

Seasonal Dynamics of Epiphytic Microbial Communities on Marine Macrophyte Surfaces

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Running title: Seasonal dynamics of epiphytic communities

1 Abstract

2 Surfaces of marine macrophytes are inhabited by diverse microbial communities. Most
3 studies focusing on epiphytic communities of macrophytes did not take into account temporal
4 changes or applied low sampling frequency approaches. The seasonal dynamics of epiphytic
5 microbial communities was determined in a meadow of *Cymodocea nodosa* invaded by *Caulerpa*
6 *cylindracea* and in a monospecific settlement of *Caulerpa cylindracea* at monthly intervals. For
7 comparison the ambient prokaryotic picoplankton community was also characterized. At the 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 et
29 al., 2011b). In such an environment a number of positive and negative interactions between the
30 macrophyte and the colonizing microorganisms have been described (Egan et al., 2013; Hollants
31 et al., 2013; Tarquinio et al., 2019). Macrophytes can promote growth of associated microbes by
32 nutrient exudation, while in return microorganisms may support macrophyte performance through
33 improved nutrient availability, phytohormone production and protection from toxic compounds,
34 oxidative stress, biofouling organisms and pathogens (Egan et al., 2013; Hollants et al., 2013;
35 Tarquinio et al., 2019). Besides these positive interactions, macrophytes can negatively impact
36 the associated microbes such as pathogenic bacteria by producing reactive oxygen species and
37 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
44 al., 2016). Recently, it was shown that even different morphological niches within the same
45 alga had a higher influence on the composition of the bacterial community than biogeography or
46 environmental factors (Morrissey et al., 2019). While the microbial community composition varies
47 between host species, metagenomic analyses revealed that the majority of the microbial functions
48 are conserved (Burke et al., 2011a; 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 et al., 2011a; Roth-Schulze et al., 2016). In addition, some of the variation
53 in the reported data could be attributed to different techniques used in these studies, such as different
54 protocols for epiphytic cell detachment and/or DNA isolation, as no standard protocol has been yet
55 established to study epiphytic communities (Ugarelli et al., 2019; Korlević et al., submitted).

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

67 **Materials and methods**

68 **Sampling**

69 Sampling was performed in the Bay of Funtana, northern Adriatic Sea (45°10'39" N,
70 13°35'42" E). Leaves and thalli were sampled in a meadow of *Cymodocea nodosa* invaded by the
71 invasive *Caulerpa cylindracea* (mixed settlement) and in a monospecific settlement of *Caulerpa*
72 *cylindracea* located in the proximity of the meadow at approximately monthly intervals from
73 December 2017 to October 2018 (Table S1). Leaves and thalli were collected by diving and
74 transported to the laboratory in containers placed on ice and filled with seawater collected at
75 the sampling site. Upon arrival to the laboratory, *C. nodosa* leaves were cut into sections of 1 –
76 2 cm, while *C. cylindracea* thalli were cut into 5 – 8 cm long sections. Leaves and thalli were
77 washed three times with sterile artificial seawater (ASW) to remove loosely attached microbial
78 cells. Ambient seawater was collected in 10 l containers by diving and transported to the laboratory
79 where 10 – 20 l were filtered through a 20 µm net. The filtrate was further sequentially filtered
80 through 3 µm and 0.2 µm polycarbonate membrane filters (Whatman, United Kingdom) using a
81 peristaltic pump. Filters were briefly dried at room temperature and stored at –80 °C. Seawater
82 samples were also collected approximately monthly from July 2017 to October 2018.

83 **DNA isolation**

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

90 from 0.2 µm polycarbonate filters according to Massana et al. (1997) with a slight modification.
91 Following the phenol-chloroform extraction, 1/10 of chilled 3 M sodium acetate (pH 5.2) was added.
92 DNA was precipitated by adding 1 volume of chilled isopropanol, incubating the mixtures overnight
93 at –20 °C and centrifuging at 20,000 × g and 4 °C for 21 min. The pellet was washed twice with
94 500 µl of chilled 70 % ethanol and centrifuged after each washing step at 20,000 × g and 4 °C for 5
95 min. Dried pellets were re-suspended in 50 – 100 µl of deionized water.

96 **Illumina 16S rRNA sequencing**

97 Illumina MiSeq sequencing of the V4 region of the 16S rRNA gene was performed as described
98 previously (Korlević et al., submitted). The V4 region of the 16S rRNA gene was amplified using a
99 two-step PCR procedure. In the first PCR, the 515F (5'-GTGYCAGCMGCCGCGTAA-3') and
100 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers from the Earth Microbiome Project (<https://earthmicrobiome.org/protocols-and-standards/16s/>) were used (Caporaso et al., 2012; Apprill et al.,
101 2015; Parada et al., 2016). These primers contained on their 5' end a tagged sequence. Purified PCR
102 products were sent for Illumina MiSeq sequencing at IMGM Laboratories, Martinsried, Germany.
103 Prior to sequencing at IMGM, the second PCR amplification of the two-step PCR procedure was
104 performed using primers targeting the tagged region incorporated in the first PCR. In addition,
105 these primers contained adapter and sample-specific index sequences. Beside samples, a positive
106 and negative control for each sequencing batch was sequenced. The negative control comprised
107 PCR reactions without DNA template, while for a positive control a mock community composed
108 of evenly mixed DNA material originating from 20 bacterial strains (ATCC MSA-1002, ATCC,
109 USA) was used. Sequences obtained in this study have been deposited in the European Nucleotide
110 Archive (ENA) at EMBL-EBI under accession number PRJEB37267.

112 **Sequence analysis**

113 Obtained sequences were analysed on the computer cluster Isabella (University Computing
114 Center, University of Zagreb) using mothur (version 1.43.0) (Schloss et al., 2009) according
115 to the MiSeq Standard Operating Procedure (MiSeq SOP; https://mothur.org/wiki/MiSeq_SOP)
116 (Kozich et al., 2013) and recommendations provided by the Riffomonas project to enhance data
117 reproducibility (<http://www.riffomonas.org/>). For alignment and classification of sequences the
118 SILVA SSU Ref NR 99 database (release 138; <http://www.arb-silva.de>) was used (Quast et al.,
119 2013; Yilmaz et al., 2014). Pipeline data processing and visualization was done using R (version
120 3.6.0) (R Core Team, 2019) combined with packages vegan (version 2.5-6) (Oksanen et al., 2019),
121 tidyverse (version 1.3.0) (Wickham et al., 2019) and multiple other packages (Neuwirth, 2014; Xie,
122 2014, 2015, 2019a, 2019b; Wilke, 2018; Xie et al., 2018; Allaire et al., 2019; Zhu, 2019). The
123 detailed analysis procedure including the R Markdown file are available in the GitHub repository
124 (https://github.com/MicrobesRovinj/Korlevic_EpiphyticDynamics_FrontMicrobiol_2021). Based
125 on the ATCC MSA-1002 mock community included in the analysis an average sequencing error
126 rate of 0.01 % was determined, which is in line with previously reported values for next-generation
127 sequencing data (Kozich et al., 2013; Schloss et al., 2016). In addition, the negative controls
128 processed together with the samples yielded on average only 2 sequences after sequence quality
129 curation.

130 **Results**

131 Sequencing of the 16S rRNA V4 region yielded a total of 1.7 million sequences after quality
132 curation and exclusion of sequences without known relatives (no relative sequences) and eukaryotic,
133 chloroplast and mitochondrial sequences (Table S1). A total of 35 samples originating from
134 epiphytic archaeal and bacterial communities associated with surfaces of the seagrass *C. nodosa*
135 and the macroalga *C. cylindracea* were analysed. In addition, 18 samples (one of the samples was
136 sequenced twice) originating from the ambient seawater were also processed for comparison. The
137 number of reads per sample ranged between 8,408 and 77,463 sequences (Table S1). Even when
138 the highest sequencing effort was applied the rarefaction curves did not level off as commonly
139 observed in high-throughput 16S rRNA amplicon sequencing approaches (Fig. S1). Following
140 quality curation and exclusion of sequences as mentioned above reads were clustered into 28,750
141 different OTUs at a similarity level of 97 %. Read numbers were normalized to the minimum
142 number of sequences (8,408, Table S1) through rarefaction resulting in 17,201 different OTUs with
143 352 to 2,062 OTUs per sample (Fig. S2). To determine seasonal changes in richness and diversity
144 the observed number of OTUs, Chao1, ACE, Exponential Shannon and Inverse Simpson (Jost,
145 2006) were calculated after normalization through rarefaction. Generally, richness estimators and
146 diversity indices showed similar trends. On average, higher values were found for *C. cylindracea*
147 (mixed [Number of OTUs, $1,688.4 \pm 136.6$ OTUs] and monospecific [Number of OTUs, $1,750.4 \pm$
148 165.7 OTUs]) than for *C. nodosa* (Number of OTUs, $1,063.7 \pm 210.6$ OTUs) and lowest values were
149 obtained for the microbial community of the ambient seawater (Number of OTUs, 531.0 ± 143.9
150 OTUs) (Fig. S2). Seasonal changes did not reveal such large dissimilarities. *C. nodosa* communities
151 showed a slow increase towards the end of the study, while *C. cylindracea* (mixed and monospecific)
152 communities were characterized by slightly higher values in spring and summer than in autumn and
153 winter (Fig. S2).

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

156 Bray-Curtis Similarity Coefficient, respectively, were calculated. Coefficients were determined after
157 normalization through rarefaction and binning of samples from the particular environment. The
158 highest proportion of shared OTUs and community was found between mixed and monospecific *C.*
159 *cylindracea* (Jaccard, 0.35; Bray-Curtis, 0.77), while lower shared values were calculated between
160 ambient seawater and epiphytic communities (Fig. 1). Shared proportion between *C. nodosa* and *C.*
161 *cylindracea* were approximately in-between the values of mixed and monospecific *C. cylindracea*.
162 To assess seasonal changes in the proportion of shared OTUs and communities the Jaccard's and
163 Bray-Curtis Similarity Coefficients were calculated between consecutive sampling points (Fig. 2).
164 Both coefficients showed similar trends. Temporal proportional changes were more pronounced for
165 ambient seawater than for *C. nodosa* and especially *C. cylindracea* associated communities (Fig. 2).
166 In addition, only 0.4 – 1.0 % of OTUs from each surface associated community were present at
167 all seasons. These persistent OTUs constituted a high proportion of total sequences (40.2 – 53.2
168 %). To further disentangle the environmental and seasonal community dissimilarity a Principal
169 Coordinates Analysis (PCoA) based on Bray-Curtis distances and OTU abundances was applied. A
170 clear separation between ambient seawater and surface associated communities was found (Fig. 3).
171 In addition, a separation of epiphytic bacterial and archaeal communities based on host species
172 was detected. This separation was further supported by ANOSIM ($R = 0.96, p < 0.001$). Seasonal
173 changes of *C. nodosa* associated communities indicated a separation between spring, summer and
174 autumn/winter samples (ANOSIM, $R = 0.56, p < 0.01$). For *C. cylindracea* associated communities
175 a separation between summer and autumn/winter/spring samples was observed that was, however,
176 not as strong as for *C. nodosa* associated communities (ANOSIM, $R = 0.30, p < 0.01$) (Fig. 3).

177 The taxonomic composition of both, macrophyte associated and ambient seawater communities
178 was dominated by bacterial (99.1 ± 2.1 %) over archaeal sequences (0.9 ± 2.1 %) (Fig. 4). Higher
179 relative abundances of chloroplast related sequences were only observed in surface associated
180 communities, with higher values in autumn/winter (37.2 ± 11.2 %) than in spring/summer
181 (20.9 ± 9.7 %) (Fig. S3). Generally, at higher taxonomic ranks (phylum-class), epiphytic and
182 ambient seawater microbial communities were composed of similar bacterial taxa. Ambient

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

190 *Cyanobacteria* related sequences comprised, on average, $5.5 \pm 4.4\%$ of total sequences (Fig. 5).
191 Higher proportions were found for *C. nodosa* ($16.4 \pm 5.3\%$) and *C. cylindracea* mixed (7.7 ± 3.9
192 %) and monospecific ($7.8 \pm 2.4\%$) associated communities in autumn and for ambient seawater
193 communities in winter ($8.8 \pm 7.5\%$). Large taxonomic differences between surface associated
194 and ambient seawater cyanobacterial communities were observed. Ambient seawater communities
195 were mainly comprised of *Cyanobium* and *Synechococcus*, while surface associated communities
196 were comprised of *Pleurocapsa* and sequences within the class *Cyanobacteriia* that could not be
197 further classified (no relative *Cyanobacteriia*) (Fig. 5). In addition, seasonal changes in surface
198 associated communities were observed in *Pleurocapsa* and no relative *Cyanobacteriia* comprising
199 larger proportions in autumn and winter and *Acrophormium*, *Phormidesmis* and sequences without
200 known relatives within the *Nodosilineaceae* (no relative *Nodosilineaceae*) in spring and summer
201 (Fig. 5).

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

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

216 On average, *Alphaproteobacteria* were in comparison to the other high rank taxa the largest
217 taxonomic group, comprising 29.2 ± 12.0 % of all sequences (Fig. 7). In accordance to the above
218 described taxa, large differences between ambient seawater and surface associated communities
219 were observed. Ambient seawater communities were composed mainly of the SAR11 clade,
220 AEGEAN-169 marine group, SAR116 clade, sequences without known relatives within the
221 *Rhodobacteraceae* (no relative *Rhodobacteraceae*), HIMB11 and the OCS116 clade, while
222 surface associated communities were composed mainly of no relative *Rhodobacteraceae* and to
223 a lesser degree of *Pseudoahrensia*, *Amylibacter* and sequences without known relatives within
224 the *Alphaproteobacteria* (no relative *Alphaproteobacteria*) and *Hyphomonadaceae* (no relative
225 *Hyphomonadaceae*). Representatives of no relative *Rhodobacteraceae* comprised on average 40.6
226 ± 23.2 % of all alphaproteobacterial sequences in the epiphytic community (Fig. 7). In addition,
227 *Amylibacter* was detected mainly in *C. nodosa* from November 2017 until March 2018.

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

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

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

252 **Discussion**

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

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

278 We observed a strong differentiation between the surface attached and ambient seawater
279 communities at the level of OTUs, in agreement with most published studies (Burke et al., 2011b;
280 Michelou et al., 2013; Mancuso et al., 2016; Roth-Schulze et al., 2016; Crump et al., 2018;
281 Ugarelli et al., 2019). This indicates that marine macrophytes are a selecting factor from the pool
282 of microbial taxa present in the ambient seawater, modifying the microbial community once the
283 macrophyte associated microbial biofilm develops (Salaün et al., 2012; Michelou et al., 2013). In
284 contrast, Fahimipour et al. (2017) report in a global study of *Zostera marina*, similarities between
285 the microbial community developed on leaves and in the ambient seawater. The discrepancy
286 between our data and the study of Fahimipour et al. (2017) could be explained by different
287 seagrass species, methodological variations or biogeographic trends as Fahimipour et al. (2017)
288 analysed samples from different locations throughout the northern hemisphere. It is possible
289 that the microbial communities in ambient seawater and on leaves from the same location are
290 differing but are still more similar to each other when compared to other sampling locations.
291 Indeed, it was found that prokaryotic communities vary substantially between different sampling
292 sites (Bengtsson et al., 2017). When the taxonomic composition at high ranks was analysed no
293 such strong differentiation was noticed. Phyla and classes such as *Actinobacteriota*, *Bacteroidota*,
294 *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobiota* were found in
295 both ambient seawater as well as macrophyte associated, in agreement with previous studies (Burke
296 et al., 2011b; Egan et al., 2013; Michelou et al., 2013). In contrast, when low taxonomic ranks
297 were analysed (i.e., family and genus) a strong differentiation was observed (Figs. 5 – 9). A similar
298 differentiation at lower microbial taxonomic ranks between ambient seawater and macrophytes was
299 described for other macrophyte species as well (Egan et al., 2013; Michelou et al., 2013; Ugarelli et
300 al., 2019).

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

305 at high taxonomic ranks no major difference between the microbial communities associated with
306 different hosts was observed. The only high rank phylum that was differing between *C. nodosa*
307 and *C. cylindracea* was *Desulfobacterota*, with more abundant sequences in the *C. cylindracea*
308 associated community. As already mentioned, the rhizoids and part of the stolon are in contact
309 with the sediment. Thus *Desulfobacterota* are probably a part of the epiphytic community that
310 was in contact with the sediment. Similar high rank taxa found in this study were described to be
311 specific for other species of macrophytes (Burke et al., 2011b; Lachnit et al., 2011; Mancuso et
312 al., 2016; Bengtsson et al., 2017). In contrast to high taxonomic ranks, a substantial differentiation
313 between host specific communities was found supporting the notion that macrophyte associated
314 microbial communities might be host-specific. Host-specificity was also observed for other species
315 of macroalgae and seagrasses (Lachnit et al., 2011; Roth-Schulze et al., 2016; Morrissey et al.,
316 2019; Ugarelli et al., 2019). Taken together, at high taxonomic ranks a core set of taxa could
317 be described that is characteristic for all or many macrophytes, while at low taxonomic ranks a
318 community specific to host species was identified (Figs. 3 and 4) (Egan et al., 2013).

319 Seasonal changes in richness in the epiphytic community were substantial as indicated by the
320 proportion of OTUs ($\leq 1.0\%$) present at every sampling date. These persistent OTUs, however,
321 were accounting for a high proportion of sequences ($\geq 40.2\%$) (Fig. 2). A very similar proportion
322 of persistent OTUs was reported in high-frequency sampling studies describing seasonal changes in
323 picoplankton (Gilbert et al., 2009, 2012). In comparison to the seawater community, a lower degree
324 of seasonal shifts was observed for the macrophyte surface associated communities. It appears that
325 microniches at the surfaces of macrophytes are providing more stable conditions than the ambient
326 seawater. At the level of OTUs seasonal changes of *C. nodosa* and *C. cylindracea* associated
327 communities were identified that could be linked to the growth cycle of the seagrass and macroalgae
328 (M. Najdek, personal communication). *C. nodosa* was characterized by a spring community
329 during maximum seagrass proliferation, a summer community during the highest standing stock of
330 *C. nodosa* and an autumn/winter community during the decay of seagrass biomass. In contrast, *C.*
331 *cylindracea* started to proliferate in late spring and was characterized only by a summer community

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

337 Chloroplast sequence abundances were higher in autumn/winter than in spring/summer. This
338 pattern is not surprising as seagrasses harbor more algal epiphytes during autumn/winter than
339 in spring/summer (Reyes and Sansón, 2001). Furthermore, we used an adapted DNA isolation
340 protocol that is known to partially co-extract DNA from planktonic eukaryotes (Korlević et al.,
341 2015). Strong seasonal fluctuations of high rank epiphytic taxa were not observed, with the
342 exception of *Cyanobacteria*. Cyanobacterial sequences were more pronounced in November and
343 December than in spring and summer. In the months of high cyanobacterial sequence abundances
344 the majority of sequences from this group were classified as *Pleurocapsa*, a group known to
345 colonized different living and non-living surfaces (Burns et al., 2004; Longford et al., 2007;
346 Mobberley et al., 2012; Reisser et al., 2014). It is possible that during periods of low metabolic
347 activity there is a reduced interaction and selection of the epiphytic community by the seagrass,
348 causing leaves to become a suitable surface for non-specific colonizers (Zavodnik et al., 1998).
349 *Pleurocapsa* was replaced in spring and summer by *Acrophormium*, *Phormidesmis* and sequences
350 without known relatives within the *Nodosilineaceae*. A study of coastal microbial mats found
351 also a higher proportion of *Nodosilineaceae* sequences in summer, while *Phormidesmis* sequences
352 were at their peak in autumn (Cardoso et al., 2019). Other high rank taxa did not exhibit strong
353 successional patterns. In every analysed group, with the exception of *Desulfobacterota*, taxa present
354 throughout the year in similar proportions and season specific taxa could be identified (Figs. 6
355 and 9). Within *Bacteroidota* different groups within the family *Sapspiraceae* (e.g. *Lewinella*,
356 *Portibacter* and *Rubidimonas*) were detected across all seasons. Members of this family are often
357 found in association with macrophytes and it is suggested that they are involved in the hydrolysis and
358 utilization of complex carbon sources (Burke et al., 2011b; McIlroy and Nielsen, 2014; Crump et al.,

359 2018). In contrast, the families *Flavobacteriaceae* and *Rhodothermaceae* showed seasonal patterns,
360 with *Flavobacteriaceae* being more pronounced from November to June and *Rhodothermaceae*
361 from June to October (Fig. 6). Within *Alphaproteobacteria* the family *Rhodobacteraceae* comprised
362 the majority of sequences throughout the year (Fig. 7). This metabolically versatile family is
363 often associated with macrophyte surfaces and usually is one of the most abundant groups (Burke
364 et al., 2011b; Michelou et al., 2013; Pujalte et al., 2014; Mancuso et al., 2016). In addition,
365 *Hyphomonadaceae* were found in all samples. Interestingly, some of the species within this group
366 contain stalks on their cells, which can be used to attach to the macrophyte surface (Weidner et al.,
367 2000; Abraham and Rohde, 2014). Within the *Gammaproteobacteria*, sequences without known
368 representatives were the most pronounced group present throughout the year (Fig. 8). In addition,
369 *Granulosicoccus* was also found in almost all samples. *Gammaproteobacteria* are often a major
370 constituent of macrophyte epiphytic communities (Burke et al., 2011b; Michelou et al., 2013;
371 Crump et al., 2018). Beside these two groups, other less abundant, taxa showed seasonal and
372 host-specific patterns. For example, *C. cylindracea* harbored *Thioploca*, a known sulfur sediment
373 bacteria and *Cellvibrionaceae*, a family with cultured members known as polysaccharide degraders
374 (Jørgensen and Gallardo, 1999; Xie et al., 2017). *Desulfobacterota* were found only associated with
375 *C. cylindracea* and no group within this phylum showed seasonal patterns (Fig. 9). The presence of
376 this phylum only on *C. cylindracea* is to be expected as part of the epiphytic community is in direct
377 contact with the sediment. The *Desulfobacterota* community was dominated by *Desulfatitalea* and
378 no relative *Desulfocapsaceae*, known sulphate sediment groups (Kuever, 2014; Higashioka et al.,
379 2015).

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

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

395 **Acknowledgments**

396 This work was funded by the Croatian Science Foundation through the MICRO-SEAGRASS
397 project (project number IP-2016-06-7118). ZZ and GJH were supported by the Austrian Science
398 Fund (FWF) project ARTEMIS (project number P28781-B21). We would like to thank Margareta
399 Buterer for technical support, Paolo Paliaga for help during sampling and the University Computing
400 Center of the University of Zagreb for access to the computer cluster Isabella.

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596 **Figure legends**

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

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

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

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

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

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

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

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

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

Jaccard's Similarity Coefficient



Bray-Curtis Similarity Coefficient

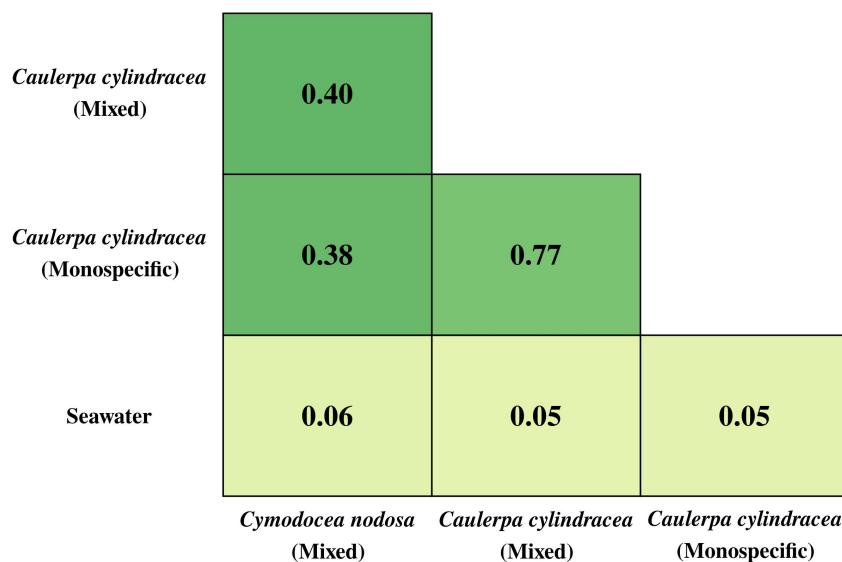


Fig. 1. Proportion of shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) and shared bacterial and archaeal communities (Bray-Curtis similarity coefficient) between assemblages associated with the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed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

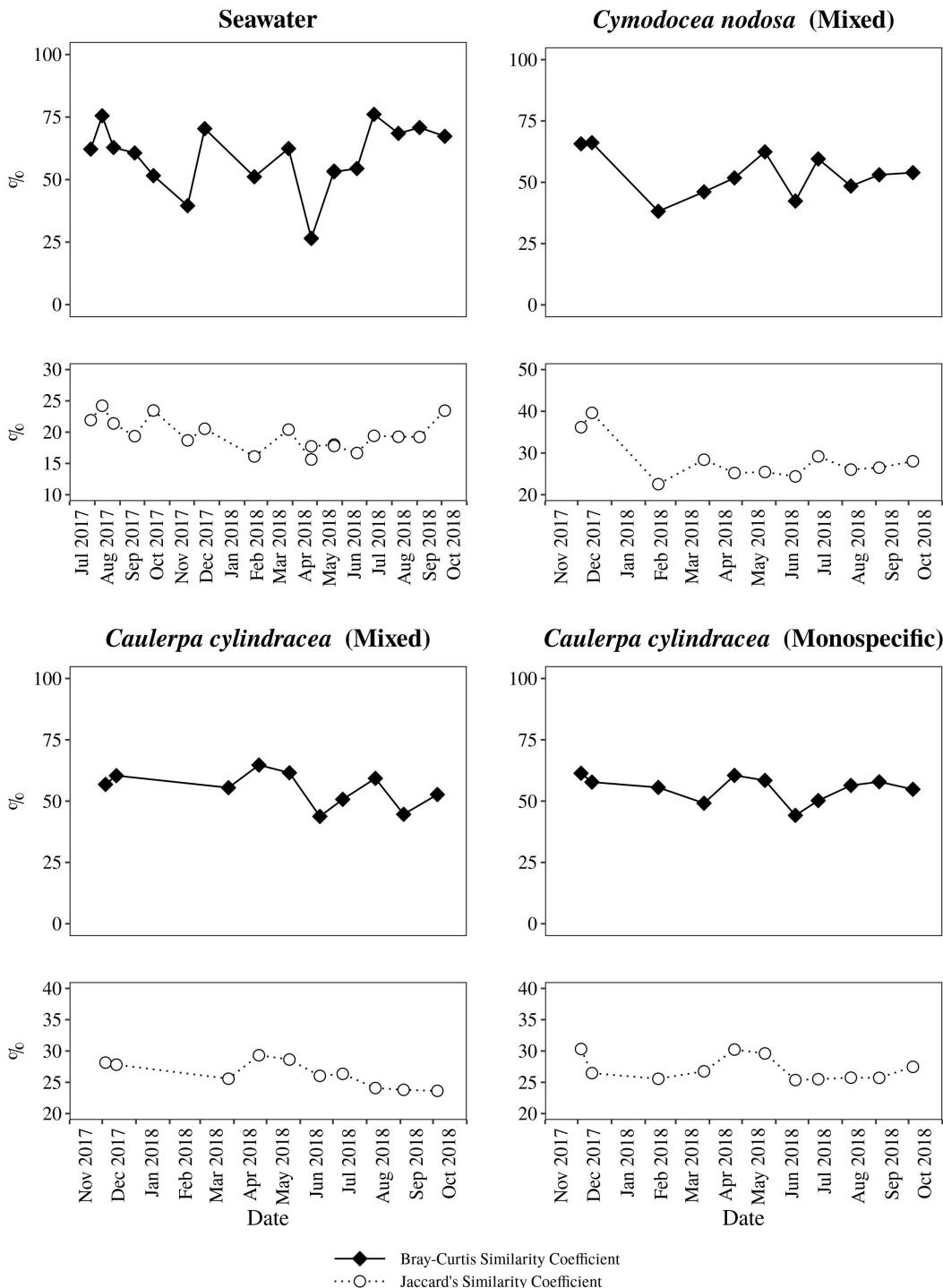


Fig. 2. Proportion of shared bacterial and archaeal communities (Bray-Curtis similarity coefficient) and shared bacterial and archaeal OTUs (Jaccard's similarity coefficient) between consecutive sampling dates and from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea*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

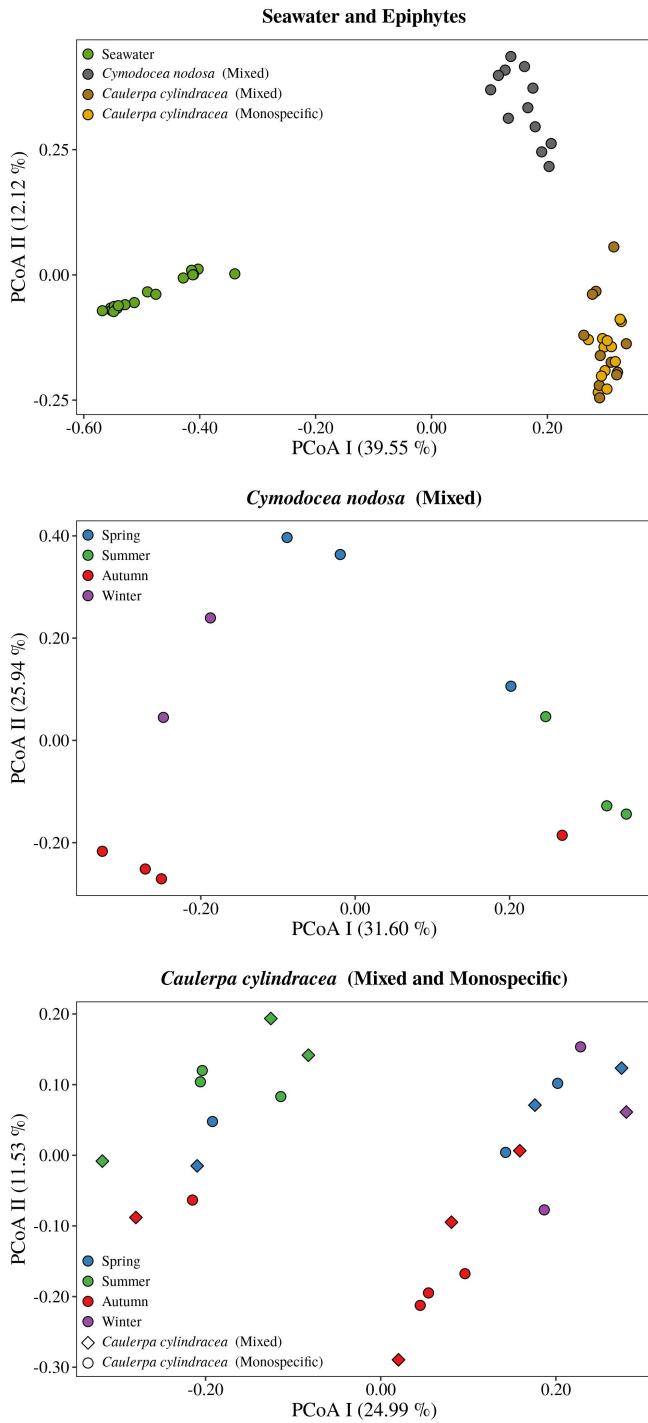


Fig. 3. Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on OTU abundances of bacterial and archaeal communities from the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. Samples from the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

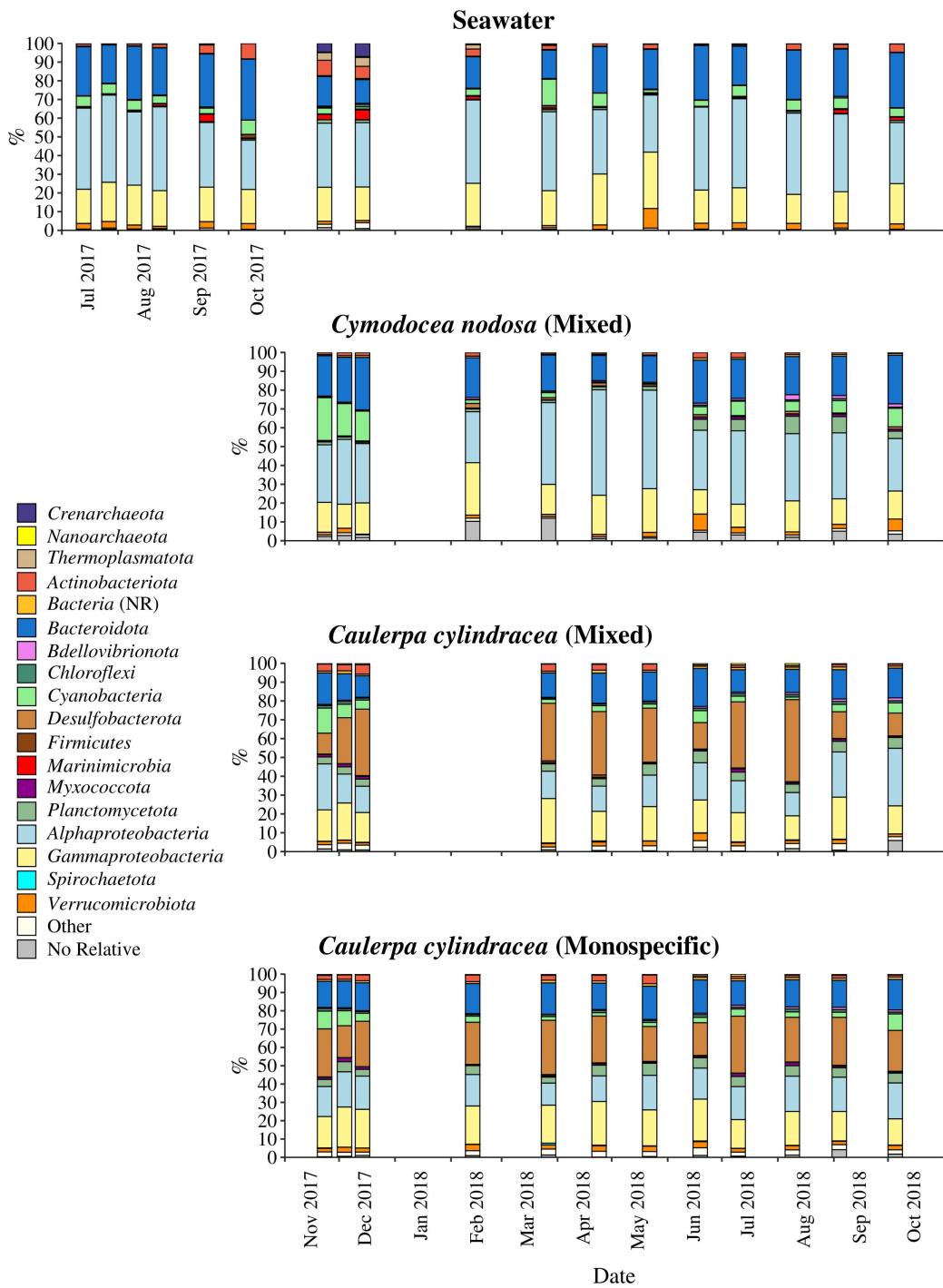


Fig. 4. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) bacterial and archaeal sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. NR – No Relative (sequences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

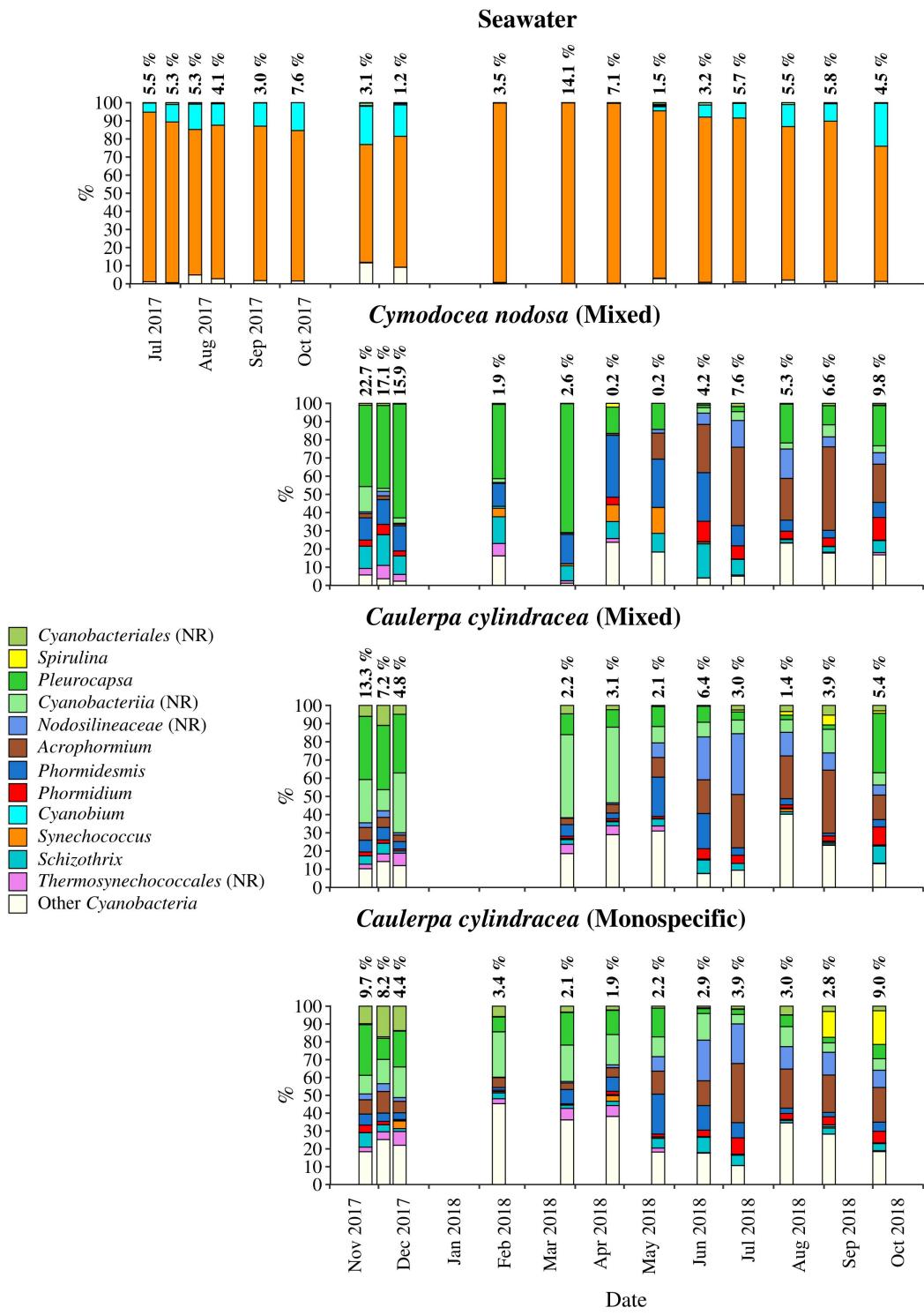


Fig. 5. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) cyanobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of cyanobacterial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

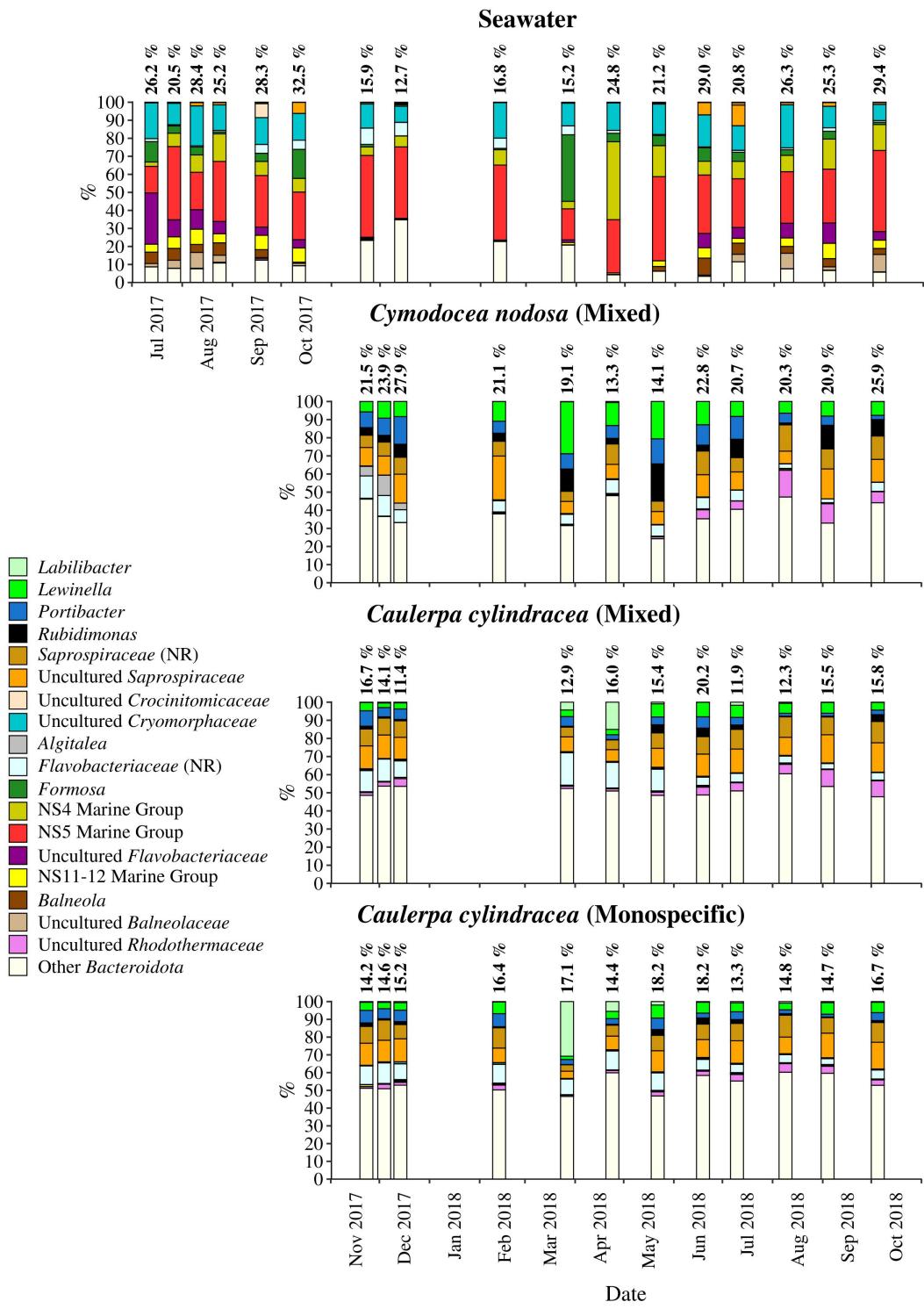


Fig. 6. Taxonomic classification and relative contribution of the most abundant ($\geq 2 \%$) sequences within the *Bacteroidota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of sequences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

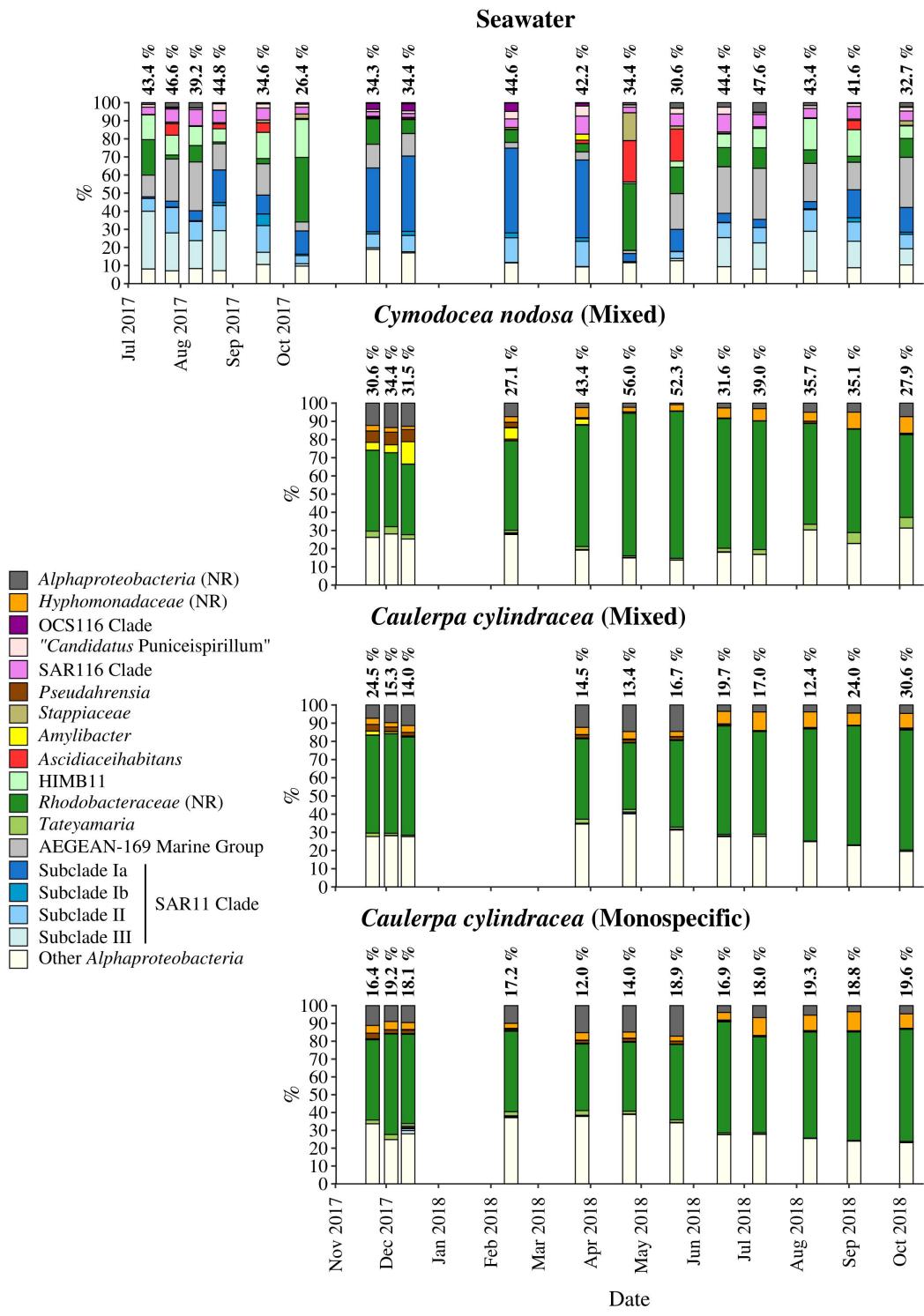


Fig. 7. Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) alphaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of alphaproteobacterial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

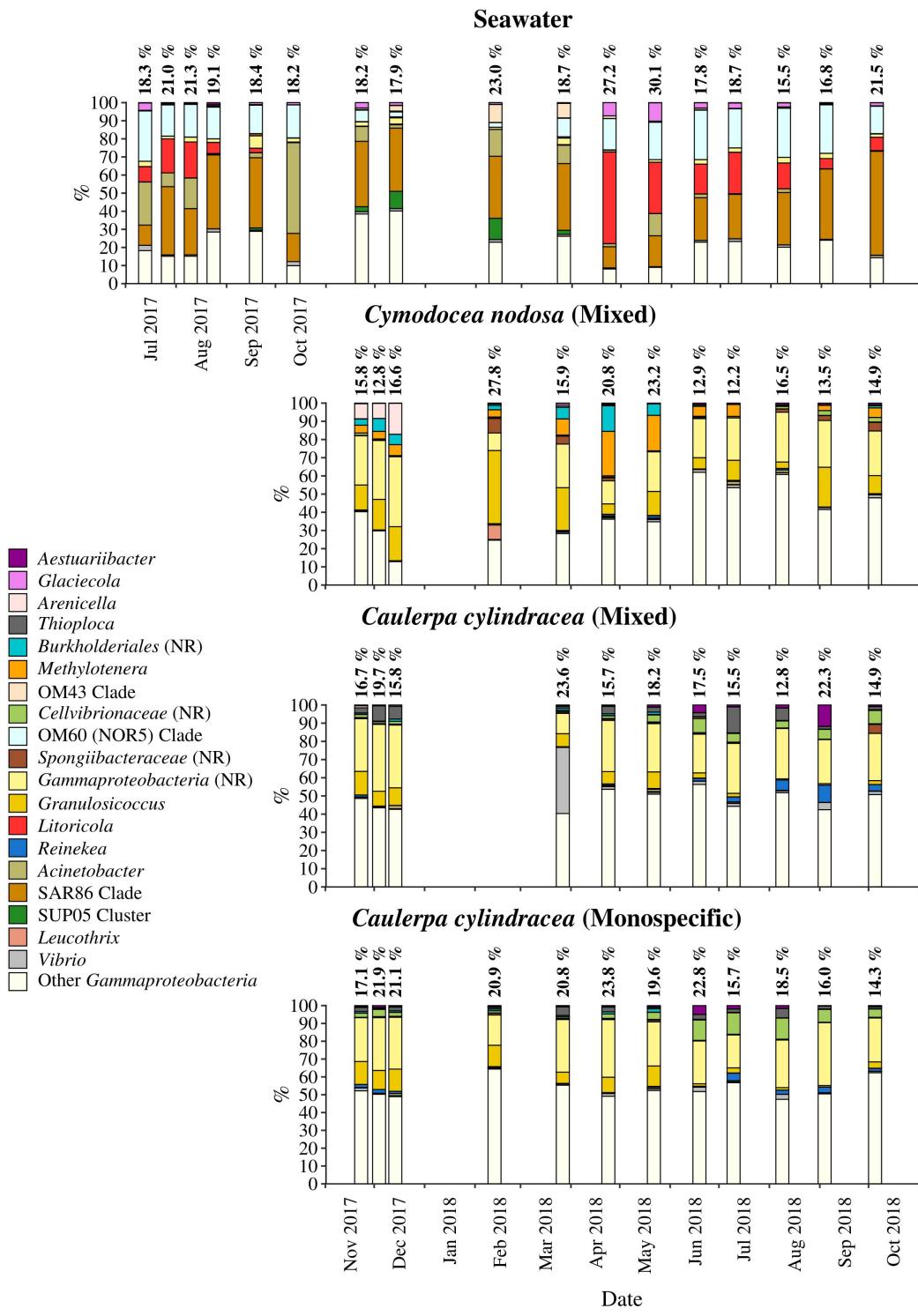


Fig. 8. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) gammaproteobacterial sequences on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of gammaproteobacterial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

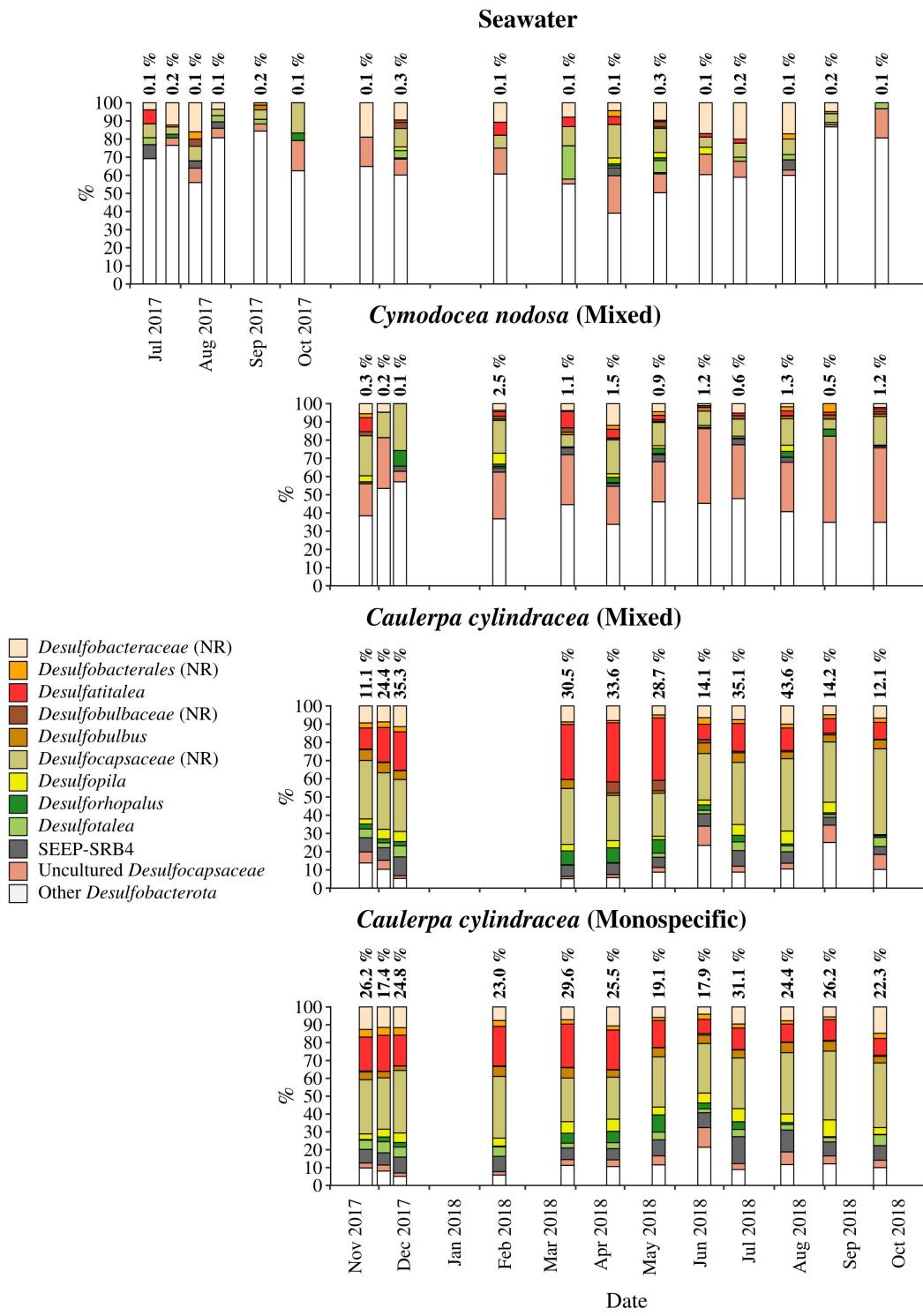


Fig. 9. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) sequences within the *Desulfobacterota* on the surfaces of the macrophytes *C. nodosa* (mixed settlement) and *C. cylindracea* (mixed and monospecific settlement) and in the ambient seawater. The proportion of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