aggregates in August, where *Vibrionales* represented 16% of all clones and 16S rRNA library from December, which was dominated by the SAR86 clade (Fig. 4D).

Most of the moderately-abundant bacterial groups (*Actinobacteria, Firmicutes* and *Planctomycetes*) and rare bacterial groups (*Acidobacteria, Deferribacteres*, *Gemmatimonadetes*, *Betaproteobacteria*, and *Epsilonproteobacteria*), i.e. those comprising less than 10 and 5% of all clones at any time and in any

sample type, respectively, were more abundant or detected only in December, when diversity was higher than in August (Fig. 3). Only *Synergistetes* were exclusively found in the 16S rRNA library of the ambient water in the summer. Members of *Chlorobi, Verrucomicrobia* and *Deltaproteobacteria* were found in both seasons and were more frequently associated with marine snow than with ambient water.

In contrast to *Bacteria*, archaeal diversity was very low in all 3 samples examined (Table 1) and was extremely low in the ambient water in August. Based on 97% sequence similarity, all the clones obtained from this sample clustered in a single *Nitrosopumilus maritimus* SCM1-related OTU. The archaeal community in December was more diverse than in August, but the majority of the clones were also affiliated with *N. maritimus*. The highest archaeal diversity (Da = 4.0) was observed in marine snow in December, with members of the euryarchaeal class Marine group II *Euryarchaeota* and the crenarchaeal class *Thermoprotei*. Interestingly, some members of *Methanomicrobia* were also detected in marine snow collected in December.

DISCUSSION

Methodological considerations

In most of the studies that have addressed compositional and/or activity differences between free-living and particle-attached prokaryotic communities, the 2 communities have been separated by filtration (Middelboe et al. 1995, Ghiglione et al. 2007, Kellogg & Deming 2009). However, this type of separation has some methodological drawbacks. On one hand, the filters can get clogged very quickly with large mucus particles, such as those present in the northern Adriatic, potentially biasing the separation of the particle-attached and free-living community. On the other hand, filtering may cause a dislodging of particle-attached cells due to suction pressure, which leads to an underestimation of the attached fraction. To avoid these biases, we used a fraction-specific sampling of marine snow, where particles >1 mm were selectively obtained by SCUBA diving using disposable syringes, and subsequently collected onto 0.22 µm pore-size filters. Thus, while marine snow was selectively collected, ambient water collected by Niskin bottles represents a mix of truly free-living and marine-snow-attached microbes, albeit the latter in low concentrations due to the patchy distribution of marine snow in the water column.

Prokaryotic activity is linked to particle developmental stage

The bulk activity of marine-snow-associated prokaryotic communities has been shown to be highly variable, depending on particle size, age and specific density (Alldredge & Silver 1988, Alldredge & Gotschalk 1990, Müller-Niklas et al. 1994). Commonly, however, the bulk activity of marine-snow-associated prokaryotes is much higher than that of ambient-water prokaryotes (Alldredge & Silver 1988, Simon et al. 2002). This has also been shown for GEA from the northern Adriatic Sea (Müller-Niklas et al. 1994) and is in agreement with our results (Fig. 1A). However, CSPP in marine snow was usually lower than in the ambient water (Fig. 1C), suggesting lower growth rates of marine-snow-associated heterotrophic prokaryotes compared to their counterparts in the ambient water. Thus, the higher bulk PHP in marine snow is mainly due to the higher total abundance of heterotrophic prokaryotes than in ambient water.

Marine snow of non-phytoplankton origin, such as appendicularian houses and faecal pellets produced by zooplankton (Alldredge & Gotschalk 1990, Simon et al. 1990), or senescent stages of marine snow might sustain lower prokaryotic growth rates compared to the ambient water (Müller-Niklas et al. 1994). In contrast, high prokaryotic growth rates have been reported for freshly formed marine snow of phytoplankton origin (Alldredge et al. 1986, Müller-Niklas et al. 1994). Based on these findings, the marine snow collected in December and mid-August would resemble freshly produced marine snow (Fig. 1A,B), as these particles exhibited a higher CSPP (Fig. 1C) than the ambient water (Alldredge & Gotschalk 1990, Simon et al. 1990, Ghiglione et al. 2007). In freshly formed marine snow, degradation rates might greatly exceed the organic carbon demand of marine-snow-attached heterotrophic bacteria (Smith et al. 1992, Kiørboe & Jackson 2001). Consequently, dissolved organic matter leaches out of the marine snow, attracting not only chemotactic prokaryotes (Stocker et al. 2008) but also their predators such as highly motile nanoflagellates (Fenchel & Blackburn 1999, Turk et al. 2010). Bacterial grazing mortality can therefore exceed bacterial growth and colonization (Kiørboe et al. 2004, Mari et al. 2004). As marine snow ages, however, it becomes more refractory due to the intense utilization of easily degradable compounds (Müller-Niklas et al. 1994, Turk et al. 2010) and the production of refractory capsular material by marine-snow-attached bacteria (Heissenberger & Herndl 1994, Heissenberger et al.

1996). This transformation of marine snow gradually leads to a decrease in cell-specific prokaryotic activity in ageing aggregates (Grossart & Ploug 2000). In our study, CSPP in marine snow was lower than in the ambient water in April, June, July and late-August (Fig. 1C), indicative of an aged marine snow.

Prokaryotic community structure is influenced by season or life mode

Freshly formed marine snow is initially colonized by motile heterotrophic prokaryotes from the surrounding water (Kiørboe et al. 2002). This free-living prokaryotic community undergoes seasonal changes associated with changing environmental conditions (Pinhassi & Hagström 2000, Alonso-Sáez et al. 2007, Ghiglione et al. 2007). In our study, the ambient-water as well as the marine-snow-associated bacterial communities clustered according to season. The bacterial life mode (ambient-water vs. marine-snow-associated), however, appears to have a smaller and non-significant effect on the bacterial community structure as assessed by T-RFLP. The higher dissimilarity between marine-snow-associated and the corresponding ambient-water community observed in spring (Fig. 2) might be explained by rapid changes in the free-living community due to rapidly changing environmental conditions, e.g. frequent freshwater inputs and accompanied varying salinity, temperature and substrate availability (Malačič & Petelin 2001). In contrast, under these rapidly changing environmental conditions marine snow might represent a more stable environment for its associated bacterial community.

Sequencing-based analysis of bacterial communities from the 2 contrasting seasons (December and late-August) only partly supported the T-RFLP based clustering (Figs. 2 & 3). Based on the 16S rDNA sequence analyses, the marine-snow-associated bacterial community collected in December clustered with the late-August communities, while T-RFLP analysis clustered the communities according to seasons (Fig. 2). This discrepancy might be caused by the higher phylogenetic resolution of the clone library approach compared to T-RFLP fingerprinting, as the former clustering is based on the phylogenetic relationship between individuals in different communities.

Furthermore, clone libraries of the marine-snow-associated bacteria revealed that the community is not only shaped by the seasonally changing environmental conditions in the ambient water as suggested by clustering (Figs. 2 & 3), but also by the physico-chemical conditions within marine snow. These conditions develop successively during the particle's ageing process which is, in turn, largely mediated by microbes (Alldredge & Silver 1988, Herndl et al. 1999). As a consequence, specific bacterial groups are exclusively present or dominate in marine snow. For instance, the presence of anaerobic bacteria, such as deltaproteobacterial sulphate-reducing bacteria (SRB), in marine snow in late August, and some specific groups, like *Bacteroides* (known for their partially anaerobic metabolism; Green et al. 2011), and the euryarchaeal methanogens of the orders *Methanosarcinales* and *Methanomicrobiales* in small December marine snow particles, point to the existence of anoxic and/or suboxic microenvironments in marine snow. Suboxic and anoxic conditions develop as a consequence of high oxygen consumption during heterotrophic degradation of marine snow organic matter and/or insufficient oxygen diffusion into the complex organic matrix (Ploug et al. 1997, Rath et al. 1998). Interestingly, the percentage of the *Deltaproteobacteria* in marine snow was higher in August than in December (Fig. 3). However, an even higher proportion of clones affiliated to *Deltaproteobacteria* (23%) was found in GEA in the northern Adriatic (Rath et al. 1998), suggesting that this bacterial group increases in abundance with age and size of marine snow, probably associated with the development of anoxic zones inside the marine snow. The presence of the methanogens in these small marine snow particles resembling marine snow commonly observed in many different marine environments might partially explain the supersaturation of methane in oceanic waters (Karl et al. 2008).

Another bacterial group suggesting niche partitioning in marine prokaryotic communities is the family *Vibrionaceae*, whose members were found to be highly abundant only in larger marine snow in August (Fig. 4). *Vibrionaceae* are rarely detected in the free-living community and in small marine snow (DeLong et al. 1993, Crump et al. 1999, Kellogg & Deming 2009). Nevertheless, they have been reported to be highly abundant in gel-like marine snow in the northern Adriatic (Danovaro et al. 2009), and are generally known for their attached life-style (Thompson et al. 2004 and references therein) and their preference for organic matter enriched environments (Fuks et al. 2005, Tinta et al. 2012).

In addition to seasonal changes and physico-chemically distinct microenvironments, the marine-snow-associated community is also defined by the particles' origin. The high abundance of *Cyanobacteria* in marine snow and the ambient water in late August

(Fig. 4) might indicate that these phototrophs substantially contribute to the extracellular polysaccharides of the marine-snow matrix, as proposed earlier (Kaltenböck & Herndl 1992, Fuks et al. 2005). In contrast, the high proportion of chloroplast-affiliated clones obtained from marine snow in December indicates a phytoplankton origin of the marine-snow matrix (Fig. 4), most likely derived from the diatom bloom that typically occurs in late autumn (Mozeti et al. 2012) and which was also observed in December 2009 (Tinta et al. 2014). However, the presence or high abundance of bacterial groups characteristic of low salinity estuarine or fresh waters, like *Betaproteobacteria, Verucomicrobia* and *Actinobacteria* (Kirchman et al. 2005, Alonso et al. 2010) might indicate a freshwater origin for a part of the bacterial community detected in marine snow in December. In addition, some bacterial groups, such as *Gemmatimonadetes* (Polymenakou et al. 2005), *Acidobacteria* and soil- or sediment-associated *Thaumarchaeota* might originate from the sediment in this shallow coastal sea. Despite the high percentage of archaeal cells observed on marine snow (Fig. S2), a very low diversity of the archaeal community was detected, with marine *Thaumarchaeota* as the only group detected in the ambient water in summer and dominating in December, which is in accordance with previous findings (Galand et al. 2008, Kellogg & Deming 2009).

The active fraction of the ambient-water community might be supported by marine-snow-associated prokaryotes

Analyses based on 16S rDNA reveal the phylogenetic structure of a given prokaryotic community, but do not provide any information about its active fraction (Moeseneder et al. 2005). In contrast, the ratio of RNA:DNA has been shown to correlate with bacterial growth rates in bacterial cultures (Kemp et al. 1993, Kerkhof & Kemp 1999). Therefore, the comparison between 16S rRNA and 16S rDNA has been suggested as an index of potential activity (Moeseneder et al. 2005, Campbell & Kirchman 2013, De Corte et al. 2013, Hugoni et al. 2013).

Our results based on T-RFLP analyses suggest that only a fraction of the detected bacterial taxa is metabolically active in the ambient water and in marine snow, since a lower richness of the active community (on the 16S rRNA level) compared to the overall richness (on the 16S rDNA level) was detected (Table S2). This difference between the richness of the potentially active and the total bacterial community was most pronounced in the large, presumably aged, marine snow collected in June and late August. Some of the OTUs detected by T-RFLP at the RNA level were not detected at the DNA level in the same sample (data not shown), indicating that some rare bacterial species, with an abundance below the detection limit of the method used here, might be highly active (Campbell et al. 2011, Campbell & Kirchman 2013). A high dissimilarity between marine-snow-associated bacterial communities and their potentially active fraction has been previously reported for the Aegean Sea (Moeseneder et al. 2001b) and could be connected to the successional transformation of particles, provoking shifts in the active bacterial taxa. Following the period of high metabolic activity, specific bacterial groups might cease their growth due to altered physical and/or chemical conditions in the particles. These bacteria, although metabolically inactive, remain entrapped in the mucous matrix at high abundances for a variable period of time (Moeseneder et al. 2001b).

The phylogenetic analysis revealed that the active fraction (on the 16S rRNA level) of the ambient-water bacterial communities in late August is related to the marine-snow-associated bacterial community (on the 16S rDNA level) (Fig. 3). This might indicate that the release of soluble organic substances from the marine snow supports the ambient-water bacterial community, resulting in a similar phylogenetic composition of the active bacterial community in both the ambient water and marine snow. Considering that oligotrophic conditions develop in the water column during summer mucilage events in the northern Adriatic (Fuks et al. 2005), dissolved organic matter released from the aggregates might represent the predominant source of substrate available for growth of the ambient-water bacterial community.

A recent study based on metagenomic analyses of marine-snow-associated assemblages revealed an up to 6-fold higher concentration of genes encoding for cell-to-cell transfer and mobile elements in the size fraction typical for marine snow as compared to the ambient water (Ganesh et al. 2014). This points to the fact that, in addition to fuelling the background community with nutrients, densely colonised marine snow might act as a hotspot for horizontal gene transfer, thereby providing new genetic combinations and facilitating the evolution of metabolic pathways in the ocean (Stewart 2013, Ganesh et al. 2014). Together with our results, these findings further emphasise the importance of marine snow for ecosystem functioning.

CONCLUSION

Our results indicate that the prokaryotic communities associated with marine snow and those in the ambient water are connected, and vary throughout the year in both environments. The presumably more stable environmental conditions in marine snow lead to less pronounced seasonal changes in the composition of the associated bacterial community. However, the composition of the marine-snow-associated community is linked to the source and developmental stage of the snow. The link between free-living and marine-snow-associated bacteria is signified by the similar composition of the active community in the ambient water and in the marine snow when the marine snow reaches a mature stage.

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