

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

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## Summary

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

## Introduction

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

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

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

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

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

## Results

Sequencing of the 16S rRNA V4 region yielded a total of 1.8 million sequences after quality curation and exclusion of eukaryotic, chloroplast, mitochondrial and no relative sequences (Table S1). A total of 35 samples originating from epiphytic archaeal and bacterial communities associated with surfaces of the seagrass *C. nodosa* and macroalga *C. cylindracea* were analyzed. In addition, 18 samples (one of the samples was sequenced two times) originating from picoplankton archaeal and bacterial communities in the ambient seawater were also processed for comparison. The number of reads per sample ranged between 8,408 and 77,463 sequences (Table S1). Even when the highest sequencing effort was applied the rarefaction curves did not level off that is a common observation in high-throughput 16S rRNA amplicon sequencing approaches (Fig. S1). Following quality curation and exclusion of sequences mentioned before reads were clustered into 28,750 different OTUs at a similarity level of 97 %. Read numbers were normalized to the minimum number of sequences, 8,408 (Table S1), through rarefaction resulting in 17,017 different OTUs that ranged from 360 to 2,108 OTUs per sample (Fig. S2). To determine seasonal changes of richness and diversity the Observed Number of OTUs, Chao1, ACE, Exponential Shannon (Jost, 2006) and Inverse Simpson were calculated after normalization through rarefaction. Generally, richness estimators and diversity indices showed similar trends. On average, higher values were found for *C. cylindracea* (mixed [Number of OTUs,  $1,675.9 \pm 144.0$  OTUs] and monospecific [Number of OTUs,  $1,735.6 \pm 175.0$  OTUs]), middle values for *C. nodosa* (Number of OTUs,  $1,052.6 \pm 204.9$  OTUs) and lower values for picoplankton communities in the ambient seawater (Number of OTUs,  $525.3 \pm 146.6$  OTUs) (Fig. S2). Seasonal changes did not show such large dissimilarities. *C. nodosa* communities showed a slow increase towards the end of the study, while *C. cylindracea* (mixed and monospecific) communities were characterized by slightly larger values in Spring and Summer in comparison to Autumn and Winter (Fig. S2).

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

data and Bray-Curtis Similarity Coefficient were, respectively, calculated. Coefficients were determined after normalization through rarefaction and binning of samples from a particular environment. The highest proportion of shared OTUs and community was found between mixed and monospecific *C. cylindracea* (Jaccard, 0.36; Bray-Curtis, 0.78), while lower shared values were calculated between seawater and epiphytic communities (Fig. 1). Shared proportion between *C. nodosa* and *C. cylindracea* were approximately in the middle between these two extremes. To assess seasonal changes in the proportion of shared OTUs and communities the Jaccard's and Bray-Curtis Similarity Coefficients were calculated between consecutive sampling points (Fig. 2). Both coefficients showed similar trends. Temporal proportional changes were more pronounced for seawater in comparison to *C. nodosa* and especially *C. cylindracea* associated communities (Fig. 2). In addition, only 0.4 – 1.0 % of OTUs from each surface associated community were found at every time point. These OTUs also made a high proportion of total sequences (40.6 – 52.6 %). To further disentangle the environmental and seasonal community dissimilarity a Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances and OTU abundances was applied. It showed a clear separation between planktonic and surface associated communities (Fig. 3). In addition, a separation of epiphytic bacterial and archaeal communities based on host species was determined. This separation was further supported by ANOSIM ( $R = 0.96, p < 0.001$ ). Seasonal changes of *C. nodosa* associated communities indicated a separation between Spring, Summer and Autumn/Winter samples (ANOSIM,  $R = 0.53, p < 0.01$ ). For *C. cylindracea* associated communities a separation between Summer and Autumn/Winter/Spring samples was observed that was not so strongly supported (ANOSIM,  $R = 0.31, p < 0.05$ ) (Fig. 3).

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

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

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

Sequences classified as *Bacteroidota* were comprising, on average,  $19.2 \pm 5.5\%$  of all sequences (Fig. 6). Similarly to *Cyanobacteria*, large differences in the taxonomic composition between seawater and surface associated communities were found (Fig. 6). The seawater community was characterized by the NS4 and NS5 marine groups, uncultured *Cryomorphaceae*, uncultured *Flavobacteriaceae*, NS11-12 marine group, *Balneola*, uncultured *Balneolaceae* and *Formosa*. In contrast, in surface associated communities *Lewinella*, *Portibacter*, *Rubidimonas*, no relative *Saprosiraceae*, uncultured *Saprosiraceae*, no relative *Flavobacteriaceae* and uncultured *Rhodothermaceae* were found. Some groups showed slight seasonal changes such as no relative *Flavobacteriaceae* that were more pronounced from November 2017 until June 2018. In contrast,

uncultured *Rhodothermaceae* showed higher proportions from June 2018 until the end of the study period. Surface associated *Bacteroidota* communities were very diverse as could be observed in the high proportion of taxa that grouped as other *Bacteroidota* (Fig. 6).

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

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

community was grouped as other *Gammaproteobacteria* indicating high diversity within this group (Fig. 8).

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

## Discussion

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

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

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

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

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

Seasonal richness changes in the epiphytic community were substantial as could be observed in the proportion of OTUs that could be found at every sampling time ( $\leq 1.0\%$ ). Interestingly, these OTUs were accounting for a high proportion of sequences ( $\leq 52.6\%$ ). A very similar proportion of persistent OTUs and their sequence contribution was reported in high frequency studies describing seasonal picoplankton changes (Gilbert *et al.*, 2009, 2012). In comparison to the seawater community, a lower degree of seasonal shifts was observed for the surface associated communities. It seems, microniches on the surfaces of macrophytes are providing more stable conditions in comparison to the seawater. At the level of OTUs seasonal changes of *C. nodosa* and *C. cylindracea* associated communities were identified that could be linked to the growth cycle of the seagrass and macroalgae (M. Najdek, personal communication). *C. nodosa* was characterized by a Spring community during maximum seagrass proliferation, a Summer community during a biomass maximum and a Autumn/Winter community during a biomass senescence. In contrast, *C. cylindracea* started to proliferate in late Spring and was characterized only by a Summer community during maximal biomass increase and by a Autumn/Winter/Spring community when the biomass was at the peak and the settlement started to subsequently decay. Similar seasonal

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

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

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

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

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

## Experimental procedures

### Sampling

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

### DNA isolation

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

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

### **Illumina 16S rRNA sequencing**

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

## Sequence analysis

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

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## Figure legends

**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 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 points and from the surfaces of 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 macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. Samples from the same environment or same season are labeled in different colors. The proportion of explained variation by each axis is shown on the corresponding axis in parentheses.

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

**Fig. 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

**Fig. 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.*

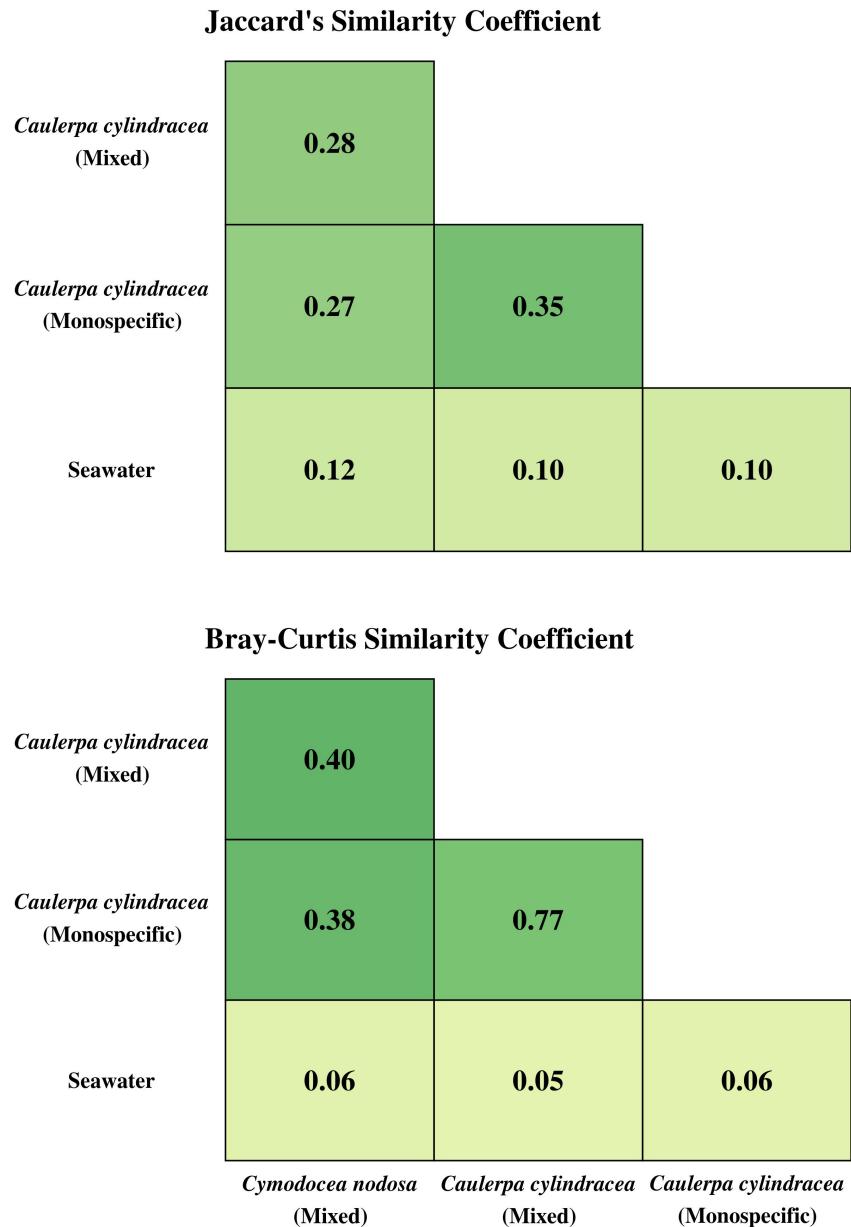
*cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion of sequences classified as *Bacteroidota* in the total bacterial and archaeal community is given above the corresponding bar. NR – No Relative

**Fig. 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

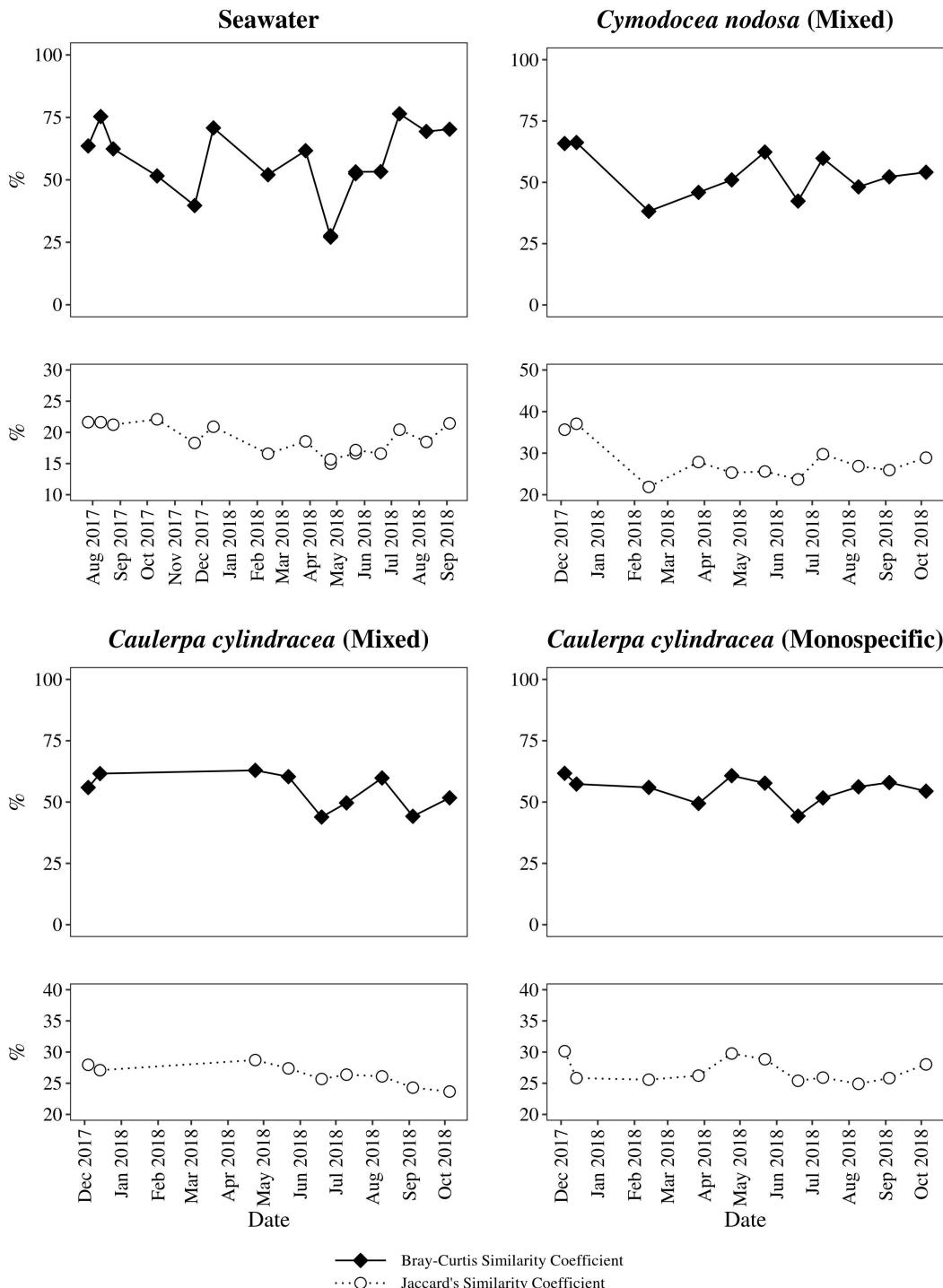
**Fig. 8.** Taxonomic classification and relative contribution of the most abundant (> 2 %) gammaproteobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and in the ambient seawater. The proportion of gammaproteobacterial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

**Fig. 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

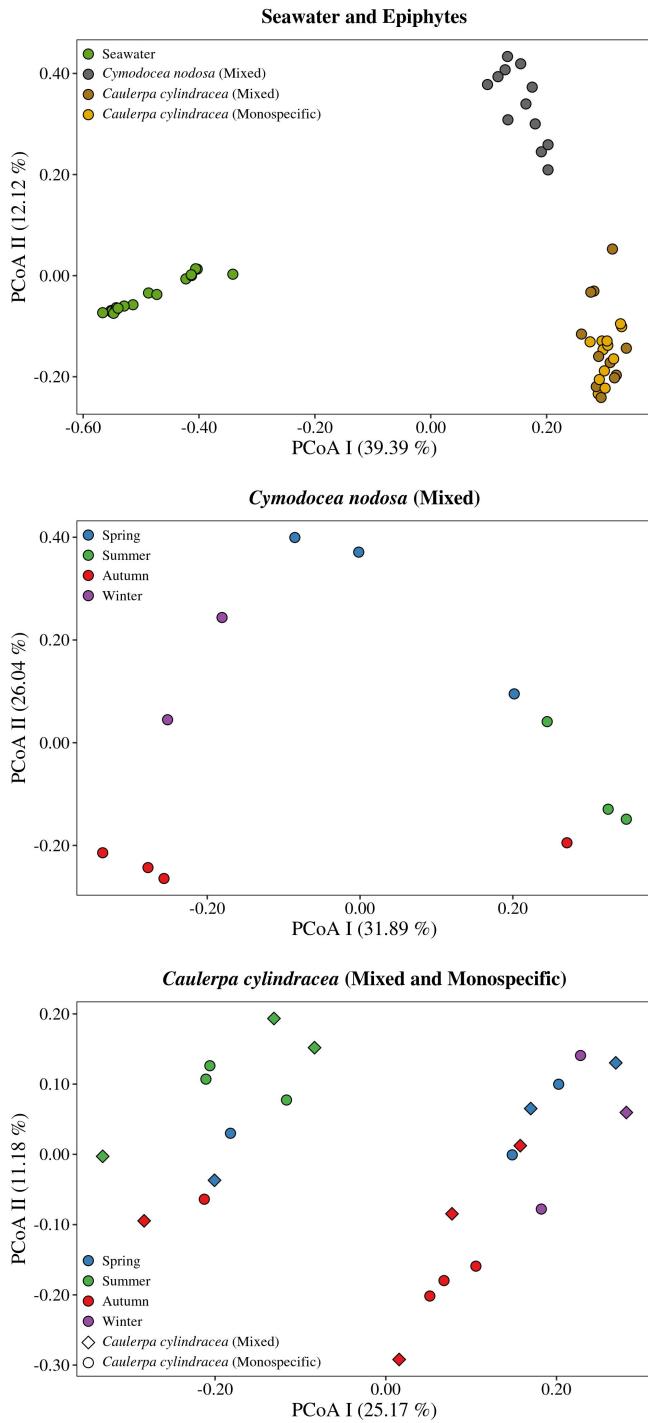
## Figures



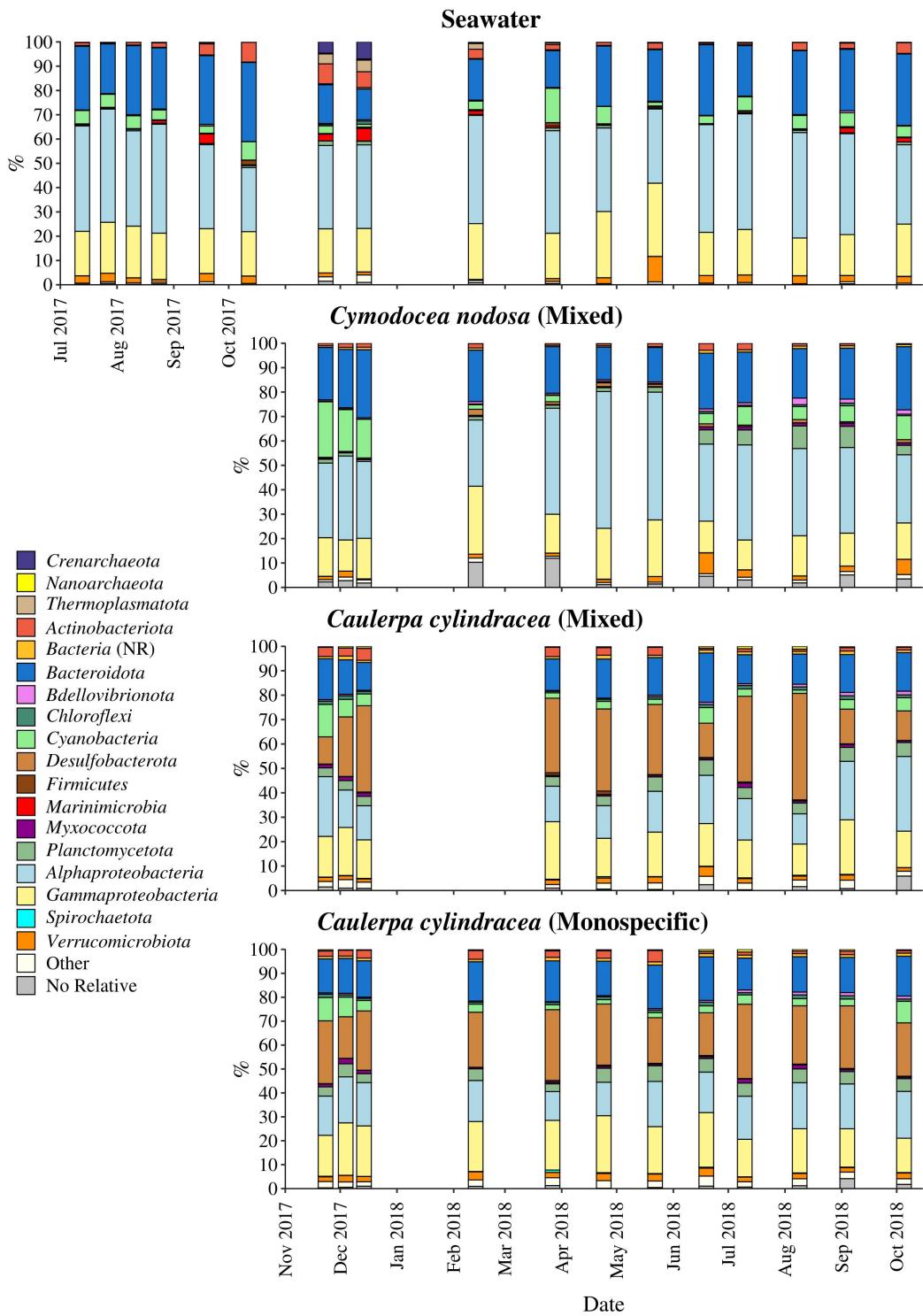
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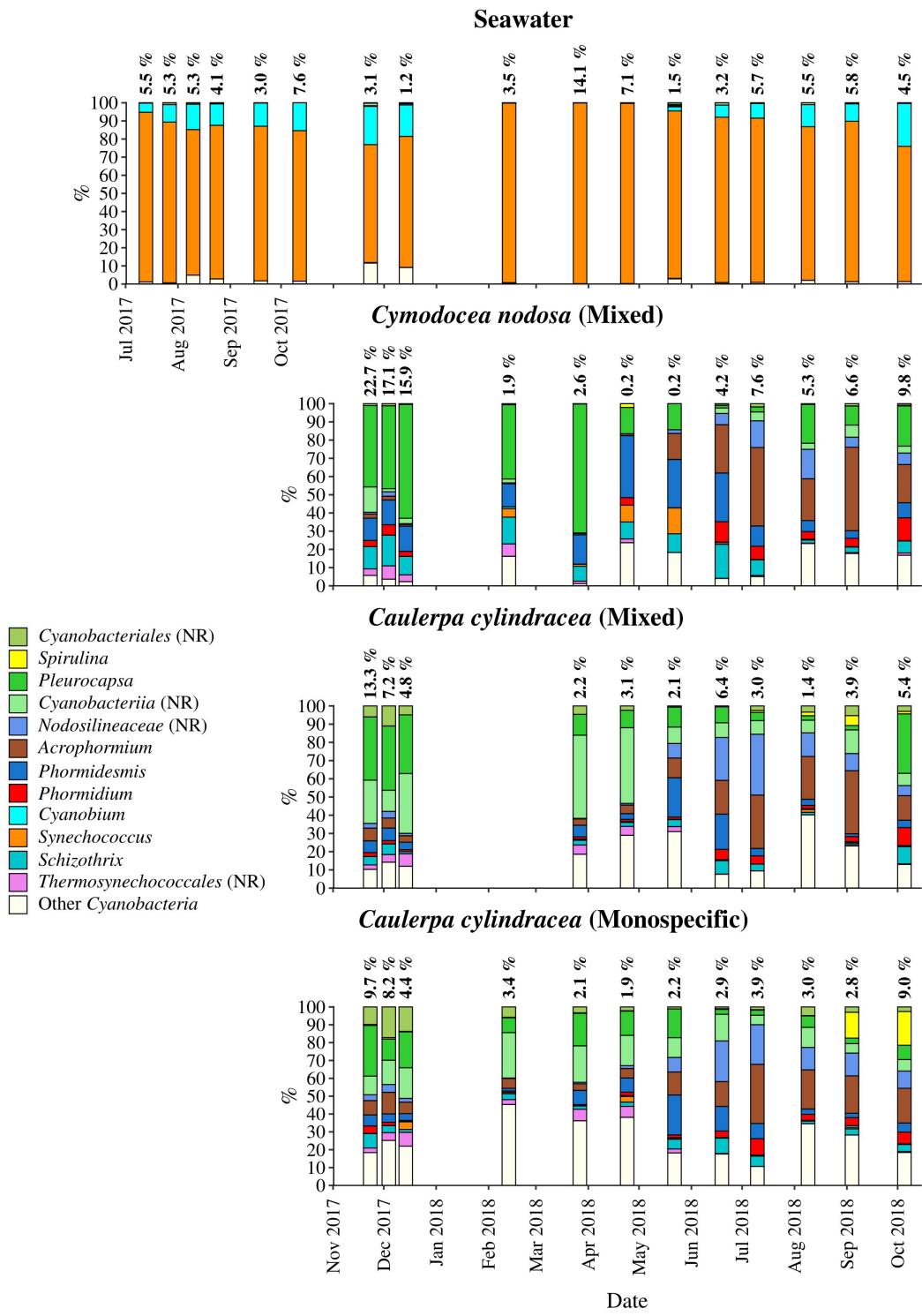
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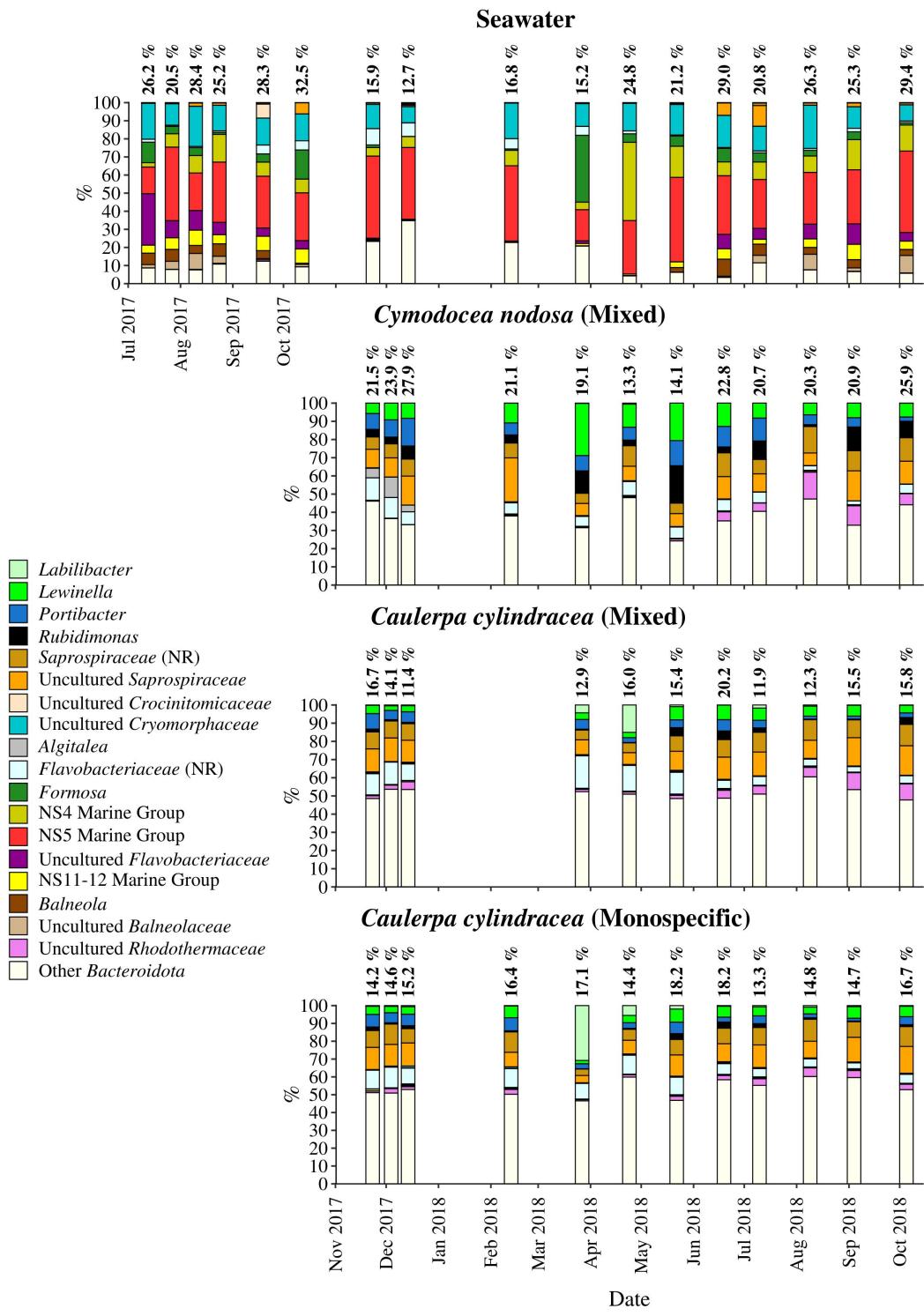
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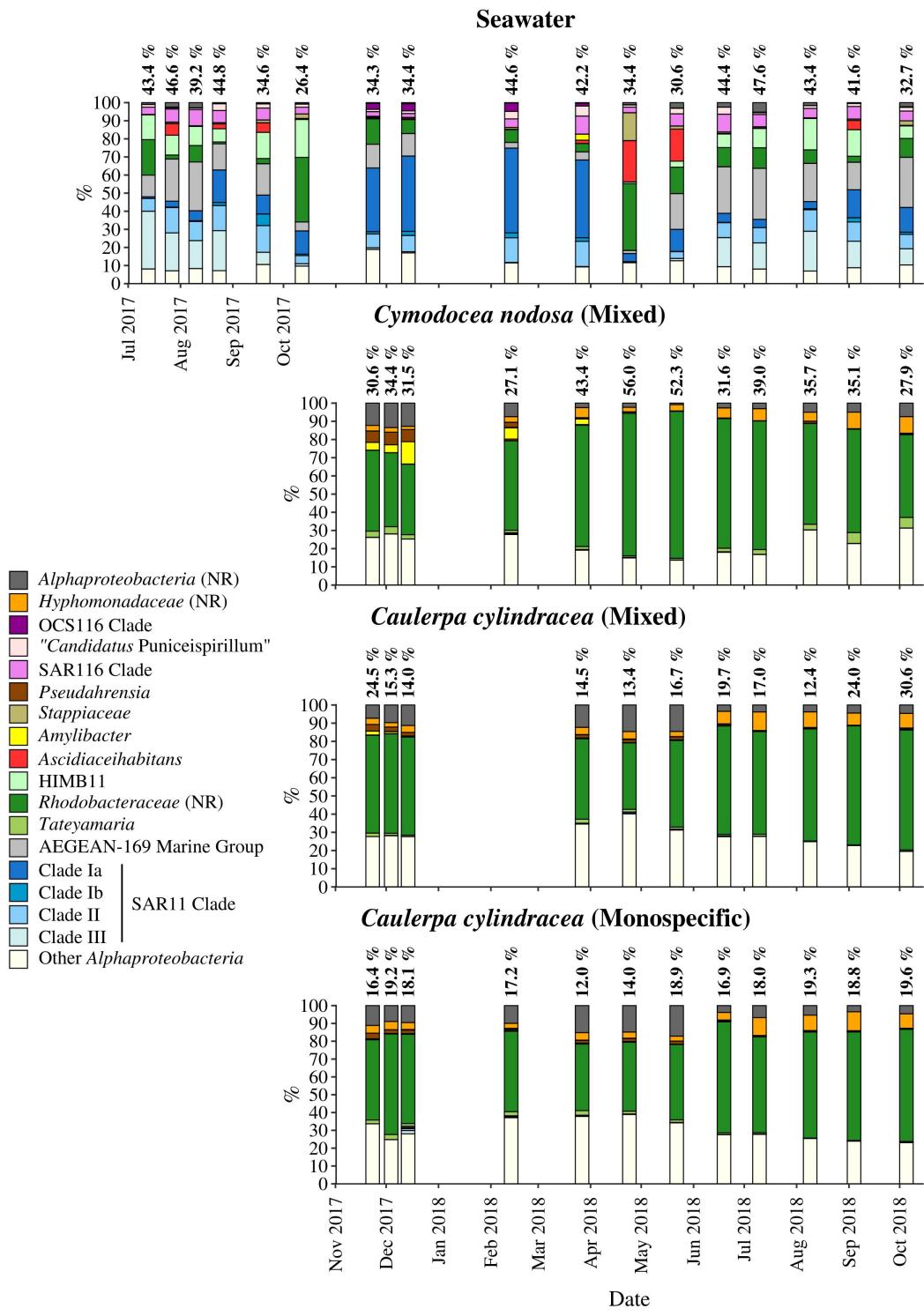
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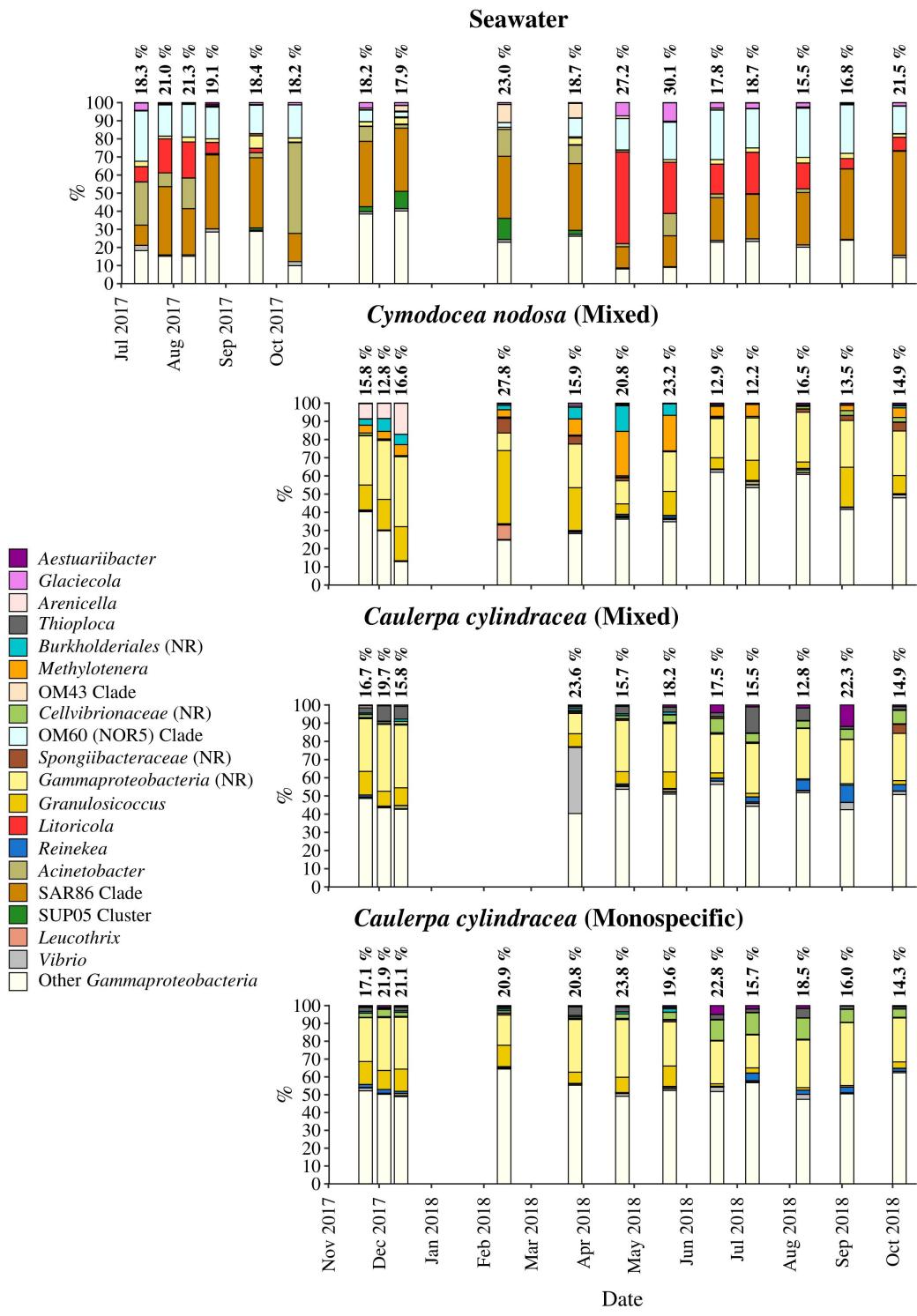
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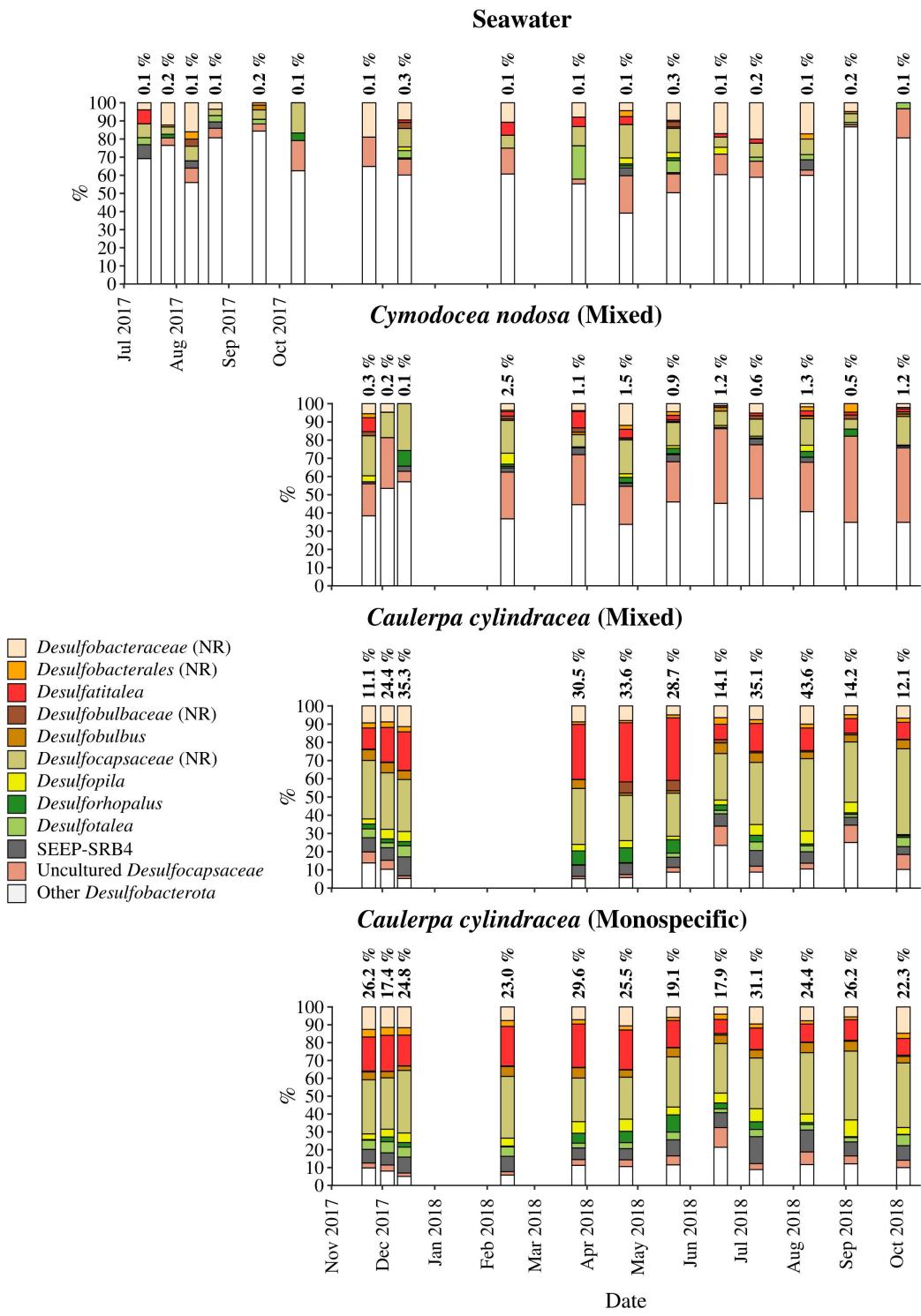
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**Fig. 7.** Taxonomic classification and relative contribution of the most abundant (> 2 %) alphaproteobacterial sequences on the surfaces of macrophytes (*C. nodosa* [Mixed Settlement] and *C. cylindracea* [Mixed and Monospecific Settlement]) and 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



**Fig. 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



**Fig. 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