

Seasonal Dynamics of Epiphytic Microbial Communities on Marine Macrophyte Surfaces

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1 Abstract

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

22 **Introduction**

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

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

43 All these ecological roles are carried out by a taxonomically diverse community of
44 microorganisms. At higher taxonomic ranks a core set of macrophyte epiphytic taxa was
45 described consisting mainly of *Alphaproteobacteria*, *Gammaproteobacteria*, *Desulfobacterota*,
46 *Bacteroidota*, *Cyanobacteria*, *Actinobacteriota*, *Firmicutes*, *Planctomycetota*, *Chloroflexi* and

47 *Verrucomicrobiota* (Crump and Koch, 2008; Tujula *et al.*, 2010; Lachnit *et al.*, 2011). In contrast,
48 at lower taxonomic ranks host specific microbial communities were described (Lachnit *et al.*,
49 2011; Roth-Schulze *et al.*, 2016). Recently, it was shown that even different morphological niches
50 within the same alga had a higher influence on bacterial community variation than biogeography
51 or environmental factors (Morrissey *et al.*, 2019). While there is high community variation
52 between host species it was observed that the majority of metagenome determined functions were
53 conserved both between host species and individuals (Burke and Peter Steinberg *et al.*, 2011;
54 Roth-Schulze *et al.*, 2016). This discrepancy between taxonomic and functional composition
55 could be explained by the lottery hypothesis. It postulates that an initial random colonization step
56 is performed from a set of functionally equivalent taxonomic groups resulting in taxonomically
57 different epiphytic communities sharing a core set of functional genes (Burke and Peter Steinberg
58 *et al.*, 2011; Roth-Schulze *et al.*, 2016). In addition, some of the variation in the observed data
59 could be attributed to different techniques used in various studies, such as different protocols
60 for epiphytic cell detachment and/or DNA isolation, as no standard protocol to study epiphytic
61 communities was established (Ugarelli *et al.*, 2019; Korlević *et al.*, submitted).

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

73 **Results**

74 Sequencing of the 16S rRNA V4 region yielded a total of 1.8 million sequences after
75 quality curation and exclusion of eukaryotic, chloroplast, mitochondrial and no relative sequences
76 (Table S1). A total of 35 samples originating from epiphytic archaeal and bacterial communities
77 associated with surfaces of the seagrass *C. nodosa* and macroalga *C. cylindracea* were analyzed. In
78 addition, 18 samples (one of the samples was sequenced two times) originating from picoplankton
79 archaeal and bacterial communities in the ambient seawater were also processed for comparison.
80 The number of reads per sample ranged between 8,409 and 77,463 sequences (Table S1). Even
81 when the highest sequencing effort was applied the rarefaction curves did not level off that is a
82 common observation in high-throughput 16S rRNA amplicon sequencing approaches (Figure S1).
83 Following quality curation and exclusion of sequences mentioned before reads were clustered
84 into 28,702 different OTUs at a similarity level of 97 %. Read numbers were normalized to the
85 minimum number of sequences, 8,409 (Table S1), through rarefaction resulting in 17,172 different
86 OTUs that ranged from 366 to 2,043 OTUs per sample (Figure S2). To determine seasonal changes
87 of richness and diversity the Observed Number of OTUs, Chao1, ACE, Exponential Shannon (Jost,
88 2006) and Inverse Simpson were calculated after normalization through rarefaction. Generally,
89 richness estimators and diversity indices showed similar trends. On average, higher values were
90 found for *C. cylindracea* (mixed [Number of OTUs, $1,683.2 \pm 130.9$ OTUs] and monospecific
91 [Number of OTUs, $1,737.2 \pm 166.4$ OTUs]), middle values for *C. nodosa* (Number of OTUs,
92 $1,058.4 \pm 216.8$ OTUs) and lower values for picoplankton communities in the ambient seawater
93 (Number of OTUs, 529.5 ± 143.0 OTUs) (Figure S2). Seasonal changes did not show such large
94 dissimilarities. *C. nodosa* communities showed a slow increase towards the end of the study,
95 while *C. cylindracea* (mixed and monospecific) communities were characterized by slightly larger
96 values in Spring and Summer in comparison to Autumn and Winter (Figure S2).

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

99 Bray-Curtis Similarity Coefficient were, respectively, calculated. Coefficients were determined
100 after normalization through rarefaction and binning of samples from a particular environment. The
101 highest proportion of shared OTUs and community was found between mixed and monospecific
102 *C. cylindracea* (Jaccard, 0.35; Bray-Curtis, 0.78), while lower shared values were calculated
103 between seawater and epiphytic communities (Figure 1). Shared proportion between *C. nodosa*
104 and *C. cylindracea* were approximately in the middle between these two extremes. To assess
105 seasonal changes in the proportion of shared OTUs and communities the Jaccard's and Bray-Curtis
106 Similarity Coefficients were calculated between consecutive sampling points (Figure 2). Both
107 coefficients showed similar trends. Temporal proportional changes were more pronounced for
108 seawater in comparison to *C. nodosa* and especially *C. cylindracea* associated communities
109 (Figure 2). In addition, only 0.4 – 1.0 % of OTUs from each surface associated community were
110 found at every time point. These OTUs also made a high proportion of total sequences (40.8
111 – 52.2 %). To further disentangle the environmental and seasonal community dissimilarity a
112 Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances and OTU abundances was
113 applied. It showed a clear separation between planktonic and surface associated communities
114 (Figure 3). In addition, a separation of epiphytic bacterial and archaeal communities based on
115 host species was determined. This separation was further supported by ANOSIM ($R = 0.96, p <$
116 0.001). Seasonal changes of *C. nodosa* associated communities indicated a separation between
117 Spring, Summer and Autumn/Winter samples (ANOSIM, $R = 0.53, p < 0.01$). For *C. cylindracea*
118 associated communities a separation between Summer and Autumn/Winter/Spring samples was
119 observed that was not so strongly supported (ANOSIM, $R = 0.30, p < 0.01$) (Figure 3).

120 The taxonomic composition of both, macrophyte associated and seawater communities,
121 was dominated by bacterial ($99.1 \pm 2.1 \%$) over archaeal sequences ($0.9 \pm 2.1 \%$) (Figure 4).
122 Higher relative abundances of chloroplast related sequences were only observed in surface
123 associated communities, with higher values in Autumn/Winter ($37.2 \pm 11.2 \%$) in comparison to
124 Spring/Summer ($20.9 \pm 9.7 \%$) (Figure S3). Generally, at higher taxonomic ranks (phylum-class)
125 epiphytic and seawater microbial communities were composed of similar bacterial taxa.

126 Seawater communities were mainly comprised of *Actinobacteriota*, *Bacteroidota*, *Cyanobacteria*,
127 *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobiota*. Communities associated
128 with *C. nodosa* consisted of same groups with the addition of *Planctomycetota* whose contribution
129 was higher in summer 2018. In addition, communities from mixed and monospecific *C.*
130 *cylindracea* were similar and characterized by same groups as seawater and *C. nodosa*
131 communities with the addition of *Desulfobacterota* (Figure 4). Larger differences between
132 environments and host species could be observed at lower taxonomic ranks (Figure 5 – 9).

133 *Cyanobacteria* related sequences were comprising, on average, $5.5 \pm 4.4\%$ of total sequences
134 (Figure 5). Higher proportions were found for *C. nodosa* ($16.4 \pm 5.3\%$) and *C. cylindracea*
135 (mixed [$(7.7 \pm 3.9\%)$] and monospecific [$(7.8 \pm 2.4\%)$]) associated communities in autumn and
136 for seawater communities in winter ($8.8 \pm 7.5\%$). Large taxonomic differences between surface
137 associated and seawater cyanobacterial communities were observed. Seawater communities
138 were mainly comprised of *Cyanobium* and *Synechococcus*, while surface associated communities
139 were consisted of *Pleurocapsa* and sequences without known relatives within *Cyanobacteriia*
140 (Figure 5). In addition, seasonal changes in surface associated communities were observed
141 with *Pleurocapsa* and no relative *Cyanobacteriia* comprising larger proportions in autumn and
142 winter and *Acrophormium*, *Phormidesmis* and no relative *Nodosilineaceae* in spring and summer
143 (Figure 5).

144 Sequences classified as *Bacteroidota* were comprising, on average, $19.2 \pm 5.5\%$ of all
145 sequences (Figure 6). Similarly to *Cyanobacteria*, large differences in the taxonomic composition
146 between seawater and surface associated communities were found (Figure 6). The seawater
147 community was characterized by the NS4 and NS5 marine groups, uncultured *Cryomorphaceae*,
148 uncultured *Flavobacteriaceae*, NS11-12 marine group, *Balneola*, uncultured *Balneolaceae* and
149 *Formosa*. In contrast, in surface associated communities *Lewinella*, *Portibacter*, *Rubidimonas*, no
150 relative *Sapspiraceae*, uncultured *Sapspiraceae*, no relative *Flavobacteriaceae* and uncultured
151 *Rhodothermaceae* were found. Some groups showed slight seasonal changes such as no relative

152 *Flavobacteriaceae* that were more pronounced from November 2017 until June 2018. In contrast,
153 uncultured *Rhodothermaceae* showed higher proportions from June 2018 until the end of the study
154 period. Surface associated *Bacteroidota* communities were very diverse as could be observed in
155 the high proportion of taxa that grouped as other *Bacteroidota* (Figure 6).

156 On average, *Alphaproteobacteria* were in comparison to other high rank taxa the largest
157 taxonomic group, comprising 29.2 ± 12.0 % of all sequences (Figure 7). In accordance to previous
158 taxa, high differences between seawater and surface associated communities were observed.
159 Picoplankton communities were composed mainly of the SAR11 clade, AEGEAN-169 marine
160 group, SAR116 clade, no relative *Rhodobacteraceae*, HIMB11 and OCS116 clade, while surface
161 associated communities were composed in high proportion of no relative *Rhodobacteraceae* and to
162 a lesser degree of *Pseudoahrensia*, no relative *Alphaproteobacteria*, no relative *Hyphomonadaceae*
163 and *Amylibacter*. Representatives of no relative *Rhodobacteraceae* were comprising on average
164 40.6 ± 23.2 % of all alphaproteobacterial sequences from the epiphytic community (Figure 7). In
165 addition, *Amylibacter* was detected mainly in *C. nodosa* from November 2017 until March 2018.

166 Sequences related to *Gammaproteobacteria* were comprising, on average, 18.6 ± 3.9 %
167 of all sequences (Figure 8). Similarly to previous taxa, large taxonomic differences between
168 seawater and surface associated communities were found. Seawater communities were mainly
169 comprised of the OM60 (NOR5) clade, *Litoricola*, *Acinetobacter* and the SAR86 clade,
170 while epiphytic communities were mainly composed of no relative *Gammaproteobacteria* and
171 *Granulosicoccus*. Beside these two groups specific to all three epiphytic communities, *C. nodosa*
172 was characterized by *Arenicella*, no relative *Burkholderiales* and *Methylotenera*, while *Thioploca*,
173 no relative *Cellvibrionaceae* and *Reinekea* were more specific to both mixed and monospecific
174 *C. cylindracea*. In addition, *Arenicella* was more pronounced in November and December 2017,
175 while no relative *Burkholderiales* and *Methylotenera* were more characteristic for the period from
176 March until May 2018. For the *C. cylindracea* specific taxa no relative *Cellvibrionaceae* and
177 *Reinekea* showed some seasonality and were characteristic for samples originating from June to

¹⁷⁸ October 2018. In addition, similarly to *Bacteroidota*, a large proportion of the surface associated
¹⁷⁹ community was grouped as other *Gammaproteobacteria* indicating high diversity within this
¹⁸⁰ group (Figure 8).

¹⁸¹ In contrast to previously described high rank taxa, *Desulfobacterota* were specific to *C.*
¹⁸² *cylindracea*. On average they comprised 11.2 ± 13.3 % of all sequences. Seawater and *C.*
¹⁸³ *nodosa* communities consisted of only 0.1 ± 0.08 % and 1.0 ± 0.7 % *Desulfobacterota* sequences,
¹⁸⁴ respectively. In the mixed and monospecific *C. cylindracea* communities their proportion was
¹⁸⁵ 25.7 ± 11.2 % and 24.0 ± 4.3 %, respectively (Figure 9). The community consisted mainly of
¹⁸⁶ no relative *Desulfobacteraceae*, *Desulfatitalea*, no relative *Desulfobulbaceae*, *Desulfobulbus*,
¹⁸⁷ no relative *Desulfocapsaceae*, *Desulfopila*, *Desulforhopalus*, *Desulfotalea*, SEEP-SRB4 and
¹⁸⁸ uncultured *Desulfocapsaceae* (Figure 9).

189 **Discussion**

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

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

215 Since the colonization of macrophyte surfaces is performed from a pool of prokaryotic cells
216 from the ambient seawater, it was interesting to see to which extent these two communities differ.
217 We observed a strong differentiation between the surface attached and seawater communities at
218 the level of OTUs that is in agreement with most published studies (Burke and Thomas *et al.*,
219 2011; Michelou *et al.*, 2013; Roth-Schulze *et al.*, 2016; Crump *et al.*, 2018; Ugarelli *et al.*, 2019).
220 These data indicate that marine macrophytes are selecting, from a pool of seawater microbial taxa,
221 the one that can colonize and proliferate on their surfaces (Salaün *et al.*, 2012; Michelou *et al.*,
222 2013). In contrast to these findings Fahimipour *et al.* (2017) found, in a global study of *Zostera*
223 *marina*, similarities between leaves and seawater samples. Discrepancies between our data and this
224 study could be explained by differences in studied seagrass species, methodological variations or
225 biogeographic trends as Fahimipour *et al.* (2017) were analyzing samples from different locations
226 throughout the northern hemisphere. It is possible that ambient seawater and leaves communities
227 from the same location are differing but are still more similar to each other when compared to
228 other sampling locations. Indeed, it was found that prokaryotic communities vary substantially
229 between different sampling sites (Bengtsson *et al.*, 2017). When the taxonomic composition at
230 high ranks was analyzed no such strong differentiation was noticed. Phyla and classes such as:
231 *Actinobacteriota*, *Bacteroidota*, *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and
232 *Verrucomicrobiota* were described that is in agreement with previously reported data (Burke and
233 Thomas *et al.*, 2011; Egan *et al.*, 2013; Michelou *et al.*, 2013). In contrast, when low taxonomic
234 ranks were analyzed (i.g. family and genus) a strong differentiation was observed. A similar
235 differentiation at lower taxonomic ranks was described for other species of macrophytes (Egan *et*
236 *al.*, 2013; Michelou *et al.*, 2013; Ugarelli *et al.*, 2019).

237 Beside differences between seawater and surface associated communities, there were
238 discussions if the prokaryotic epiphytic community is host-specific or there are generalists taxa
239 characteristic to all or many macrophytes (Egan *et al.*, 2013). Similarly to previously described
240 differences between seawater and surface attached communities, at high taxonomic ranks no
241 strong differentiation between communities associated with different host was observed. The only

242 high rank phylum that was differing between *C. nodosa* and *C. cylindracea* was *Desulfobacterota*,
243 whose sequences were more abundant in the *C. cylindracea* associated community. As already
244 mentioned, the rhizoids and part of the stolon are in contact with the sediment, so *Desulfobacterota*
245 are probably a part of the epiphytic community that was in contact with the sediment. Similar
246 high rank taxa found in this study were described to be specific for other species of macrophytes
247 (Burke and Thomas *et al.*, 2011; Lachnit *et al.*, 2011; Bengtsson *et al.*, 2017). In contrast to
248 high taxonomic ranks, a substantial differentiation between host specific communities was found,
249 which supports the host-specific hypothesis. Similar host-specificity was observed for different
250 species of macroalgae and seagrasses (Lachnit *et al.*, 2011; Roth-Schulze *et al.*, 2016; Ugarelli *et*
251 *al.*, 2019; Morrissey *et al.*, 2019). Taken together, at high taxonomic ranks a core set of taxa could
252 be described that is characteristic for all or many macrophytes, while at low taxonomic ranks a
253 community specific to host species could be identified (Egan *et al.*, 2013).

254 Seasonal richness changes in the epiphytic community were substantial as could be observed
255 in the proportion of OTUs that could be found at every sampling time ($\leq 1.0\%$). Interestingly,
256 these OTUs were accounting for a high proportion of sequences ($\leq 52.2\%$). A very similar
257 proportion of persistent OTUs and their sequence contribution was reported in high frequency
258 studies describing seasonal picoplankton changes (Gilbert *et al.*, 2009, 2012). In comparison to
259 the seawater community, a lower degree of seasonal shifts was observed for the surface associated
260 communities. It seems, microniches on the surfaces of macrophytes are providing more stable
261 conditions in comparison to the seawater. At the level of OTUs seasonal changes of *C. nodosa* and
262 *C. cylindracea* associated communities were identified that could be linked to the growth cycle of
263 the seagrass and macroalgae (M. Najdek, personal communication). *C. nodosa* was characterized
264 by a Spring community during maximum seagrass proliferation, a Summer community during a
265 biomass maximum and a Autumn/Winter community during a biomass senescence. In contrast,
266 *C. cylindracea* started to proliferate in late Spring and was characterized only by a Summer
267 community during maximal biomass increase and by a Autumn/Winter/Spring community when
268 the biomass was at the peak and the settlement started to subsequently decay. Similar seasonal

269 changes in the epiphytic community was also described for other macroalgae (Tujula *et al.*,
270 2010; Lachnit *et al.*, 2011). Higher temporal stability of *C. cylindracea* surface communities
271 in comparison to *C. nodosa* were also observed in the higher proportion of shared communities
272 between two consecutive sampling points.

273 Analysis of seasonal chloroplast sequence abundances showed higher values in Autumn/Winter
274 in comparison to Spring/Summer. This pattern is not surprising as seagrasses are known to harbor
275 more epiphytes during Autumn/Winter (Reyes and Sansón, 2001). Furthermore, we used an
276 adapted DNA isolation protocol that is known to partially coextract DNA from planktonic
277 eukaryotes (Korlević *et al.*, 2015). Strong seasonal fluctuations of high rank epiphytic taxa
278 were not observed, with the exception of *Cyanobacteria*. Cyanobacterial sequences were more
279 pronounced in November and December in comparison to Spring and Summer. Interestingly,
280 in these high proportion months the majority of cyanobacterial sequences were classified as
281 *Pleurocapsa*, a group known to colonized different living and nonliving surfaces (Burns *et al.*,
282 2004; Longford *et al.*, 2007; Mobberley *et al.*, 2012; Reisser *et al.*, 2014). It is possible that during
283 periods of low metabolic activity there is a reduced interaction and selection of the epiphytic
284 community by the seagrass, causing leaves to become a suitable surface for nonspecific colonizers
285 (Zavodnik *et al.*, 1998). *Pleurocapsa* was replaced in Spring and Summer by *Acrophormium*,
286 *Phormidesmis* and no relative *Nodosilineaceae*. A study of coastal microbial mats found also
287 higher proportion of *Nodosilineaceae* sequences in Summer, while *Phormidesmis* sequences
288 were at their peak in Autumn (Cardoso *et al.*, 2019). Other high rank taxa did not show strong
289 successional patterns. In every analyzed group, with the exception of *Desulfobacterota*, taxa
290 present throughout the year in similar proportions and season specific taxa could be identified.
291 Within *Bacteroidota* different groups withing the family *Saprospiraceae* (i.g. *Lewinella*,
292 *Portibacter* and *Rubidimonas*) were detected through the year. Members of this family are
293 often found in association with macrophytes and it is suggested that they are involved in the
294 hydrolysis and utilization of complex carbon sources (Burke and Thomas *et al.*, 2011; McIlroy
295 and Nielsen, 2014; Crump *et al.*, 2018). On the other hand, families *Flavobacteriaceae* and

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

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

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

331 **Experimental Procedures**

332 **Sampling**

333 Sampling was performed in the Bay of Funtana, northern Adriatic Sea (45°10'39" N,
334 13°35'42" E). Leaves and thalli were sampled in a meadow of *Cymodocea nodosa* invaded by the
335 invasive *Caulerpa cylindracea* (mixed settlement) and in a monospecific settlement of *Caulerpa*
336 *cylindracea* located in the proximity of the meadow at approximately monthly intervals from
337 December 2017 to October 2018 (Table S1). Leaves and thalli were collected by diving and
338 transported to the laboratory in containers placed on ice and filled with site seawater. Upon arrival
339 to the laboratory, *C. nodosa* leaves were cut into sections of 1 – 2 cm, while *C. cylindracea* thalli
340 were cut into 5 – 8 cm long sections. Leaves and thalli were washed three times with sterile
341 artificial seawater (ASW) to remove loosely attached microbial cells. Ambient seawater was
342 collected in 10 l containers by diving and transported to the laboratory where the whole container
343 volume was filtered through a 20 µm net. The filtrate was further sequentially filtered through 3
344 µm and 0.2 µm polycarbonate membrane filters (Whatman, United Kingdom) using a peristaltic
345 pump. Filters were briefly dried at room temperature and stored at –80 °C. Seawater samples
346 were also collected approximately monthly from July 2017 to October 2018.

347 **DNA Isolation**

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

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

360 **Illumina 16S rRNA Sequencing**

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

377 **Sequence Analysis**

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

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578 **Figure Captions**

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

583 **Figure 2.** Proportion of shared bacterial and archaeal communities (Bray-Curtis Similarity
584 Coefficient) and shared bacterial and archaeal OTUs (Jaccard's Similarity Coefficient) between
585 consecutive sampling points and from the surfaces of macrophytes (*C. nodosa* [Mixed Settlement]
586 and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater.

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

592 **Figure 4.** Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial
593 and archaeal sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C.*
594 *cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. NR – No Relative

595 **Figure 5.** Taxonomic classification and relative contribution of the most abundant (> 1 %)
596 cyanobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C.*
597 *cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion
598 of cyanobacterial sequences in the total bacterial and archaeal community is given above the
599 corresponding bar. NR – No Relative

600 **Figure 6.** Taxonomic classification and relative contribution of the most abundant (> 2 %)
601 sequences within the *Bacteroidota* on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement]

602 and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The
603 proportion of sequences classified as *Bacteroidota* in the total bacterial and archaeal community
604 is given above the corresponding bar. NR – No Relative

605 **Figure 7.** Taxonomic classification and relative contribution of the most abundant (> 2 %)
606 alphaproteobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement]
607 and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The
608 proportion of alphaproteobacterial sequences in the total bacterial and archaeal community is
609 given above the corresponding bar. NR – No Relative

610 **Figure 8.** Taxonomic classification and relative contribution of the most abundant (> 2 %)
611 gammaproteobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement]
612 and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The
613 proportion of gammaproteobacterial sequences in the total bacterial and archaeal community is
614 given above the corresponding bar. NR – No Relative

615 **Figure 9.** Taxonomic classification and relative contribution of the most abundant (> 1 %)
616 sequences within the *Desulfobacterota* on the surfaces of macrophytes (*C. nodosa* [Mixed
617 Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient
618 seawater. The proportion of sequences classified as *Desulfobacterota* in the total bacterial and
619 archaeal community is given above the corresponding bar. NR – No Relative

620 **Figures**

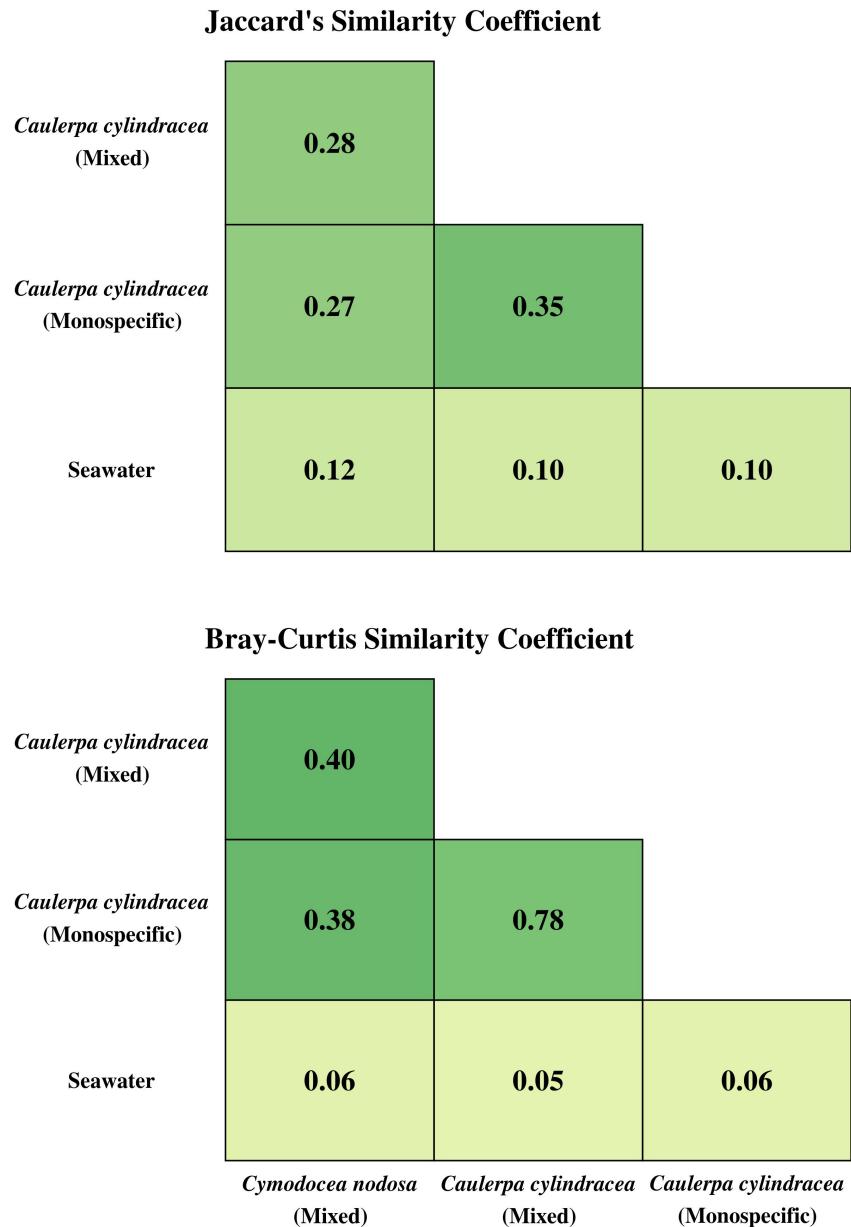


Figure 1. Proportion of shared bacterial and archaeal OTUs (Jaccard's Similarity Coefficient) and shared bacterial and archaeal communities (Bray-Curtis Similarity Coefficient) between assemblages associated with the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed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

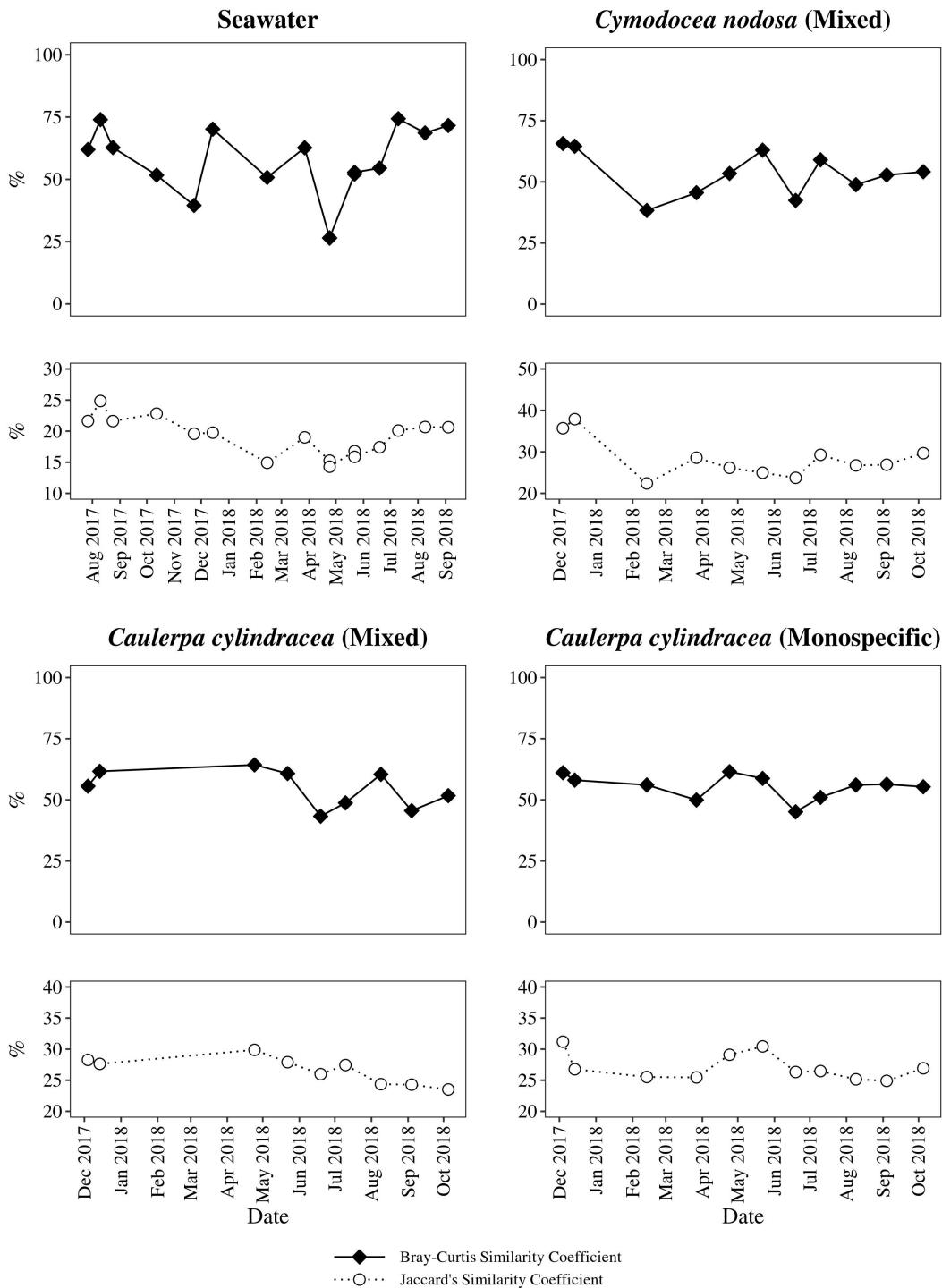


Figure 2. Proportion of shared bacterial and archaeal communities (Bray-Curtis Similarity Coefficient) and shared bacterial and archaeal OTUs (Jaccard's Similarity Coefficient) between consecutive sampling points and from the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea*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

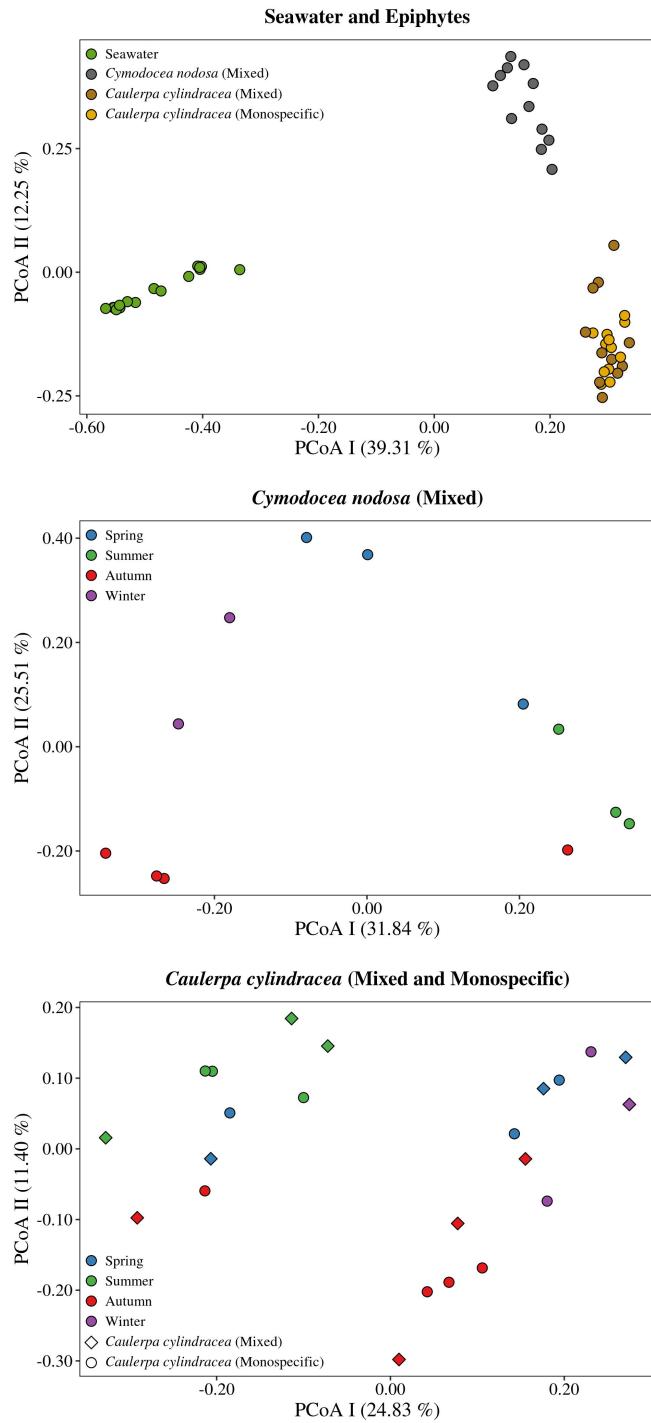


Figure 3. Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU abundances of bacterial and archaeal communities from the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. Samples from the 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.

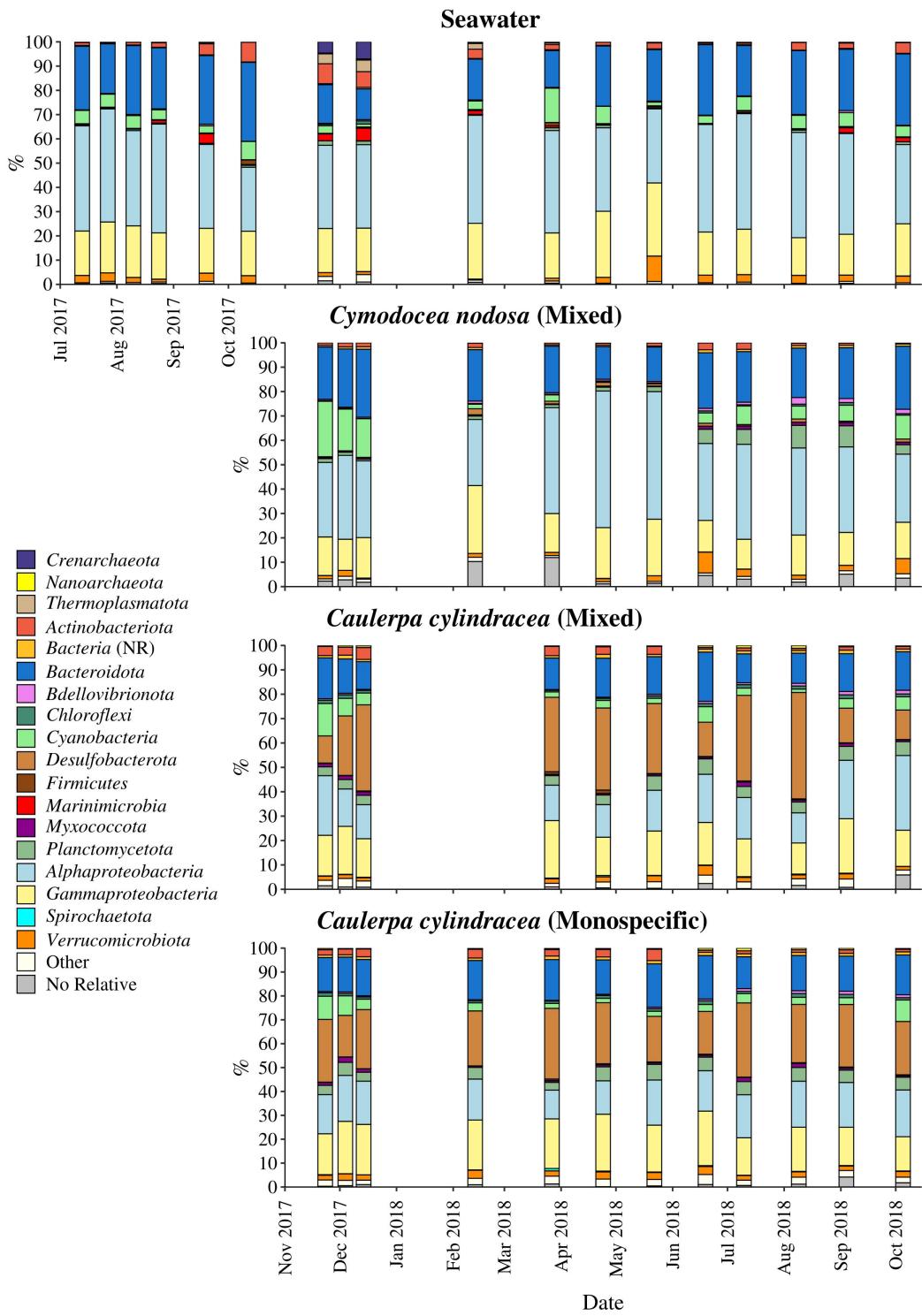


Figure 4. Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial and archaeal sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. NR – No Relative

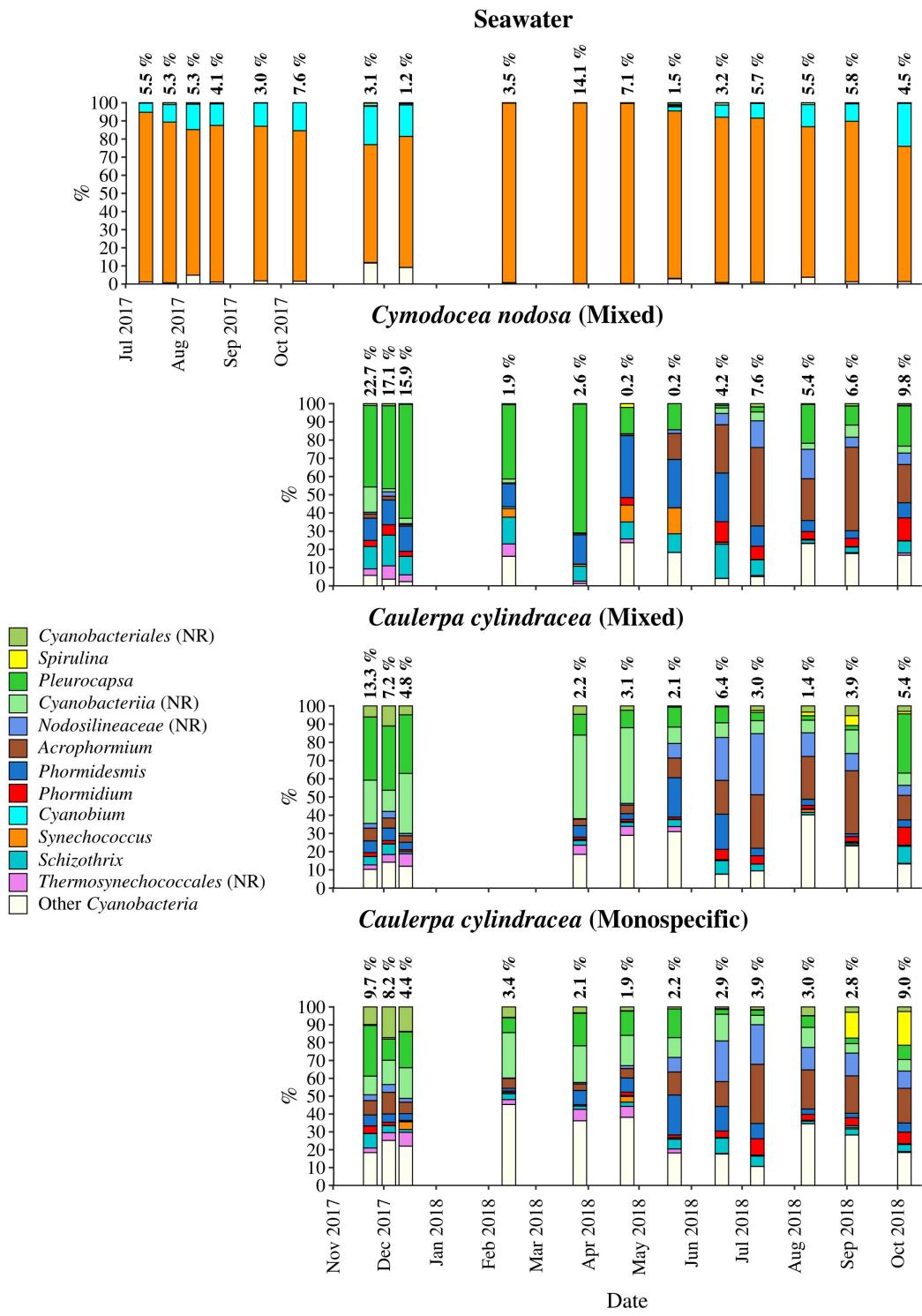


Figure 5. Taxonomic classification and relative contribution of the most abundant (> 1 %) cyanobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion of cyanobacterial sequences in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative

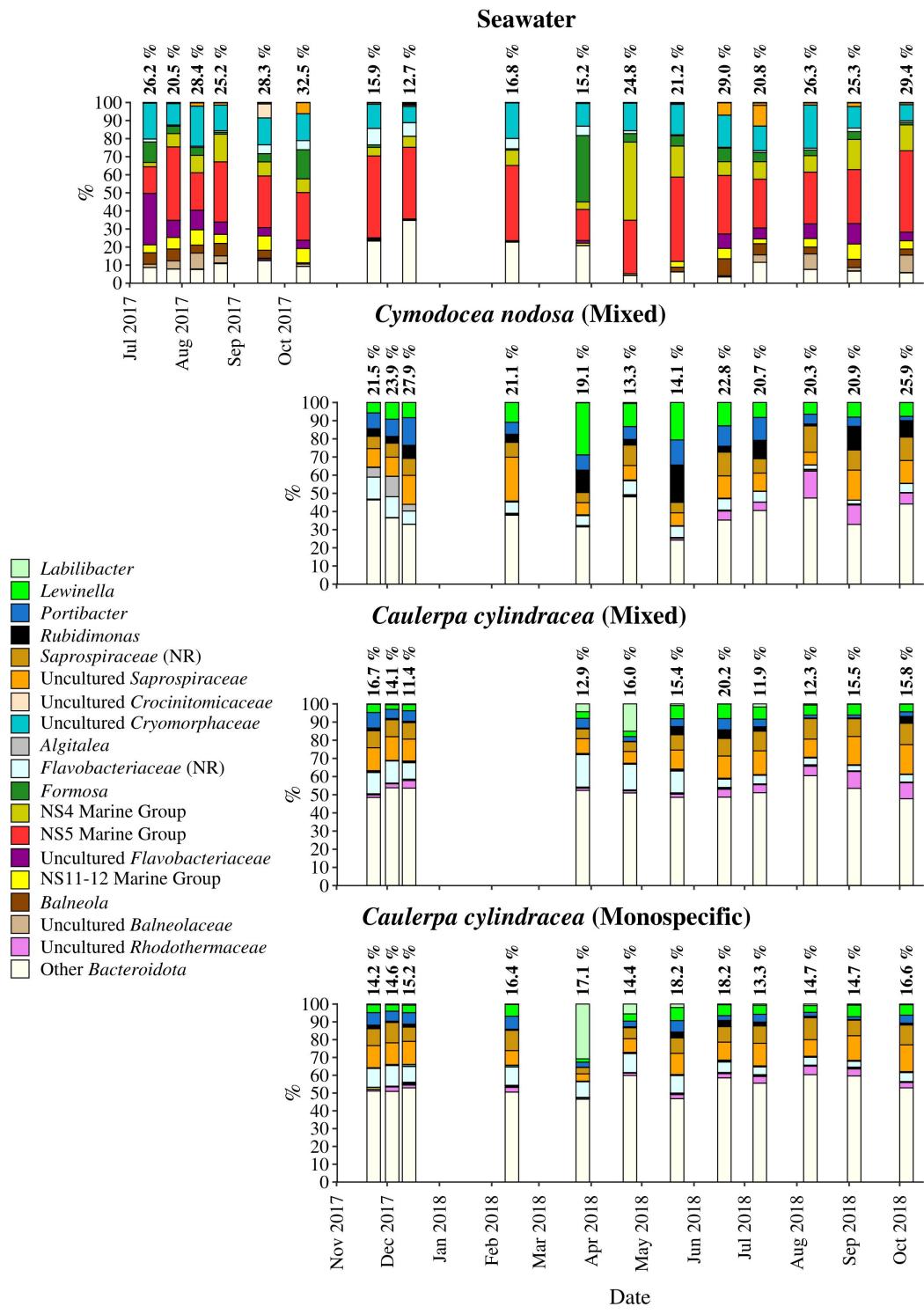


Figure 6. Taxonomic classification and relative contribution of the most abundant (> 2 %) sequences within the *Bacteroidota* on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion of sequences classified as *Bacteroidota* in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative

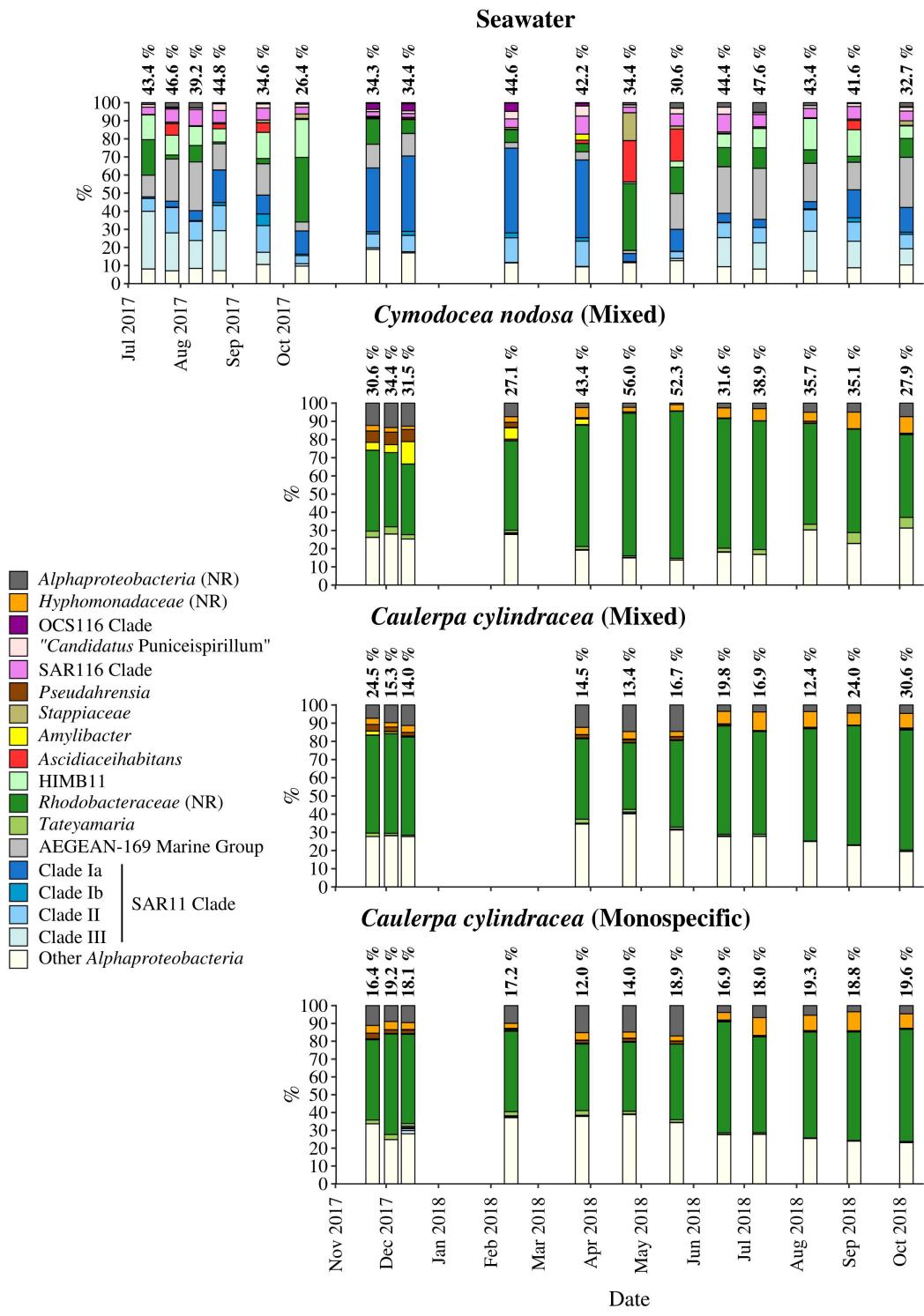


Figure 7. Taxonomic classification and relative contribution of the most abundant (> 2 %) alphaproteobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion of alphaproteobacterial sequences in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative

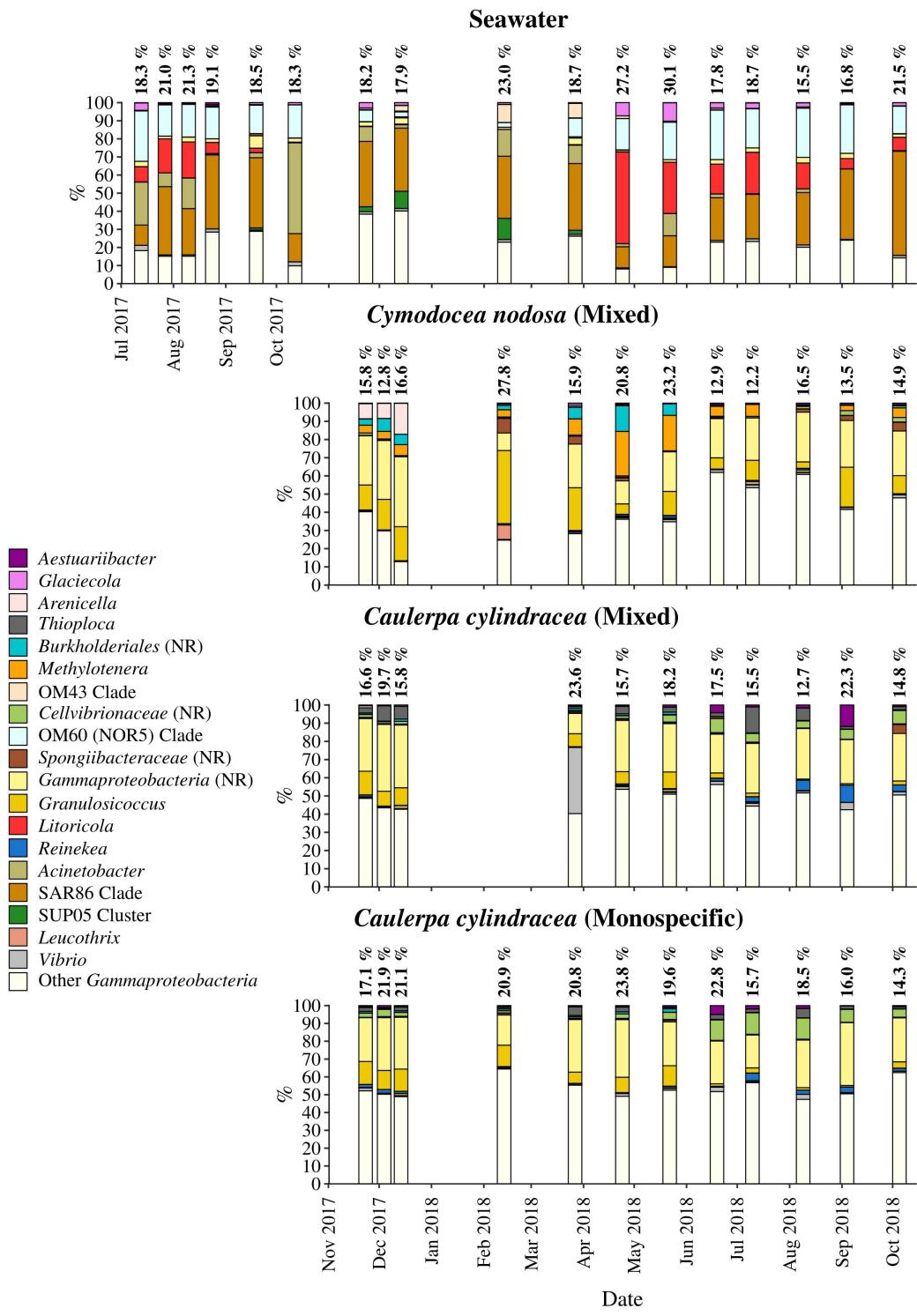


Figure 8. Taxonomic classification and relative contribution of the most abundant (> 2 %) gammaproteobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion of gammaproteobacterial sequences in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative

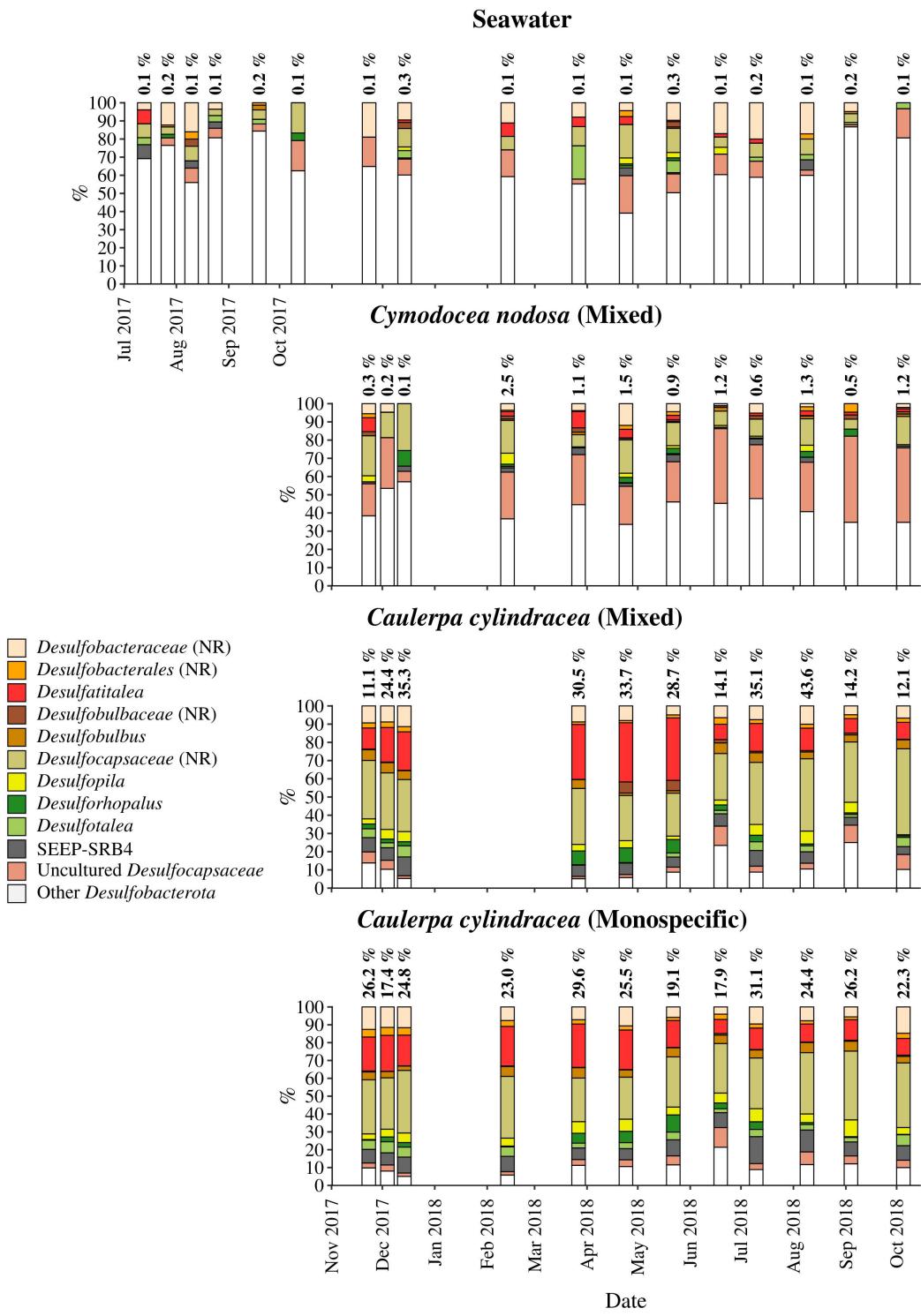


Figure 9. Taxonomic classification and relative contribution of the most abundant (> 1 %) sequences within the *Desulfobacterota* on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion of sequences classified as *Desulfobacterota* in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative