

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 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 OTU
8 level, the microbial community composition differed between the ambient water and the epiphytic
9 communities exhibiting host-specificity. Also, successional changes were observed connected to
10 the macrophyte growth cycle. Taxonomic analysis, however, showed similar high rank groups in
11 the ambient water and the epiphytic communities, with the exception of *Desulfobacterota*, which
12 were only found on *Caulerpa cylindracea*. *Cyanobacteria* showed seasonal changes while other
13 high rank taxa were present throughout the year. Phylogenetic groups present throughout the year
14 constituted most of the sequences, while less abundant taxa showed seasonal patterns connected
15 to the macrophyte growth cycle. Taken together, epiphytic microbial communities of the seagrass
16 *Cymodocea nodosa* and the macroalga *Caulerpa cylindracea* appear to be host-specific and contain
17 taxa that undergo successional changes.

18 **Introduction**

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

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

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

43 ranks host specific microbial communities were found (Lachnit *et al.*, 2011; Roth-Schulze *et al.*,
44 2016). Recently, it was shown that even different morphological niches within the same alga had a
45 higher influence on the composition of the bacterial community than biogeography or environmental
46 factors (Morrissey *et al.*, 2019). While the microbial community composition varies between host
47 species, metagenomic analyses revealed that the majority of the microbial functions are conserved
48 (Burke and Peter Steinberg *et al.*, 2011; Roth-Schulze *et al.*, 2016). This discrepancy between the
49 microbial taxonomic and functional composition might be explained by the lottery hypothesis. It
50 postulates that an initial random colonization step takes places from a set of functionally equivalent
51 taxonomic groups resulting in taxonomically different epiphytic communities sharing a core set of
52 functional genes (Burke and Peter Steinberg *et al.*, 2011; Roth-Schulze *et al.*, 2016). In addition,
53 some of the variation in the reported data could be attributed to different techniques used in these
54 studies, such as different protocols for epiphytic cell detachment and/or DNA isolation, as no
55 standard protocol has been yet established to study epiphytic communities (Ugarelli *et al.*, 2019;
56 Korlević *et al.*, submitted).

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

68 **Results**

69 Sequencing of the 16S rRNA V4 region yielded a total of 1.8 million sequences after quality
70 curation and exclusion of sequences without known relatives (no relative sequences) and eukaryotic,
71 chloroplast and mitochondrial sequences (Table S1). A total of 35 samples originating from
72 epiphytic archaeal and bacterial communities associated with surfaces of the seagrass *C. nodosa*
73 and the macroalga *C. cylindracea* were analysed. In addition, 18 samples (one of the samples was
74 sequenced twice) originating from the ambient seawater were also processed for comparison. The
75 number of reads per sample ranged between 8,408 and 77,463 sequences (Table S1). Even when
76 the highest sequencing effort was applied the rarefaction curves did not level off as commonly
77 observed in high-throughput 16S rRNA amplicon sequencing approaches (Fig. S1). Following
78 quality curation and exclusion of sequences as mentioned above reads were clustered into 28,750
79 different OTUs at a similarity level of 97 %. Read numbers were normalized to the minimum
80 number of sequences (8,408, Table S1) through rarefaction resulting in 17,079 different OTUs with
81 384 to 2,062 OTUs per sample (Fig. S2). To determine seasonal changes in richness and diversity
82 the observed number of OTUs, Chao1, ACE, Exponential Shannon and Inverse Simpson (Jost,
83 2006) were calculated after normalization through rarefaction. Generally, richness estimators and
84 diversity indices showed similar trends. On average, higher values were found for *C. cylindracea*
85 (mixed [Number of OTUs, $1,684.1 \pm 133.8$ OTUs] and monospecific [Number of OTUs, $1,731.9 \pm$
86 163.8 OTUs]) than for *C. nodosa* (Number of OTUs, $1,058.2 \pm 214.5$ OTUs) and lowest values were
87 obtained for the microbial community of the ambient seawater (Number of OTUs, 523.9 ± 138.7
88 OTUs) (Fig. S2). Seasonal changes did not reveal such large dissimilarities. *C. nodosa* communities
89 showed a slow increase towards the end of the study, while *C. cylindracea* (mixed and monospecific)
90 communities were characterized by slightly higher values in spring and summer than in autumn and
91 winter (Fig. S2).

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

94 Bray-Curtis Similarity Coefficient, respectively, were calculated. Coefficients were determined after
95 normalization through rarefaction and binning of samples from the particular environment. The
96 highest proportion of shared OTUs and community was found between mixed and monospecific *C.*
97 *cylindracea* (Jaccard, 0.35; Bray-Curtis, 0.78), while lower shared values were calculated between
98 ambient seawater and epiphytic communities (Fig. 1). Shared proportion between *C. nodosa* and *C.*
99 *cylindracea* were approximately in-between the values of mixed and monospecific *C. cylindracea*.
100 To assess seasonal changes in the proportion of shared OTUs and communities the Jaccard's and
101 Bray-Curtis Similarity Coefficients were calculated between consecutive sampling points (Fig. 2).
102 Both coefficients showed similar trends. Temporal proportional changes were more pronounced for
103 ambient seawater than for *C. nodosa* and especially *C. cylindracea* associated communities (Fig. 2).
104 In addition, only 0.4 – 1.0 % of OTUs from each surface associated community were present at
105 all seasons. These persistent OTUs constituted a high proportion of total sequences (39.5 – 52.3
106 %). To further disentangle the environmental and seasonal community dissimilarity a Principal
107 Coordinates Analysis (PCoA) based on Bray-Curtis distances and OTU abundances was applied. A
108 clear separation between ambient seawater and surface associated communities was found (Fig. 3).
109 In addition, a separation of epiphytic bacterial and archaeal communities based on host species
110 was detected. This separation was further supported by ANOSIM ($R = 0.96, p < 0.001$). Seasonal
111 changes of *C. nodosa* associated communities indicated a separation between spring, summer and
112 autumn/winter samples (ANOSIM, $R = 0.54, p < 0.01$). For *C. cylindracea* associated communities
113 a separation between summer and autumn/winter/spring samples was observed that was, however,
114 not as strong as for *C. nodosa* associated communities (ANOSIM, $R = 0.30, p < 0.05$) (Fig. 3).

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

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

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

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

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

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

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

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

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

190 **Discussion**

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

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

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

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

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

257 Seasonal changes in richness in the epiphytic community were substantial as indicated by the
258 proportion of OTUs ($\leq 1.0\%$) present at every sampling date. These persistent OTUs, however,
259 were accounting for a high proportion of sequences ($\geq 39.5\%$) (Fig. 2). A very similar proportion
260 of persistent OTUs was reported in high-frequency sampling studies describing seasonal changes in
261 picoplankton (Gilbert *et al.*, 2009, 2012). In comparison to the seawater community, a lower degree
262 of seasonal shifts was observed for the macrophyte surface associated communities. It appears that
263 microniches at the surfaces of macrophytes are providing more stable conditions than the ambient
264 seawater. At the level of OTUs seasonal changes of *C. nodosa* and *C. cylindracea* associated
265 communities were identified that could be linked to the growth cycle of the seagrass and macroalgae
266 (M. Najdek, personal communication). *C. nodosa* was characterized by a spring community
267 during maximum seagrass proliferation, a summer community during the highest standing stock of
268 *C. nodosa* and an autumn/winter community during the decay of seagrass biomass. In contrast, *C.*
269 *cylindracea* started to proliferate in late spring and was characterized only by a summer community

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

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

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

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

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

333 **Experimental procedures**

334 **Sampling**

335 Sampling was performed in the Bay of Funtana, northern Adriatic Sea (45°10'39" N,
336 13°35'42" E). Leaves and thalli were sampled in a meadow of *Cymodocea nodosa* invaded by the
337 invasive *Caulerpa cylindracea* (mixed settlement) and in a monospecific settlement of *Caulerpa*
338 *cylindracea* located in the proximity of the meadow at approximately monthly intervals from
339 December 2017 to October 2018 (Table S1). Leaves and thalli were collected by diving and
340 transported to the laboratory in containers placed on ice and filled with seawater collected at
341 the sampling site. Upon arrival to the laboratory, *C. nodosa* leaves were cut into sections of 1 –
342 2 cm, while *C. cylindracea* thalli were cut into 5 – 8 cm long sections. Leaves and thalli were
343 washed three times with sterile artificial seawater (ASW) to remove loosely attached microbial
344 cells. Ambient seawater was collected in 10 l containers by diving and transported to the laboratory
345 where 10 – 20 l were filtered through a 20 µm net. The filtrate was further sequentially filtered
346 through 3 µm and 0.2 µm polycarbonate membrane filters (Whatman, United Kingdom) using a
347 peristaltic pump. Filters were briefly dried at room temperature and stored at –80 °C. Seawater
348 samples were also collected approximately monthly from July 2017 to October 2018.

349 **DNA isolation**

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

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

362 **Illumina 16S rRNA sequencing**

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

378 **Sequence analysis**

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

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582 **Figure legends**

583 **Fig. 1.** Proportion of shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) and
584 shared bacterial and archaeal communities (Bray-Curtis similarity coefficient) between assemblages
585 associated with the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea*
586 (mixed and monospecific settlement) and communities in the ambient seawater.

587 **Fig. 2.** Proportion of shared bacterial and archaeal communities (Bray-Curtis similarity coefficient)
588 and shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) between consecutive
589 sampling dates and from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C.*
590 *cylindracea* (mixed and monospecific settlement) and in the ambient seawater.

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

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

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

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

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

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

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

630 **Figures**

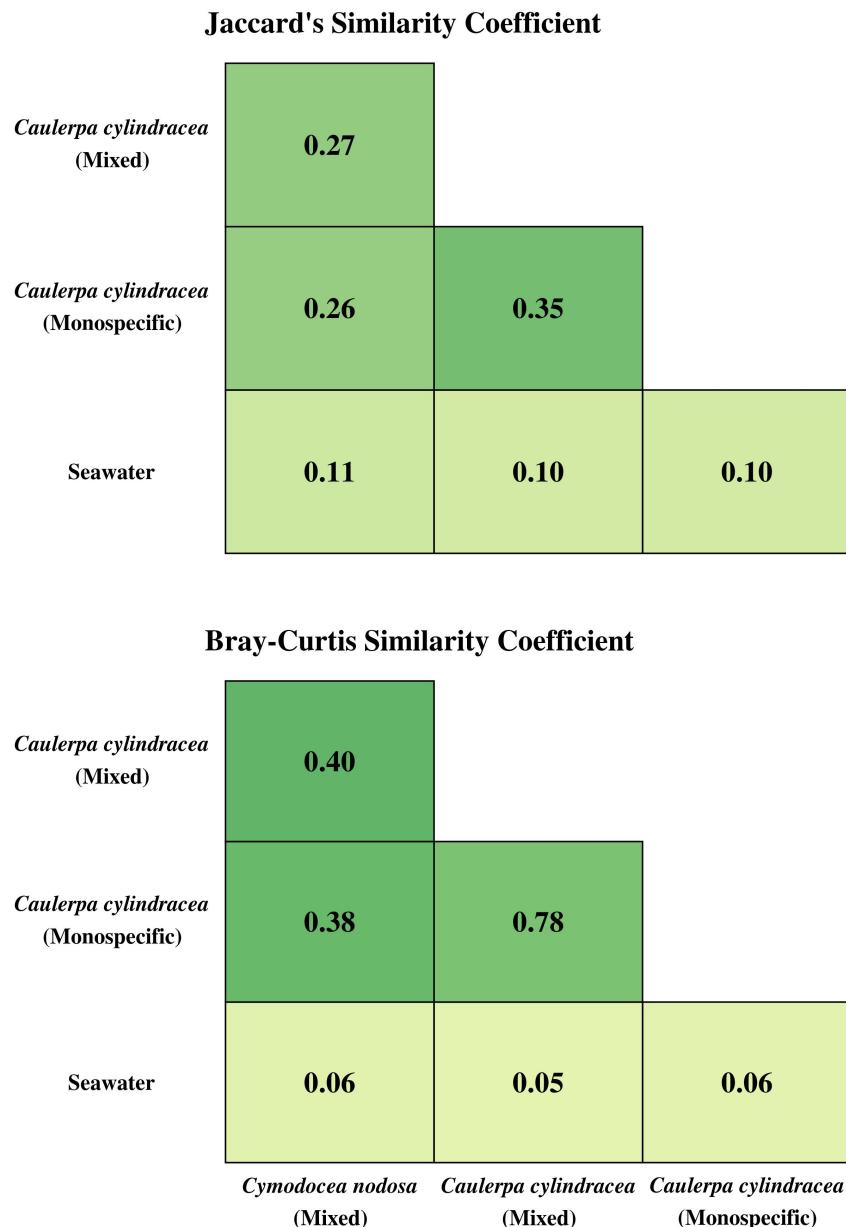


Fig. 1. Proportion of shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) and shared bacterial and archaeal communities (Bray-Curtis similarity coefficient) between assemblages associated with the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and communities in the ambient seawater.

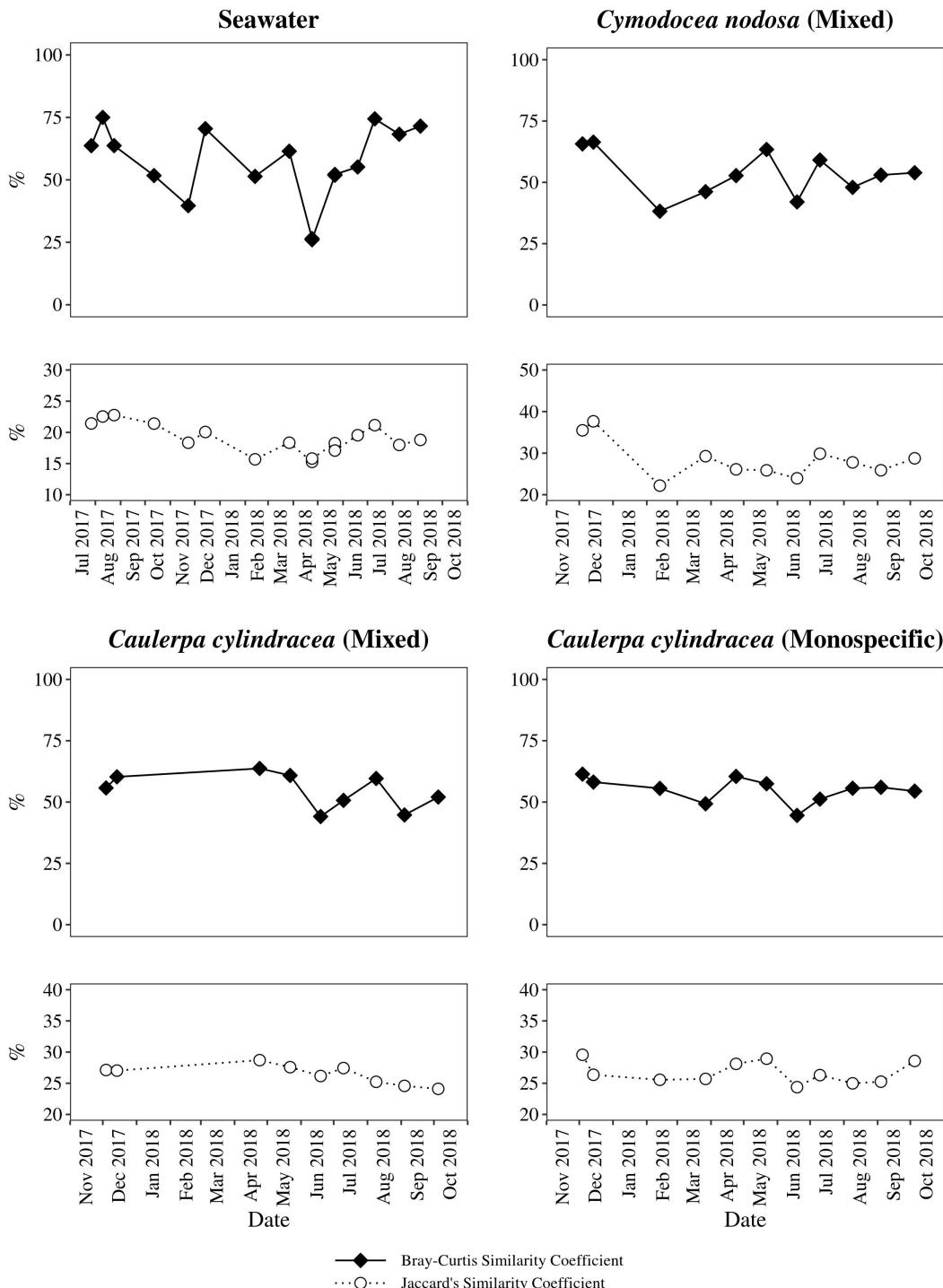


Fig. 2. Proportion of shared bacterial and archaeal communities (Bray-Curtis similarity coefficient) and shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) between consecutive sampling dates and from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater.

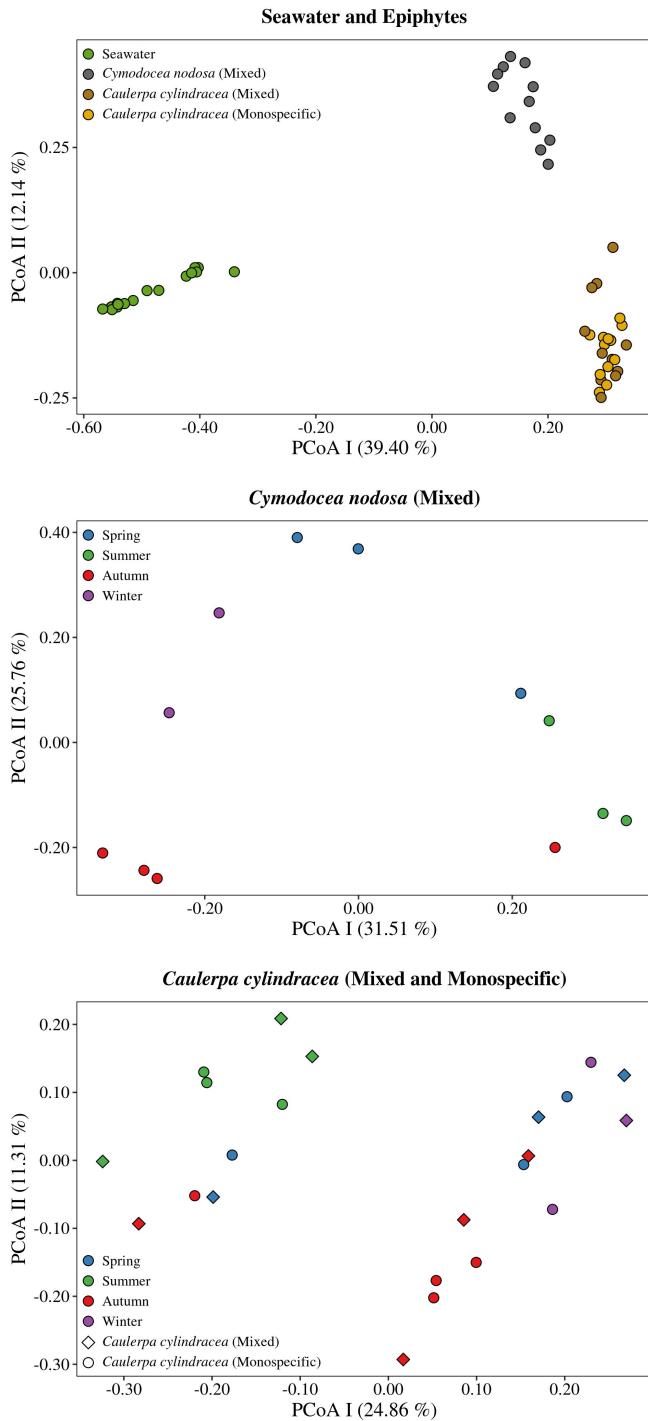


Fig. 3. Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU abundances of bacterial and archaeal communities from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. Samples from the 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.

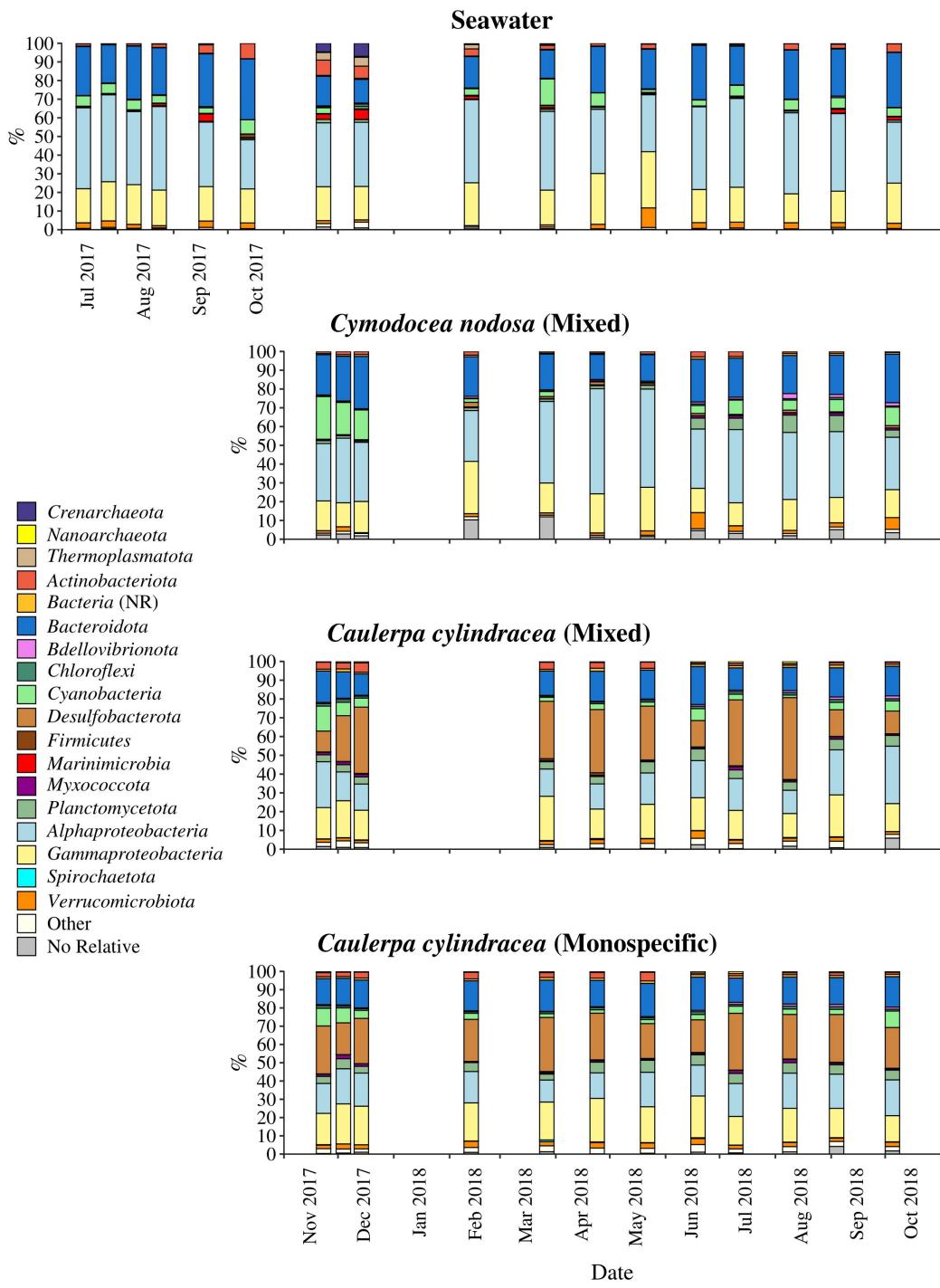


Fig. 4. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) bacterial and archaeal sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. NR – No Relative (sequences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

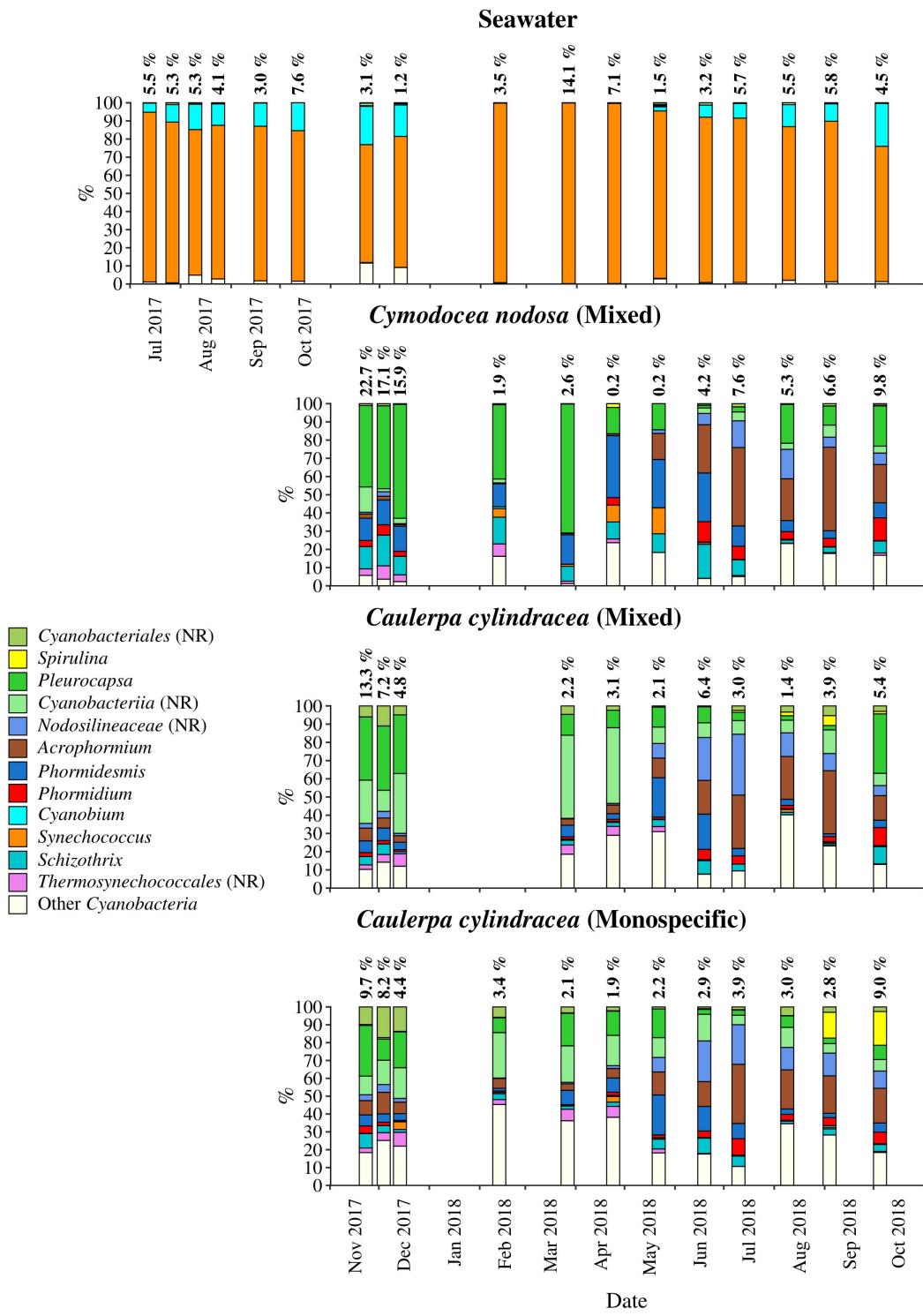


Fig. 5. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) cyanobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of cyanobacterial 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)

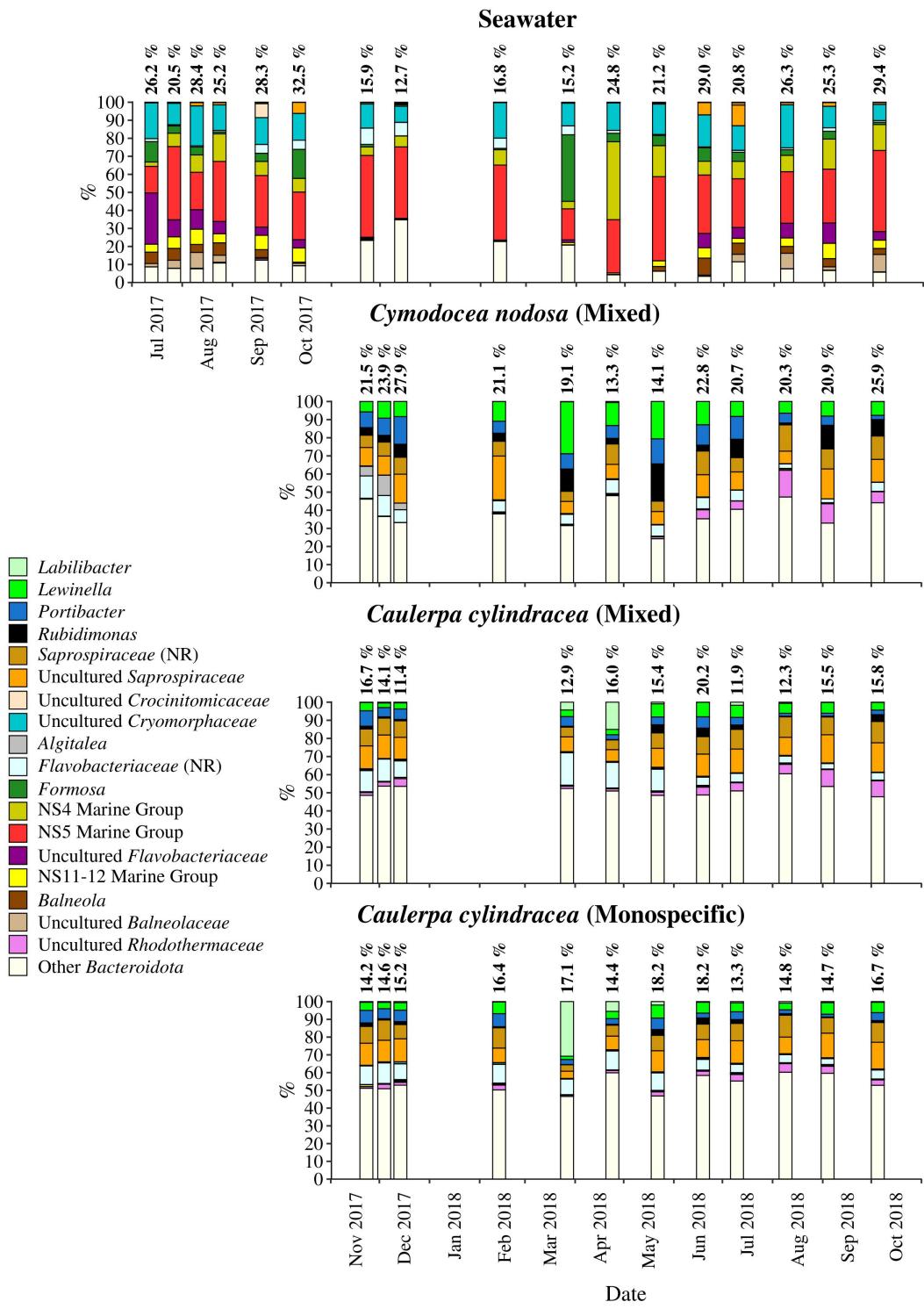


Fig. 6. Taxonomic classification and relative contribution of the most abundant ($\geq 2 \%$) sequences within the *Bacteroidota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of sequences 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)

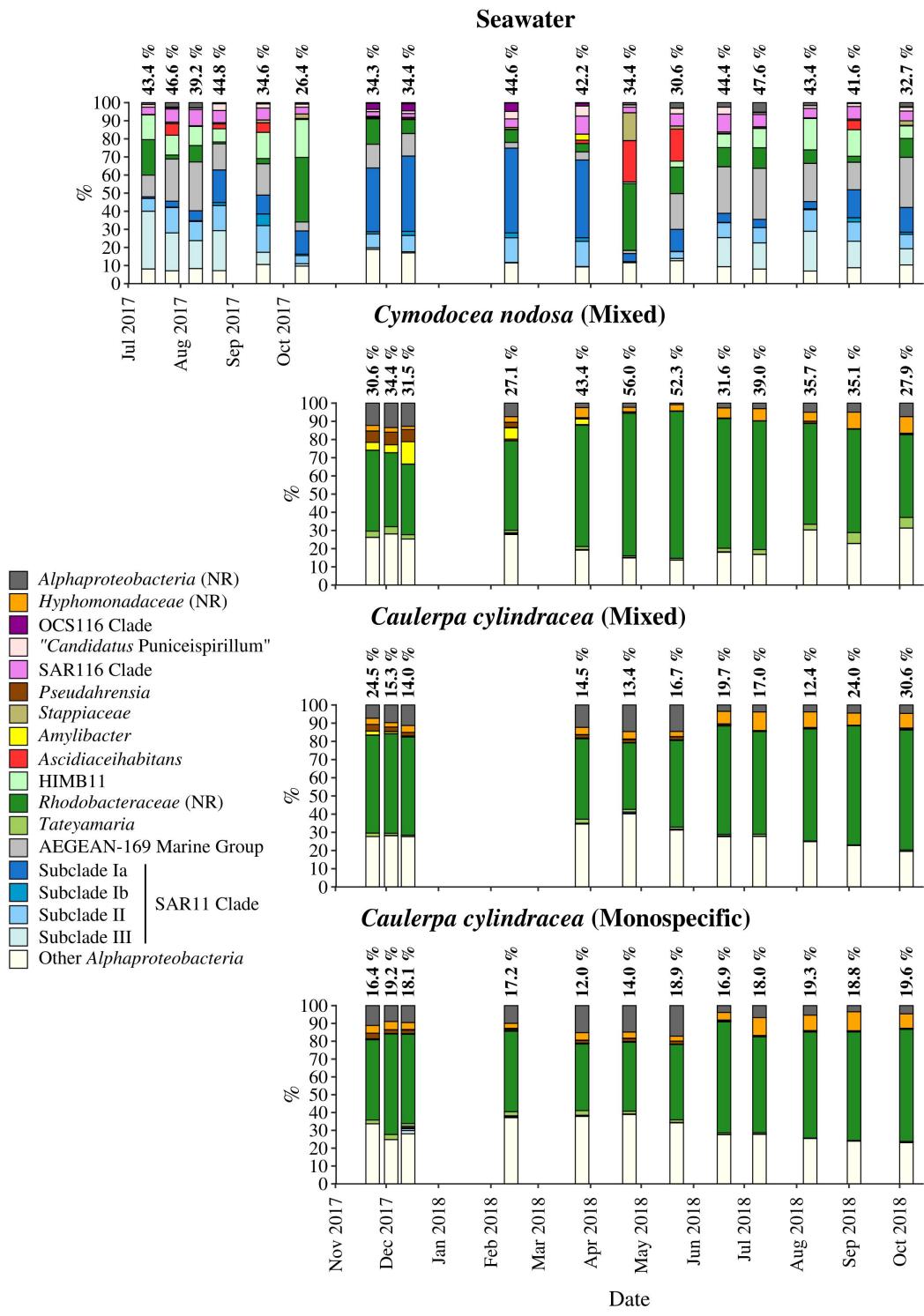


Fig. 7. Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) alphaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of alphaproteobacterial 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)

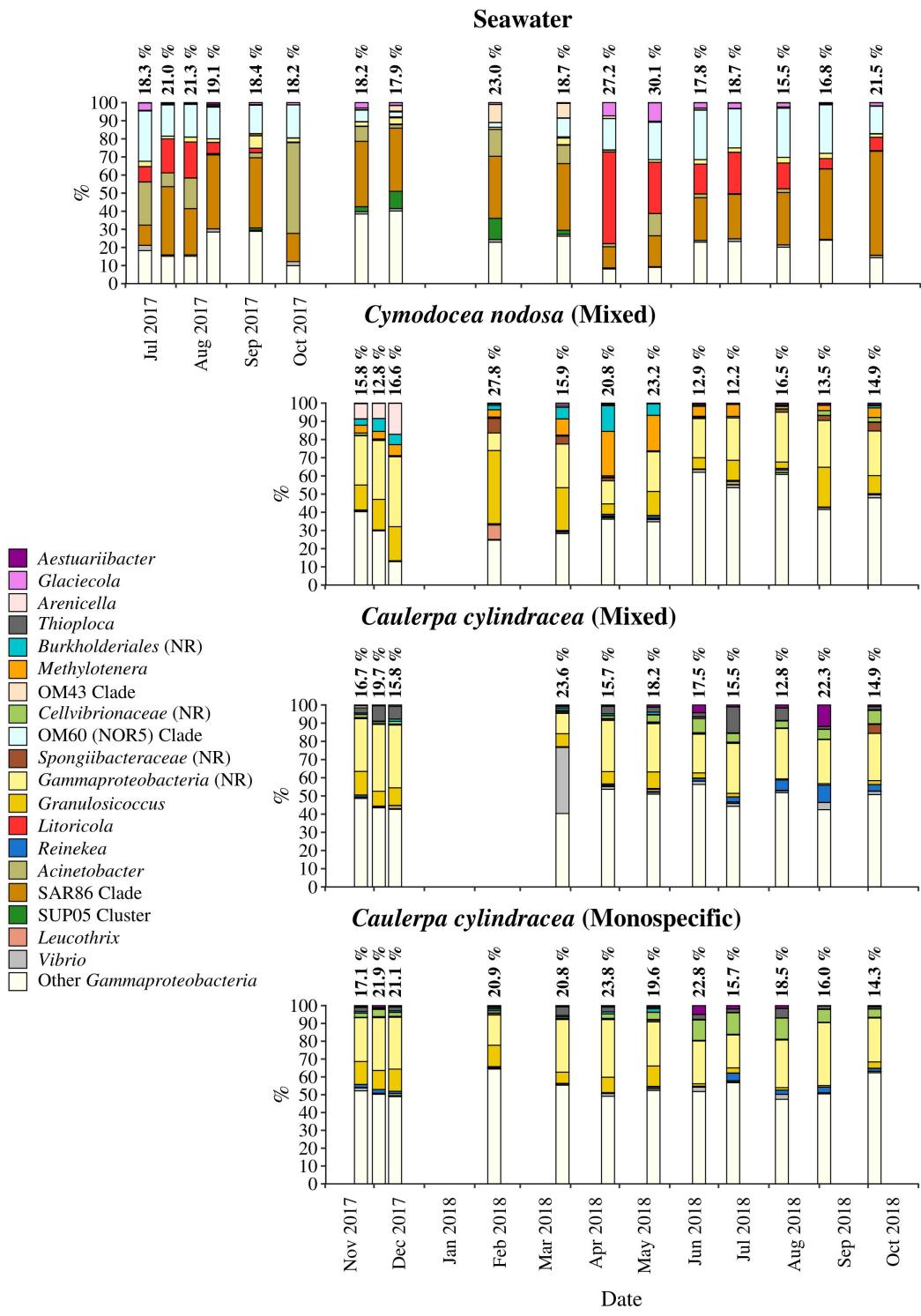


Fig. 8. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) gammaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of gammaproteobacterial 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)

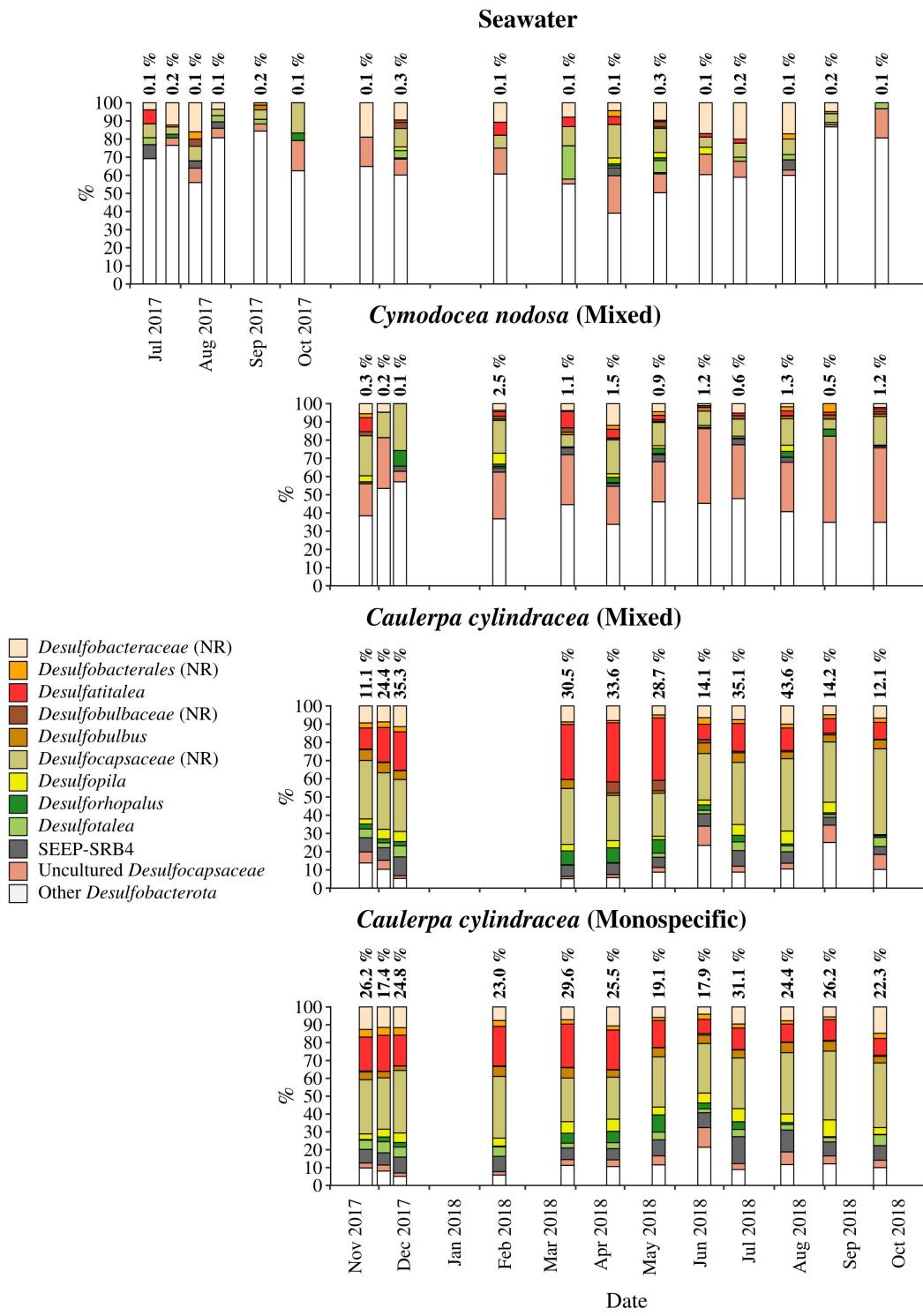


Fig. 9. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) sequences within the *Desulfobacterota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of 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)