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Seasonal variation of extracellular enzymatic activity in marine snow-associated microbial communities and their impact on the surrounding water

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Running title: Seasonality of ectoenzymatic activity in marine snow

ABSTRACT

Seasonal changes of microbial abundance and associated extracellular enzymatic activity in marine snow and in seawater were studied in the northern Adriatic during a three year period. Marine snow was present during the entire investigated period, although with higher concentrations during summer than during winter. Microorganisms densely colonized marine snow and aggregate-associated enzymatic activity was substantially higher (up to 10^5 times) than in seawater. Alkaline phosphatase activity (APA) and aminopeptidase activity in marine snow showed seasonal variation with higher activities in late spring-summer than in autumn-winter, probably in response to changes in the quantity and quality of organic matter. The highest cell-specific bacterial activity was found for phosphatase, followed by peptidase, and the lowest for glucosidases. Differential hydrolysis of marine snow-derived organic matter points to the well-known P-limitation of the northern Adriatic and indicates preferential utilization of phosphorus- and nitrogen-rich organic compounds by microbes, while hydrolysis of polysaccharides seemed to be less important. In oligotrophic conditions during summer, organic matter released from marine snow might represent a significant source of substrate for free-living bacteria in seawater. For the first time microorganisms producing APA in marine snow were identified revealing that dense populations of bacteria expressed APA, while cyanobacteria did not. Cyanobacteria proliferating in marine snow could benefit from phosphorus release by bacteria and nanoflagellates.

INTRODUCTION

Flocculent organic aggregates known as marine snow are a common feature in coastal and open ocean waters. They represent 'microbial hotspots' characterized by high microbial abundance (review Simon *et al.* 2002; Vojvoda *et al*. 2014; Thiele *et al*. 2015). This is presumably due to the favourable nutritive conditions in marine snow with respect to seawater, and may be partly explained by microbial colonization through chemotactic localization of high concentration of organic matter (Kiørboe and Jackson 2001; Grossart *et al*. 2003). Furthermore, microbial communities are regulated by growth and predator-prey interactions, and such interactions may interplay with colonization and detachment to regulate the dynamics of marine snow-associated microbial communities (Kiørboe *et al*. 2003).

Moreover, marine snow is an important site of organic matter solubilisation and remineralisation. Many studies have shown that the extracellular enzymatic activity (EEA) of attached bacteria is at times one to two orders of magnitude higher than of free-living bacteria (Karner and Herndl 1992; Smith *et al*. 1992; Grossart *et al*. 2003, 2007; Ziervogel *et al*. 2010; Lyons and Dobbs 2012; Kellog and Deming 2014). According to Lyons and Dobbs (2012) functional redundancy, and not strictly species diversity is responsible for different activity level between aggregate-associated and free-living bacteria. A laboratory study demonstrated that bacterial strains are quickly capable of up-regulating protease activity upon attachment to artificial aggregates and cultures of bacteria isolated from marine snow showed a 20-fold increase in enzyme activity within 2 h of particle attachment (Grossart *et al*. 2007). Elevated EEA on aggregates may result from bacterial quorum sensing that has been demonstrated to occur in bacterial strains isolated from marine snow (Jatt *et al*. 2015).

The northern Adriatic is specific for the appearance of giant mucilaginous aggregates during the summer (up to few meters in diameter). This phenomenon has been rather well investigated from many aspects, since it is very unusual and provokes socioeconomic

consequences on the region (STOTEN, 2005 and citation therein). However, smaller marine snow (up to 5 mm), common for many ocean and coastal areas, are sporadically studied and only during summer of mucilage events. Consequently changes in EEA activity are also only sparsely studied (e.g. Karner and Herndl 1992; Müller-Niklas *et al*. 1994). Globally, the importance of EEA of marine snow associated-bacteria is well documented, however, little is known about seasonal changes. In this study, the EEA of marine snow was determined over a three-year period covering 53 sampling dates. The EEA of alkaline phosphatase, aminopeptidase, α- and β- glucosidase on marine snow was compared with the corresponding EEA in the seawater. Additionally, the variation in cell-specific bacterial EEA and the relationships between the activities of enzymes were studied in two contrasting seasons (summer and autumn) differing in hydrodynamic and trophic conditions. The identification of APA expressing microorganisms was performed using Enzyme Labelled Fluorescence (ELF) on marine snow collected *in situ*. Alkaline phosphatase is the key enzyme in natural environments (Jones, 1997) and a significant component of most marine algae and bacteria (Hoppe, 2003).

Furthermore, it was demonstrated from theoretical considerations and experiments with aggregates that interactions through small-scale fluxes of microorganisms, solutes, and nutrients to and from the surrounding water are substantial (Kiørboe and Jackson 2001; Kiørboe *et al*. 2001; Ziervogel *et al*. 2010; Lombard *et al*. 2013). Therefore, the effects of marine snow on the surrounding seawater were studied on field samples. EEA of free-living bacteria around marine snow and their counterpart in seawater was compared on samples collected in the water column of the northern Adriatic. Moreover, the release of free enzymes from aggregates in the surrounding water was determined over different seasons.

MATERIAL AND METHODS

The study was conducted in the northern Adriatic Sea about 3 km off Rovinj (Croatia), at a

89 long-term monitoring site (RV001) where hydrographical, chemical and biological data have
90 been collected at about monthly intervals since the year 1972. Marine snow was collected
91 from June 2014 to November 2016, representing a total of 53 sampling dates spread over all
92 seasons. Samples of marine snow aggregates and ambient seawater were taken by SCUBA
93 divers from depths between 15 and 17 m using 0.1 M HCl-rinsed 100 mL disposable syringes.
94 The diameter of marine snow in the water column, based on visual observation varied
95 between 2 to 5 mm using the opening of the syringe as a reference. Although aggregates were
96 easily recognizable, a certain amount of seawater was unavoidably sampled along with the
97 aggregates. Generally 50-90 aggregates (70 on average) were collected in one syringe giving
98 the concentration of about 0.7 aggregates/mL. Within one hour, the samples were brought to
99 the laboratory and processed within the next hour. Samples from different syringes containing
100 marine snow were pooled and subsamples were taken for further measurements.

101 Water temperature, salinity and in vivo fluorescence were determined with a SBE25
102 conductivity-temperature-depth probe (Sea-Bird Electronics, Washington, USA). Chlorophyll
103 *a* (chl *a*) was determined by fluorescence after calibration of the probe with known chl *a*
104 concentrations. Samples for nutrient analyses and transparent exopolymer particles were taken
105 (5 L Niskin bottles) from February to November 2016 at the depth where aggregates were
106 collected.

107 Analytical protocol

108 **Inorganic nutrients (NH_4, NO_2, NO_3, PO_4,).** Samples for nutrient analyses in seawater were
109 filtered (Whatman GF/F, precombusted at 380ºC for 4h) and stored in polyethylene bottles at
110 -30ºC. Analyses were performed within one month using spectrophotometric methods
111 (Parsons *et al*. 1984; Ivančić and Degobbis 1984).

112 **Transparent exopolymer particles (TEP).** TEP was measured as an indicator for marine

snow formation as they often control the formation of marine snow (Alldredge *et al*. 1993). For the concentration of TEP, samples (20 ml) were filtered through polycarbonate filters of 0.4 µm pore size, stained with a pre-filtered (0.2 µm pore size) 0.02% solution of Alcian blue in 0.06% acetic acid (pH 2.5) for 3 s, transferred to beakers with 80% H_2SO_4 and determined spectrophotometrically at 787 nm (Passow and Alldredge 1995). For the 4 sampling campaigns (7 days in February, 7 days in May, 9 days in July-August, 8 days in November 2016), 3 samples per day were analyzed and all data averaged per campaign.

Extracellular enzymatic activity (EEA). Measurements were performed for alkaline phosphatase activity (APA), aminopeptidase activity (AMA), as well as α- and β-glucosidase activity (α-GLUA and β-GLUA, respectively). EEA was measured in seawater without aggregates (SW) and in marine snow aggregates suspended in seawater (about 0.7 aggregate/mL) (AGG+SW, Table 1). Measurements were performed on unfiltered samples (total activity) and in the filtrate (0.22 µm mixed cellulose esters Millipore filter; activity of free enzymes). In addition to measurements in SW and AGG+SW, during the summer (26 July-2 August) and autumn (8-15 November) of 2016, the EEA of individual aggregates and the EEA of free-living bacteria in seawater without aggregates and seawater surrounding marine snow were measured. To measure the EEA of aggregates, individual marine snow particles were placed in 1.5 mL of 0.22µm filtered and sterilised seawater. To determine the EEA of free-living bacteria, samples of SW and AGG+SW were filtered through a 2 µm pore-size filter (polycarbonate) and activity measured in the filtrate. The EEA of free-living bacteria was calculated by subtracting the EEA measured in the 0.22 µm filtrate from the EEA in the 2 µm filtrate. All filtrations were carried out gently using Millipore filtration units.

Table 1

All EEA measurements were performed in triplicate with the respective fluorogenic substrate at saturation concentrations (Hoppe 1983; Hoppe *et al*. 1988). The following substrates were

used: APA methylumbelliferyl-phosphate (MUF-P; final concentration 50 µmol L^{-1}); AMA L-leucine 7-amino-4-methylcoumarin (MCA-leucine; final concentration 100 µmol L^{-1}); α- and β-GLUA MUF-α-D-glucopyranoside and MUF-β-D-glucopyranoside, respectively (final concentration 25 µmol L^{-1}). Substrates were dissolved in methylcellosolve and diluted with water immediately before addition. Incubations were performed in the dark at *in situ* temperature and pH. Fluorescence was measured immediately after substrate addition and after 15 min to 1 h of incubation using a Turner TD-700 fluorometer (2014-2015) or Tecan Infinite 200 Pro microplate reader (2016) at an excitation of 365 nm and emission of 460 nm. The fluorescence increased linearly over the incubation time (maximum time tested 1 h 40 min; data not shown). EEA (pmol mL^{-1} h^{-1}) was calculated as the difference between these two measurements divided by the incubation time after calibration of instruments with MUF and MCA. For individual aggregates in autoclaved seawater all fluorescence was attributed to aggregates since the fluorescence in autoclaved seawater did not increase over time upon substrate addition. Cell-specific activity was calculated by dividing the respective EEA by the bacterial abundance.

Additionally, APA was also resolved on a single cell level on individual aggregates utilizing the ELF®97 Endogenous Phosphatase Detection Kit (E6601) (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's recommendations. ELF substrate was diluted 20-fold in detection buffer and mixed with aggregates to reach finally a 40-fold dilution. Chloroplast autofluorescence was recorded at an emission wavelength of 580-600 nm (555 nm excitation). The ELF signal was detected with the following filter setup: excitation filter 340/26 nm, split mirror at 400 nm, emission filter 525/50 nm, Zeiss (Oberkochen, Germany). An AxioImager bright field fluorescence microscope was used.

Microbial abundance and aggregate dimensions. The microbial abundance was determined during the summer (26 July-2 August) and autumn (8-15 November) of 2016. Measurements

were performed on individual aggregates, SW, as well as in the filtrate through 2 μm of SW and AGG+SW. The microbial abundance was determined after EEA measurements on the same samples. A Leitz Laborlux D epifluorescence microscope was used for enumeration at 1000 × magnification.

Seawater and filtrate (1.5 mL) were fixed with formaldehyde (final concentration 2%) and stored at 4°C until analysis within one month. Samples were stained with DAPI for 10 min (Porter and Feig 1980) and filtered onto black 0.2 μm Nuclepore polycarbonate filters (Whatman, UK). For heterotrophic bacteria (bacteria) count at least 500 cells per sample were counted. The cyanobacteria (CB) abundance was determined using green light excitation, with a minimum of 300 cells counted per sample (Takahashi *et al*. 1985). The observed CB had the same shape, dimensions and autofluorescence as the CB that have been previously identified as *Synechococcus* (Šilović *et al*. 2012). Heterotrophic nanoflagellates (HNF) abundance was determined by counting a minimum of 100 cells per sample (Sherr *et al*. 1993).

The microbial abundance on individual aggregates was determined after fixing the samples with formaldehyde (final concentration 2%) for 1 h, staining with DAPI (1 μg mL^{-1} final concentration) for 10 min, and collecting the single aggregates on a 0.2 μm black polycarbonate filter. The DAPI stain made the aggregate-attached bacteria clearly visible under the epifluorescence microscope (Fig. S1). Aggregates were spread upon the polycarbonate filter and then gently compressed with the cover slip flattening their structure. This resulted in rather flat aggregates which allowed a straight forward counting of the bacteria. For the parts of the aggregate that were thicker, the counting was performed by carefully focusing on multiple layers (by moving the objective a few micrometres from the top to the bottom). Each field view containing the aggregate was photographed and the surface of each aggregate section was measured using the program AxioVision 4.7.2. The total aggregate surface was obtained by summing the surfaces of each section. The volume of

188 the respective aggregates was calculated using dimensions determined microscopically and
189 assuming a cylindrical shape usually observed on collected aggregates. Bacteria on the
190 aggregates were counted on a minimum of 10 random fields (141x110 µm) and at least 500
191 cells were counted. The HNF and CB were counted simultaneously with bacteria on 10
192 random fields and at least 300 CB and 30 HNF were counted. The average number of
193 microbes/µm^2 was then multiplied by the total surface of the aggregate to obtain the bacterial
194 abundance on a specific aggregate. The microbial abundance per volume of aggregate was
195 obtained dividing the total number of cells by the estimated volume of the analysed aggregate.
196 Since marine snow components have often a loose structure and typically have high porosities
197 (up to 99%) (Alldredge and Gotschalk 1988) it can be assumed that during the filtration, the
198 aggregates might have lost a considerable part of their pore water and have collapsed on the
199 filters. Thus, the calculated volumes of the investigated fragments are smaller than they would
200 have been in their original hydrated state. Therefore, the microbial cell numbers per volume
201 of aggregate might be overestimated.

202 **Statistical analyses**. Results of EEA and microbial abundances are presented in box-and-
203 whisker statistical plots. Differences of EEA and microbial abundances between two selected
204 seasons or two media were tested using the Mann-Whitney *U* test (Supplementary table 1).
205 Results were considered significant if $p < 0.05$. EEA between three different fractions were
206 tested using the Kruskal-Wallis *H* test. Differences between three fractions found to be
207 statistically significant ($p < 0.05$) were pairwise compared using the Mann-Whitney *U* test
208 followed by the Bonferroni correction (Supplementary table 2). Results were considered
209 significant if $p < 0.05$. All analyses were performed in the R software environment
210 (http://www.r-project.org/).

211 # RESULTS

212 APA and AMA showed a distinct seasonal cycle with higher values during the late

spring-summer than during autumn-winter period (Fig. 1a,b). Activity in marine snow aggregates suspended in seawater (AGG+SW) was generally several times higher than in seawater (SW) during the entire period of investigation. Daily variations of activity for both enzymes were less pronounced than seasonal changes. In contrast, α- and β-GLUA did not show a clear seasonal trend, neither in AGG+SW nor in SW (Fig. 1c,d). Daily variations in activity of these enzymes were more pronounced than seasonal variations. Although activity in AGG+SW was also generally higher than in SW, differences were less pronounced than for APA and AMA.

Fig. 1

In the following chapters, data for two contrasting seasons during 2016 (summer: 26 July-2 August and autumn: 8-16 November) are compared to evaluate factors that drive changes in microbial activities. The selected seasons are characterised by completely different environmental conditions (see text below: Environmental conditions). EEA in the chosen seasons fit the measurements performed during three years (Fig. 1).

Summer and autumn 2016

Environmental conditions

During the sampling in July-August, the water column was, as common in the summer, stratified with the halocline and primary thermocline at 15-17 m (Fig. S2a,b). Salinity and temperature of this depth were 38.02-38.10 and 22-23°C, respectively. In November, the water column was mixed with uniform temperature (~ 16.5°C) and salinity (37.81) throughout the column (Fig. S2a,b). Marine snow aggregates were collected at 15-17 m depth (layer of the pycnocline), where according to visual observations by the SCUBA divers the highest abundance of marine snow was found. Marine snow was always present in the water column during the entire year. The lowest TEP concentrations were found during

237 winter and spring (118.3±18.5 ng mL^{-1}; Fig. 2). The highest and the most variable TEP
238 concentrations were found during the summer (303.1±141.2 ng mL^{-1}), decreasing in the
239 autumn (Fig 2). TEP indicate the potential for marine snow formation; inferring that marine
240 snow abundance was lower in the winter-spring compared to the summer-autumn season.

241 Fig. 2

242 Inorganic nutrient concentrations at the sampling depth (PO_4 0.04-0.14 µmol L^{-1};
243 DIN 0.35-2.34 µmol L^{-1}) were markedly higher in November than in July-August (Fig.
244 S2d,e). Consequently, the phytoplankton biomass in November (chl *a* 0.70-1.03 µg L^{-1}) was
245 about twice as high as in July (0.37-0.40 µg L^{-1}; Fig. S2c). Exhaustion of nutrients during the
246 summer and markedly lower phytoplankton biomass than during the autumn and winter are
247 characteristic for the sampling site, as shown by the long-term data set (period 1972-2017)
248 (Fig S2d,e,f).

249 *Microbial abundance*

250 Microbial abundance on aggregates was much higher (10^3-10^4 times) than in seawater
251 in both seasons (Fig. 3). Generally, microbial abundance on aggregates (10^8-10^9 bacteria mL^{-1};
252 10^6-10^9 CB mL^{-1}; 10^6-10^8 HNF mL^{-1}) was higher in November than in July-August.
253 Differences between seasons were statistically significant only for bacterial abundance. In
254 seawater, microbial abundances (10^5-10^6 bacteria mL^{-1}; 10^3-10^5 CB mL^{-1}; 10^3-10^4 HNF mL^{-1})
255 were also generally higher in November than in July-August, although differences were
256 statistically significant only for CB.

257 Fig. 3

258 *Extracellular enzymatic activity (EEA)*

259 On a volumetric basis, EEA was about 10^4-10^5 times higher in marine snow than in
260 seawater (Fig. 4). Although microbial abundance was higher in November, APA (10^5-10^7

pmol mL^{-1} h^{-1}) and AMA (10^4-10^6 pmol mL^{-1} h^{-1}) on aggregates were, on average, about an order of magnitude higher in July-August (Fig. 4a,b). A similar trend was observed for APA in seawater, although values were much lower (10-10^2 pmol mL^{-1} h^{-1}). AMA in seawater (13.2-39.0 pmol mL^{-1} h^{-1}) was relatively similar in both seasons.

Fig. 4

In both seasons, the major part of APA and AMA was found in the particulate fraction. Activity of free enzymes (APA: 1.1-25.9 pmol mL^{-1} h^{-1}; AMA: 0.2-8.2 pmol mL^{-1} h^{-1}) in seawater was similar as in seawater surrounding aggregates, both being an order of magnitude lower than the total activity in seawater (Fig. 4a,b). A low contribution of free enzymes (generally < 10%) to the total activity was observed in all seasons during the entire investigated period (2014-2016; data not shown).

In July-August, APA was significantly higher than AMA, both on aggregates and in seawater (average APA/AMA ratio about 4), while in November APA and AMA were similar (Fig. 4e).

Seasonal differences of α-GLUA were statistically not significant, both on aggregates (10^3-10^4 pmol mL^{-1} h^{-1}) and in seawater (0.9-4.6 pmol mL^{-1} h^{-1}) (Fig. 4c). In contrast β-GLUA on aggregates (10^3-10^6 pmol mL^{-1} h^{-1}) was significantly higher in July-August than in November, while in seawater differences were not significant (Fig. 4d).

The major part of GLUA in seawater was in the dissolved fraction. Free α-GLUA (0.1-5.9 pmol mL^{-1} h^{-1}) and β-GLUA (0.2-16.6 pmol mL^{-1} h^{-1}) in seawater were similar as in seawater surrounding aggregates, and close to the total activity in seawater (Fig. 4c,d). This pattern was observed during the entire investigated period (2014-2016; data not shown).

In aggregates, β-GLUA was generally higher than α-GLUA, especially in July-August (on average 4 times higher) (Fig. 4h). In seawater, α-GLUA and β-GLUA were similar.

APA and AMA were markedly higher than sum of GLUA (up to 100 times), both on

aggregates and in seawater (Fig. 4f,g), particularly in July-August.

Cell-specific extracellular enzymatic activity

The highest cell-specific bacterial EEA (Fig. 5) was found for phosphatase followed by peptidase, while for glucosidases was markedly lower (10-100 times). Cell-specific activities in aggregates (10^1-10^4 amol cell^{-1} h^{-1}) were noticeably higher than in seawater (2-4 times for glucosidases and 10-20 times for phosphatase and peptidase). Cell-specific APA and AMA were >10 times higher in July-August than in November, except for less pronounced (but statistically significant) differences of cell-specific AMA in seawater (Fig. 5a,b). For cell-specific GLUA differences between seasons were less evident (Fig. 5c,d) and statistically significant only for aggregates.

Fig. 5

Cell-specific APA and AMA of free-living bacteria in seawater surrounding the aggregates and in seawater without aggregates were markedly higher in July-August (10^2-10^3 amol cell^{-1} h^{-1} and 30-120 amol cell^{-1} h^{-1}, respectively) than in November (3-100 amol cell^{-1} h^{-1} and 7-50 amol cell^{-1} h^{-1}, respectively) (Fig. 6a,b). Differences were statistically significant. Generally in July-August, cell-specific activity of free-living bacteria for both enzymes was 2-3 times higher in the seawater around aggregates than in seawater without aggregates (Fig. 6a,b), while in November values were similar, except in the third day of sampling.

Fig. 6

At some sampling dates (27 August and 10 November 2016), cell-specific APA of free-living bacteria in the seawater around the aggregates was about an order of magnitude higher than in seawater (Fig. 6a).

Calculating cell-specific α- and β-GLUA of free-living bacteria was not possible since the major part of GLUA was in the dissolved fraction.

APA localization

Within aggregates, a large number of bacterial cells were ELF-labelled and hence, expressed APA (Fig. 7; S3). Cyanobacteria were readily detected by their autofluorescence, however, did not show any ELF signal (Fig. 7a,d; S1a,d). Live phytoplankton were virtually absent from the aggregates, while empty frustules of diatoms were occasionally observed (Fig. 7f). ELF signal was almost always found localized on bacteria within aggregates (in 52 of 53 cases). Only at one occasion (8 September 2014), the ELF signal, and consequently APA, was localized on bacteria and additionally in the matrix of the aggregates, indicative for the presence of free enzymes attached to the aggregate matrix (Fig. 7e,h).

Fig. 7

DISCUSSION

Microorganisms densely colonized marine snow and aggregate-associated EEA were far higher than in seawater, both in terms of volume-normalized and cell-specific EEA. Although highly variable, EEA were within ranges reported for the northern Adriatic and other areas, as well as in laboratory studies (Karner and Herndl 1992; Smith *et al*. 1992; Grossart *et al*. 2003; 2007; Ziervogel *et al*. 2010; Lyons and Dobbs 2012).

Microbial dynamics

The most abundant organisms within marine snow were bacteria (10^8-10^9 cell mL^{-1}) followed by cyanobacteria (10^7-10^8 cell mL^{-1}) and heterotrophic nanoflagellates (10^6-10^8 cell mL^{-1}). Although calculation of the total number of microorganism based on volume estimated from parts of deflated aggregates may have led to an overestimation of total cell numbers per aggregate, the obtained results are within the ranges reported for the northern Adriatic (Müller-Niklas *et al*. 1994; Vojvoda *et al*. 2014), and other areas (Simon *et al*. 2002 and references therein; Thiele *et al*. 2015).

The enrichment factor of microbes in marine snow compared to seawater was about 10^3 for all the microbial groups and did not vary between seasons. The abundance of all organisms was higher in autumn than in summer in both media.

In summer, oligotrophic conditions with low phytoplankton biomass prevailed, while a shift toward more eutrophic conditions, provoking an increase in phytoplankton biomass due to the import of nutrients from the bottom, occurred in autumn. Such a pattern is characteristic for the northern Adriatic (Ivančić *et al*. 2012 and references therein). Consequently, freshly produced organic matter in autumn supported higher bacterial biomass compared to summer.

Marine snow is typically rapidly colonized by bacteria followed by the increase of heterotrophic flagellates, as described elsewhere (Simon *et al*. 2002). Protozoans are important in regenerating phosphorus (P), ammonium and primary amines (Gotschalk and Alldredge 1989; Grossart and Ploug 2001). Therefore heterotrophic flagellates were probably important in the remineralisation of marine snow-associated nitrogen (N) and P, predominantly by grazing on bacteria and cyanobacteria. In reverse, cyanobacteria could benefit from regenerated nutrients and proliferate in marine snow reaching observed 10^3 times higher abundance than in seawater. Remineralisation of nutrients by bacteria and flagellates could allow cyanobacteria to thrive in marine snow without expressing EEA (energy expensive), as observed in this study for APA. In aggregates, cyanobacteria, mainly *Synechococcus*, did not produce APA, since the ELF signal was found localized only on associated bacteria. The ELF method is appropriate to test APA in *Synechococcus* since ELF labelling of *Synechococcus* was observed in another study reporting an increased fraction of labelled cells (up to 95%) when exposed to P-limiting conditions (Duhamel *et al*. 2010).

Contrary to cyanobacteria, which heavily colonized marine snow, intact and live phytoplankton cells were not found, and only dead cells heavily colonized by bacteria were observed.

Seasonal changes in EEA

APA and AMA followed a distinct seasonal pattern with the highest values during water column stratification. Although activity was much higher in marine snow (volume normalized 10^4-10^5 times, cell-specific 10-10^2 times), the seasonal changes paralleled those in seawater indicating that in both media they were governed by the same factors (temperature, nutrients' concentrations, concentration and quality of organic matter, biological activity). A similar seasonal trend of APA in seawater of the investigated area was observed in earlier studies with low values during autumn-winter (0.1-57 pmol mL^{-1} h^{-1}) and about an order of magnitude higher values during late spring-summer (40-357 pmol mL^{-1} h^{-1}) (Ivančić *et al*. 2016 and citation therein), strongly indicating a seasonally recurring pattern. In contrast to APA and AMA, for α- and β-GLUA daily variations were more pronounced than seasonal pattern. Only cell-specific GLUA on marine snow showed significant difference between summer and autumn. Marine snow-associated GLUA was much higher than in seawater, particularly in summer (volume normalized ~10^4 times, cell-specific ~10-30 times).

Although bacteria were more abundant in autumn, volume normalized and cell-specific EEA on marine snow was for up to one order of magnitude higher in summer. For cell-specific APA and AMA a similar trend was observed also in seawater. Changes in the quality and quantity of substrate produced during these two contrasting seasons were probably the main reason for the observed differences. In summer, characterised by low nutrient concentration and low phytoplankton biomass, organic matter in marine snow and seawater of the northern Adriatic becomes more refractory, due to the intense utilization of easily degradable compounds (Müller-Niklas *et al*. 1994; Turk *et al*. 2010). In autumn the production of fresh organic matter is usually higher than in summer indicated by the higher phytoplankton biomass. Lower β-/α- GLUA in marine snow in autumn also indicated more easily degradable organic matter than in summer. However, changes in organic matter quality

could also trigger changes in bacterial community composition (Ortega-Retuerta *et al*. 2013). One study in the northern Adriatic indicated that the marine snow-associated and seawater-associated bacterial communities undergo similar seasonal changes associated with changing environmental conditions (Vojvoda *et al*. 2014). Hence, differences in cell-specific EEA between summer and autumn were probably a response to different quality of organic matter, either by triggering colonization of bacteria capable to use given substrates, and/or by adjusting their metabolic activity to the organic matter composition. According to Ziervogel *et al*. (2010) microbes change their extracellular enzyme production in response to aggregate composition. Furthermore, Lyons and Dobbs (2012) suggest that aggregate-associated microbial communities exhibit not only high metabolic activity, but also high metabolic versatility and functional redundancy, compared to free-living bacteria.

Lower cell-specific APA in autumn was probably to some extent also caused by mitigation of the P-limitation, regularly observed in the northern Adriatic during this period (Ivančić *et al.* 2016 and citations therein). In summer, the APA/AMA ratio was higher than 1, suggesting P-limitation (Sala *et al*. 2001). The relaxation of the P-limitation in autumn reduced the ratio to ~ 1.

Lower cell-specific AMA might also point to a higher availability of mono- and oligomers originating from freshly produced organic matter during autumn, which do not require prior enzymatic hydrolysis (Grossart and Ploug 2001). Rath *et al*. (1993) found that cell-specific AMA is inversely correlated with the trophic status of the environment, i.e. in oligotrophic waters (characteristic for summer in this study) cell-specific AMA was higher than in more eutrophic waters (characteristic for autumn in this study).

GLUA in marine snow was much lower than APA and AMA, specifically in summer (on average APA/GLUA~ 50 and AMA/GLUA ~ 20), indicating that hydrolysis of organic P, proteins and peptides was the prevalent potential activity in marine snow. Similar results were

409 reported also for other marine sites (Smith *et al.* 1992; Grossart and Ploug 2001; Azam and
410 Malfatti 2007) and for marine snow and larger aggregates in the northern Adriatic (Karner
411 and Herndl 1992; Müller-Niklas *et al*. 1994; Del Negro *et al.* 2005; Zoppini *et al*. 2005).
412 Another interesting feature arises from the different partitioning of APA and AMA in
413 seawater compared to GLUA. While APA and AMA were mostly cell-attached, the major
414 part of GLUA was in the dissolved fraction. This indicates a greater bacterial need for P and
415 N than for C source. Duhamel *et al*. (2010) reported a shift from cell-free APA dominating
416 under N-limitation and P-stress (i.e. physiological response) to cell-bound APA dominating
417 under P-limitation (i.e. growth rate limitation).

418 ## Influence of marine snow on the surrounding waters

419 In summer, cell-specific APA and AMA of free-living bacteria in the seawater
420 containing marine snow were higher than of their counterpart in the seawater without
421 aggregates included. This indicates that field populations of free-living bacteria in the intimate
422 vicinity of marine snow were metabolically more active than those populations further away
423 from aggregates. Bacteria residing on aggregates solubilize particulate material faster than
424 they absorb the resulting solutes, i.e. exhibiting a loose hydrolysis-uptake coupling (Smith *et*
425 *al*. 1992). This causes leaching of DOM from marine snow into the water (Grossart and
426 Simon 1998; Alldredge 2000; Grossart and Ploug 2001). The volume of the plume with high
427 DOM concentrations extends the aggregate length and enlarges its volume many times
428 (Kiørboe *et al*. 2001). Such plumes of potential substrates can be detected by chemotactic
429 bacteria and might stimulate EEA of free-living bacteria (Kiørboe *et al*. 2001; Kiørboe and
430 Jackson 2001).
431 In autumn, cell-specific APA and AMA of free-living bacteria around marine snow
432 were similar to that of their counterpart in seawater. Mixing in the water column might have
433 disrupted DOM plumes and dispersed the bacterial structure around marine snow formed

under more stable, less turbulent conditions during the summer. This point to the importance of DOM released from marine snow as a significant source of substrate available for the growth of free-living bacteria in seawater under the oligotrophic summer conditions.

Occasionally, very high cell-specific APA of free-living bacteria around marine snow was found, similar as in marine snow. This could be explained by significant detachment of bacteria from aggregates. According to Bettarel *et al.* (2016) bacteria can attach and detach from aggregates. Laboratory experiments showed that attached bacteria initially detached at high specific rates, and although natural assemblages remain permanently attached after less than one day, recently colonized cells detached (Kiørboe *et al.*, 2003).

The activity of free enzymes in seawater in the intimate vicinity of marine snow was similar as in seawater. This indicates that the release of free enzymes from aggregates into the surrounding water was not important. According to Ziervogel *et al*. (2010), formation of aggregates triggers extracellular enzyme production by aggregate-associated microorganisms and some of these enzymes escape the aggregates, adding to the total hydrolytic activity in seawater. In this study, APA was almost always found bound to bacterial cells in marine snow (in 52 of 53 cases), and only once it was additionally found in the marine snow matrix, the latter indicating free enzymes entrapped in the matrix. Yet, this does not mean that free enzymes are not produced in marine snow since it was shown that marine bacteria release enzymes into the surrounding media (Alderkamp *et al*., 2007). However, free enzymes produced inside marine snow, including via active release, cell lysis, grazing or viral infection, are targeted by proteases. Thus, in a media heavily colonised by bacteria with protease activity, as indicated by high AMA, the active lifetime of free enzymes was probably too short for being released in significant amounts in the surrounding water.

Concluding, marine snow in the northern Adriatic shows marked seasonal variation with the largest appearance during summer. They are densely colonized microbial

459 microenvironments, seasonally variable in their importance in organic matter regeneration. In
460 marine snow as well as in seawater, APA and AMA were more important during the
461 stratification period when P and N in the water column are limited by intensive biological
462 activity and their availability depends on their recycling within these waters. In these
463 conditions, DOM released from marine snow might represent a significant source of P and N
464 available for growth of the free-living bacteria in seawater.

465 The major producers of APA in marine snow were bacteria. *Synechococcus* probably
466 benefited from P (and probably also N) regenerated by bacteria and/or heterotrophic
467 nanoflagellates, since it proliferated on marine snow without APA expression, and it is known
468 that it produces APA when P-limited.

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587

588 **Table 1.** Description of samples and their abbreviation.

589

590 Samples	Abbreviation
591	
592 Free enzymes in marine snow aggregates suspended in seawater	$(AGG+SW)_{FE}$
593 Free enzymes in seawater without aggregates	SW_{FE}
594 Marine snow aggregates	AGG
595 Marine snow aggregates suspended in seawater	AGG+SW
596 Seawater without aggregates	SW

597
598

599 Figure captions

600 Fig. 1. Seasonal changes of extracellular enzymatic activity (EEA) on aggregates of marine
601 snow suspended in seawater (AGG+SW) and in seawater (SW) during 2014-2016. (a)
602 alkaline phosphatase activity (APA); (b) leucine aminopeptidase activity (AMA); (c) α-
603 glucosidase activity (α-GLUA); (d) β-glucosidase activity (β-GLUA). Sampling dates in
604 which additional measurements were performed are denoted by light grey areas.

605 Fig. 2. Seasonal changes of transparent exopolymer particles (TEP) in seawater during 2016.
606 Filled circles show average values of daily triplicates throughout the sampling days (7 days
607 in February, 7 days in May, 9 days in July-August, 8 days in November). Error bars show
608 standard deviations.

609 Fig. 3. Microbial abundance on marine snow aggregates (AGG) and in seawater (SW) during
610 summer and autumn 2016. (a) bacteria; (b) cyanobacteria (CB); (c) heterotrophic
611 nanoflagellates (HNF).

612 Fig. 4. (a-d) Total EEA on marine snow aggregates (AGG_{Tot}) and in seawater (SW_{Tot}), as well
613 as activity of free enzymes in seawater with aggregates ($(AGG+SW)_{FE}$) and seawater alone
614 (SW_{FE}) during summer and autumn 2016. (a) APA; (b) AMA; (c) α-GLUA; (d) β-GLUA.
615 (e-f) Ratios between enzymes on marine snow aggregates and in seawater. (e) APA/AMA;
616 (f) APA/(α+β)-GLUA; (g) AMA/(α+β)-GLUA; (h) β-GLUA/α-GLUA. For explanation of
617 abbreviation see Fig. 1.

618 Fig. 5. Cell specific bacterial EEA on marine snow aggregates (AGG) and in seawater (SW)
619 during summer and autumn 2016. (a) cell specific APA (sAPA); (b) cell specific AMA
620 (sAMA); (c) cell specific α-GLUA (sα-GLUA); (d) cell specific β-GLUA (sβ-GLUA). For
621 explanation of abbreviation see Fig. 1.

622 Fig. 6. Daily changes of cell specific EEA of free living bacteria (freeB) in seawater with
623 aggregates (AGG+SW) and seawater alone (SW) during summer and autumn 2016. (a) cell

624 specific APA ($sAPA_{freeB}$); (b) cell specific AMA ($sAMA_{freeB}$). For explanation of
625 abbreviation see Fig. 1.
626 Fig. 7. (a-d) Marine snow aggregate sampled on 27 July 2016. (a) chlorophyll
627 autofluorescence (red). Notable are a dense chlorophyll aggregation of unidentifiable
628 origin (arrows) and several cyanobacterial signal (e.g. arrowhead). (b) APA as
629 demonstrated by ELF. ELF positive bacteria are dispersed across the aggregate. (c) phase
630 contrast micrograph. (d) overlay of chlorophyll signal (red), ELF signal (green) and phase
631 contrast image of the aggregate. (e-g) Marine snow aggregate sampled on 8 September
632 2014. (e) overlay of ELF signal for APA (green) and phase contrast of the aggregate
633 (grey). Notably next to a few ELF positive small eukaryotes (e.g. arrow) and ELF positive
634 bacterial cells, the entire matrix of the aggregate shows APA. (f) phase contrast
635 micrograph of the aggregate. The arrow points towards an empty diatom frustule. (g) ELF
636 signal for APA (green). Bars represent 100 μm.
637 Supplementary figure captions
638 Fig. S1. Microscopic picture (1000x magnification) of DAPI stained cells on the aggregate.
639 Aggregate surface calculated by the program AxioVision 4.7.2. is reported.
640 Fig. S2. (a) Temperature (T), (b) salinity (S) and (c) chlorophyll a (chl *a*) profiles at RV001 in
641 July (filled triangles) and November (filled circles) 2016. Sampling positions are circled.
642 Seasonal changes of (d) orthophosphate (PO_4), (e) dissolved inorganic nitrogen (DIN) and
643 (f) chl *a* for the period 1972-2017. Data measured during the aggregate collection in
644 summer and autumn 2016 are represented by filled triangles and filled circles, respectively.
645 Fig. S3. Marine snow aggregate sampled on 8 November 2016. (a) chlorophyll
646 autofluorescence (red). Notable are two chloroplast signals (arrows) and one
647 cyanobacterial signal (arrowhead). (b) APA as demonstrated by ELF. ELF positive
648 bacteria are located in the right half of the field of view demonstrating an uneven

649 distribution of ELF positive bacteria and hence APA. (c) phase contrast micrograph. (d)
650 overlay of chlorophyll signal (red), ELF signal (green) and phase contrast image of the
651 aggregate. Bars represent 100 μm.