

Seasonal Dynamics of Epiphytic Microbial Communities on Marine Macrophyte Surfaces

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Running title: Seasonal dynamics of epiphytic communities

1 Abstract

2 Surfaces of marine macrophytes are inhabited by diverse microbial communities. Most
3 studies focusing on epiphytic communities of macrophytes did not take into account temporal
4 changes or applied low sampling frequency approaches. The seasonal dynamics of epiphytic
5 microbial communities was determined in a meadow of *Cymodocea nodosa* invaded by *Caulerpa*
6 *cylindracea* and in a monospecific settlement of *Caulerpa cylindracea* at monthly intervals. For
7 comparison the ambient prokaryotic picoplankton community was also characterized. At the
8 OTU level, the microbial community composition differed between the ambient water and the
9 epiphytic communities exhibiting host-specificity. Also, successional changes were observed
10 connected to the macrophyte growth cycle. Taxonomic analysis, however, showed similar high rank
11 taxa (phyla and classes) in the ambient water and the epiphytic communities, with the exception
12 of *Desulfobacterota*, which were only found on *Caulerpa cylindracea*. *Cyanobacteria* showed
13 seasonal changes while other high rank taxa were present throughout the year. In months of high
14 *Cyanobacteria* presence the majority of cyanobacterial sequences were classified as *Pleurocapsa*.
15 Phylogenetic groups present throughout the year (e.g. *Saprosiraceae*, *Rhodobacteraceae*, members
16 without known relatives within *Gammaproteobacteria*, *Desulfatitalea* and members without known
17 relatives within *Desulfocapsaceae*) constituted most of the sequences, while less abundant taxa
18 showed seasonal patterns connected to the macrophyte growth cycle. Taken together, epiphytic
19 microbial communities of the seagrass *Cymodocea nodosa* and the macroalga *Caulerpa cylindracea*
20 appear to be host-specific and contain taxa that undergo successional changes.

21 **Introduction**

22 Marine macrophytes (seagrasses and macroalgae) are important ecosystem engineers forming
23 close associations with microorganisms belonging to all three domains of life (Egan et al., 2013;
24 Tarquinio et al., 2019). Microbes can live within macrophyte tissue as endophytes or form epiphytic
25 communities on surfaces of leaves, roots, rhizomes and thalli (Egan et al., 2013; Hollants et al.,
26 2013; Aires et al., 2015; Tarquinio et al., 2019). Epiphytic and endophytic microbial communities
27 exhibit a close functional relationship with the macrophyte host. It has been proposed that this
28 close relationship constitutes a holobiont, an integrated community where the macrophyte organism
29 and its symbiotic partners support each other (Margulis, 1991). In addition, as suggested by the
30 hologenome theory endophytic microbes play a critical role in the adaptation and evolution of the
31 host species (Aires et al., 2015).

32 Biofilms of microbial epiphytes can contain diverse taxonomic groups and harbour cell
33 abundances from 10^2 to 10^7 cells cm^{-2} (Armstrong et al., 2000; Bengtsson et al., 2010; Burke
34 et al., 2011b). In such an environment a number of positive and negative interactions between the
35 macrophyte and the colonizing microorganisms have been described (Egan et al., 2013; Hollants
36 et al., 2013; Tarquinio et al., 2019). Macrophytes can promote growth of associated microbes
37 by nutrient exudation (Wood and Hayasaka, 1981), while in return microorganisms may support
38 macrophyte performance through improved nutrient availability (Nielsen et al., 2001; de Oliveira
39 et al., 2012), phytohormone production (Matsuo et al., 2003; Celdrán et al., 2012) and protection
40 from toxic compounds (Küsel et al., 2006), oxidative stress (Sanchez-Amat et al., 2010), biofouling
41 organisms (Dobretsov and Qian, 2002) and pathogens (Penesyan et al., 2009). Besides these positive
42 interactions, macrophytes can negatively impact the associated microbes by producing reactive
43 oxygen species (Weinberger, 2007) and secondary metabolites (Saha et al., 2011).

44 All these ecological roles are carried out by a taxonomically diverse community of
45 microorganisms. At higher taxonomic ranks (phyla and classes) microbial taxa, such as

46 *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidota* and *Cyanobacteria*, have been
47 associated with surfaces of seagrass leaves and macroalgal thalli (Crump and Koch, 2008; Tujula et
48 al., 2010; Lachnit et al., 2011; Egan et al., 2013; Tarquinio et al., 2019; Ugarelli et al., 2019). While
49 similar high rank taxa have been found on surfaces of different macrophyte species, in order to
50 describe new ecological patterns it is also necessary to focus on lower taxonomic ranks (genus and
51 OTUs) which tend to be host-specific (Lachnit et al., 2011; Hollants et al., 2013; Roth-Schulze et al.,
52 2016). While the microbial community composition can vary between host species, metagenomic
53 analyses revealed that the majority of microbial functions are conserved, showing that different
54 epiphytic microbial species could be functionally similar (Burke et al., 2011a; Roth-Schulze et
55 al., 2016; Cúcio et al., 2018). This discrepancy between the microbial taxonomic and functional
56 composition might be explained by the lottery hypothesis (Sale, 1976). It postulates that an initial
57 random colonization step takes place from a set of functionally equivalent taxonomic groups
58 resulting in taxonomically different epiphytic communities sharing a core set of functional genes
59 (Burke et al., 2011a; Stratil et al., 2013; Schmidt et al., 2015; Roth-Schulze et al., 2016).

60 Seagrasses are known to form close relationships with microbial communities associated with
61 the surfaces of leaves, roots and rhizomes (Cúcio et al., 2016; Crump et al., 2018; Ugarelli et
62 al., 2019; Ettinger and Eisen, 2020; Wang et al., 2020). For different seagrass species a distinct
63 microbial community from ambient seawater or bulk sediment has been reported, however no
64 species specific communities have been found (Cúcio et al., 2016; Crump et al., 2018; Ugarelli et al.,
65 2019). It seems that seagrasses are selecting the associated microbial community but these microbes
66 have not coevolved with their seagrass plant host. Similar to seagrasses, siphonous macroalgae
67 of the genus *Caulerpa* are also closely associated with their microbial communities (Aires et al.,
68 2013, 2015; Rizzo et al., 2016b; Stabili et al., 2017; Morrissey et al., 2019). While some studies
69 have found similar culturable bacterial groups associated with the surface of a *Caulerpa* species
70 from different geographic locations (Stabili et al., 2017), others have reported large compositional
71 differences that were mainly attributed to different host species of this genus, biogeography and
72 nutrient levels (Morrissey et al., 2019) again raising the question to which extent are associated

73 communities host-specific.

74 Since marine macrophytes in temperate zones are exhibiting seasonal changes in growth and
75 physiology (Agostini et al., 2003; Najdek et al., 2020) it is important to verify if and how surface
76 associated microbial communities are affected by these changes. The majority of studies describing
77 macrophyte epiphytic microbial communities have not included possible seasonal changes (Crump
78 and Koch, 2008; Lachnit et al., 2009; Burke et al., 2011b; Roth-Schulze et al., 2016; Ugarelli et
79 al., 2019). If seasonal changes have been taken into account, low temporal frequency, applied
80 methodologies and/or limited number of analysed host species did not allow for a detailed taxonomic
81 analysis (Tujula et al., 2010; Lachnit et al., 2011; Bengtsson et al., 2012; Michelou et al., 2013;
82 Miranda et al., 2013; Mancuso et al., 2016). In the present study we performed a descriptive analysis
83 of seasonal bacterial and archaeal community dynamics on the surfaces of the seagrass *Cymodocea*
84 *nodososa*, an abundant seagrass species in the Mediterranean (Short et al., 2001), and siphonous
85 macroalga *Caulerpa cylindracea*, one of the most invasive macroalgal species (Klein and Verlaque,
86 2008; Boudouresque et al., 2009). Bacterial and archaeal epiphytes were sampled in a meadow of
87 *C. nodosa* invaded by the invasive *C. cylindracea* and in a locality of only *C. cylindracea* located in
88 the proximity of the seagrass meadow. For comparison, the microbial community of the ambient
89 seawater was also characterized. The presence of both macrophytes in the same area enabled (i)
90 the assessment of differences in the bacterial and archaeal communities between host species and
91 settlements of *C. cylindracea* and (ii) the evaluation of differences between surface associated and
92 free living (ambient seawater) communities. In addition, these differences were evaluated on a
93 monthly scale providing insight into seasonal changes (iii).

94 **Materials and methods**

95 **Sampling**

96 Sampling was performed in the Bay of Funtana, northern Adriatic Sea ($45^{\circ}10'39''$ N, $13^{\circ}35'42''$ E). The sea floor in the bay is partly covered by the invasive macroalga *C. cylindracea* that can be found in a monospecific settlement or mixed with the seagrass *C. nodosa* (Figure 1). *C. nodosa* leaves were retrieved from a meadow of *C. nodosa* invaded by the invasive *C. cylindracea* (mixed settlement; depth, 2 – 2.5 m), while *C. cylindracea* thalli were sampled in the same invaded meadow (mixed settlement; depth, 2 – 2.5 m) and in a monospecific settlement (depth, 1 – 1.5 m) of *C. cylindracea* located in the proximity (20 – 50 m) of the invaded meadow at approximately monthly intervals from November 2017 to October 2018 (Table S1). Leaves and thalli were collected by diving and transported to the laboratory in containers placed on ice and filled with seawater collected at the sampling site. Upon arrival to the laboratory, *C. nodosa* leaves were cut into sections of 1 – 2 cm, while *C. cylindracea* thalli were cut into 5 – 8 cm long sections. Leaves and thalli were washed three times with sterile artificial seawater (ASW) to remove loosely attached microbial cells. Ambient seawater was collected in 10 l containers by diving and transported to the laboratory where 10 – 20 l were filtered through a 20 μm net. The filtrate was further sequentially filtered through 3 μm and 0.2 μm polycarbonate membrane filters (Whatman, United Kingdom) using a peristaltic pump. Filters were briefly dried at room temperature and stored at -80°C . Seawater samples were also collected approximately monthly from July 2017 to October 2018.

113 **DNA isolation**

114 DNA from surfaces of *C. nodosa* and *C. cylindracea* was isolated from a pool of leaves or thalli 115 on the sampling day using a previously modified and adapted protocol that allows for a selective 116 epiphytic DNA isolation (Massana et al., 1997; Korlević et al., 2021). Briefly, leaves and thalli were

117 incubated in a lysis buffer and treated with lysozyme and proteinase K. Following the incubations,
118 the mixture containing lysed epiphytic cells was separated from the leaves and thalli and extracted
119 using phenol-chloroform. Finally, the extracted DNA was precipitated using isopropanol. DNA
120 from seawater picoplankton was extracted from 0.2 μ m polycarbonate filters according to Massana
121 et al. (1997) with a slight modification. Following the phenol-chloroform extraction, 1/10 of
122 3 M sodium acetate (pH 5.2) was added. DNA was precipitated by adding 1 volume of chilled
123 isopropanol, incubating the mixtures overnight at -20 °C and centrifuging at 20,000 \times g and 4 °C
124 for 20 min. The pellet was washed twice with 500 μ l of chilled 70 % ethanol and centrifuged after
125 each washing step at 20,000 \times g and 4 °C for 5 min. Dried pellets were re-suspended in 50 – 100 μ l
126 of deionized water.

127 **Illumina 16S rRNA sequencing**

128 Illumina MiSeq sequencing of the V4 region of the 16S rRNA gene was performed as described
129 previously (Korlević et al., 2021). The V4 region of the 16S rRNA gene was amplified using a
130 two-step PCR procedure. In the first PCR, the 515F (5'-GTGYCAGCMGCCGCGTAA-3') and
131 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers from the Earth Microbiome Project (<https://earthmicrobiome.org/protocols-and-standards/16s/>) were used (Caporaso et al., 2012; Apprill et al.,
132 2015; Parada et al., 2016). These primers contained on their 5' end a tagged sequence. Purified PCR
133 products were sent for Illumina MiSeq sequencing at IMGM Laboratories, Martinsried, Germany.
134 Prior to sequencing at IMGM, the second PCR amplification of the two-step PCR procedure was
135 performed using primers targeting the tagged region incorporated in the first PCR. In addition,
136 these primers contained adapter and sample-specific index sequences. Beside samples, a positive
137 and negative control for each sequencing batch was sequenced. The negative control comprised
138 PCR reactions without DNA template, while for a positive control a mock community composed
139 of evenly mixed DNA material originating from 20 bacterial strains (ATCC MSA-1002, ATCC,
140 USA) was used. Sequences obtained in this study have been deposited in the European Nucleotide
141

142 Archive (ENA) at EMBL-EBI under the accession number PRJEB37267 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB37267>).

144 **Sequence and data analysis**

145 Obtained sequences were analysed on the computer cluster Isabella (University Computing
146 Center, University of Zagreb) using mothur (version 1.43.0) (Schloss et al., 2009) according to the
147 MiSeq Standard Operating Procedure (MiSeq SOP; https://mothur.org/wiki/MiSeq_SOP) (Kozich et
148 al., 2013) and recommendations provided by the Riffomonas project to enhance data reproducibility
149 (<http://www.riffomonas.org/>). For alignment and classification of sequences the SILVA SSU Ref
150 NR 99 database (release 138; <http://www.arb-silva.de>) was used (Quast et al., 2013; Yilmaz et al.,
151 2014). Sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97
152 %.

153 Pipeline data processing and visualization was done using R (version 3.6.0) (R Core Team,
154 2019) combined with packages vegan (version 2.5-6) (Oksanen et al., 2019), tidyverse (version
155 1.3.0) (Wickham, 2017; Wickham et al., 2019) and multiple other packages (Neuwirth, 2014;
156 Xie, 2014, 2015, 2019a, 2019b, 2019c; Xie et al., 2018; Allaire et al., 2019; Wilke, 2019; Zhu,
157 2019). Observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity index and
158 Inverse Simpson index were calculated after normalization to the minimum number of reads per
159 sample using vegan's function **rrarefy** to account for different sequencing depths (Oksanen et
160 al., 2019). Chao1 and ACE estimators were calculated using vegan's function **estimateR**, while
161 Shannon and Inverse Simpson diversity indices were retrieved using vegan's function **diversity**
162 (Oksanen et al., 2019). To express both diversity indices in terms of effective number of OTUs the
163 exponential of the Shannon diversity index was calculated (Jost, 2006). The proportions of shared
164 OTUs and communities between samples and community types (seawater, *C. nodosa* [mixed],
165 *C. cylindracea* [mixed] and *C. cylindracea* [monospecific]) were expressed as the Jaccard's (on
166 presence/absence data) and Bray-Curtis similarity coefficient, respectively. The coefficients were

167 calculated on the OTU data table using vegan's function `vegdist` and converted from dissimilarities
168 to similarities (Borcard et al., 2011; Legendre and Legendre, 2012; Oksanen et al., 2019). The
169 Principal Coordinates Analysis (PCoA) was performed on Bray-Curtis dissimilarities based on
170 OTU abundances using the function `cmdscale` (Borcard et al., 2011; Legendre and Legendre,
171 2012). Differences between communities were tested by performing the Analysis of Similarities
172 (ANOSIM) using the vegan's function `anosim` and 1000 permutations (Oksanen et al., 2019), while
173 differences in relative contributions or proportions of shared OTUs and communities were tested
174 by applying the Mann–Whitney U test using the function `wilcox.test`. In addition, differences
175 between community type estimators or indices were tested by performing the Kruskal-Wallis H
176 test (function `kruskal.test`) followed by a pairwise comparison using the Mann-Whitney U test
177 (function `pairwise.wilcox`). Bonferroni correction was used to address the problem of multiple
178 comparisons.

179 A total of 1.7 million sequences after quality curation and exclusion of sequences without
180 known relatives (no relative sequences) and eukaryotic, chloroplast and mitochondrial sequences
181 were obtained (Table S1). The number of reads per sample ranged between 8,408 and 77,463
182 sequences (Table S1). Even when the highest sequencing effort was applied the rarefaction curves
183 did not level off as commonly observed in high-throughput 16S rRNA amplicon sequencing
184 approaches (Figure S1). Following quality curation and exclusion of sequences as mentioned above
185 reads were clustered into 28,750 different OTUs. Read numbers were normalized to the minimum
186 number of sequences (8,408, Table S1) using previously mentioned vegan's function `rrarefy`
187 resulting in 17,201 different OTUs ranging from 352 to 2,062 OTUs per sample (Figure S2). Based
188 on the ATCC MSA-1002 mock community included in the analysis an average sequencing error
189 rate of 0.01 % was determined, which is in line with previously reported values for next-generation
190 sequencing data (Kozich et al., 2013; Schloss et al., 2016). In addition, the negative controls
191 processed together with the samples yielded only 2 sequences after sequence quality curation. The
192 detailed analysis procedure including the R Markdown file is available as a GitHub repository
193 (https://github.com/MicrobesRovinj/Korlevic_EpiphyticDynamics_FrontMicrobiol_2021).

194 **Results**

195 A total of 35 samples originating from epiphytic archaeal and bacterial communities associated
196 with surfaces of the seagrass *C. nodosa* and the macroalga *C. cylindracea* were analysed. In addition,
197 18 samples (one of the samples was sequenced twice) originating from the ambient seawater were
198 also processed for comparison. Generally, richness estimators and diversity indices showed similar
199 trends. On average, higher values were found for *C. cylindracea* (mixed [Number of OTUs, 1,688.4
200 \pm 136.6 OTUs] and monospecific [Number of OTUs, 1,750.4 \pm 165.7 OTUs]) than for *C. nodosa*
201 (Number of OTUs, 1,063.7 \pm 210.6 OTUs) and lowest values were obtained for the microbial
202 community of the ambient seawater (Number of OTUs, 531.0 \pm 143.9 OTUs) (Kruskal-Wallis, $p <$
203 0.0001) (Figure S2 and Tables S2 and S3). Temporal changes did not reveal such large dissimilarities.
204 *C. nodosa* communities showed a slow increase in all calculated richness estimators towards the
205 end of the study, while *C. cylindracea* (mixed and monospecific) communities were characterized
206 by slightly higher values in spring and summer than in autumn and winter (Figure S2).

207 A clear separation between ambient seawater and surface associated communities was found
208 (Figure 2). In addition, a separation of epiphytic bacterial and archaeal communities based on
209 host species was detected. This separation was further supported by ANOSIM ($R = 0.96$, $p < 0.001$). The highest proportion of shared OTUs and community was found between mixed
210 and monospecific *C. cylindracea* (Jaccard, 0.35; Bray-Curtis, 0.77), while lower shared values
211 were calculated between ambient seawater and epiphytic communities (Jaccard, 0.10 – 0.11;
212 Bray-Curtis, 0.05 – 0.06). Shared proportions of OTUs and communities between *C. nodosa* and
213 *C. cylindracea* (either mixed or monospecific) were approximately in-between the values obtained
214 for the comparison of ambient seawater with all other communities and for the comparison of the
215 mixed and monospecific *C. cylindracea* associated community. Seasonal changes of *C. nodosa*
216 associated communities indicated a separation between spring, summer and autumn/winter samples
217 (ANOSIM, $R = 0.56$, $p < 0.01$). For *C. cylindracea* associated communities a separation between
218 summer and autumn/winter/spring samples was observed that was, however, not as strong as for *C.*

nodosa associated communities (ANOSIM, $R = 0.30$, $p < 0.05$) (Figure 2). Shared proportions of OTUs between consecutive sampling points were lower for ambient seawater ($19.6 \pm 2.5 \%$) than for *C. nodosa* ($28.3 \pm 5.2 \%$) and *C. cylindracea* (mixed [$26.3 \pm 2.1 \%$] and monospecific [$27.2 \pm 2.0 \%$]) associated communities ($p < 0.0001$), while mean proportions of shared communities between consecutive sampling points did not show such a difference (seawater, $57.4 \pm 14.7 \%$; *C. nodosa*, $53.4 \pm 9.3 \%$; *C. cylindracea* [mixed], $55.0 \pm 7.0 \%$; *C. cylindracea* [monospecific], $55.1 \pm 5.2 \%$) ($p = 0.1$), although in ambient seawater higher fluctuations could be observed (Figure 3). In addition, only $0.4 - 1.0 \%$ of OTUs from each surface associated community were present at all seasons. These persistent OTUs constituted a high proportion of total sequences ($40.2 - 53.2 \%$) and were mainly contributing to abundant phylogenetic groups present throughout the year, e.g. the no relative *Rhodobacteraceae* in the case of *C. nodosa* or taxa within *Desulfobacterota* in the case of *C. cylindracea* (see below) (Table S4).

The taxonomic composition of both, macrophyte associated and ambient seawater community, was dominated by bacterial ($99.1 \pm 2.1 \%$) over archaeal sequences ($0.9 \pm 2.1 \%$) (Figure 4). Higher relative abundances of chloroplast related sequences were only observed in surface associated communities, with higher values in autumn/winter ($37.2 \pm 11.2 \%$) than in spring/summer ($20.9 \pm 9.7 \%$) ($p < 0.0001$) (Figure S3). Generally, at higher taxonomic ranks (phylum-class), epiphytic and ambient seawater microbial communities were composed of similar bacterial taxa. Ambient seawater communities were mainly comprised of *Actinobacteriota*, *Bacteroidota*, *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobiota*. Communities associated with *C. nodosa* consisted additionally of *Planctomycetota* contributing more in summer 2018 than in other seasons. In addition, communities from mixed and monospecific *C. cylindracea* were similar and characterized by the same groups as ambient seawater and *C. nodosa* communities with the addition of *Desulfobacterota* (Figure 4). Larger differences between environments and host species were observed at lower taxonomic ranks (Figures 5 – 9).

Cyanobacteria related sequences comprised, on average, $5.5 \pm 4.4 \%$ of total sequences

246 (Figure 5). Higher proportions were found for *C. nodosa* ($16.4 \pm 5.3 \%$) and *C. cylindracea* mixed
247 ($7.7 \pm 3.9 \%$) and monospecific ($7.8 \pm 2.4 \%$) associated communities in autumn ($p < 0.0001$)
248 and for ambient seawater communities in winter ($8.8 \pm 7.5 \%$). Large taxonomic differences
249 between surface associated and ambient seawater cyanobacterial communities were observed.
250 Ambient seawater communities were mainly comprised of *Cyanobium* and *Synechococcus*, while
251 surface associated communities were comprised of *Pleurocapsa* and sequences within the class
252 *Cyanobacteriia* that could not be further classified (no relative *Cyanobacteriia*) (Figure 5). In
253 addition, seasonal changes in surface associated cyanobacterial communities were observed with
254 *Pleurocapsa* and no relative *Cyanobacteriia* comprising larger proportions of *Cyanobacteria* in
255 autumn and winter and *Acrophormium*, *Phormidesmis* and sequences without known relatives
256 within the *Nodosilineaceae* (no relative *Nodosilineaceae*) in spring and summer (Figure 5).

257 Sequences classified as *Bacteroidota* comprised, on average, $19.2 \pm 5.5 \%$ of all sequences
258 (Figure 6). Similar to *Cyanobacteria*, large differences in the taxonomic composition between
259 ambient seawater and surface associated communities were found (Figure 6). The ambient seawater
260 community was characterized by the NS4 and NS5 marine groups, uncultured *Cryomorphaceae*,
261 uncultured *Flavobacteriaceae*, NS11-12 marine group, *Balneola*, uncultured *Balneolaceae* and
262 *Formosa*. In contrast, in surface associated communities *Lewinella*, *Portibacter*, *Rubidimonas*,
263 sequences without known relatives within the *Saprospiraceae* (no relative *Saprospiraceae*),
264 uncultured *Saprospiraceae*, sequences without known relatives within the *Flavobacteriaceae*
265 (no relative *Flavobacteriaceae*) and uncultured *Rhodothermaceae* were found. Some groups
266 showed minor seasonal changes such as no relative *Flavobacteriaceae* whose sequences were
267 more abundant from November 2017 until June 2018. In contrast, uncultured *Rhodothermaceae*
268 showed higher proportions from June 2018 until the end of the study period. Surface associated
269 *Bacteroidota* communities were very diverse as observed in the high proportion of taxa clustering
270 as other *Bacteroidota* (Figure 6).

271 On average, *Alphaproteobacteria* were in comparison to the other high rank taxa the largest

272 taxonomic group, comprising 29.2 ± 12.0 % of all sequences (Figure 7). In accordance to
273 the above described taxa, large differences between ambient seawater and surface associated
274 communities were observed. Ambient seawater communities were composed mainly of the SAR11
275 clade, AEGEAN-169 marine group, SAR116 clade, sequences without known relatives within
276 the *Rhodobacteraceae* (no relative *Rhodobacteraceae*), HIMB11 and the OCS116 clade, while
277 surface associated communities were composed mainly of no relative *Rhodobacteraceae* and to
278 a lesser degree of *Pseudoahrensia*, *Amylibacter* and sequences without known relatives within
279 the *Alphaproteobacteria* (no relative *Alphaproteobacteria*) and *Hyphomonadaceae* (no relative
280 *Hyphomonadaceae*). Representatives of no relative *Rhodobacteraceae* comprised on average 54.7
281 ± 11.5 % of all alphaproteobacterial sequences in the epiphytic community (Figure 7). In addition,
282 *Amylibacter* was detected mainly in *C. nodosa* from November 2017 until March 2018.

283 Sequences related to *Gammaproteobacteria* comprised on average 18.6 ± 3.9 % of all
284 sequences (Figure 8). Similar to above mentioned taxa, large taxonomic differences between
285 ambient seawater and surface associated communities were found. Ambient seawater communities
286 were mainly comprised of the OM60 (NOR5) clade, *Litoricola*, *Acinetobacter* and the SAR86
287 clade, while epiphytic communities were mainly composed of sequences without known relatives
288 within the *Gammaproteobacteria* (no relative *Gammaproteobacteria*) and *Granulosicoccus*. Beside
289 these two groups specific to all three epiphytic communities, *C. nodosa* was characterized by
290 *Arenicella*, *Methylotenera* and sequences without known relatives within the *Burkholderiales* (no
291 relative *Burkholderiales*), while *Thioploca*, *Reinekea* and sequences without known relatives within
292 *Cellvibrionaceae* (no relative *Cellvibrionaceae*) were more specific to both mixed and monospecific
293 *C. cylindracea*. In addition, *Arenicella* was more pronounced in November and December 2017,
294 while no relative *Burkholderiales* and *Methylotenera* were characteristic for the period from March
295 until May 2018. For the *C. cylindracea* specific taxa no relative *Cellvibrionaceae* and *Reinekea*
296 showed seasonality and were characteristic for samples originating from June to October 2018.
297 In addition, similar to *Bacteroidota*, a large proportion of the surface associated community was
298 grouped as other *Gammaproteobacteria* indicating high diversity within this group (Figure 8).

299 *Desulfobacterota* were specific for *C. cylindracea*. In the mixed and monospecific *C.*
300 *cylindracea* communities the proportion of *Desulfobacterota* was $25.7 \pm 11.2 \%$ and $24.0 \pm$
301 4.3% , respectively (Figure 9). In contrast, in ambient seawater and *C. nodosa* communities
302 the contribution of *Desulfobacterota* was only $0.1 \pm 0.08 \%$ and $1.0 \pm 0.7 \%$, respectively. In
303 *C. cylindracea* the community consisted mainly of *Desulfatitalea*, *Desulfobulbus*, *Desulfopila*,
304 *Desulforhopalus*, *Desulfotalea*, SEEP-SRB4, uncultured *Desulfocapsaceae* and sequences without
305 known relatives within the *Desulfobacteraceae* (no relative *Desulfobacteraceae*), *Desulfobulbaceae*
306 (no relative *Desulfobulbaceae*) and *Desulfocapsaceae* (no relative *Desulfocapsaceae*) (Figure 9).

307 **Discussion**

308 In the present study, we applied a selective epiphytic DNA isolation procedure based on
309 direct cellular lysis (Korlević et al., 2021) coupled with a monthly sampling and Illumina amplicon
310 sequencing to describe in detail the bacterial and archaeal communities associated with the surfaces
311 of two marine macrophytes, *C. nodosa* and *C. cylindracea*. Highest richness was observed for *C.*
312 *cylindracea* (mixed and monospecific) followed by *C. nodosa* and lowest richness was found in
313 ambient seawater microbial communities. Higher richness of microbial communities associated with
314 macrophytes than in ambient seawater has been described earlier (Mancuso et al., 2016; Ugarelli et
315 al., 2019) and could be attributed to a larger set of inhabitable microniches existing on macrophyte
316 surfaces than in the ambient seawater. The highest richness observed for *C. cylindracea* might be
317 partly due to its contact with the sediment. The stolon of *C. cylindracea* is attached to the sediment
318 surface with rhizoids and thus, the stolon and rhizoids are in direct contact with the sediment.
319 Also, studies have shown that the presence of *C. cylindracea* can alter the content and biochemical
320 composition of sedimentary organic matter (Pusceddu et al., 2016; Rizzo et al., 2017, 2020) possibly
321 further expanding the number of inhabitable microniches and thus causing the observed increase in
322 richness. Seasonal differences in richness observed for surface attached communities indicated a
323 slightly higher richness in spring and summer. This pattern could be explained by a more intense
324 macrophyte growth in these two seasons than in autumn and winter (Zavodnik et al., 1998; Ruitton et
325 al., 2005; Najdek et al., 2020). During their main growth season in spring and summer macrophytes
326 exhibit a more dynamic chemical interaction with the surface community probably causing an
327 increase in the number of inhabitable microniches (Borges and Champenois, 2015; Rickert et al.,
328 2016). Proportions of shared epiphytic OTUs between consecutive sampling points were low also
329 indicated by the proportion of OTUs ($\leq 1.0\%$) present at every sampling date (Figure 3). These
330 persistent OTUs, however, accounted for a high proportion of sequences ($\geq 40.2\%$), as is often
331 the case with similar high-frequency sampling studies (Gilbert et al., 2009; Gilbert et al., 2012).
332 In comparison to the seawater community, higher values of shared OTUs between consecutive

333 sampling points were observed for the macrophyte surface associated communities. It appears that
334 macrophyte surfaces are providing more stable conditions than the ambient seawater.

335 We observed a strong differentiation between the surface attached and ambient seawater
336 communities at the level of OTUs which is in agreement with most published studies (Burke et
337 al., 2011b; Michelou et al., 2013; Mancuso et al., 2016; Roth-Schulze et al., 2016; Crump et al.,
338 2018; Ugarelli et al., 2019; Sanders-Smith et al., 2020). This indicates that marine macrophytes
339 are selecting microorganisms from the pool of microbial taxa present in the ambient seawater,
340 modifying the microbial community once the macrophyte associated microbial biofilm develops
341 (Salaün et al., 2012; Michelou et al., 2013). In addition, similar to the study of Roth-Schulze
342 et al. (2016) seagrass and macroalgae specific microbial communities were identified, while no
343 difference between *C. cylindracea* settlements was observed indicating that seagrass and macroalgae
344 specific metabolism is involved in the selection and development of the associated biofilm. At the
345 level of OTUs seasonal changes of *C. nodosa* and *C. cylindracea* associated communities were
346 identified that could be linked to the growth cycle of the seagrass and macroalgae (Agostini et al.,
347 2003; Najdek et al., 2020). *C. nodosa* was characterized by a spring community during maximum
348 seagrass proliferation, a summer community during the highest standing stock of *C. nodosa* and an
349 autumn/winter community during the decay of seagrass biomass. In contrast, *C. cylindracea* started
350 to proliferate in late spring and was characterized only by a summer community during high growth
351 rates and by an autumn/winter/spring community when the biomass was at the peak and decaying
352 thereafter. Similar seasonal changes in the epiphytic community have also been described for other
353 macroalgae (Tujula et al., 2010; Lachnit et al., 2011).

354 The taxonomic analysis showed higher chloroplast sequence abundances in autumn/winter
355 than in spring/summer. This pattern is not surprising as seagrasses harbour more algal epiphytes
356 during autumn/winter than in spring/summer (Reyes and Sansón, 2001). Furthermore, we used
357 an adapted DNA isolation protocol that is known to partially co-extract DNA from planktonic
358 eukaryotes (Korlević et al., 2015). In general, the taxonomic analysis identified epiphytic

359 phylogenetic groups present throughout the year comprising most of the reads, and taxa present
360 in lower proportions showing seasonal patterns. The first group was comprised of members
361 of the *Bacteroidota* family *Saprospiraceae*, the alphaproteobacterial *Rhodobacteraceae* and
362 *Hyphomonadaceae*, the gammaproteobacterial genus *Granulosicoccus*, sequences without known
363 relatives within *Gammaproteobacteria* and various taxa within *Desulfobacterota* (Figures 6 – 9).
364 All these groups were found on all host species, with the exception of *Desulfobacterota* that
365 was characteristic for *C. cylindracea*. In addition, the persistence of *Rhodobacteraceae* in the
366 case of *C. nodosa* and *Desulfobacterota* in the case of *C. cylindracea* could be observed in the
367 taxonomic classification of OTUs present at every sampling date. Within the *Bacteroidota* different
368 groups within *Saprospiraceae* (e.g. *Lewinella*, *Portibacter* and *Rubidimonas*) were identified to be
369 persistent. It has been suggested that members of this family are important in the hydrolysis and
370 utilization of complex organic sources (McIlroy and Nielsen, 2014). Surface attached life style
371 would be beneficial to these microbes as they could thrive on products of host cellular breakdown
372 or by-products of host metabolism, so it not surprising that they are often found associated with
373 macrophyte surfaces (Burke et al., 2011b; McIlroy and Nielsen, 2014; Crump et al., 2018).
374 *Rhodobacteraceae* are often detected on macrophyte surfaces and usually are one of the most
375 abundant groups (Burke et al., 2011b; Michelou et al., 2013; Mancuso et al., 2016). The functional
376 association between macrophytes and members of this groups is difficult to assess based on 16S
377 rRNA analysis as this family is phenotypically, metabolically, and ecologically very diverse (Pujalte
378 et al., 2014). However, some interesting metabolic capacities linked to this group were described.
379 Genomic analysis of *Rhodobacteraceae* strains and metatranscriptomic sequencing of seagrass
380 microbiomes revealed the potential for biosynthesis of indole-3-acetic acid (IAA), a plant hormone
381 (Simon et al., 2017), indicating a possible intake by seagrasses. However, another study found no
382 effect of IAA on *C. nodosa* growth showing the complexity of macrophyte–microbes interactions
383 (Muñoz, 1995). Another persistent alphaproteobacterial family was the *Hyphomonadaceae*, a group
384 that contain species with stalks used to attach cells to different surfaces (Abraham and Rohde,
385 2014). This group has been previously associated with seagrass surfaces (Weidner et al., 2000)

386 and it is believed that possessing stalks could be an advantage to keep the cells in the proximity of
387 exudate excreted by the host (Weidner et al., 2000; Abraham and Rohde, 2014).

388 Within the *Gammaproteobacteria*, sequences without known representatives were the most
389 pronounced group present throughout the year. *Gammaproteobacteria* are often a major constituent
390 of macrophyte epiphytic communities (Burke et al., 2011b; Michelou et al., 2013; Crump et al.,
391 2018). A study has attributed the expression of enzymes for the degradation of galactose-based
392 algal polymers to this class indicating their possible involvement into epibiotic algal biofilm control
393 (Crump et al., 2018). In addition, *Granulosicoccus* was also found in almost all samples. A species
394 of this genus has been isolated from the leaf surface of the seagrass *Zostera marina* (Kurilenko et al.,
395 2010), while sequences related to this genus have been found on the surfaces of macroalgae (Lachnit
396 et al., 2011; Bengtsson et al., 2012), including *C. cylindracea* (Rizzo et al., 2016a), indicating this
397 group preference for macrophyte surfaces. It is possible that bacteria of this genus can thrive on
398 exudates of different macrophytes as it is known from cultivated members that they can utilise
399 various sugars and amino acids (Ivanova and Webb, 2014). The presence of *Desulfobacterota* only
400 on *C. cylindracea* is to be expected as part of the epiphytic community is in direct contact with the
401 sediment. The *Desulfobacterota* community was comprised of known sulphate sediment groups
402 such as the *Desulfatitalea* and no relative *Desulfocapsaceae* (Kuever, 2014; Higashioka et al., 2015).
403 Sequences related to sulphur cycling bacterial groups have been found in *Caulerpa* endophytic
404 and epiphytic communities (Aires et al., 2013). It is possible that these groups are involved into
405 enhanced sulphate reduction rates observed in sediments underlying *Caulerpa* settlements causing
406 unsuitable conditions to sulphide-sensitive seagrasses (Holmer et al., 2009).

407 The only high rank taxonomic group showing strong seasonal fluctuations was *Cyanobacteria*.
408 Cyanobacterial sequences were more pronounced in November and December than in spring and
409 summer. In the months of high cyanobacterial sequence abundances the majority of sequences from
410 this group were classified as *Pleurocapsa*, a group known to colonize different living and non-living
411 surfaces (Burns et al., 2004; Longford et al., 2007; Mobberley et al., 2012; Reisser et al., 2014;

412 Kolda et al., 2020). While we observed a strong temporal pattern for this group, a study of surface
413 sediment cyanobacterial communities did not find any seasonal dynamics for *Pleurocapsa* (Kolda
414 et al., 2020), indicating a possibility that there is a reduced selection of the epiphytic community
415 by the seagrass during periods of low photosynthetic activity (Zavodnik et al., 1998), causing
416 leaves to become a suitable surface for non-specific colonizers. Beside all these groups comprising
417 most of the sequences, a set of taxa present in lower proportions and showing seasonal patterns
418 was identified. This group was comprised of e.g. *Bacteroidota* sequences without known relatives
419 within *Flavobacteriaceae* and *Rhodothermaceae*, the alphaproteobacterial *Amylibacter* and the
420 gammaproteobacterial *Methylotenera*, *Reinekea* and sequences without known relatives within
421 *Cellvibrionaceae* (Figures 6 and 8).

422 It is possible that *Flavobacteriaceae* and *Rhodothermaceae* are occupying similar niches
423 with *Rhodothermaceae* being more adapted to higher temperatures as it is known that culturable
424 members of this family exhibit mesophilic and thermophilic characteristics (Park et al., 2014).
425 This would explain why we observed a higher presence of *Rhodothermaceae* in the warmer period
426 of the year. A strain belonging to the *Rhodobacteraceae* genus *Amylibacter* has been isolated
427 from the surface of a green macroalga indicating that members of this group can exhibit surface
428 attached life style (Nedashkovskaya et al., 2016). In addition, since this is a relatively novel genus
429 it is possible that novel taxa within *Rhodobacteraceae* will be described in the future elucidating
430 the taxonomy of the currently high proportion of *Rhodobacteraceae* sequences without known
431 relatives. The genus *Methylotenera* belongs to the methylotrophic family *Methylophilaceae*, a group
432 capable of oxidising non-methane single-carbon compounds such as methanol and methylamine
433 (Chistoserdova and Kalyuzhnaya, 2018). Interestingly, angiosperms produce methanol during
434 cell-wall synthesis (Nemecek-Marshall et al., 1995; Dorokhov et al., 2018), so it is not surprising
435 that we found members of this genus only on *C. nodosa* and in spring, during a period of maximum
436 seagrass proliferation. Other studies have also found *Methylotenera*-specific sequences associated
437 with seagrass roots and leaves indicating that this group members are important constituents of the
438 seagrass microbiome (Crump et al., 2018; Sanders-Smith et al., 2020). Genomic and physiological

439 analyses of cultivated *Cellvibrionaceae* and *Reinekea* members showed the capabilities to use
440 important algal polysaccharides (Avci et al., 2017; Xie et al., 2017) indicating their possible
441 involvement into the degradation of *C. cylindracea* polysaccharides and/or the control of its algal
442 epiphytes.

443 The epiphytic microbial community associated with marine macrophytes is undergoing
444 seasonal changes that can be attributed to the fluctuations of environmental conditions, the growth
445 cycle of macrophytes inhabiting temperate zones or the combined effect of both. In the present
446 study, we could identify in analysed high rank taxa phylogenetic groups present throughout the
447 year, comprising most of the sequences and a lower proportion of taxa showing seasonal patterns
448 connected to the macrophyte growth cycle (Figures 4 and 9). Studies focusing on functional
449 comparisons between communities associated with different hosts showed that the majority of
450 functions could be found in every community, indicating functional redundancy (Roth-Schulze
451 et al., 2016). This difference between phylogenetic variability and functional stability has been
452 explained by the lottery hypothesis assuming an initial random colonization step performed by a
453 set of functionally equivalent taxonomic groups (Burke et al., 2011a; Roth-Schulze et al., 2016).
454 It is possible that functional redundancy is a characteristic of high abundance taxa detected to be
455 present throughout the year, while seasonal and/or host-specific functions are an attribute of taxa
456 displaying successional patterns. Further studies connecting taxonomy with functional properties
457 will be required to elucidate the degree of functional redundancy or specificity in epiphytic microbial
458 communities.

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785 **Figure legends**

786 **Figure 1.** Location of the mixed (*C. nodosa* and *C. cylindracea*) and monospecific (*C. cylindracea*)
787 settlement in the Bay of Funtana, northern Adriatic Sea (© OpenStreetMap contributors,
788 www.openstreetmap.org/copyright).

789 **Figure 2.** Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU
790 abundances of bacterial and archaeal communities from the surfaces of the macrophytes *C. nodosa*
791 (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient
792 seawater. Samples from different environments or seasons are labeled in different color and
793 shape. The proportion of explained variation by each axis is shown on the corresponding axis in
794 parentheses.

795 **Figure 3.** Proportion of shared bacterial and archaeal communities (Bray-Curtis similarity
796 coefficient) and shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) between
797 consecutive sampling dates and from the surfaces of the macrophytes *C. nodosa* (mixed settlement)
798 and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater.

799 **Figure 4.** Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) bacterial
800 and archaeal sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C.*
801 *cylindracea* (mixed and monospecific settlement) and in the ambient seawater. NR – No Relative
802 (sequences without known relatives within the corresponding group)

803 **Figure 5.** Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$)
804 cyanobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and
805 *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion
806 of cyanobacterial sequences in the total bacterial and archaeal community is given above the
807 corresponding bar. NR – No Relative (sequences without known relatives within the corresponding
808 group)

809 **Figure 6.** Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) sequences
810 within the *Bacteroidota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C.*
811 *cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of
812 sequences classified as *Bacteroidota* in the total bacterial and archaeal community is given above the
813 corresponding bar. NR – No Relative (sequences without known relatives within the corresponding
814 group)

815 **Figure 7.** Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$)
816 alphaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and
817 *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of
818 alphaproteobacterial sequences in the total bacterial and archaeal community is given above the
819 corresponding bar. NR – No Relative (sequences without known relatives within the corresponding
820 group)

821 **Figure 8.** Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$)
822 gammaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement)
823 and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion
824 of gammaproteobacterial sequences in the total bacterial and archaeal community is given above the
825 corresponding bar. NR – No Relative (sequences without known relatives within the corresponding
826 group)

827 **Figure 9.** Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) sequences
828 within the *Desulfobacterota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and
829 *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion
830 of sequences classified as *Desulfobacterota* in the total bacterial and archaeal community is given
831 above the corresponding bar. NR – No Relative (sequences without known relatives within the
832 corresponding group)

833 **Figures**

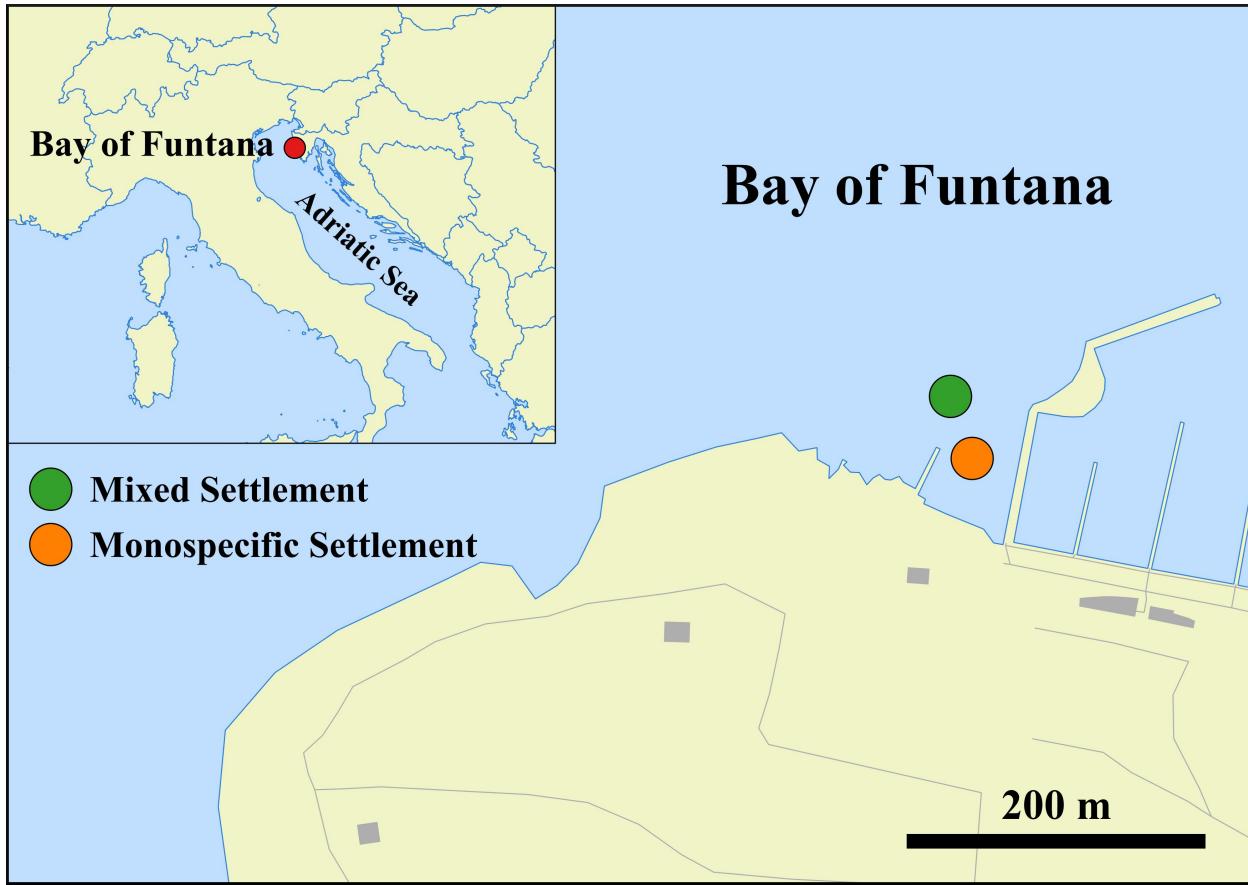


Figure 1. Location of the mixed (*C. nodosa* and *C. cylindracea*) and monospecific (*C. cylindracea*) settlement in the Bay of Funtana, northern Adriatic Sea (© OpenStreetMap contributors, www.openstreetmap.org/copyright).

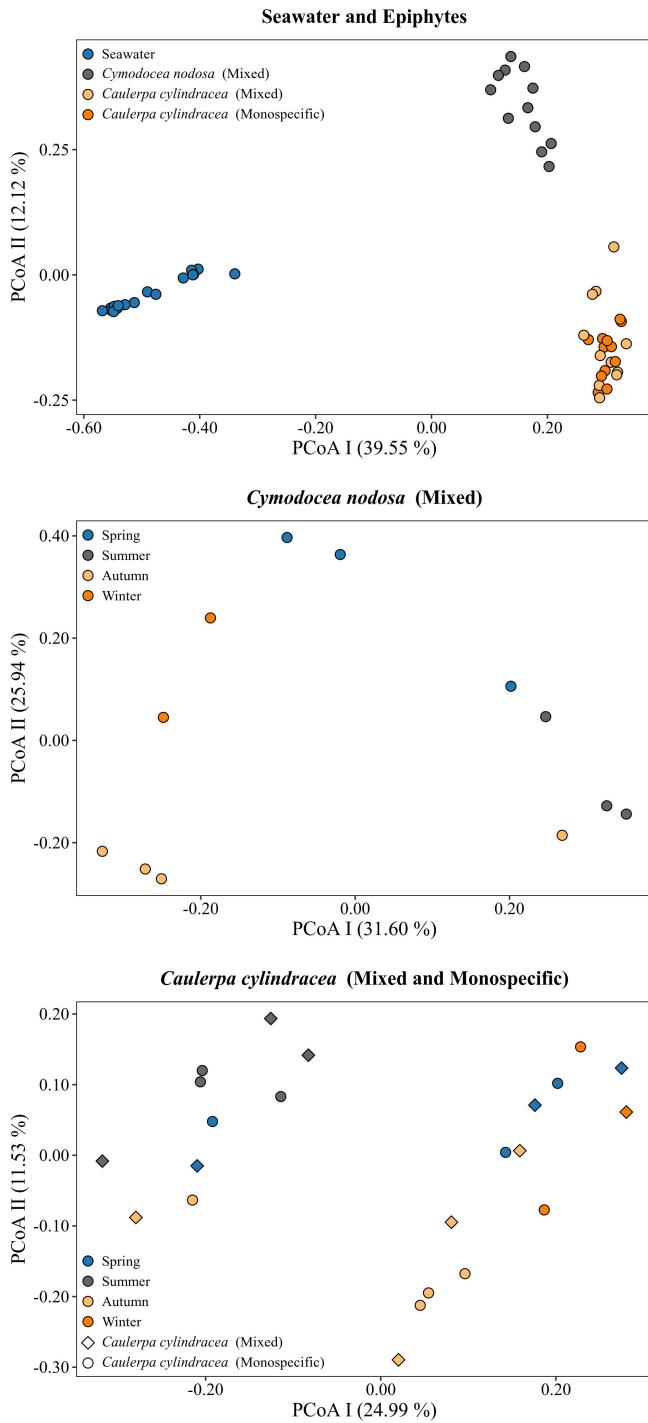


Figure 2. Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU abundances of bacterial and archaeal communities from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. Samples from different environments or seasons are labeled in different color and shape. The proportion of explained variation by each axis is shown on the corresponding axis in parentheses.

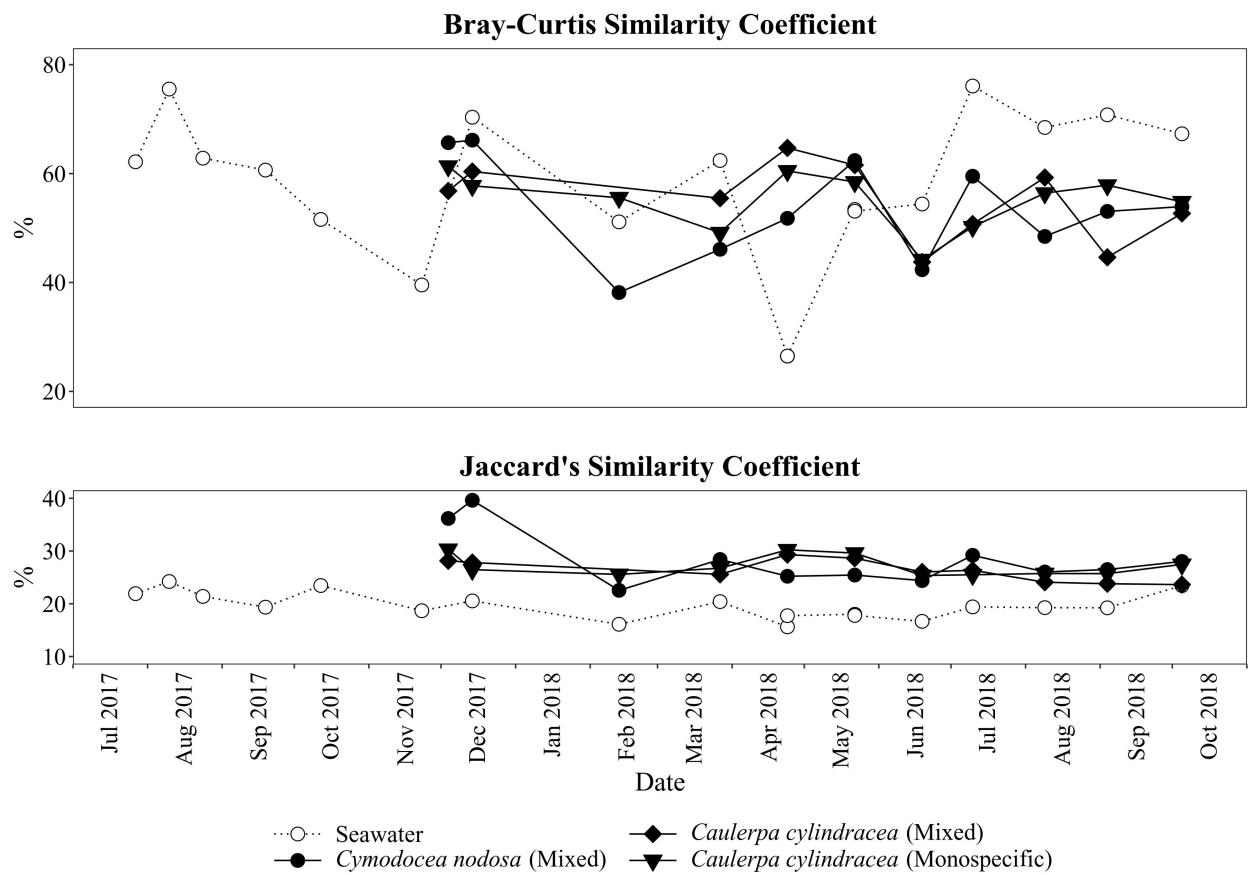


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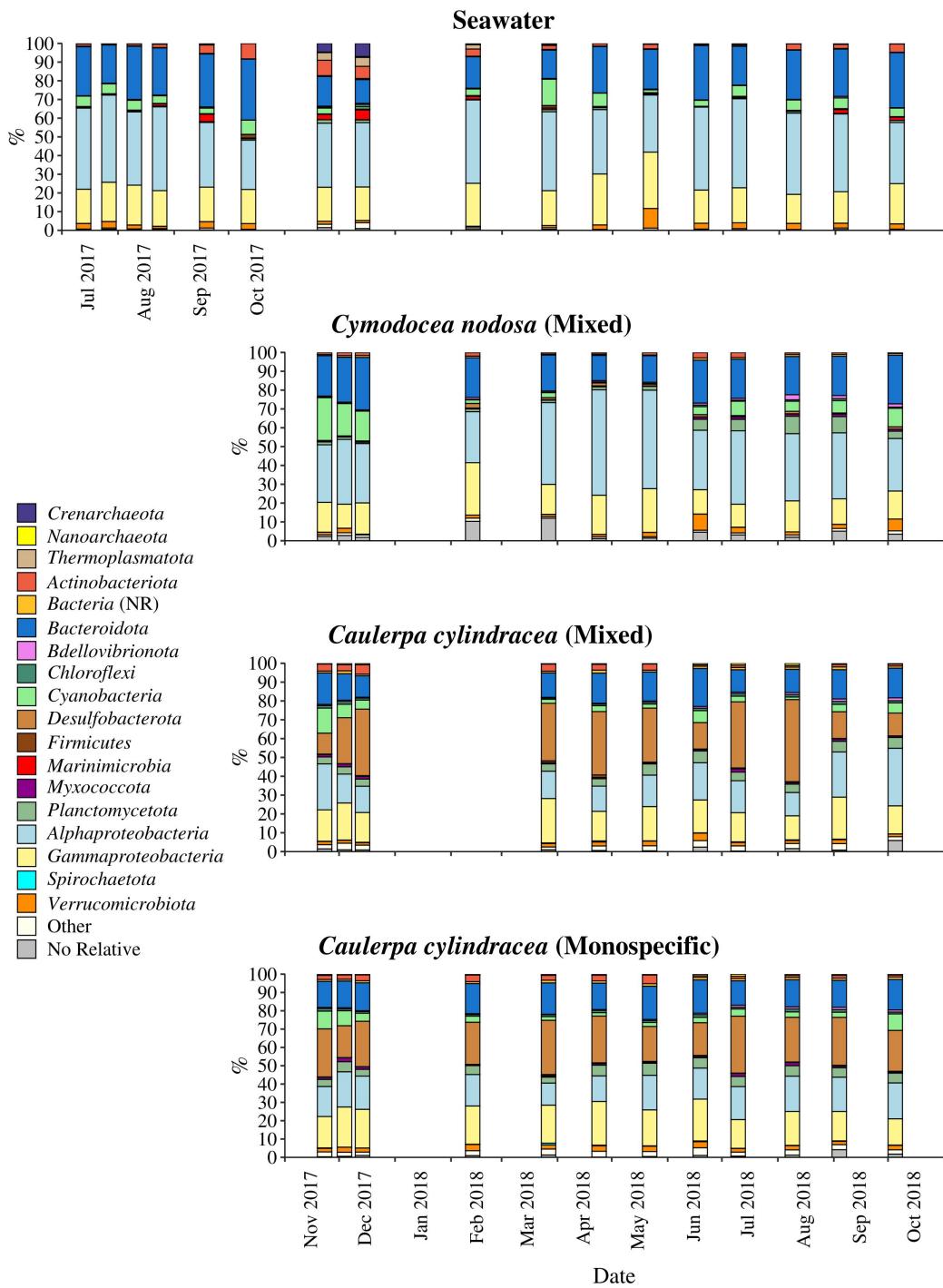


Figure 4. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) bacterial and archaeal sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. NR – No Relative (sequences without known relatives within the corresponding group)

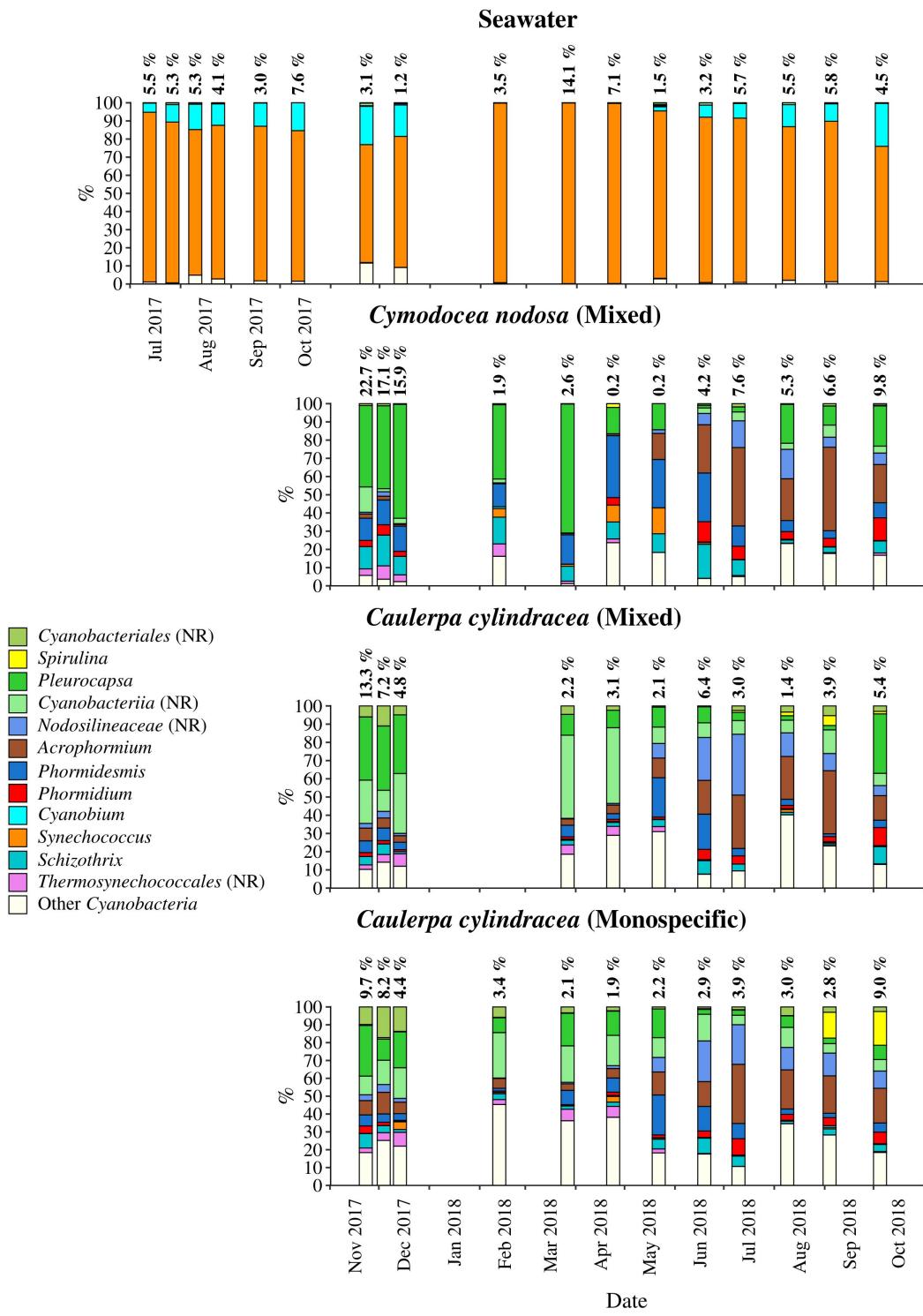


Figure 5. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) cyanobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of cyanobacterial sequences in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative (sequences without known relatives within the corresponding group)

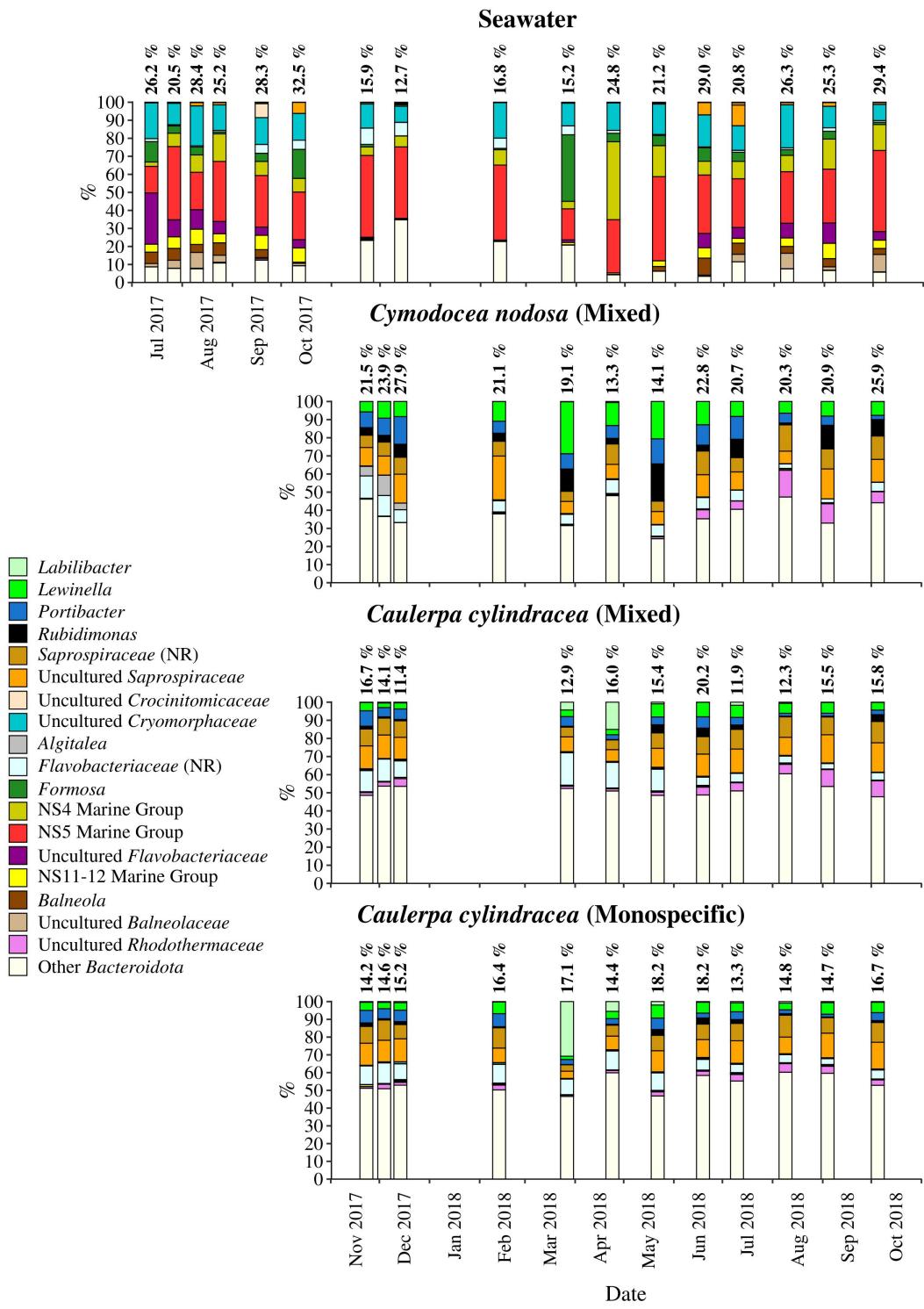


Figure 6. Taxonomic classification and relative contribution of the most abundant ($\geq 2 \%$) sequences within the *Bacteroidota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of sequences classified as *Bacteroidota* in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative (sequences without known relatives within the corresponding group)

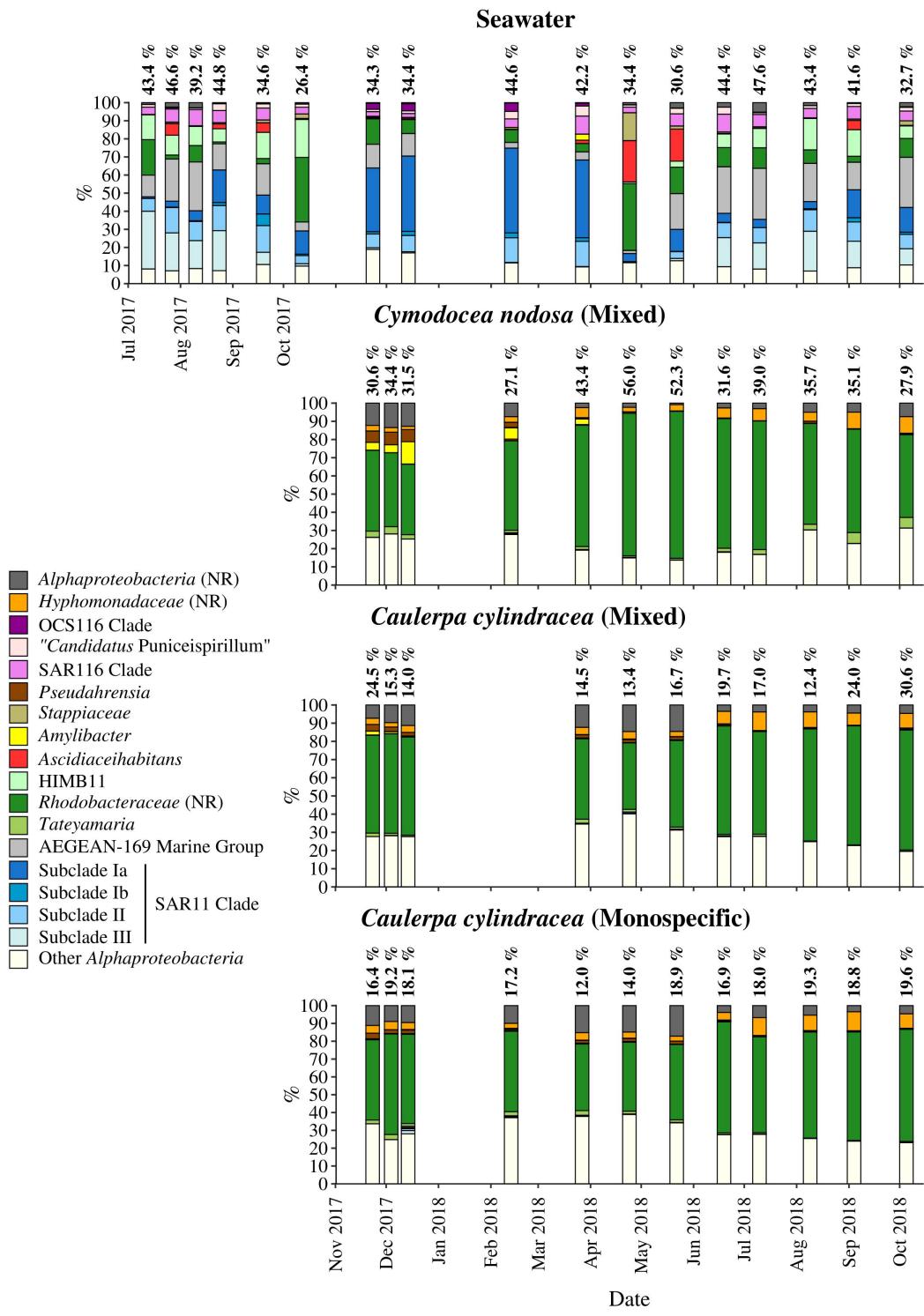


Figure 7. Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) alphaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of alphaproteobacterial sequences in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative (sequences without known relatives within the corresponding group)

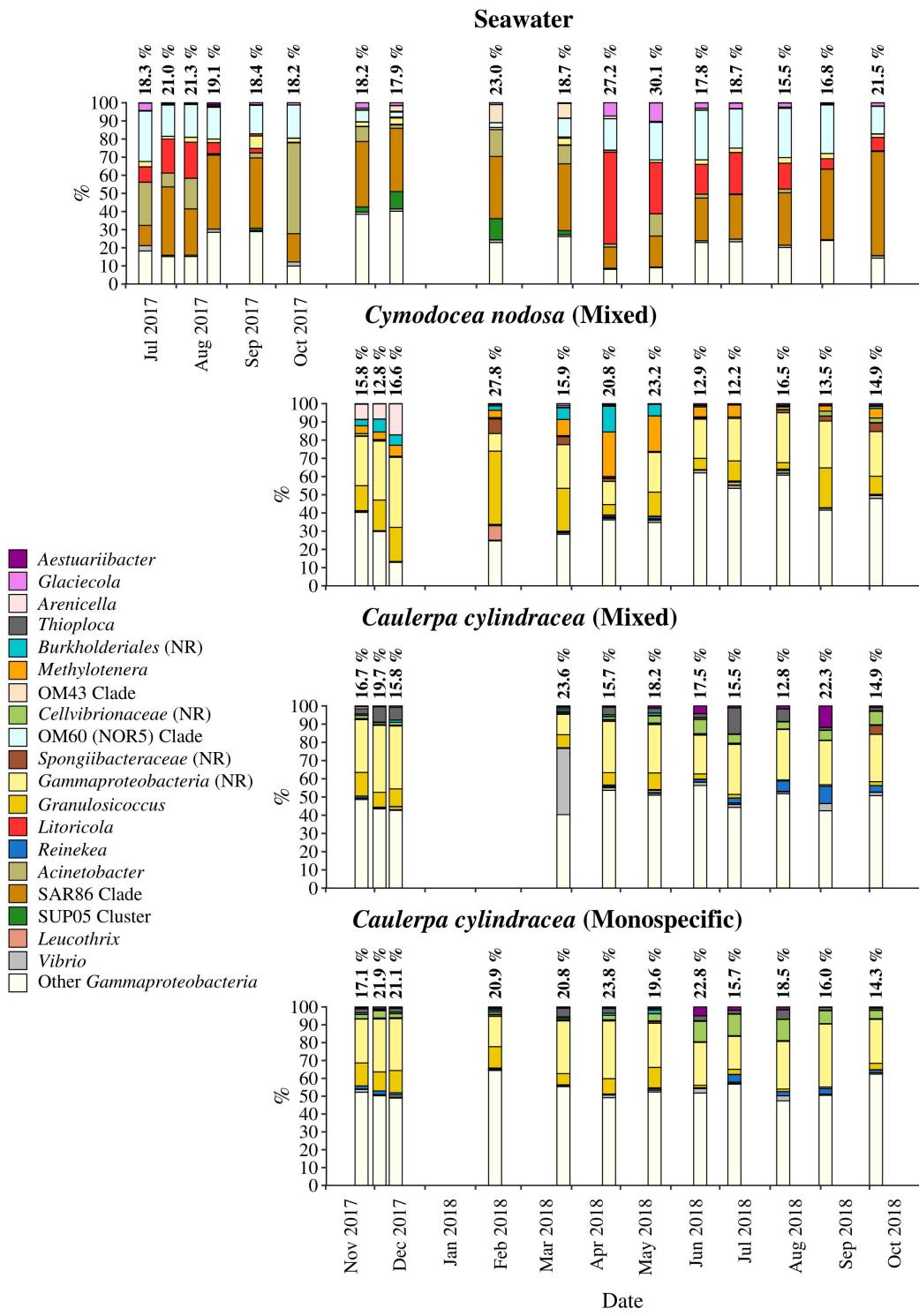


Figure 8. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) gammaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of gammaproteobacterial sequences in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative (sequences without known relatives within the corresponding group)

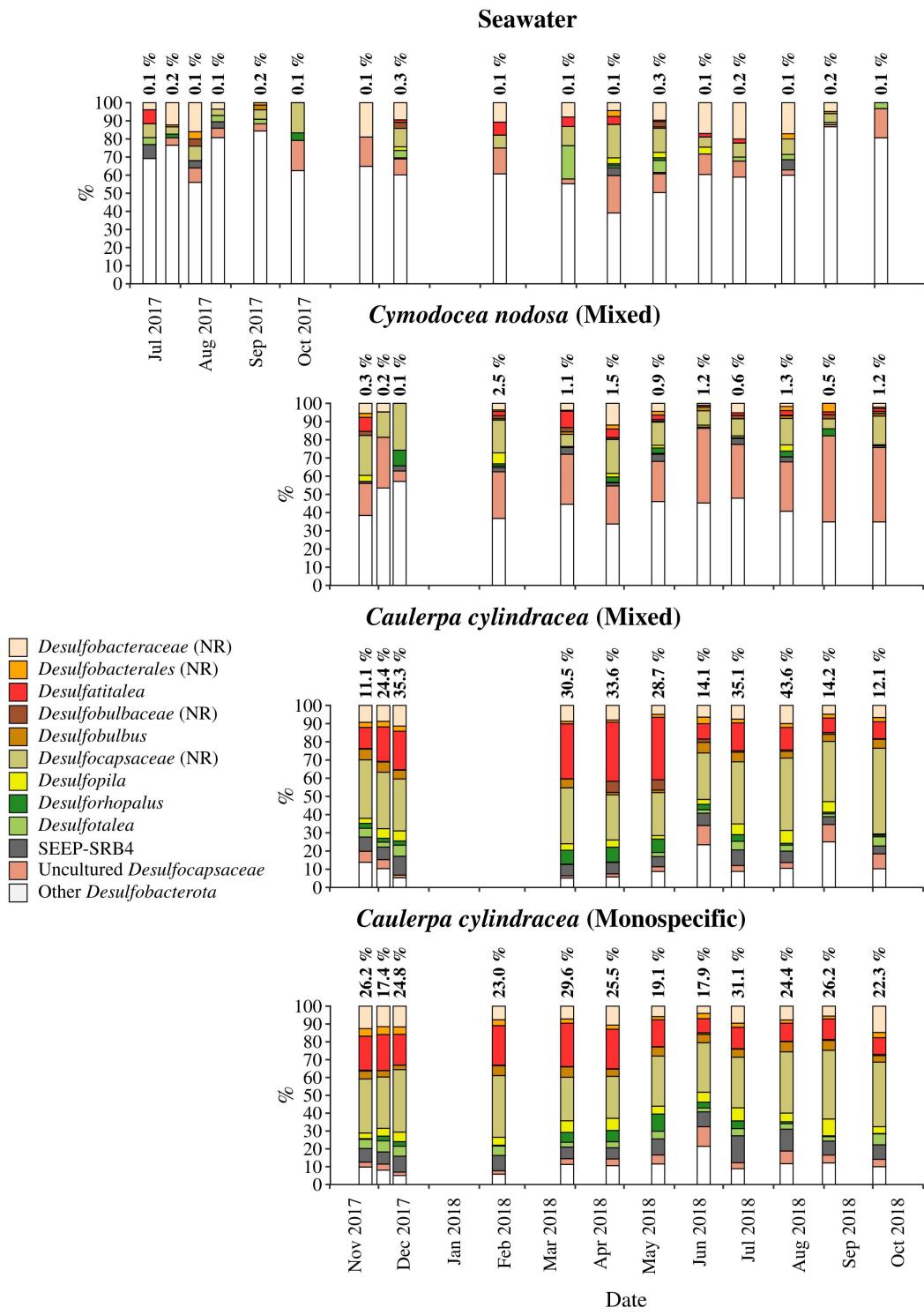


Figure 9. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) sequences within the *Desulfobacterota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of sequences classified as *Desulfobacterota* in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative (sequences without known relatives within the corresponding group)