

Compositional stability of sediment microbial communities during a seagrass meadow decline

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Running title: Compositional stability of sediment communities

1 Abstract

2 The presence of seagrass shapes surface sediments and forms a specific environment for
3 diverse and abundant microbial communities. A severe decline of *Cymodocea nodosa*, a widespread
4 seagrass species in the Mediterranean Sea, has been documented. To characterise and assess
5 the changes in microbial community composition during the decline of a *Cymodocea nodosa*
6 meadow, Illumina MiSeq sequencing of the V4 region of the 16S rRNA gene was performed.
7 Samples of surface sediments from two sites, one without any vegetation and one with a declining
8 *Cymodocea nodosa* meadow, were collected at monthly intervals from July 2017 to October 2018.
9 Microbial communities were stratified by sediment depth and differed between the vegetated
10 and the nonvegetated site. Although the *Cymodocea nodosa* meadow declined to a point where
11 almost no leaves were present, no clear temporal succession in the community was observed.
12 Taxonomic analysis revealed a dominance of bacterial over archaeal sequences, with most archaeal
13 reads classified as *Nanoarchaeota*, *Thermoplasmata*, *Crenarchaeota*, and *Asgardarchaeota*.
14 The bacterial community was mainly composed of *Desulfobacterota*, *Gammaproteobacteria*,
15 *Bacteroidota*, *Chloroflexi*, *Planctomycetota*, and *Campylobacterota*. Our results show that sediment
16 microbial communities are remarkably stable and may resist major disturbances such as seagrass
17 meadow decline.

18 **Introduction**

19 Shallow coastal sediments are often colonized by seagrasses, which cover approximately 0.1
20 to 0.2 % of the global ocean (Duarte, 2002). Seagrasses penetrate the sediment with their roots and
21 rhizomes forming extensive meadows. The presence of seagrass meadows shapes surface sediments
22 and provides a specific environment for diverse and abundant microbial communities (Duarte et al.,
23 2005). Sediments colonized by seagrasses are considered hotspots for microbial activity as seagrass
24 meadows enrich the underlying sediment with organic matter (Duarte et al., 2005). High organic
25 matter content is mainly achieved by releasing dissolved organic carbon from seagrass roots and by
26 trapping organic particles from the water column (Duarte, 2002). Moreover, seagrasses stabilize the
27 underlying sediment, promoting the accumulation of organic matter and sediment particles (Fonseca
28 and Kenworthy, 1987; Terrados and Duarte, 2000; van Katwijk et al., 2010). In addition, seagrass
29 beds can also increase the availability of organic matter through the decomposition of detached
30 leaves, roots and rhizomes (Jensen et al., 2007; Liu et al., 2017).

31 Studies of marine sediment microbial communities primarily focus on changes in microbial
32 abundance and activity with sediment depth (Jørgensen and Marshall, 2016; Petro et al., 2017;
33 Starnawski et al., 2017; Orsi, 2018). Depth-dependent changes in taxonomic composition have been
34 well described differentiating surface sediment communities dominated by *Bacteria*, especially
35 *Proteobacteria*, from deeper communities characterized by *Archaea* (Orcutt et al., 2011; Chen et al.,
36 2017; Petro et al., 2017). Coastal surface sediments colonized by seagrass are not as well investigated
37 due to studies focusing primarily on rhizosphere communities and only occasionally including
38 sediment communities for comparison (Cúcio et al., 2016; Rabbani et al., 2021). Communities
39 in the rhizosphere are not species-specific and differ from those in the sediment (Cúcio et al.,
40 2016; Ettinger et al., 2017; Zhang et al., 2020). One of the main differences is the higher relative
41 abundance of *Desulfobacterota*, one of the most abundant sulphate reducing bacteria in seagrass
42 sediments, in contrast to the rhizosphere, which is characterized by *Epsilonproteobacteria* (Ettinger
43 et al., 2017). When sediment microbial communities were described, the main focus was on the

44 differences between vegetated and nonvegetated sites (Zheng et al., 2019; Sun et al., 2020). In
45 addition, these studies showed that communities differ even with respect to the meadow edge
46 (Ettinger et al., 2017). However, little is known about the response of these communities to seagrass
47 decline. As only limited information is available on the succession of microbial communities
48 in seagrass sediments it is hard to predict how and if seagrass decline influences the underlying
49 sediment communities. It was reported that the sulphate-reducing community in seagrass sediments
50 changes over time (Smith et al., 2004) and that seagrass sediment microbial communities change
51 according to nutrient availability (Guevara et al., 2014). Furthermore, seagrass restoration was also
52 found to alter the sediment microbial community (Bourque et al., 2015). These studies suggest that
53 a temporal community pattern may be observed in sediment communities of seagrass meadows and
54 that these communities could also change as a result of seagrass decline.

55 In the Mediterranean Sea, *Cymodocea nodosa* is a widespread seagrass species declining in
56 coastal areas (Ruiz Fernandez et al., 2009; Tuya et al., 2014; Orlando-Bonaca et al., 2015). The
57 rhizosphere and epiphytic communities of *C. nodosa* have been described (Cúcio et al., 2016;
58 Korlević et al., 2021a), however, little is known about sediment communities underlying *C. nodosa*
59 meadows. The aim of the present study was to characterize the taxonomic composition of sediment
60 communities of a *C. nodosa* meadow and to assess the temporal dynamics of these communities.
61 As the studied meadow experienced a major decline (Najdek et al., 2020), we investigated whether
62 this event affected the sediment microbial community structure.

63 **Materials and methods**

64 **Sampling**

65 Sediment cores were sampled in a declining *C. nodosa* meadow (vegetated site) and at an
66 adjacent area without any vegetation (nonvegetated site) both located in the Bay of Saline, east
67 coast of the northern Adriatic Sea ($45^{\circ}7'5''$ N, $13^{\circ}37'20''$ E) (Figure 1). One sediment core from
68 each site was collected monthly from July 2017 to October 2018 (Supplementary Table S1) by
69 diving using 15 cm long plastic core samplers. Sediment samples were immediately transported
70 on ice to the laboratory and stored at -80°C until further processing. A detailed description of
71 the study site, the decline of the *C. nodosa* meadow and the dynamics of environmental conditions
72 during the decline are provided in Najdek et al. (2020). Briefly, at the beginning of the study the
73 seagrass *C. nodosa* formed a large and dense meadow at the vegetated site. Seagrass roots and
74 rhizomes penetrated into slightly gravelly sandy mud, while shoots and leaves were present from
75 the southwestern coastal area up to the central part of the bay which was without any vegetation.
76 Following the regular vegetation minimum in November 2017, shoots and leaves started to decline,
77 while roots and rhizomes persisted longer. At the end of the study, after a severe meadow decline at
78 the vegetated site only very small patches persisted along the shoreline.

79 **DNA isolation**

80 Total DNA from sediment samples was extracted following a modified (Pjevac et al., 2018)
81 isolation protocol of Zhou et al. (1996). Prior to DNA isolation, cores were cut into four different
82 1 cm sections: top (0 – 1 cm), bottom (7 – 8 cm), and two middle sections: upper middle (1 – 3
83 cm) and lower middle (3 – 6 cm) section. Sediment samples were weighted (2 g) avoiding roots
84 and rhizomes from vegetated cores, mixed with 5.4 ml of extraction buffer (100 mM Tris [pH 8.0],
85 100 mM sodium EDTA [pH 8.0], 100 mM Na₃PO₄ [pH 8.0], 1.5 M NaCl, 1 % CTAB) and 10 µl

86 of proteinase K (20 mg ml⁻¹) and incubated by horizontal shaking at 225 rpm at 37°C for 30 min.
87 Thereafter 1.2 ml of 10 % SDS was added and the mixture incubated again by horizontal shaking at
88 225 rpm at 65°C for 60 min. The supernatant was collected after centrifugation at 3220 × g at room
89 temperature for 10 min and mixed with an equal volume of chloroform:isoamyl alcohol (1:1). The
90 aqueous phase was retrieved after centrifugation at 3220 × g at room temperature for 10 min. The
91 extraction procedure with the organic solvent mixture was repeated twice. After the final extraction
92 0.6 volumes of isopropanol were added to precipitate the DNA. The mixture was incubated at 22°C
93 for 60 min and centrifuged at 3220 × g at room temperature for 45 min. The obtained pellet was
94 washed twice with 10 ml of chilled 70 % ethanol, centrifuged at 3220 × g at room temperature for
95 10 min after each washing, and finally resuspended in 100 µl of deionized water.

96 Illumina 16S rRNA sequencing

97 The V4 region of the 16S rRNA gene was sequenced using a two-step PCR approach
98 described previously (Korlević et al., 2021b). Briefly, the V4 region was amplified using the 515F
99 (5'-GTGYCAGCMGCCGCGTAA-3') and 806R (5'-GGACTACNVGGTWTCTAAT-3') primers
100 from the Earth microbiome project (<https://earthmicrobiome.org/protocols-and-standards/16s/>),
101 which contained a sequence tag on the 5' end (Caporaso et al., 2011, 2012; Apprill et al., 2015;
102 Parada et al., 2016). Purified samples were sent for Illumina MiSeq sequencing (2 × 250 bp) at
103 IMGM Laboratories (Martinsried, Germany) where the second PCR of the two-step PCR approach
104 was performed using primers targeting the tag region incorporated in the first PCR. These primers
105 also contained adapter and sample-specific index sequences. For each sequencing batch, a positive
106 and a negative control were also sequenced. The positive control consisted of a mock community
107 composed of uniformly mixed DNA from 20 different bacterial strains (ATCC MSA-1002, ATCC,
108 USA), while PCR reactions without DNA template served as the negative control. Sequences
109 obtained in this study have been deposited in the European Nucleotide Archive at EMBL-EBI under
110 the accession numbers SAMEA11293274 – SAMEA11293412 and SAMEA6648825.

111 **Sequence and data analysis**

112 Sequences were analysed on the computer cluster Isabella (University Computing Centre,
113 University of Zagreb) using version 1.45.2 of mothur (Schloss et al., 2009) according to the MiSeq
114 Standard Operating Procedure (MiSeq SOP; https://mothur.org/wiki/miseq_sop/) (Kozich et al.,
115 2013) and recommendations given by the Riffomonas project (<https://riffomonas.org>) to foster data
116 reproducibility. Alignment and classification were performed using the 138.1 release of the SILVA
117 SSU Ref NR 99 database (<https://www.arb-silva.de>) (Quast et al., 2013; Yilmaz et al., 2014). A
118 cut-off of 97 % was used to cluster sequences into operational taxonomic units (OTUs).

119 Pipeline data processing and visualization were done using R (version 3.6.3) (R Core Team,
120 2020) combined with packages vegan (version 2.5.7) (Oksanen et al., 2020) tidyverse (version
121 1.3.1) (Wickham et al., 2019) and multiple other packages (Neuwirth, 2014; Xie, 2014, 2015, 2019,
122 2021a, 2021b; Xie et al., 2018, 2020; Edwards, 2020; Wilke, 2020; Allaire et al., 2021; Zhu, 2021).
123 Observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity index and Inverse
124 Simpson diversity index were calculated after normalization to the minimum number of reads per
125 sample to account for different sequencing depths using vegan's function `rrarefy` (Oksanen et
126 al., 2020). Chao1 and ACE estimators were calculated using vegan's function `estimateR`, while
127 Shannon and Inverse Simpson diversity indices were obtained using vegan's function `diversity`
128 (Oksanen et al., 2020). To express both diversity indices in terms of effective number of OTUs the
129 exponential of the Shannon diversity index was retrieved (Jost, 2006). The proportions of shared
130 community members between different sediment layers and the two sites were expressed as the
131 Bray-Curtis similarity coefficient calculated on the OTU data table using vegan's function `vegdist`
132 and transformed from dissimilarities to similarities (Legendre and Legendre, 2012; Borcard et
133 al., 2018; Oksanen et al., 2020). The Principal Coordinate Analysis (PCoA) was performed on
134 Bray-Curtis dissimilarities based on OTU abundances using the function `wcmdscale` (Legendre and
135 Legendre, 2012; Oksanen et al., 2020). Differences between communities of different layers, sites,
136 years, and decay periods were tested by performing the Analysis of Similarities (ANOSIM) using

137 vegan's function `anosim` and 1000 permutations (Oksanen et al., 2020). When differences between
138 years or decay periods were tested samples were grouped based on sampling year (2017 and 2018)
139 and decay of roots and rhizomes (before and after decay). The period prior to the decay included
140 samples retrieved from the begining of the study until and including February 2018, while the
141 period after the decay included samples taken after February 2018. To calculate the proportion of
142 OTU community variation explained by environmental variables (redox potential [E_h], oxygen [O_2],
143 hydrogen sulfide [H_2S], sulfur [S^0], organic matter content, and prokaryotic abundance) reported in
144 Najdek et al. (2020) vegan's function `RsquareAdj` was applied on the results of the distance-based
145 Redundancy Analysis (db-RDA) (Borcard et al., 2018; Oksanen et al., 2020). To calculate the
146 db-RDA vegan's function `capscale` on OTU data and explanatory environmental variables was
147 performed. The analysis was computed using the Bray-Curtis dissimilarity index and the Lingoës
148 correction for negative eigenvalues (Legendre and Legendre, 2012; Borcard et al., 2018; Oksanen
149 et al., 2020). In addition, differences between richness estimators, diversity indices, and relative
150 sequence abundances were tested by performing the Mann-Whitney U test (function `wilcox.test`),
151 when two groups were compared, or the Kruskal-Wallis H test (function `kruskal.test`) followed
152 by a pairwise comparison using the Mann-Whitney U test (function `pairwise.wilcox.test`),
153 when more than two groups were compared. Bonferroni correction was applied to address the
154 problem of multiple comparisons.

155 In total 3.3 million sequences were obtained after quality curation and exclusion of sequences
156 without known relatives (no relative sequences), and eukaryotic, chloroplast, and mitochondrial
157 sequences. Altogether, 68 samples from the vegetated site and 68 from the nonvegetated site
158 were analysed. The number of reads per sample ranged from 9,722 to 55,381 (Supplementary
159 Table S1). Even with the highest sequencing effort the rarefaction curves did not level off as
160 commonly observed in high-throughput 16S rRNA amplicon sequencing approaches (Supplementary
161 Figure S1, and S2). After quality curation and exclusion of sequences as mentioned above, reads
162 were clustered into 89,488 different OTUs. Normalization to the minimum number of sequences
163 (9,722) described earlier resulted in 64,335 distinct OTUs ranging from 1,774 to 3,576 OTUs per

¹⁶⁴ sample (Supplementary Figure S3). Based on the positive control, a sequencing error rate of 0.01 %
¹⁶⁵ was calculated which is in line with previously reported values for high-throughput sequencing data
¹⁶⁶ (Kozich et al., 2013; Schloss et al., 2016). Following quality curation, the negative controls yielded
¹⁶⁷ on average 34.2 ± 62.6 sequences. The detailed analysis procedure is available in a Github repository
¹⁶⁸ (https://github.com/MicrobesRovinj/Markovski_SalineSediment16S_FrontMarSci_2022).

169 **Results**

170 To assess the richness and diversity of microbial communities in sediments of the Bay of Saline
171 the observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity index, and Inverse
172 Simpson diversity index were calculated (Figure 2). The observed number of OTUs was similar
173 between the vegetated ($2,746.7 \pm 398.4$ OTUs) and the nonvegetated site ($2,883.0 \pm 353.1$ OTUs)
174 and showed no statistical difference ($p = 0.06$). Interestingly, both the highest and lowest number of
175 OTUs were observed at the vegetated site, more specifically the highest number was found in the
176 top layer ($2,976.1 \pm 262.0$ OTUs) and lowest in the bottom layer ($2,500.4 \pm 462.7$ OTUs). These
177 layers were also the only ones showing statistical difference at the vegetated site (Figure 2 and
178 Supplementary Table S2). In contrast, the observed number of OTUs at the nonvegetated site was
179 similar across sediment layers and did not show significant differences (Figure 2 and Supplementary
180 Table S3), although the lowest value was also observed in the bottom layer ($2,700.8 \pm 378.8$ OTUs).
181 During the study period, the observed number of OTUs was variable, with no clear temporal trend
182 observed (Supplementary Figure S3). Chao1, ACE, exponential of the Shannon diversity index and
183 the Inverse Simpson diversity index of sediment communities at the site with and without vegetation
184 were very similar, with no estimate or index showing a statistically significant difference (all $p > 0.1$).
185 In addition, the Chao1 and ACE richness estimators also showed no significant differences between
186 sediment layers (Figure 2 and Supplementary Tables S2 and S3). In contrast, diversity indices at
187 the vegetated site showed a difference between the top and bottom layer and between the upper
188 middle and bottom layer, with exponential of the Shannon diversity index also showing a significant
189 difference between the top and lower middle layer (Figure 2 and Supplementary Table S2). At the
190 nonvegetated site, the different sediment layers showed no statistical difference in either richness or
191 diversity (Figure 2 and Supplementary Table S3). Temporal variability in richness estimates and
192 diversity indices was high at both sites, with no clear trend (Supplementary Figures S3 and S4).

193 To evaluate the dynamics of sediment microbial communities Principal Coordinate Analyses
194 (PCoA) of Bray-Curtis distances based on OTU community data were performed. PCoA of all

samples differentiated communities based on sediment depth along the first axis, whereas samples from the vegetated and nonvegetated site were separated along the second axis (Figure 3). ANOSIM confirmed that sediment communities in the Bay of Saline differed between sediment layers with some overlap ($R = 0.48, p < 0.001$), while the communities of the vegetated and nonvegetated site showed a higher degree of overlap ($R = 0.27, p < 0.001$). When communities of different sediment layers were analysed separately, a clearer differentiation between communities of the vegetated and nonvegetated site was observed ($R = 0.45 – 0.49$, all $p < 0.001$). Interestingly, when samples from the same layer of the vegetated and nonvegetated site were compared, the top layers of the sediment showed the highest degree of similarity (Bray-Curtis, 0.64), while the lowest degree of similarity was observed in samples from the upper middle and bottom layers (Bray-Curtis, 0.59) (Figure 3 and Supplementary Figure S5). When samples from each site were analysed separately, the previously observed differentiation of samples based on sediment depth was noted (Figure 4) (ANOSIM; vegetated, $R = 0.50, p < 0.001$ and nonvegetated $R = 0.49, p < 0.001$) with the highest degree of similarity observed between samples from middle layers (Bray-Curtis; vegetated, 0.71 and nonvegetated, 0.71) and between lower middle and bottom layers (Bray-Curtis; vegetated, 0.69 and nonvegetated, 0.71) (Supplementary Figure S5). Also, to determine the main environmental parameters governing community changes OTU data were linked to a set of environmental variables reported by Najdek et al. (2020) using db-RDA. Only a small proportion ($R_a^2 = 18.3\%$) of the observed community variation could be explained by the environmental variables. To determine whether there is a temporal succession in the community pattern, samples from each layer and site were analysed separately to exclude the effects of sediment depth and vegetation, which have been shown to primarily influence sediment community structure (Figure 4). No grouping of samples by month was observed in any of the layers and sites analysed. Although Najdek et al. (2020) described a sharp decline in aboveground biomass in the same meadow since the beginning of 2018, we did not detect a clearly defined grouping of samples based on sampling year in all the analysed layers (ANOSIM; vegetated, $R = 0.06 – 0.26, p = 0.05 – 0.18$ and nonvegetated, $R = 0.03 – 0.18, p = 0.05 – 0.29$). In addition, we also analysed the samples according to the reported decline of

222 roots and rhizomes, as belowground biomass showed a later onset of decline than the aboveground
223 biomass (Najdek et al., 2020). However, this analysis also did not reveal a grouping in any of the
224 tested layers (ANOSIM; vegetated, $R = 0.07 - 0.19$, $p = 0.05 - 0.18$ and nonvegetated, $R = 0.16 -$
225 0.20 , $p = 0.05 - 0.06$). Furthermore, as with the community analysis, taxonomic classification of all
226 samples also did not indicate a temporal succession but a fairly stable community composition was
227 detected in all layers both at the vegetated and nonvegetated site (Supplementary Figure S6).

228 Archaeal sequences comprised $9.5 \pm 4.7\%$ of all reads. Sequences classified as *Archaea*
229 increased in relative abundance from the top ($4.5 \pm 1.6\%$) to the bottom sediment layer (14.1 ± 4.0
230 %). The archaeal community was comprised of *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota*,
231 and *Asgardarchaeota* (Figure 5). *Nanoarchaeota* comprised $3.6 \pm 1.3\%$ of all sequences and were
232 evenly distributed across the different sediment layers, whereas all other archaeal phyla showed a
233 depth-related pattern. All *Nanoarchaeota* related sequences were classified as *Woesearchaeales*,
234 with $28.2 \pm 13.5\%$ of sequences further classified as SCGC AAA011-D5. A particularly pronounced
235 depth-related pattern was found in *Thermoplasmatota*. Sequences classified as *Thermoplasmatota*
236 comprised $4.1 \pm 1.2\%$ of all sequences in the bottom sediment layer and only $0.7 \pm 0.6\%$ in the
237 top layer. The majority of sequences related to this group was further classified as Marine Benthic
238 Group D and DHVEG-1. *Crenarchaeota* comprised $1.8 \pm 2.3\%$ of all reads. This group had a
239 higher relative sequence abundance at the nonvegetated ($2.7 \pm 2.8\%$) than at the vegetated site (1.0
240 $\pm 0.9\%$) ($p < 0.0001$). The vast majority of *Crenarchaeota* related sequences were classified as
241 *Bathyarcheia*. Out of all reads, *Asgardarchaeota* comprised $0.9 \pm 0.7\%$ of sequences that could all
242 be further classified as *Lokiarchaeia*.

243 Overall, bacterial sequences ($90.5 \pm 4.7\%$) dominated over archaeal ones and were mainly
244 comprised of *Desulfobacterota*, *Gammaproteobacteria*, *Bacteroidota*, *Chloroflexi*, *Planctomycetota*,
245 and *Campylobacterota* (Figure 5). Of all reads, *Desulfobacterota* was the most abundant taxon
246 in the middle (upper middle, $19.4 \pm 2.0\%$ and lower middle, $20.2 \pm 3.2\%$) and bottom layers
247 ($18.3 \pm 3.1\%$) (Figures 5 and 6). *Desulfobacterota* consisted mainly of *Desulfosarcinaceae*,

Desulfatiglandaceae, *Desulfocapsaceae*, *Desulfobulbaceae*, and uncultured members of the order *Syntrophobacterales* (Figure 6). Sequences classified as *Desulfocapsaceae* showed affinity for the top sediment layer, where they comprised $26.3 \pm 8.2\%$ of *Desulfobacterota* reads compared to the bottom layer where they constituted only $3.8 \pm 3.3\%$ of *Desulfobacterota* reads. *Desulfosarcinaceae* and *Desulfobulbaceae* varied depending on the site. In the whole microbial community, *Desulfosarcinaceae* reads were more abundant at the vegetated ($8.6 \pm 2.7\%$) than nonvegetated site ($6.1 \pm 2.7\%$) ($p < 0.0001$), while sequences classified as *Desulfobulbaceae* were less represented at the vegetated ($0.8 \pm 0.7\%$) than at the nonvegetated site ($1.3 \pm 0.7\%$) ($p < 0.0001$).

Gammaproteobacteria comprised most of the *Proteobacteria* sequences ($87.6 \pm 4.1\%$) and made up the majority of all reads in the top sediment layer ($23.2 \pm 6.2\%$) (Figures 5 and 6). This group was represented with more sequences at the nonvegetated ($14.8 \pm 8.9\%$) than at the vegetated site ($9.5 \pm 7.4\%$) ($p < 0.001$). Out of all gammaproteobacterial sequences, $25.2 \pm 8.3\%$ of reads could not be further classified than to the class *Gammaproteobacteria* (Figure 6). Sequences that could be further classified were mainly assigned to *Thiotrichaceae*, B2M28, *Woeseiaceae*, *Halieaceae* and *Thioalkalispiraceae* (Figure 6). The observed difference between the relative abundance in *Gammaproteobacteria* at the two sites was particularly pronounced for *Thioalkalispiraceae*. Sequences of this group were more abundant at the nonvegetated ($1.1 \pm 0.8\%$) than at the vegetated site ($0.3 \pm 0.3\%$) ($p < 0.0001$).

Sequences classified as *Bacteroidota* were more abundant in the top sediment layer ($16.9 \pm 2.7\%$) with their relative abundance decreasing with sediment depth and reaching a minimum in the bottom layer ($6.7 \pm 2.2\%$) (Figures 5 and 6). A higher relative abundance of *Bacteroidota* sequences was observed at the vegetated site ($12.0 \pm 4.5\%$) than at the nonvegetated site ($9.8 \pm 4.6\%$) ($p < 0.01$). *Bacteroidota* were mainly composed of sequences without known relatives within *Bacteroidales*, *Bacteroidetes* BD2-2, *Cyclobacteriaceae*, *Flavobacteriaceae*, *Prolixibacteraceae* and *Saprospiraceae* (Figure 6). In contrast to *Bacteroidota*, sequences classified as *Chloroflexi* increased

²⁷⁴ with sediment depth (top layer, $4.8 \pm 2.0\%$ and bottom layer, $13.8 \pm 2.7\%$) (Figures 5 and 7).

²⁷⁵ *Chloroflexi* were mainly composed of *Anaerolineaceae*, while SBR1031, uncultured *Anaerolineae*,

²⁷⁶ sequences without known relatives within *Anaerolineae* and *Dehalococcoidia*, AB-539-J10, and

²⁷⁷ *Ktedonobacteraceae* made up the remainder of the *Chloroflexi* community (Figure 7).

²⁷⁸ *Planctomycetota* were evenly represented in the middle (upper middle, $7.3 \pm 0.9\%$ and lower

²⁷⁹ middle, $7.6 \pm 0.9\%$) and bottom layers ($7.5 \pm 1.0\%$), and less abundant in the top layer ($6.0 \pm 0.7\%$)

²⁸⁰), showing no difference between the sites (vegetated, $7.0 \pm 1.2\%$ and nonvegetated, $7.1 \pm 1.0\%$)

²⁸¹ (Figures 5 and 7). The *Planctomycetota* community consisted mainly of SG8-4, *Pirellulaceae*,

²⁸² 4572-13, and sequences that could not be further classified (no relative *Planctomycetota*) (Figure 7).

²⁸³ A high proportion of *Planctomycetota* reads ($39.1 \pm 5.1\%$) were assigned to other *Planctomycetota*,

²⁸⁴ indicating a high diversity within this group. *Campylobacterota* comprised on average $3.1 \pm 3.0\%$

²⁸⁵ of all sequences (Figures 5 and 7). Overall, no pattern related to sediment depth was observed

²⁸⁶ for this group. Slightly higher values were characteristic for the vegetated ($3.9 \pm 3.5\%$) than the

²⁸⁷ nonvegetated site ($2.3 \pm 2.3\%$) ($p < 0.001$). When differences between sites were tested for all

²⁸⁸ sediment layers, only the difference in the top layer between the two sites (vegetated, $3.4 \pm 1.8\%$

²⁸⁹ and nonvegetated, $1.5 \pm 1.6\%$) was significant ($p < 0.01$). Reads related to *Campylobacterota*

²⁹⁰ could be further classified into two families, *Sulfurimonadaceae* and *Sulfurovaceae* (Figure 7). Of

²⁹¹ these two families, *Sulfurimonadaceae* showed an area-related difference in relative abundance.

²⁹² Higher values were found at the vegetated ($2.3 \pm 3.4\%$) than at the nonvegetated site ($0.7 \pm 1.8\%$)

²⁹³ ($p < 0.0001$). *Sulfurimonadaceae* consisted of the genus *Sulfurimonas*, while *Sulfurovaceae*

²⁹⁴ consisted of the genus *Sulfurovum*.

295 **Discussion**

296 Sediments of seagrass meadows harbour diverse, abundant, and active microbial communities
297 (Smith et al., 2004; Duarte et al., 2005; Sun et al., 2015). Although research on microbial
298 communities of seagrass meadows mainly focused on rhizosphere communities, some studies
299 also included the underlying and surrounding sediment (Jensen et al., 2007; Cúcio et al., 2016;
300 Zhang et al., 2020). As with most sediments, a vertical structuring has been found in the microbial
301 communities of seagrass meadow sediments (Sun et al., 2020). Furthermore, a difference between
302 prokaryotic communities of seagrass meadow sediments and nonvegetated sediments has been
303 observed (Ettinger et al., 2017; Zheng et al., 2019). Temporal studies of these communities are
304 generally rare, and little is known about how microbial communities in seagrass meadow sediments
305 change with meadow decline and loss. In this study, we assessed the microbial communities in the
306 sediment of a declining *C. nodosa* meadow to gain further insights into the taxonomic composition,
307 vertical structuring, and dynamics of microbial communities in seagrass meadow sediments while
308 at the same time comparing them with bare sediments of a nearby site.

309 Shannon and Simpson indices account for both richness and evenness and are less sensitive
310 to rare taxa than richness estimators such as ACE and Chao1 (Bent and Forney, 2008). We found
311 no difference in richness (Chao1 and ACE) between sediment layers, suggesting that the observed
312 rare taxa did not play a key role in the vertical structuring of the sediment community in the Bay
313 of Saline (Figure 2). In contrast, diversity indices at the vegetated site showed a depth related
314 pattern (Figure 2). Diversity was highest in the first centimetre of the sediment and differed from
315 the deepest layer (7 – 8 cm). This is consistent with previous studies of marine sediments that
316 describe a decrease in community diversity from the surface to deeper sediment layers, even at
317 small scales within the first few meters (Petro et al., 2017; Hoshino et al., 2020). Seagrasses are
318 known to stabilize the sediment and reduce sediment resuspension (Terrados and Duarte, 2000; van
319 Katwijk et al., 2010). It is possible that the presence of the seagrass, especially roots and rhizomes,
320 increase diversity differences between the top and bottom layer by stabilizing the sediment. In

321 contrast, mechanical mixing may homogenise the sediment together with microbial cells causing
322 more similar microbial diversity in different layers. In addition, seagrass meadows increase the
323 organic matter content of the sediment through the decay of dead tissue (Jensen et al., 2007; Liu
324 et al., 2017), which may have further contributed to the observed differences between sediment
325 layers. Vertical structuring of sediment communities is typically achieved through burial, which is
326 accompanied by selection based on successive changes in environmental conditions (Petro et al.,
327 2017; Kirkpatrick et al., 2019; Marshall et al., 2019). Specific environmental conditions surrounding
328 roots and rhizomes may act as a filter during burial, separating the top from the bottom layer. In
329 contrast, the sediment of the nonvegetated site remained vertically more stable in terms of richness
330 and diversity.

331 Another component known to differentiate communities in marine sediments besides depth
332 stratification is site-specificity (Polymenakou et al., 2005; Hamdan et al., 2013), which is even
333 more pronounced in seagrass meadows where sediment microbial communities differ not only
334 between the vegetated and nonvegetated area, but also towards the edge of the seagrass patch
335 (Ettinger et al., 2017). In this study, we also observed a grouping of samples according to the
336 two sites (Figure 3), while the microbial communities of both the vegetated and nonvegetated site
337 were stratified according to sediment depth. This is in line with Sun et al. (2020) who noted that
338 the seagrass *Zostera marina* and *Zostera japonica* influence the vertical organisation of microbial
339 communities in the sediment. Although the microbial communities at the vegetated site were distinct
340 from the ones at the nonvegetated site, a high degree of overlap was present. Given that the two
341 sampling sites were in close proximity to each other, a high degree of similarity is not surprising.
342 The microbial communities in the Bay of Saline most likely originate from the same source and
343 only through burial undergo a specific selection characteristic for each site. This type of community
344 structuring (Hamdan et al., 2013; Walsh et al., 2016; Petro et al., 2019) is further supported by
345 the highest degree of similarity between the vegetated and nonvegetated site observed in the top
346 sediment layer. Also, such a high similarity of the top sediment layer may be attributed to imports of
347 seagrass detritus to the nonvegetated site. As one of the main carbon sources in *C. nodosa* meadows

(Holmer et al., 2004), seagrass detritus may easily be transported to the adjacent nonvegetated site forming similar communities in the top sediment layer. To assess the temporal dynamics of the microbial community, we analysed each sediment layer and site separately to exclude the influence of sediment depth and site-specificity. Because microbial communities of surface sediments have shorter generation times and higher biomass than communities at deeper sediment strata, and seagrass meadow sediments are hotspots for microbial activity (Duarte et al., 2005; Starnawski et al., 2017), successional changes during the decline of a seagrass meadow could be expected. Surprisingly, the decline of the *C. nodosa* meadow in the Bay of Saline appeared to have little or no effect on the microbial community, as we did not observe any grouping of communities according to month, year, or meadow condition (Figure 4). In addition, no temporal patterns were observed in the taxonomic composition, richness, or diversity of the microbial community. Such a stable community structure and low proportion of community variation explained by the available environmental variables (Najdek et al., 2020) could be caused by a greater proportion of dormant or dead microbial cells remaining in the sediment, leading to a perceived taxonomic stability (Luna et al., 2002; Jones and Lennon, 2010; Cangelosi and Meschke, 2014; Carini et al., 2016; Torti et al., 2018; Bradley et al., 2019). Taxonomic identification by molecular methods such as sequencing of the 16S rRNA gene cannot distinguish between active and dormant cells, nor whether the cell is alive or dead (Cangelosi and Meschke, 2014). Indeed, it has been reported that in coastal marine sediments dead cells account for 70 % of all bacterial cells, while among living bacterial cells only 4 % grow actively (Luna et al., 2002). Furthermore, it is possible that the change in community composition may be delayed given that microbial communities in marine sediments often have very long generation times (Jørgensen and Marshall, 2016; Starnawski et al., 2017) and that some recognizable remnants of roots and rhizomes were still observed at the end of the study (Najdek et al., 2020). High metabolic versatility of microbial community members which allows functional continuity to be maintained despite changes in composition (Louca et al., 2018), may also allow for some degree of compositional stability despite changing environmental conditions. Indeed, a decoupling of microbial composition and biogeochemical processes has been

375 observed in sediments. Bowen et al. (2011) have shown that microbial communities in sediments
376 are able to resist compositional changes despite significant variations in external nutrient supply,
377 while Marshall et al. (2021) found that the composition of the nitrogen cycling community might
378 change but these compositional changes are not reflected in functional changes.

379 The archaeal community of both sites was comprised of *Nanoarchaeota*, *Thermoplasmatota*,
380 *Crenarchaeota* and *Asgardarchaeota* which are all typical sediment members (Zheng et al., 2019;
381 Sun et al., 2020). We found a nearly threefold increase in the relative abundance of *Archaea* in the
382 deepest sediment layer compared to the top layer (Figure 5). This is not surprising as it has been well
383 documented that *Bacteria* dominate the upper sediment layers while at deeper layers the distribution
384 between *Bacteria* and *Archaea* is more uniform (Chen et al., 2017). A particularly pronounced
385 increase in relative abundance with depth was observed for *Thermoplasmatota* at both sites. It is
386 possible that oxygen penetration in the uppermost sediment layer caused such a pronounced change
387 as representatives of the Marine Benthic Group D and DHVEG-1, accounting for the majority of
388 sequences within the phylum *Thermoplasmatota* (Rinke et al., 2019), are known to be restricted to
389 anoxic environments (Lloyd et al., 2013).

390 The main difference between the archaeal community of the vegetated and nonvegetated
391 site was the increased presence of *Crenarchaeota* in the nonvegetated sediment (Figure 5). This
392 difference resulted from a much greater increase in the relative abundance of *Bathyarcheia* with
393 increasing depth at the nonvegetated site (Figure 5). In a study comparing archaeal communities
394 in the sediment of a *Zostera marina* meadow with those of bare sediment, a higher presence of
395 *Bathyarchaeota* was found in the vegetated sediment, which is not consistent with our results
396 (Zheng et al., 2019). This discrepancy could have been caused by patchiness and different sampling
397 strategies. In contrast to the three samples per vegetated and nonvegetated sediment in the study of
398 Zheng et al. (2019), we analysed sixty-eight samples from each site. *Bathyarcheia*, formerly known
399 as the Miscellaneous Crenarchaeotal Group (MCG), are typically present in deeper sediment layers
400 as they are well adapted to energy limitation (Kubo et al., 2012). Since seagrasses are known to

401 directly and indirectly enrich the underlying sediment with organic matter (Terrados and Duarte,
402 2000; Duarte, 2002; Duarte et al., 2005; Jensen et al., 2007; van Katwijk et al., 2010; Liu et al.,
403 2017), it is possible that the presence of *C. nodosa* caused the observed lower relative abundance of
404 this group in the sediment at the vegetated site.

405 The sediment bacterial community of both sites consisted of taxonomic groups commonly
406 found in marine sediments such as *Desulfobacterota*, *Gammaproteobacteria*, *Bacteroidota*,
407 *Chloroflexi*, and *Planctomycetota* (Walsh et al., 2016; Hoshino et al., 2020), along with
408 *Campylobacterota*, characteristic of seagrass meadows (Jensen et al., 2007). These major groups
409 showed different patterns in relative abundance depending on sediment depth (Figures 6 and
410 7). The proportion of *Gammaproteobacteria* and *Bacteroidota* decreased with sediment depth,
411 while the relative abundance of *Chloroflexi* increased (Figure 6). Although the proportion of
412 *Desulfobacterota* remained similar in all sediment layers, *Desulfocapsaceae*, a major constituent of
413 the *Desulfobacterota* community, decreased with sediment depth (Figure 6). *Gammaproteobacteria*
414 and *Desulfobacterota* (formerly known as *Deltaproteobacteria*), were reported to decrease with
415 sediment depth, while *Chloroflexi* increased (Petro et al., 2017). Also, Smith et al. (2004)
416 documented no vertical trend in sulphate-reducing prokaryotes (*Desulfobacterota*) over a similarly
417 small depth range. Reduction of sulphate is one of many processes that affects pH in sediments,
418 while oxygen penetration controls the depth of pH minima (Silburn et al., 2017). Because
419 *Desulfocapsaceae* are neutrophilic (Galushko and Kuever, 2021) it is possible that depletion of
420 oxygen below the first centimetre and an increase in hydrogen sulphide with sediment depth
421 (Najdek et al., 2020) contributed to the observed vertical trend of this group. The pronounced
422 decline in *Gammaproteobacteria* after the top centimetre could also be attributed to the oxygen
423 penetration depth observed in the Bay of Saline (Najdek et al., 2020) coinciding with the abrupt
424 change in the relative abundance of this class. Oxygen availability could also influence the vertical
425 distribution of *Chloroflexi* and *Planctomycetota* (Figure 7), as these phyla are known to be prevalent
426 in anoxic sediments (Hoshino et al., 2020). In addition to oxygen availability, the decline of
427 *Gammaproteobacteria* and *Bacteroidota* with sediment depth may also be related to the lower

428 availability of fresh organic matter in deeper layers (Middelburg, 1989), as both of these groups are
429 known to break down and assimilate fresh detritus in coastal sediments (Gihring et al., 2009).

430 The differences in taxonomic composition of microbial communities from the vegetated
431 and nonvegetated site were not as pronounced as those influenced by sediment depth.
432 *Gammaproteobacteria* made up a large proportion of the microbial community at the nonvegetated
433 site, and as with vertical structuring, their higher presence at this site could be explained by oxygen
434 availability. This class contains representatives with a wide range of metabolisms, including
435 aerobic species (Gutierrez, 2019), which could benefit from the higher oxygen availability at
436 the nonvegetated site (Najdek et al., 2020). Indeed, a study by Ettinger et al. (2017) also found
437 a higher presence of *Gammaproteobacteria* in the sediment outside a seagrass meadow. The
438 most pronounced difference in the taxonomic composition of this class between the vegetated
439 and nonvegetated site is the higher relative abundance of *Thioalkalipiraceae* in the nonvegetated
440 sediment (Figure 6). This higher relative abundance could be due to differences in organic matter
441 content. In fact, *Thioalkalipiraceae* are known to be chemolithoautotrophs (Mori et al., 2011; Mori
442 and Suzuki, 2014) and thus may rely on inorganic compounds rather than organic matter supplied by
443 the seagrass. Slight differences were also observed in the *Desulfobacterota* community between the
444 vegetated and nonvegetated site. *Desulfosarcinaceae* were more pronounced at the vegetated site,
445 while *Desulfobulbaceae* were more pronounced at the nonvegetated site (Figure 6). Although both
446 families have been associated with the rhizosphere of seagrasses (Cúcio et al., 2016), our results are
447 consistent with previous studies that reported a high presence of *Desulfosarcinaceae* in vegetated
448 sediments and higher relative abundances of *Desulfobulbaceae* in the nonvegetated sediment (Smith
449 et al., 2004; García-Martínez et al., 2009). The most abundant *Desulfobacterota* family at both
450 the vegetated and nonvegetated site was *Desulfosarcinaceae*. The high metabolic versatility of
451 this group (Watanabe et al., 2020) may have lead to its even greater proliferation at the vegetated
452 site (Figure 6) where high concentrations of different carbon substrates may become available
453 during decomposition of organic matter. In contrast to *Gammaproteobacteria* and *Desulfobacterota*,
454 a higher relative abundance of *Bacteroidota* at the vegetated site may be influenced by the

presence of the plant itself. Seagrass cell walls contain polysaccharides like cellulose (Pfeifer and Classen, 2020) and *Bacteroidota* have been identified as decomposers of macromolecules such as cellulose (Thomas et al., 2011). The differences between the vegetated and nonvegetated sediment communities were also reflected in the higher proportion of *Campylobacterota* related sequences at the vegetated site. *Campylobacterota*, formerly known as *Epsilonproteobacteria*, are known to be closely associated with roots and rhizomes of seagrasses, particularly *Sulfurimonadaceae* (Jensen et al., 2007). In this study, the family *Sulfurimonadaceae* also contributed highly to *Campylobacterota* at the vegetated site (Figure 7). This high contribution may be caused by close proximity of the sampled sediment to roots and rhizomes. The seagrass selects the rhizosphere microbial community from the surrounding bulk sediment by enrichment of certain taxa and depletion of others (Cúcio et al., 2016; Zhang et al., 2020, 2022). It may be possible that roots and rhizomes to some degree also alter the composition of the community in the surrounding sediment in their close proximity forming the observed structure of *Campylobacterota* in our samples.

Taken together, sediment microbial communities in the Bay of Saline were depth stratified, and differed between the vegetated and nonvegetated site, however, remained temporally stable. Although the *C. nodosa* meadow experienced a sharp decline during the investigation period, no pronounced change in the microbial community was observed. The characterization of the sediment microbial community of the declining *C. nodosa* meadow in the Bay of Saline forms the basis for further studies based on methods that can differentiate active communities or methods that can provide insight into the prevailing metabolic processes during the period of seagrass decline.

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726 **Figure legends**

727 **Figure 1.** Location of the vegetated (declining *Cymodocea nodosa* meadow) and nonvegetated site
728 in the Bay of Saline, northern Adriatic Sea, together with visual representations of vegetated and
729 nonvegetated sediment cores (© OpenStreetMap contributors, www.openstreetmap.org/copyright).

730 **Figure 2.** The observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity
731 index, and Inverse Simpson diversity index of sediment microbial communities sampled in different
732 sediment layers of the vegetated and nonvegetated site in the Bay of Saline.

733 **Figure 3.** Principal Coordinates Analysis (PCoA) of Bray-Curtis dissimilarities based on OTU
734 abundances of sediment microbial communities sampled in the Bay of Saline. Samples from
735 different sites are labelled with different symbols while samples from different sediment layers
736 are indicated by colour. The proportion of explained variation by each axis is shown on the
737 corresponding axis in parentheses.

738 **Figure 4.** Principal Coordinates Analyses (PCoA) of Bray-Curtis dissimilarities based on OTU
739 abundances of sediment microbial communities, of all and individual sediment layers, sampled at
740 the vegetated and nonvegetated site in the Bay of Saline. The proportion of explained variation by
741 each axis is shown on the corresponding axis in parentheses.

742 **Figure 5.** Taxonomic classification and relative contribution of the most abundant bacterial and
743 archaeal ($\geq 3\%$) sequences in sediment communities sampled at the vegetated and nonvegetated
744 site in the Bay of Saline. NR – sequences without known relatives

745 **Figure 6.** Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) taxonomic
746 groups within *Desulfobacterota*, *Gammaproteobacteria*, and *Bacteroidota* in sediment communities
747 sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known
748 relatives

749 **Figure 7.** Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) taxonomic
750 groups within *Chloroflexi*, *Planctomycetota*, and *Campylobacterota* in sediment communities
751 sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known
752 relatives

753 **Figures**

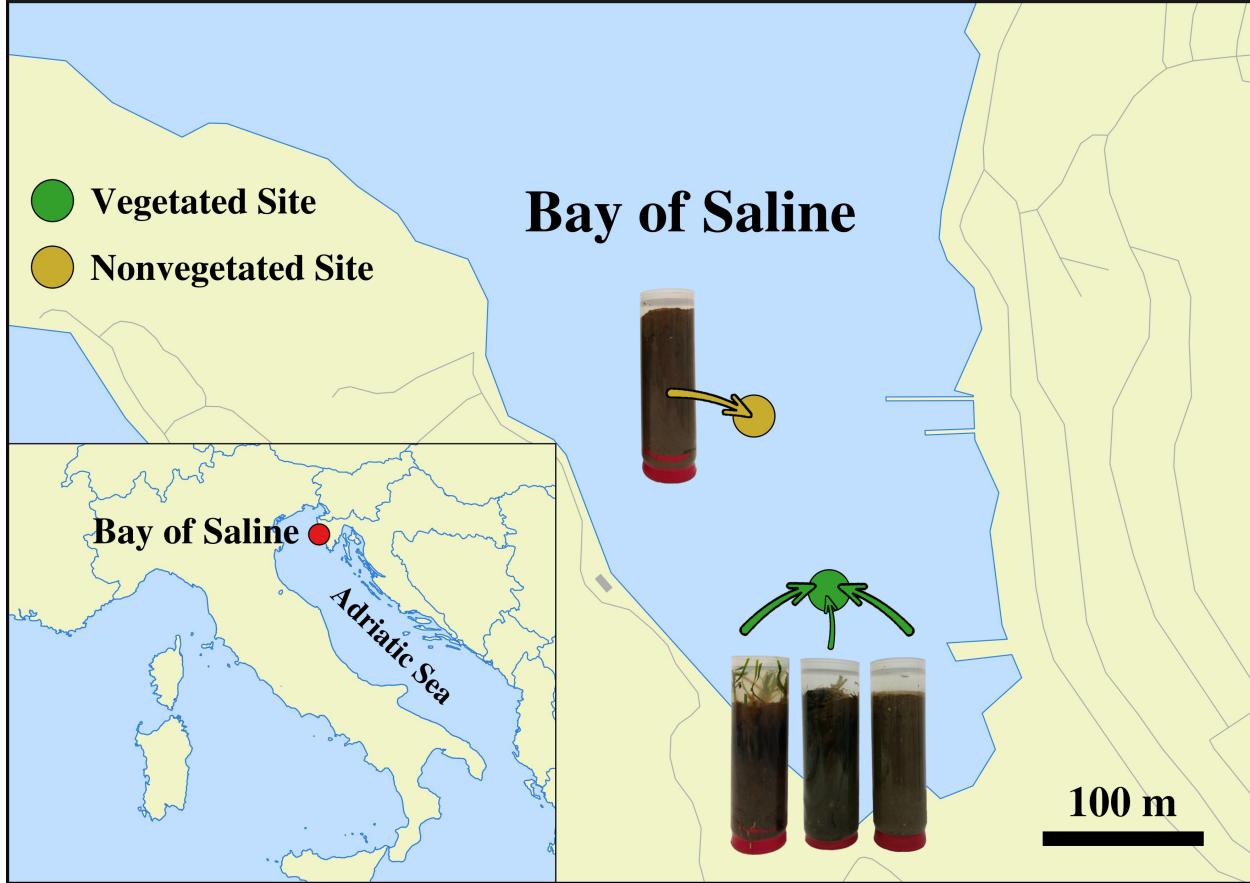


Figure 1. Location of the vegetated (declining *Cymodocea nodosa* meadow) and nonvegetated site in the Bay of Saline, northern Adriatic Sea, together with visual representations of vegetated and nonvegetated sediment cores (© OpenStreetMap contributors, www.openstreetmap.org/copyright).

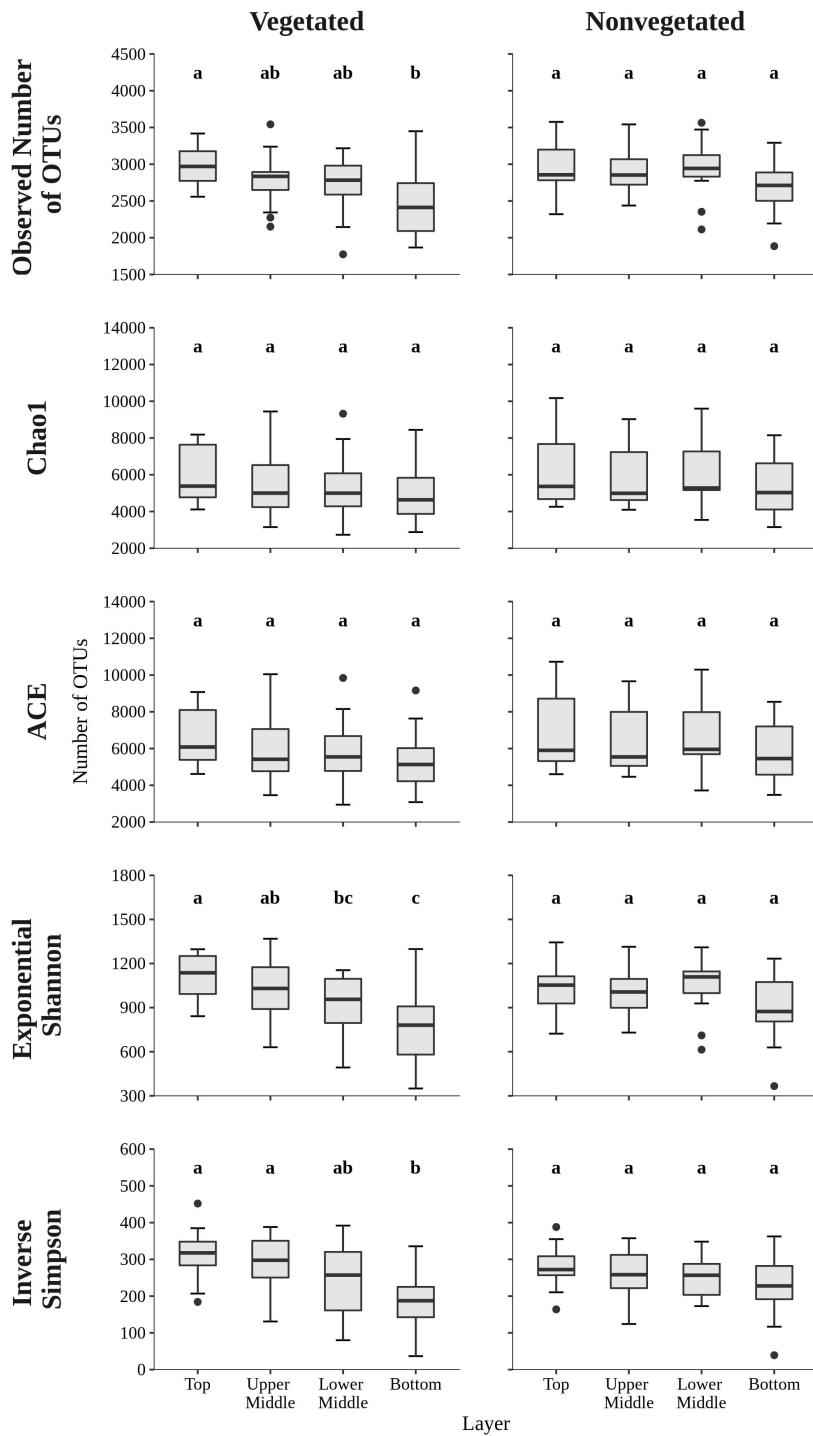


Figure 2. The observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity index, and Inverse Simpson diversity index of sediment microbial communities sampled in different sediment layers of the vegetated and nonvegetated site in the Bay of Saline.

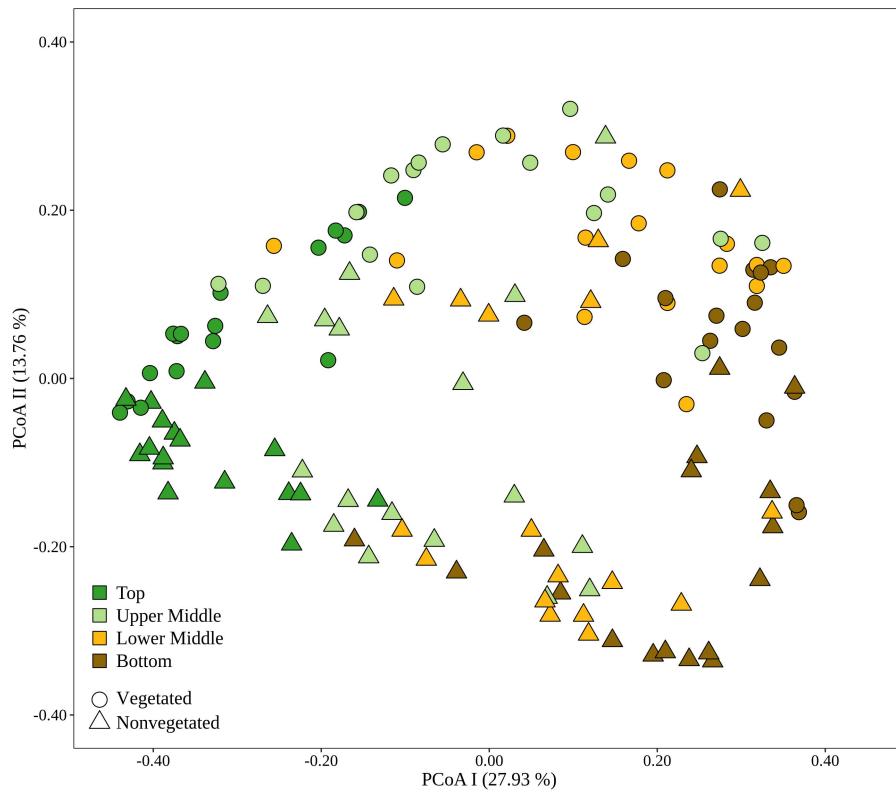


Figure 3. Principal Coordinates Analysis (PCoA) of Bray-Curtis dissimilarities based on OTU abundances of sediment microbial communities sampled in the Bay of Saline. Samples from different sites are labelled with different symbols while samples from different sediment layers are indicated by colour. The proportion of explained variation by each axis is shown on the corresponding axis in parentheses.

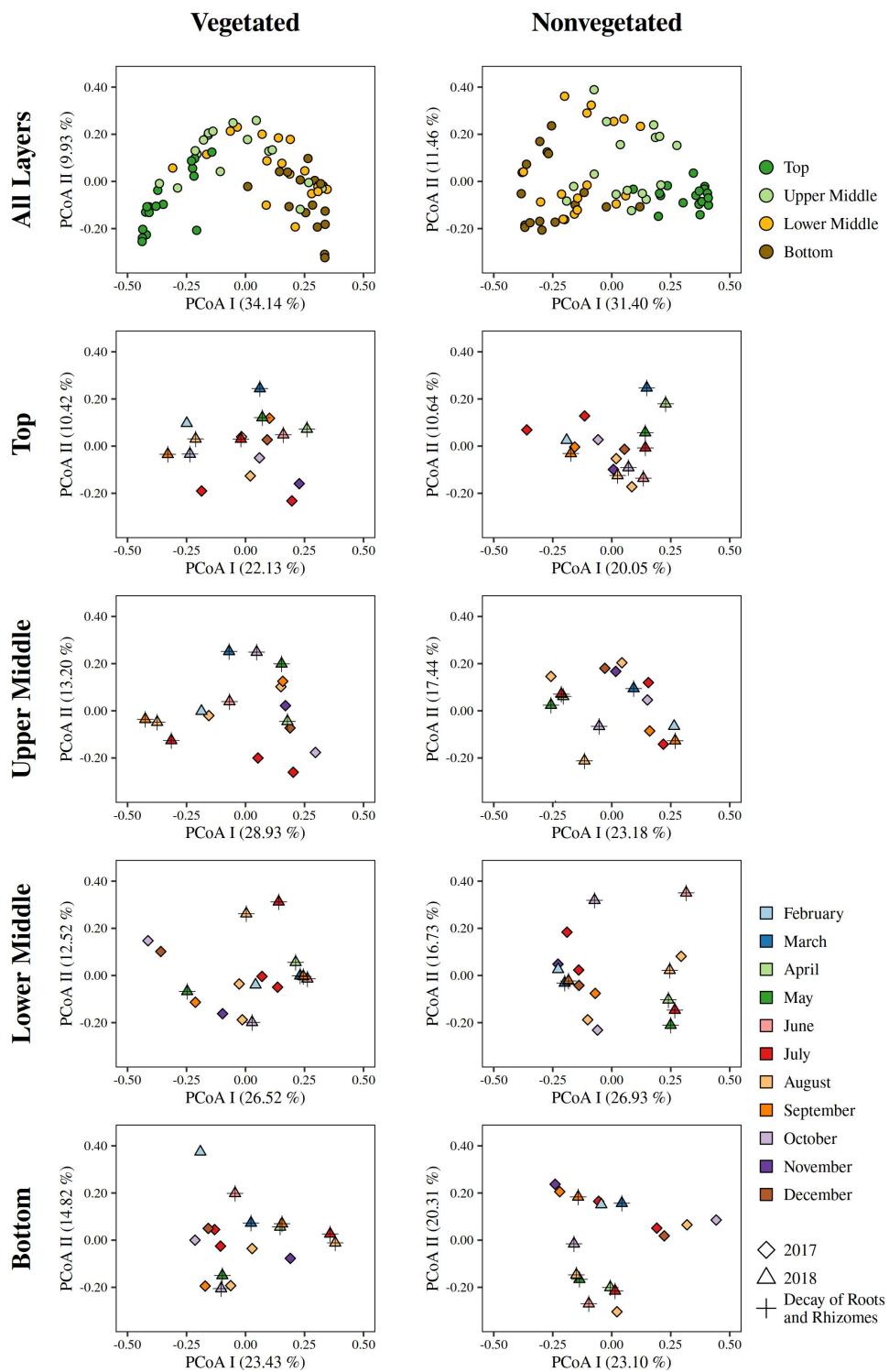


Figure 4. Principal Coordinates Analyses (PCoA) of Bray-Curtis dissimilarities based on OTU abundances of sediment microbial communities, of all and individual sediment layers, sampled at the vegetated and nonvegetated site in the Bay of Saline. The proportion of explained variation by each axis is shown on the corresponding axis in parentheses.

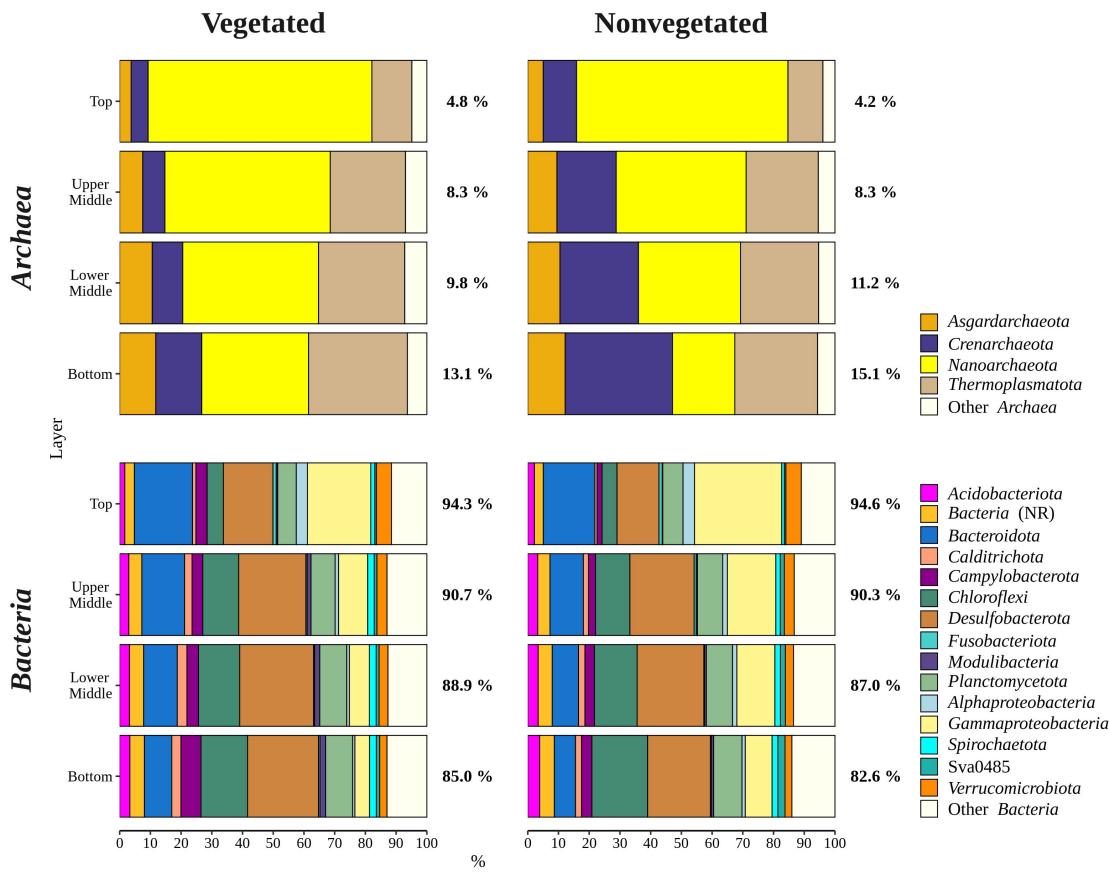


Figure 5. Taxonomic classification and relative contribution of the most abundant bacterial and archaeal ($\geq 3\%$) sequences in sediment communities sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known relatives

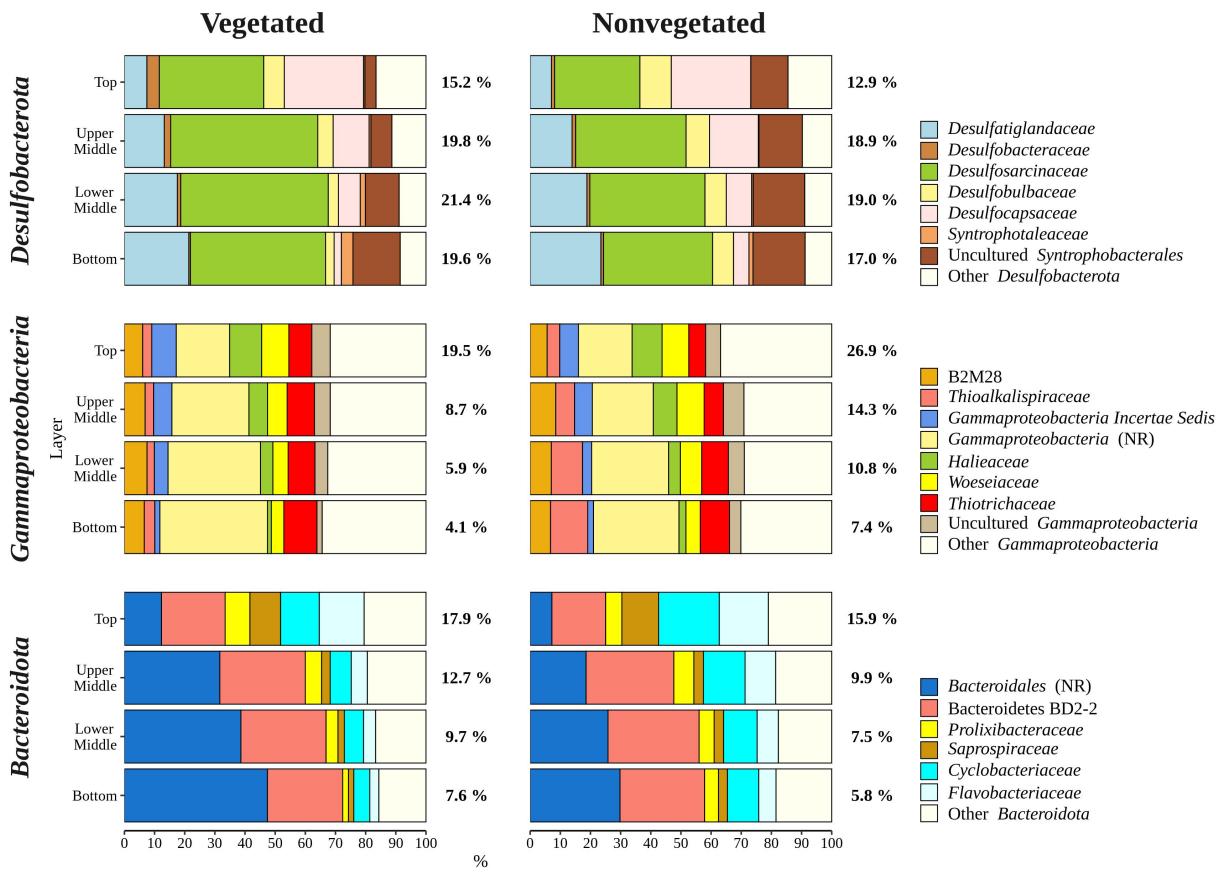


Figure 6. Taxonomic classification and relative contribution of the most abundant ($\geq 2\%$) taxonomic groups within *Desulfobacterota*, *Gammaproteobacteria*, and *Bacteroidota* in sediment communities sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known relatives

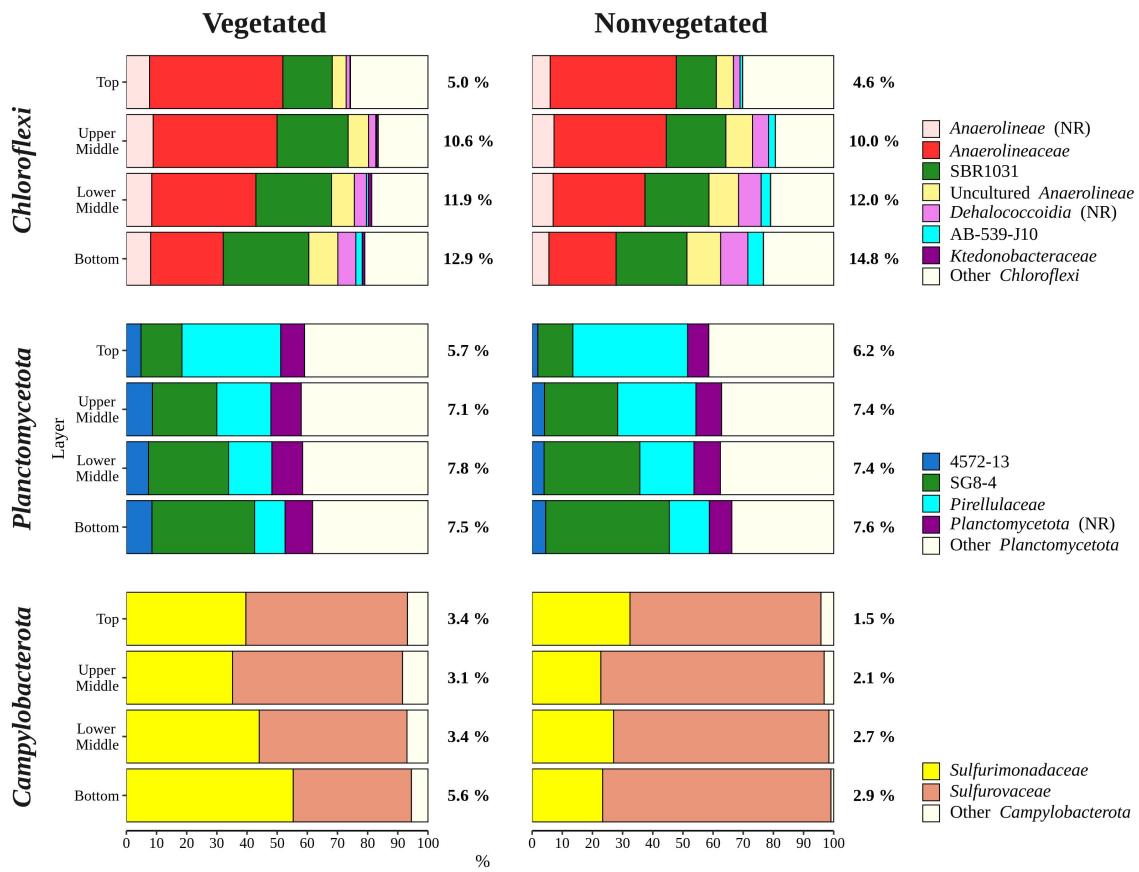


Figure 7. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) taxonomic groups within *Chloroflexi*, *Planctomycetota*, and *Campylobacterota* in sediment communities sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known relatives