

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 17,142 different OTUs with
81 365 to 2,038 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, $1,694.5 \pm 135.8$ OTUs] and monospecific [Number of OTUs, $1,729 \pm$
86 159.6 OTUs]) than for *C. nodosa* (Number of OTUs, $1,061.9 \pm 209.5$ OTUs) and lowest values were
87 obtained for the microbial community of the ambient seawater (Number of OTUs, 527.8 ± 146.1
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
103 for ambient seawater than for *C. nodosa* and especially *C. cylindracea* associated communities
104 (Fig. 2). In addition, only 0.4 – 1.0 % of OTUs from each surface associated community were
105 present at all seasons. These persistent OTUs constituted a high proportion of total sequences
106 (41.8 – 51.6 %). To further disentangle the environmental and seasonal community dissimilarity a
107 Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances and OTU abundances was
108 applied. A clear separation between ambient seawater and surface associated communities was
109 found (Fig. 3). In addition, a separation of epiphytic bacterial and archaeal communities based
110 on host species was detected. This separation was further supported by ANOSIM ($R = 0.96, p <$
111 0.001). Seasonal changes of *C. nodosa* associated communities indicated a separation between
112 spring, summer and autumn/winter samples (ANOSIM, $R = 0.53, p < 0.001$). For *C. cylindracea*
113 associated communities a separation between summer and autumn/winter/spring samples was
114 observed that was, however, not as strong as for *C. nodosa* associated communities (ANOSIM, $R =$
115 0.31, $p < 0.05$) (Fig. 3).

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

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

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

141 Sequences classified as *Bacteroidota* comprised, on average, 19.2 ± 5.5 % of all sequences
142 (Fig. 6). Similarly to *Cyanobacteria*, large differences in the taxonomic composition between
143 ambient seawater and surface associated communities were found (Fig. 6). The ambient seawater
144 community was characterized by the NS4 and NS5 marine groups, uncultured *Cryomorphaceae*,
145 uncultured *Flavobacteriaceae*, NS11-12 marine group, *Balneola*, uncultured *Balneolaceae* and
146 *Formosa*. In contrast, in surface associated communities *Lewinella*, *Portibacter*, *Rubidimonas*,

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

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

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

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

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

191 **Discussion**

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

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

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

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

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

258 Seasonal changes in richness in the epiphytic community were substantial as indicated by the
259 proportion of OTUs ($\leq 1.0\%$) present at every sampling date. These persistent OTUs, however,
260 were accounting for a high proportion of sequences ($\geq 41.8\%$) (Fig. 2). A very similar proportion
261 of persistent OTUs was reported in high-frequency sampling studies describing seasonal changes in
262 picoplankton (Gilbert *et al.*, 2009, 2012). In comparison to the seawater community, a lower degree
263 of seasonal shifts was observed for the macrophyte surface associated communities. It appears that
264 microniches at the surfaces of macrophytes are providing more stable conditions than the ambient
265 seawater. At the level of OTUs seasonal changes of *C. nodosa* and *C. cylindracea* associated
266 communities were identified that could be linked to the growth cycle of the seagrass and macroalgae
267 (M. Najdek, personal communication). *C. nodosa* was characterized by a spring community
268 during maximum seagrass proliferation, a summer community during the highest standing stock of
269 *C. nodosa* and an autumn/winter community during the decay of seagrass biomass. In contrast, *C.*
270 *cylindracea* started to proliferate in late spring and was characterized only by a summer community

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

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

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

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

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

334 **Experimental procedures**

335 **Sampling**

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

350 **DNA isolation**

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

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

363 **Illumina 16S rRNA sequencing**

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

379 **Sequence analysis**

380 Obtained sequences were analysed on the computer cluster Isabella (University Computing
381 Center, University of Zagreb) using mothur (version 1.43.0) (Schloss *et al.*, 2009) according to the
382 MiSeq Standard Operating Procedure (MiSeq SOP; https://mothur.org/wiki/MiSeq_SOP) (Kozich *et*
383 *al.*, 2013) and recommendations provided by the Riffomonas project to enhance data reproducibility
384 (<http://www.riffomonas.org/>). For alignment and classification of sequences the SILVA SSU Ref
385 NR 99 database (release 138; <http://www.arb-silva.de>) was used (Quast *et al.*, 2013; Yilmaz *et*
386 *al.*, 2014). Pipeline data processing and visualization was done using R (version 3.6.0) (R Core
387 Team, 2019) combined with packages vegan (version 2.5-6) (Oksanen *et al.*, 2019), tidyverse
388 (version 1.3.0) (Wickham *et al.*, 2019) and multiple other packages (Xie, 2014, 2015, 2019, 2020;
389 Neuwirth, 2014; Xie *et al.*, 2018; Wilke, 2018; Allaire *et al.*, 2019; Zhu, 2019). The detailed
390 analysis procedure including the R Markdown file are available in the GitHub repository (https://github.com/MicrobesRovinj/Korlevic_EpiphyticDynamics_EnvironMicrobiol_2020). Based on
391 the ATCC MSA-1002 mock community included in the analysis an average sequencing error rate
392 of 0.01 % was determined, which is in line with previously reported values for next-generation
393 sequencing data (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative controls
394 processed together with the samples yielded on average only 2 sequences after sequence quality
395 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 ($\geq 1\%$) 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 ($\geq 1\%$)
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 ($\geq 2\%$) 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 ($\geq 2\%$)
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 ($\geq 1\%$)
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 ($\geq 1\%$) 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)	0.27		
<i>Caulerpa cylindracea</i> (Monospecific)	0.26	0.35	
Seawater	0.11	0.10	0.10

Bray-Curtis Similarity Coefficient

<i>Caulerpa cylindracea</i> (Mixed)	0.40		
<i>Caulerpa cylindracea</i> (Monospecific)	0.38	0.78	
Seawater	0.06	0.05	0.06
<i>Cymodocea nodosa</i>	(Mixed)	<i>Caulerpa cylindracea</i> (Mixed)	<i>Caulerpa cylindracea</i> (Monospecific)

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.

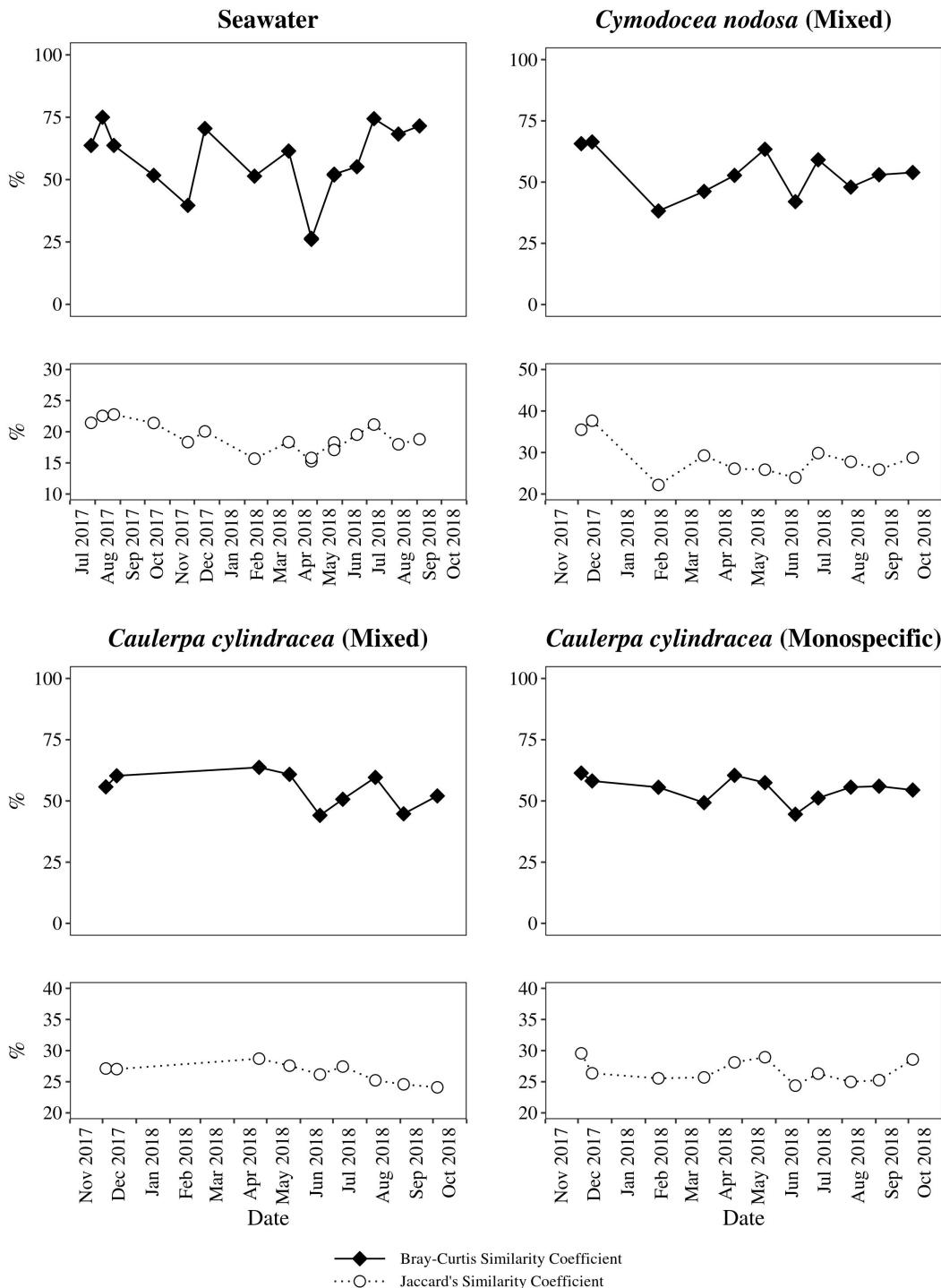


Fig. 2. Proportion of shared bacterial and archaeal communities (Bray-Curtis similarity coefficient) and shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) between consecutive sampling dates and from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea*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

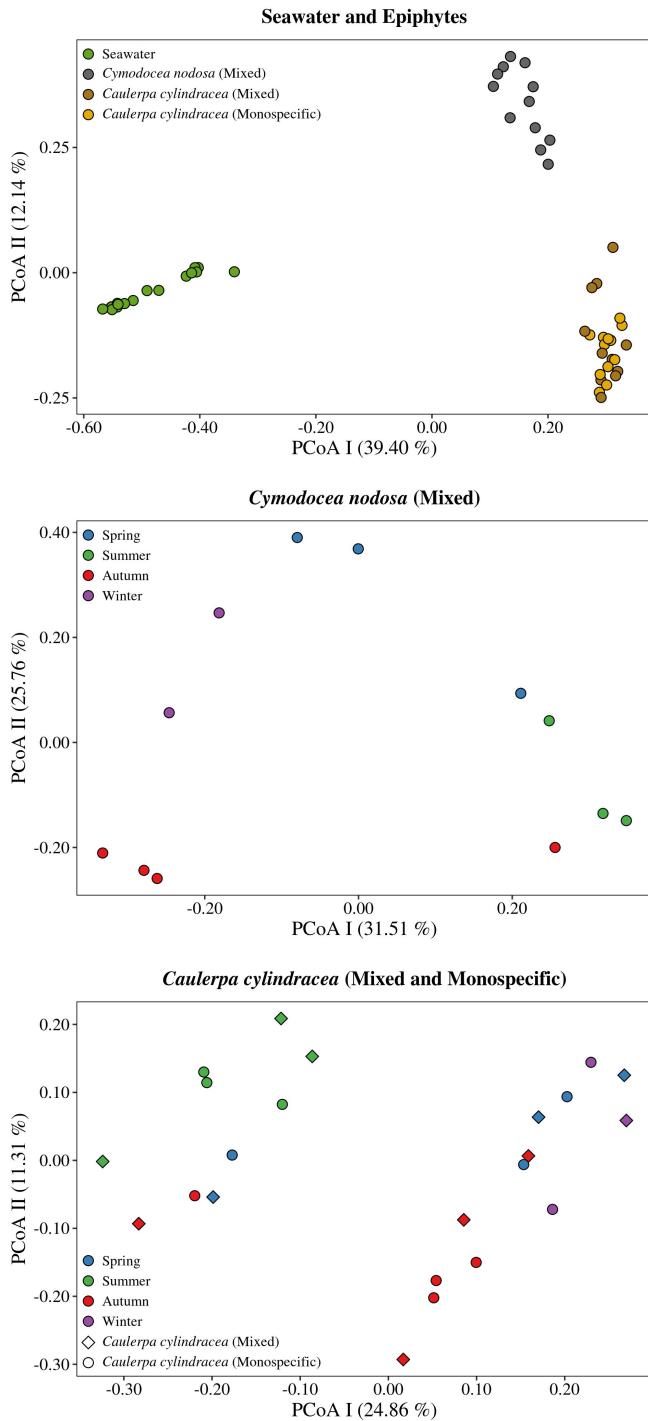


Fig. 3. Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU abundances of bacterial and archaeal communities from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. Samples from the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

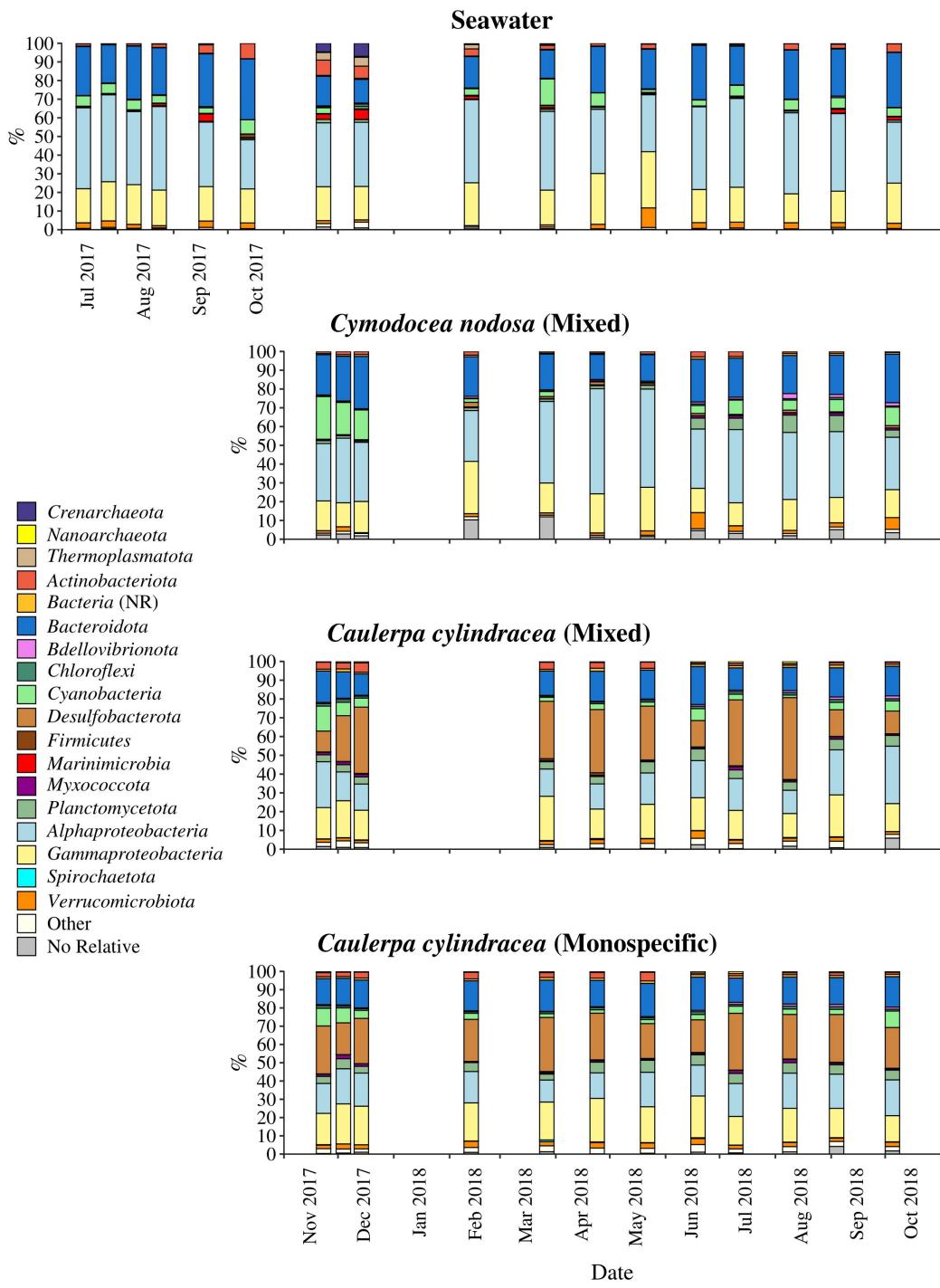


Fig. 4. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) bacterial and archaeal sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. NR – No Relative (sequences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

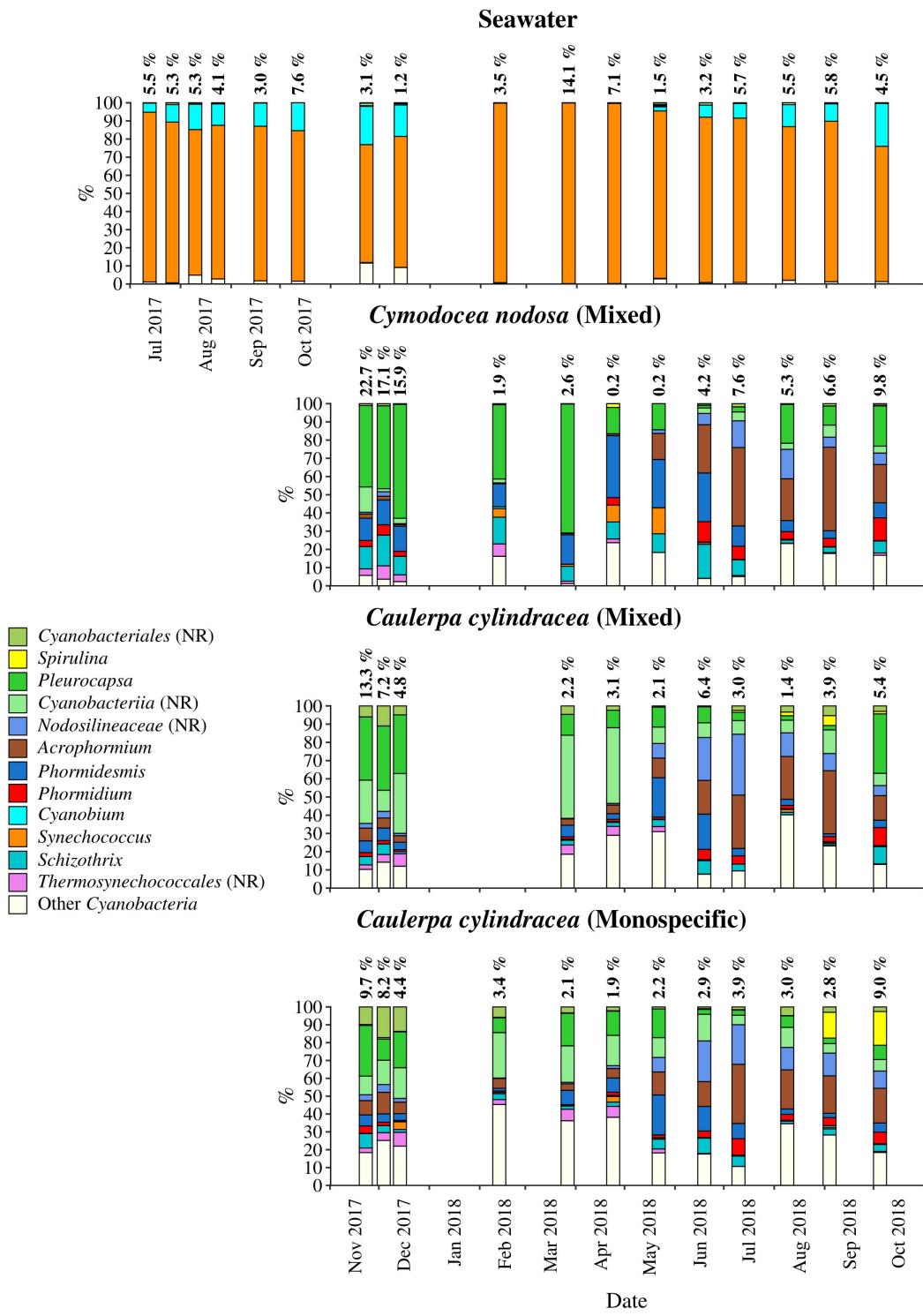


Fig. 5. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) cyanobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of cyanobacterial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

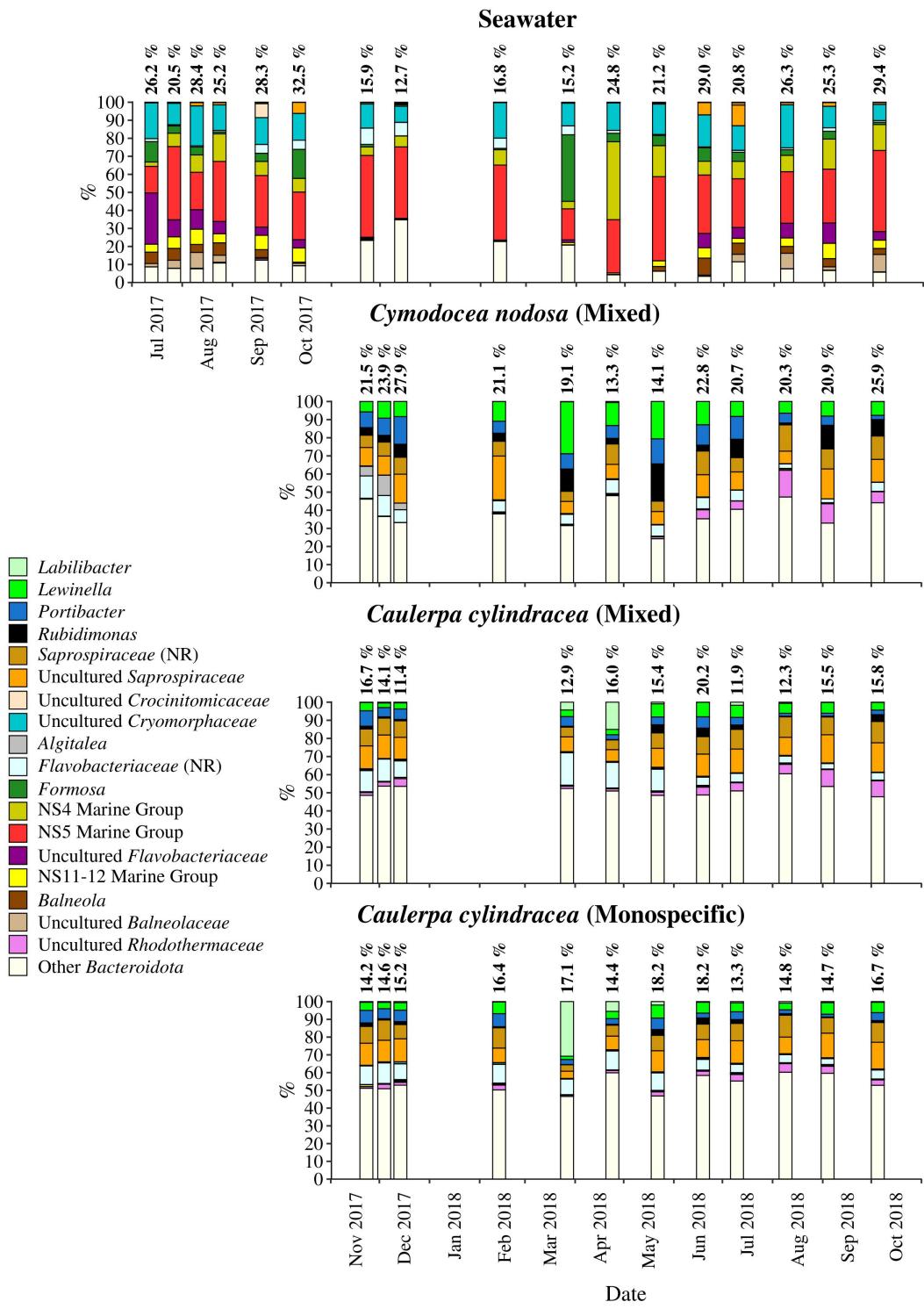


Fig. 6. Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) sequences within the *Bacteroidota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of sequences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

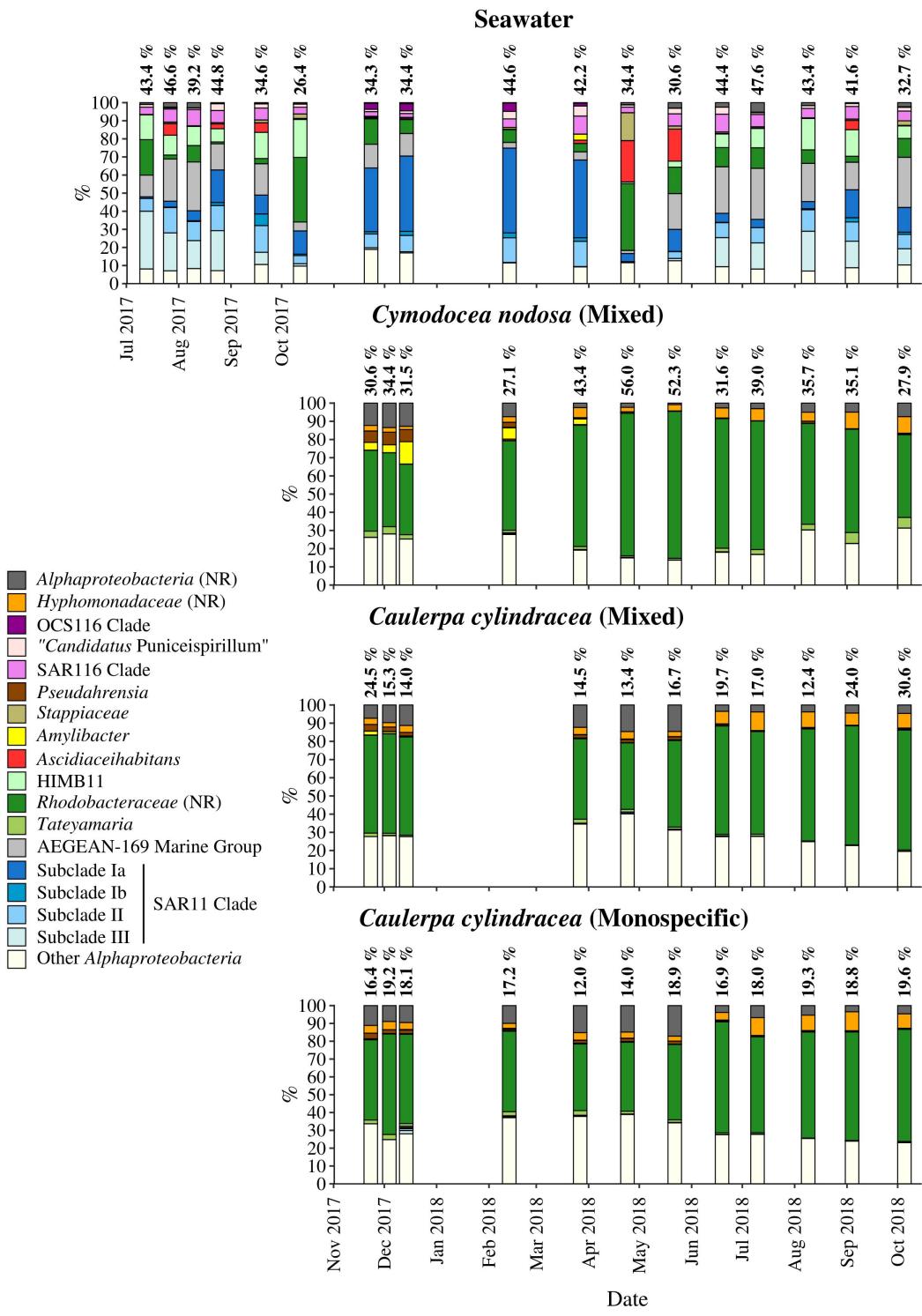


Fig. 7. Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) alphaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of alphaproteobacterial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

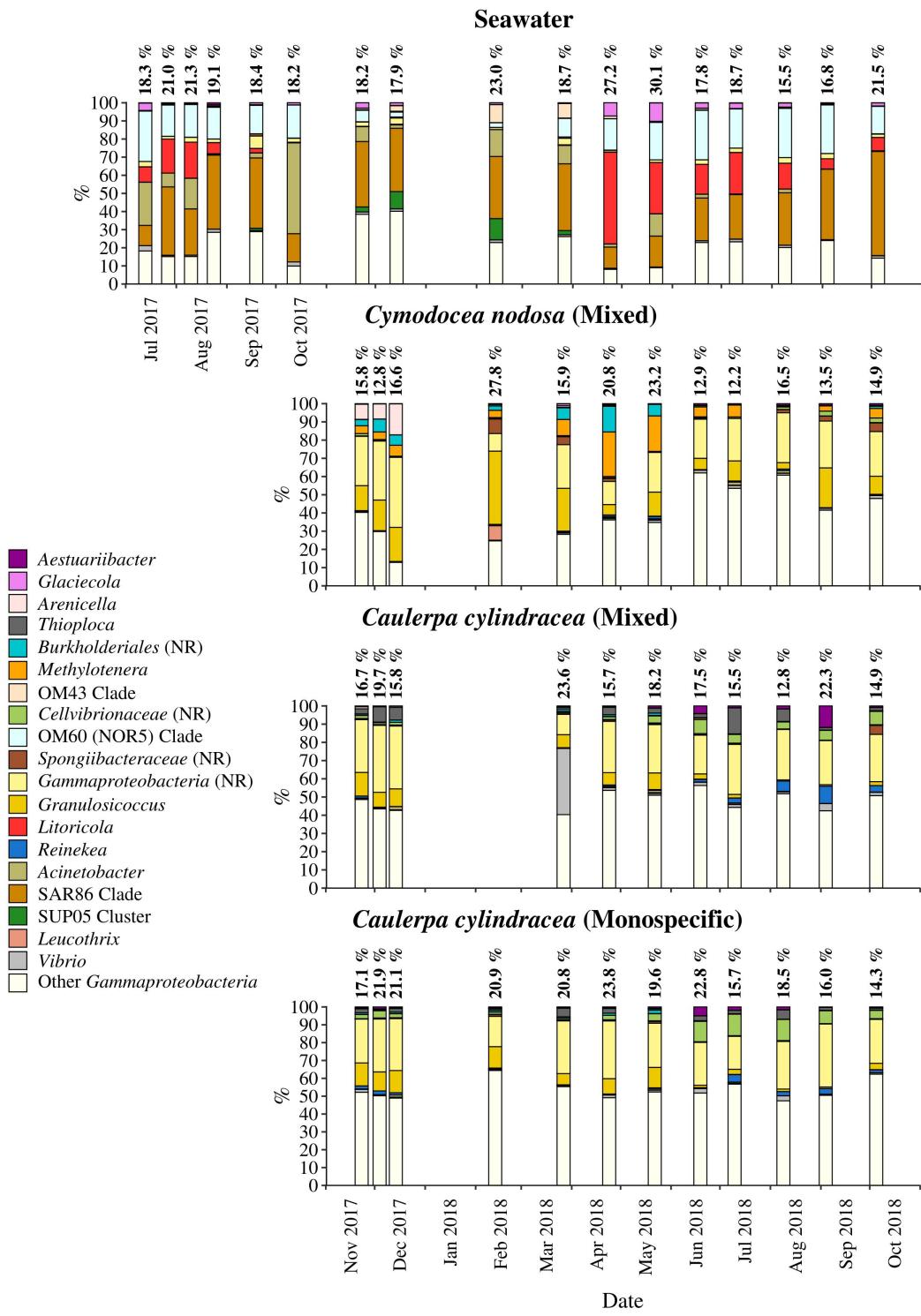


Fig. 8. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) gammaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of gammaproteobacterial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

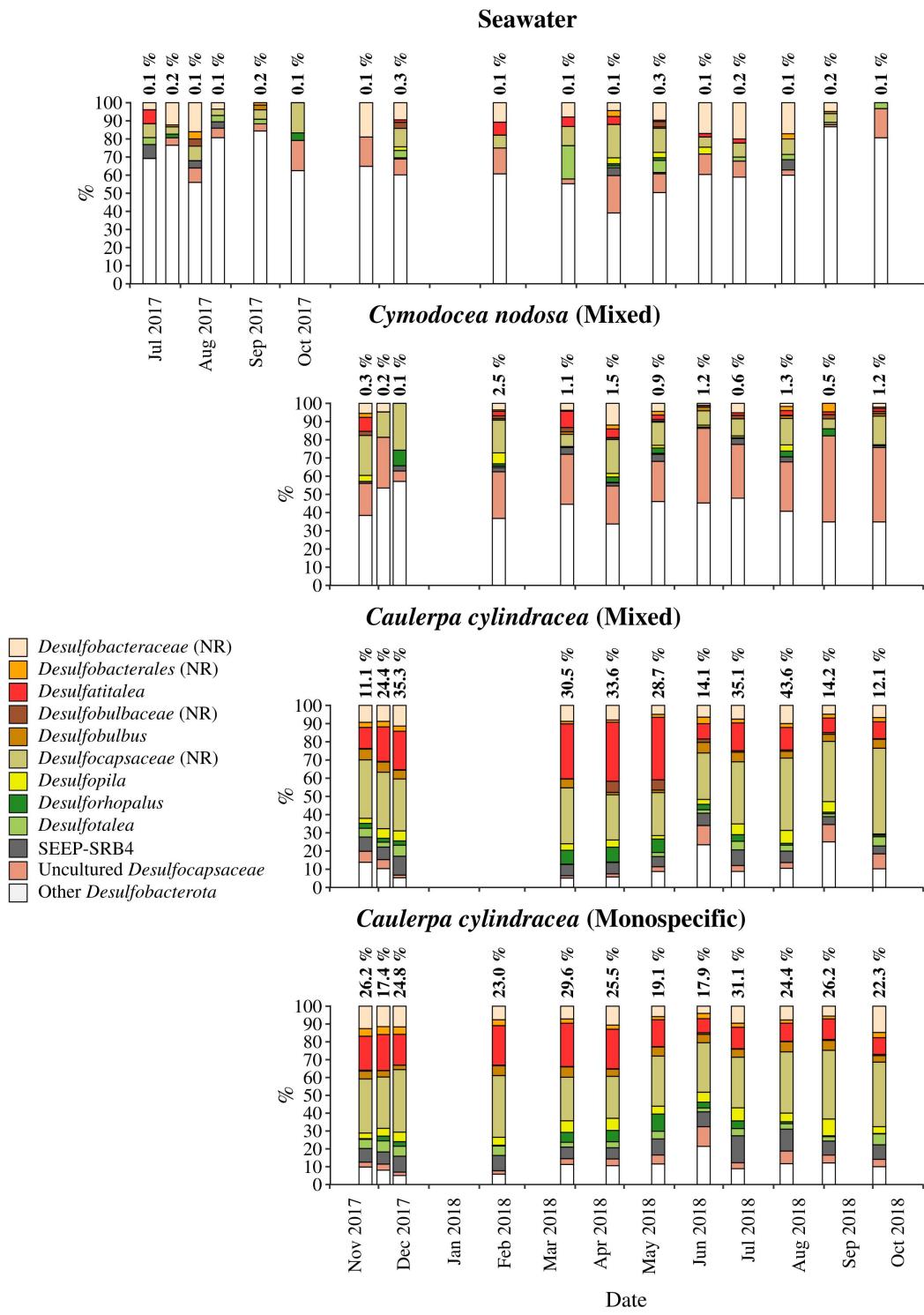


Fig. 9. Taxonomic classification and relative contribution of the most abundant ($\geq 1 \%$) sequences within the *Desulfobacterota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion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