

# **Seasonal Dynamics of Epiphytic Microbial Communities on Marine Macrophyte Surfaces**

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Running title: Seasonal dynamics of epiphytic communities

## 1 Summary

2 Surfaces of marine macrophytes (seagrasses and macroalgae) are inhabited by diverse microbial  
3 communities. Most studies focusing on epiphytic communities of macrophytes did not take into  
4 account temporal changes or applied low sampling frequency approaches. Illumina sequencing  
5 of the V4 16S rRNA region was performed to determine the seasonal dynamics of epiphytic  
6 communities of the seagrass *Cymodocea nodosa* and invasive macroalga *Caulerpa cylindracea*.  
7 Leaves and thalli were sampled in a meadow of *Cymodocea nodosa* invaded by the invasive *Caulerpa*  
8 *cylindracea* and in a monospecific settlement of *Caulerpa cylindracea* located in the proximity of  
9 the meadow at monthly intervals. For comparison the ambient prokaryotic plankton community was  
10 also characterized. At the OTU level, the microbial community composition differed between the  
11 ambient water and the epiphytic communities exhibiting host-specificity. Also, successional changes  
12 were observed connected to the macrophyte growth cycle. Taxonomic analysis, however, showed  
13 similar high rank groups in the ambient water and the epiphytic communities, with the exception  
14 of *Desulfobacterota*, which were only found on *Caulerpa cylindracea*. *Cyanobacteria* showed  
15 seasonal changes while other high rank taxa were present throughout the year. Phylogenetic groups  
16 present throughout the year constituted most of the sequences, while less abundant taxa showed  
17 seasonal patterns connected to the macrophyte growth cycle. Taken together, epiphytic microbial  
18 communities of the seagrass *Cymodocea nodosa* and the macroalgae *Caulerpa cylindracea* appear  
19 to be host-specific and contain taxa that undergo successional changes.

20 **Introduction**

21       Marine macrophytes (seagrasses and macroalgae) are important ecosystem engineers forming  
22 close associations with microorganism belonging to all three domains of life (Egan *et al.*, 2013;  
23 Tarquinio *et al.*, 2019). Microbes can live within macrophyte tissue as endophytes or can form  
24 epiphytic communities on surfaces of leaves and thalli (Egan *et al.*, 2013; Hollants *et al.*, 2013;  
25 Tarquinio *et al.*, 2019). Epiphytic and endophytic microbial communities exhibit a close functional  
26 relationship with the macrophyte host. It was proposed that this close relationship constitutes a  
27 holobiont, an integrated community where the macrophyte organism and its symbiotic partners  
28 support each other (Margulis, 1991; Egan *et al.*, 2013; Tarquinio *et al.*, 2019).

29       Biofilms of microbial epiphytes can contain diverse taxonomic groups and harbor cell  
30 abundances from  $10^2$  to  $10^7$  cells  $\text{cm}^{-2}$  (Armstrong *et al.*, 2000; Bengtsson *et al.*, 2010; Burke  
31 and Thomas *et al.*, 2011). In such an environment a number of positive and negative interactions  
32 between the macrophyte and the colonizing microorganisms have been described (Egan *et*  
33 *al.*, 2013; Hollants *et al.*, 2013; Tarquinio *et al.*, 2019). Macrophytes can promote growth of  
34 associated microbes by nutrient exudation, while in return microorganisms may support macrophyte  
35 performance through improved nutrient availability, phytohormone production and protection from  
36 toxic compounds, oxidative stress, biofouling organisms and pathogens (Egan *et al.*, 2013; Hollants  
37 *et al.*, 2013; Tarquinio *et al.*, 2019). Besides these positive interactions, macrophytes can negatively  
38 impact the associated microbes such as pathogenic bacteria by producing reactive oxygen species  
39 and secondary metabolites (Egan *et al.*, 2013; Hollants *et al.*, 2013; Tarquinio *et al.*, 2019).

40       All these ecological roles are carried out by a taxonomically diverse community of  
41 microorganisms. At higher taxonomic ranks a core set of macrophyte epiphytic taxa was described  
42 consisting mainly of *Alphaproteobacteria*, *Gammaproteobacteria*, *Desulfobacterota*, *Bacteroidota*,  
43 *Cyanobacteria*, *Actinobacteriota*, *Firmicutes*, *Planctomycetota*, *Chloroflexi* and *Verrucomicrobiota*  
44 (Crump and Koch, 2008; Tujula *et al.*, 2010; Lachnit *et al.*, 2011). In contrast, at lower taxonomic

45 ranks host specific microbial communities were found (Lachnit *et al.*, 2011; Roth-Schulze *et al.*,  
46 2016). Recently, it was shown that even different morphological niches within the same alga had a  
47 higher influence on the composition of the bacterial community than biogeography or environmental  
48 factors (Morrissey *et al.*, 2019). While the microbial community composition varies between host  
49 species, metagenomic analyses revealed that the majority of the microbial functions are conserved  
50 (Burke and Peter Steinberg *et al.*, 2011; Roth-Schulze *et al.*, 2016). This discrepancy between the  
51 microbial taxonomic and functional composition might be explained by the lottery hypothesis. It  
52 postulates that an initial random colonization step takes places from a set of functionally equivalent  
53 taxonomic groups resulting in taxonomically different epiphytic communities sharing a core set of  
54 functional genes (Burke and Peter Steinberg *et al.*, 2011; Roth-Schulze *et al.*, 2016). In addition,  
55 some of the variation in the reported data could be attributed to different techniques used in these  
56 studies, such as different protocols for epiphytic cell detachment and/or DNA isolation, as no  
57 standard protocol has been yet established to study epiphytic communities (Ugarelli *et al.*, 2019;  
58 Korlević *et al.*, submitted).

59 The majority of studies describing macrophyte epiphytic microbial communities did not include  
60 possible seasonal changes (Crump and Koch, 2008; Lachnit *et al.*, 2009; Burke and Thomas *et*  
61 *al.*, 2011; Roth-Schulze *et al.*, 2016; Ugarelli *et al.*, 2019). If seasonal changes were taken into  
62 account, low temporal frequency, applied methodologies and/or limited number of analyzed host  
63 species did not allow a high taxonomic resolution (Tujula *et al.*, 2010; Lachnit *et al.*, 2011; Miranda  
64 *et al.*, 2013; Michelou *et al.*, 2013; Mancuso *et al.*, 2016). In the present study we describe the  
65 seasonal dynamics of bacterial and archaeal communities on the surfaces of the seagrass *Cymodocea*  
66 *nodosa* and siphonous macroalgae *Caulerpa cylindracea* determined on a mostly monthly scale.  
67 Bacterial and archaeal epiphytes were sampled in a meadow of *C. nodosa* invaded by the invasive  
68 *C. cylindracea* and in a locality of only *C. cylindracea* located in the proximity of the seagrass  
69 meadow. For comparison, the microbial community of the ambient seawater was also characterized.

70 **Results**

71 Sequencing of the 16S rRNA V4 region yielded a total of 1.8 million sequences after quality  
72 curation and exclusion of sequences without known relatives (no relative sequences) and eukaryotic,  
73 chloroplast and mitochondrial sequences (Table S1). A total of 35 samples originating from  
74 epiphytic archaeal and bacterial communities associated with surfaces of the seagrass *C. nodosa*  
75 and the macroalga *C. cylindracea* were analyzed. In addition, 18 samples (one of the samples was  
76 sequenced twice) originating from the ambient seawater were also processed for comparison. The  
77 number of reads per sample ranged between 8,408 and 77,463 sequences (Table S1). Even when  
78 the highest sequencing effort was applied the rarefaction curves did not level off as commonly  
79 observed in high-throughput 16S rRNA amplicon sequencing approaches (Fig. S1). Following  
80 quality curation and exclusion of sequences as mentioned above reads were clustered into 28,750  
81 different OTUs at a similarity level of 97 %. Read numbers were normalized to the minimum  
82 number of sequences (8,408, Table S1) through rarefaction resulting in 17,087 different OTUs with  
83 361 to 2,028 OTUs per sample (Fig. S2). To determine seasonal changes in richness and diversity  
84 the observed number of OTUs, Chao1, ACE, Exponential Shannon (Jost, 2006) and Inverse Simpson  
85 were calculated after normalization through rarefaction. Generally, richness estimators and diversity  
86 indices showed similar trends. On average, higher values were found for *C. cylindracea* (mixed  
87 [Number of OTUs,  $1,681.8 \pm 124.2$  OTUs] and monospecific [Number of OTUs,  $1,746 \pm 161.4$   
88 OTUs]) than for *C. nodosa* (Number of OTUs,  $1,052.8 \pm 209.5$  OTUs) and lowest values were  
89 obtained for the microbial community of the ambient seawater (Number of OTUs,  $527.8 \pm 140.4$   
90 OTUs) (Fig. S2). Seasonal changes did not reveal such large dissimilarities. *C. nodosa* communities  
91 showed a slow increase towards the end of the study, while *C. cylindracea* (mixed and monospecific)  
92 communities were characterized by slightly higher values in spring and summer than in autumn and  
93 winter (Fig. S2).

94 To determine the proportion of shared archaeal and bacterial OTUs and communities sampled  
95 in different environments the Jaccard's Similarity Coefficient on presence-absence data and

96 Bray-Curtis Similarity Coefficient, respectively, were calculated. Coefficients were determined after  
97 normalization through rarefaction and binning of samples from the particular environment. The  
98 highest proportion of shared OTUs and community was found between mixed and monospecific *C.*  
99 *cylindracea* (Jaccard, 0.35; Bray-Curtis, 0.78), while lower shared values were calculated between  
100 ambient seawater and epiphytic communities (Fig. 1). Shared proportion between *C. nodosa* and *C.*  
101 *cylindracea* were approximately in-between the values of mixed and monospecific *C. cylindracea*.  
102 To assess seasonal changes in the proportion of shared OTUs and communities the Jaccard's and  
103 Bray-Curtis Similarity Coefficients were calculated between consecutive sampling points (Fig. 2).  
104 Both coefficients showed similar trends. Temporal proportional changes were more pronounced for  
105 ambient seawater than for *C. nodosa* and especially *C. cylindracea* associated communities (Fig. 2).  
106 In addition, only 0.4 – 1.0 % of OTUs from each surface associated community were present at  
107 all seasons. These persistent OTUs constituted a high proportion of total sequences (39.5 – 52.4  
108 %). To further disentangle the environmental and seasonal community dissimilarity a Principal  
109 Coordinates Analysis (PCoA) based on Bray-Curtis distances and OTU abundances was applied. A  
110 clear separation between ambient seawater and surface associated communities was found (Fig. 3).  
111 In addition, a separation of epiphytic bacterial and archaeal communities based on host species  
112 was detected. This separation was further supported by ANOSIM ( $R = 0.96, p < 0.001$ ). Seasonal  
113 changes of *C. nodosa* associated communities indicated a separation between spring, summer and  
114 autumn/winter samples (ANOSIM,  $R = 0.54, p < 0.01$ ). For *C. cylindracea* associated communities  
115 a separation between summer and autumn/winter/spring samples was observed that was, however,  
116 not as strong as for *C. nodosa* associated communities (ANOSIM,  $R = 0.31, p < 0.01$ ) (Fig. 3).

117 The taxonomic composition of both, macrophyte associated and ambient seawater communities  
118 was dominated by bacterial ( $99.1 \pm 2.1 \%$ ) over archaeal sequences ( $0.9 \pm 2.1 \%$ ) (Fig. 4). Higher  
119 relative abundances of chloroplast related sequences were only observed in surface associated  
120 communities, with higher values in autumn/winter ( $37.2 \pm 11.2 \%$ ) than in spring/summer  
121 ( $20.9 \pm 9.7 \%$ ) (Fig. S3). Generally, at higher taxonomic ranks (phylum-class), epiphytic and  
122 ambient seawater microbial communities were composed of similar bacterial taxa. Ambient

123 seawater communities were mainly comprised of *Actinobacteriota*, *Bacteroidota*, *Cyanobacteria*,  
124 *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobiota*. Communities associated with  
125 *C. nodosa* consisted additionally of *Planctomycetota* contributing more in summer 2018 than in  
126 other seasons. In addition, communities from mixed and monospecific *C. cylindracea* were similar  
127 and characterized by the same groups as ambient seawater and *C. nodosa* communities with the  
128 addition of *Desulfobacterota* (Fig. 4). Larger differences between environments and host species  
129 were observed at lower taxonomic ranks (Figs. 5 – 9).

130 *Cyanobacteria* related sequences comprised, on average,  $5.5 \pm 4.4\%$  of total sequences (Fig. 5).  
131 Higher proportions were found for *C. nodosa* ( $16.4 \pm 5.3\%$ ) and *C. cylindracea* mixed ( $7.7 \pm 3.9$   
132 %) and monospecific ( $7.8 \pm 2.4\%$ ) associated communities in autumn and for ambient seawater  
133 communities in winter ( $8.8 \pm 7.5\%$ ). Large taxonomic differences between surface associated  
134 and ambient seawater cyanobacterial communities were observed. Ambient seawater communities  
135 were mainly comprised of *Cyanobium* and *Synechococcus*, while surface associated communities  
136 were comprised of *Pleurocapsa* and sequences within the class *Cyanobacteriia* that could not be  
137 further classified (no relative *Cyanobacteriia*) (Fig. 5). In addition, seasonal changes in surface  
138 associated communities were observed in *Pleurocapsa* and no relative *Cyanobacteriia* comprising  
139 larger proportions in autumn and winter and *Acrophormium*, *Phormidesmis* and sequences without  
140 known relatives within the *Nodosilineaceae* (no relative *Nodosilineaceae*) in spring and summer  
141 (Fig. 5).

142 Sequences classified as *Bacteroidota* comprised, on average,  $19.2 \pm 5.5\%$  of all sequences  
143 (Fig. 6). Similarly to *Cyanobacteria*, large differences in the taxonomic composition between  
144 ambient seawater and surface associated communities were found (Fig. 6). The ambient seawater  
145 community was characterized by the NS4 and NS5 marine groups, uncultured *Cryomorphaceae*,  
146 uncultured *Flavobacteriaceae*, NS11-12 marine group, *Balneola*, uncultured *Balneolaceae* and  
147 *Formosa*. In contrast, in surface associated communities *Lewinella*, *Portibacter*, *Rubidimonas*,  
148 sequences without known relatives within the *Saprospiraceae* (no relative *Saprospiraceae*),

149 uncultured *Saprospiraceae*, (sequences without known relatives within the *Flavobacteriaceae*  
150 (no relative *Flavobacteriaceae*)and uncultured *Rhodothermaceae* were found. Some groups  
151 showed minor seasonal changes such as no relative *Flavobacteriaceae* whose sequences were  
152 more abundant from November 2017 until June 2018. In contrast, uncultured *Rhodothermaceae*  
153 showed higher proportions from June 2018 until the end of the study period. Surface associated  
154 *Bacteroidota* communities were very diverse as observed in the high proportion of taxa clustering  
155 as other *Bacteroidota* (Fig. 6).

156 On average, *Alphaproteobacteria* were in comparison to the other high rank taxa the largest  
157 taxonomic group, comprising  $29.2 \pm 12.0$  % of all sequences (Fig. 7). In accordance to the above  
158 described taxa, large differences between ambient seawater and surface associated communities  
159 were observed. Ambient seawater communities were composed mainly of the SAR11 clade,  
160 AEGEAN-169 marine group, SAR116 clade, sequences without known relatives within the  
161 *Rhodobacteraceae* (no relative *Rhodobacteraceae*), HIMB11 and the OCS116 clade, while  
162 surface associated communities were composed mainly of no relative *Rhodobacteraceae* and to  
163 a lesser degree of *Pseudoahrensia*, *Amylibacter* and sequences without known relatives within  
164 the *Alphaproteobacteria* (no relative *Alphaproteobacteria*) and *Hyphomonadaceae* (no relative  
165 *Hyphomonadaceae*). Representatives of no relative *Rhodobacteraceae* comprised on average  $40.6$   
166  $\pm 23.2$  % of all alphaproteobacterial sequences in the epiphytic community (Fig. 7). In addition,  
167 *Amylibacter* was detected mainly in *C. nodosa* from November 2017 until March 2018.

168 Sequences related to *Gammaproteobacteria* comprised on average  $18.6 \pm 3.9$  % of all  
169 sequences (Fig. 8). Similar to above mentioned taxa, large taxonomic differences between ambient  
170 seawater and surface associated communities were found. Ambient seawater communities were  
171 mainly comprised of the OM60 (NOR5) clade, *Litoricola*, *Acinetobacter* and the SAR86 clade,  
172 while epiphytic communities were mainly composed of sequences without known relatives within  
173 the *Gammaproteobacteria* (no relative *Gammaproteobacteria*) and *Granulosicoccus*. Beside  
174 these two groups specific to all three epiphytic communities, *C. nodosa* was characterized by

175 *Arenicella*, *Methylotenera* and sequences without known relatives within the *Burkholderiales* (no  
176 relative *Burkholderiales*), while *Thioploca*, *Reinekea* and sequences without known relatives within  
177 *Cellvibrionaceae* (no relative *Cellvibrionaceae*) were more specific to both mixed and monospecific  
178 *C. cylindracea*. In addition, *Arenicella* was more pronounced in November and December 2017,  
179 while no relative *Burkholderiales* and *Methylotenera* were characteristic for the period from March  
180 until May 2018. For the *C. cylindracea* specific taxa no relative *Cellvibrionaceae* and *Reinekea*  
181 showed seasonality and were characteristic for samples originating from June to October 2018.  
182 In addition, similar to *Bacteroidota*, a large proportion of the surface associated community was  
183 grouped as other *Gammaproteobacteria* indicating high diversity within this group (Fig. 8).

184 *Desulfobacterota* were specific for *C. cylindracea*. In the mixed and monospecific *C.*  
185 *cylindracea* communities the proportion of *Desulfobacterota* was  $25.7 \pm 11.2\%$  and  $24.0 \pm 4.3\%$ ,  
186 respectively (Fig. 9). In contrast, in ambient seawater and *C. nodosa* communities the contribution  
187 of *Desulfobacterota* was only  $0.1 \pm 0.08\%$  and  $1.0 \pm 0.7\%$ , respectively. In *C. cylindracea* the  
188 community consisted mainly of *Desulfatitalea*, *Desulfobulbus*, *Desulfopila*, *Desulforhopalus*,  
189 *Desulfotalea*, SEEP-SRB4, uncultured *Desulfocapsaceae* and sequences without known relatives  
190 within the *Desulfobacteraceae* (no relative *Desulfobacteraceae*), *Desulfobulbaceae* (no relative  
191 *Desulfobulbaceae*) and *Desulfocapsaceae* (no relative *Desulfocapsaceae*) (Fig. 9).

192 **Discussion**

193 Surfaces of marine macrophytes harbor biofilms consisting of diverse microbial taxa (Egan  
194 *et al.*, 2013; Tarquinio *et al.*, 2019). No standard protocol has been developed to study these  
195 macrophyte-associated microbes (Ugarelli *et al.*, 2019). Different procedures for removal of  
196 microbial cells from host surfaces are described, such as host tissue shaking (Nõges *et al.*, 2010),  
197 scraping (Uku *et al.*, 2007), swabbing (Mancuso *et al.*, 2016) and ultrasonication (Cai *et al.*,  
198 2014). All these methods result in different removal efficiencies but none was enabling a complete  
199 removal of attached microbial cells based on our experience. In the present study, we applied a  
200 removal protocol (Korlević *et al.*, submitted) based on direct cellular lysis (Burke *et al.*, 2009).  
201 The application of a direct lysis procedure coupled with a high sampling frequency and Illumina  
202 amplicon sequencing has enabled us to described in detail the bacterial and archaeal communities  
203 associated with the surfaces of two marine macrophytes, *C. nodosa* and *C. cylindracea*.

204 In the present study, highest richness was observed for *C. cylindracea* (mixed and monospecific)  
205 followed by *C. nodosa* and lowest richness was found in ambient seawater microbial communities.  
206 Higher richness of microbial communities associated with seagrasses than in ambient seawater  
207 was described earlier and could be attributed to a larger set of inhabitable microniches existing  
208 on macrophyte surfaces than in the ambient seawater (Ugarelli *et al.*, 2019). The highest richness  
209 observed for *C. cylindracea* might be partly due to its contact with the sediment. The stolon of *C.*  
210 *cylindracea* is attached to the sediment surface with rhizoids and thus, the stolon and rhizoids are in  
211 a direct contact with the sediment. In addition, seasonal differences in richness observed for surface  
212 attached communities indicated a slightly higher richness in spring and summer. This pattern could  
213 be explained by a higher macrophyte growth in these two seasons than in autumn and winter (M.  
214 Najdek, personal communication; Zavodnik *et al.*, 1998; Ruitton *et al.*, 2005). During their main  
215 growth season in spring and summer macrophytes exhibit a more dynamic chemical interaction  
216 with the surface community probably causing an increase in the number of inhabitable microniches  
217 (Borges and Champenois, 2015; Rickert *et al.*, 2016).

218 We observed a strong differentiation between the surface attached and ambient seawater  
219 communities at the level of OTUs, in agreement with most published studies (Burke and Thomas *et*  
220 *al.*, 2011; Michelou *et al.*, 2013; Roth-Schulze *et al.*, 2016; Mancuso *et al.*, 2016; Crump *et al.*,  
221 2018; Ugarelli *et al.*, 2019). This indicates that marine macrophytes are a selecting factor from  
222 the pool of microbial taxa present in the ambient seawater, modifying the microbial community  
223 once the macrophyte associated microbial biofilm develops (Salaün *et al.*, 2012; Michelou *et*  
224 *al.*, 2013). In contrast, Fahimipour *et al.* (2017) report in a global study of *Zostera marina*,  
225 similarities between the microbial community developed on leaves and in the ambient seawater.  
226 The discrepancy between our data and the study of Fahimipour *et al.* (2017) could be explained  
227 by different seagrass species, methodological variations or biogeographic trends as Fahimipour  
228 *et al.* (2017) analyzed samples from different locations throughout the northern hemisphere. It is  
229 possible that the microbial communities in ambient seawater and on leaves from the same location  
230 are differing but are still more similar to each other when compared to other sampling locations.  
231 Indeed, it was found that prokaryotic communities vary substantially between different sampling  
232 sites (Bengtsson *et al.*, 2017). When the taxonomic composition at high ranks was analyzed no  
233 such strong differentiation was noticed. Phyla and classes such as *Actinobacteriota*, *Bacteroidota*,  
234 *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobiota* were found  
235 in both ambient seawater as well as macrophyte associated, in agreement with previous studies  
236 (Burke and Thomas *et al.*, 2011; Egan *et al.*, 2013; Michelou *et al.*, 2013). In contrast, when low  
237 taxonomic ranks were analyzed (i.e., family and genus) a strong differentiation was observed (Figs.  
238 5 – 9). A similar differentiation at lower microbial taxonomic ranks between ambient seawater and  
239 macrophytes was described for other macrophyte species as well (Egan *et al.*, 2013; Michelou *et al.*,  
240 2013; Ugarelli *et al.*, 2019).

241 Beside differences between ambient seawater and surface associated microbial communities,  
242 it is unclear whether the prokaryotic epiphytic community is host-specific or whether there are  
243 generalist taxa characteristic to all or many macrophytes (Egan *et al.*, 2013). Similar to previously  
244 described differences between microbial communities in the ambient seawater and on macrophytes,

245 at high taxonomic ranks no major difference between the microbial communities associated with  
246 different hosts was observed. The only high rank phylum that was differing between *C. nodosa*  
247 and *C. cylindracea* was *Desulfobacterota*, with more abundant sequences in the *C. cylindracea*  
248 associated community. As already mentioned, the rhizoids and part of the stolon are in contact  
249 with the sediment. Thus *Desulfobacterota* are probably a part of the epiphytic community that  
250 was in contact with the sediment. Similar high rank taxa found in this study were described to be  
251 specific for other species of macrophytes (Burke and Thomas *et al.*, 2011; Lachnit *et al.*, 2011;  
252 Mancuso *et al.*, 2016; Bengtsson *et al.*, 2017). In contrast to high taxonomic ranks, a substantial  
253 differentiation between host specific communities was found supporting the notion that macrophyte  
254 associated microbial communities might be host-specific. Host-specificity was also observed for  
255 other species of macroalgae and seagrasses (Lachnit *et al.*, 2011; Roth-Schulze *et al.*, 2016; Ugarelli  
256 *et al.*, 2019; Morrissey *et al.*, 2019). Taken together, at high taxonomic ranks a core set of taxa  
257 could be described that is characteristic for all or many macrophytes, while at low taxonomic ranks  
258 a community specific to host species was identified (Figs. 3 and 4) (Egan *et al.*, 2013).

259 Seasonal changes in richness in the epiphytic community were substantial as indicated by the  
260 proportion of OTUs ( $\leq 1.0\%$ ) present at every sampling date. These persistent OTUs, however,  
261 were accounting for a high proportion of sequences ( $\leq 52.4\%$ ) (Fig. 2). A very similar proportion  
262 of persistent OTUs was reported in high-frequency sampling studies describing seasonal changes in  
263 picoplankton (Gilbert *et al.*, 2009, 2012). In comparison to the seawater community, a lower degree  
264 of seasonal shifts was observed for the macrophyte surface associated communities. It appears that  
265 microniches at the surfaces of macrophytes are providing more stable conditions than the ambient  
266 seawater. At the level of OTUs seasonal changes of *C. nodosa* and *C. cylindracea* associated  
267 communities were identified that could be linked to the growth cycle of the seagrass and macroalgae  
268 (M. Najdek, personal communication). *C. nodosa* was characterized by a spring community  
269 during maximum seagrass proliferation, a summer community during the highest standing stock of  
270 *C. nodosa* and an autumn/winter community during the decay of seagrass biomass. In contrast, *C.*  
271 *cylindracea* started to proliferate in late spring and was characterized only by a summer community

272 during high growth rates and by an autumn/winter/spring community when the biomass was at  
273 the peak and decaying thereafter. Similar seasonal changes in the epiphytic community were also  
274 described for other macroalgae (Tujula *et al.*, 2010; Lachnit *et al.*, 2011). Higher seasonal stability  
275 of *C. cylindracea* surface communities than in *C. nodosa* was also observed in the higher proportion  
276 of shared communities between two consecutive sampling dates in *C. cylindracea*.

277 Chloroplast sequence abundances were higher in autumn/winter than in spring/summer. This  
278 pattern is not surprising as seagrasses harbor more algal epiphytes during autumn/winter than in  
279 spring/summer (Reyes and Sansón, 2001). Furthermore, we used an adapted DNA isolation protocol  
280 that is known to partially co-extract DNA from planktonic eukaryotes (Korlević *et al.*, 2015).  
281 Strong seasonal fluctuations of high rank epiphytic taxa were not observed, with the exception of  
282 *Cyanobacteria*. Cyanobacterial sequences were more pronounced in November and December than  
283 in spring and summer. In the months of high cyanobacterial sequence abundances the majority of  
284 sequences from this group were classified as *Pleurocapsa*, a group known to colonized different  
285 living and non-living surfaces (Burns *et al.*, 2004; Longford *et al.*, 2007; Mobberley *et al.*, 2012;  
286 Reisser *et al.*, 2014). It is possible that during periods of low metabolic activity there is a reduced  
287 interaction and selection of the epiphytic community by the seagrass, causing leaves to become  
288 a suitable surface for nonspecific colonizers (Zavodnik *et al.*, 1998). *Pleurocapsa* was replaced  
289 in spring and summer by *Acrophormium*, *Phormidesmis* and sequences without known relatives  
290 within the *Nodosilineaceae*. A study of coastal microbial mats found also a higher proportion  
291 of *Nodosilineaceae* sequences in summer, while *Phormidesmis* sequences were at their peak in  
292 autumn (Cardoso *et al.*, 2019). Other high rank taxa did not exhibit strong successional patterns. In  
293 every analyzed group, with the exception of *Desulfobacterota*, taxa present throughout the year in  
294 similar proportions and season specific taxa could be identified (Figs. 6 and 9). Within *Bacteroidota*  
295 different groups within the family *Saprospiraceae* (e.g. *Lewinella*, *Portibacter* and *Rubidimonas*)  
296 were detected across all seasons. Members of this family are often found in association with  
297 macrophytes and it is suggested that they are involved in the hydrolysis and utilization of complex  
298 carbon sources (Burke and Thomas *et al.*, 2011; McIlroy and Nielsen, 2014; Crump *et al.*, 2018).

299 In contrast, the families *Flavobacteriaceae* and *Rhodothermaceae* showed seasonal patterns, with  
300 *Flavobacteriaceae* being more pronounced from November to June and *Rhodothermaceae* from  
301 June to October (Fig. 6). Within *Alphaproteobacteria* the family *Rhodobacteraceae* comprised the  
302 majority of sequences throughout the year (Fig. 7). This metabolically versatile family is often  
303 associated with macrophyte surfaces and usually is one of the most abundant groups (Burke and  
304 Thomas *et al.*, 2011; Michelou *et al.*, 2013; Pujalte *et al.*, 2014; Mancuso *et al.*, 2016). In addition,  
305 *Hyphomonadaceae* were found in all samples. Interestingly, some of the species within this group  
306 contain stalks on their cells, which can be used to attach to the macrophyte surface (Weidner *et al.*,  
307 2000; Abraham and Rohde, 2014). Within the *Gammaproteobacteria*, sequences without known  
308 representatives were the most pronounced group present throughout the year (Fig. 8). In addition,  
309 *Granulosicoccus* was also found in almost all samples. *Gammaproteobacteria* are often a major  
310 constituent of macrophyte epiphytic communities (Burke and Thomas *et al.*, 2011; Michelou *et al.*,  
311 2013; Crump *et al.*, 2018). Beside these two groups, other less abundant, taxa showed seasonal and  
312 host-specific patterns. For example, *C. cylindracea* harbored *Thioploca*, a known sulfur sediment  
313 bacteria and *Cellvibrionaceae*, a family with cultured members known as polysaccharide degraders  
314 (Jørgensen and Gallardo, 1999; Xie *et al.*, 2017). *Desulfobacterota* were found only associated with  
315 *C. cylindracea* and no group within this phylum showed seasonal patterns (Fig. 9). The presence of  
316 this phylum only on *C. cylindracea* is to be expected as part of the epiphytic community is in direct  
317 contact with the sediment. The *Desulfobacterota* community was dominated by *Desulfatitalea* and  
318 no relative *Desulfocapsaceae*, known sulfate sediment groups (Kuever, 2014; Higashioka *et al.*,  
319 2015).

320 In temperate zones, marine macrophytes are exhibiting growth cycles, so it is not surprising that  
321 the associated epiphytic microbial community is undergoing partial seasonal changes. In the present  
322 study, we could identify in every analyzed high rank taxa phylogenetic groups present throughout  
323 the year, comprising most of the sequences and a lower proportion of taxa showing seasonal  
324 patterns connected to the macrophyte growth cycle (Figs. 4 and 9). Studies focusing on functional  
325 comparisons between communities associated with different hosts showed that the majority of

326 functions could be found in every community, indicating functional redundancy (Roth-Schulze *et*  
327 *al.*, 2016). This difference between phylogenetic variability and functional stability was explained by  
328 the lottery hypothesis assuming an initial random colonization step performed by a set of functionally  
329 equivalent taxonomic groups (Burke and Peter Steinberg *et al.*, 2011; Roth-Schulze *et al.*, 2016).  
330 It is possible that functional redundancy is a characteristic of high abundance taxa detected to be  
331 present throughout the year, while seasonal and/or host-specific functions are an attribute of taxa  
332 displaying successional patterns. Further studies connecting taxonomy with functional properties  
333 will be required to elucidate the degree of functional redundancy or specificity in epiphytic microbial  
334 communities.

335 **Experimental procedures**

336 **Sampling**

337 Sampling was performed in the Bay of Funtana, northern Adriatic Sea (45°10'39" N,  
338 13°35'42" E). Leaves and thalli were sampled in a meadow of *Cymodocea nodosa* invaded by the  
339 invasive *Caulerpa cylindracea* (mixed settlement) and in a monospecific settlement of *Caulerpa*  
340 *cylindracea* located in the proximity of the meadow at approximately monthly intervals from  
341 December 2017 to October 2018 (Table S1). Leaves and thalli were collected by diving and  
342 transported to the laboratory in containers placed on ice and filled with seawater collected at  
343 the sampling site. Upon arrival to the laboratory, *C. nodosa* leaves were cut into sections of 1 –  
344 2 cm, while *C. cylindracea* thalli were cut into 5 – 8 cm long sections. Leaves and thalli were  
345 washed three times with sterile artificial seawater (ASW) to remove loosely attached microbial cells.  
346 Ambient seawater was collected in 10 l containers by diving and transported to the laboratory where  
347 the whole container volume was filtered through a 20 µm net. The filtrate was further sequentially  
348 filtered through 3 µm and 0.2 µm polycarbonate membrane filters (Whatman, United Kingdom)  
349 using a peristaltic pump. Filters were briefly dried at room temperature and stored at –80 °C.  
350 Seawater samples were also collected approximately monthly from July 2017 to October 2018.

351 **DNA isolation**

352 DNA from surfaces of *C. nodosa* and *C. cylindracea* was isolated using a previously modified  
353 and adapted protocol that allows for a selective epiphytic DNA isolation (Massana *et al.*, 1997;  
354 Korlević *et al.*, submitted). Briefly, leaves and thalli are incubated in a lysis buffer and treated  
355 with lysozyme and proteinase K. Following the incubations, the mixture containing lysed epiphytic  
356 cells was separated from the leaves and thalli and extracted using phenol-chloroform. Finally, the  
357 extracted DNA was precipitated using isopropanol. DNA from seawater picoplankton was extracted

358 from 0.2 µm polycarbonate filters according to Massana *et al.* (1997) with a slight modification.  
359 Following the phenol-chloroform extraction, 1/10 of chilled 3 M sodium acetate (pH 5.2) was added.  
360 DNA was precipitated by adding 1 volume of chilled isopropanol, incubating the mixtures overnight  
361 at –20 °C and centrifuging at 20,000 × g and 4 °C for 21 min. The pellet was washed twice with  
362 500 µl of chilled 70 % ethanol and centrifuged after each washing step at 20,000 × g and 4 °C for 5  
363 min. Dried pellets were re-suspended in 50 – 100 µl of deionized water.

364 **Illumina 16S rRNA sequencing**

365 Illumina MiSeq sequencing of the V4 region of the 16S rRNA gene was performed as described  
366 previously (Korlević *et al.*, submitted). The V4 region of the 16S rRNA gene was amplified using  
367 a two-step PCR procedure. In the first PCR, the 515F (5'-GTGYCAGCMGCCGCGTAA-3')  
368 and 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers from the Earth Microbiome Project  
369 (<http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>) were used (Caporaso *et*  
370 *al.*, 2012; Apprill *et al.*, 2015; Parada *et al.*, 2016). These primers contained on their 5' end a tagged  
371 sequence. Purified PCR products were sent for Illumina MiSeq sequencing at IMGM Laboratories,  
372 Martinsried, Germany. Prior to sequencing at IMGM, the second PCR amplification of the two-step  
373 PCR procedure was performed using primers targeting the tagged region incorporated in the first  
374 PCR. In addition, these primers contained adapter and sample-specific index sequences. Beside  
375 samples, a positive and negative control for each sequencing batch was sequenced. The negative  
376 control comprised PCR reactions without DNA template, while for a positive control a mock  
377 community composed of evenly mixed DNA material originating from 20 bacterial strains (ATCC  
378 MSA-1002, ATCC, USA) was used. Sequences obtained in this study have been deposited in the  
379 European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB37267.

380 **Sequence analysis**

381       Obtained sequences were analyzed on the computer cluster Isabella (University Computing  
382       Center, University of Zagreb) using mothur (version 1.43.0) (Schloss *et al.*, 2009) according  
383       to the MiSeq Standard Operating Procedure (MiSeq SOP; [https://mothur.org/wiki/MiSeq\\_SOP](https://mothur.org/wiki/MiSeq_SOP))  
384       (Kozich *et al.*, 2013) and recommendations provided by the Riffomonas project to enhance data  
385       reproducibility (<http://www.riffomonas.org/>). For alignment and classification of sequences the  
386       SILVA SSU Ref NR 99 database (release 138; <http://www.arb-silva.de>) was used (Quast *et al.*,  
387       2013; Yilmaz *et al.*, 2014). Pipeline data processing and visualization was done using R (version  
388       3.6.0) (R Core Team, 2019) combined with packages vegan (version 2.5-6) (Oksanen *et al.*, 2019),  
389       tidyverse (version 1.3.0) (Wickham *et al.*, 2019) and multiple other packages (Xie, 2014, 2015,  
390       2020; Neuwirth, 2014; Xie *et al.*, 2018; Y. Xie, 2019b, 2019a; Allaire *et al.*, 2019; Zhu, 2019). The  
391       detailed analysis procedure including the R Markdown file are available in the GitHub repository  
392       ([https://github.com/mkorlevic/Korlevic\\_EpiphyticDynamics\\_EnvironMicrobiol\\_2020](https://github.com/mkorlevic/Korlevic_EpiphyticDynamics_EnvironMicrobiol_2020)). Based on  
393       the ATCC MSA-1002 mock community included in the analysis an average sequencing error rate  
394       of 0.01 % was determined, which is in line with previously reported values for next-generation  
395       sequencing data (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative controls  
396       processed together with the samples yielded on average only 2 sequences after sequence quality  
397       curation.

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583 **Figure legends**

584 **Fig. 1.** Proportion of shared bacterial and archaeal OTUs (Jaccard's Similarity Coefficient) and  
585 shared bacterial and archaeal communities (Bray-Curtis Similarity Coefficient) between assemblages  
586 associated with the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C. cylindracea*  
587 (Mixed and Monospecific Settlement) and communities in the ambient seawater.

588 **Fig. 2.** Proportion of shared bacterial and archaeal communities (Bray-Curtis Similarity Coefficient)  
589 and shared bacterial and archaeal OTUs (Jaccard's Similarity Coefficient) between consecutive  
590 sampling dates and from the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C.*  
591 *cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater.

592 **Fig. 3.** Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU abundances  
593 of bacterial and archaeal communities from the surfaces of the macrophytes *C. nodosa* (Mixed  
594 Settlement) and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater.  
595 Samples from the same environment or same season are labeled in different colors. The proportion  
596 of explained variation by each axis is shown on the corresponding axis in parentheses.

597 **Fig. 4.** Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial  
598 and archaeal sequences on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C.*  
599 *cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. NR – No Relative  
600 (sequences without known relatives within the corresponding group)

601 **Fig. 5.** Taxonomic classification and relative contribution of the most abundant (> 1 %)  
602 cyanobacterial sequences on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C.*  
603 *cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. The proportion  
604 of cyanobacterial sequences in the total bacterial and archaeal community is given above the  
605 corresponding bar. NR – No Relative (sequences without known relatives within the corresponding  
606 group)

607 **Fig. 6.** Taxonomic classification and relative contribution of the most abundant (> 2 %) sequences  
608 within the *Bacteroidota* on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C.*  
609 *cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. The proportion of  
610 sequences classified as *Bacteroidota* in the total bacterial and archaeal community is given above the  
611 corresponding bar. NR – No Relative (sequences without known relatives within the corresponding  
612 group)

613 **Fig. 7.** Taxonomic classification and relative contribution of the most abundant (> 2 %)  
614 alphaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement)  
615 and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. The  
616 proportion of alphaproteobacterial sequences in the total bacterial and archaeal community is given  
617 above the corresponding bar. NR – No Relative (sequences without known relatives within the  
618 corresponding group)

619 **Fig. 8.** Taxonomic classification and relative contribution of the most abundant (> 2 %)  
620 gammaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement)  
621 and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. The  
622 proportion of gammaproteobacterial sequences in the total bacterial and archaeal community is  
623 given above the corresponding bar. NR – No Relative (sequences without known relatives within  
624 the corresponding group)

625 **Fig. 9.** Taxonomic classification and relative contribution of the most abundant (> 1 %) sequences  
626 within the *Desulfobacterota* on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and  
627 *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. The proportion  
628 of sequences classified as *Desulfobacterota* in the total bacterial and archaeal community is given  
629 above the corresponding bar. NR – No Relative (sequences without known relatives within the  
630 corresponding group)

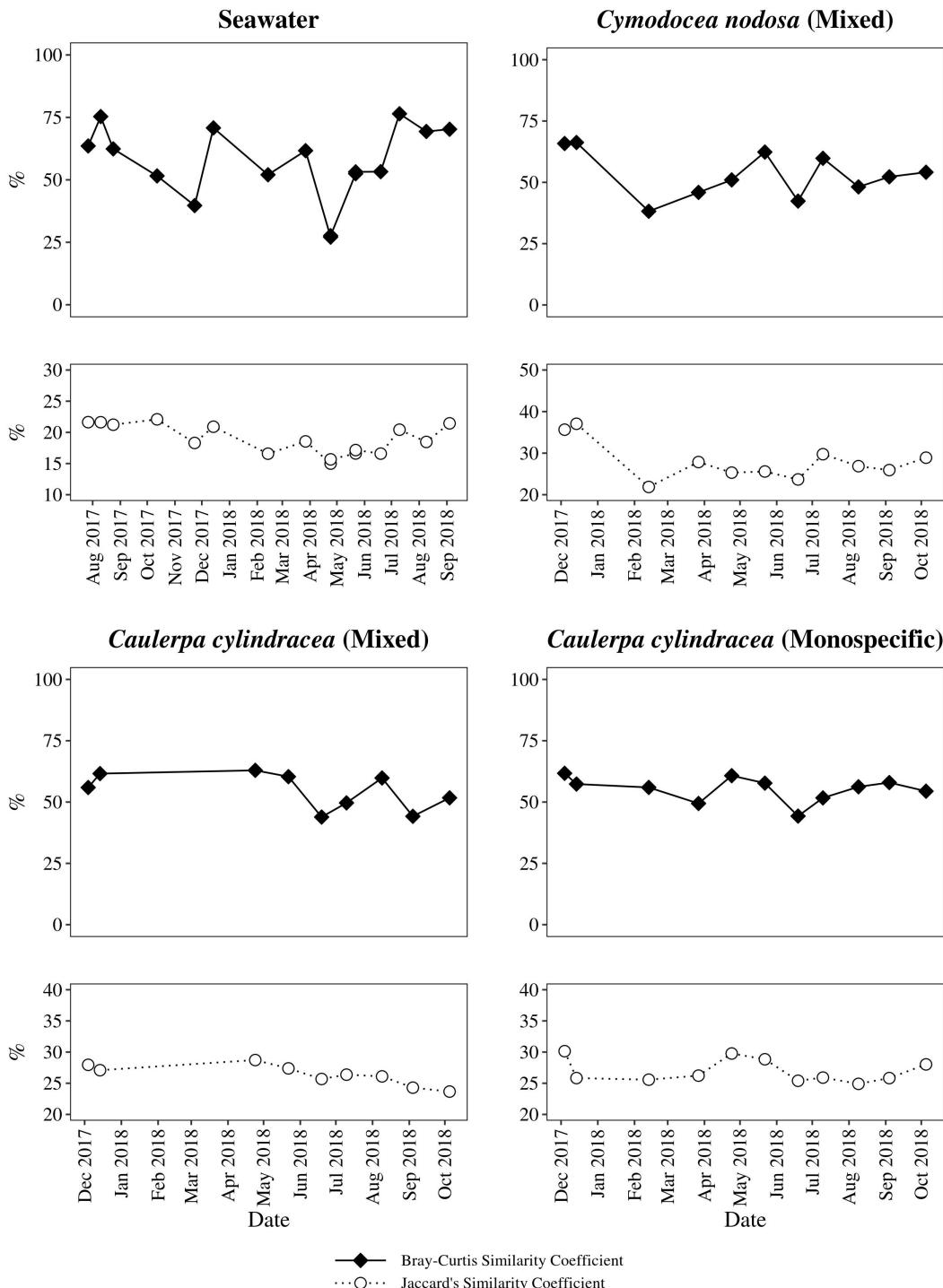
## Jaccard's Similarity Coefficient

<i>Caulerpa cylindracea</i> (Mixed)	<b>0.28</b>		
<i>Caulerpa cylindracea</i> (Monospecific)	<b>0.27</b>	<b>0.35</b>	
Seawater	<b>0.12</b>	<b>0.10</b>	<b>0.10</b>

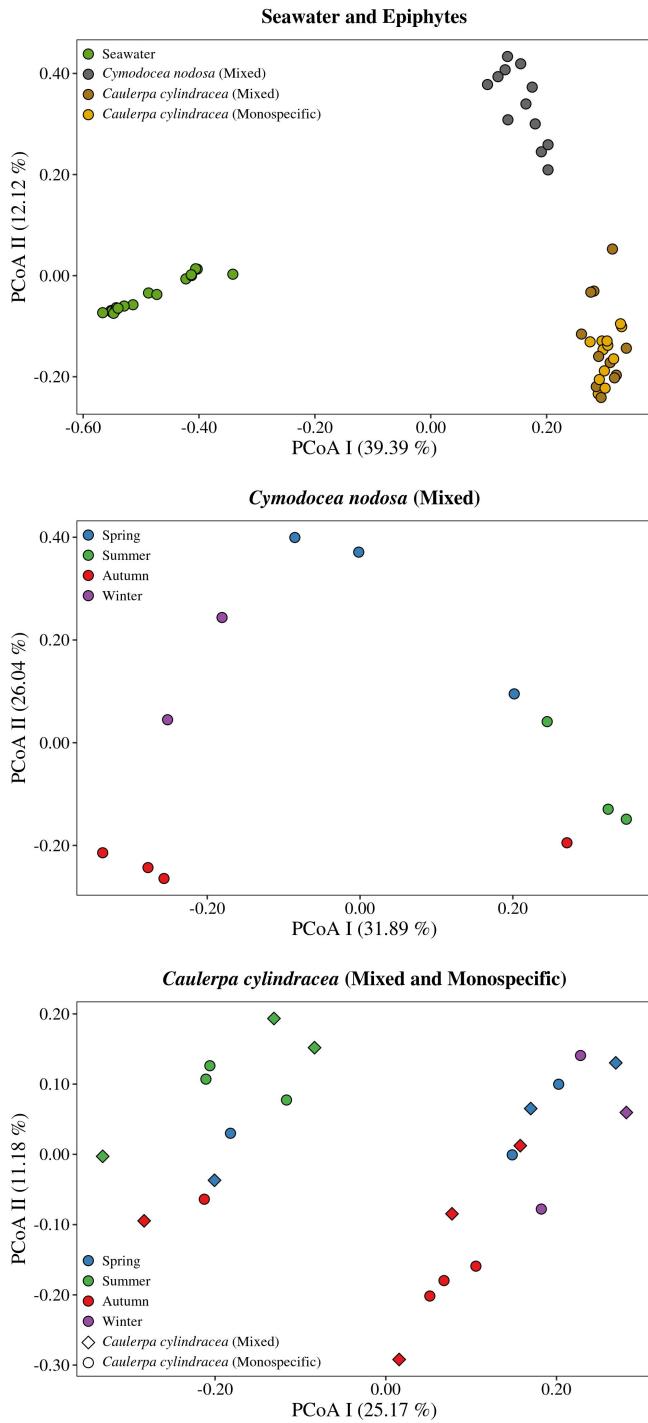
## Bray-Curtis Similarity Coefficient

	<i>Cymodocea nodosa</i> (Mixed)	<i>Caulerpa cylindracea</i> (Mixed)	<i>Caulerpa cylindracea</i> (Monospecific)
<i>Caulerpa cylindracea</i> (Mixed)	<b>0.40</b>		
<i>Caulerpa cylindracea</i> (Monospecific)	<b>0.38</b>	<b>0.77</b>	
Seawater	<b>0.06</b>	<b>0.05</b>	<b>0.06</b>

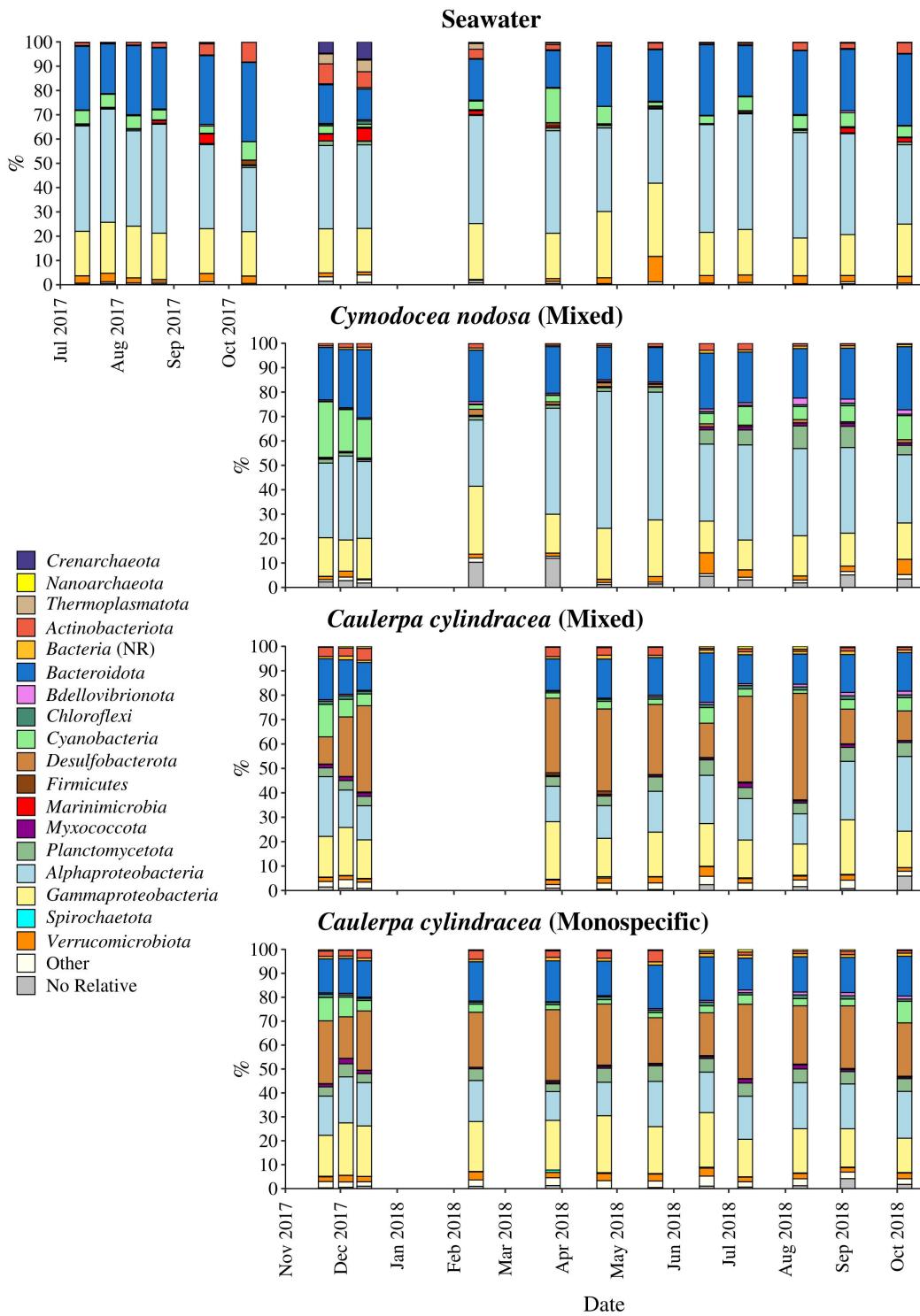
**Fig. 1.** Proportion of shared bacterial and archaeal OTUs (Jaccard's Similarity Coefficient) and shared bacterial and archaeal communities (Bray-Curtis Similarity Coefficient) between assemblages associated with the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C. cylindracea* (Mixed and Monospecific Settlement) and communities in the ambient seawater.



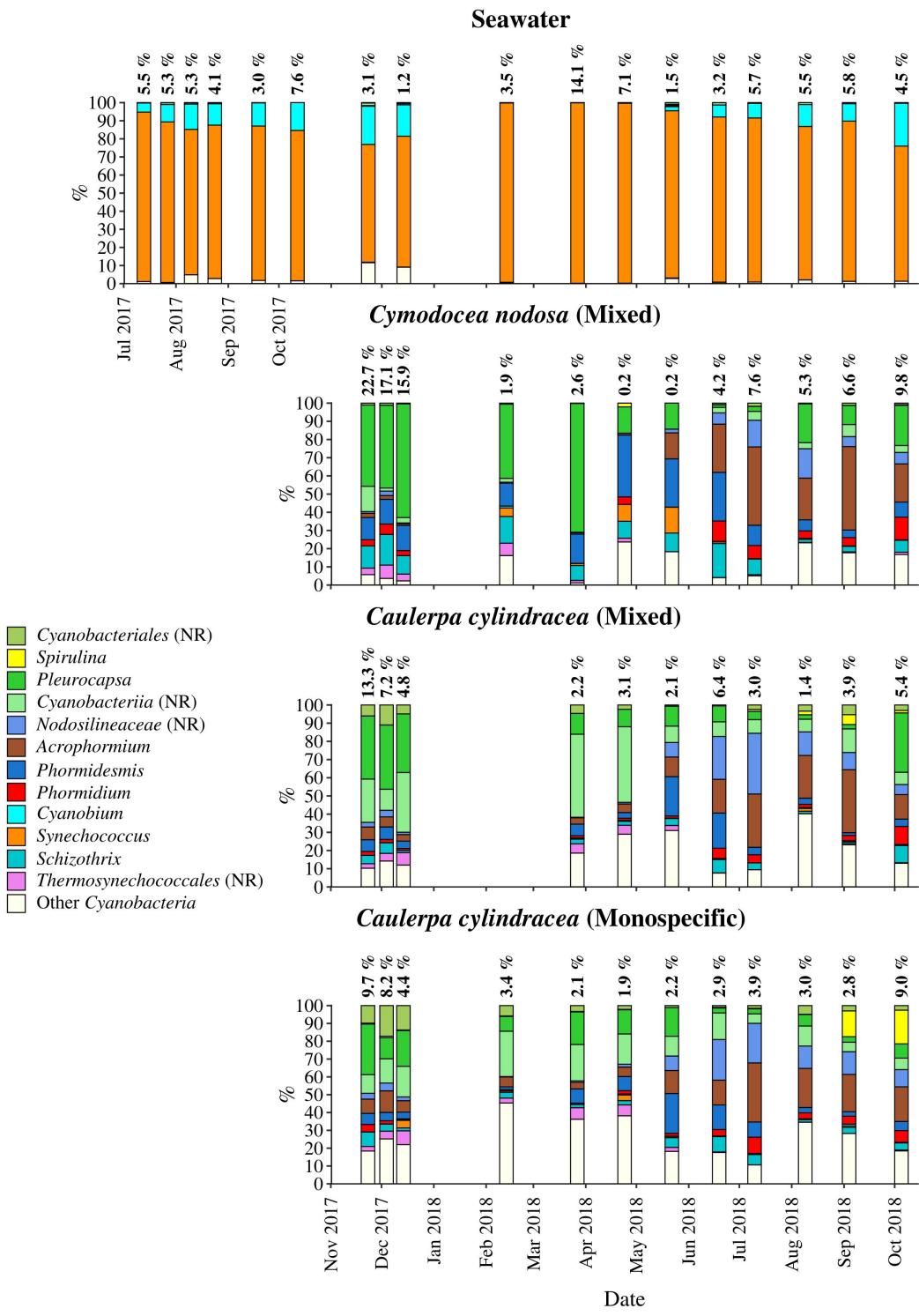
**Fig. 2.** Proportion of shared bacterial and archaeal communities (Bray-Curtis Similarity Coefficient) and shared bacterial and archaeal OTUs (Jaccard's Similarity Coefficient) between consecutive sampling dates and from the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater.



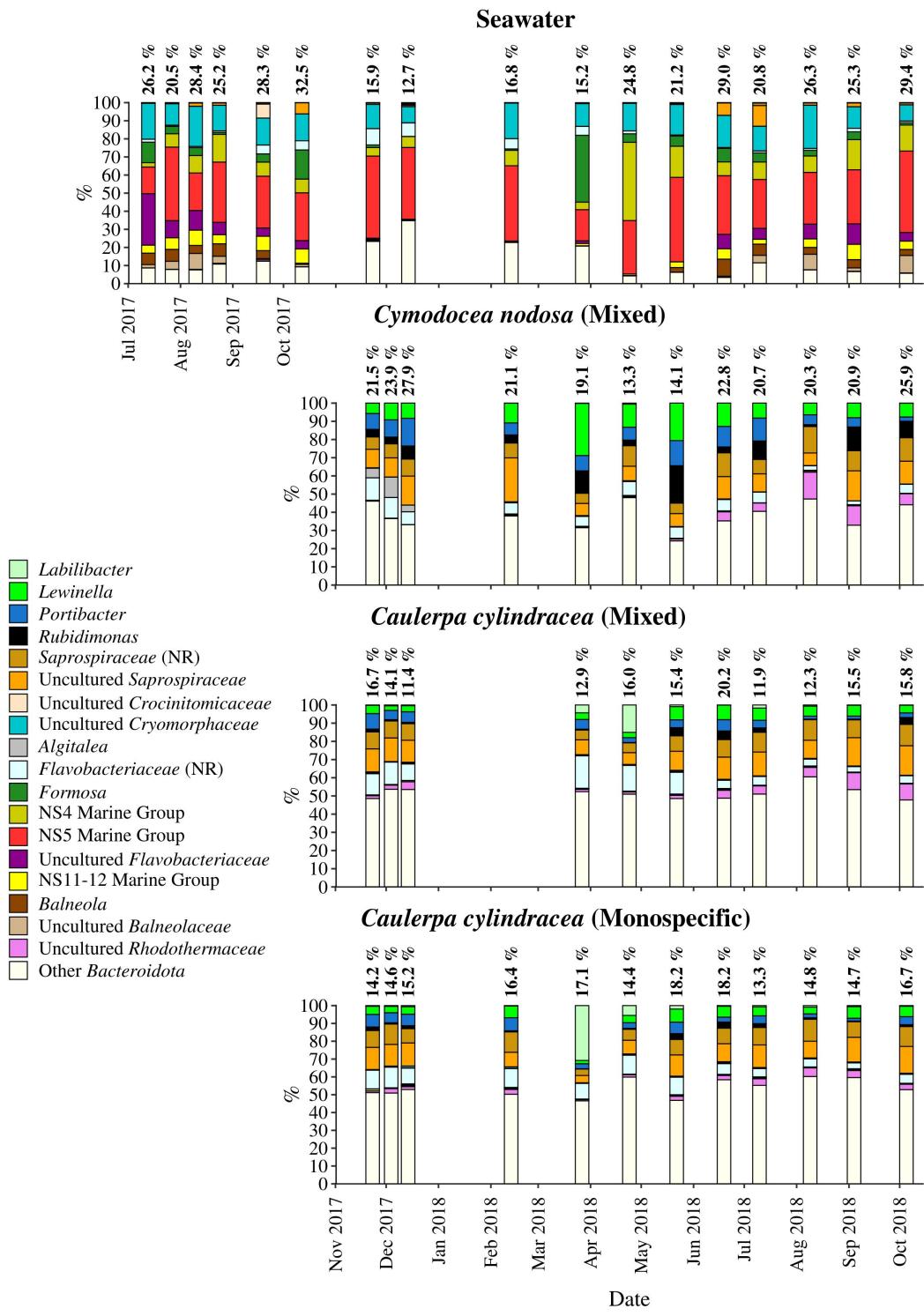
**Fig. 3.** Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU abundances of bacterial and archaeal communities from the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. Samples from the same environment or same season are labeled in different colors. The proportion of explained variation by each axis is shown on the corresponding axis in parentheses.



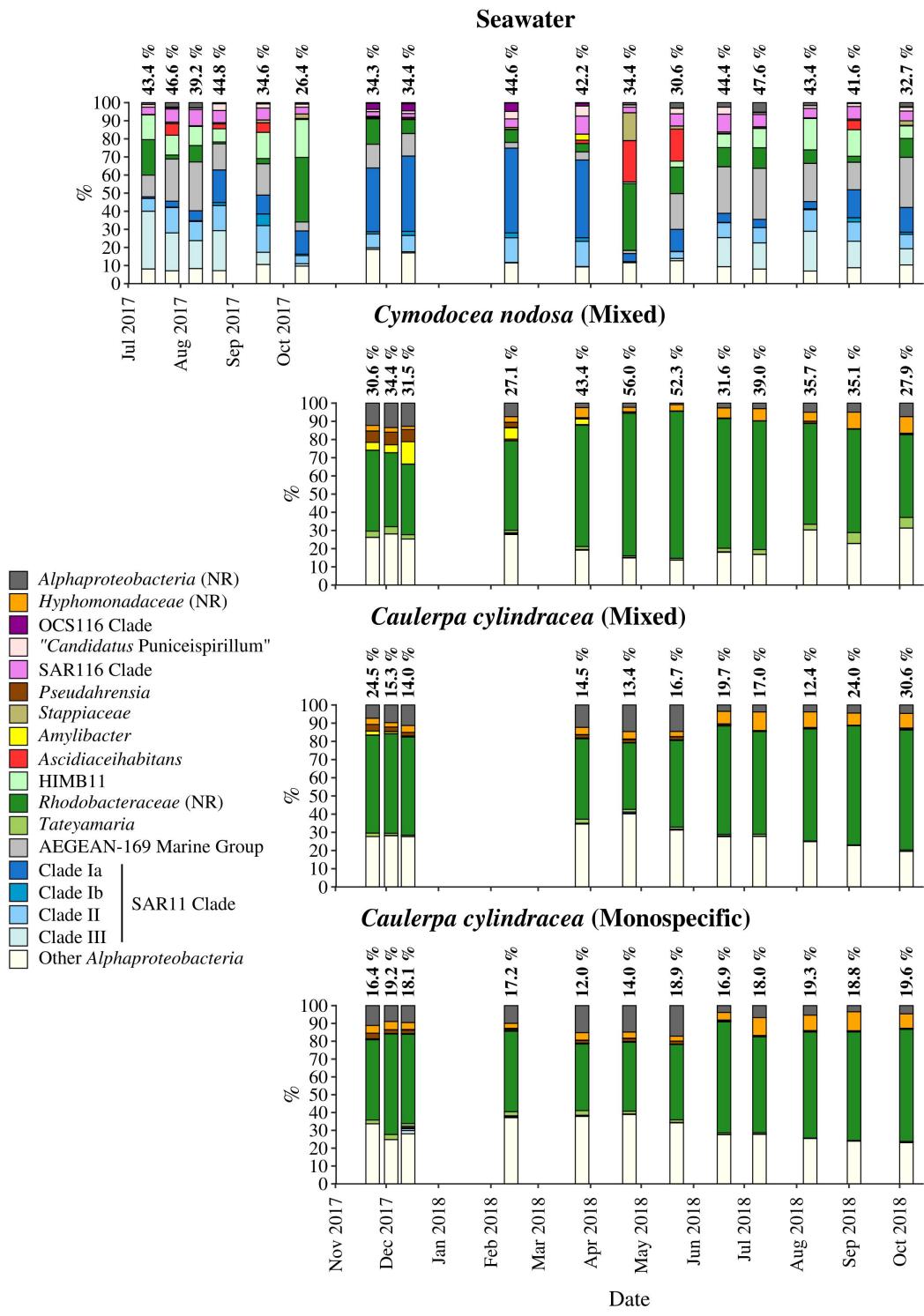
**Fig. 4.** Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial and archaeal sequences on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. NR – No Relative (sequences without known relatives within the corresponding group)



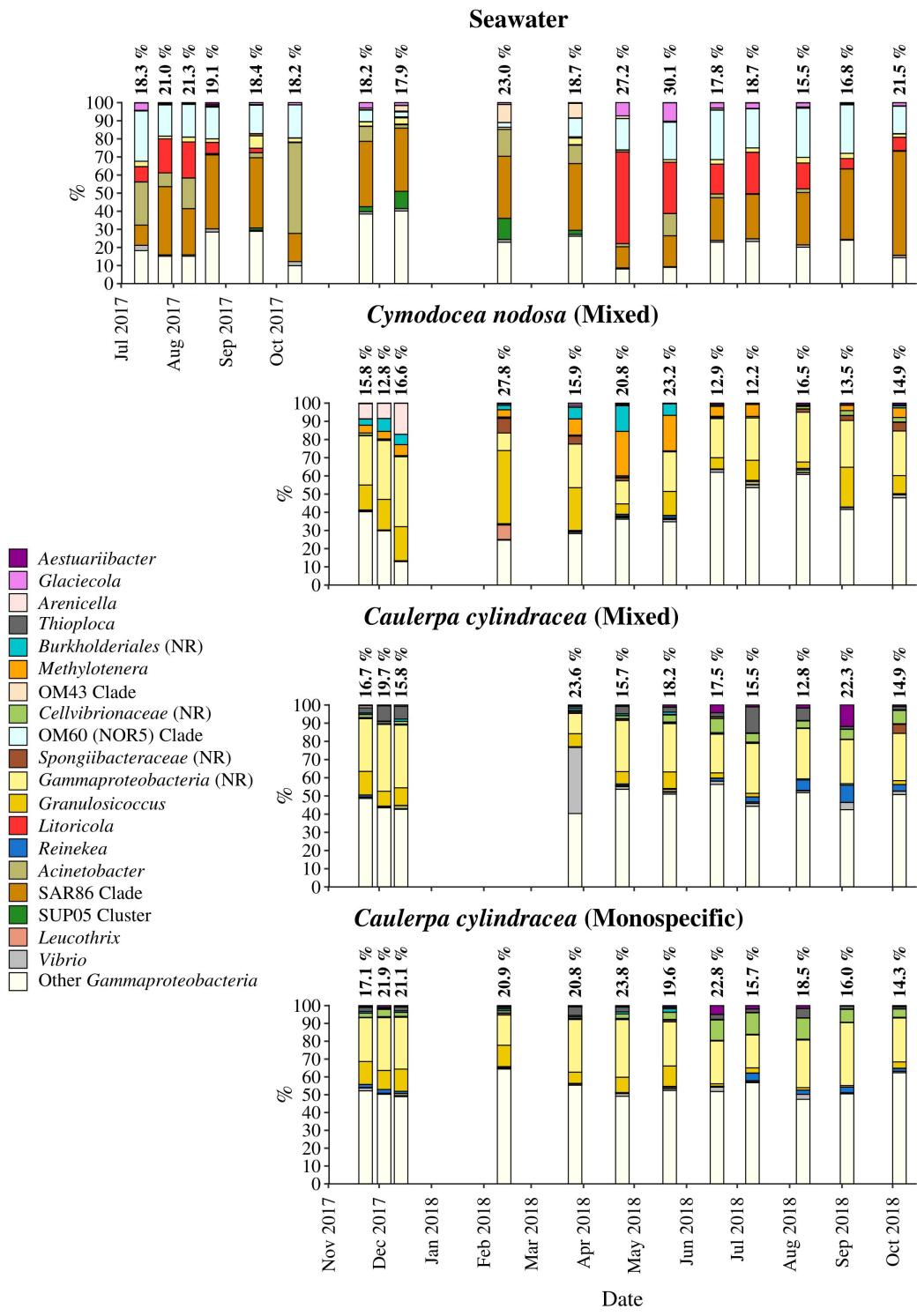
**Fig. 5.** Taxonomic classification and relative contribution of the most abundant (> 1 %) cyanobacterial sequences on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. The proportion of cyanobacterial 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)



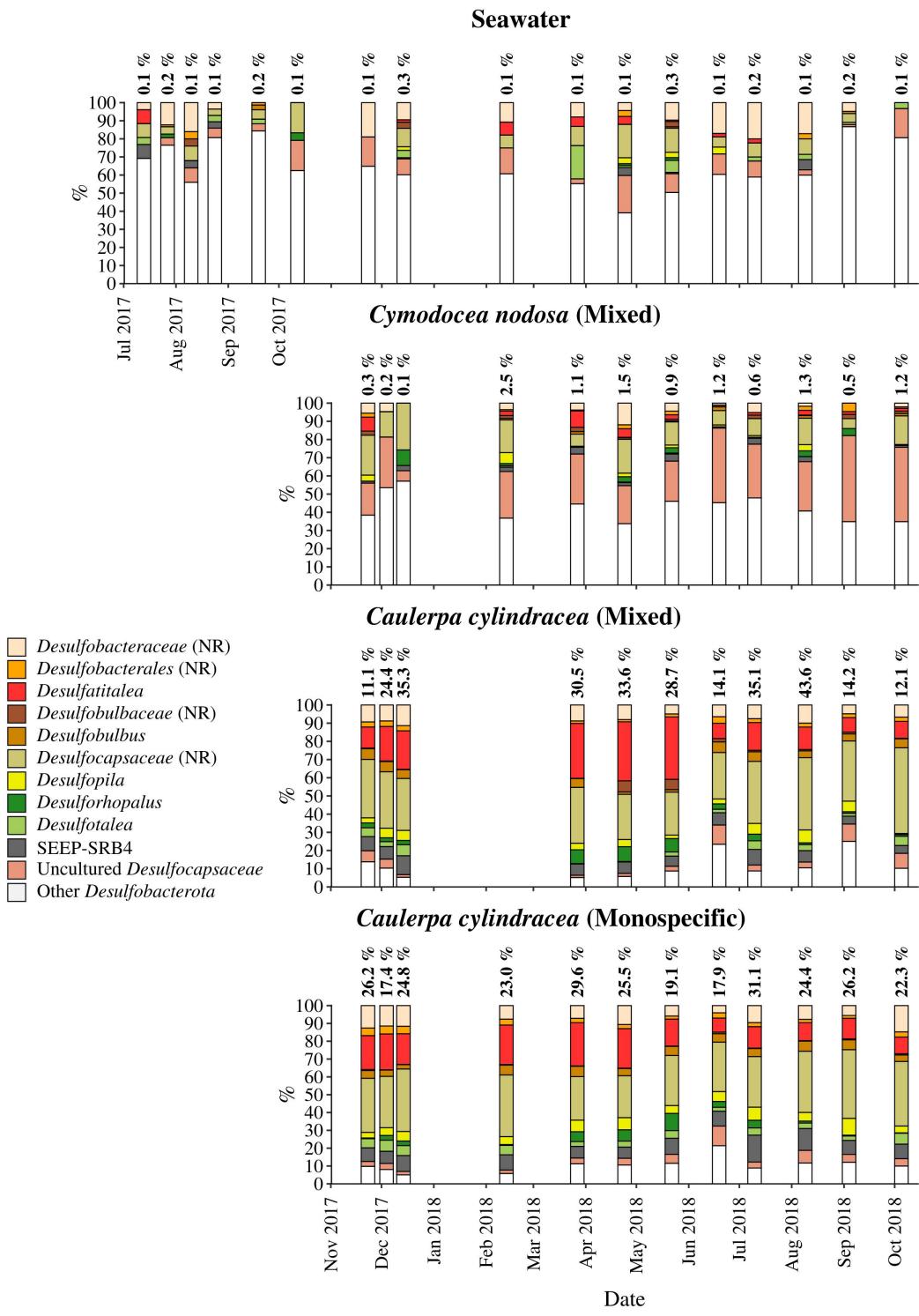
**Fig. 6.** Taxonomic classification and relative contribution of the most abundant (> 2 %) sequences within the *Bacteroidota* on the surfaces of the macrophytes *C. nodosa* (Mixed Settlement) and *C. cylindracea* (Mixed and Monospecific Settlement) and in the ambient seawater. The proportion of sequences 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)



**Fig. 7.** Taxonomic classification and relative contribution of the most abundant (> 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)



**Fig. 8.** Taxonomic classification and relative contribution of the most abundant (> 2 %) 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)



**Fig. 9.** Taxonomic classification and relative contribution of the most abundant (> 1 %) sequences within the *Desulfovibro* 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 *Desulfovibro* in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative (sequences without known relatives within the corresponding group)