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„Successional changes in marine snow in the northern Adriatic Sea “

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Introduction

Formation and characteristics of marine snow

Marine particulate organic matter exists in a size continuum ranging from entangled macromolecules to large, visible aggregates (Alldredge & Silver 1988, Verdugo et al 2004). The smallest forms of particulate organic matter (< 0.1µm) are networks of biopolymers of spontaneously assembled dissolved organic matter, so-called marine gels (Verdugo & Santschi 2010). Marine gels represent the base for micro- and macroaggregates (Simon et al 2002). Additionally, transparent exopolymer particles (TEP) are directly released from phytoplankton and bacteria and are important in the formation of larger aggregates with fecal pellets and phytodetritus (Alldredge et al 1993, Engel et al 2020, Turner 2002, Turner 2015, Verdugo et al 2004) (Fig. 1). Particulate organic matter represents the largest source of organic carbon to the deep sea via vertical transport (Boyd et al 2019, Burd et al 2010). Sinking macroaggregates from the surface to the dark ocean are coined ‘marine snow’ (Silver 2015). Marine snow represents the upper end of the continuum ranging in size from 500µm to centimeters (Alldredge & Silver 1988, Herndl & Peduzzi 1988, Simon et al 2002) and can reach sizes up to meters in coastal basins (Kaltenböck & Herndl 1992). Long periods of calm weather conditions create a stratified water column where particulate organic matter is retained at the pycnocline and large ‘clouds’ of organic matter might form (Kaltenböck & Herndl 1992, MacIntyre et al 1996).

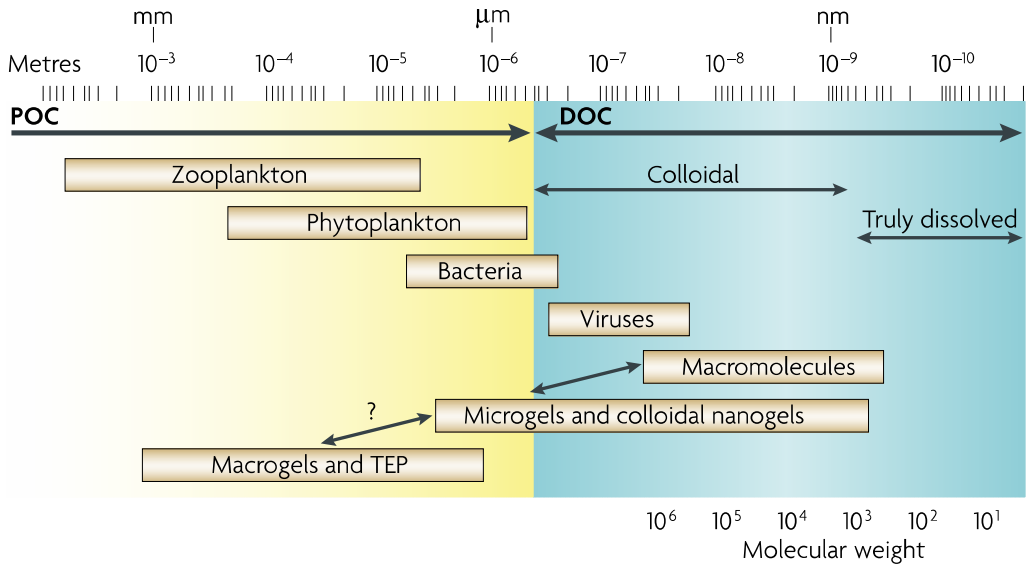

Figure 1. Size continuum of organic matter (Azam & Malfatti 2007)

Methodological considerations

Due to the large variety of particulate organic matter and the difficulty of sampling marine snow, a large variety of methods to collect marine snow has been developed. Most commonly, free-living and particle-associated microbes are separated by size fractionation via filtration, with the drawback of fractionation according to artificial pore sizes of the filters and loss of associated microbes, not firmly attached to the marine snow (Mestre et al 2017). Other methods such as sediment traps and marine snow catchers were deployed to collect sinking marine snow, thereby neglecting non-sinking particles (Buesseler et al 2008, De La Rocha & Passow 2007, Riley et al 2012, van der Jagt et al 2018). By selectively collecting marine snow with syringes by SCUBA divers (Fig. 2) sinking and non-sinking marine snow particles are sampled including the associated but not attached microbial community (Kaltenböck & Herndl 1992, Müller-Niklas et al 1994, Rath et al 1998). However, due to the manual collection by SCUBA divers primarily large visible particles are collected and sampling is constrained to shallow surface waters (~15m). In this PhD thesis, we aimed at collecting large and aged cloud-like marine snow in the coastal northern Adriatic Sea. The first field trips were in June, August and September 2014 and were intended for testing methods. However, we neither observed large cloud-like marine snow in 2014 nor in the following field trips, despite reports of occurrences off Trieste, Italy. However, we observed a constant presence of abundant marine snow ranging in size from 0.5 to ~2cm (Fig. 2). Hence, we adjusted and optimized the sampling procedure and tested a variety of methods. This included determining the suitability of meta-transcriptomics to assess prokaryotic metabolic pathways in environmental communities. Since the introduction of chain-termination sequencing by Sanger et al (1977), DNA sequencing became an important tool to characterize prokaryotes and their metabolic potential (Acinas et al 2019, Duarte 2015, Venter et al 2004). With the evolution of sequencing technologies, attention refocused on transcribed DNA, giving valuable insights into microbial community dynamics and responses to the environment (Cottrell & Kirchman 2016, McCarren et al 2010). Nevertheless, few drawbacks concerning its use in activity profiling remained (Greenbaum et al 2003, Hambraeus et al 2003, Kristoffersen et al 2012, Steglich et al 2010). Whereas transcriptomics based on experiments conducted *in vitro* can provide reliable results, this technique might misrepresent prokaryotic metabolic activity of envi-

ronmental samples, mainly due to the short half-life time of some transcripts (Steglich et al 2010) and its potentially high variability within communities (Clouet-d'Orval et al 2018, Condon 2003, Jain 2002). The assessment of RNA half-life time and the factors controlling it were of considerable importance to implement the use of transcriptomics in further studies.

Figure 2. Marine snow sampling with syringes by SCUBA divers

Seasonal dynamics of free-living and particle-associated microbes

The high abundance of marine snow in the Adriatic Sea in the late 1980s led to intensified research in the following years (Bochdansky & Herndl 1992a, Bochdansky & Herndl 1992b, Cozzi et al 2004, Degobbis et al 1995, Herndl & Peduzzi 1988, Kaltenböck & Herndl 1992, Müller-Niklas et al 1994). The distinct physical and biological characteristics from seawater and the biogeochemical impact of marine snow were thoroughly studied in the northern Adriatic Sea and many other ocean regions (Burd & Jackson 2009, Herndl & Reinthaler 2013, Simon et al 2002, Turner 2002, Turner 2015). The studies revealed that marine snow is enriched in organic and inorganic nutrients and populated by distinct and highly active heterotrophic and autotrophic microbes, primarily bacteria (Kaltenböck & Herndl 1992, Müller-Niklas et al 1994, Najdek et al 2002, Rath et al 1998, Simon et al 2002). The high metabolic activity of prokaryotes within marine snow can generate anoxic microenvironments allowing anaerobic metabolism with important biogeochemical implications (Shanks & Reeder 1993, Stocker et al 2008, Swan et al 2011). A particularly high potential for anoxic microenvironments has been suggested in particles larger than 1mm in diameter (Bianchi et al 2018, Bristow 2018, Lehto et al 2014). Furthermore, the substrate and nutrient concentrations within marine snow can directly and indirectly affect the associated prokaryotic community composition (Müller-Niklas et al 1994). Microenvironments and high nutrient concentrations might allow the development of a prokaryotic community that is largely independent of the conditions in the bulk seawater (Bianchi et al 2018, Bochdansky et al 2016, Mestre et al 2017, Yung et al 2016). Despite the biogeochemical importance of aging and maturation processes of marine snow, successional and seasonal studies on marine snow associated communities are scarce (Vojvoda et al 2014, Yung et al 2016). In the course of this thesis, we collected marine snow seasonally over a 1.5-year period and compared the microbial community composition and functional potential to that of the ambient water.

Free-living and particle-associated DMSP demethylating bacteria

Nutrient concentrations in marine snow are occasionally orders of magnitude higher than in the surrounding water (Kaltenböck & Herndl 1992, Stocker 2012). Labile substances, such as amino acids, are released by senescent phytoplankton trapped within the marine snow matrix, and leach out of the particles, consequently creating microgradients of nutrients in the intimate vicinity of marine snow (Stocker 2012, Stocker et al 2008). This plume of nutrients attracts motile chemotactic organisms (Kiørboe & Jackson 2001, Son et al 2015). One of the substances with strongest attraction to chemotactic organisms is dimethylsulfoniopropionate (DMSP) (Seymour et al 2010, Tout et al 2015). DMSP is an organic sulfur compound mainly produced by marine phytoplankton with various important physiological and ecological functions (Moran et al 2012). When released into the environment, DMSP is utilized by prokaryotes as an osmolyte and is processed via two competing metabolic pathways (Kiene et al 2000, Kiene et al 1999, Sun et al 2016) (Fig. 3). DMSP cleavage regulates intracellular DMSP concentrations yielding the carbon compound acrylate or 3-hydroxypropionate (Kiene et al 2000, Sun et al 2016), while the sulfur compound is lost from the system via the climatically relevant gas dimethylsulfide (DMS) (Kiene et al 2000) (Fig. 3). DMSP demethylation, in contrast, yields methanethiol (MeSH) and the carbon and sulfur moieties enter the marine food web (Kiene et al 1999, Moran et al 2012) (Fig. 3). The requirement of carbon and sulfur to synthesize sulfur containing proteins is of greater importance to bacteria than the benefits gained by DMSP cleavage. Therefore, ~90% of dissolved DMSP is demethylated (Kiene et al 1999, Moran et al 2012) and ~10% of the carbon demand of heterotrophic bacteria is met via DMSP demethylation (Archer et al 2001, Howard et al 2006). DMSP demethylation genes are 2 to 5-fold more abundant than genes of the cleavage pathway (Moran et al 2012, Varaljay et al 2012) and are present in a wide range of bacterial subclades, comprising mainly Alphaproteobacteria (members of the SAR11 clade, SAR116 cluster and Roseobacter) and Gammaproteobacteria (Nowinski et al 2019, Varaljay et al 2012). Their temporal distribution is associated to phytoplankton blooms and specific phytoplankton taxa (Howard et al 2011, Nowinski et al 2019, Varaljay et al 2015). Demethylating bacteria utilize the DMSP which typically increases in concentration after phytoplankton blooms (Archer et al 2001, González et al 2000, Zubkov et al 2001) depending on the phytoplankton species (Keller et al 1989, Stefels 2000). The substantial contribution of phytoplankton to both, DMSP production and marine snow formation led us to hypothesize that ma-

rine snow represents a hot-spot of DMSP in the water column. We related the demethylating bacterial community to DMSP concentrations in marine snow and the ambient water over a seasonal cycle. To obtain a complete picture of the environment to which particle-associated and free-living bacteria are exposed to, we determined a variety of parameters including nutrients, DMSP concentrations, phytoplankton community composition and abundance, prokaryotic community composition, abundance and activity (as leucine incorporation) and the potentially demethylating bacterial community.

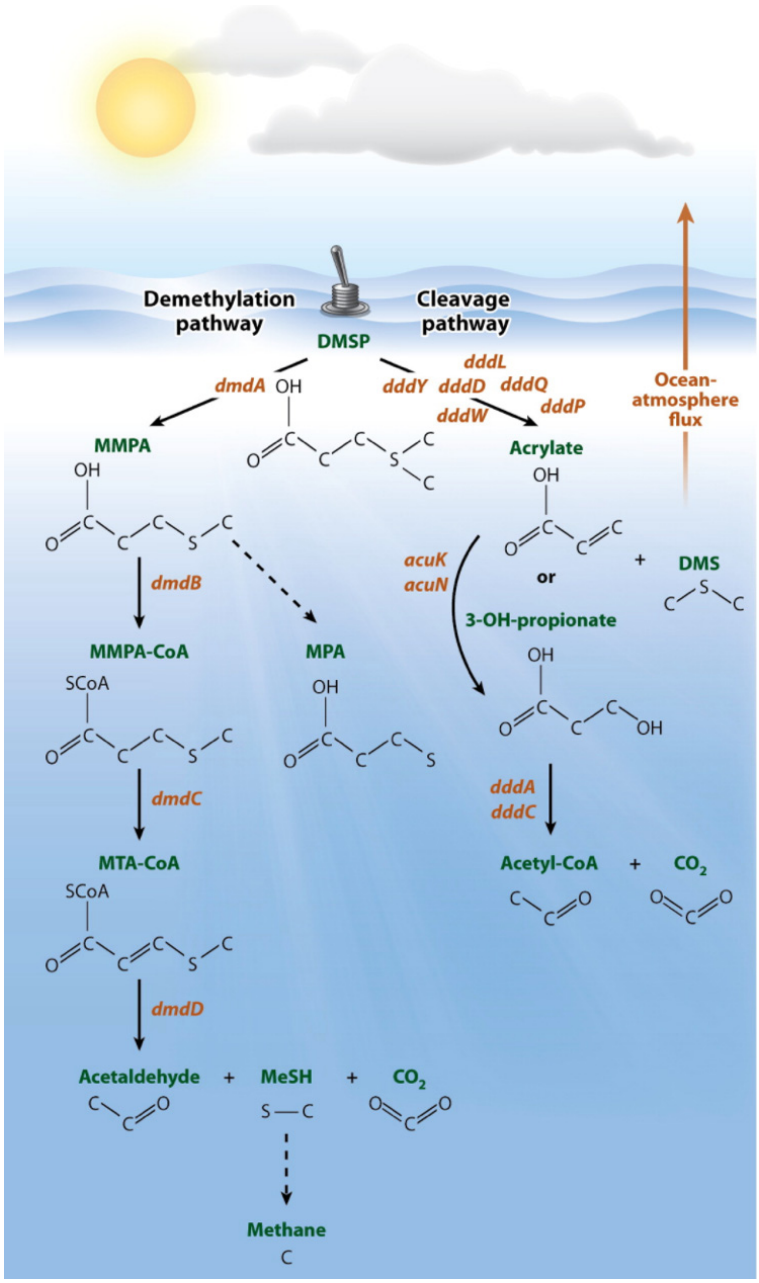

Figure 3. Dimethylsulfoniopropionate (DMSP) degradation pathways in the ocean (Moran et al 2012)

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Chapter overview

Chapter I: Highly variable mRNA half-life time within marine bacterial taxa and functional genes

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Author contributions:

ES conceived and together with DDC and PAS carried out the experiments. PAS and CM extracted RNA. ES designed qPCR primers. PAS carried out qPCR. DDC prepared the metatranscript library and TY coordinated and supervised the sequencing. JG and TR provided bioinformatics support. PAS analyzed the data and wrote the manuscript. GJH and ES provided project oversight. All co-authors read and approved the final manuscript.

Chapter II: Functional seasonality of free-living and particle-associated prokaryotic communities in the coastal Adriatic Sea

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To be submitted

Author contributions:

PAS, JG, TR, ES and GJH conceived the research. PAS conducted DNA extraction and prepared 16SrRNA and metagenome sequencing. JG, AO and PAS analyzed the data under supervision and following the advices of TR and EF. PAS wrote the manuscript. All co-authors revised the draft version and approved the final version.

Chapter III: Seasonal dynamics of marine snow-associated and free-living demethylating bacterial communities in the coastal northern Adriatic Sea

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Author contributions:

ES, PAS and GJH conceived the research. MN arranged field trips, wet lab and housing issues. ES measured prokaryotic leucine incorporation, RS determined DMSPt+DMS concentrations, DMP identified the phytoplankton community composition and II measured nutrient concentrations. PAS determined prokaryotic abundance, extracted prokaryotic DNA, prepared 16SrRNA Illumina sequencing and performed qPCRs. PAS conducted data analysis following the advices of ES and DDC. PAS wrote the manuscript and specific methodological details were added by the respective co-authors. The final version was approved by all co-authors.

Chapter I Highly variable mRNA half-life time within marine bacterial taxa and functional genes

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Originality-Significance Statement

Messenger RNA (mRNA) is increasingly used to look beyond the metabolic potential and explore the activity of microorganisms in culture and in the environment. However, the extent of RNA instability and the factors affecting RNA decay are uncertain, particularly in environmental communities. In this study we determine the half-life time of the 16S rRNA and the mRNA of 10 housekeeping genes of three isolated bacteria. Additionally we determine the mRNA half-life time of a metatranscriptome of an environmental sample and explore factors affecting mRNA decay.

Summary

Messenger RNA can provide valuable insights into the variability of metabolic processes of microorganisms. However, due to uncertainties that include the stability

of RNA, its application for activity profiling of environmental samples is questionable. We explored different factors affecting the decay rate of transcripts of three marine bacterial isolates using qPCR and determined mRNA half-life time of specific bacterial taxa and of functional genes by metatranscriptomics of a coastal environmental prokaryotic community. The half-life time of transcripts from 11 genes from bacterial isolates ranged from 1 to 46 min. About 80% of the analyzed transcripts exhibited half-live times shorter than 10 min. Significant differences were found in the half-life time between mRNA and rRNA. The half-life time of mRNA obtained from a coastal metatranscriptome ranged from 9 to 400 min. The shortest half-life times of the metatranscriptome corresponded to transcripts from the same clusters of orthologous groups (COGs) in all bacterial classes. The prevalence of short mRNA half-life time in genes related to defense mechanisms and motility indicate a tight connection of RNA decay rate to environmental stressors. The short half-life time of RNA and its high variability needs to be considered when assessing metatranscriptomes especially in environmental samples.

Introduction

In contrast to the genome, which illustrates the metabolic potential of microorganisms, the transcriptome indicates metabolic activity. Identification and quantification of expressed genes at a particular moment can provide valuable insights into the response of organisms to environmental conditions (Cottrell & Kirchman 2016) or microbial community dynamics (McCarren et al 2010). However, poor correlations between mRNA and protein levels raised concerns about the use of mRNA for activity profiling (Greenbaum et al 2003, Wang et al 2019). Moreover, capturing the expression profile of an organism at a specific moment is typically challenging due to the rapid turnover and decay of mRNA (Hambraeus et al 2003, Kristoffersen et al 2012, Selinger et al 2003, Steglich et al 2010). RNA half-life times ($T_{1/2}$) as short as a few seconds for single transcripts (Steglich et al 2010) and few minutes for cultured prokaryotic species (Hambraeus et al 2003, Selinger et al 2003, Steglich et al 2010) have been reported. However, stable mRNAs have also been observed (Hambraeus et al 2003), indicating a high complexity of the mechanisms regulating RNA degradability. RNA $T_{1/2}$ has been mostly determined on model microorganisms such as *Escherichia coli* and *Bacillus subtilis* to understand the mechanisms causing the instability (Condon 2003, Jain 2002, Kushner 2002). Ribonucleases (RNAses) are

main players in RNA metabolism and enable controlled degradation of RNA (Deutscher 2006, Deutscher 2015). One of the cells' strategies to deal with RNAses is to isolate them from the RNA molecules by locating RNAses in the periplasmic space, bound to the inner cell membrane or in the cell periphery (Deutscher 2015, Taghbalout et al 2014). However, after cell lysis or damage, RNAses can get access and degrade RNA molecules in an uncontrolled way, both inside and outside the cell (Deutscher 2015). A multi-protein complex termed RNA degradosome has been shown to play a major role in RNA degradation (Deutscher 2006). Other factors such as RNA type (Deutscher 2003, Deutscher 2006) and gene position (Hambraeus et al 2003, Steglich et al 2010) suggest a structural base in the RNA degradation patterns. However, less attention was given to environmental factors, such as temperature, and whether or how it influences RNA decay (as reviewed in (Takayama & Kjelleberg 2000). Reports of both extremely short decay rates and stable mRNAs in selected organisms, e.g., the longer half-life times reported for archaeal mRNAs as compared to bacterial mRNAs (Clouet-d'Orval et al 2018) indicate potentially high variability of RNA decay rates within complex communities.

In this study, we analyzed RNA degradation patterns of three marine bacterial isolates and related RNA $T_{1/2}$ to RNA type (mRNA, rRNA), optimal growth temperature and growth rate. Furthermore, we used metatranscriptomics to assess the mRNA $T_{1/2}$ from a coastal prokaryotic community on a phylogenetic and a functional level to examine possible ecological factors such as competition or nutrient stress causing RNA degradation and determining RNA degradation rates.

Results and discussion

RNA half-life time of bacterial isolates determined by qPCR

The three bacterial isolates had a query cover of 99% to 100% and an identity between 98% and 99% to *Alcanivorax jadensis* (Accession Nr.: NR_025271.1), *Colwellia polaris* (Accession Nr.: NR_043462.1) and *Croceibacter atlanticus* (Accession Nr.: NR_074636.1). The respective isolates are subsequently referred to as *Alcanivorax*, *Colwellia*, and *Croceibacter. Alcanivorax* and *Croceibacter* are marine bacteria typically found in the water column of the Mediterranean Sea and Atlantic (Cho & Giovannoni 2003, Fernández-Martínez et al 2003). *Colwellia polaris* is a psychrotolerant marine bacterium first isolated from Arctic sea-ice (Cho & Giovannoni 2003, Fernández-Martínez et al 2003, Zhang et al 2008).

RNA degradation was assessed during the early stationary phase of the isolates grown at 4°C, 15°C and 25°C. At the early stationary phase, cell abundance varied between 3.7 x 10^8 and 8.0 x 10^8 cells mL^{-1} in *Alcanivorax* cultures, 3.8 x 10^7 and 6.6 x 10^8 cells mL^{-1} in *Colwellia,* and 1.5 x 10^9 and 1.8 x 10^9 cells mL^{-1} in *Croceibacter* cultures (Fig. S1).

Overall, the $T_{1/2}$ of transcripts of 11 genes varied from 1 min up to 46 min at all temperatures, as revealed by qPCR (Fig. 1, Fig. S2). Eighty percent had a $T_{1/2}$ shorter than 10 min, in agreement with the short half-life times previously reported for bacterial RNA (Hambraeus et al 2003, Kristoffersen et al 2012, Selinger et al 2003, Steglich et al 2010).

The $T_{1/2}$ of 16S rRNA was significantly longer than of the mRNAs at all temperatures, except for *Alcanivorax* at 25°C (Table 1). A higher stability of non-coding RNA (ncRNA) as compared to mRNA has also been found in previous studies (Deutscher 2003, Deutscher 2006, Steglich et al 2010). The mechanisms of resistance of ncRNA to degradation are unclear but likely due to physical protection (Deutscher 2003, Deutscher 2006). Different degradation patterns were observed at different temperatures. At 4°C and 15°C the $T_{1/2}$ of *hfl*B and *pss*A of *Alcanivorax* was shorter (2.2 to 7.9 min) than of 16S rRNA $T_{1/2}$ (>40 min), while at 25°C a longer $T_{1/2}$ for these two genes was obtained than for 16S rRNA $T_{1/2}$ (Fig.1, Table 1). Such contrasting $T_{1/2}$ patterns of mRNA and rRNA at different temperatures might arise from the flexibility and adaptability of the degradosome to environmental conditions (Deutscher 2006, Prud'homme-Généreux et al 2004). All components of the degradosome need to function to ensure normal mRNA turnover (Bernstein et al 2004). Different transcript types have been shown to be affected differently by failures in degradosome components (Bernstein et al 2004). The long $T_{1/2}$ of mRNA in *Alcanivorax* at 25°C might result from temperature sensitivity of some components of the degradosome machinery (Sulthana et al 2016). Typically 16S rRNA is degraded only under stress or in response to mis-assembly and deficiency (Deutscher 2003, Sulthana et al 2016). The degradation of 16S rRNA determined in this experiment might have been caused by the addition of the antibiotic rifampicin or, alternatively, temperature stress to the bacterial isolates (Sulthana et al 2016).

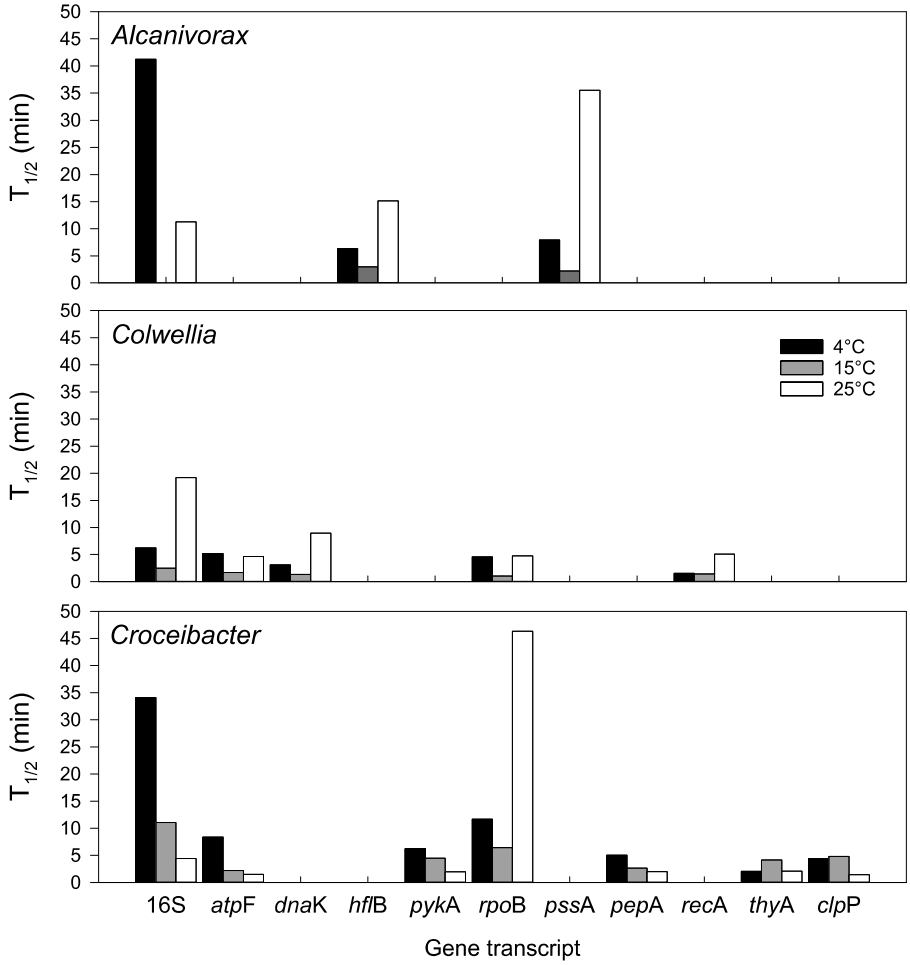

Figure 1. Half-life time ($T_{1/2}$) of gene transcripts of the genes 16SrRNA, *atp*F (ATP synthase B chain), *dna*K (chaperone Hsp70), *hfl*B (ATP-dependent protease), *pyk*A (pyruvate kinase), *rpo*B (RNA polymerase ß subunit), *pss*A (phosphatidylserine synthase), *pep*A (aminopeptidase A/I), *rec*A (DNA repair protein), *thy*A (thymidylate synthase) and *clp*P (clp protease subunit 2) of the bacterial isolates *Alcanivorax jadensis* (n = 9) *, Colwellia polaris* (n = 15) and *Croceibacter atlanticus* (n = 21) at 4°C, 15°C and 25°C.

It has been hypothesized that the rate of transcription and hence, the growth rate of an organism, affects the $T_{1/2}$ of mRNA (Takayama & Kjelleberg 2000, Vytvytska et al 1998). However, it has also been suggested that the $T_{1/2}$ depends more on temperature than on growth rate (Hundt et al 2007). The generation time of *Alcanivorax* correlated inversely to temperature (r = -0.97, p = 0.001, n = 6). The shortest generation time (3.17 h) was obtained at 30°C and the longest generation time (13.32 h) at 4°C (Fig. S3), in agreement with the classification of *Alcanivorax jadensis* as mesophilic in 'The Bacterial Diversity Metadatabase' (Söhngen et al 2015) of the Leibniz Institute DMSZ. Consequently, the average mRNA $T_{1/2}$ of *Al-*

canivorax was long when generation time was short (r = -0.48, p = 0.68, n = 3) and temperature was high (r = 0.74, p = 0.47, n = 3) and *vice versa*. This is in contrast to the findings reported in other studies (Hundt et al 2007, Vytvytska et al 1998).

Table 1. Half-life time ($T_{1/2}$) of transcripts of specific genes for the different isolates (Alc. = *Alcanivorax*, Col. = *Colwellia*, Cro. = *Croceibacter*) at 4ºC, 15ºC and 25ºC. Slope (m) coefficient of determination (R^2) of the fitted curve is also indicated. The *p* value (one-sample t-test) denoting the significance of the difference between the half-life time of the 16S rRNA and other genes is also shown. Asterisk (*) indicates that 100 min was replaced by 'no decay' in the statistical test. Two asterisks (**) indicate that the $T_{1/2}$ of *rpo*B was excluded from the one-sample t-test

Gene		*Alc*. 4°C	*Alc*. 15°C	*Alc*. 25°C	*Col*. 4°C	*Col*. 15°C	*Col*. 25°C	*Cro*. 4°C	*Cro*. 15°C	*Cro*. 25°C
	R^2	0.32	0.10	0.58	0.59	0.22	0.92	0.70	1.00	0.88
16S rRNA	m	–0.01	0.01	–0.05	–0.10	–0.21	–0.03	–0.02	–0.06	–0.17
	$T_{1/2}$	41.25	no decay	11.29	6.23	2.46	19.22	34.09	11.06	4.41
	R^2				0.91	0.52	0.94	0.99	0.99	0.99
*atp*F	m				–0.14	–0.35	–0.15	–0.08	–0.32	–0.45
	$T_{1/2}$				5.17	1.69	4.63	8.39	2.20	1.51
	R^2				0.60	0.50	0.997			
*dna*K	m				–0.20	–0.44	–0.13			
	$T_{1/2}$				3.07	1.32	8.97			
	R^2	0.99	0.99	0.98						
*hfl*B	m	–0.22	–0.23	–0.05						
	$T_{1/2}$	6.33	2.98	15.15						
	R^2							0.97	0.90	0.99
*pyk*A	m							–0.12	–0.15	–0.36
	$T_{1/2}$							6.20	4.47	1.97
	R^2				1.00	0.90	0.99	1.00	0.94	0.98
*rpo*B	m				–0.15	–0.72	–0.15	–0.06	–0.10	–0.02
	$T_{1/2}$				4.57	1.03	4.73	11.70	6.45	46.34
	R^2	0.99	0.92	0.98						
*pss*A	m	–0.09	–0.34	–0.02						
	$T_{1/2}$	7.91	2.19	35.51						
	R^2							0.86	0.72	0.96
*pep*A	m							–0.14	–0.23	–0.36
	$T_{1/2}$							5.04	2.65	2.01
	R^2				0.94	0.36	0.93			
*rec*A	m				–0.47	–0.38	–0.13			
	$T_{1/2}$				1.54	1.45	5.10			
	R^2							0.98	0.90	0.95
*thy*A	m							–0.36	–0.18	–0.32
	$T_{1/2}$							2.06	4.16	2.11
	R^2							0.99	0.76	0.96
*clp*P	m							–0.16	–0.13	–0.49
	$T_{1/2}$							4.37	4.81	1.47
t-test	*p* value	0.01	0.003*	0.40	0.05	0.004	0.001	0.000	0.000	0.00004**

Colwellia, however, exhibited the shortest generation time (3.77 h) at 15°C and long generation times at 4°C and 25°C (11.68 h and 9.50 h, respectively) (Fig. S3). It has been shown previously that *Colwellia polaris* reached its growth optimum at 20°C – 21°C (Zhang et al 2008). The increase of generation time beyond 15°C indicates a psychrophilic life style of *Colwellia polaris*. In *C. Polaris*, this is also indicated by ceased growth at 26°C in a previous study (Zhang et al 2008) and at 30°C in this study (Fig. S3). The average $T_{1/2}$ of transcripts of four genes (*atp*F, *dna*K,

*rpo*B and *rec*A) of *C. polaris* were more strongly correlated with generation time (r = 0.69, p = 0.51, n = 3) than with temperature (r = 0.48, p = 0.68, n = 3), as both average $T_{1/2}$ and generation time were shortest at 15°C (Fig. S3). The long $T_{1/2}$ at high temperatures might result from a malfunctioning degradosome.

The generation times of *Croceibacter* correlated inversely to temperature (r = -0.80, p = 0.06, n = 6) with the longest generation time (14.05 h) obtained at 4°C (Fig. S3). However, generation times were similar at 15°C and 30°C averaging 4.48 ± 0.47 h (mean ± SD), indicating a wide range of optimal temperature growth. The type strain *Croceibacter atlanticus* has an optimum growth temperature of 20-23ºC (Krieg et al 2010) and, in contrast to our isolate, does not grow at 4ºC or 30ºC. The average $T_{1/2}$ of six gene transcripts (*atp*F, *pyk*A, *rpo*B, *pep*A, *thy*A and *clp*P) of *Croceibacter* correlated negatively to temperature (r = -0.999, p = 0.03, n = 3) and positively to generation time (r = 0.89, p = 0.30, n = 3).

Correlations of $T_{1/2}$ of individual gene transcripts to temperature and generation time (Table S3) varied strongly among the bacterial strains indicating that mRNA $T_{1/2}$ is probably regulated by a combined effect of temperature and generation time among other factors such as the position within an operon (Hambraeus et al 2003, Steglich et al 2010) or the secondary structure and interactions with other molecules (Hambraeus et al 2003).

Variability of RNA $T_{1/2}$ in complex environmental communities determined by transcriptomics

The number of reads ranged between 3.2 x 10^5 and 2.2 x 10^6 per metatranscriptome and the fraction of mRNA between 56.3% and 93.5% (data not shown). However, after quality control, the disagreement between the transcriptomes at time points *t2.5*, *t10* and *t20* was higher than 30%. Consequently, these samples were considered outliers and were excluded from further analysis (Conesa et al (2016). Therefore, $T_{1/2}$ was calculated using only the time points *t0*, *t5*, *t40* and *t60*. The transcript abundance at time point *t0* (without rifampicin addition) was on average 6.1 ± 8.0 times lower than at time point *t5*. A similar pattern was observed in the transcript abundances of isolates where *t0* samples (without rifampicin) had on average 9.3 ± 7.4 times lower mRNA abundance than *t2.5* samples (Fig. S2). Lower abundance of transcripts prior to the addition of rifampicin has been reported for other bacterial cultures (Selinger et al 2003, Steglich et al 2010). These lags in tran-

scription inhibition could result from the different rate of entry of rifampicin into the cells and/or from the insensitivity of RNA polymerase to rifampicin (Laguerre et al 2018).

Vibrionaceae exhibited the highest relative transcript abundance (19.7 ± 3 %) followed by *Enterobacteriaceae* (14.3 ± 2.4 %) and *Rhodospirillaceae* (10.4 ± 0.9 %) (Fig. 2), but no significant correlations between the relative abundance and $T_{1/2}$ were found. The families *Vibrionaceae* and *Rhodospirillaceae* are among the most abundant bacterial families in the coastal northern Adriatic Sea (Steiner et al., unpublished), and frequently are the most active taxa in marine environmental communities (Bergauer et al 2018, McCarren et al 2010, Vorobev et al 2018). The high transcript abundance of *Enterobacteriaceae* and other bacteria detected in this study might be due to the close vicinity of the sampling location to a fish cannery, releasing high amounts of untreated wastewater (Paliaga et al 2017). In addition, the original bacterial community composition might have shifted during the five-day incubation prior to initiating the experiment (Massana et al 2001).

mRNA did not decay in 16 bacterial families in this study. The very slow or lack of decay might indicate resistance to the antibiotic rifampicin, as reported for clinical isolates and specific laboratory strains showing mutations in *rpo*B codons (Goldstein 2014). *Mycobacterium tuberculosis* and *Staphylococcus aureus* of the families *Mycobacteriaceae* and *Staphylococcaceae*, respectively, have been reported to be resistant to rifampicin (Goldstein 2014), in agreement with the lack of decay of mRNA of these two families (Table S4). Moreover, we could not determine mRNA decay rates for members of the orders *Burkholderiales*, *Pseudomonadales*, *Enterobacteriales*, *Actinomycetales*, *Rhizobiales* and *Shpingomonadales* (Table S4), as reported for soil isolates from these orders not only being resistant but can grow on several antibiotics as the sole carbon source (Dantas et al 2008).

Exponential mRNA decay was determined in 44 families, with $T_{1/2}$ ranging from 9 min to 400 with a median of 28 min (Fig. 2). The shortest $T_{1/2}$ times, based on the whole transcriptome of a bacterial family, were longer than in the experiments with the bacterial strains, where decay rates of single gene transcripts of individual isolates were assessed. Most studies on RNA $T_{1/2}$ are based on model organisms, such as *Escherichia coli* or *Bacillus subtilis*. The $T_{1/2}$ of 329 known and predicted operons of *E. coli* vary strongly from less than 2 min to more than 20 min and rifampicin insensitivity has been reported (Selinger et al 2003). Although about 80% of mRNAs

of *Bacillus subtilis* have a $T_{1/2}$ of less than 7 min, also extremely stable mRNAs have been detected in the same organism (Hambraeus et al 2003). The transcriptome of *Enterobacteriaceae* (which includes *E. coli*) has a $T_{1/2}$ of 29.5 min, while that of the family *Bacillaceae* (which includes *B. subtilis*) has a relatively long $T_{1/2}$ of 147.5 min, indicating a faster mRNA turnover for *Enterobacteriaceae* in coastal Adriatic Sea waters.

To address the variability of the $T_{1/2}$ linked to different functional groups of mRNAs, the $T_{1/2}$ was calculated for COG subcategories at the class taxa level. $T_{1/2}$ of COG subcategories ranged from 9 min to 134 min, with a median of 24 min (Fig. 3) and 28% of all $T_{1/2}$ were shorter than 20 min. This substantially longer $T_{1/2}$ as compared to previous reports on decay of single gene transcripts and single organisms (Hambraeus et al 2003, Hundt et al 2007, Kristoffersen et al 2012, Selinger et al 2003, Steglich et al 2010) might be related to the broad grouping of bacteria into classes. However, mRNA assignments to lower taxonomic levels were generally too low to calculate $T_{1/2}$ of COG subcategories, in agreement with the low levels of transcript abundance (< 1 per cell) for most genes recently reported in abundant marine bacteria (Cottrell & Kirchman 2016).

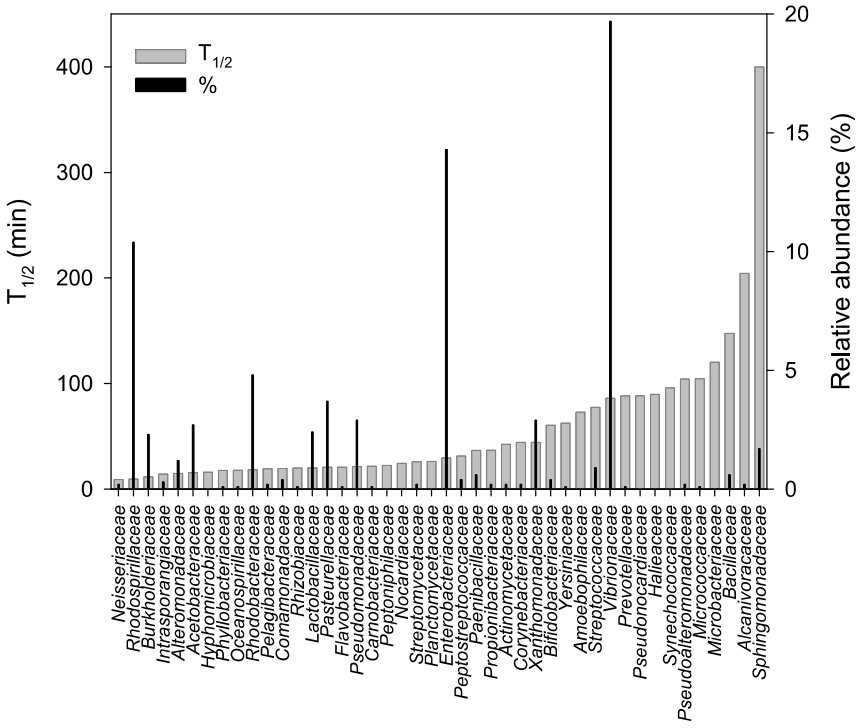

Figure 2. Half-life time ($T_{1/2}$) and relative abundance of rRNA (%) assigned to 44 bacterial families in the metatranscriptome from the coastal Adriatic Sea.

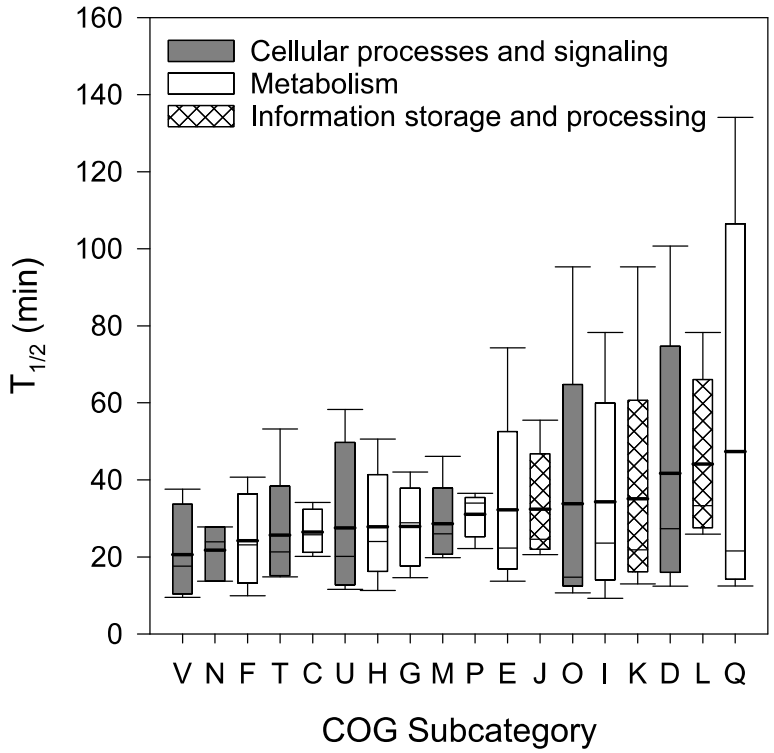

Figure 3. Half-life time ($T_{1/2}$) of COG categories and subcategories. The median is indicated by the thin horizontal line, the mean by the thick horizontal line. Whiskers indicate the 10th and 90th percentiles, and the edge of the box represent 25th, 75th percentiles. COG category 'cellular processes and signaling' includes the subcategories: **[V]** Defence mechanisms, **[N]** Cell motility, **[T]** Signal transduction mechanisms, **[U]** Intracellular trafficking, secretion, and vesicular transport, **[M]** Cell wall/membrane/envelope biogenesis, **[O]** Post-translational modification, protein turnover, and chaperones and **[D]** Cell cycle control, cell division, chromosome partitioning. COG category 'metabolism' includes the subcategories: **[F]** Nucleotide transport and metabolism, **[C]** Energy production and conversion, **[H]** Coenzyme transport and metabolism, **[G]** Carbohydrate transport and metabolism, **[P]** Inorganic ion transport and metabolism, **[E]** Amino acid transport and metabolism, **[I]** Lipid transport and metabolism and **[Q]** Secondary metabolites biosynthesis, transport, and catabolism. COG category 'information storage and processing' includes the subcategories: **[J]** Translation, ribosomal structure and biogenesis, **[K]** Transcription and **[L]** Replication, recombination and repair.

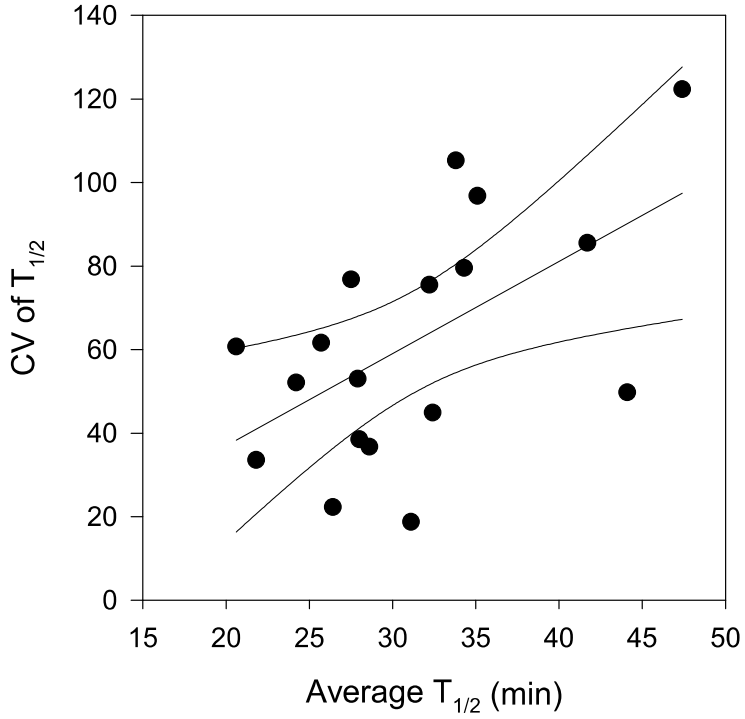

Figure 4. Linear regression with 95% confidence intervals between the coefficient of variance (CV) of the half-life time ($T_{1/2}$) and the average half-life time of COG subcategories based on the five most abundant bacterial classes (*Gammaproteobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria* and *Actinobacteria)*. Based on 18 COG categories, the correlation coefficient was $r = 0.56$ and p value = 0.01.

The average $T_{1/2}$ and the coefficient of variance (CV) of the different COG subcategories were weakly positively correlated ($r = 0.56$, $p = 0.01$, $n = 18$), indicating that COG subcategories with shorter $T_{1/2}$ had a less variable mRNA $T_{1/2}$ in the five bacterial classes analyzed than subcategories with longer $T_{1/2}$ (Fig. 4). The significance of a conserved short $T_{1/2}$ in specific functions derives from the necessity of rapidly responding to changes in the environment (Takayama & Kjelleberg 2000). The transcripts from information storage and processing COG category generally had long $T_{1/2}$ while cellular processes and signaling and metabolism had variable $T_{1/2}$ (Fig. 3). The shortest $T_{1/2}$ corresponded to the COG subcategories related to defence mechanisms and cell motility (Fig. 3). The four most abundant COGs within the defence mechanisms were ABC transporters of the ABC-type multidrug transport system involved in the transport of nutrients or toxins through the cell membranes (Davidson et al 2008). The most frequently expressed gene within the cell motility COG subcategory was the protein flagellin, a component of the bacterial flagellum (Vonderviszt & Namba 2013). These results suggest that shorter $T_{1/2}$ of mRNA and

thus, rapid turnover time prevail for functions related to the response of the cells to environmental conditions, such as patches of nutrients stimulating chemotactic responses or presence of toxins. The widespread pattern in the five bacterial classes analyzed here further indicate the significance of these functions for the survival and fitness of most, if not all, bacteria in the environment.

Conclusions

Overall, the mRNA $T_{1/2}$ of 11 genes of the three marine bacterial isolates grown at different temperatures was commonly shorter than 10 min, supporting the need of fast processing of samples when studying transcription in natural communities. Sample collection of surface waters (100 to 200 m) with traditional oceanographic methods (e.g. Niskin bottles) can take as long as 30 min, allowing for substantial, possibly stress-induced changes in the metatranscriptome (Edgcomb et al 2016). A variety of recently developed *in situ* samplers, implemented with optional RNA fixation mechanisms, permit a more reliable sampling of environmental RNA (Feike et al 2012, McQuillan & Robidart 2017). Nevertheless, activity profiling of microorganisms based on mRNA remains questionable due to the poor correlations of mRNA with protein levels (Greenbaum et al 2003, Wang et al 2019). Recently, it has been shown that eukaryal and archaeal mRNA have a longer $T_{1/2}$ as compared to bacterial mRNA (Clouet-d'Orval et al 2018). Hence, care has to be taken when drawing conclusions based on metatranscriptomes, likely including organisms from the three domains of life. Furthermore, we demonstrate here that the $T_{1/2}$ of mRNA varied widely within a complex environmental bacterial community, potentially biasing the interpretation of activity profiles.

Different factors affected the half-life time of RNA in the marine bacterial isolates and the bacterial community studied here. There were clear differences in $T_{1/2}$ of RNA with distinct functions (mRNA *vs.* ncRNA) likely due to differences in physical protection (Deutscher 2003, Deutscher 2006). Moreover, short mRNA $T_{1/2}$ prevails in genes related to the response to environmental stressors. However, the relationship of $T_{1/2}$ with temperature and growth rates varied among the different isolates, suggesting that these two factors interact affecting RNA degradation. Our findings emphasize the need to consider the short $T_{1/2}$ and its high variability when assessing metatranscriptomes especially in environmental samples and highlight the requirement to minimize processing and handling time.

Experimental Procedures

Sample collection, bacterial isolation and sequencing

The bacteria *Alcanivorax jadensis, Croceibacter atlanticus* and *Colwellia polaris* were isolated from the mesopelagic (250m) and bathypelagic (3200m) North Atlantic (67.35ºN 4.94ºW) and from the surface waters (~2 m) of the Adriatic Sea off the coast of Piran (Slovenia), respectively. Seawater from the different locations was amended with media (15 g/L Select Agar and 25 g/L LB Broth), autoclaved and distributed in petri dishes. Fifty µL of the respective seawater was spread on the plates and incubated in the dark at 20°C. Three of the growing bacterial colonies were picked with a sterile toothpick and preserved after flash-freezing in liquid N_2 for subsequent experiments. The 16S rRNA gene from the different isolates was PCR amplified using the primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3') and the following thermocycling conditions: 94°C for 4 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 30 min and hold at 4°C. PCR products were checked on a 2% agarose gel, stained with SYBR Gold (Invitrogen) and purified using a PCR extract mini kit (5-PRIME). The DNA concentration from the PCR products was quantified on a Nanodrop 2000 spectrophotometer (Thermo Scientific) prior to Sanger sequencing (Applied Biosystems 3130 x l genetic analyzer). Sequences were analyzed using FinchTV and aligned with CodonCode Aligner. Taxonomic identification was conducted with BLAST against the NCBI nucleotide database.

Growth characteristics of the different isolates

The three bacterial isolates were inoculated in duplicate 50 mL LB liquid media and incubated at 4°C, 10°C, 15°C, 20°C, 25°C and 30°C. Growth rates and growth stages (lag, log, stationary and death phase) of the different isolates were determined for each temperature. Subsamples for bacterial abundance (1 mL) were taken at approximately 12h, 8h and 4h intervals, depending on the growth curve for the specific culture and temperature. Subsamples for bacterial abundance were fixed with glutaraldehyde (0.5% final concentration) and kept at 4°C in the dark for 10 min. Subsequently, the samples were flash-frozen in liquid nitrogen and stored at -80°C. Prior to cell enumeration, samples were diluted 1:10 with TRIS-EDTA-buffer and

stained with SYBRGreen I (Invitrogen, 1x final concentration) in the dark for 10 min. Polystyrene 1µm beads (FluoSpheres®, Fisher scientific, ~ 10^5 beads mL^{-1} final concentration) were added as internal standard. Afterwards, samples were vortexed and run in an Accuri C6 flow cytometer (BD Biosciences). Bacterial cells were distinguished based on their green *versus* side scatter signals (Brussaard 2004).

The generation time (*G*) was calculated as follows: G= t / ((log(N)-log(N0))/log(2)) where *t* is the time from the beginning of the exponential phase to the beginning of the stationary phase; *N0* is the cell abundance at the beginning of the exponential phase; and *N* is the cell abundance at the beginning of the stationary phase.

RNA degradation experiment using bacterial isolates

The RNA degradation experiment started at the beginning of the stationary phase for each of the bacterial isolates growing at 4°C, 15°C and 25°C. The three bacterial isolates were inoculated in duplicate 50 mL liquid media. Rifampicin was used to inhibit bacterial DNA-dependent RNA polymerase (Calvori et al 1965) in order to determine RNA half-life time (Hambraeus et al 2003, Selinger et al 2003, Steglich et al 2010). An initial *t0* subsample (1 mL) was taken in duplicate prior to the addition of rifampicin and used as a control. Then, 150 µg/mL rifampicin (final concentration) was added to the 50 mL liquid duplicate cultures. One mL subsamples were collected after 2.5 min, 5 min, 10 min, 20 min, 40 min and 60 min of incubation (subsequently referred to as *t2.5*, *t5*, *t10*, *t20*, *t40* and *t60*) following the rifampicin addition. All subsamples were immediately fixed with 1 mL RNA later (Invitrogen) and flash-frozen in liquid nitrogen.

RNA degradation experiment using a complex coastal bacterial community

Seawater was collected with an HCl cleaned 10 L carboy from the coastal northern Adriatic Sea at Valdibora Bay (Rovinj, Croatia) on 07 Nov 2016. The collected water was pre-filtered through a sterile 0.8 µm polycarbonate filter (Millipore) with HCl cleaned tubes and filtration devices. The 0.8µm filtered seawater was stored at 20.5°C in the dark for five days to allow acclimatization of the prokaryotic community. The first sample was taken prior to the addition of rifampicin (initial RNA *t0*) and used as a control. After adding rifampicin to 1.2 L of seawater (150 µg/mL final concentration) samples were taken at 0 min, 2.5 min, 5 min, 10 min, 20 min, 40

min and 60 min (subsequently referred to as time point *t0*, *t2.5*, *t5*, *t10*, *t20*, *t40* and *t60*). At each sampling, 150 mL were collected and fixed with 150 mL RNA storage solution (200 mM sucrose, 5 mM EDTA, 10 mM sodium acetate pH 5.2) (Steglich et al 2010) and subsequently filtered onto 0.2 µm Millipore GTTP filters. Immediately after filtration, the filters were flash-frozen in liquid nitrogen and stored at -80°C.

RNA extraction and cDNA preparation

The pipets and the surface of the lab bench used during the extractions were cleaned with 70% ethanol and RNase Killer (5Prime) prior to use. All the material used was RNase/DNase free and all the stock buffer solutions and water were pre-treated with diethyl pyrocarbonate (DEPC). RNA was extracted using a hot phenol/chloroform extraction modified from Kramer et al (1996). Detailed information is available in the Supplementary Material. No negative controls for RNA extraction and sequencing were included in this study.

Primer design, PCR and qPCR

Taxa-specific primers were designed for genes encoding proteins that belonged to distinct categories or subcategories to cover a diversity of protein functions. In order to choose genes that are present in most bacterial cells, genes predicted to be part of the core gene set of bacterial cells were selected (Gil et al., 2004). The selected genes were ATP synthase B chain (*atp*F), chaperone Hsp70 (*dna*K), cytoskeletal cell division protein (*fts*Z), ATP-dependent protease (*hfl*B), aminopeptidase A/I (*pep*A), pyruvate kinase (*pyk*A), clp protease subunit 2 (*clp*P), phosphatidylserine synthase (*pss*A), RNA polymerase ß subunit (*rpo*B) and thymidylate synthase (*thy*A). Primer sets were designed for all the genes and isolates, however, only those that successfully amplified the specific gene are shown in Table S1. The isolates were identified solely based on their 16S rRNA sequences. Subsequently, we used available data on full-genome sequenced cultures that corresponded to our isolates according to the identity of the 16SrRNA and on other close relatives. However, some variation in the genome of the isolates might occur compared to the available data. Potentially, variation in some positions of the primer sets designed might have hindered efficient amplification of some of the genes and isolates. The taxa-specific primers were designed in Geneious 6.1.8 (Biomatters, Ltd) using the sequences from close relatives to the isolates (Table S2). Specificity was confirmed

by gel electrophoresis using the extracted DNA from the isolates. 16S rRNA (Suzuki et al., 2000) and *rec*A (Holmes et al 2004) genes were amplified with available non-taxa specific primer sets (Table S1). Yet, the *rec*A primer set only successfully amplified the *Colwellia* isolate. The corresponding amplified fragments were kept as short as possible to minimize PCR efficiency differences (Debode et al 2017). Due to the varying location of conserved sites in the different genes, however, fragment size varied between 123 and 532 bp (Table S1). Regardless of the different fragment size, PCR efficiency varied between 68.7% and 98.9% (Table S1).

Annealing temperatures for all specific primers were determined by gradient PCR, and the amplified products were checked by electrophoresis on a 2% agarose gel. The annealing temperatures with best results were used in qPCR (Table S1). Standard dilutions from 10^7 to 1 gene for quantification were prepared from purified PCR products of the isolated bacteria as previously described (Sintes et al 2013). The standard dilution was loaded to each qPCR plate (Bio-Rad) together with the cDNA samples, RNA samples, a positive and a negative control. All samples were loaded in triplicate and the plates were closed with optical tape (Bio-Rad) and run on a Light Cycler 480 (Roche). Successful amplification in the RNA samples indicates the presence of undigested DNA. On average 5% ±11% SD of cDNA gene abundance corresponded to undigested DNA. The gene abundance in the RNA sample was subtracted from cDNA gene abundance. The reaction mixture for each sample contained 1x Mastermix (LightCycler 480 SYBRGreen I Master, Roche), 0.5 µM of the forward and reverse specific primers, 1 µL of sample and ultrapure sterile water (Roche) up to 10 µL. Thermocycling for all genes was initiated by a denaturation step at 95°C for 10 min, followed by 50 cycles consisting of a denaturation step at 95°C for 5 sec; annealing temperatures and times for each gene as listed in Table S1; extension at 72°C for 15 sec, and a plate read at 74°C for 3 sec.

Metatranscript library preparation

The transcribed cDNA obtained from the natural bacterial community was fragmented (~500 bp) with a Covaris focused-ultrasonicator. The concentration of the obtained cDNA fragments was measured with a QUBIT spectrophotometer (ThermoFisher) following the manufacturer's protocol. The library preparation was performed with a KAPA Hyper kit (KAPA Biosystems). Briefly, 10 ng of cDNA were end repaired and A-tailed. Subsequently, the genetic material was ligated and puri-

fied using a bead-based cleanup method. The obtained libraries were PCR amplified with following conditions: 98°C for 45 sec, 12 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 1 min and hold at 4°C. The PCR products were further purified using a bead-based cleanup method. The libraries concentrations were measured by qPCR (KAPA hyper kit) following the manufacturer's recommendations and 2.5 nM cDNA of each sample was sequenced using an Illumina MiSeq high throughput sequencing (2x250 paired-end platform) at JAMSTEC, Japan. The sequence data generated are publically available in the DDBJ sequence read archive (DRA) under the accession number DRA008144 for sample t0, DRA008145 for sample t5, DRA008146 for sample t40 and DRA008147 for sample t60.

Bioinformatics and statistical analyses

The transcriptomes were analyzed using a read-based approach. The program SortmeRNA (Kopylova et al 2012) was used to sort mRNA reads from rRNA reads with all available databases (RFAM (December 22, 2015) and SILVA (July 23, 2015): rfam-5.8s-database-id98.fasta, rfam-5s-database-id98.fasta, silva-arc-16s-id95.fasta, silva-arc-23s-id98.fasta, silva-bac-16s-id90.fasta, silva-bac-23s-id98.fasta, silva-euk-18s-id95.fasta and silva-euk-28s-id98.fasta). Adapters were identified and removed with AdapterRemoval version 2.1.7. and the following settings: --identify-adapters, --file1, --file2) (Schubert et al 2016). Paired-end reads were merged with PEAR (v0.9.10) (Zhang et al 2013) and diamond (Buchfink et al 2014) was performed using the nr database (April 2018) for transcriptome annotation with the following settings: -b 12.0, -e 0.00001, -k 1. The diamond blast results were analyzed with MEGAN6 v6.13 (Huson et al 2007). Taxonomic affiliation was assigned using the database prot_acc2tax-Oct2017X1.abin and the 'Naive LCA' algorithm, with default parameters. Functional assignment was assessed with the database acc2eggnog-Oct2016X.abin. The absolute comparison mode in EGGNOG Viewer (Powell et al 2014) of MEGAN6 was used to extract functional gene counts with the 'Best-Hit' algorithm for binning. Further analysis was performed only in transcriptomes keeping more than 50% of the original mRNA read abundance after quality control.

The transcriptomes were analyzed using two approaches. The first approach consisted in determining the $T_{1/2}$ of bacteria at the 'family' taxonomic level. Function-

al genes (mRNA) were successfully assigned to 112 families (127,472 assignments), while phylogenetic genes (16S rRNA gene) were successfully assigned to 182 families (186,993 assignments) with MEGAN6. Functional and phylogenetic genes could be jointly assigned to 100 families. Forty families were excluded from further analysis due to ineffective detection in all time points, and 16 families due to non-exponential decay of their mRNA. Consequently, 44 families were used for $T_{1/2}$ calculations.

The second approach consisted in determining the $T_{1/2}$ of functional categories from bacterial taxonomic classes. We used the EggNOG database (Huerta-Cepas et al 2015) and COG categories (Galperin et al 2014, Tatusov et al 1997) to classify the mRNA. Five bacterial classes (*Gammaproteobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria* and *Actinobacteria*) comprised more than 1000 rRNA assignments and more than 200 mRNA assignments on average. These were subsequently used to calculate the $T_{1/2}$ of COG subcategories.

The relative abundance of transcripts was assessed as the ratio of mRNA to rRNA at each time point for both approaches. Therefore, the number of mRNA assignments was divided by the number of rRNA assignments to a bacterial family or COG category. The relative abundance of bacterial families was calculated by dividing the number of rRNA assignments to each bacterial family by the total rRNA in each sample. The mean and standard deviation were calculated for each family for all merged samples. Tests of significance were calculated with Mann-Whitney pairwise test and one-sample t-test using the program Past3 v3.20 (Hammer et al 2001).

RNA half-life time ($T_{1/2}$)

$T_{1/2}$ was calculated with the 'relative two phase decay model' according to Steglich et al (2010). Briefly, an exponential decay was fitted to the RNA expression values *versus* time after rifampicin addition. The time point with maximal expression (N_0) was selected as the initial time point of the decay (t_0). The expression value (N) at the last time point (t) of the exponential decay was chosen that the best fit was achieved: $T_{1/2} = (t - t_0) / (\log_2(N_0) - \log_2(N))$. This method was chosen to target the previously described two phases of RNA decay (delay and decay phase) (Steglich et al 2010).

Availability of data and material

The datasets generated and/or analyzed during the current study are available in the DDBJ Sequence Read Archive (DRA): http://ddbj.nig.ac.jp/DRASearch/
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Competing interests

The authors declare that they have no competing interests

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Authors' contributions

E.S. conceived and together with D.D.C. and P.A.S carried out the experiments. P.A.S. analyzed the data and wrote the paper. P.A.S and C.M. extracted RNA. E.S. designed qPCR primers. P.A.S. carried out qPCR. D.D.C. prepared the metatranscript library and T.Y. coordinated and supervised the sequencing. J.G. and T.R. provided bioinformatics support. G.J.H. and E.S. provided project oversight. All authors read and approved the final manuscript.

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Supplementary materials

RNA extraction and cDNA preparation

Lysis of bacterial cells was initiated by thawing the RNA Later preserved sub-samples and concentrating them to 100 µL by centrifugation at 3220x *g* and 4°C with 10 KDa Amicon Ultra 2 mL Centrifugal filters (Ultracel 10K, Millipore). Samples of the RNA degradation experiment with a complex coastal bacterial community were collected on filters and flash-frozen. For RNA extraction, the filters were thawed and cut into small pieces and subsequently incubated with the lysis buffer. Lysis buffer (0.1 mL Tris 100 mM pH 8, 0.1 mL EDTA 250 mM pH8, 0.02 mL NaCl 5 M, made up to 1 mL with water) and lysozyme solution (8.75 U/mL final concentration) (Lysozyme Ready-Lyse, Epicenter) were added to the concentrated isolate or the filter pieces. Samples were incubated at 37°C for 45 min. Thereafter, SDS (final conc.: 1 %) and proteinase K (final conc.: ~8 U/mL) (Proteinase K from Tritirachium album, Sigma-Aldrich) were added and incubated at 55°C for 1h. Thereafter, combusted zirconium beads ~200 µL (0.1 mm diameter Zirconia/Silica, BioSpec Products) were added. The samples were subsequently vortexed for 10 min at maximum speed and incubated at 70°C for 30 min. The lysate (supernatant) was recovered after centrifugation at 4500x *g* for 5 min and pipetted into clean 2 mL Eppendorf tubes. After the lysis, 90 µL of 1 M NaOAc (pH 5.2) per tube (final conc. 100 mM) and an equal volume (~1 mL) of water saturated phenol (pH 4.3, Sigma-Aldrich) were added, mixed with the lysate and incubated at 64°C for 6 min. Samples were chilled on ice and centrifuged at 21,000x *g* at 4°C for 15 min. The aqueous layer was transferred to a Phase Lock

Gel (Phase Lock Gel Heavy 2 mL, 5Prime) with an equal volume of chloroform (~0.9 mL) (Sigma-Aldrich) and centrifuged at 21,000x *g* at 4°C for 10 min. The aqueous layer was split into two 1.5 mL eppendorf tubes and ethanol precipitation was initiated by adding 1/10 volume of 3 M NaOAc (pH 5.2), 1 mM EDTA and 2 volumes cold ethanol (for molecular biology, Merck) followed by incubation at -80°C overnight. Subsequently, samples were thawed and centrifuged at 21,000x *g* at 4°C for 25 min. The pellets were washed with 1 mL ice cold 80% ethanol and centrifuged at 21,000x *g* at 4°C for 20 min. Afterwards, the pellets were air-dried at 45°C for 3-4 h and resuspended in 100 µL DEPC-water at 4ºC overnight (10-18h). Thereafter, all the RNA extracts from the same sample were combined into one microfuge tube and stored at -80°C until further analysis. Residual DNA from the nucleic acid extract was digested and the RNA was purified with RNeasy MiniElute Cleanup Kit (RNase free DNase set, Qiagen) following the manufacturer's protocol. Elution was carried out twice with 14 µL RNAse/DNAse free water (final volume of ~28 µL of eluate). RNA concentration and quality were determined spectrophotometrically with a Nanodrop 2000 (Thermo Scientific) and subsequently by electrophoresis with an Experion (Bio-Rad) using the RNA HighSens Kit (Bio-Rad). RNA quality is indicated by the 23S rRNA to 16S rRNA ratio (Sambrook et al 1989). However, it has been shown that this method might be misleading (Bhagwat et al 2013), hence we mainly checked the quality by visual inspection of the electropherograms. Purified RNA was transcribed into cDNA using Super Script III First-Strand Synthesis System (Invitrogen) with random hexamers. The resulting cDNA was diluted 1:10 prior to qPCR analysis.

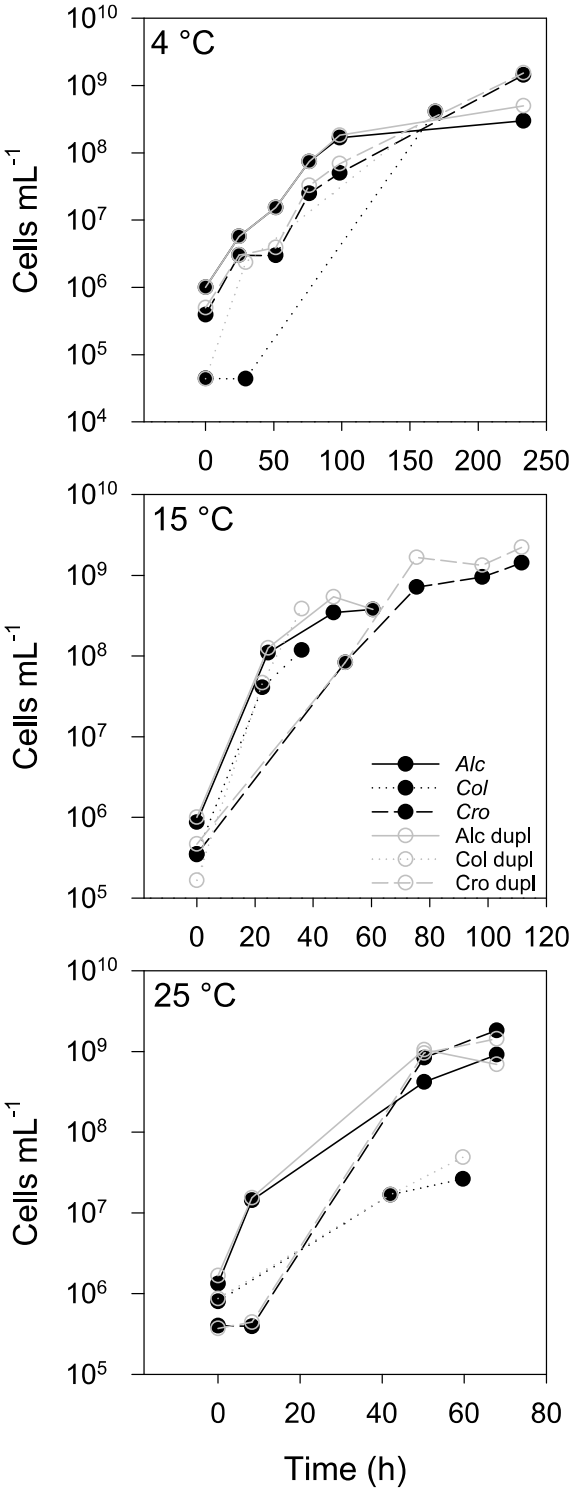

Figure S1. Development of the cell abundance of the bacterial strains *Alc*: *Alcanivorax jadensis, Col: Colwellia polaris* and Cro: *Croceibacter atlanticus* grown in duplicates at 4°C, 15°C and 25°C.

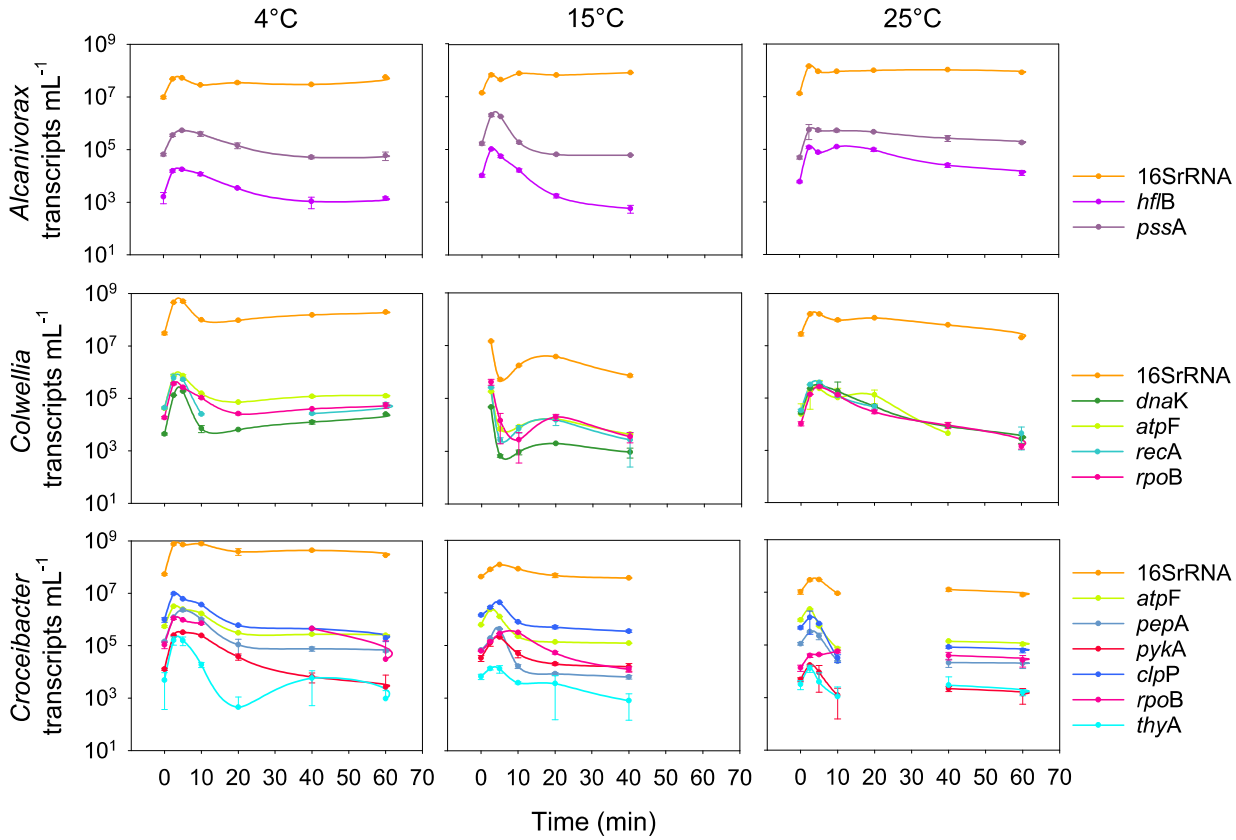

Figure S2. Transcript abundance of different genes over time of the bacterial isolates *Alcanivorax jadensis* (n = 9) , *Colwellia polaris* (n = 15) and *Croceibacter atlanticus* (n = 21) at 4°C, 15°C and 25°C. Average ± standard deviation (SD) of triplicate measurements is shown.

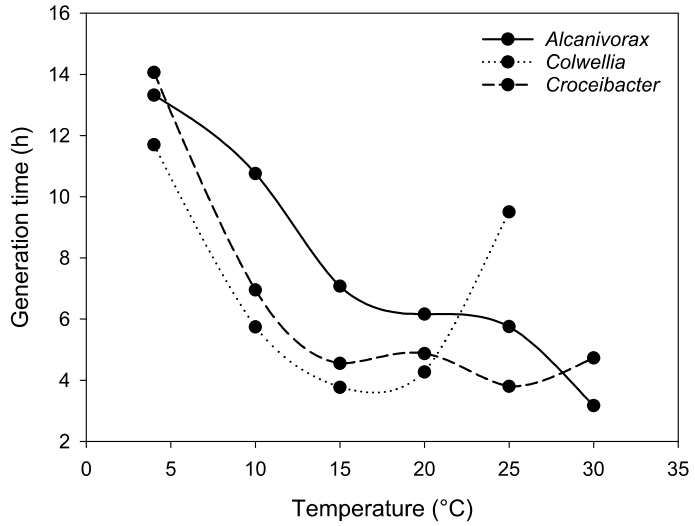

Figure S3. Generation time of the bacterial isolates *Alcanivorax jadensis, Colwellia polaris* and *Croceibacter atlanticus* grown at 4°C, 10°C, 15°C, 20°C, 25°C and 30°C.

Supplementary tables

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Chapter II Functional seasonality of free-living and particle-associated prokaryotic communities in the coastal Adriatic Sea

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To be submitted

Abstract

Marine snow is an important habitat for microbes, characterized by chemical and physical properties contrasting those of the ambient water. The higher nutrient concentrations in marine snow lead to compositional differences between the ambient water and the marine snow-associated prokaryotic community. Whether these compositional differences vary due to seasonal environmental changes, however, remains unclear. Thus, we investigated the seasonal patterns of the free-living and marine snow-associated microbial community composition and their functional potential in the northern Adriatic Sea. Our data revealed seasonal patterns in both, the free-living and marine snow-associated prokaryotes. The two assemblages were more similar to each other in spring and fall than in winter and summer. The taxonomic distinctness resulted in a contrasting functional potential. Motility and adapta-

tions to low temperature in winter and partly anaerobic metabolism in summer characterized the marine snow-associated prokaryotes. Free-living prokaryotes were enriched in genes indicative for functions related to phosphorus limitation in winter and in genes tentatively supplementing heterotrophic growth with proteorhodopsins and CO-oxidation in summer. Taken together, the results suggest a strong influence of environmental parameters on both free-living and marine snow-associated prokaryotic communities in spring and fall leading to higher similarity between the communities, and an increased influence of the specific microenvironment in marine snow in winter and summer leading to a distinctly different prokaryotic community in marine snow in these two seasons.

Importance

The specific microenvironment of marine snow allows for a prokaryotic metabolism distinctly different from that in the ambient water. High nutrient concentrations typically found in marine snow might shape the microbial community towards competition and motility. Here we analyze whether the community composition and functional potential of marine snow-associated prokaryotes persist over different seasons compared to those in the ambient water as environmental conditions change.

Introduction

Marine snow, described as detrital particles larger than 500 µm, plays an important role in the export of organic carbon to the deep sea and the sequestration via the biological carbon pump (Cho & Azam 1988, Ducklow et al 2001, Herndl & Reinthaler 2013). Chemical characterization, microbial community composition and activity of marine snow in a large variety of oceanic regions have been extensively studied (Alldredge & Silver 1988, Simon et al 2002, Turner 2002, Turner 2015). However, the majority of studies represent snapshots of the biotic and abiotic characteristics of marine snow captured with sediment traps (Buesseler et al 2008, De La Rocha & Passow 2007), marine snow catchers (Riley et al 2012, van der Jagt et al 2018), SCUBA divers (Kaltenböck & Herndl 1992, Müller-Niklas et al 1994, Rath et al 1998) or filtration systems (Mestre et al 2017, Salazar et al 2015). Only a few studies investigated the dynamics of marine snow and its associated microbial community over a few weeks (Kaltenböck & Herndl 1992, Müller-Niklas et al 1994). Recently,

the importance of increasing the spatial and temporal resolution of research studies to understand the dynamics in the community composition and functioning of ecosystems has been emphasized (Chow et al 2013, Fuhrman et al 2015, Ward et al 2017). Marine snow-associated microbial communities are considered to be relatively insensitive to changes in environmental parameters of the bulk seawater (Yung et al 2016). In contrast, a certain influence of changes of physico-chemical parameters on marine snow-associated microbial communities has also been reported (Vojvoda et al 2014).

Free-living microbial communities are exposed to rapidly changing environmental parameters, resulting in seasonal changes in taxa and closely related ecotypes (Eren et al 2013, Fuhrman et al 2015, Steiner et al 2019, Ward et al 2017, Yung et al 2015). Additionally, organic matter leaching from marine snow (Stocker 2012) links and shapes the free-living community composition (Vojvoda et al 2014). In contrast, the marine snow-associated microbial community is primarily affected by the origin, composition and developmental stage of the marine snow, and secondarily by the seasonal changes of environmental parameters (Duret et al 2019, Vojvoda et al 2014, Yung et al 2016).

Marine snow represents a specific microhabitat with organic and inorganic nutrient concentrations potentially orders of magnitude higher than in the ambient water (Alldredge & Silver 1988, Kaltenböck & Herndl 1992, Müller-Niklas et al 1994). In addition, it also provides plenty of surfaces for microbial attachment. Therefore, it is not surprising that the prokaryotic community associated with marine snow is distinctly different from that of the ambient water under such conditions (Kaltenböck & Herndl 1992, Rath et al 1998, Simon et al 2014, Simon et al 2002). However, these reports have been performed during extensive marine snow formation mainly in the summer months of temperate seas. Whether these differences between the free-living and marine snow-associated prokaryotic community composition are maintained throughout the seasonal cycle in temperate seas, however, remains unknown.

In this study, we analyzed the dynamics of the composition of the free-living and particle-associated microbial community based on 16S rRNA gene amplicon sequences over a seasonal cycle in the northern Adriatic Sea. Furthermore, the functional potential of marine snow-associated and free-living prokaryotic community at two contrasting seasons (summer 2015 and winter 2016) was assessed using metagenomics analysis. Finally, the eukaryotic and prokaryotic community composi-

tion was related to the environmental parameters and the implications of the prokaryotic metabolic potential for biogeochemical cycles are discussed.

Results and Discussion

Prokaryotic taxa enriched in marine snow and ambient water

Taxonomic analysis revealed on average 891 ± 232 amplicon sequence variants (ASVs) hit by 46797 ± 13521 sequences in the ambient water (AW) and 437 ± 219 ASVs hit by 31548 ± 17230 sequences in marine snow (MS) (Fig. S1). In total 1711 ASVs had a prevalence of more than 10%, implying the presence of these ASVs in at least two samples. Species richness (number of taxa) was on average 1.5 ± 0.6 times higher in the AW than MS in spring, summer and fall (Table S1 Fig. S2). In the winter, however, species richness in the AW was 5.4 ± 6.9 times higher than in MS (Table S1, Fig. S2). The observed low species richness of MS-associated communities as compared to AW is in agreement with previous findings and was linked to the high nutrient concentrations these microhabitats offer (Acinas et al 1999, DeLong et al 1993). The noticeably lower species richness in MS in winter compared to the other seasons and to AW concurred with lower Shannon diversity (3.3 ± 0.2) as compared to the average diversity in MS (4.9 ± 0.4) (Fig. S2). This is likely due to the dominance of *Synechococcus* ASVs in the winter MS communities (Fig. S3). Summer-spring communities were distinct from winter-fall prokaryotic communities, with the free-living communities showing larger seasonal dissimilarities than the MS-associated prokaryotic communities (Fig. 1). This suggests a more pronounced impact of seasonality on the free-living than on MS-associated prokaryotes possibly due to the more stable microenvironmental conditions in MS than in the AW (Vojvoda et al 2014, Yung et al 2016).

Overall, the free-living and MS-associated communities differed significantly (one-way ANOSIM $p < 0.05$) (Fig. 1). Yet, the low number of samples precluded to statistically test whether the free-living and MS-associated communities differed in particular seasons. However, ASVs with significantly different sequence abundances between seasons were obtained. The AW sample collected at 30 July 2016 was identified as an outlier and excluded from the differential sequence abundance analysis (DESeq2) due to high compositional similarity to the summer MS community (Fig. 1).

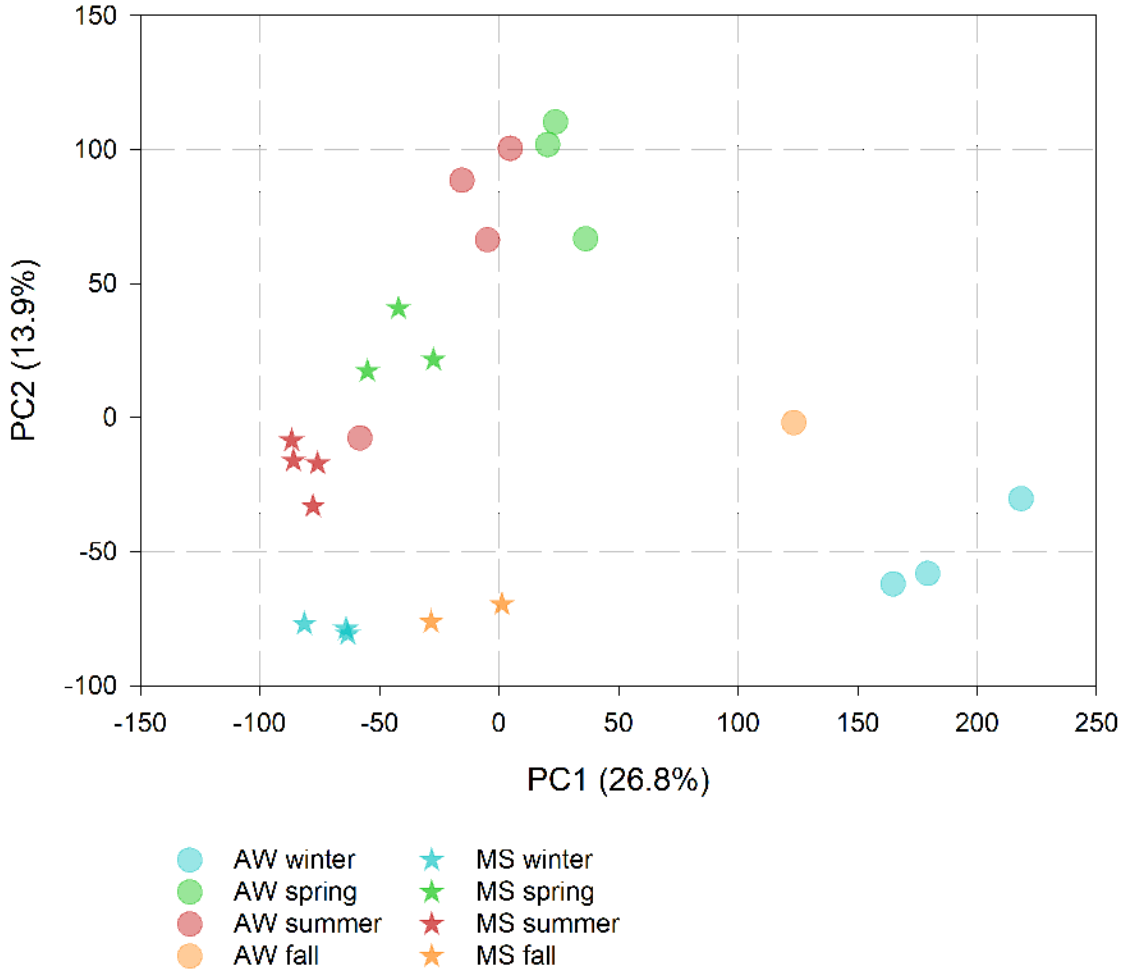

Figure 1. Principal component analysis showing the seasonal differences in the marine snow (MS)-associated and ambient water (AW) prokaryotic community composition based on amplicon sequencing (symbols represent data collected on 12 and 11 days, respectively) labeled according to the season. The percentage of variation explained by each axis is indicated in parenthesis.

In winter, 589 ASV were enriched in the AW as compared to MS, and 19 ASVs in the MS as compared to the AW. Enriched ASVs in the AW represented 91% of the total AW community in winter, enriched ASVs in MS accounted for 26% of the total MS community. Twelve of the 589 AW-enriched ASVs had a mean abundance > 1% and were assigned to Synechococcales, SAR11, Thiomicrospirales, SAR86, SAR406 and Actinomarinales (Fig. 2A). These taxa comprise groups typically found in the free-living community (Mestre et al 2017, Preston et al 2019), some consisting of many ecotypes, including ecotypes characteristic for the winter in temperate marine waters (Giovannoni 2017, Hoarfrost et al 2020). Seven MS-ASVs exhibited a

mean relative abundance > 1% in winter (Fig. 2A). These MS-ASVs were affiliated to orders including diverse phenotypes, such as Caulobacterales, capable of attachment and motility (Poindexter 1964), Obscuribacterales, an order of non-phototrophic fermentative and motile cyanobacteria (Soo et al 2014), the symbiotic nitrogen fixing Rhizobiales (Summers et al 2013), pigmented photoautotrophs of the order Sphingomonadales (Siddaramappa et al 2018), and Propionibacteriales, usually found on human skin (Barka et al 2016) and in liver and kidney tissue of marine fish (Meron et al 2020) (Fig. 2A). The high abundance of potential symbionts and pathogens in MS might be related to the influx of waste water from a fish cannery about 1.5 km away the sampling location (Paliaga et al 2017). No ASVs were found enriched in spring AW and MS, indicating freshly generated and hence, recently populated MS, or a strong effect of environmental parameters on both AW and MS communities, favoring copiotrophic opportunistic prokaryotes such as Rhodobacterales (ASV4), Flavobacteriales (ASV5, 11 and 140) and Opitutales (ASV9) (Chafee et al 2018, Jain et al 2020, Wagner-Döbler & Biebl 2006). In summer, 50 ASVs were enriched in the AW as compared to MS and 68 ASVs in the MS over AW. AW-enriched ASVs had a mean relative abundance < 1% during the summer, however, together accounting for 7% of the total AW community. All MS-enriched ASVs together accounted for 31% of the total MS-associated community in summer. Noticeably, ASVs of the orders Vibrionales, Pirellulales and Caulobacterales (Fig. 2B) were prominently enriched in MS, in agreement with previous findings of members of these taxa attached to particles, particularly in coastal systems in the summer (DeLong et al 1993, Duret et al 2019, Mestre et al 2017). The only two enriched ASVs in MS in fall were putatively assigned to Gemmatales and Alteromonadales, together accounting for 3% of the total MS-associated community (Fig. 2C). Both taxa belong to previously reported particle-associated classes (Planctomycetacia and Gammaproteobacteria) (Mestre et al 2017). The low number of enriched ASVs in fall might be explained by similar reasons as described above for spring.

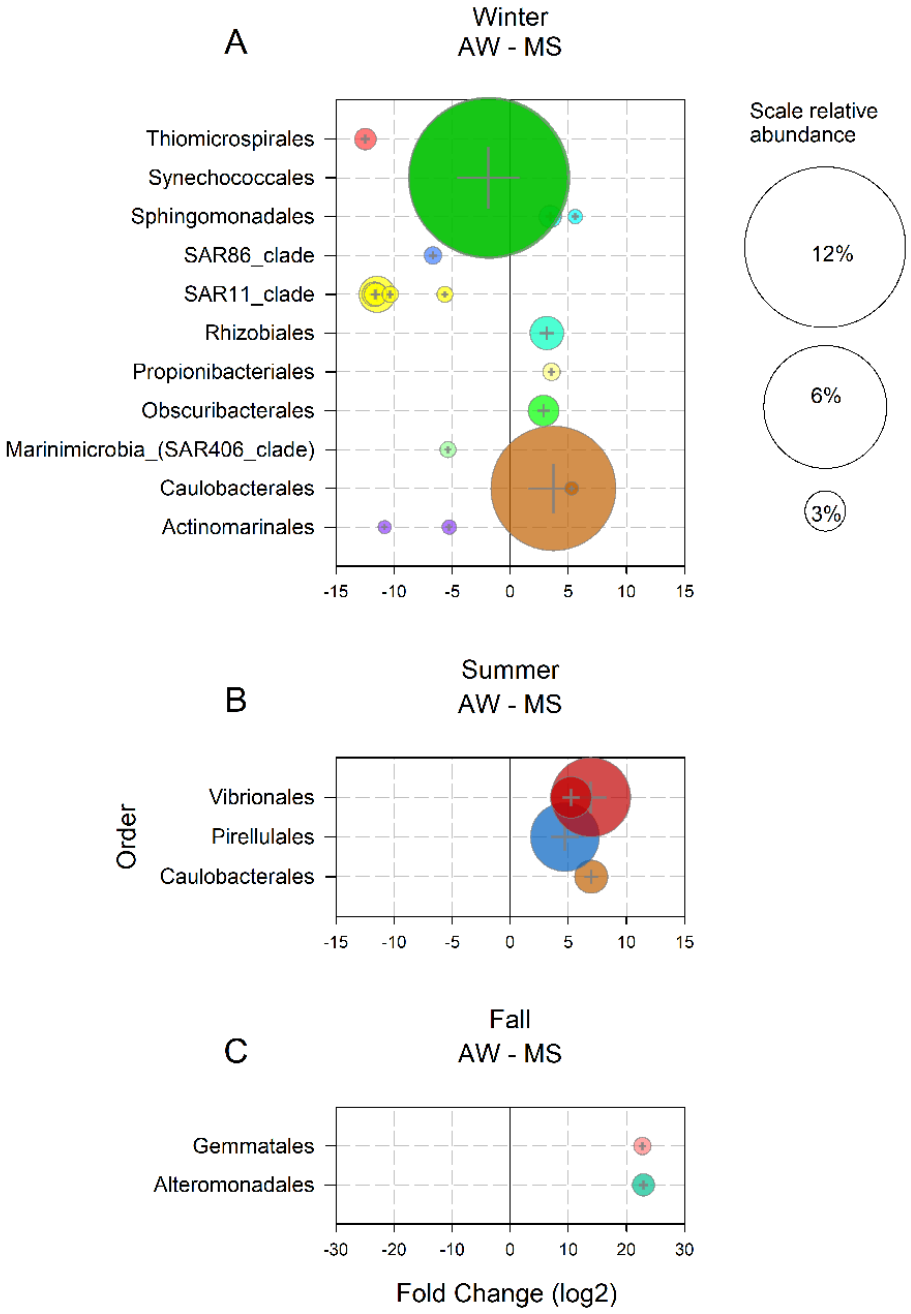

Figure 2. Amplicon sequence variants (ASVs) with relative abundance > 1% and a $\log_2$ fold change > 1 significantly ($p < 0.05$) enriched in the ambient water (AW) and in marine snow (MS) in **(A)** winter, **(B)** summer and **(C)** fall. Bubble size represents the relative abundance and colors indicate the assigned taxonomy at the order level.

Enriched taxa in different seasons

Significant enrichment of ASVs in a specific season as compared to the previous and the following season (e.g. fall *vs*. winter and winter *vs.* spring) was tested in the AW and MS communities separately. In total, 964 ASVs were enriched in one

season compared to the previous or the following season and 58 out of the 964 ASVs exhibited an average relative abundance > 1% (Fig. S4). The pattern of ASVs enriched in a specific season compared to the previous or following season was similar in the AW and MS (Mantel test: $r = 0.7$, $p < 0.05$). This indicates that seasonal conditions shape the taxonomic distribution of both, free-living and MS-associated communities.

In the following, only ASVs with a relative abundance > 1% and those with significant enrichment in a particular season as compared to the previous and the following season are considered. Hence, only truly season-specific ASVs were selected (Fig. 3). In the winter, the free-living community was largely characterized by one abundant *Synechococcus* ASV3, Marinimicrobia (SAR406 clade) ASV65 and Actinomarinales ASV77 (Fig. 3A, D). Temperature, nutrients and light regime have been shown to structure *Synechococcus* into a large number of ecotypes, potentially including a free-living winter ecotype (Ahlgren & Rocap 2006, Sohm et al 2016). Marinimicrobia are ubiquitous and typically found in high abundance in the mesopelagic but also in the euphotic zone in the Adriatic Sea in winter (Mucko et al 2018, Yilmaz et al 2016). Actinomarinales persist throughout the year in the open Mediterranean Sea and are found below the season pycnocline during the summer (Haro-Moreno et al 2018). Hence, the high abundance and specific occurrence of Marinimicrobia and Actinomarinales in the winter in the shallow waters of the northern Adriatic Sea are presumably due to water column mixing and lateral advection.

No ASVs were specific for the winter in the MS-associated community (Fig. 3E, H). However, compared to the fall, *Synechococcus* ASV2 and ASV3, both highly abundant, were enriched and dominated the MS-associated and free-living prokaryotic community in winter (Fig. S4). The high relative abundance of these two *Synechococcus* ASVs (Fig. S4) in the AW as well as in MS in winter might be due to a variety of reasons: i) inefficient separation between MS-attached and free-living prokaryotes, particularly of abundant taxa due to sampling biases introduced by using syringes when MS is small in size; ii) possible frequent change between the free-living and MS-associated lifestyle of *Synechococcus* cells as described for *Pseudomonas aeruginosa* (Son et al 2015) via RTX proteins. RTX proteins are related to cell motility in cyanobacteria (Linhartová et al 2010) and were highly abundant in winter MS (see below). iii) Integration of copepod fecal pellets containing *Synechococcus* DNA into MS. iv) Additionally, *Synechococcus* could initiate particle formation

via release of extracellular polymeric substances (Deng et al 2016, Kaltenböck & Herndl 1992).

The SAR86 ASV39, Rhodobacterales ASV4, Opitutales ASV9 and two Flavobacteriales ASV34 and ASV35 were enriched in the AW in spring as compared to winter and summer (Fig. 3A, B). Rhodobacterales, Flavobacteriales and Opitutae are flexible and generalist heterotrophs (Chen et al 2020, Choo et al 2007, Cohan 2016, Newton et al 2010). The SAR86 clade comprises aerobic heterotrophs with the potential of energy acquisition via proteorhodopsin (Dupont et al 2012). Flavobacteriales ASV35 and Puniceispirillales ASV27 were also enriched in MS in spring as compared to winter and summer (Fig. 3E, F). The enrichments of the Flavobacteriales ASV35 specifically in spring and of largely the same prokaryotic groups in the AW and MS in spring as compared to winter or summer (Fig. S4) indicate the similarity of AW and MS communities in spring. This suggests freshly produced MS originating from the spring phytoplankton bloom or a strong effect of environmental parameters on the prokaryotic communities in spring, as mentioned above. Typically, the increasing temperature and solar radiation in spring promote a phytoplankton bloom in the Mediterranean Sea, and hence opportunistic copiotroph prokaryotes such as *Flavobacteria* (Chafee et al 2018) experience favorable conditions for growth.

In the summer, Flavobacteriales ASV71 and ASV17 were enriched in the AW community as compared to spring and fall (Fig. 3B, C). Rhodobacterales ASV25 was enriched in the AW and MS-associated community (Fig. 3). Vibrionales ASV24, ASV7 and Pirellulales ASV18 were enriched in MS and were previously reported as primarily MS-associated prokaryotes (Salazar et al 2015, Smith et al 2013, Steiner et al 2019). The seasonal re-occurrence of *Vibrio* in the Adriatic and Mediterranean Sea has been linked to water stratification, elevated dissolved organic carbon concentrations and production of transparent exopolymeric particles (Tinta et al 2015, Zaccone et al 2002).

Similar to spring, the fall was characterized by the enrichment of the same prokaryotic groups in both the AW and MS in contrast to prokaryotic community composition in summer and winter. Actinomarinales ASV102 (Fig. 3) and the abundant prokaryotic groups of the orders Rhodobacterales, Flavobacteriales, SAR11 and Pirellulales were enriched in both, the free-living and MS-associated prokaryotic community in fall (Fig. S4). Fall-specific free-living ASVs were Pirellulales ASV231 and Thiomicrospirales ASV142 (Fig. 3C, D). Thiomicrospirales has been reported

from oxygen depleted coastal sites and deep oxygen minimum zones linked to nitrate and the sulfur cycle (Aldunate et al 2018, Muck et al 2019). Pirellulales is a heterotrophic order within the class Planctomycetacia (Dedysh et al 2020). MS-associated ASVs in fall were Planctomycetales ASV314, Cellvibrionales ASV179, Pirellulales ASV265 and Flavobacteriales ASV140 (Fig. 3G, H). Planctomycetales produce secondary metabolites such as bacteriocins and ectoines (Wiegand et al 2020) and are typically found in MS (Duret et al 2019, Mestre et al 2017). Some Planctomycetes convert ammonium and nitrite to dinitrogen gas under low oxygen concentrations (<10 μM) and account for up to half of all N_2 loss to the atmosphere (Francis et al 2007, Jogler et al 2012, Kuypers et al 2005). Cellvibrionales consist of oligotrophs as well as copiotrophs and have been reported from coastal systems and distinct nutrient-rich niches (Spring et al 2015).

Overall, enrichment analysis revealed that 'transition seasons', i.e., spring and fall, exhibited a high abundance of enriched ASVs compared to winter and summer (1182 in total) and low abundance of habitat-specific ASVs (2 in total). Whereas 'peak seasons', i.e., winter and summer, showed the opposite pattern, with a comparably low number of enriched ASVs compared to spring and fall (827 in total) and a large number of habitat-specific ASVs (728 in total). High habitat specificity in AW *vs*. MS particularly in winter might be caused by large difference between the two habitats in terms of nutrient and possibly, oxygen availability (e.g., low oxygen microzones in MS). In the summer, MS is generally larger in size than in winter due to low turbulence conditions in the stratified water column facilitating aggregation of colloidal organic matter. Differences in the substrate availability between AW and MS are higher in summer and winter than in spring and fall, when phytoplankton blooms are providing labile substrate to the AW prokaryotic community.

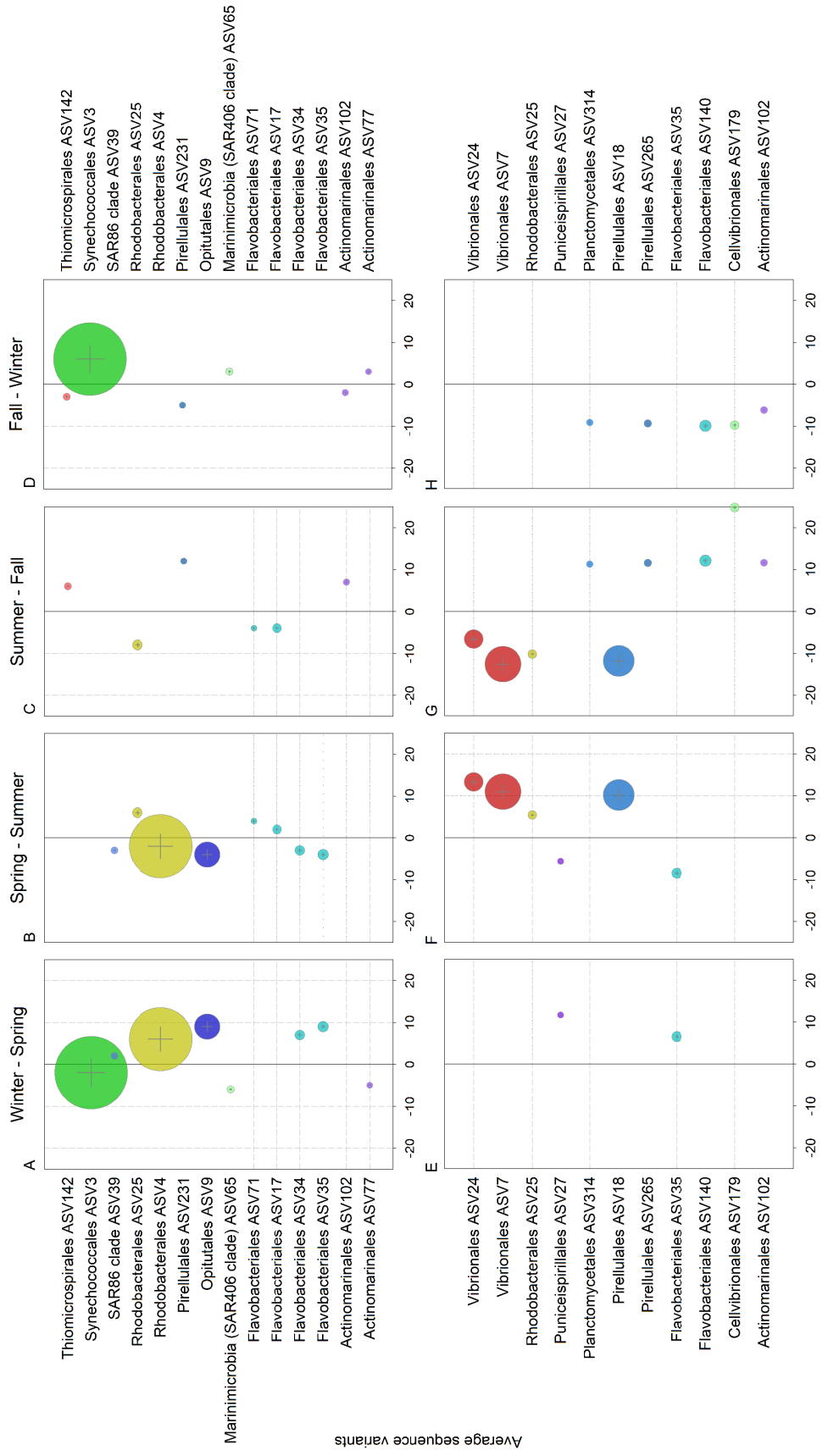

Figure 3. Amplicon sequence variants (ASVs) with a relative abundance > 1% and a $\log_2$ fold change > 1 significantly ($p < 0.05$) enriched when compared to both, the previous and the following seasons

in **(A, B, C, D)** the ambient water (AW) and **(E, F, G, H)** in marine snow (MS). Bubble size represents the relative abundance and colors indicate the assigned taxonomy at the order level.

Free-living and MS-associated eukaryotic community composition

The largest fraction of eukaryotic reads retrieved from the metagenomes was assigned to copepods (on average 49% ± 27%, Table S2 and Fig. 4), with *Acartia clausii* (on average 15 ± 15%) as the most abundant eukaryote. However, copepods are not able to efficiently feed on phytoplankton embedded in transparent exopolymer particles and gel-like structures like marine snow (Bochdansky & Herndl 1992a). The high diversity and larger proportion (89 ± 6%) of metazoan reads in MS compared to AW (73 ± 12%) (Tables S2 and S3) suggest an important contribution of metazoans to the remineralization of marine snow in coastal systems (Bochdansky & Herndl 1992a). Conversely, protists were relatively more abundant in the AW (27 ± 12%) than in MS (11 ± 6%). AW protists were mostly assigned to Alveolata (including dinoflagellates), Rhizaria (including radiolaria), Stramenopiles (including diatoms) and Archaeplastida (including red and green algae), all taxa commonly found in the northern Adriatic Sea (Monti et al 2012, Piredda et al 2016, Steiner et al 2019). Hydrozoa comprised a substantial fraction of assigned reads in AW in the summer (35 ± 6%). Certain Hydrozoa reproduce during the summer period, reaching high abundances in the Mediterranean Sea (Boero & Fresi 1986, Di Camillo et al 2012), suggesting that Hydrozoa sequences in summer originated from free-living hydrozoan larvae. Sequences assigned to Polychaeta (on average 24%) characterized the winter MS community and Platyhelminthes the summer MS community (on average 12%, Table S2 and Fig. 4). These findings support the notion that MS represents an important microenvironment for eukaryotes, particularly larvae of Polychaeta and juvenile turbellarians (Platyhelminthes) (Bochdansky et al 2017, Bochdansky & Herndl 1992b). Larvae use MS as a food source and to enhance dispersal since a considerable fraction of MS is only slowly sinking or neutrally buoyant hence being laterally transported over large distances (Bochdansky & Herndl 1992a). Furthermore, a high number of ‘not assigned’ metazoa (NA_Metazoa), Gastropoda, Bivalvia and Tunicata were also present in the MS eukaryotic community (Table S2 and Fig.

4).

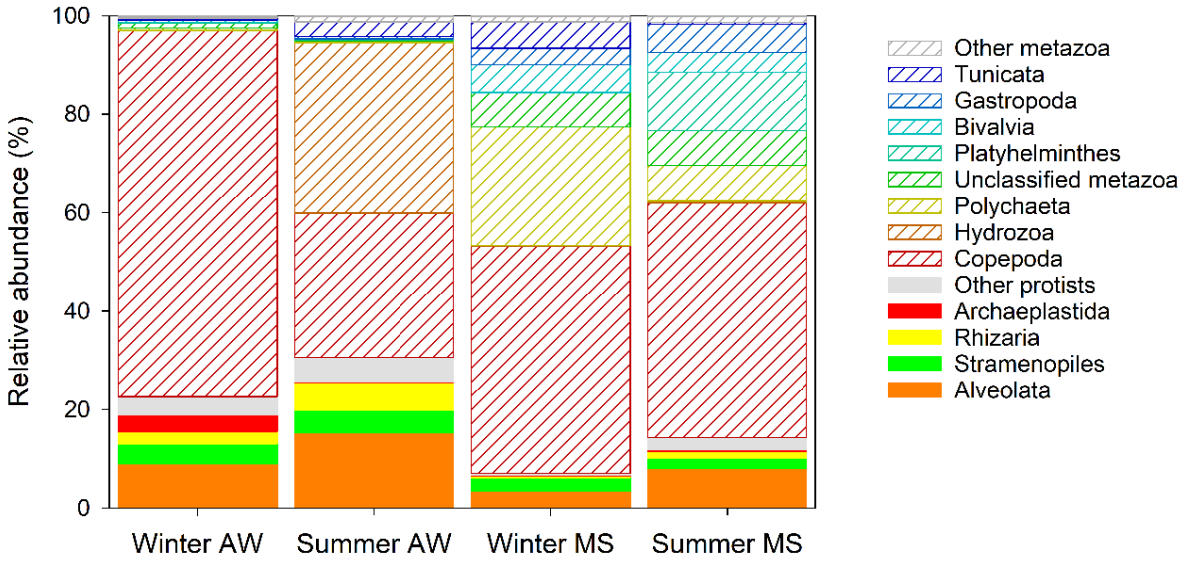

Figure 4. Averaged relative abundance of reads assigned to eukaryotes. The fraction of protists has color filling and the fraction of metazoa is shown as white bars with colored shading. Eukaryotic groups with lower relative abundance than 1% are grouped under “other protists” and “other metazoa”.

Functional assignment of prokaryotic communities

The majority of reads from the metagenomes were assigned to the domain Bacteria, ranging from 43% to 56% (Table S3), followed by ‘not assigned’ reads (on average 41 ± 4% for all samples). Reads mapping to Archaea were more abundant in winter, both in AW (3-4%) and in MS (2%) (Table S3), in accordance with previous reports on seasonality and lifestyles of Archaea in surface waters (Galand et al 2010, Haro-Moreno et al 2017, Smith et al 2013). Eukaryotes contributed substantially less with a maximum contribution of 7-13% in MS in summer (Table S3) in the coastal Adriatic than previously reported for MS-associated microbes in coastal (up to 44%) and deep waters (27% - 40% of all small subunit rRNA genes) (Boeuf et al 2019, Fontanez et al 2015, Smith et al 2013). The prokaryotic community composition revealed by 16SrRNA amplicon sequences was similar to the prokaryotic community composition obtained by the metagenomes (Mantel test: $r = 0.55$, $p < 0.05$, $n = 8$). This allows linking the taxonomic analysis to functional analysis based on metagenomes.

The 4181 identified COGs comprised on average 11 ± 4.4% of all reads with functional assignment. The AW community was defined by 189 significantly enriched COGs as compared to the MS-associated community, accounting for 5.2% of all COGs assigned to AW prokaryotes. A particularly high relative abundance (4%) was observed for COGs involved in metabolism, indicating that metabolic capabilities led to a differentiation of the AW from the MS-associated communities. The most abundant COGs (> 0.1%) of the supergroup 'metabolism' were related to 'C - Energy production and conversion', including enzymes participating in terpenoid backbone biosynthesis (COG1304), precursors of carotenoids, the building blocks of the proteorhodopsin chromophore retinal (Béja et al 2001, DeLong & Beja 2010) (Fig. 5A). The latter suggests that photoheterotrophy is a survival strategy of the AW community, related to oligotrophy and carbon limitation (Moran & Miller 2007). The vast majority of reads (65%) aligned to COGs and enriched in the AW community over MS were assigned to *Candidatus* Pelagibacter sp. HTCC7211, the alpha proteobacterium HIMB114 and *Candidatus* Puniceispirillum marinum, all abundant proteorhodopsin-containing Alphaproteobacteria (Giovannoni et al 2005, Lee et al 2019) (Table S4). Other genes enriched in AW communities encoded for oxidoreductases and transferases involved in the acquisition of energy from organic compounds via the citric acid cycle (COG1894, COG0074 and COG0045) (Fig. 5A). An acyl-CoA transferase (COG1804) involved in the catabolism of compatible solutes, such as carnitine, a betaine structurally similar to dimethylsulfoniopropionate (DMSP) (Curson et al 2011, Elssner et al 2001, Todd et al 2007) was abundant as well (Fig. 5A). Compatible solutes are either generated by or imported into bacterial cells. Three of the most abundant AW specific COGs (category 'E - Amino acid transport and metabolism') were components of the ABC-type proline/glycine betaine transport system (COG4176, COG2113 and COG4175), indicating potential utilization of compatible solutes as substrate or adaptations to salt stress (Fig. 5A). A phytanoyl-CoA dioxygenase (COG5285) involved in the biosynthesis of polyketides (mitomycin antibiotics/fumonisin) indicated the production of antibiotics by the AW prokaryotes, possibly as a survival strategy (Fig. 5A) (Karimi et al 2019).

The MS-associated community was characterized by 219 enriched COGs, accounting for 2.9% of all COGs assigned to MS-associated prokaryotes. Half of the MS-enriched COGs (1.5%) were assigned to functions involved in metabolism. Particularly, a choline-glycine betaine transporter (COG1292) and a Ca^{2+} binding protein

RTX toxin-related domain (COG2931) were abundant (Fig. 5A). RTX proteins are involved in cell defense acting as cytotoxins in *Vibrio* or contribute to motility in *Cyanobacteria* (Linhartová et al 2010). Furthermore, RTX proteins may form bacterial surface layers as protection and have been observed in pathogenic and nonpathogenic heterotrophic bacteria and cyanobacteria (Linhartová et al 2010). Most reads (55%) aligned to COGs enriched in MS were assigned to diverse *Synechococcus* species (CC9311, CC9902, BL107 and WH 7805) and *Vibrio caribbeanicus* (Table S5).

The functional potential of the AW prokaryotic community in the summer as compared to winter was characterized by 396 enriched COGs constituting 4% of the total summer COGs of the AW prokaryotes, of which approximately half (2.3%) were related to metabolism. The most abundant gene encoded the malic enzyme (COG0281), important in various metabolic pathways including the citric acid cycle (Fig. 5B) (Takahashi-Íñiguez et al 2016). The main fraction of all reads aligned to enriched COGs in the summer was assigned to Pelagibacterales (53%) and unclassified Alphaproteobacteria (24%) (Table S6).

In the AW prokaryotes in winter, 531 COGs (accounting for 8.2% of COGs of the AW community) were enriched compared to the summer, and were mainly associated to Synechococcales (59%) and Pelagibacterales (24%) (Table S7). More than half (5.9%) of all winter-specific COGs were related to metabolism. Specific functions were assigned to oxidative phosphorylation (COG1009), the biosynthesis of amino acids and other secondary metabolites (COG0147), a cobalamin biosynthesis protein (COG1429) involved in porphyrin and chlorophyll metabolism, an exopolyphosphatase (COG0248), a polyphosphate kinase (COG0855) and a phosphate/phosphonate transport system (COG3639) indicating phosphorus stress (Fig. 5B).

The MS-associated community showed pronounced differences in the relative abundance of enriched COGs when comparing summer and winter. Enriched COGs in summer represented 2% compared to the winter and in winter 47.3% compared to the summer. However, a similar number of enriched COGs (853 in summer and 806 in winter) were found in both seasons. The reads aligned to summer-specific COGs were mainly assigned to Vibrionales (28%), Pelagibacterales (22%) and Rhodobacterales (13%), and mostly related to metabolism (1.2% of all enriched summer MS COGs, Table S8). However, no single COG contributed more than 0.1% to the total

COGs in MS in the summer. In winter, almost all enriched COGs were assigned to Synechococcales (92%), reflecting the main difference in the taxonomic assignment of COGs between summer and winter MS-associated communities (compare Table S8 and Table S9). The most abundant COGs (> 0.1%) of the supergroup ‘metabolism’ (57 COGs) spanned eight COG categories (Fig. 5C). Of particularly high relative abundance were COGs involved in nitrogen assimilation and storage (COG0436, COG0070 and COG0458), pointing to an adaptation to changing nitrogen availability (Eisenberg et al 2000, Zhang et al 2018). Fatty acid biosynthesis genes (COG0304 and COG0439) were enriched in the winter MS-associated community, particularly in Synechococcales members (Table S10). Unsaturated membrane lipids in cyanobacteria protect the photosynthetic machinery from photoinhibition associated with low temperatures (Nishida & Murata 1996). A high relative abundance of enzymes involved in photosynthesis including carotenoid biosynthesis (COG1233), terpenoid backbone biosynthesis (COG1154 and COG0142) and porphyrin and chlorophyll metabolism (COG1429 and COG0155) underlines the impact of Synechococcales on the functional potential of the MS-associated community in the winter (Fig. 5C).

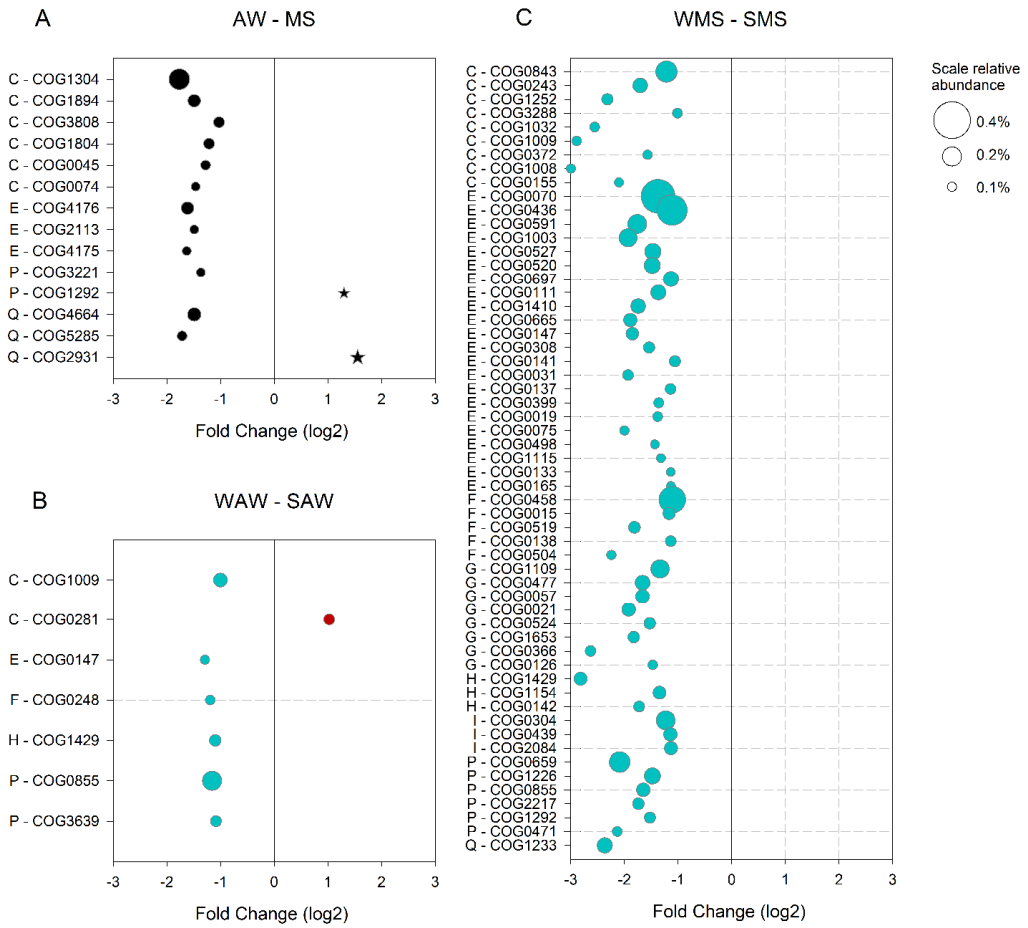

Figure 5. Significantly (p < 0.05) enriched COGs in **(A)** the ambient water (AW) *versus* marine snow (MS), **(B)** in winter (WAW) *versus* summer (SAW) ambient water communities; and **(C)** in winter (WMS) *versus* summer (SMS) marine snow. Only COGs with a $\log_2$ fold change > 1 and a relative abundance > 0.1% (indicated by bubble size) are shown. COG numbers and corresponding COG categories are indicated: E - Amino acid transport and metabolism, H - Coenzyme transport and metabolism, P - Inorganic ion transport and metabolism, C - Energy production and conversion, I - Lipid transport and metabolism, G - Carbohydrate transport and metabolism, F - Nucleotide transport and metabolism and Q - Secondary metabolites biosynthesis, transport and catabolism.

Transporters

Transporters provide insights into the dynamics of nutrient and organic matter uptake of microbial communities (Bergauer et al 2018). COGs associated with substrate transport comprised 6.6% of the MS- and AW-COGs combined. ATP-binding cassette (ABC) transporters were the majority (44.4 ± 0.6%) of transporters, in agreement with results from diverse open ocean regions (Bergauer et al 2018). Highest dissimilarity (determined via SIMPER analysis) between summer MS, winter

MS, summer AW and winter AW was reflected in COGs (ENOG4111IMY and ENOG4111FRH) involved in transport of the compatible solute ectoine in winter AW (Table S11), which acts as an osmoprotectant and is synthesized in conditions of salinity or temperature stress (Kuhlmann et al 2011). Another ABC transporter (COG3638) found in high abundance particularly in winter in both AW and MS is associated to phosphonate/phosphite/phosphate transport and linked to phosphorus limitation (Gebhard & Cook 2008) (Table S11). Many ABC transporters responsible for the dissimilarity between summer and winter were associated to iron transport and mostly present in summer in both AW and MS (COG4181 and COG3127) and in MS in the summer (COG4594) (Table S11). This includes high affinity Fe^{3+}-citrate siderophores and uptake systems to pirate siderophores of other microbes (Banerjee et al 2016, Gaballa & Helmann 2007), pointing to iron limitation and high competition for iron in MS. Other ABC transporters characteristic for the MS-associated community in summer were important in the assembly of cytochrome *bd* terminal oxidase which is responsible for micro-aerophilic respiration and is overexpressed under nitric oxide stress (Jünemann 1997, Mason et al 2009).

The second most abundant transporter subfamily was the major facilitator superfamily (MFS) accounting for 21 ± 0.2% averaged over all seasons and habitats (AW and MS). The MFS is the largest family of secondary transport carriers (Reddy et al 2012, Saier Jr et al 1999). Unfortunately, due to poor annotation a detailed functional description was not possible. However, a strong seasonal separation was observed, with a range of COGs seasonally enriched in summer or winter independent of the habitat, and specific COGs linked to summer MS or to summer AW (Table S12). Other abundant superfamilies were the tripartite ATP-independent periplasmic transporter (TRAP-T) family comprising 5.7 ± 0.8% of transporter COGs averaged over all seasons and habitats, and the resistance-nodulation-cell division (RND) superfamily with 4.2 ± 0.3% of transporter COGs, also averaged over all seasons and habitats. The presence of TRAP transporters implicates preferential utilization of organic compounds and an energy saving lifestyle, which is advantageous under oligotrophic conditions (Bergauer et al 2018, Kelly & Thomas 2001). In this study, the few TRAP transporters showed low dissimilarity between samples and were mainly associated to a high affinity transport system encoded by the dct locus (Forward et al 1997) (Table S13). This system transports the C4-dicarboxylates malate, succinate and fumarate (Forward et al 1997). The RND family includes transporters for multi-

drug efflux in Gram-negative bacteria (Nikaido & Takatsuka 2009). Almost all COGs identified as members of the RND family were assigned to the membrane fusion protein HlyD (Table S14, Table S15), a specific transporter for the RTX hemolytic toxin HlyA (Pimenta et al 2005). HlyA is a pore-forming toxin released by many pathogens (including *Vibrio* spp.) into the medium or directly into the host cell (Costa et al 2015). However, it has been suggested that HlyD is involved in sync1217 secretion, a RTX toxin domain protein that creates an outer layer membrane barrier to toxins in *Synechococcus* (Stuart et al 2013). In this study, 66% of all reads of all seasons and habitats combined targeting COGs of RND family transporters were aligned to COGs of the HlyD protein of *Synechococcus* (Table S15). The large variety of HlyD assigned COGs and the heterogenous distribution through seasons and habitats indicate an important role of defense mechanisms for prokaryotic communities in this coastal system.

Functions of prokaryotic communities – Marker gene analysis

Functional prokaryotic community analysis based on 69 selected key marker genes revealed the presence of 39 KOs corresponding to diverse genes involved in carbon, nitrogen, sulfur, methane and carbon monoxide metabolism. Genes involved in H_2 oxidation were not present in the dataset. Interestingly, AW prokaryotes were characterized by a high relative abundance of anaerobic methanogenesis (*mtt*B, Fig. 6) which has been typically described for marine sediments and linked to Archaea (Ferry & Lessner 2008, Sun et al 2019). However, anaerobic methanogens have also been found in oxygenated coastal marine environments and have been hypothesized to inhabit anoxic microenvironments within organic particles such as MS (Acinas et al 2019, Alldredge & Cohen 1987, Bianchi et al 2018, DeLong 1992, Ditchfield et al 2012, Ploug et al 1997, Repeta et al 2016, Shanks & Reeder 1993). Our results indicate a larger potential for methanogenesis in the AW than in MS. The AW also includes small particles, which might harbor methanogens. Another possible explanation could be frequent attachment and detachment from MS by methanogenic prokaryotes, being active in microzones of MS and detaching as MS becomes dispersed (Son et al 2015). The *apr*A and *apr*B genes were very abundant in the AW, particularly in the winter (Fig. 6). The *apr*A and *apr*B genes are involved in anaerobic dissimilatory sulfate reduction by sulfate reducing organisms (SRO) such as Syntrophobacterales, Thermodesulfobacterium, Thermodesulfovibrio, Archaeoglo-

bus and some deltaproteobacterial lineages (Meyer & Kuever 2007b). SRO have been found to be frequently associated with methanogens (Grein et al 2013) which were abundant in winter AW as mentioned above. However, apr genes are also found in anoxygenic phototrophic and chemolithoautotrophic sulfur oxidizing bacteria (SOB) (Frigaard & Dahl 2008), as well as chemolithoheterotrophic sulfur oxidizing bacteria (Meyer & Kuever 2007a), including the abundant Pelagibacterales (Giovannoni et al 2005) and thus, are widespread throughout the oxygenated oceanic water column (De Corte et al., submitted).

Anaerobic metabolisms such as dissimilatory nitrate reduction (*nrf*A, *nap*A and *nap*B) were only present in summer MS (Fig. 6), indicating that large marine snow present in summer was colonized by prokaryotes specialized in attachment, biofilm formation and anaerobic nitrate respiration. Other genes involved in denitrification and dissimilatory nitrate reduction (*nir*K, *nar*I and *nar*V), the assimilatory nitrate reduction gene *nar*B and genes encoding nitrate reductases - nitrite oxidoreductases (*nar*H, *nar*Y, *nxr*B, *nar*G, *nar*Z and *nxr*A) and methane/ammonia monooxygenases (*pmo*A-*amo*A) were generally more frequent in winter than in summer in both habitats (Fig. 6), although in low abundances. This is in agreement with the widespread distribution of nitrogen related metabolisms (Kuypers et al 2018). Summer AW prokaryotes exhibited the highest relative abundance of genes involved in CO-oxidation (*cox*L, *cox*S, *cox*M and *cut*M) (Fig. 6). These genes occur mostly in *Roseobacter* to supplement heterotrophic growth with inorganic carbon (Moran & Miller 2007). In this study, however, most reads (46%) assigned to CO-oxidation COGs (COG1529, COG2080, COG1319) affiliated to the Alphaproteobacterium HIMB114 and *Candidatus* Puniceispirillum marinum (Table S16) indicating supplementing heterotrophic growth with energy derived from carbon monoxide (Moran & Miller 2007, Oh et al 2010). Winter MS exhibited the highest abundance of genes from the Calvin cycle (*rbc*S, *rbc*L and *prk*B) (Fig. 6) in accordance with the high abundance of cyanobacteria in MS (Fig. S3).

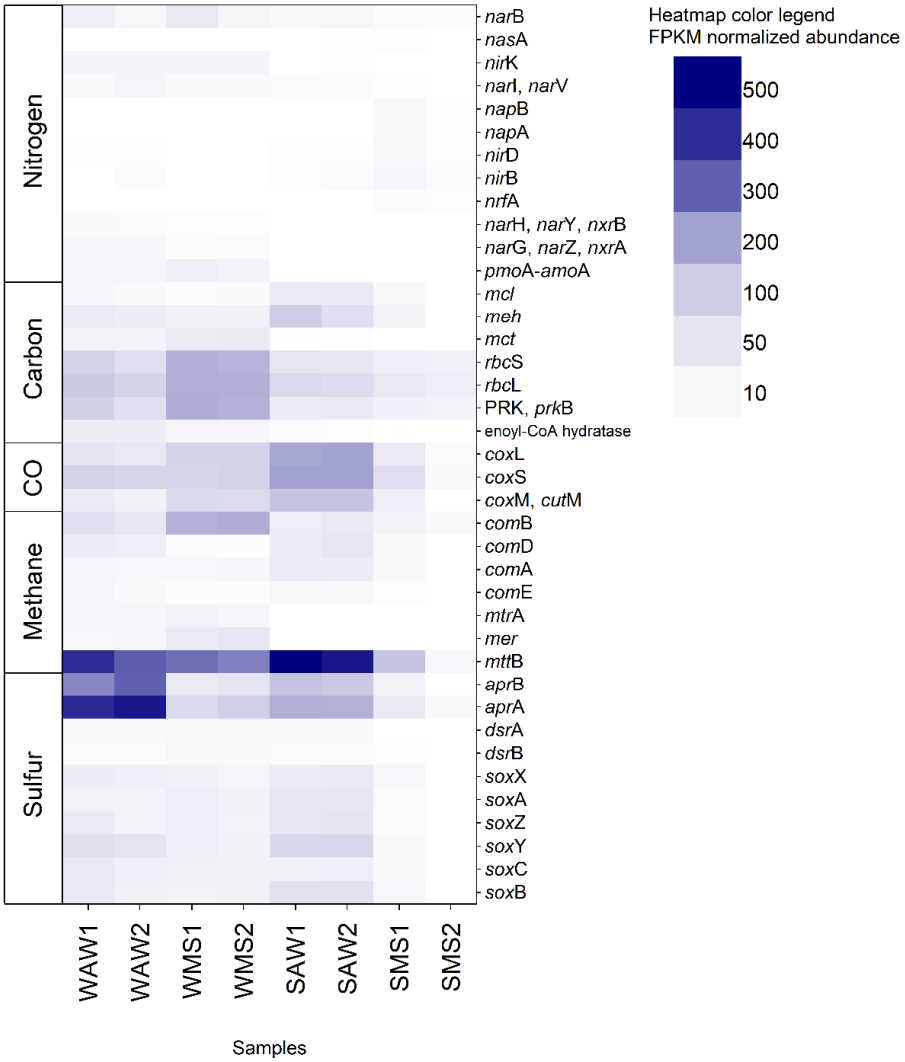

Figure 6. Fragments per kilo base million (FPKM) normalized abundance of the 39 KOs (Kyoto Encyclopedia of Genes and Genomes Orthology) identified and corresponding to genes involved in the carbon, nitrogen, sulfur, methane and carbon monoxide (CO) metabolism. Gene names are listed and described in Table S17. WAW1 and WAW2 indicate winter ambient water duplicates, SAW1 and SAW2 indicate summer ambient water duplicates, WMS1 and WMS2 correspond to winter marine snow duplicates, SMS1 and SMS2 indicate summer marine snow duplicates.

Summary and conclusions

We analyzed the community composition and functional potential of the AW and MS-associated communities over a seasonal cycle. The winter and summer were characterized by habitat specific prokaryotes, while the 'transition seasons', i.e., spring and fall were defined by season-specific prokaryotes, similar in the AW and MS-associated communities.

Differences in the nutrient availability between AW and MS are larger in winter and summer than in spring and fall favoring pronounced differences in the microbial community composition in the winter and summer between AW and MS. A selective advantage of prokaryotes associated to MS possibly includes the utilization of RTX toxin-related proteins for protection and motility. Adaptations to low temperature in winter might involve protection of the photosynthetic machinery from photoinhibition by low temperature. *Synechococcus* was not significantly enriched in MS, however, most of the enriched potential functions were assigned to *Synechococcus* in MS in the winter. The prominent appearance of *Synechococcus* associated to MS suggests that MS represents a hotspot for autotrophy in winter. In contrast, the development of low oxygen microzones within MS during water column stratification and low turbulence conditions in summer offers suitable conditions for anaerobic metabolism such as nitrite respiration.

Adaptations to a free-living life-style in winter included coping with phosphorus limitation and energy acquisition from labile organic matter such as compatible solutes. In the summer, prokaryotes supplement heterotrophic growth with solar radiation energy harvested with proteorhodopsins (Gómez-Consarnau et al 2019) or with CO-oxidation (Moran & Miller 2007).

The ‘transition seasons’ spring and fall were largely characterized by similar composition and functional potential of the AW and MS-associated prokaryotic communities in contrast to winter and summer. The fact that the same ASVs were enriched in the AW and MS indicates that environmental parameters of the ‘transition seasons’ influence both, the AW and MS-associated communities in the same way leading to an overall convergence of the microbial community composition and functional potential between AW and MS during these ‘transition seasons’.

Overall, a pronounced seasonality in both, the AW and the MS-associated communities was detectable. The MS-associated microbial community, however, was less sensitive to seasonal changes than the AW microbial community. Thus, the MS-associated community was more stable over the seasonal cycle than the AW microbial community. The large diversity and proportion of metazoans associated with MS might substantially affect the substrate composition of marine snow and thus, prokaryotic community composition and function, particularly in the summer. In contrast, the relatively low abundance of protists (including eukaryotic phytoplankton)

together with the high abundance of *Synechococcus* in winter MS suggests a major prokaryotic contribution to marine snow in this season.

Methods

Sampling

Samples were collected at twelve days covering a 1.5-year period from April 2015 to July 2016 in the coastal northern Adriatic Sea off Rovinj, Croatia (45.08347°N, 13.60518°E). Marine snow was collected with 100 mL sterile syringes at 15 ± 2 m depth by SCUBA diving and processed as described elsewhere (Steiner et al 2019). In brief, the marine snow collected in several syringes was pooled into a 0.1M HCl-rinsed glass bottle on deck and stored at *in situ* temperature in the dark. The marine snow included the pore water and a small amount of the water surrounding marine snow. The ambient water was collected at 15 m depth with 5 L Niskin bottles. The samples were transferred to the Center for Marine Research of the Ruder Bošković Institute at Rovinj within 30 min after sample collection.

DNA extraction

Marine snow (150 mL in June and 500 mL in February) and 1 L of ambient water were filtered onto 0.2 µm polyethersulfone filters (47 mm diameter, Supor, PALL Gelman) using an aspirator pump (Cole-Parmer). Filters were placed in cryovials (Biozym), flash-frozen in liquid nitrogen and stored at -80°C. DNA was extracted using a standard phenol-chloroform method as described in Steiner et al (2019). Hereinafter, ambient water and marine snow are abbreviated as AW and MS, respectively. Metagenome samples are labeled indicating the season, winter (W) or summer (S), followed by AW or MS, and the duplicate sample number 1 or 2. Duplicates collected in winter (W) correspond to samples collected on 1 and 2 February 2016 and summer (S) duplicates correspond to samples collected on 24 and 25 June 2015. All sequence data are publicly available the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB38662 (https://www.ebi.ac.uk/ena/data/view/PRJEB38662). Data was deposited using brokerage service of the German Federation for Biological Data (GFBio) (Diepenbroek et al 2014) in compliance with the Minimal Information about any (x) Sequence (MIxS) standard (Yilmaz et al 2011).

Prokaryotic community composition using amplicon sequencing

Twelve MS and 11 AW samples were collected for amplicon sequencing of the 16S rRNA gene at the same location as the metagenomics samples. Water samples were collected seasonally over 1.5 years and processed as described elsewhere (Steiner et al 2019). In brief, PCR amplification was performed with the primers 341_ill forward (TCGTCGGCAGATGTGTATAAGAGACAGCCTACGGG-NGGCWGCAG) and 802_ill reverse (GTCTCGTGGGCTCGGAGATGTGTATAA-GAGACAGGACTACHVGGGTATCTAATCC) containing adaptors and a KAPAHiFi Mastermix (Peqlab) using the following program: initial denaturation at 94°C for 3 min, followed by 20 cycles of 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 90 s. A final extension step was carried out at 72°C for 7 min, followed by cooling at 4°C. PCR products were purified using Agencourt AMPure XP magnetic particles (Beckman Coulter) and quantified with a Quant-IT PicoGreen® Assay (Invitrogen). Subsequently, Nextera PCR was performed with the same thermocycling conditions as described above for 10 additional cycles, followed by pooling and sequencing on an Illumina MiSeq system (Microsynth AG, Balgach, Switzerland) using v2 chemistry. Amplicon sequences were demultiplexed and trimmed by Microsynth AG (Balgach, Switzerland).

Bioinformatics analysis followed the 'DADA2 1.12' (Callahan et al 2016) pipeline tutorial with specific adaptations when filtering and trimming: truncLen = c(225, 210), maxN = 0, minQ = 2, maxEE = c(3, 3), truncQ = 0. Singletons, mitochondria and chloroplast sequences were removed. Statistical analysis of the amplicon sequences was conducted using R (v.3.6.2, https://www.R-project.org/) in RStudio v.1.2.5033 (RStudio-Team 2015) with the package phyloseq v.1.30.0 (McMurdie & Holmes 2013). The obtained amplicon sequence variants (ASVs) were variance stabilized (McMurdie & Holmes 2014) and a differential gene expression analysis was conducted using the R package DESeq2 v.1.26.0 (Love et al 2014). Differences in variance stabilized abundance of ASVs were considered significant if $p < 0.05$. Only ASVs with a $\log_2$ fold change higher than 1 in sequence abundance were further analyzed.

Metagenomics analysis of prokaryotes

Metagenomic DNA was quantified fluorometrically using the Quant-iT™ PicoGreen® Assay (Invitrogen) on a plate reader (Infinite 200). The library prepara-

tion of metagenomic DNA was done with the Westburg kit and sequencing was performed on an Illumina HiSeq 2500 (2x150 paired-end platform) at the Vienna BioCenter Core Facilities GmbH (VBCF). Metagenome sequences were quality checked using fastQC v.0.11.8. Sequences were trimmed with trimmomatic v.0.39 (Bolger et al 2014) and a sliding window size of 3 bases and a quality of 30. Assembly was done with megahit v.1.1.2 (Li et al 2015).

The program phyloFlash v.3.3 (Gruber-Vodicka et al 2019) was used to analyze the prokaryotic community composition based on 16S rRNA obtained from metagenomes with the following settings: -lib LIB -emirge -poscov -treemap. The correlation between the 16SrRNA gene amplicon data matrix and the extracted 16SrRNA genes from the metagenome was tested using Mantel test implemented in the R package vegan v.2.5.6. Prokaryotic OTUs of eight metagenomes were correlated to metataxonomic ASVs determined in the corresponding MS and AW samples collected on 24 and 25 June 2015 and 1 and 3 Feb 2016.

Further taxonomic and functional analyses were performed with MEGAN Community Edition v.6.17.0 (Huson et al 2007) with default settings (min score: 50, max expected: 0.01, min percent identity: 0, top percent: 10, min support percent: 0.05, min support: 0, LCA algorithm: naive, percent cover: 100, read assignment mode: readCount). Reads were blasted against the NCBI nr database using DIAMOND with short reads settings: -b 12.0 -k 1 -f 100 -e 0.00001 -p 20. The taxonomic affiliation was determined by aligning the reads to the database prot_acc2tax-Jul2019X1.abin. The database acc2eggnog-Oct2016X.abin was used for functional assignment in MEGAN6. Reads putatively assigned to prokaryotes were extracted from the MEGAN6 file using the ‘extract to new document’ function. The prokaryotic reads were visualized using taxonomy and EGGNOG viewer (Powell et al 2014) of MEGAN6 in absolute comparison mode. Taxonomic and gene assignments to clusters of orthologous groups (COGs) were extracted using the ‘extract to new document’ function to determine the taxonomic affiliation of the COGs. Subsequently, prokaryotes were sorted out from eukaryotic and other reads. The ‘export’ function was used to extract absolute numbers of reads assigned to COGs. Absolute read counts were variance stabilized (McMurdie & Holmes 2014) and differential gene expression analysis was conducted using the R package DESeq2 v.1.26.0 (Love et al 2014). COGs were selected for further analysis if differences in their variance-stabilized abundance were significant ($p < 0.05$) and the $\log_2$ fold change of se-

quence abundance was > 1. COGs with assignment to transport function were extracted from the variance-stabilized data set and used for further analyses.

Marker gene analysis was performed based on the Kyoto Encyclopedia of Genes and Genomes Orthology (KOs) functional classification of metabolisms (Table S17). The presence of 69 selected key marker genes (Acinas et al 2019) was checked against the aligned metagenomic reads. Predicted genes of aligned reads were determined with MetaProdigal v.2.6.3 (Hyatt et al 2012) and quantified using FPKM (Fragments Per Kilobase Million) normalization.

Eukaryotic community composition

The eukaryotic community composition was analyzed as described elsewhere Obiol et al (2020). Briefly, a BLAST search against a subset of the *eukaryotesV4* database with >90% similarity and >70% alignment coverage was performed to retrieve fragments of the 18S rRNA V4 region from the metagenomes. The extracted fragments were mapped to the full *eukaryotesV4* database using USEARCH v.11 local alignment at 97% identity. All hits with maximal score for every fragment were selected. All top hits of each read were merged into one, keeping the minimum consensus taxonomic level for taxonomic classification. Reads which resulted in no consensus taxonomic level remained unclassified.

Statistics

Diversity indices were calculated using the package vegan v.2.5.6 in R on rarefied data to the lowest number of ASVs. Diversity indices, one-way ANOSIM, PERMANOVA, SIMPER and principal component analysis (PCA) were calculated in PAST3 (Hammer et al 2001) using Euclidean similarity index. Graphs were created with the program SigmaPlot 14.0.

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Supplementary information

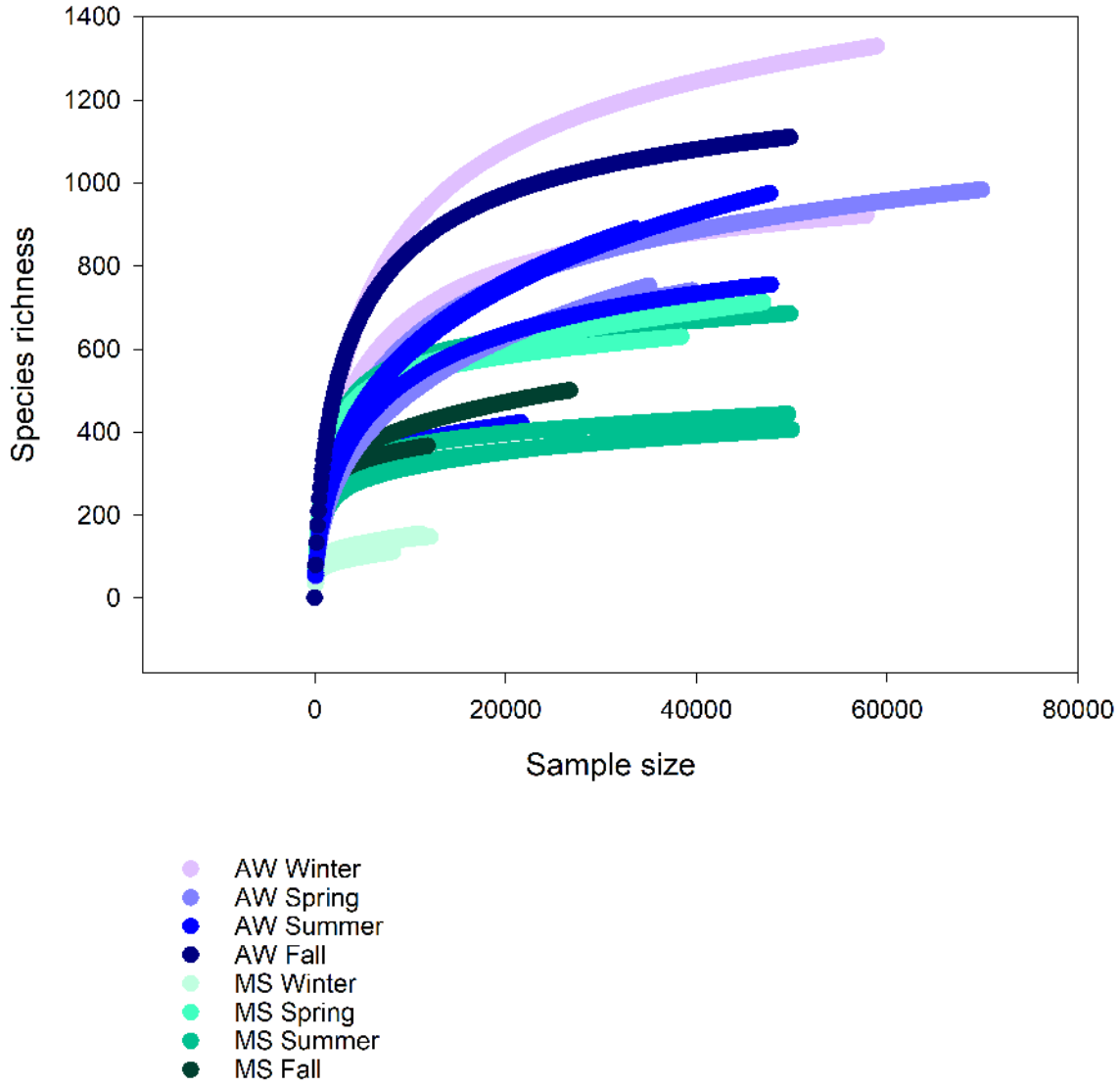
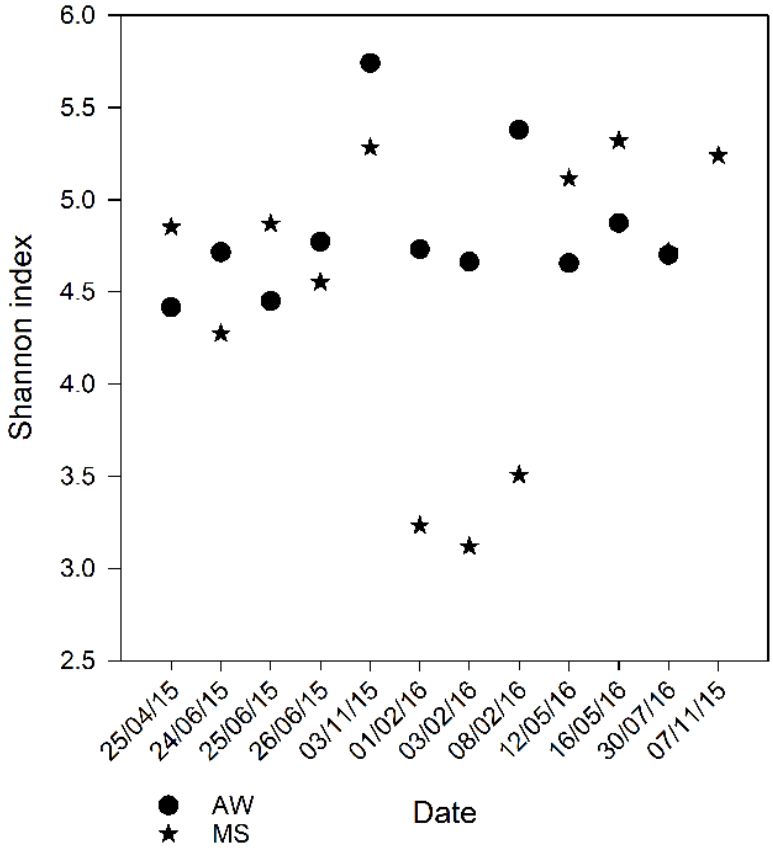


Figure S1. Rarefaction curves of amplicon sequences normalized to the sample with smallest number of sequences (08 Feb. 2016 marine snow).


Shannon index
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5.5
5.0
4.5
4.0
3.5
3.0
2.5
25/04/15
24/06/15
25/06/15
26/06/15
03/11/15
01/02/16
03/02/16
08/02/16
12/05/16
16/05/16
30/07/16
07/11/15
AW
MS
Date

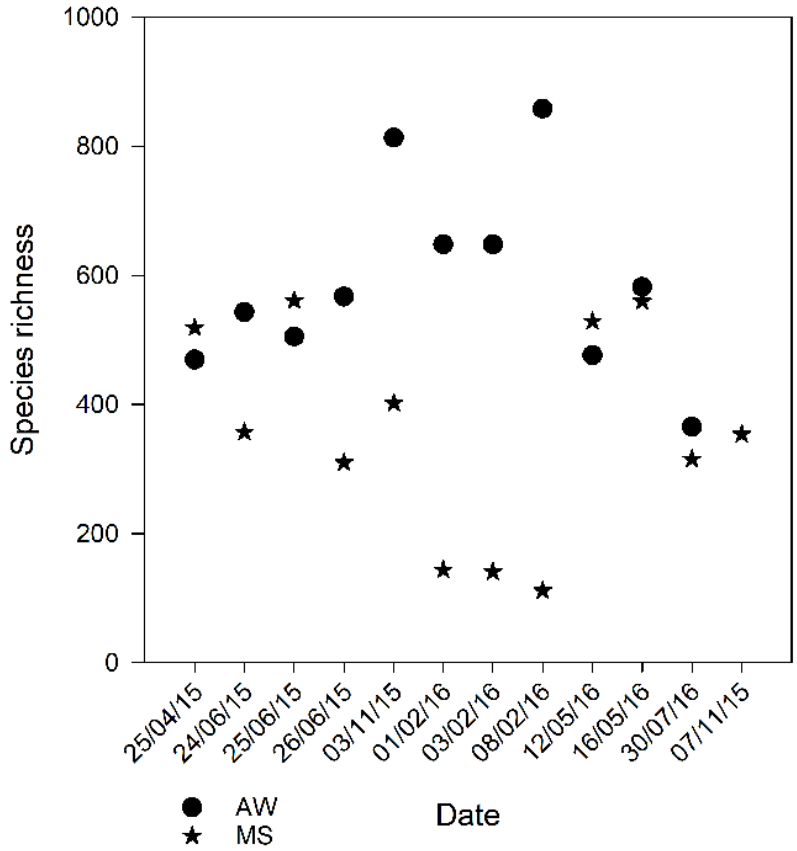
Species richness
1000
800
600
400
200
0
25/04/15
24/06/15
25/06/15
26/06/15
03/11/15
01/02/16
03/02/16
08/02/16
12/05/16
16/05/16
30/07/16
07/11/15
AW
MS
Date

Figure S2. Shannon diversity index (A) and species richness (number of taxa) (B) of rarified prokaryotic communities in different sampling days based 16S rRNA amplicon sequence variant analysis. Symbols indicate ambient water (AW) and marine snow (MS) habitats.

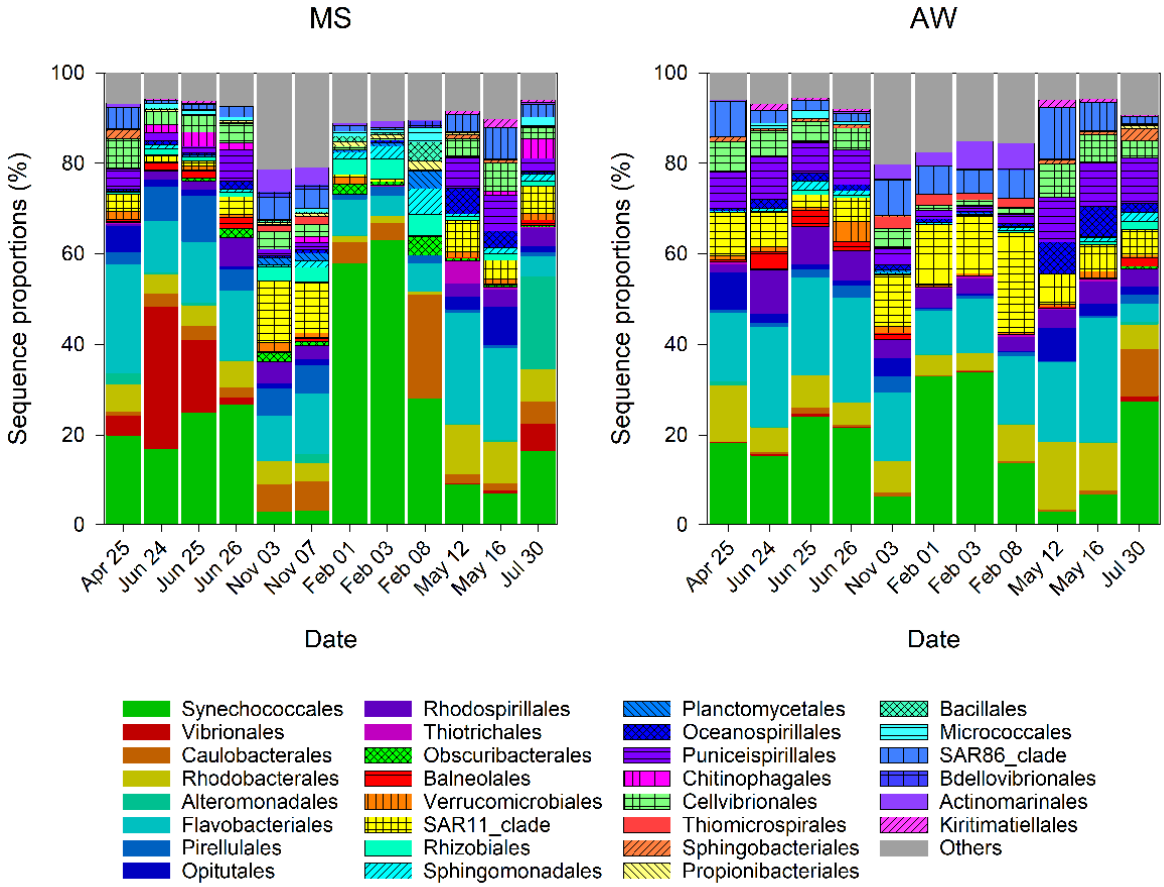

Figure S3. Contribution of the 30 most abundant MS-associated (MS) and ambient water (AW) prokaryotic orders to the communities based on variance-stabilized 16S rRNA amplicon sequences. Less abundant prokaryotic orders are grouped under “Others”. Samples from April to November were taken in 2015 and samples from February to July were taken in 2016.

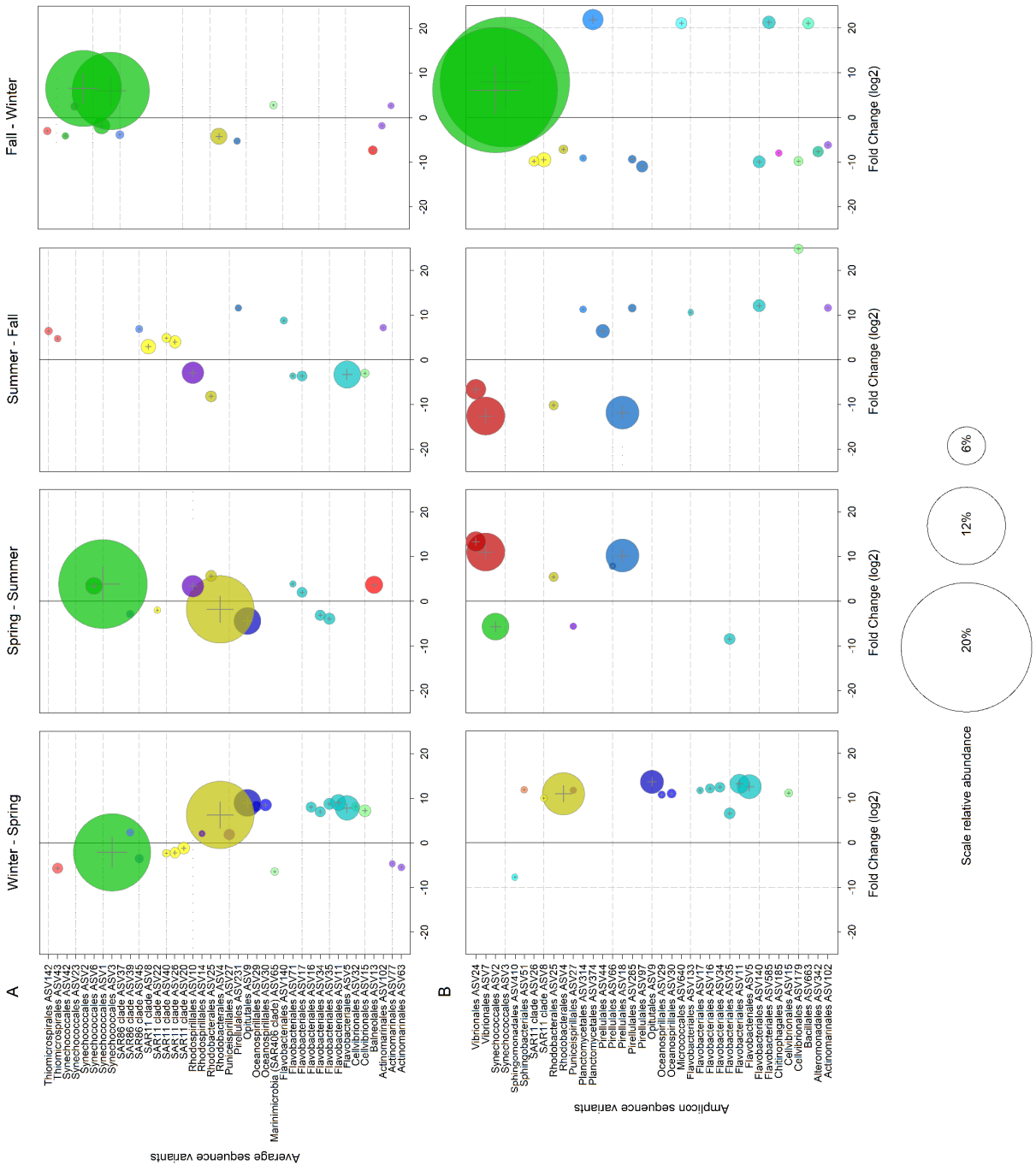

Figure S4. Amplicon sequence variants (ASVs) with a relative abundance > 1% and a $\log_2$ fold change > 1 significantly ($p < 0.05$) enriched in one of two consecutive seasons in **(A)** the ambient water and **(B)** in marine snow (MS). Bubble size represents the relative abundance and colors indicate the assigned taxonomy at the order level.

Table Legends

Table S1. Date, sample type and diversity indices of samples rarified to the lowest number of ASVs.

Table S2. SIMPER analysis based on relative abundance of reads assigned to eukaryotes. Winter ambient water: WAW, summer ambient water: SAW, winter marine snow: WMS, summer marine snow: SMS.

Table S3. Abundance and taxonomic assignment of trimmed reads, and diversity indexes of the metagenomes.

Table S4. Taxonomy, number of reads and relative abundance of reads aligned to significantly enriched species in the AW.

Table S5. Taxonomy, number of reads and relative abundance of reads aligned to significantly enriched species in MS.

Table S6. Taxonomy, number of reads and relative abundance of reads aligned to significantly enriched species in summer AW.

Table S7. Taxonomy, number of reads and relative abundance of reads aligned to significantly enriched species in winter AW.

Table S8. Taxonomy, number of reads and relative abundance of reads aligned to significantly enriched species in summer MS.

Table S9. Taxonomy, number of reads and relative abundance of reads aligned to significantly enriched species in winter MS.

Table S10. Taxonomy, number of reads and relative abundance of reads aligned to COG0304 and COG0439 in winter MS.

Table S11. SIMPER analysis based on variance stabilized abundance of reads aligned to ATP-binding cassette (ABC) transporter COGs. Winter ambient water: WAW, summer ambient water: SAW, winter marine snow: WMS, summer marine snow: SMS.

Table S12. SIMPER analysis based on variance stabilized abundance of reads aligned to major facilitator superfamily (MFS) transporter COGs. Winter ambient water: WAW, summer ambient water: SAW, winter marine snow: WMS, summer marine snow: SMS.

Table S13. SIMPER analysis based on variance stabilized abundance of reads aligned to tripartite ATP-independent periplasmic transporter (TRAP-T) family COGs. Winter ambient water: WAW, summer ambient water: SAW, winter marine snow: WMS, summer marine snow: SMS.

Table S14. SIMPER analysis based on variance stabilized abundance of reads aligned to resistance-nodulation-cell division (RND) superfamily COGs. Winter ambient water: WAW, summer ambient water: SAW, winter marine snow: WMS, summer marine snow: SMS.

Table S15. List of COGs associated with the hylD gene and a table showing the taxonomic affiliation (Taxa), average (AV), sum and relative abundance (%) of reads aligned to COGs associated with the gene hylD of all samples.

Table S16. Taxonomy (Taxa), number of reads (Reads) and relative abundance (%) of reads aligned to COGs associated to CO-oxidation (COG1529, COG2080, COG1319) in winter MS.

Table S17. Marker genes used normalized abundance. The presence (+) in at least one sample of the dataset is indicated. Winter ambient water duplicates: WAW1 and WAW2, summer ambient water

duplicates: SAW1 and SAW2, winter marine snow duplicates: WMS1 and WMS2, summer marine snow duplicates: SMS1 and SMS2.

The supplementary excel file that includes all supplementary tables is deposited at the digital longtime archive of the University of Vienna and at https://ucloud.univie.ac.at/index.php/s/VdM3lz9VGtcXlgn/download

Chapter III Seasonal dynamics of marine snow-associated and free-living demethylating bacterial communities in the coastal northern Adriatic Sea

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Summary

The extent of DMSP demethylation has been hypothesized to depend on DMSP availability and bacterial sulfur demand, which might lead to niche differentiation of the demethylating bacterial community. In this study, we determined DMSP concentrations in marine snow and the ambient water over a seasonal cycle and linked DMSP concentrations to the abundance of bacteria harboring the demethylation *dmd*A gene in the Adriatic Sea. In marine snow, DMSP concentrations were up to four times higher than in the ambient water and three times higher in marine snow in summer than in winter. The average *dmd*A:*rec*A gene ratio over the sampling period was 0.40 ± 0.24 in marine snow and 0.48 ± 0.21 in the ambient water. However, at the subclade level, differences in the demethylating bacterial community of marine

snow and the ambient water were apparent. Seasonal patterns of potentially demethylating bacteria were best visible at the oligotype level. In the ambient water, the SAR116 and the OM60/NOR5 clade were composed of oligotypes that correlated to high DMSP concentrations, while oligotypes of the *Rhodospirillales* correlated to low DMSP concentrations. Our results revealed a pronounced seasonal variability and spatial heterogeneity in DMSP concentrations and the associated demethylating bacterial community.

Introduction

Dimethylsulfoniopropionate (DMSP) is a sulfur-containing metabolite mainly produced by marine phytoplankton in species-specific concentrations (Stefels 2000). DMSP acts primarily as an osmolyte but also serves as an antioxidant, cryoprotectant or represents an overflow metabolite (Karsten et al 1996, Malin & Erst 1997, Simó 2001, Stefels 2000, Sunda et al 2002). It is released from phytoplankton into the ambient water and is subsequently degraded by bacteria either via the DMSP cleavage or demethylation pathway (Kiene et al 2000, Moran et al 2012). Diverse taxa of marine bacteria can mediate one or the other, or both processes (Moran et al 2012, Nowinski et al 2019, Reisch et al 2011, Simó 2001, Varaljay et al 2015), thereby controlling the fate of dissolved DMSP in the ocean. Genes of the demethylation pathway have been found in 2- to about 5-fold higher abundance than genes of the cleavage pathway in metagenomes obtained from the Global Ocean Survey (Moran et al 2012), at Station ALOHA (Varaljay et al 2012) and Monterey Bay (Nowinski et al 2019). Approximately 90% of dissolved DMSP in the ocean is demethylated (Kiene & Linn 2000b, Kiene et al 2000), implicating that the sulfur moieties are not lost to the atmosphere via dimethylsulfide (DMS), but rather enter the marine food web (Moran et al 2012). Total DMSP concentrations in the ocean depend largely on the abundance and intracellular DMSP concentrations of the main DMSP-producing phytoplankton taxa (Keller et al. 1989) but also on environmental factors such as salinity, grazing pressure and nutrient concentrations (Stefels et al 2007). Responses of demethylating bacteria to phytoplankton blooms (Howard et al 2011), specific phytoplankton taxa (Howard et al 2011, Varaljay et al 2015) and to DMSP concentrations (Frade et al 2016, Nowinski et al 2019) indicate that demethylating bacteria can occupy a wide range of niches. Specific subclades of the *dmd*A gene, responsible for the demethylation of DMSP, have been found in different size

fractions of coastal waters, suggesting that bacteria with contrasting lifestyles, such as free-living *versus* particle-attached, could potentially demethylate DMSP (Varaljay et al 2010).

In this study we analyzed the spatial and temporal distribution patterns of the demethylating bacterial community and determined DMSP concentrations in marine snow (MS) and the ambient water (AW) over a seasonal cycle. We expected substantially higher DMSP concentrations in MS than in the AW as MS originates mainly from transparent exopolymeric particles (TEPs) released by phytoplankton into the ambient water where they randomly collide (Alldredge et al 1993, Herndl 1992, Schuster & Herndl 1995). To determine the factors affecting DMSP concentrations in MS and the AW, we characterized the phytoplankton community and measured contextual environmental parameters over a seasonal cycle. We hypothesized that the demethylating bacterial community in MS differs from that in the AW and varies over seasons. Changes in the bacterial community might have important implications for the cycling of DMSP, as demethylation of DMSP prevents the formation of the climatically relevant gas DMS (Moran et al 2012).

Results

Phytoplankton and DMSPt + DMS concentrations

Four main phytoplankton groups (diatoms, silicoflagellates, dinoflagellates and coccolithophores) were present during the seasonal cycle (Fig. S1). Total phytoplankton abundance integrated over the upper 10 m of the ambient water (AW) was highest on 15 November (1.1 x 10^9 cells m^{-2}) due to a diatom bloom (8.7 x 10^8 cells m^{-2}) and high abundance of coccolithophores (1.6 x 10^8 cells m^{-2}) (Fig. S1). Diatoms were generally the most abundant phytoplankton group. Coccolithophores were abundant on 15 June (1.1 x 10^8 cells m^{-2}) and 19 July (1.4 x 10^8 cells m^{-2}). Silicoflagellates were the least abundant group (Fig. S1).

Particulate and dissolved DMSP + DMS (DMSPt + DMS) concentrations averaged over all samples were significantly higher in marine snow (MS) (80.46 ± 52.44 nmol L^{-1}) than in AW (33.62 ± 12.57 nmol L^{-1}) with the monthly averaged enrichment factor (EF) ranging from 2 to 4 (Fig. 1). DMSP concentrations in MS and the AW showed seasonal variations with lowest monthly average in November and February and highest concentrations in May, June, and July (Fig. 1, Table S1).

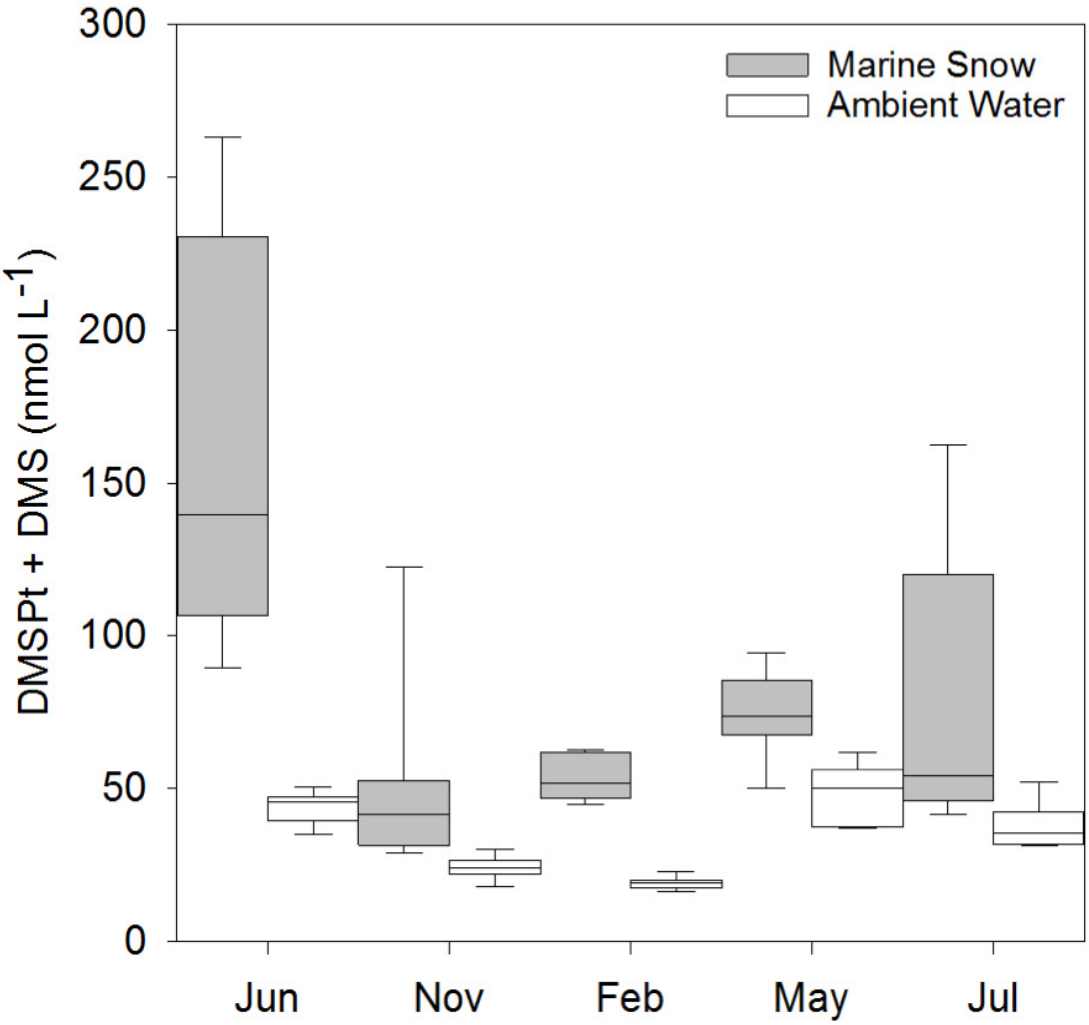

Figure 1. Box-plot of DMSP total + DMS concentrations (median, 10th, 25th, 75th and 90th percentiles) in marine snow (grey) as compared to the ambient water (white).

*dmd*A *subclades determined by qPCR*

Five of the nine specific primer pairs (A/2, B/3, B/4, D/3 and E/2) for the *dmd*A subclades generated clear signals in PCR and hence, were subsequently used for qPCR. On average, 40.0 ± 24% of the bacterial community in MS and 48 ± 21% of the AW bacterial community harbored the *dmd*A gene. Subclades D/3 and B/3 were only detected in the AW. The *dmd*A:*rec*A ratio of subclade D/3 was on average 0.19 ± 0.27, while subclade B/3 had the lowest (0.02 ± 0.04) contribution to the total demethylating bacterial community (Fig. 2). The subclades E/2 and B/4 contributed similarly to the DMSP demethylating bacterial community in AW and MS (Fig. 2). Subclade E/2 was significantly more abundant in June than in any other month. The

relative abundance of subclade A/2 was approximately twice as high in MS (0.15 ± 0.24) than in AW (0.06 ± 0.1). Subclade A/2 was mainly present in November and February in MS and AW (Fig. 2).

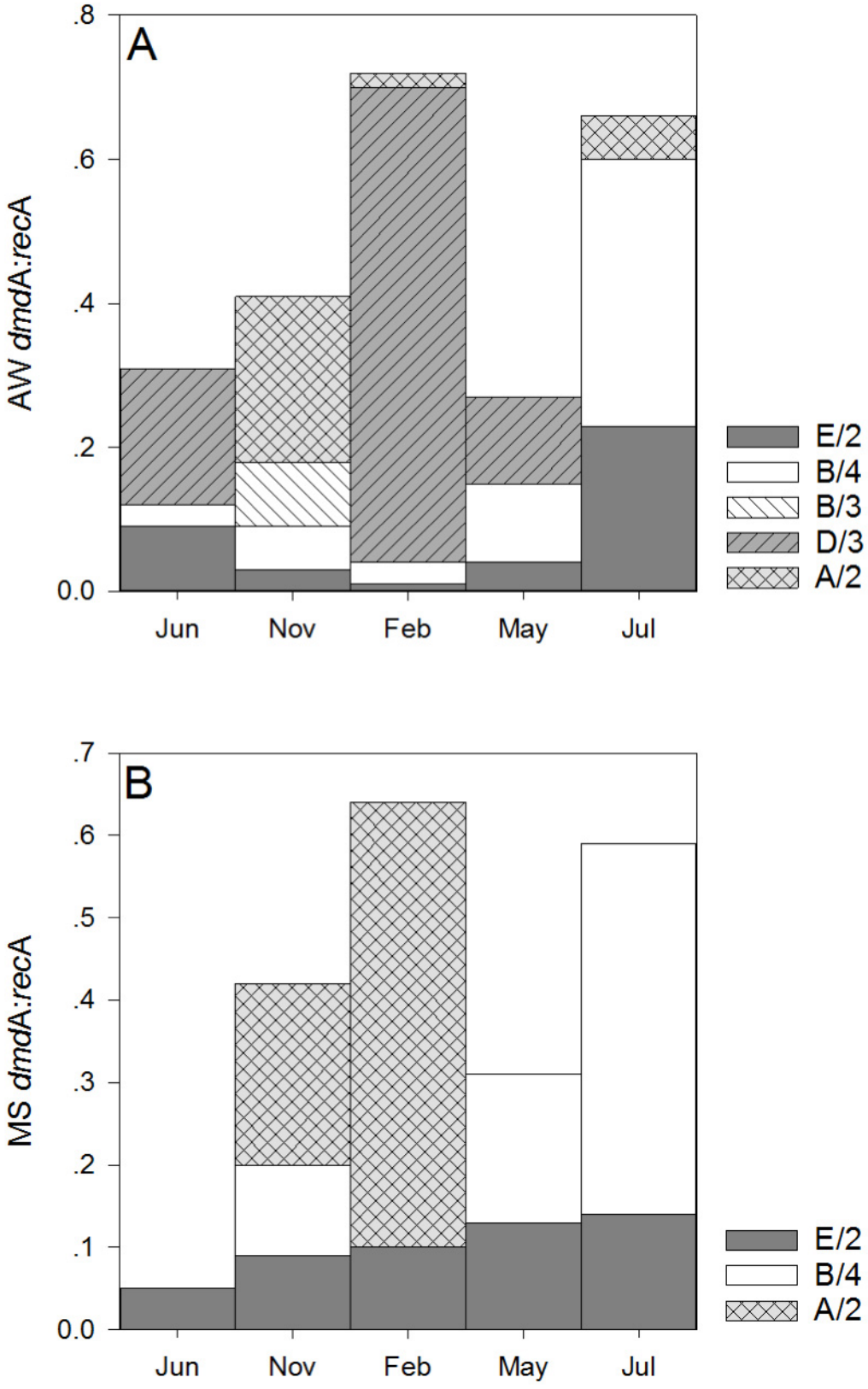

Figure 2. Seasonal variation of the monthly average of *dmd*A subclades (E/2, B/4, B/3, D/3 and A/2) normalized to *rec*A gene abundance assessed by qPCR in AW (A) and MS (B). SD was always below 27% of the respective subclades normalized to recA gene abundance.

Bacterial community composition

All bacterial groups specifically targeted by the qPCR primers of the *dmd*A subclades A/2, B/3, B/4, D/3 and E/2 were observed in both, the AW and MS. Clade A primers target *Rhodospirillales* and *Roseobacter*. *Roseobacter* was present only in MS and accounting for 0.1% of the OTUs there, hence subclade A/2 was mostly represented by *Rhodospirillales*. The SAR11 clade (with members representative for *dmd*A subclade D/3) amounted in total to 4.6% in MS and 10.6% in AW, *Rhodospirillales* (with members representing the subclade A/2) contributed 3.5% to MS and 6.2% to AW, the SAR116 clade (with members representative for subclades B/3 and B/4) constituted 3.4% to MS and 5.7% to AW, and the OM60 clade (with members representative for subclade E/2) contributed 2.5% in MS and 3.3% in AW (Fig. 3).

The variation of the bacterial community composition in MS and the AW in June and July coincided with the abundance of dinoflagellates, coccolithophores and DMSPt + DMS concentrations and the abundance of the *dmd*A subclade E/2 (Fig. 4A, B). In contrast, silicoflagellates, diatoms and the subclades B/3, D/3 and A/2 corresponded to the AW community composition in November and February (Fig. 4A). Between November and May, the bacterial community in MS was related to a higher contribution of diatoms, silicoflagellates and the *dmd*A subclade A/2. The MS bacterial community in July corresponded mainly to the *dmd*A subclade B/4 (Fig. 4B).

The OTUs targeted by the primers of the *dmd*A subclades and appearing in both, MS and AW, were further analyzed with oligotyping to obtain more detailed insights into their temporal distribution and preferred life styles. Oligotyping revealed 40 SAR116 oligotypes, 26 OM60 clade oligotypes and 48 Rhodospirillales oligotypes (Fig. 5). In the AW, the 9 most abundant oligotypes of the SAR116 clade (representative of subclade B/4) exhibited seasonal dynamics. Oligotypes SAR116_3, 7 and 8 were more abundant in November and February and oligotypes SAR116_1, 2, 4, 5, 6 and 9 were more abundant in June, July and May (Fig. 5A). Oligotype SAR116_6 and SAR116_9 correlated with DMSPt + DMS concentrations in the AW (Fig. S2). The dynamics of the MS oligotypes of SAR116 showed a similar seasonal pattern as in the AW, however, in a less pronounced way (Fig. 5B). Oligotype

SAR116_5 correlated significantly to DMSPt + DMS concentrations in MS (Fig. S2). The Gammaproteobacteria clade OM60, representative of demethylating subclade E/2 bacteria, was present in all seasons with highest relative abundances in May (Fig. 3). The oligotypes OM60_1 and OM60_2 correlated significantly with DMSPt + DMS concentrations. OM60 oligotypes did not correlate with DMSPt + DMS concentrations in MS (Fig. S2). The four most abundant oligotypes were specifically dominant in May, June and July (Fig. 5C, D). *Rhodospirillales* and *Roseobacteriales* are the representative group of subclade A/2, however, *Roseobacter* was not present in the AW. The most abundant OTU was AEGEAN-169 in the AW in February (Fig. 3A).

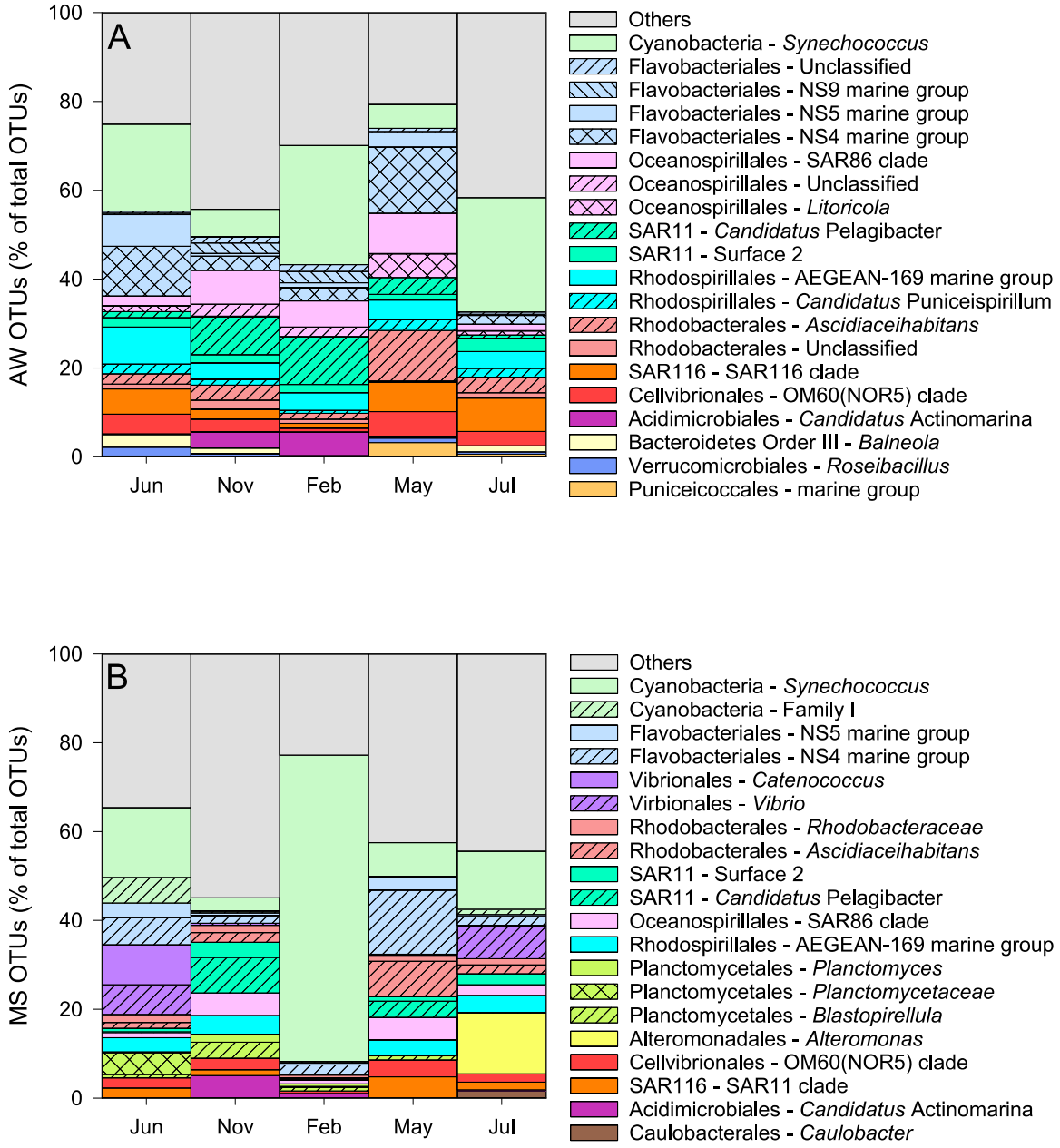

Figure 3. Relative contribution of the 20 most abundant OTUs in the AW (A) and MS (B) expressed as % of total OTUs averaged over each month. All other OTUs are summed up and plotted together as “Others” in grey.

The *Rhodospirillales* oligotypes showed a strong seasonality in the AW with oligotypes Rhodospirillales_6 and Rhodospirillales _7 appearing in November and February, while all others were more abundant in June, May and July (Fig. 5E). Oligotypes Rhodospirillales_6 and Rhodospirillales_7 were negatively related to DMSPt + DMS concentrations (Fig. S2). The seasonality of this group was also less pronounced in

MS than in AW (Fig. 5F). Oligotypes Rhodospirillales_1 and Rhodospirillales_5 correlated significantly to DMSPt + DMS concentrations in MS (Fig. S2).

Discussion

Demethylating bacterial community in ambient water vs. marine snow

Typically, ca. 90% of DMSP occurs in plankton cells, and only between 2.3% to 10% is actually dissolved (Kiene & Slezak 2006). The DMS fraction of the measured DMSPt + DMS is presumably small as average DMS concentrations of 12 nmol L^{-1} in a coccolithophore bloom in the Northeast Atlantic represent rather high concentrations (Malin et al 1993). The DMSPt + DMS concentrations in AW were comparable to previously reported concentrations for the coastal Mediterranean Sea (Belviso et al 1990, Vila-Costa et al 2008). DMSPt + DMS in MS reached high concentrations, comparable to concentrations measured in phytoplankton blooms (Matrai & Keller 1993). The observed high DMSPt + DMS concentrations in MS (Fig. 1) are likely due to DMSP accumulation caused by high abundance of MS-associated phytoplankton (Turner 2002). Higher DMSP concentrations in MS, however, might also be caused by lower DMSP degradation rates. The overall bacterial activity determined as leucine incorporation was up to 7 times higher in MS as compared to the AW (Fig. S2B, Table S1), indicating a highly active bacterial community in MS. Consequently, it is unlikely that lower DMSP degradation rates are not responsible for the higher DMSP concentrations in MS. Bacterial activity has been shown to correlate with dissolved DMSP turnover rate (Kiene & Linn 2000a). Hence, despite potentially high dissolved DMSP turnover, DMSP accumulated in MS. However, further work is required to determine dissolved DMSP concentrations and turnover rates to obtain better insights into the dynamics of DMSP utilization.

While the contribution of demethylating bacteria to the bulk bacterial community was similar in MS and AW (40 and 48%, respectively) and over the seasons, with only few exceptions, the composition of the demethylating bacterial community varied considerably between MS and AW (Fig. 2A, B). The *dmd*A subclades D/3 and B/3 exclusively occurred in the AW, suggesting that these subclades of demethylating bacteria prefer a free-living lifestyle (Fig. 2A, B). This is in agreement with the known representative of subclade D/3, the SAR11 clade (Acinas et al 1999). However, it contradicts the typically particle-associated lifestyle of the SAR116 clade (Jain & Krishnan 2017, Salazar et al 2015) affiliated to subclades B/3 and B/4. Subclade

B/3 was significantly more abundant in the free-living fraction in coastal waters of the south eastern US, indicating that some members of the SAR116 clade, particularly the OTUs capable of demethylation, might exhibit a free-living life style (Varaljay et al 2010). The *dmd*A subclade A/2 was twice as abundant in MS than in AW (Table S1). This is in agreement with the finding of the demethylating subclade A/2 colonizing the surface mucus of corals (Frade et al 2016) but contradicts the findings of Varaljay et al (2010) indicating that subclade A/2 is composed of bacteria with contrasting life-styles. The high abundance of the *dmd*A subclade A/2 and the low abundance of *Rhodospirillales* in MS further indicate that this subclade is composed of diverse taxa (Fig. 2B, 3B). In this study, the most abundant *Rhodospirillales* OTU was affiliated to the AEGEAN-169 marine group (Fig. 3A), typically found in the AW (Salazar et al 2015). Subclades E/2 and B/4 occurred in both, MS and AW in this coastal system, similarly as previously reported for the south eastern US coast (Varaljay et al 2010). Taken together, the *dmd*A gene-harboring bacterial community constitutes a similar fraction to the total bacterial community in MS as in AW. However, the community composition of the *dmd*A gene harboring community on the subclade level differs substantially between MS and AW. AW and MS are characterized by contrasting DMSPt + DMS concentrations, which might favor niche differentiation of demethylating bacteria. However, the observed differences in MS-associated and free-living demethylating communities also reflect phylogenetically conserved general patterns of particle-attached and free-living bacteria (Salazar et al 2015).

Temporal variability of the demethylating bacterial community

Seasonal dynamics of the demethylating bacterial community were evident on the *dmd*A subclade level for most subclades (Fig. 2A, B). Some subclades appeared episodically throughout the year indicating that the fluctuations in the targeted bacterial groups are not necessarily related to environmental changes on a seasonal scale but related to sporadic events. The northern Adriatic Sea is a highly dynamic system, as indicated by the variation in the nutrient concentrations over the investigation period (Fig. S3) and as shown previously (Cantoni et al 2003, Cozzi et al 2004). The appearance of subclade B/3 might not be related to seasonal changes but rather to a diatom bloom in November (Fig. S1). The patchy distribution of subclade D/3 in the AW might also indicate that the representative bacterial group (SAR11 clade) consists of many ecotypes with fine tuned adaptations to physical and chemical gradi-

ents (Carlson et al 2009). The SAR11 clade consists of members highly active in DMSP degradation correlating with haptophyte abundance (including coccolithophores) (Nowinski et al 2019). In this study subclade D/3 was present in June, when coccolithophores were abundant and DMSPt + DMS concentrations high. However, subclade D/3 was also present in February, coinciding with high relative abundance of SAR11 16SrRNA genes (Fig. 3A). Seasonality was observed in the temporal distribution of the *dmd*A subclades E/2 and B/4 in the AW and subclade A/2 present mostly in November and February in MS and AW (Fig. 2). The gammaproteobacterial clade OM60, targeted by E/2 primers, has been found in nutrient rich coastal areas (Xia et al 2015) and was shown to vary with DMSPt + DMS concentrations (Nowinski et al 2019). In this study, the relative abundance of subclade E/2 correlated to DMSPt + DMS concentrations in MS ($r=0.89$, $p<0.05$) (Fig. 4B). Subclade A/2 occurred mostly in samples with low DMSPt + DMS concentrations in MS and AW pointing to diverse life styles within this subclade as reported in other studies (Frade et al 2016, Nowinski et al 2019, Varaljay et al 2010). Conclusions based on normalized bacterial communities might not reflect actual community dynamics. In this study, however, the bacterial abundance remained fairly stable over the seasons in the AW. MS exhibited significantly higher prokaryotic abundance only in November as compared to all other months (Fig. S4). The rather stable bacterial abundance over seasons strengthens the conclusions drawn from normalized gene abundances.

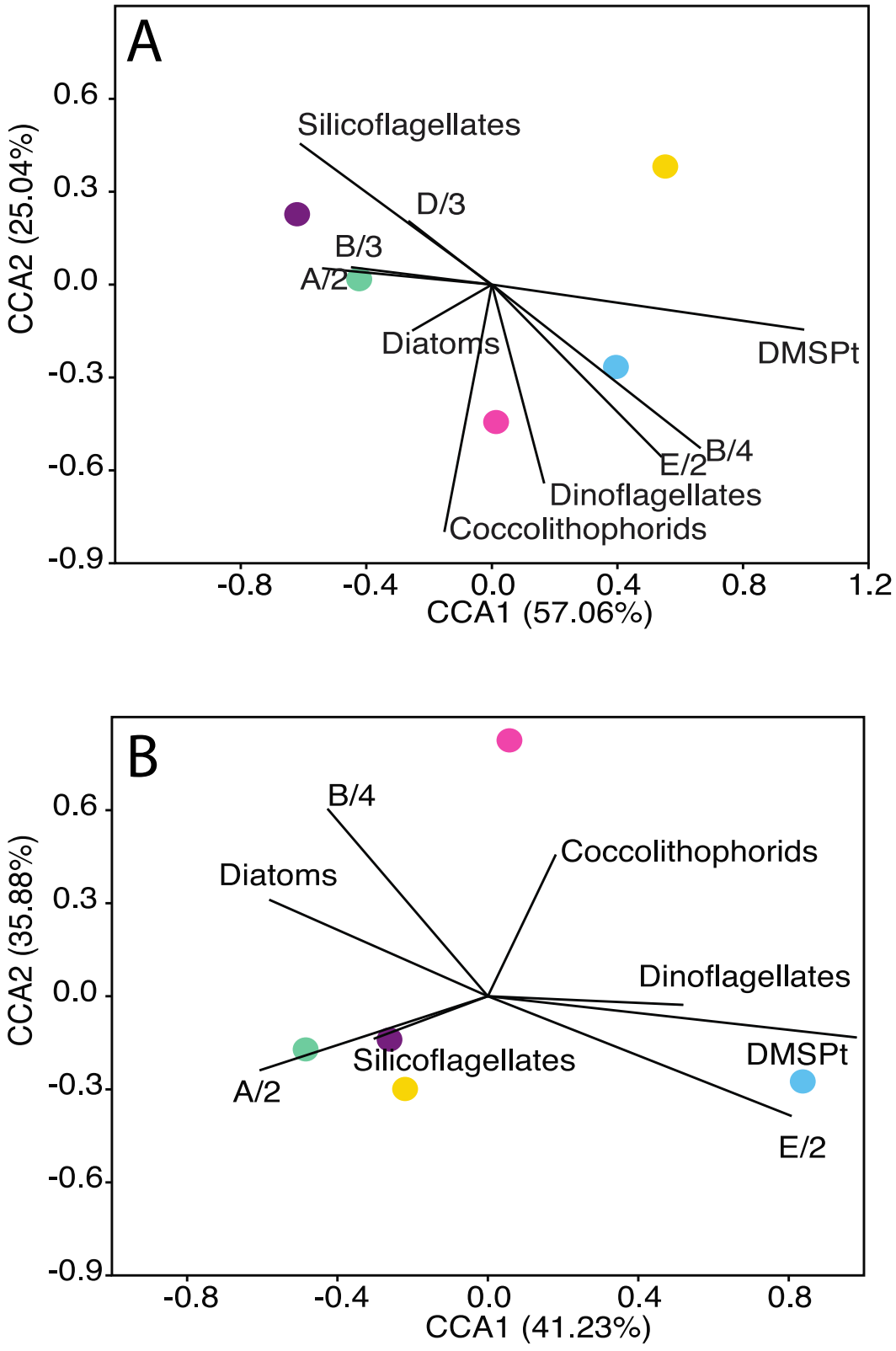

Figure 4. Canonical correspondence analysis (CCA) based on the seasonal variation of the bacterial community in the AW (A) and MS (B). The percentage of variation of the bacterial community explained by each axis is shown in parenthesis in the axis title. *Synechococcus* was excluded from the

CCAs. Environmental variables (DMSPt concentration, diatoms, coccolithophores, dinoflagellates, silicoflagellates abundances and the *dmd*A subclades E/2, B/4, B/3, D/3 and A/2 relative gene abundances) are depicted as triplot. The color-coding for the sampling months is as follows: cyan: June; green: November; purple: February; yellow: May; pink: July.

The phenomenon of seasonal variation of closely related bacteria has been observed previously in other studies (Ward et al 2017, Yung et al 2015). However, here we show for the first time seasonality of potentially demethylating bacteria at the oligotype level. We documented a seasonal switch of potentially demethylating oligotypes along with changes in environmental factors such as DMSPt + DMS concentrations (Fig. 1, Fig. 5, Table S1). The high abundance of potentially demethylating oligotypes and the significant correlations to DMSPt + DMS concentrations of some oligotypes (Fig. S2) suggest a wide range of niches for DMSP demethylation. DMSP demethylation of oligotypes at various DMSP concentrations might have major biogeochemical and ecological implications, by preventing the formation of the climatically relevant gas DMS and by retaining the sulfur compounds in the marine food web (Moran et al 2012). While closely related *dmd*A gene harboring oligotypes revealed a strong seasonality in their occurrence, their preferential lifestyle (free-living *vs*. particle-associated) was less obvious using oligotyping. This stresses the finding of the particle-association lifestyle as a deeply rooted trait in the phylogenetic tree (Salazar et al 2015) and suggests that traits defining a narrow niche might have only recently been acquired. A less obvious differentiation of lifestyles between oligotypes might have also been caused by the method used to collect MS with syringes, resulting in a dilution of MS with small amounts of AW including the pore water of MS (supplementary video). This inefficient separation of MS and AW might have caused a less pronounced differentiation of free-living *vs*. particle-associated oligotypes. However, a strict separation of MS and AW is difficult unless the pore-water (including biological and chemical compounds) is removed by filtration. Moreover, the bacterial community is not strictly separated into free-living and particle-associated bacteria, but rather into a gradient of particle association and sporadic attachment and detachment (Gibiansky et al 2010, Salazar et al 2015, Son et al 2015).

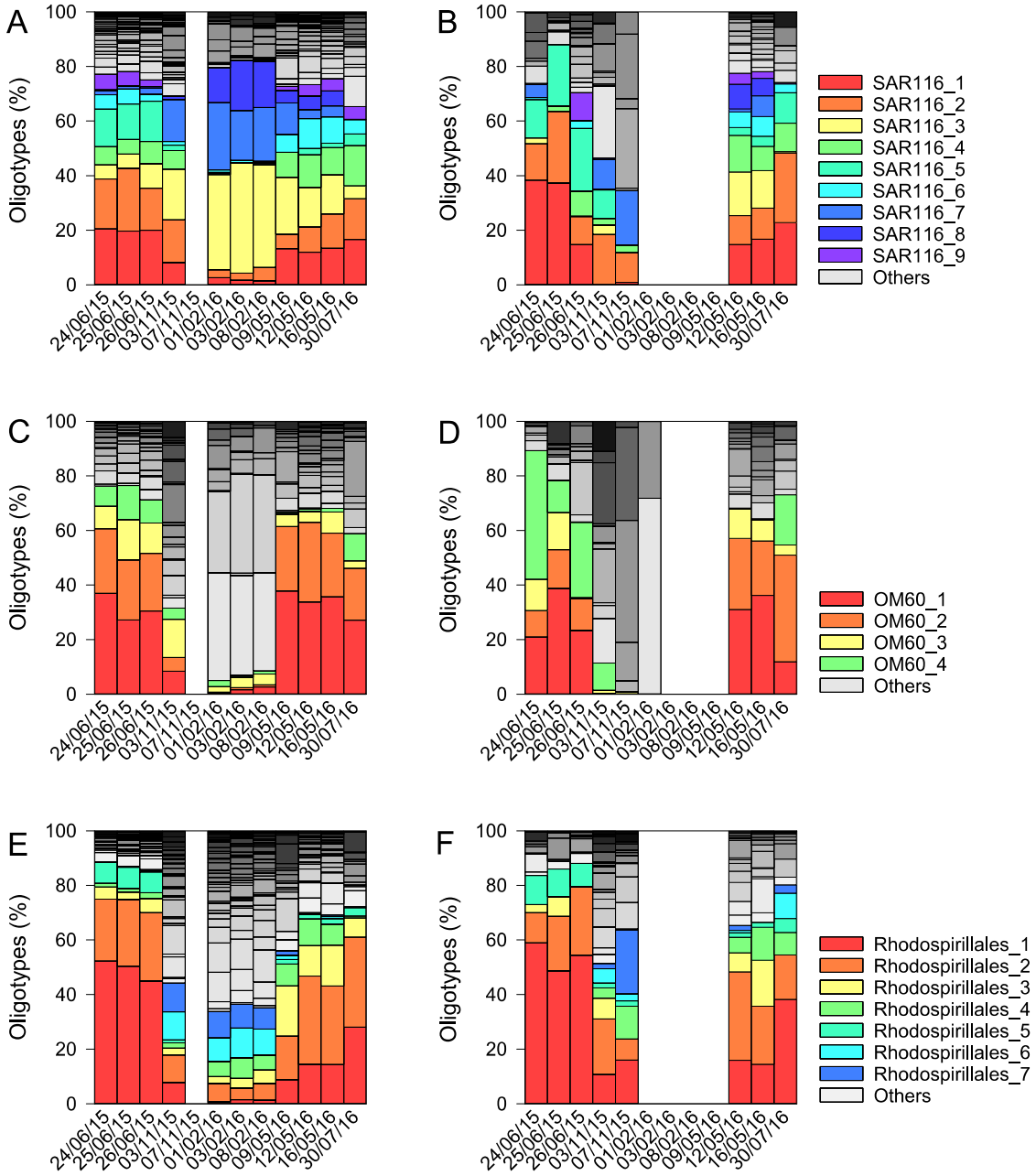

Figure 5. Distribution of oligotypes expressed as % of the corresponding group for each sampling day: SAR116 oligotypes in AW (A); SAR116 oligotypes in MS (B); OM60 oligotypes in AW (C); OM60 oligotypes in MS (D); Rhodospirillales oligotypes in AW (E); Rhodospirillales oligotypes in MS (F). All

oligotypes with a total abundance larger than 1000 reads over all sampling days are displayed in color and numbered, oligotypes with less than a 1000 reads are depicted in grey and summed up as “Others”.

The existence of two competing DMSP degradation pathways leading to contrasting ecologically and climatically relevant compounds has raised the question on the mechanisms controlling the ‘bacterial switch’ to one or the other pathway (Cantoni & Anderson 1956, Kiene et al 2000, Moran et al 2012). Sulfur demand and DMSP availability have been suggested to control DMSP demethylation, while sulfur saturation and carbon demand have been suggested to control the cleavage pathway (Kiene et al 2000, Simó 2001). UV radiation has also been hypothesized to affect the ‘bacterial switch’ and the fate of sulfur (Levine et al 2012, Vallina & Simó 2007) as DMS acts as a radical scavenger (Brugger et al 1998, Levine et al 2012, Sunda et al 2002). However, it was also suggested that even at high DMSP concentrations demethylating and DMSP-cleaving bacteria compete (Levine et al 2012), contrasting the scenario formulated in the sulfur demand hypothesis (Kiene et al 2000). In this study we analyzed DMSP demethylating bacteria and environmental factors potentially shaping the demethylating community. Our results suggest that MS might be a hotspot of competition between demethylating and DMSP cleaving bacteria due to high DMSPt + DMS concentrations and high abundance of demethylating bacteria. However, further work is needed to determine the potential for DMSP cleavage and the abundance of demethylation and cleavage gene transcripts to test this assumption. We confirmed the seasonality of the demethylating community by analyzing the demethylating bacterial community beyond the commonly used subclade level and found a high diversity of closely related oligotypes with distribution patterns that tentatively indicate a wide range of niches. However, we did not see differences of lifestyles (particle-attached *vs*. free-living) at the oligotype level, but rather at the coarser subclade level. A broad potential for DMSP demethylation is of particular importance in highly dynamic systems such as coastal areas. The confirmation of the existence of low and high affinity ecotypes, however, requires further experimental approaches. We suggest that seasonality as well as DMSP availability are the main factors shaping the demethylating bacterial community in both MS and the AW in the coastal Mediterranean Sea.

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Supporting information

Experimental procedures

Sampling

Marine snow (MS) and ambient water (AW) were collected about 1 km off the coast of Rovinj, Croatia (45.08347°N, 13.60518°E) (Fig. S5) at 15 ± 2 m depth in June 2015 (8 days), November 2015 (10 days), February 2016 (8 days), May 2016 (7 days) and July 2016 (8 days), thus covering a full seasonal cycle (summer, fall, winter, spring, summer respectively). Each sampling campaign consisted of seven to ten consecutive sampling days (Table S1). MS was collected with 100 mL syringes (0.1M HCl-rinsed prior to use) at 15 ± 2 m depth by SCUBA diving as described previously (Herndl & Peduzzi 1988, Rath et al 1998) (supplementary video). Although MS particles were selectively collected, a small amount of AW was collected along with the MS. Therefore in this study the term 'marine snow' includes the highly porous MS particle and the water in close proximity to it. Separating MS and AW without loosing the pore-water, which includes biological and chemical compounds of the MS, is difficult as there are no clear biological and chemical boundaries. The bacterial community is not strictly separated into free-living and particle-attached bacteria, but rather into a gradient of particle association and sporadic attach- and detachment of bacteria (Gibiansky et al 2010, Salazar et al 2015, Son et al 2015), which are included in MS as sampled via syringes. Due to its fragile and sticky nature, MS changes in size after collection, making a volume determination and quantification difficult. However, due to the narrow opening of the syringe (~3mm diameter) MS was sampled with high efficiency. For each sampling day, the MS collected in the different syringes was pooled into a pre-rinsed glass bottle and stored in the dark at in situ temperature until further processing (~30 min) in the lab. The pooled MS sample is considered representative for the quantity and quality of the MS at the day of sampling, as exact size and volume determination was not possible. However, various concentrations determined in MS therefore represent conservative values and were compared with that on the AW. All measurements on MS were done on subsamples of the pooled MS. Care was taken to homogenize the MS particles by carefully mixing and slowly inverting the glass bottle containing the pooled MS before collecting subsamples. Due to the heterogeneous nature of MS particles, however, the standard deviations of measurements on MS are generally higher than those of

the AW. AW was collected with 5 L Niskin bottles at the same depth as MS and filled into 0.1M HCl-rinsed 2L polycarbonate bottles. The collected AW includes a small proportion of MS particles, however large and fast sinking particles were naturally omitted as they sink into the space below the spout of the niskin bottle (Suter et al 2017). The samples were transferred to the laboratory of the Center for Marine Research at Rovinj (Ruder Bošković Institute) within 30 min for further processing as detailed below.

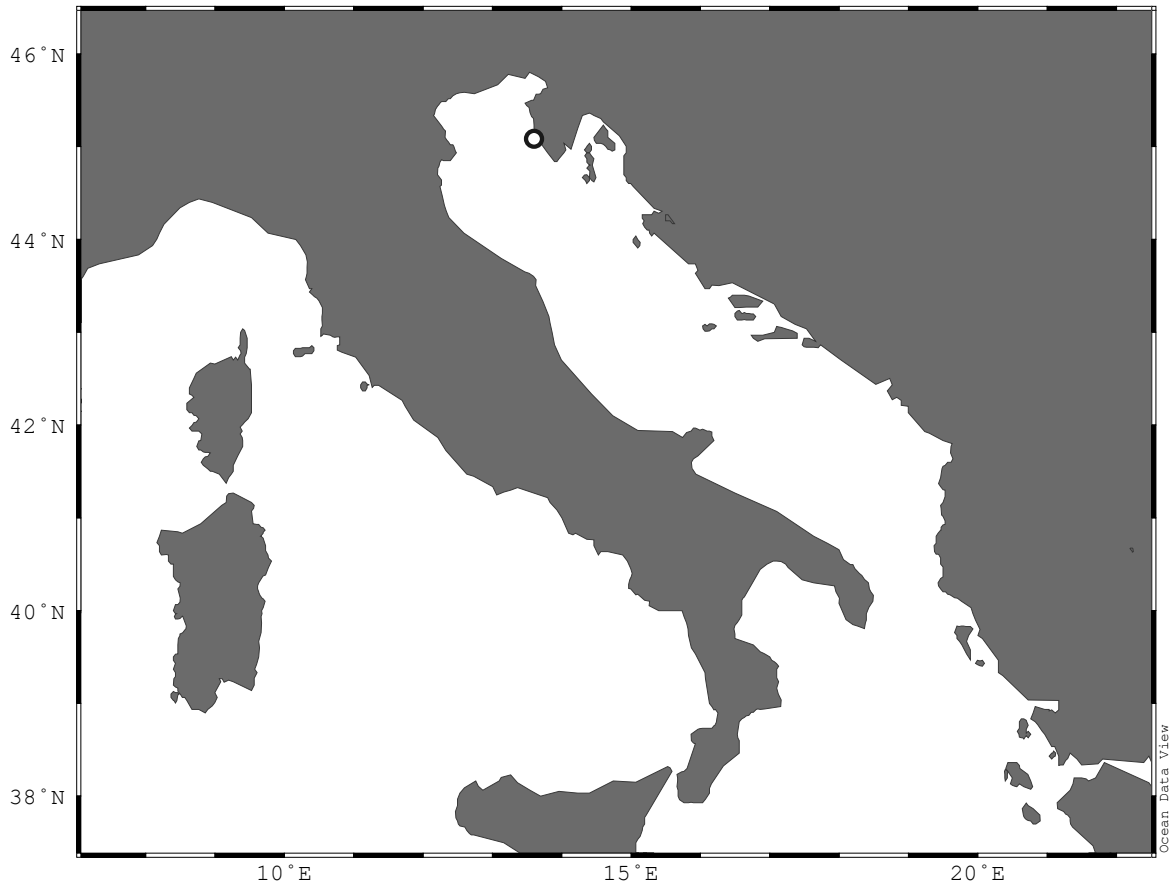

Figure S5. Map of sampling area in the northern Adriatic Sea. The diamond marks the location of station RV001 where MS, AW and phytoplankton samples were collected seasonally.

Inorganic nutrient analyses

Inorganic nutrient analyses of NO_3^-, NO_2^-, Si, NH_4^+, PO_4^{3-} and DOP were performed only on AW samples. Seawater samples were filtered through pre-combusted Whatman GF/F filters and stored in polyethylene bottles at -20°C. Analyses were performed within one month following standard protocols (Ivančič & Degobbis 1984, Strickland & Parsons 1972).

Phytoplankton abundance and community composition

To determine phytoplankton community composition 200 mL samples were collected at 0m, 5m and 10m depth (except in November 0 and 5m only) on 15 Jun 2015, 15 Nov 2015, 20 Feb 2016, 06 May 2016 and 19 Jul 2016. Phytoplankton samples were fixed with neutralised formaldehyde (2% final concentration). Phytoplankton were counted in 50 mL subsamples after allowing the cells to settle for 40 h (Hasle 1978) using an Axiovert 200 inverted microscope (Zeiss GmbH, Oberkochen, Germany) and following the Utermöhl (1958) method. Phytoplankton community composition was determined using standard identification keys in the laboratory of the Center for Marine Research (Ruder Bošković Institute). The measured phytoplankton abundance was integrated over the upper 10m water column using the trapezoidal approach.

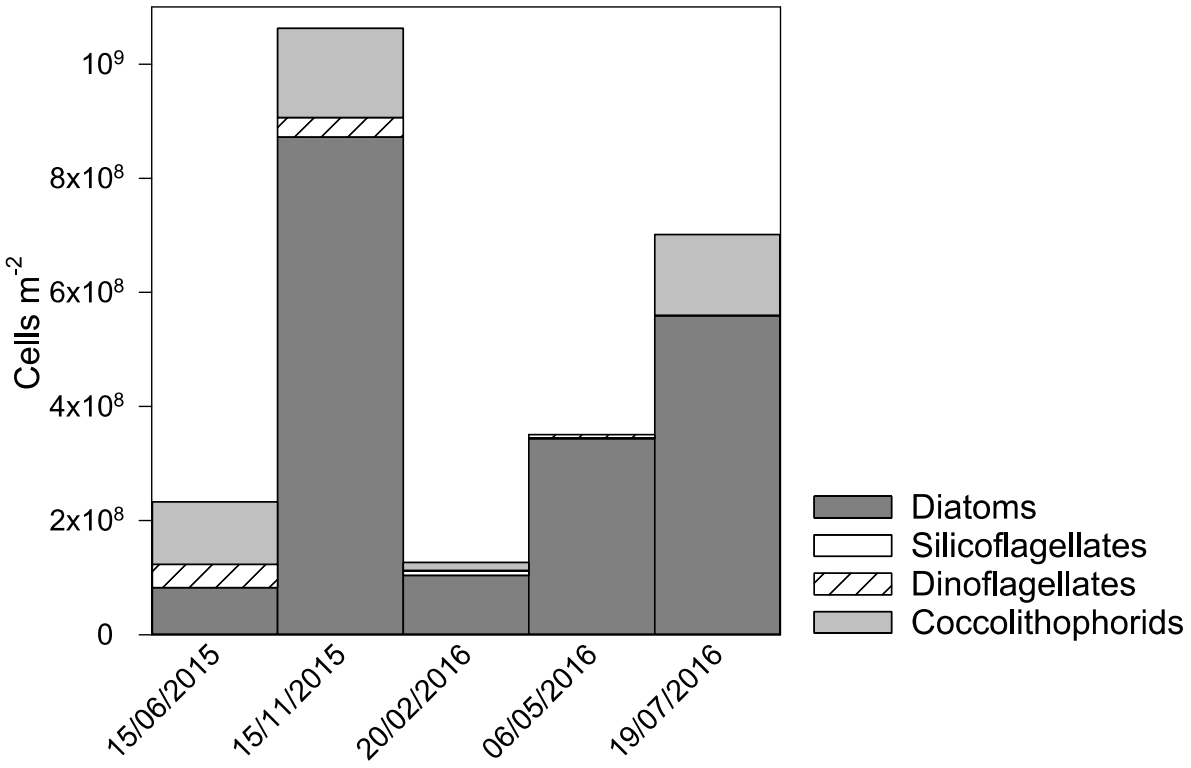

Figure S1. Depth-integrated abundance of phytoplankton groups over the upper 10m water column.

Prokaryotic abundance

Ten mL of MS and AW were fixed with 0.2 µm-filtered formaldehyde (final concentration 2%) and stored overnight at 4°C. Subsequently, the samples were filtered at low pressure (< 0.2 bar) onto 0.2 µm GTTP polycarbonate filters (Millipore, filter diameter 25 mm) supported by 0.45 µm HAWP (Millipore) filters. The filters

were air-dried and stored in 2 mL cryovials (Biozym) at -80°C until analysis in the home lab. The filters were stained with DAPI (4', 6-diamidino-2-phenylindole, 2 µg mL^{-1} final concentration) for 10 min and observed under an epifluorescence microscope (Axio Imager M2, Carl Zeiss, 1,250x magnification). DAPI-stained cells were counted in 20 randomly selected fields of view per sample with > 200 cell counts per sample. The field of view was 1.72 x 10^3 μm^2.

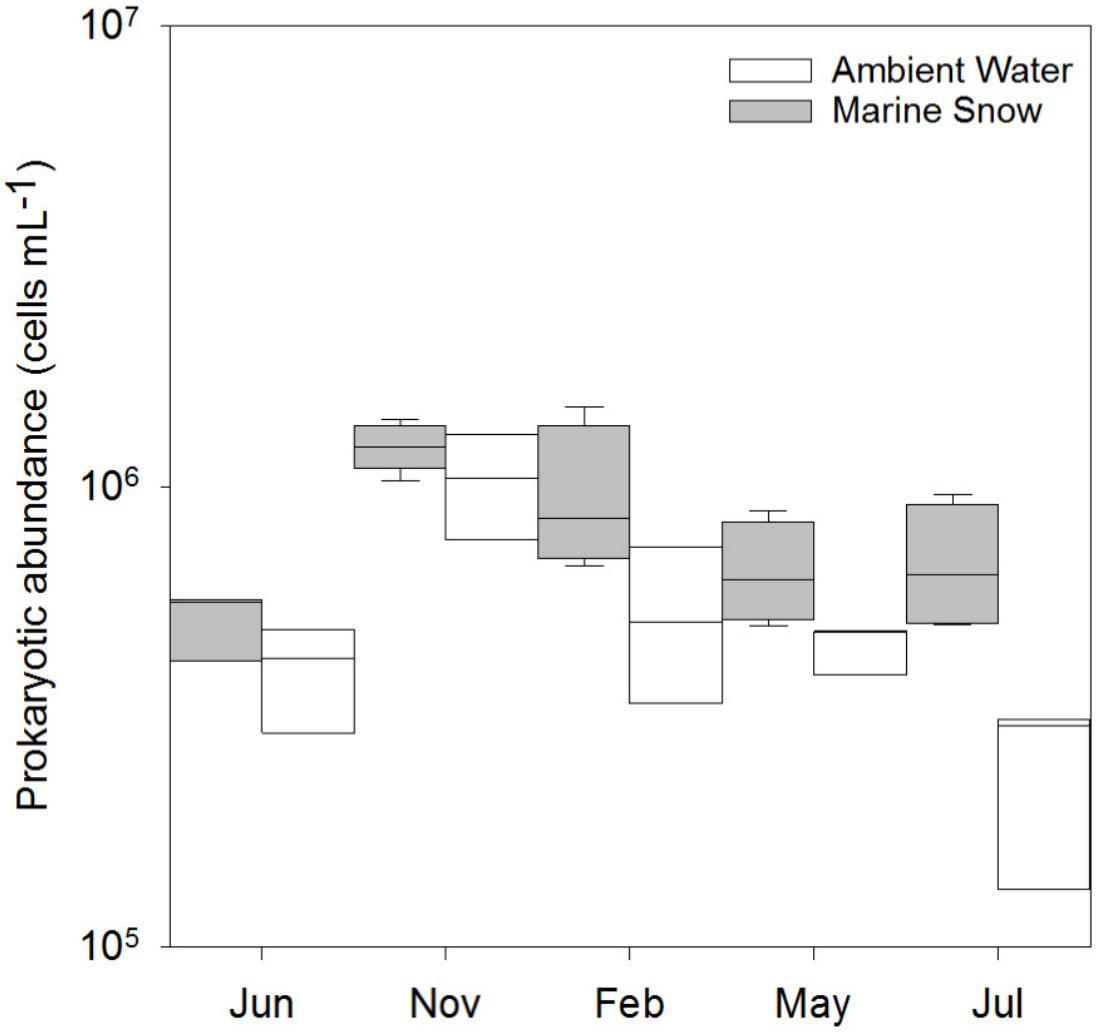

Figure S4. Box-plot of prokaryotic abundance (median, 10th, 25th, 75th and 90th percentiles) in marine snow (grey) and ambient water (white).

Leucine incorporation into prokaryotic biomass

Leucine incorporation as a proxy for heterotrophic biomass production of prokaryotes was measured using 1.5 mL of MS or AW sample following the protocol of Smith and Azam (1992). Briefly, four replicates and two trichloroacetic acid (TCA)-

killed blanks were amended with 40 nmol L^{-1} (final concentration) of [^{3}H]-leucine (specific activity 120 Ci $mmol^{-1}$, BioTrend Chemicals) and incubated at in situ temperature in the dark for 2-4 h. Incubations were terminated by adding TCA (5% final concentration). Subsequently, the samples were centrifuged at 20,000x *g* for 10 min. The supernatant was discarded, while the resulting pellet was rinsed with TCA (5%), centrifuged (20,000x *g*, 10 min) and 1mL of Ultima-GOLD (Canberra-Packard) scintillation cocktail added to the pellet. After 18 h, the samples were measured in a liquid scintillation counter (Canberra Packard TriCarb 2900 TR, Perkin Elmer Packard, USA). The samples were corrected for quenching and the mean of the disintegrations per minute (DPM) of the TCA-killed blank was subtracted from the mean of the live samples. The resulting DPM were converted into leucine incorporation rates.

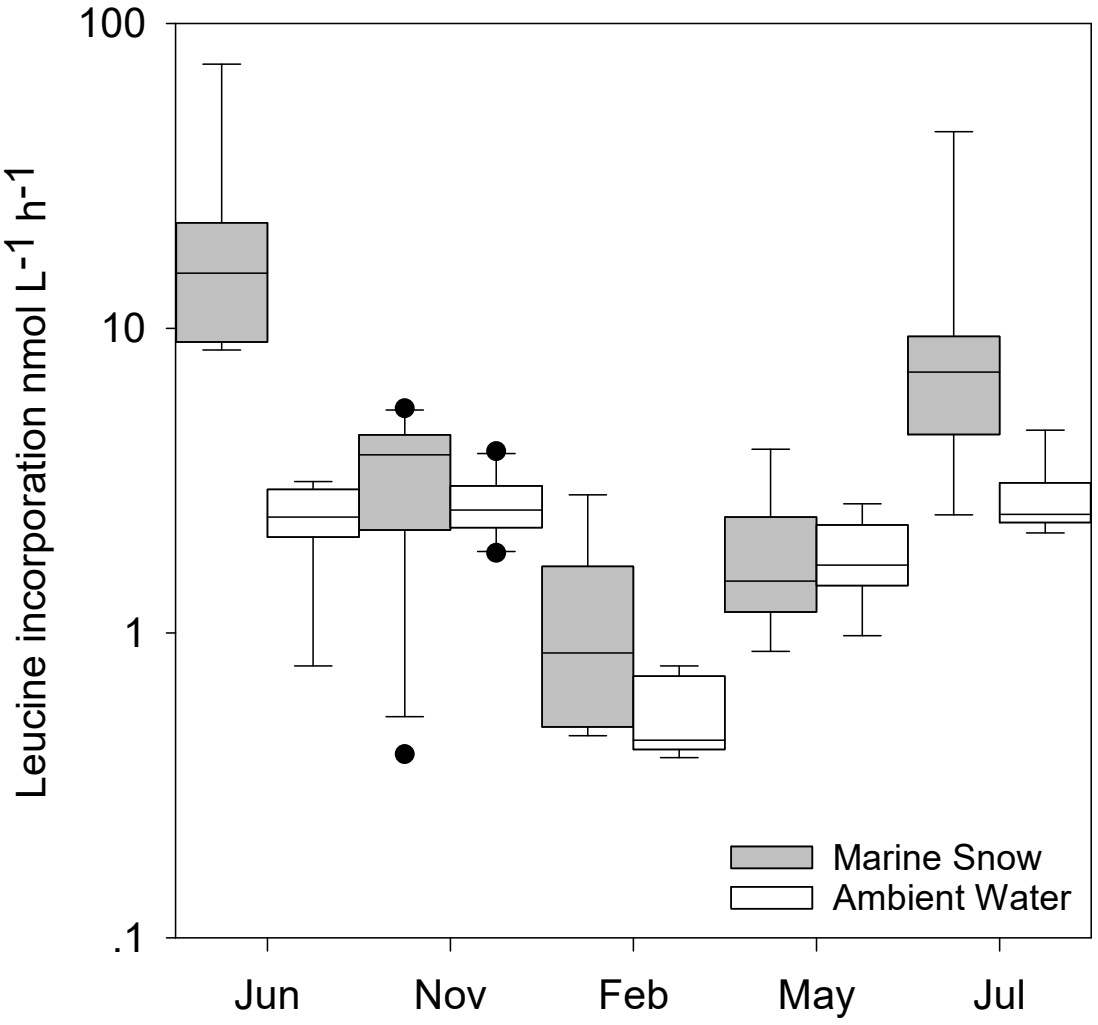

Figure S3. Box-plot of leucine incorporation into heterotrophic microbes (median, 10th, 25th, 75th and 90th percentiles) in marine snow (grey) and ambient water (white).

DMSP

MS and AW were carefully filled into 10 mL crimp-top glass vials leaving no headspace. Duplicate samples for total DMSPt + DMS measurements (particulate DMSP + dissolved DMSP + DMS) were fixed by adding one NaOH pellet. Samples were stored at room temperature in the dark until analysis at the Institute of Marine Sciences (CSIC, Barcelona, Spain). DMSPt + DMS concentrations were measured using a purge-and-trap method and sulfur-specific gas chromatography as described elsewhere (Galí et al 2011).

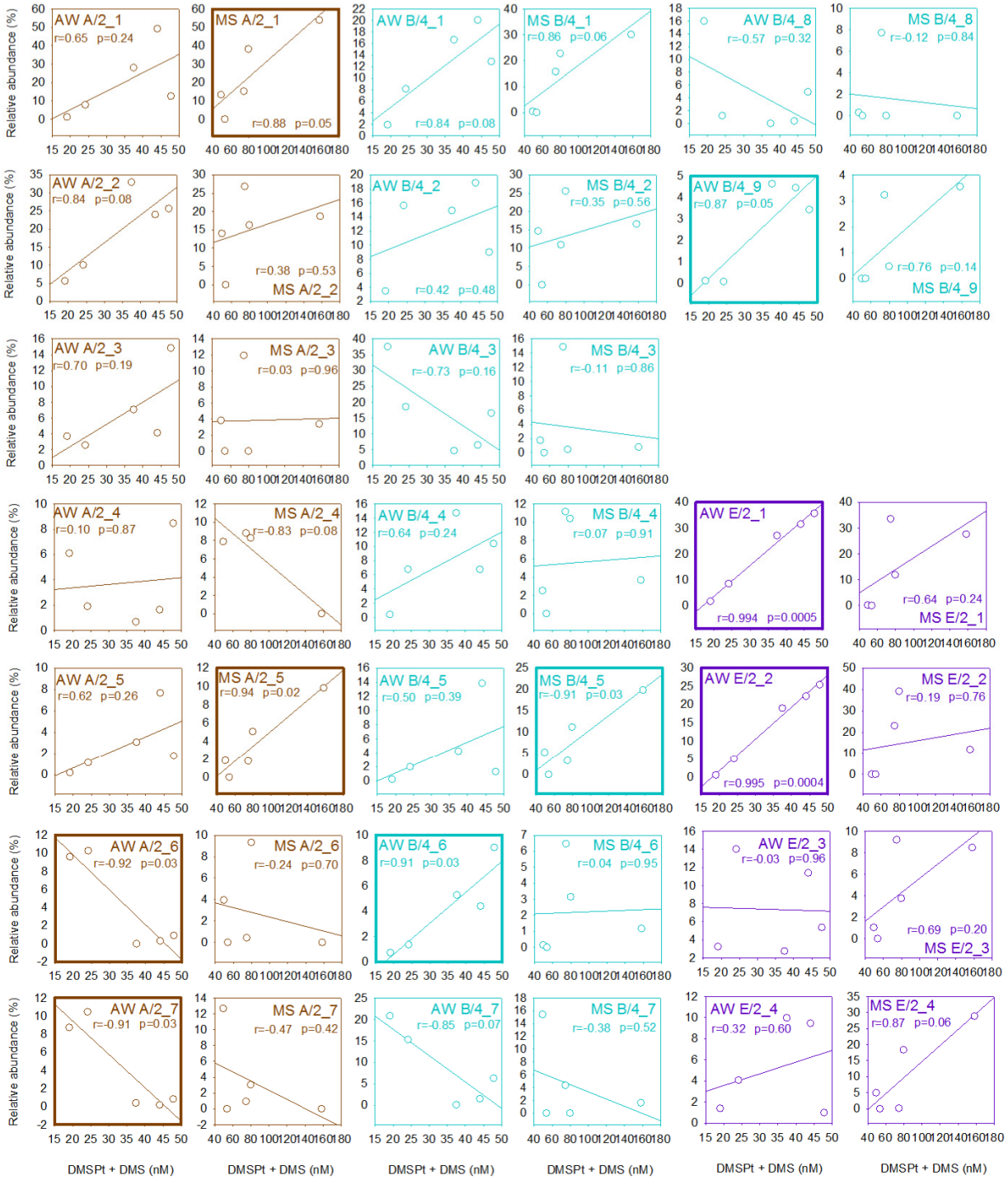

Figure S2. Correlation plots of the most abundant oligotypes of phylogenetic groups representative of *dmd*A harboring bacteria and DMSPt concentrations. Correlation plots of Rhodospirillales oligotypes (A/2) are shown in brown, SAR11 oligotypes (subclade B/4) in turquois and OM60 oligotypes (subclade E/2) are shown in purple. Correlation coefficient (r) and p values are indicated, correlation plots with p values ≤ 0.05 are in bold.

DNA extraction

One liter of AW and 150 to 500 mL of MS were filtered onto 0.2 µm polyethersulfone filters (47 mm diameter, Supor, PALL Gelman) using an aspirator pump (Cole-Parmer) immediately after sampling. The filters were stored in cryovials (Biozym), flash-frozen in liquid nitrogen and subsequently stored at -80°C until DNA extraction in the home lab. DNA was extracted using the standard phenol-chloroform method with slight modifications as described below.

Briefly, the filters were thawed and cut with sterile scissors into small pieces. After adding 2 mL of lysis buffer (0.2 mL Tris 100 mM pH8, 0.2 mL EDTA 250 mM pH8, 0.04 mL NaCl 5M, 1.56 mL RNAse /DNAse-free water from Sigma-Aldrich) and 8.75 U/mL final concentration of Lysozyme Ready-Lyse (Epicenter), the filters were incubated at 37°C for 45 min. Subsequently, sodium dodecyl sulfate (1% final concentration) and 8 U/mL of proteinase K (from *Tritirachium album*, Sigma-Aldrich) were added and the samples incubated at 55°C for 1 h. Combusted (450°C) zirconium beads (0.1 mm diameter Zirconia/Silica, BioSpec Products) were added to the samples and the tubes were vortexed at maximum speed for 10 min. Samples were heated up to 70°C for 30 min and subsequently, the lysate was aspirated and split into two 2 mL microcentrifuge tubes. An equal volume of water-saturated phenol pH 8 (Sigma-Aldrich) was added to the samples, mixed and centrifuged for 10 min. All centrifugation steps were done at 21,000x *g* at 4°C. The aqueous layer was recovered and an equal volume of PCI (phenol-chloroform-isoamylalcohol 25:24:1, Sigma-Aldrich) was added, mixed and centrifuged for 10 min. The aqueous layer was aspirated, mixed with an equal amount of ice-cold chloroform (Sigma-Aldrich) and centrifuged for 10 min. Subsequently, DNA from the aqueous layer was precipitated by adding 10 µL of 5 M NaCl and 2 volumes of 100% ethanol (Merk) at -20°C overnight, followed by centrifugation for 25 min. The pellet was washed with 70% ethanol and centrifuged for 20 min. Thereafter, the pellet was dried in a Concentrator plus (Eppendorf) at 45°C for 30 min. The split pellets from each sample were re-suspended in 50 µL of RNAse/DNAse-free water, pooled and stored at -80°C until analysis.

Preparation of qPCR standards

Analysis of the demethylating bacterial community was done using the primer sets described by Varaljay et al (2010) (Table S2) targeting different clades (A to E) of the *dmd*A gene-harboring bacteria. Clade A is represented by *Roseobacter* and

Rhodospirillales species, clade B by the SAR116 group member "*Candidatus* Puniceispirillum marinum". Clade C is represented by the SAR11 strain *Pelagibacter ubique* HTCC7211, and clade D by the SAR11 strains *Pelagibacter ubique* HTCC1002, HTCC1062 and another homolog to HTCC7211. Clade E includes sequences from the marine Gammaproteobacterium clade OM60/NOR5 clade (thereafter referred to as OM60 clade). *Dmd*A genes of all described subclades were PCR amplified in a Master cycler (Eppendorf) via gradient PCR with the following settings: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, annealing at a gradient from 40°C to 60°C for 30 s, extension at 68°C for 30 s, followed by a final extension step at 68°C for 10 min and cooling at 4°C. Only those subclades with positive PCR were further used for qPCR analysis. PCR of the *rec*A gene was carried out with the following settings: 4 min initial denaturation at 94°C, 30 cycles at 94°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 45 s, followed by a final extension at 72°C for 10 min and cooling at 4°C. Each 25 µL reaction of the Master-mix contained 1.25 U/µL picomaxx high fidelity polymerase and 2.5 µL of 10 x picomaxx buffer (Agilent Technologies), 2.5 µL dNTPs (dNTP mix containing 2 mM of each dNTP resulting in 0.2 mM final concentration), 0.5 µL of 20 mg mL^{-1} BSA, 0.5 µL of 25 mM $MgCl_2$ (Thermo Scientific) and 0.5 µM of each primer. The PCR products were purified with the PCRExtract MiniKit (5-PRIME) and the DNA concentration was measured with a Nanodrop® spectrophotometer. Standards for the *dmd*A genes A/2-sp, B/3-sp, B/4-sp, D/3-sp and E/2-sp and the *rec*A gene (Holmes et al 2004) (Table S2) were prepared from the purified PCR products. The gene abundance was calculated from the concentration of the purified DNA and the size of the fragment. Serial dilutions were prepared for each gene with TRIS buffer (10 mM, pH 8.0) from 10^7 to 10^0 gene copies per mL and added as a standard in triplicate to each qPCR run.

qPCR analysis

The samples were loaded in triplicate and duplicate (*rec*A and *dmd*A, respectively) to a 96-well qPCR plate (Bio-Rad), closed with optical tape (Bio-Rad) and run on a Light Cycler 480 (Roche). The AW samples were diluted 1:10 and the MS samples to 1:100 with RNAse/DNAse-free water. The reaction mixture for each sample contained 1x Mastermix (LightCycler 480 SYBR Green I Master, Roche), 0.5 µM of each primer, 1 µL of diluted sample and ultrapure sterile water (Roche) made up to

10 µL. Thermocycling for *rec*A and *dmd*A genes was initiated by a denaturation step at 95°C for 10 min and followed by 50 cycles consisting of a denaturation step at 95°C for 5 s; annealing at specific temperatures for each gene and subclades (Table S2) for 5 s and for 10 s for *rec*A and *dmd*A subclades, respectively, extension at 72°C for 15 s, and a plate read at 74°C for 3 s.

Quantitative PCR analysis was performed on all samples for *rec*A and on at least three samples from each sampling campaign for the *dmd*A subclades. The total *dmd*A gene abundance was calculated as the sum of all *dmd*A gene subclades. The sub-clade specificity of the primers was assessed *in silico* and experimentally by Varaljay et al (2010). The total *dmd*A gene abundance and the abundance of each subclade are presented as their respective ratio to *rec*A gene abundance. Normalization to *rec*A was used as a proxy of bacterial abundance and to account for possible variations in DNA extraction efficiency between different samples. The housekeeping gene *rec*A is commonly used for this purpose in qPCR studies, as it is a single copy gene present in all bacteria (Miller & Kokjohn 1990).

Bacterial community composition assessed by next generation sequencing

The 16S rRNA genes of the bacterial community inhabiting MS and the AW were PCR-amplified from samples collected at the beginning, middle and end of each sampling period using a Mastercycler (Eppendorf) (in total 30 samples). One negative control (RNAse/DNAse-free water) was also PCR amplified. Thermocycling consisted of a 1st-step PCR with the primers 341_ill forward and 802_ill reverse containing adaptors (Table S2) and a KAPAHiFi Mastermix (Peqlab) with the following program: initial denaturation at 94°C for 3 min, followed by 20 cycles of 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 90 s; a final extension step was done at 72°C for 7 min followed by cooling at 4°C. PCR products were purified with Agencourt AMPure XP magnetic particles (Beckman Coulter) and quantified with a Quant-IT PicoGreen® Assay (Invitrogen). The Nextera 2nd-step PCR was performed under the same thermocycling conditions as described above for 10 additional cycles, followed by pooling and 2 x 250 v2 sequencing on an Illumina MiSeq system (Microsynth AG, Balgach, Switzerland).

Sequence data processing and analysis

The 16S rRNA sequences were analyzed using the program Mothur (Schloss et al 2009) against the Silva v128 reference database with a cut-off value of 80. Operational taxonomic units (OTUs) were defined as sequences with 97% similarity. OTUs sequences identified in the negative control were subtracted from the OTUs. Also, singletons were removed. Samples with less than 1000 reads or with less than 80% of the reads remaining in each sample after quality control were removed from the dataset. This procedure resulted in the exclusion of 9 out of the 30 samples from further analysis (Table S3). The community composition is given as percentage of the total community in each sample to allow comparison between different samples.
Oligotyping (Eren et al 2013) was performed following the “best practices” pipeline of A. Murat Eren (http://merenlab.org/2013/11/04/oligotyping-best-practices/). The reads of interest were extracted using the “mothur2oligo” script (courtesy of Michelle Berry) after running the Mothur pipeline (MiSeq SOP) until the taxonomy assignment step, and skipping the “pre-clustering” step to avoid the removal of unique sequences. Oligotyping was carried out at the taxonomic level of the order. Shannon entropy level of 0.2 was used to recover true and relevant ecological oligotypes and omit random sequencing errors. The minimum substantive abundance of an oligotype (-M parameter) was set to 50. This method revealed a large set of oligotypes for each OTU. However, further analysis was done only on those oligotypes with an overall read abundance of more than 1000. We focused on the representative bacterial groups of those *dmd*A subclades that appeared throughout the seasonal cycle or in both sample types (MS and AW). We linked the relative abundances of the *dmd*A subclades determined by qPCR to the relative abundances of oligotypes of the bacterial community and to DMSPt + DMS concentrations. This approach was used to provide insights into fine scale differences of highly similar bacterial groups and their ecological relevance as ecotypes.

Statistical analysis

All statistical analyses were conducted with the software Past 3.15 (Hammer et al 2001) and SigmaPlot Version 13. A t-test was used if a normal distribution was verified. Otherwise, a Kruskal-Wallis test was used to compare MS versus AW samples and Mann-Whitney pairwise post-hoc test to compare sampling months within MS or AW samples. Differences were considered statistically significant if $p < 0.05$. All values are expressed as mean ± SD if not stated otherwise. The enrichment fac-

tor (EF) is presented as monthly average ratio of the respective value in MS to that in the AW. A canonical correspondence analysis (CCA) was used to analyze the temporal distribution patterns of the bacterial community in response to environmental parameters. The integrated abundance of phytoplankton groups, DMSPt + DMS concentrations, *dmd*A subclades and oligotypes were used as environmental variables. The *dmd*A subclades and the different oligotype abundances are expressed as percentage of their respective total. The MS samples exhibited an extremely high relative abundance of *Synechococcus* in February (70%). Due to this high abundance and its un-relatedness to demethylating bacteria, *Synechococcus* was excluded from the CCA analysis in the MS and AW dataset.

Supplementary results

Environmental parameters

Marine snow (MS) was present in all sampling months ranging from 0.5 to 5 mm in diameter as estimated by *in situ* visual examination. On average, 71 ± 9 MS particles were collected in each of the 100-mL disposable syringes.

The averaged abundance of prokaryotes was higher in MS ($8.45 \times 10^5 \pm 3.29 \times 10^5$ cells mL^{-1}) than in AW ($5.42 \times 10^5 \pm 2.87 \times 10^5$ cells mL^{-1}) with enrichment factors ranging from 1 to 4 (Fig. S5, Table S1). Leucine incorporation in MS showed the same trend as in the AW, however MS (7.75 ± 13.25 nmol Leu L^{-1} h^{-1} averaged over all samples) exhibited significantly higher leucine incorporation than AW (2.04 ± 1.02 nmol Leu L^{-1} h^{-1} averaged over all samples). The EF of leucine incorporation ranged from 1 to 7 (Fig. S3, Table S1). Cell-specific leucine incorporation was higher in MS than in AW in June (28.65 ± 6.54 amol Leu $cell^{-1}$ h^{-1}, EF=5) and July (19.63 ± 18.30 amol Leu $cell^{-1}$ h^{-1}, EF=2). In contrast, from November to May cell-specific leucine incorporation was similar in MS and AW (data not shown).

*rec*A *gene abundance and dmd*A *subclades determined by qPCR*

The *rec*A gene abundance in MS was not significantly different from that in AW. The EF ranged from 0.2 in February to 3 in June. Bacterial abundance as determined by *rec*A gene abundance in MS and AW was higher in June than in all other months (Table S1).

Bacterial community composition

The number of reads after quality control and removal of singletons ranged from 3,056 to 64,244 per sample, and the sequencing coverage ranged from 0.98 to 1 (Table S3). Bacterial community diversity indices were similar for MS and AW. The highest diversity (Simpson and Shannon diversity index) for both AW and MS was found in November and the lowest in February (Table S4). Of the 20 most abundant OTUs in the AW and MS, *Synechococcus* and Flavobacteria mainly of the genus marine group NS4 dominated the bacterial community (Fig. 3A, B). Besides *Synechococcus* and Flavobacteria, the representative bacterial groups of the *dmd*A subclades were highly abundant in the AW, particularly the SAR11 clade and Rhodospirillales. The SAR11 clade was particularly abundant in the AW in November and February with *Candidatus* Pelagibacter being the most abundant OTU. Bacteria of the order Rhodospirillales were most abundant in June. The SAR116 clade was most abundant in June, May and July in the AW (Fig. 3A). Within the 20 most abundant OTUs in MS, Vibrionales and Planctomycetes were highly abundant. The SAR11 clade was also abundant in MS particularly in November (Fig. 3B).

Tables

Table S2. Primer pairs used for amplification of *rec*A, 16S rRNA gene and five specific *dmd*A subclades. Annealing temperatures for PCR and qPCR, and efficiency of the qPCR are indicated.

Gene/subclade	Position	Amplicon length (bp)	Primer sequence 5' to 3'	Annealing temp (°C) PCR	Annealing temp (°C) qPCR	Efficiency (%)	Reference
*rec*A		212	RECAF: TGTGCITTTATWGATGCIGAGC ATGC RECAR: CCCATGTCICCTTCKATTTCIGC TTT	53	53	91 - 93.5	Holmes et al. 2004
A/2-sp	339 - 486	147	A/2-spFP: CGATGAACATTGGTGGGTTTC TA A/2-spRP: GCCATTAGGTCGTCTGATTTT GG	59 - 62	62	93.5	Varaljay et al. 2010
B/3-sp	169 - 323	154	B/3-spFP: GATGTCTCCTGCCAACGTCAG GTCGA B/3-spRP: ACCGGGTCATTGATCATGCCT GCG	62	62	88.5	Varaljay et al. 2010
B/4-sp	361 - 553	192	B/4-spFP: ATTGCCGACTCGGATGTTCT B/4-spRP: CAAGAAGGTCAAACATGGCA AAC	58 - 62	62	93	Varaljay et al. 2010
D/3-sp	347 - 473	126	D/3-spFP: AATGGTGGATTTCTATTGCAG ATAC D/3-spRP: GATTTTGGACCTTGTACAGCC A	54	54	97.5	Varaljay et al. 2010
E/2-sp	154 - 287	133	E/2-spFP: CATGTTCAGATCTGGGACGT E/2-spRP: AGCGGCACATACATGCACT	57 - 62	62	94	Varaljay et al. 2010
16S rDNA	V34	460	341ill NGS forward: TCGTCGGCAGATGTGTATAAG AGACAGCCTACGGGNGGCWG CAG 802 ill NGS reverse: GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAGGACTACH VGGGTATCTAATCC	56			Illumina

Table S3. Characteristics of the NGS dataset. Singletons and reads that appeared in the negative control were subtracted from the presented values. Percent of reads indicates the percentage of reads remaining after quality filtering as compared to the original reads in each sample. The asterisk indicates that the sample was removed from the analysis due to low number or percentage of reads after quality filtering, as indicated in the Methods section.

Sample	Coverage	Nr of reads	Percent of reads
AW_24/6/2015	1.00	42892	98.7
AW_25/6/2015	1.00	43523	99.0
AW_26/6/2015	1.00	29405	98.5
AW_3/11/2015	1.00	43517	98.9
AW_7/11/2015*	0.99	1874	71.1
AW_11/11/2015*	0.83	78	44.1
AW_1/2/2016	1.00	50794	99.1
AW_3/2/2016	1.00	45193	98.9
AW_8/2/2016	1.00	52662	99.0
AW_9/5/2016	0.98	3056	90.6
AW_12/5/2016	1.00	32792	98.7
AW_16/5/2016	1.00	64244	99.1
AW_27/07/2016*	0.97	371	19.4
AW_28/07/2016*	0.98	509	30.7
AW_30/7/2016	1.00	17873	92.5
MS_24/6/2015	1.00	41855	94.8
MS_25/6/2015	1.00	41875	96.0
MS_26/6/2015	1.00	22969	92.8
MS_3/11/2015	1.00	21624	91.0
MS_7/11/2015	1.00	8734	84.3
MS_11/11/2015*	0.86	160	55.6
MS_1/2/2016	1.00	8235	84.8
MS_3/2/2016	1.00	9303	85.8
MS_8/2/2016*	1.00	5012	69.1
MS_9/5/2016*	1.00	1543	46.4
MS_12/5/2016	1.00	33053	95.1
MS_16/5/2016	1.00	39617	95.4
MS_27/07/2016*	0.99	648	38.5
MS_28/07/2016*	0.99	849	32.3
MS_30/7/2016	1.00	42422	94.2

Table S4. Diversity indices of the NGS dataset.

Sample	Simpson index	Shannon index	Evenness
AW_24/6/2015	0.9	3.4	0.1
AW_25/6/2015	0.9	3.2	0.1
AW_26/6/2015	0.9	3.4	0.1
AW_3/11/2015	1.0	4.0	0.2
AW_7/11/2015	-	-	-
AW_11/11/2015	-	-	-
AW_1/2/2016	0.9	3.1	0.1
AW_3/2/2016	0.9	3.1	0.1
AW_8/2/2016	0.9	3.8	0.1
AW_9/5/2016	-	-	-
AW_12/5/2016	0.9	3.1	0.1
AW_16/5/2016	0.9	3.3	0.1
AW_27/07/2016	-	-	-
AW_28/07/2016	-	-	-
AW_30/7/2016	0.9	3.6	0.2
MS_24/6/2015	0.9	3.3	0.2
MS_25/6/2015	0.9	3.8	0.2
MS_26/6/2015	0.9	3.3	0.2
MS_3/11/2015	1.0	4.0	0.4
MS_7/11/2015	1.0	4.2	0.5
MS_11/11/2015	-	-	-
MS_1/2/2016	0.6	1.8	0.1
MS_3/2/2016	0.5	1.6	0.1
MS_8/2/2016	0.8	2.6	0.3
MS_9/5/2016	-	-	-
MS_12/5/2016	0.9	3.7	0.2
MS_16/5/2016	1.0	3.7	0.2
MS_27/07/2016	-	-	-
MS_28/07/2016	-	-	-
MS_30/7/2016	0.9	3.6	0.3

Supplementary table (excel file) and video

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General Discussion

Marine snow plays an essential role in the global carbon cycle contributing to the carbon flow by sequestering atmospheric carbon to the deep ocean (Boyd et al 2019, Turner 2015) and sustaining deep ocean organisms (Herndl & Reinthaler 2013). The major fraction of the marine snow organic carbon is remineralized by prokaryotes at the surface and the mesopelagic realm of the ocean, and subsequently returns to the atmosphere as CO_2 (Herndl & Reinthaler 2013). The marine snow associated prokaryotes performing this process differ from the free-living community, and typically exhibit specialized metabolisms (Rath et al 1998, Simon et al 2014, Simon et al 2002). However, whether the distinction between the marine snow associated and the free-living communities persists when environmental characteristics change is currently under debate. Additionally, marine snow associated communities frequently exhibit higher activity rates than free-living organisms (Simon et al 2002). The messenger RNA can give valuable insights into the relationships of microorganisms with their environment, and potentially enables to quantify the processes that are occurring in the ecosystem as well as their ecological significance (Cottrell & Kirchman 2016, McCarren et al 2010). However, the applicability of this approach to environmental samples remains controversial. This thesis contributes to enhance the current understanding of the effect of seasonality on marine snow associated and free-living prokaryotes, and of prokaryotic activity profiling via messenger RNA.

Activity profiling via mRNA characterization

A comprehensive understanding of the ecosystem´s functions (e.g.: DMSP utilization) carried out by environmental communities requires to complementing the determination of genomic potential with actually realized pathways. This can be accomplished by determining the transcribed genes via transcriptomics (Güell et al 2011). Messenger RNA characterization is a straightforward approach that benefits from the recent advances in sequencing technologies and informatics, allowing high throughput analysis as compared to other often laborious techniques with low throughput, such as nanoSIMS, (micro)-CARD-FISH or proteomics (Musat et al 2012, Pernthaler & Amann 2004, Tyers & Mann 2003). The high variability of mRNA

half-life time ranging from minutes to hours, however, offers opportunities and limitations (Chapter I). In addition to the variability between species, the half-life time of mRNA also varies between genes and is affected by temperature and growth rate (chapter I) (Hambraeus et al 2003, Steglich et al 2010). Taken together, these findings suggest that the use of reverse transcription quantitative PCR and transcriptomics is useful for cultures under controlled laboratory conditions, however, has limitations due to interspecific variability in the half-life of mRNA.

Patterns of seasonality of free-living and marine snow associated prokaryotes

The importance of long-term studies in revealing recurring patterns is nowadays widely acknowledged (Fuhrman et al 2015). Noticeably, temporal dynamics of relevant members of the free-living prokaryotic community are observed at fine-scale phylogenetic resolution (Eren et al 2013, Vergin et al 2013, Ward et al 2017, Yung et al 2015) linked to environmental changes, such as solar radiation angle and nutrient availability caused by stratification, seasonal upwelling or weather patterns (Fuhrman et al 2015). However, the seasonal patterns in such environmental variables have been reported to induce little or no effect on prokaryotes associated to large particles (Yung et al 2016). Yet, under certain conditions, the free-living and marine snow associated communities are intimately connected and both influenced by environmental parameters (Vojvoda et al 2014). Our results indicate that these two scenarios are not mutually exclusive but alternate over the annual cycle, as described in Chapter II. Prokaryotic communities associated to marine snow and to the ambient water differ profoundly in peak seasons (winter and summer), whereas they exhibit a higher connectivity in transition seasons (spring and fall). Hence, the sensitivity of marine snow associated communities to environmental parameters is determined by the seasonally changing particle characteristics, maturity and environmental conditions.

Environmental condition changes, such as increased nutrient supply rates associated to water column mixing, increased solar radiation exposure and the subsequent phytoplankton blooms predominantly occur in spring and late summer (Aubry et al 2004, Bernardi Aubry et al 2006, Chafee et al 2018). This environmental set-up possibly fuels opportunistic copiotrophs in both, the marine snow and the ambient

water, resulting in increased similarity between the communities in both habitats in transition seasons (Chapter II). The similarity between the marine snow associated and the free-living communities is evident by the enrichment of the same prokaryotic taxa (e.g.: *Flavobacteriales* and *Rhodobacterales*) in both habitats during specific seasons (Chafee et al 2018, Chen et al 2020, Cohan 2016, Newton et al 2010).

In contrast, the distinct marine snow community compared to the ambient water community in peak seasons originates from the different chemical composition of the marine snow compared to the ambient water, including high nutrient concentrations or reduced oxygen concentrations in marine snow (Shanks & Reeder 1993, Stocker et al 2008, Swan et al 2011). These changes provide microenvironments and expand the available niches for microbes in marine snow (Bianchi et al. 2018). The chemical characteristics and formation of microenvironments within marine snow largely depend on the retention time in the water column and on the phytoplankton species that generated them (Kaltenböck & Herndl 1992, Wolf et al 2016). Summer environmental conditions favor the development of microenvironments in non-sinking, mature marine snow, resulting in a distinct marine snow associated community (Bianchi et al 2018, Mestre et al 2017, Yung et al 2016). The establishment of microenvironments, such as low oxygen concentrations, sustains alternative prokaryotic metabolisms (Shanks & Reeder 1993, Swan et al 2011). When oxygen is depleted, nitrate becomes the most favorable electron acceptor. Oxygen limitation allows a process known as denitrification, where nitrate is reduced to nitrite (via the dissimilatory nitrate reduction), which subsequently can be further reduced to nitrous oxide or dinitrogen gas, subsequently lost from the system (Kuypers et al 2018). Consequently, denitrification processes should be typically confined to oxygen minimum zones and anoxic waters, thus, representing a rather small part of the global ocean's metabolisms (Lam & Kuypers 2011). However, if the potential anoxic microenvironments within marine snow are considered, the estimated loss of nitrogen from the system is doubled compared to oxic conditions (Bianchi et al 2018, Bristow 2018). In accordance with the establishment of anoxic microzones within marine snow, dissimilatory nitrate reduction marker genes (nrfA, napA and napB) were exclusively found in summer marine snow in the Adriatic Sea (Chapter II).

Seasonality and life-styles of the demethylating bacterial community

The organosulfur compound dimethylsulfoniopropionate (DMSP) plays an important role in the ecology and biogeochemical cycles of marine ecosystems, playing physiological (e.g., as osmolyte, antioxidant or deterrent of predators) and/or metabolic (e.g. assimilation or degradation) roles in marine organisms (Kiene et al 2000, Moran et al 2012). DMSP concentrations can reach up to 400 mM in dinoflagellates and coccolithophores (Keller et al 1989, Stefels 2000). After cell lysis, DMSP is released and becomes an important carbon and sulfur source for prokaryotes (Kiene et al 2000). High concentrations of DMSP in the ambient water typically occur during senescing phytoplankton blooms (up to 368 nM) (Matrai & Keller 1993) and in coral mucus (up to 409 nmol cm^{-2} coral surface) (Frade et al 2016). In marine snow, DMSP concentrations reach four times higher concentrations (80.46 ± 52.44 nM) than in the ambient water (Chapter III). Furthermore, marine snow exhibits a pronounced seasonality in DMSP concentrations as reported in coastal and open ocean waters (Chapter III), linked to the prevalent phytoplankton species being abundant in different seasons and their contribution to marine snow formation (Chapter III). The marine snow associated demethylating bacterial community differs from the free-living demethylating community, characterized by exclusively free-living demethylating subclades (Chapter III). The contrasting demethylating bacterial subclade composition in the two habitats results from the different DMSP concentrations in marine snow and ambient water, and the complex and phylogenetically conserved trait of lifestyle (i.e., particle association vs. free-living) (Martiny et al 2013, Salazar et al 2015). However, demethylating bacteria contributed similarly to the free-living and marine snow associated bacterial communities, suggesting that DMSP demethylation, which is based on a small set of genes, might not have contributed to the lifestyle selection (Chapter III).

Furthermore, the response to seasonally changing DMSP concentrations is more pronounced in the free-living than in the marine snow associated demethylating community, indicating niche diversification in this habitat related to DMSP availability (Chapter III). DMSP concentrations are tightly connected to recurring seasonal variation of phytoplankton communities and their blooms (Howard et al 2011, Matrai & Keller 1993, Nowinski et al 2019, Varaljay et al 2015). Dinoflagellates and cocco-

lithophores are typically abundant in late spring and summer, when they release copious amounts of DMSP, triggering DMSP demethylating bacteria to thrive. High abundance of phytoplankton in spring and fall, mainly of diatoms (Aubry et al 2004, Bernardi Aubry et al 2006), typically leads to growth of copiotrophs and opportunistic prokaryotes (Chafee et al 2018) (Chapter II, III). However, the low release rates of DMSP by diatoms might create a niche for demethylating bacteria with high affinity to DMSP during this season (Chapter III).

Future directions

This thesis unravels the seasonal patterns of marine snow associated and free-living prokaryotic and the bacterial demethylating communities, and discusses the connectivity between both habitats. The development of distinct communities in marine snow *vs.* the ambient water in specific seasons is largely influenced by the environmental and more specifically, micro-environmental conditions. Future research on coastal marine snow should focus on elucidating the dynamics of the communities during the time frame between transition and peak seasons, i.e., when similar communities develop into distinct ones. The mechanisms responsible during the establishment and development of a distinct marine snow community remain a relevant open question. High DMSP concentrations in marine snow might attract chemotactic and motile prokaryotes, such as *Vibrios*, with important implications on the health and ecosystem management research. As a consequence of increased prokaryotic abundance and activity, anoxic microenvironments within marine snow might develop, selecting for prokaryotes with adapted metabolisms, and affecting the biogeochemical cycles and carbon fluxes. Increased sampling resolution complemented with laboratory experiments, and the use of state-of-the-art methodological approaches (e.g., DNA and protein sequencing), together with microscopy, chemical composition analyses and rate measurements on marine snow and its associated microbial community will help answering the above-mentioned questions and possibly discover previously unknown microbe – marine snow interactions.

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Summary

In the oceanic realm, aggregates of organic matter larger than 500µm are coined ‘marine snow’ due to the resemblance to the meteorological phenomenon as the particles sink from the surface to the deep sea. Marine snow is mainly formed by aggregation of phytoplankton and bacterial exudates with other compounds such as fecal pellets or detritus. High nutrient concentrations in and surrounding marine snow cause a dense population of highly active microbes. High heterotrophic activity and respiration, in turn, lead to maturation of the marine snow, typically resulting in an increased nitrogen limitation and development of anoxic microenvironments. In addition to maturation processes, changes of environmental parameters can potentially affect marine snow substrate composition via compositional changes of the phytoplankton community. A compound that is produced by phytoplankton in species-specific concentrations and occurs in marine snow is dimethylsulfoniopropionate (DMSP). It acts as a chemoattractant, is a carbon and sulfur source and hence, potentially affects the prokaryotic community composition. The substrate composition and effects of maturation have important implications on the microbial community associated to marine snow, which have far reaching biogeochemical consequences. In this thesis, we investigated the microbial community associated to marine snow and in the ambient water and determined environmental factors controlling it over a seasonal cycle. We determined nutrient concentrations, DMSP concentrations and the DMSP demethylating community, abundance and composition of the phytoplankton, the eukaryotic and the prokaryotic community, prokaryotic activity and functional potential. This allowed us to draw conclusions on the formation and realization of ecological niches over time and potential biogeochemical implications. Furthermore, we determined the half-life time of prokaryotic RNA in three isolates and in an environmental community using qPCR and metatranscriptomics, respectively. We related the half-life time to RNA type, optimal growth temperature, growth rate and possible ecological factors such as competition or nutrient stress and discussed the applicability of metatranscriptomics to environmental samples.

Zusammenfassung

Der Ausdruck „Meeres-Schnee“ bezeichnet in der Ozeanographie Aggregate aus organischem Material (Phytoplankton- und Bakterien-Sekrete, Kot-Kügelchen, Detritus), die eine Größe von 500 µm überschreiten und von der Wasseroberfläche in die Tiefe sinken. Hohe Nährstoffkonzentrationen in Meeres-Schnee und in dessen Umgebung ermöglichen dichten Bewuchs mit hoch aktiven Mikroben. Die resultierende hohe heterotrophe Aktivität und Respiration wiederum führen zur Alterung des Meeres-Schnee, was sich in einer steigenden Stickstoff-Limitierung und der Entwicklung von anoxischen Mikro-Zonen zeigt. Zusätzlich zu diesen Alterungsprozessen kann eine Veränderung von Umweltbedingungen und die einhergehende Veränderung der Zusammensetzung der Phytoplankton-Gemeinschaft des Meeres-Schnee führen. Dimethylsulfoniopropionat (DMSP) ist ein Substrat, das von Phytoplankton je nach Art in unterschiedlichen Konzentrationen produziert wird und daher auch in Meeres-Schnee vorkommt. Es agiert als chemischer Lockstoff, Kohlenstoff- und Schwefelquelle und beeinflusst daher möglicherweise die Zusammensetzung der Prokaryoten-Gemeinschaft. Die Zusammensetzung des Meeres-Schnee-Substrates und dessen Alterung haben wichtige Auswirkungen auf die mikrobielle Gemeinschaft und daraus entstehende biogeochemische Reaktionen. In dieser Dissertation untersuchten wir die zeitlichen Entwicklungen der dem Meeres-Schnee zugehörigen und der freilebenden mikrobiellen Gemeinschaft und die sie kontrollierenden Faktoren. Wir ermittelten Nährstoffkonzentrationen, DMSP-Konzentrationen und die DMSP-demethylierende Gemeinschaft, Abundanz und Zusammensetzung des Phytoplanktons, der eukaryotischen und der prokaryotischen Gemeinschaft, prokaryotische Aktivität und das funktionelle Potential über 1,5 Jahre, alle Jahreszeiten abdeckend. Dadurch konnten wir Rückschlüsse auf die Entstehung und Realisierung von ökologischen Nischen und möglichen biogeochemischen Konsequenzen in diesem Zeitraum ziehen. Zusätzlich bestimmten wir in drei Bakterien-Kulturen und in einer natürlichen Gemeinschaft die Halbwertszeit von prokaryotischer RNA mittels qPCR und metatranscriptomics. Wir ordneten die Halbwertszeit der Art der RNA, der optimalen Wachstumstemperatur und der Wachstumsrate, möglichen ökologischen Faktoren sowie Konkurrenz oder Nährstoff-Stress zu und diskutieren die Anwendbarkeit von metatranscriptomics bei Umweltproben.