

Selective DNA and Protein Isolation from Marine Macrophyte Surfaces

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Running title: DNA and protein isolation from macrophyte surfaces

1 Summary

2 Studies of unculturable microbes often combine methods based on DNA, such as 16S rRNA
3 sequencing and metagenomics, with methods that allow insight into the metabolic status, such as
4 metaproteomics. To apply these techniques to the microbial community inhabiting the surfaces of
5 marine macrophytes it is advisable to perform, prior to the analysis, a selective DNA and protein
6 isolation so that the host material, present in higher quantities, is not hampering the analysis.
7 Two protocols, for DNA and protein isolation, were adapted for selective extractions of DNA
8 and proteins from epiphytic communities inhabiting the surfaces of two marine macrophytes: the
9 seagrass *Cymodocea nodosa* and the macroalga *Caulerpa cylindracea*. Protocols showed an almost
10 complete removal of the epiphytic community regardless of the sampling season, station, settlement
11 or host species. The obtained DNA was suitable for metagenomic and 16S rRNA sequencing, while
12 isolated proteins could be identified by mass spectrometry, showing that protocols can be used in 16S
13 rRNA, metagenomic and metaproteomic analysis. Low presence of host DNA and proteins, observed
14 in isolated samples, indicated a selective nature of the protocols. Furthermore, the procedures are
15 based on universally available laboratory chemicals making the protocols widely applicable. Taken
16 together, the adapted protocols ensure an almost complete removal of the macrophyte epiphytic
17 community, are selective for microbes inhabiting macrophyte surfaces and are providing DNA and
18 proteins applicable in 16S rRNA sequencing, metagenomics and metaproteomics.

19 **Introduction**

20 Surfaces of marine macrophytes are inhabited by a diverse microbial community whose
21 structure and function are poorly understood (Egan *et al.*, 2013). As less than 1 % of all prokaryotic
22 species are culturable, to study these organisms, molecular methods such as 16S rRNA sequencing,
23 metagenomics and metaproteomics are indispensable (Amann *et al.*, 1995; Su *et al.*, 2012). Applying
24 these techniques requires an initial isolation step, with the purpose of obtaining high quality DNA
25 and proteins.

26 Biological material (i.e. proteins and DNA) from pelagic microbial communities is usually
27 isolated by collecting cells onto filters and subsequently isolating the aimed compound (Gilbert *et*
28 *al.*, 2009). If a specific microbial size fraction is aimed sequential filtration is applied (Massana *et*
29 *al.*, 1997; Andersson *et al.*, 2010). In contrast, obtaining biological materials from microorganism
30 inhabiting surfaces requires either a cell detachment procedure prior to isolation or the host material
31 is coextracted together with the targeted material. Methods for separating microbial cells form the
32 host include shaking of host tissue (Gross *et al.*, 2003; Nõges *et al.*, 2010), scraping of macrophyte
33 surfaces (Uku *et al.*, 2007) or the application of ultrasonication (Weidner *et al.*, 1996; Cai *et al.*,
34 2014). It was shown that shaking alone is not sufficient to remove microbial cells, at least from
35 plant root surfaces (Richter-Heitmann *et al.*, 2016). Manual separation methods, such as scraping
36 and brushing, are time consuming and subjective, as the detachment efficiency depends on host
37 tissue and the person performing the procedure (Cai *et al.*, 2014). Ultrasonication was proposed as
38 an alternative method as it is providing better results in terms of detachment efficiency (Cai *et al.*,
39 2014; Richter-Heitmann *et al.*, 2016). The downside of this procedure is that complete cell removal
40 was still not obtained and tissue disruption was observed especially after the application of probe
41 ultrasonication (Richter-Heitmann *et al.*, 2016). An alternative to these cell detachment procedures
42 is the isolation of targeted epiphytic compounds together with host materials (Staufenberger *et*
43 *al.*, 2008; Jiang *et al.*, 2015). This procedure can lead to problems in the following processing
44 steps such as mitochondrial and chloroplast 16S rRNA sequence contaminations from the host

45 (Longford *et al.*, 2007; Staufenberger *et al.*, 2008). In addition, when performing metagenomics
46 and metaproteomics host material can cause biased results towards more abundant host DNA and
47 proteins.

48 An alternative to these procedures is a direct isolation of the targeted material by incubating
49 macrophyte tissues in an extraction buffer. After the incubation, the undisrupted host tissue is
50 removed and the isolation procedure continues, omitting host material contaminations. To our
51 knowledge the only procedure describing a direct and selective epiphytic DNA isolation from the
52 surfaces of marine macrophytes was provided by Burke *et al.* (2009). In contrast to previously
53 described methods this protocol enables an almost complete removal of the surface community and
54 it was used for 16S rRNA gene clone library construction (Burke and Thomas *et al.*, 2011) and
55 metagenome sequencing (Burke and Peter Steinberg *et al.*, 2011). This method, thought providing a
56 selective isolation procedure, uses a rapid multienzyme cleaner (3M) that is not available worldwide
57 and without a known composition (Burke *et al.*, 2009). Also to our knowledge, no selective isolation
58 protocol for proteins from epiphytic communities inhabiting marine macrophytes was established.

59 In the present study, we adapted a protocol widely used for DNA isolation from filters (Massana
60 *et al.*, 1997) and a protocol used for protein isolation from sediments (Chourey *et al.*, 2010; Hultman
61 *et al.*, 2015) for selective extractions of DNA and proteins from epiphytic communities inhabiting the
62 surfaces of two marine macrophytes: the seagrass *Cymodocea nodosa* and the macroalga *Caulerpa*
63 *cylindracea*. In addition, we tested the removal efficiency of the protocol and the suitability of
64 obtained DNA and proteins for 16S rRNA sequencing, metagenomics and metaproteomics.

65 **Results**

66 To assess the removal efficiency of DNA and protein isolation procedures leaves and thalli were
67 examined under a confocal microscope before and after treatments were performed. Developed
68 procedures showed an almost complete removal of the surface community of both, *C. nodosa* and
69 *C. cylindracea*. In addition, a similar removal efficiency was observed for communities sampled
70 in contrasting seasons, December 2017 (Fig. 1) and June 2018 (Fig. 2). Also, no effect of station,
71 settlement or isolation procedure (DNA or protein) on the removal efficiency was observed (Figs. 1
72 and 2).

73 To evaluate if the obtained DNA is suitable to determine the microbial community structure
74 an Illumina sequencing of the V4 16S rRNA region was performed. Sequencing yielded a total
75 of 336,937 sequences after quality curation and exclusion of eukaryotic, mitochondrial and no
76 relative sequences. The number of sequences classified as chloroplast was 97,328. After excluding
77 these sequences the total number of retrieved reads dropped to 239,609, ranging from 22,587 to
78 52,958 sequences per sample (Table S1). Even when the highest sequencing effort was applied the
79 rarefaction curves did not level off which is commonly observed in high-throughput 16S rRNA
80 amplicon sequencing procedures (Fig. S1). Sequence clustering at a similarity level of 97 % yielded a
81 total of 8,360 different OTUs. Taxonomic classification of reads allowed for a macrophyte associated
82 epiphytic community determination that was mainly composed of: *Alphaproteobacteria* (14.9 ± 3.5
83 %), *Bacteroidota* (12.5 ± 2.4 %), *Gammaproteobacteria* (11.6 ± 4.3 %), *Desulfobacterota* ($7.8 \pm$
84 7.4 %), *Cyanobacteria* (6.5 ± 4.7 %) and *Planctomycetota* (2.9 ± 1.7 %) (Fig. 3).

85 Primers used in this study, as in many other 16S rRNA amplicon procedures, also amplified
86 chloroplast 16S rRNA genes. We observed a high proportion of chloroplast sequences in all
87 analyzed samples (33.4 ± 9.4 %) (Fig. 3). To determine if chloroplast sequences originate from
88 hosts or eukaryotic epiphytic organisms, we exported SILVA classified chloroplast sequences
89 and reclassified them using the RDP (Ribosomal Database Project) training set that allows for a

more detailed chloroplast classification. The largest proportion of sequences were classified as Bacillariophyta ($89.7 \pm 5.7\%$) indicating that the DNA removal procedure did not coextract larger quantities of host DNA (Fig. 4). Chloroplast sequences classified as Streptophyta constituted $3.3 \pm 2.8\%$ of all chloroplast sequences originating from *C. nodosa* samples, while sequences classified as Chlorophyta comprised only $0.02 \pm 0.01\%$ of all chloroplast sequences associated with *C. cylindracea* samples.

To determine if the extracted DNA can be used for metagenomic sequencing four samples containing epiphytic DNA were selected and shotgun sequenced using an Illumina platform. Metagenomic sequencing yielded from 207,149,524 to 624,029,930 sequence pairs (Table S2). Obtained sequences were successfully assembled into contigs whose L50 ranged from 654 to 1,011 bp. In addition, predicted coding sequences were successfully functionally annotated (9,066,667 – 20,256,215 annotated sequences; Fig. 5) and taxonomically classified. Functional annotation allowed for an assessment of the relative contribution of each COG (Clusters of Orthologous Groups) functional category to the total number of annotated coding sequences (Fig. 5a). Functional categories containing the highest number of sequences were: C (Energy production and conversion), E (Amino acid transport and metabolism), M (Cell wall/membrane/envelope biogenesis), L (Replication, recombination and repair) and P (Inorganic ion transport and metabolism). If host DNA was coextracted with epiphytic it should be detected in larger proportions in sequenced metagenomes. Indeed, no higher proportions of coding sequences classified into phylum Streptophyta or Chlorophyta were detected (Table S3). Sequenced metagenomic DNA originating from the surface of *C. nodosa* contained 1.3 % of coding sequences classified into the phylum Streptophyta in December 2017 and 0.7 % in June 2018. Furthermore, the summed RPKM (Reads Per Kilobase Million) of these sequences constituted 1.7 % of total RPKM of all successfully classified sequences in December 2017 and 1.1 % in June 2018. Similar low proportions of host coding sequences were detected in metagenomic samples originating from the surfaces of *C. cylindracea*. Of all successfully classified coding sequences 0.2 % were classified into Chlorophyta in December 2017 and 0.1 % in June 2018. A relatively higher proportion of these sequences'

¹¹⁷ RPKM than in the case of *C. nodosa* was observed, indicating a higher coextraction of host DNA in
¹¹⁸ *C. cylindracea*. In December, the proportion of RPKM of sequences classified into Chlorophyta
¹¹⁹ was 8.2 %, while in June 2018 it reached 13.6 %.

¹²⁰ To evaluate if the procedure for protein extraction is suitable for metaproteomic analysis,
¹²¹ obtained proteins were trypsin digested and sequenced using a mass spectrometer. Obtained
¹²² MS/MS spectra were searched against a protein database from sequenced metagenomes. From
¹²³ 14,219 to 16,449 proteins were identified in isolated protein samples (Fig. 5b). In addition,
¹²⁴ successful identification of proteins allowed for an assessment of the relative contribution of
¹²⁵ each COG functional category to the total number of identified proteins (Fig. 5b). Functional
¹²⁶ categories containing the highest number of identified proteins were: C (Energy production and
¹²⁷ conversion), G (Carbohydrate transport and metabolism), P (Inorganic ion transport and metabolism),
¹²⁸ O (Posttranslational modification, protein turnover, chaperones) and E (Amino acid transport and
¹²⁹ metabolism). Isolated proteins could derive from epiphytic organisms inhabiting the macrophyte
¹³⁰ surface or from macrophyte tissue underlying them. The contribution of proteins originating from
¹³¹ host tissues was evaluated by identifying all the proteins predicted to belong to any taxonomic group
¹³² within the phyla Streptophyta and Chlorophyta and by calculating their contribution to the number
¹³³ and abundance of all identified proteins. On average, proteins isolated from the surface of *C. nodosa*
¹³⁴ contained 1.8 ± 0.06 % of proteins associated with Streptophyta, contributing to 2.2 ± 0.8 % of
¹³⁵ total proteins. Similar to metagenomes, proteins associated with Chlorophyta contributed more to
¹³⁶ *C. cylindracea* than proteins associated with Streptophyta to *C. nodosa*. Chlorophyta associated
¹³⁷ proteins comprised 5.2 ± 0.06 % of all identified proteins in *C. cylindracea*, contributing to $19.2 \pm$
¹³⁸ 1.5 % of all protein abundances.

139 **Discussion**

140 The study of marine macrophyte epiphytic communities using culture independent techniques,
141 such as 16S rRNA analysis, metagenomics and metaproteomics, requires an initial step of biological
142 material isolation. Methods that have been developed for selective isolation of epiphytic biological
143 material can be divided into two groups: (i) procedures involving, prior to extraction, a cell
144 detachment step such as shaking (Gross *et al.*, 2003; Nōges *et al.*, 2010), scraping (Uku *et al.*, 2007)
145 and ultrasonication (Weidner *et al.*, 1996; Cai *et al.*, 2014) and (ii) procedures involving a host
146 tissue incubation aiming at direct lysis of epiphytic microbial cells (Burke *et al.*, 2009). Protocols
147 that include a cell detachment procedure do not provide complete cell removal (Cai *et al.*, 2014;
148 Richter-Heitmann *et al.*, 2016) and in the case of probe ultrasonication tissue disruption can also be
149 observed (Richter-Heitmann *et al.*, 2016). Though, host tissue incubation procedures provide an
150 almost complete cell removal and selective isolation, existing protocols like the one developed for
151 DNA isolation by Burke *et al.* (2009) use in the extraction buffer a rapid multienzyme cleaner (3M)
152 not available worldwide and without a known composition (Burke *et al.*, 2009). To circumvent
153 these problems we developed and tested two protocols for selective DNA and protein isolation from
154 marine macrophyte epiphytic communities.

155 To test if the developed DNA and protein isolation procedures could be applied on a variety
156 of macrophyte species we selected *C. nodosa* and *C. cylindracea* as representatives of seagrass
157 and macroalgal species, on which the procedures were tested. These species especially differ
158 morphologically. While *C. nodosa* leaves are flat, *C. cylindracea* thallus is composed of uneven
159 surfaces (Kuo and den Hartog, 2001; Verlaque *et al.*, 2003). The developed procedures showed an
160 almost complete removal of epiphytic cells from the surfaces of both species comparable to the
161 result of Burke *et al.* (2009) and indicating that structural differences do not impact the removal
162 efficiency. In addition, isolation protocols were tested in two contrasting season as it is known that
163 macrophytes are harboring more algal epiphytes during autumn and winter (Reyes and Sansón,
164 2001). No differences in the removal efficiency was observed between seasons suggesting that

¹⁶⁵ protocols can be used on macrophyte samples retrieved throughout the year. Also, no removal
¹⁶⁶ differences were observed on samples derived from the same host but from different localities or
¹⁶⁷ settlements demonstrating the stability of the protocol in cell removal efficiency.

¹⁶⁸ Successful amplification and sequencing of the V4 16S rRNA gene region proved that the
¹⁶⁹ isolated DNA can be used to estimate the microbial epiphytic diversity. Taxonomic groups detected
¹⁷⁰ in this step can also be often found in epiphytic communities associated with other macrophytes
¹⁷¹ (Burke and Thomas *et al.*, 2011; Morrissey *et al.*, 2019). A problem often encountered in studies
¹⁷² focusing on epiphytic communities is the presence of large proportions of chloroplast 16S rRNA
¹⁷³ sequences in the pool of amplified molecules, especially if the epiphytic DNA was isolated without
¹⁷⁴ prior selection (Staufenberger *et al.*, 2008). These sequences can derive from host chloroplasts or
¹⁷⁵ from eukaryotic epiphytic chloroplast DNA. Although the proportion of obtained chloroplast 16S
¹⁷⁶ rRNA sequences in our samples was substantial they derived almost exclusively from eukaryotic
¹⁷⁷ epiphytes. High proportion of chloroplast 16S rRNA sequences in studies applying selective
¹⁷⁸ procedures that include direct cellular lysis on host surfaces were observed before (Michelou *et*
¹⁷⁹ *al.*, 2013). It is possible that chloroplast specific sequences even in these studies are originating
¹⁸⁰ from eukaryotic epiphytic cells and not host chloroplast. Indeed, it is common during 16S rRNA
¹⁸¹ profiling of pelagic microbial communities to observe high proportions of chloroplast sequences
¹⁸² (Gilbert *et al.*, 2009; Korlević *et al.*, 2016). In addition, a very low proportion of chloroplast 16S
¹⁸³ rRNA sequences in samples originating from *C. cylindracea* in comparison to *C. nodosa* could be
¹⁸⁴ explained by the presence of three introns in the gene for 16S rRNA in some members of the genus
¹⁸⁵ *Caulerpa* that could hamper the amplification process (Lam and Lopez-Bautista, 2016).

¹⁸⁶ Beside 16S rRNA sequencing high quality DNA is needed for metagenomics. The obtained
¹⁸⁷ number of metagenomic sequences and assembly statistics were comparable to metagenomes and
¹⁸⁸ metatranscriptomes derived from similar surface associated communities (Crump *et al.*, 2018; Cúcio
¹⁸⁹ *et al.*, 2018). In addition, functional annotation of predicted coding sequences to COG functional
¹⁹⁰ categories showed that the obtained metagenomes can be used to determine the metabolic capacity

191 of surface associated communities (Leary *et al.*, 2014; Cúcio *et al.*, 2018). The proportion of
192 coding sequences, including their RPKM, originating from *C. nodosa* metagenomes and classified
193 as Streptophyta was low indicating a good selectivity of the isolation procedure towards epiphytic
194 cells. In the case of DNA samples isolated from the surface of *C. cylindracea* the proportion of
195 Chlorophyta coding sequences was also low but their RPKM was higher than in the case of *C.*
196 *nodosa*. One of the causes for this elevated RPKM of Chlorophyta sequences in *C. cylindracea*
197 could lay in tissue structure differences between these two host species. While *C. nodosa* leaves
198 are composed of individual cells, the thallus of *C. cylindracea* is, like in other siphonous algal
199 species, composed of a single large multinucleate cell (Coneva and Chitwood, 2015). The absence
200 of individual cells in *C. cylindracea* could cause a leakage of genetic material into the extraction
201 buffer causing an elevated presence of host sequences in metagenomic data.

202 To obtain an insight into the metabolic status of uncultivated members, a metaproteomic
203 approach is required (Saito *et al.*, 2019). The applied protocol for epiphytic protein isolation
204 followed by a metaproteomic analysis identified between 14,219 and 16,449 proteins, which is
205 higher than previously reported for e.g. soils (Chourey *et al.*, 2010; Hultman *et al.*, 2015), seawater
206 (Williams *et al.*, 2012) and biofilms (Leary *et al.*, 2014). The functional annotation of identified
207 proteins into COG functional categories showed that the protein isolation procedure can be used to
208 assess the metabolic status of the macrophyte epiphytic community (Leary *et al.*, 2014). Similar
209 to metagenomes, the number of identified proteins, including their abundances, associated with
210 Streptophyta in *C. nodosa* samples were low indicating that the procedure is selective for epiphytic
211 cell proteins. In addition, a higher number of identified proteins, and especially their abundances,
212 associated with Chlorophyta was observed in *C. cylindracea* samples. The cause of this elevated
213 presence of Chlorophyta associated proteins can be, as in the case of the DNA isolation procedure,
214 explained by the absence of individual cells in this siphonous alga (Coneva and Chitwood, 2015).

215 In conclusion, the developed protocols for DNA and protein isolation from macrophyte surfaces
216 are providing an almost complete removal of the epiphytic community and are shown to ensure

*removal from both, *C. nodosa* and *C. cylindracea*, in different seasons. Also, the obtained DNA and proteins are suitable for 16S rRNA sequencing, metagenomics and metaproteomics, while the obtained material contains low quantities of host DNA or proteins making the procedures epiphyte selective. Furthermore, the procedures are based on universally available laboratory chemicals making the protocols widely applicable.*

222 **Experimental procedures**

223 **Sampling**

224 Leaves of *C. nodosa* were sampled in a *C. nodosa* meadow in the Bay of Saline (45°7'5''
225 N, 13°37'20'' E) and in a *C. nodosa* meadow invaded by *C. cylindracea* in the proximity of the
226 village of Funtana (Bay of Funtana; 45°10'39'' N, 13°35'42'' E). Thalli of *C. cylindracea* were
227 sampled in the same *C. nodosa* invaded meadow in the Bay of Funtana and on a locality of
228 only *C. cylindracea* located in the proximity of the invaded meadow. Leaves and thalli for 16S
229 rRNA analysis, metagenomics and metaproteomics were collected in two contrasting seasons, on 4
230 December 2017 (16S rRNA analysis and metaproteomics), 14 December 2017 (metagenomics) and
231 18 June 2018 (16S rRNA analysis, metagenomics and metaproteomics). During spring 2018 the *C.*
232 *nodosa* meadow in the Bay of Saline decayed to an extent that no leaves could be retrieved (Najdek
233 *et al.*, 2020). In addition, as not enough DNA for both metagenomic and 16S RNA analysis were
234 obtained during the sampling on 4 December 2017, an additional sampling on 14 December 2017
235 was carried out in the Bay of Funtana. Leaves and thalli were collected by diving and transported to
236 the laboratory in containers placed on ice and filled with site seawater. Upon arrival to the laboratory,
237 *C. nodosa* leaves were cut into sections of 1 – 2 cm, while *C. cylindracea* thalli were cut into 5 – 8
238 cm long sections. Leaves and thalli were washed three times with sterile artificial seawater (ASW)
239 to remove loosely attached microbial cells.

240 **DNA isolation**

241 The DNA was isolated according to the protocol for isolation from filters described in Massana
242 *et al.* (1997). This protocol was modified and adapted for microbial DNA isolation from macrophyte
243 surfaces as described below. 5 ml of lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose;
244 pH 8.3) was added to 1 g wet weight of leaves or 2 g wet-weight of thalli. For every sample

245 duplicate isolations were performed. Lysozyme was added (final concentration 1 mg ml⁻¹) and the
246 mixture was incubated at 37 °C for 30 min. Subsequently, proteinase K (final concentration 0.5
247 mg ml⁻¹) and SDS (final concentration 1 %) were added and the samples were incubated at 55 °C
248 for 2 h. Following the incubation, tubes were vortexed for 10 min and the mixture containing lysed
249 epiphytic cells was separated from host leaves or thalli by transferring the solution into a clean tube.
250 The lysate was extracted twice with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1; pH
251 8) and once with chloroform:isoamyl alcohol (24:1). After each organic solvent mixture addition
252 tubes were slightly vortexed and centrifuged at 4,500 × g for 10 min. Following each centrifugation
253 aqueous phases were retrieved. After the final extraction 1/10 of chilled 3 M sodium acetate (pH
254 5.2) was added. DNA was precipitated by adding 1 volume of chilled isopropanol, incubating
255 the mixtures overnight at -20 °C and centrifuging at 16,000 × g and 4 °C for 20 min. The pellet
256 was washed twice with 1 ml of chilled 70 % ethanol and centrifuged after each washing step at
257 20,000 × g and 4 °C for 10 min. After the first washing step duplicate pellets form the same sample
258 were pooled and transferred to a clean 1.5 ml tube. The dried pellet was resuspended in 100 µl of
259 deionized water.

260 Illumina 16S rRNA sequencing

261 An aliquot of isolated DNA was treated with RNase A (final concentration 200 µg ml⁻¹) for 2
262 h at 37 °C. The DNA concentration was determined using the Quant-iT PicoGreen dsDNA Assay
263 Kit (Invitrogen, USA) according to the manufacturer's instructions and diluted to 1 ng µl⁻¹. The V4
264 region of the 16S rRNA gene was amplified using a two-step PCR procedure. In the first PCR the
265 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3')
266 primers from the Earth Microbiome Project (<http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>) were used to amplify the target region (Caporaso *et al.*, 2012; Apprill *et al.*,
267 2015; Parada *et al.*, 2016). These primers contained on their 5' end a tagged sequence. Each
268 sample was amplified in four parallel 25 µl reactions of which each contained: 1 × Q5 Reaction
269

270 Buffer , 0.2 mM of dNTPmix, 0.7 mg ml⁻¹ BSA (Bovine Serum Albumin), 0.2 µM of forward
271 and reverse primers, 0.5 U of Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA)
272 and 5 ng of DNA template. Cycling conditions were: initial denaturation at 94 °C for 3 min, 20
273 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s and elongation at 72 °C for
274 90 s, finalized by an elongation step at 72 °C for 10 min. The four parallel reactions volumes
275 were pooled and PCR products were purified using the GeneJET PCR Purification Kit (Thermo
276 Fisher Scientific, USA) according to the manufacturer's instructions and following the protocol
277 that included isopropanol addition for better small DNA fragment yield. The column was eluted
278 in 30 µl of deionized water. Purified PCR products were sent for Illumina MiSeq sequencing
279 (2 × 250 bp) at IMGM Laboratories, Martinsried, Germany. Before sequencing at IMGM, the
280 second PCR amplification of the two-step PCR procedure was performed using primers targeting
281 the tagged region incorporated in the first PCR. In addition, these primers contained adapter and
282 sample-specific index sequences. The second PCR was carried out for 8 cycles. Beside samples, a
283 positive and negative control were sequenced. A negative control was comprised of four parallel
284 PCR reactions without DNA template, while for a positive control a mock community composed
285 of evenly mixed DNA material originating from 20 bacterial strains (ATCC MSA-1002, ATCC,
286 USA) was used. Partial 16S rRNA sequences obtained in this study have been deposited in the
287 European Nucleotide Archive (ENA) at EMBL-EBI under accession numbers SAMEA6786270,
288 SAMEA6648792 – SAMEA6648794, SAMEA6648809 – SAMEA6648811.

289 Obtained sequences were analyzed on the computer cluster Isabella (University Computing
290 Center, University of Zagreb) using mothur (version 1.43.0) (Schloss *et al.*, 2009) according
291 to the MiSeq Standard Operating Procedure (MiSeq SOP; https://mothur.org/wiki/MiSeq_SOP)
292 (Kozich *et al.*, 2013) and recommendations given from the Riffomonas project to enhance data
293 reproducibility (<http://www.riffomonas.org/>). For alignment and classification of sequences the
294 SILVA SSU Ref NR 99 database (release 138; <http://www.arb-silva.de>) was used (Quast *et al.*,
295 2013; Yilmaz *et al.*, 2014). Sequences classified as chloroplasts by SILVA were exported and
296 reclassified using mothur and the RDP (<http://rdp.cme.msu.edu/>) training set (version 16) reference

297 files adapted for mothur (Cole *et al.*, 2014). In comparison to SILVA, RDP allows a more detailed
298 classification of chloroplast sequences. Pipeline data processing and visualization was done using
299 R (version 3.6.0) (R Core Team, 2019), package tidyverse (version 1.3.0) (Wickham *et al.*, 2019)
300 and multiple other packages (Xie, 2014, 2015, 2020; Neuwirth, 2014; Xie *et al.*, 2018; Wilke,
301 2018; Y. Xie, 2019b, 2019a; Allaire *et al.*, 2019; Zhu, 2019; Bengtsson, 2020). The detailed
302 analysis procedure including the R Markdown file for this paper are available as a GitHub repository
303 (https://github.com/mkorlevic/Korlevic_SelectiveRemoval_EnvironMicrobiol_2020). Based on the
304 ATCC MSA-1002 mock community included in the analysis a sequencing error rate of 0.009 %
305 was determined, which is in line with previously reported values for next-generation sequencing
306 data (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative control processed together
307 with the samples yielded only 2 sequences after sequence quality curation.

308 **Metagenomics**

309 Four DNA samples were selected and sent on dry ice to IMGM Laboratories, Martinsried,
310 Germany for metagenomic sequencing. DNA was purified using AMPure XP Beads (Beckman
311 Coulter, USA) applying a bead:DNA ratio of 1:1 (v/v), quantified with a Qubit dsDNA HS Assay
312 Kit (Thermo Fisher Scientific, USA) and check for integrity on a 1 % agarose gel. Metagenomic
313 sequencing libraries were generated from 300 ng of input DNA using a NEBNext Ultra II FS
314 DNA Library Prep Kit for Illumina (New England Biolabs, USA) according to the manufacturer's
315 instructions. Fragments were selected (500 – 700 bp) using AMPure XP Beads, PCR enriched for 3
316 – 5 cycles and quality controlled. Libraries generated from different DNA samples were pooled and
317 sequenced on an Illumina NovaSeq 6000 sequencing system (2 × 250 bp).

318 Obtained sequences were analyzed on a Life Science Compute Cluster (LiSC) (CUBE –
319 Computational Systems Biology, University of Vienna). Individual sequences were assembled using
320 MEGAHIT (version 1.1.2) (Li *et al.*, 2015) under default settings. Putative genes were predicted
321 from contings longer than 200 bp using Prodigal (version 2.6.3) (Hyatt *et al.*, 2010) in metagenome

mode (-p meta). Abundances of predicted genes were expressed as Reads Per Kilobase Million (RPKM) and calculated using the BWA algorithm (version 0.7.16a) (Li and Durbin, 2009). All predicted genes were functionally annotated using the eggNOG-mapper (Huerta-Cepas *et al.*, 2017) and eggNOG database (version 5.0) (Huerta-Cepas *et al.*, 2019). Sequence taxonomy classification was determined using the lowest common ancestor algorithm adapted from DIAMOND (version 0.8.36) (Buchfink *et al.*, 2015) and by searching against the NCBI non-redundant database (NR). To determine phylogeny the top 10 % hits with an e-value $< 1 \times 10^{-5}$ were used (--top 10). Sequence renaming, coverage information computing and metagenomic statistics calculations were performed using software tools from BBTools (<https://jgi.doe.gov/data-and-tools/bbtools>). Metagenomic sequences obtained in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession numbers **TO BE ADDED**.

333 Protein isolation

334 Proteins were isolated according to the protocol for protein isolation from soil described in
335 Chourey *et al.* (2010) and modified by Hultman *et al.* (2015). This protocol was further modified
336 and adapted for microbial protein isolation from macrophyte surfaces as described below. 20 ml
337 of protein extraction buffer (4 % SDS, 100 mM Tris-HCl [pH 8.0]) was added to 5 g wet weight
338 of leaves or 10 g wet weight of thalli. The mixture was incubated in boiling water for 5 min,
339 vortexed for 10 min and incubated again in boiling water for 5 min. After a brief vortex the lysate
340 was transferred to a clean tube separating the host leaves or thalli from the mixture containing
341 lysed epiphytic cells. Dithiothreitol (DTT; final concentration 24 mM) was added and proteins were
342 precipitated with chilled 100 % trichloroacetic acid (TCA; final concentration 20 %) overnight at
343 -20°C . Precipitated proteins were centrifuged at $10,000 \times g$ and 4°C for 40 min. The obtained
344 protein pellet was washed three times with chilled acetone. During the first washing step the pellet
345 was transferred to a clean 1.5 ml tube. After each washing step samples were centrifuged at $20,000$
346 $\times g$ and 4°C for 5 min. Dried pellets were stored at -80°C until further analysis.

347 **Metaproteomics**

348 Isolated proteins were whole trypsin digested using the FASP (filter-aided sample preparation)
349 Protein Digestion Kit (Expedeon, UK) according to the manufacturer's instructions with small
350 modifications (Wiśniewski *et al.*, 2009). Before the solution was loaded to the column, protein
351 pellets were solubilized in a urea sample buffer included in the kit amended with DTT (final
352 concentration 100 mM) for 45 min at room temperature and centrifuged at 20,000 × g for 2 – 5 min
353 at room temperature to remove larger particles. The first washing step after protein solution loading
354 was repeated twice. In addition, centrifugation steps were prolonged if the column was clogged.
355 Trypsin digestion was performed on column filters at 37 °C overnight for 18 h. The final filtrate
356 containing peptides was acidified with 1 % (final concentration) trifluoroacetic acid, freezed at –80
357 °C for 15 min, lyophilized and sent to the Vienna Metabolomics Center (University of Vienna) for
358 metaproteomic analysis. Peptides were resuspended in 1 % (final concentration) trifluoroacetic
359 acid (TFA), desalted using the Pierce C18 Tips (Thermo Fisher Scientific, USA) according to the
360 manufacturer's instructions and sequenced on a Q Exactive Hybrid Quadrupole-Orbitrap Mass
361 Spectrometer (Thermo Fisher Scientific, USA). Obtained MS/MS spectra were searched against a
362 protein database composed of combined sequenced metagenomes using SEQUEST-HT engines
363 and validated with Percolator in Proteome Discoverer 2.1 (Thermo Fisher Scientific, USA). The
364 target-decoy approach was used to reduce the probability of false peptide identification. Results
365 whose false discovery rate at the peptide level was <1 % were kept. For protein identification a
366 minimum of two peptides and one unique peptide were required. For protein quantification, a
367 chromatographic peak area-based free quantitative method was applied.

368 **Confocal microscopy**

369 Host leaves and thalli from DNA and protein isolation steps were washed seven times in
370 deionized water and fixed with formaldehyde (final concentration ~ 3 %). In addition, nontreated

³⁷¹ leaves and thalli, washed three times in ASW to remove loosely attached microbial cells, were fixed
³⁷² in the same concentration of formaldehyde and used as a positive control. For long therm storage,
³⁷³ fixed leaves and thalli were immersed in a mixture of phosphate-buffered saline (PBS) and ethanol
³⁷⁴ (1:1) and stored at –20 °C. Treated and untreated segemnts of leaves and thalli were stained in a 2
³⁷⁵ × solution of SYBR Green I and examined under a Leica TCS SP8 X FLIM confocal microscope
³⁷⁶ (Leica Microsystems, Germany).

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545 **Figure Captions**

546 **Fig. 1.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of
547 Saline and the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 4 December 2017
548 and stained with SYBR Green I. Scale bar in all images is 60 µm.

549 **Fig. 2.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of
550 Funtana (Mixed and Monospecific Settlements) sampled on 19 June 2018 and stained with SYBR
551 Green I. Scale bar in all images is 60 µm.

552 **Fig. 3.** Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial
553 and archaeal sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*)
554 sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in
555 two contrasting seasons (4 December 2017 and 19 June 2018).

556 **Fig. 4.** Taxonomic classification and relative contribution of chloroplast sequences from surfaces
557 of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the
558 Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting seasons (4 December
559 2017 and 19 June 2018).

560 **Figures**

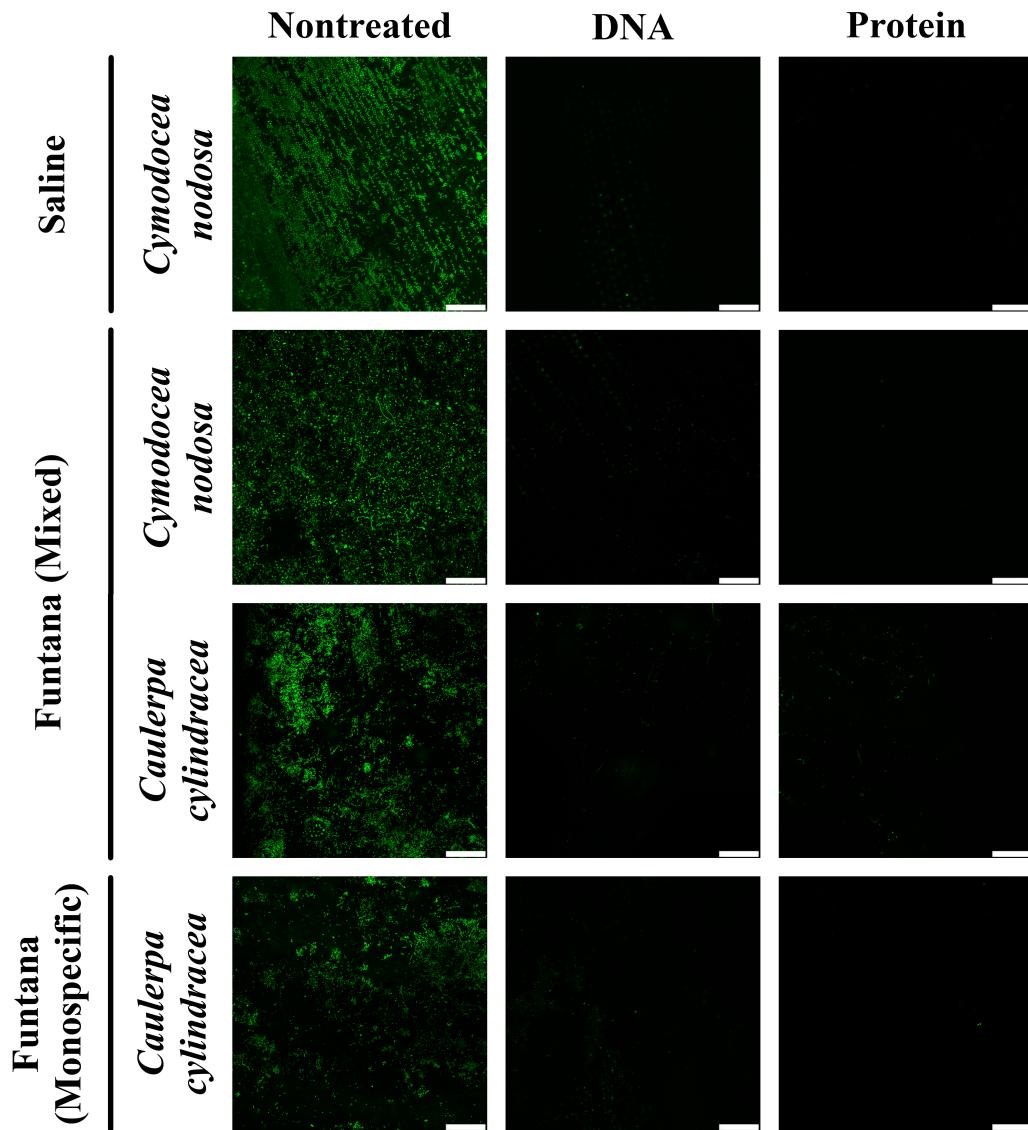


Fig. 1. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 4 December 2017 and stained with SYBR Green I. Scale bar in all images is 60 µm.

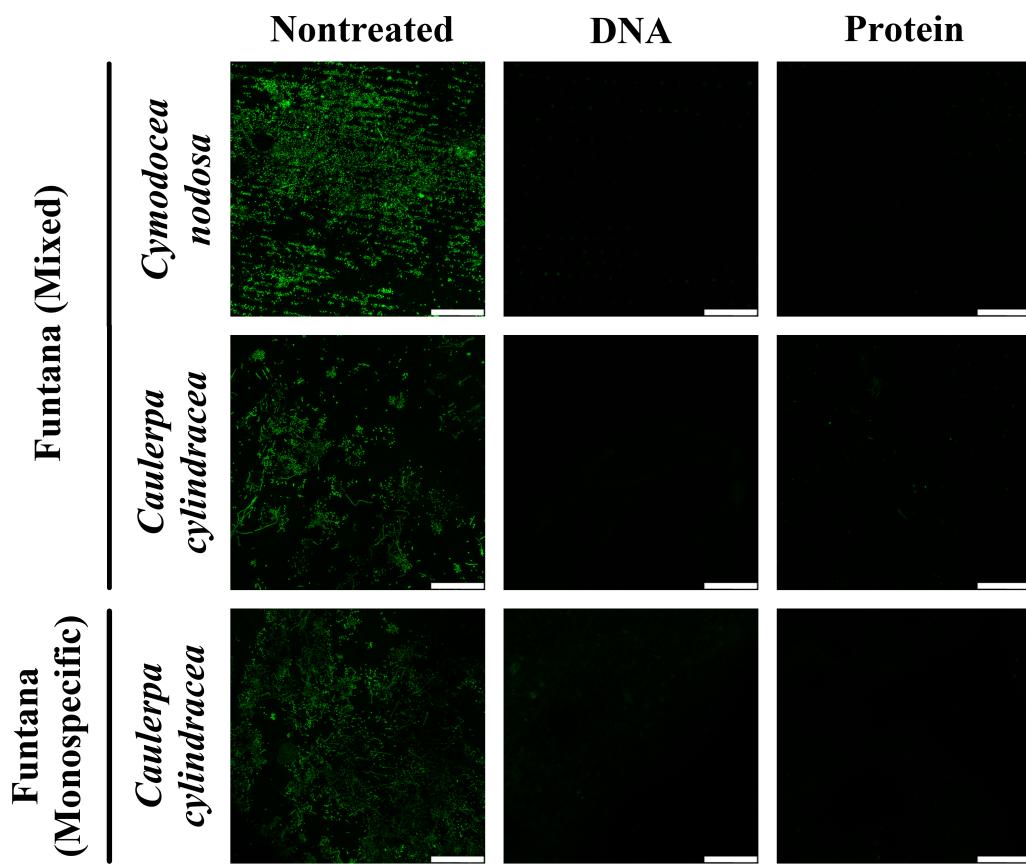


Fig. 2. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 19 June 2018 and stained with SYBR Green I. Scale bar in all images is 60 μm .

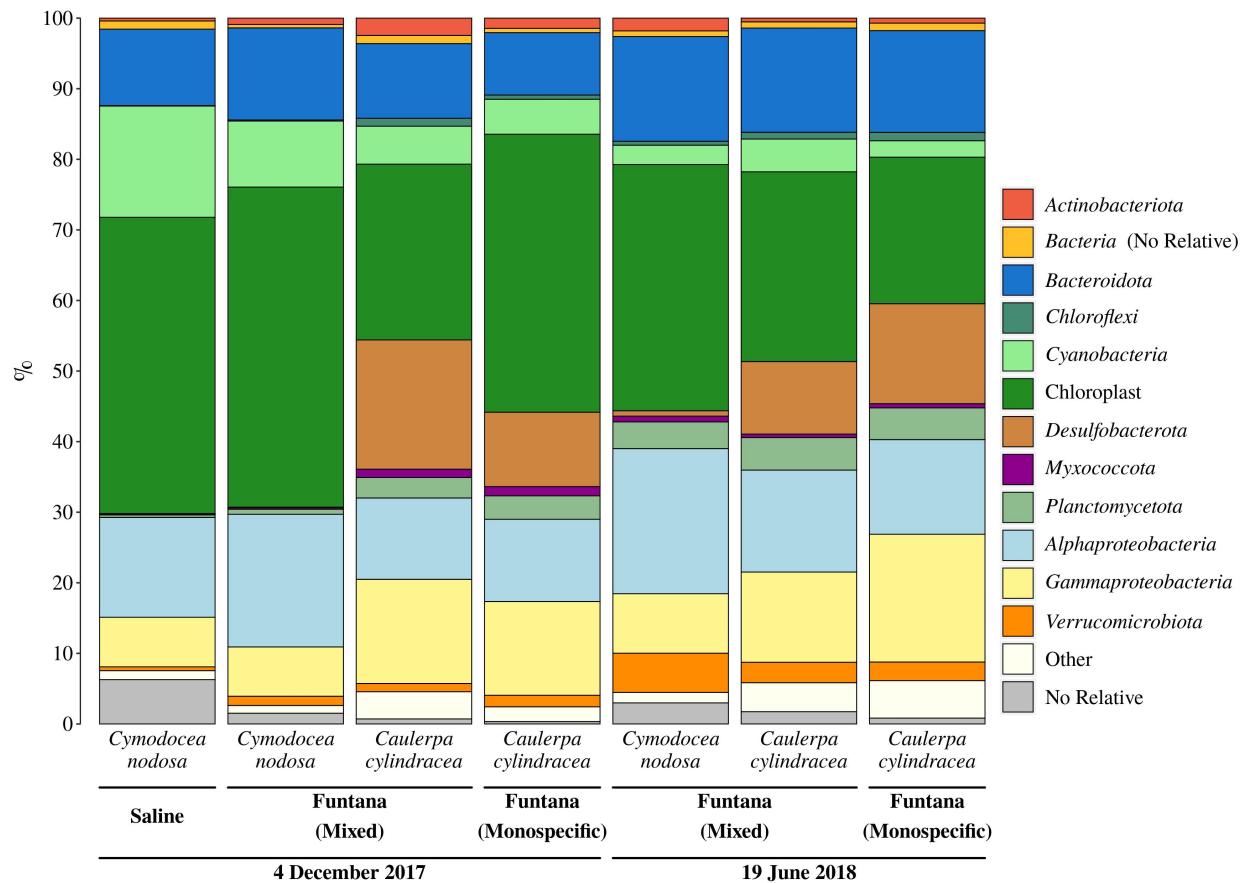


Fig. 3. Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial and archaeal sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting seasons (4 December 2017 and 19 June 2018).

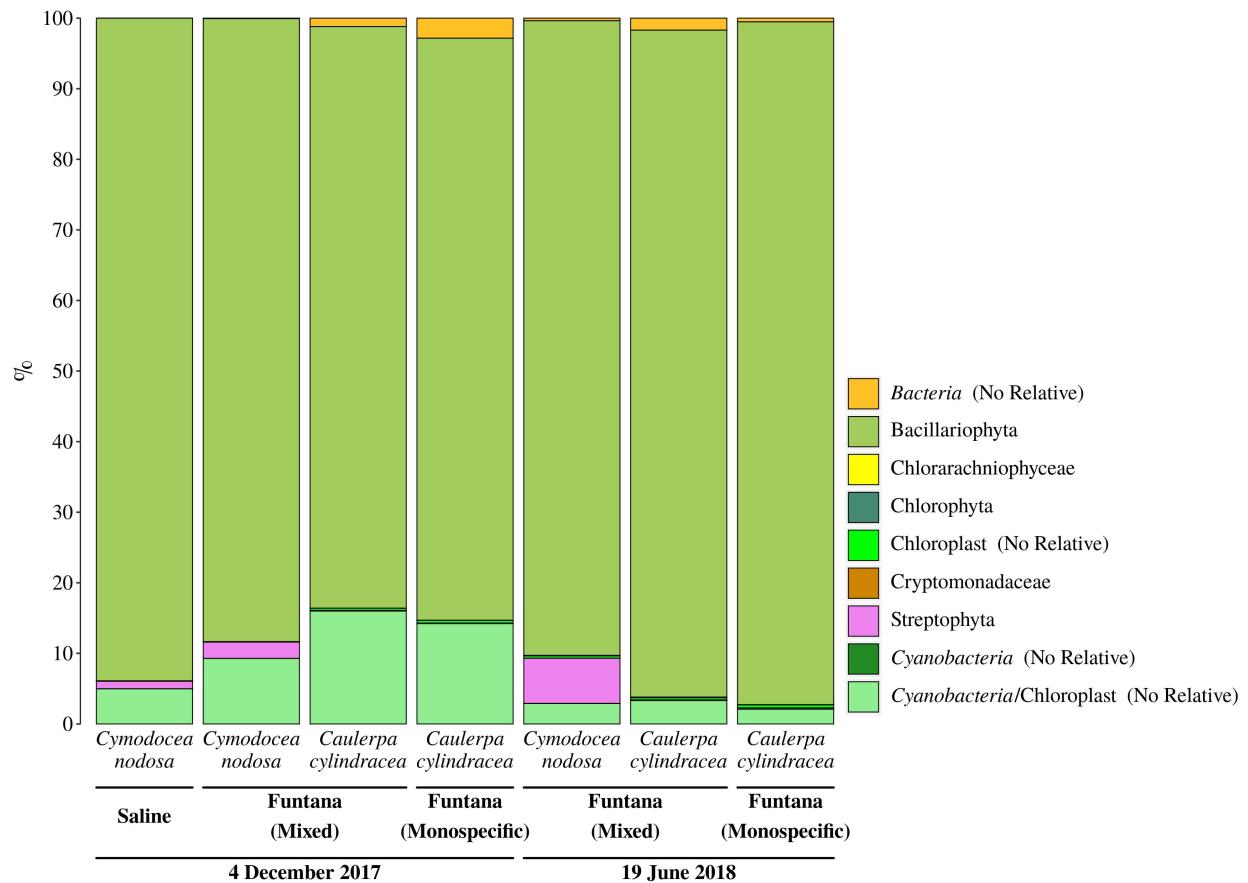


Fig. 4. Taxonomic classification and relative contribution of chloroplast sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting seasons (4 December 2017 and 19 June 2018).

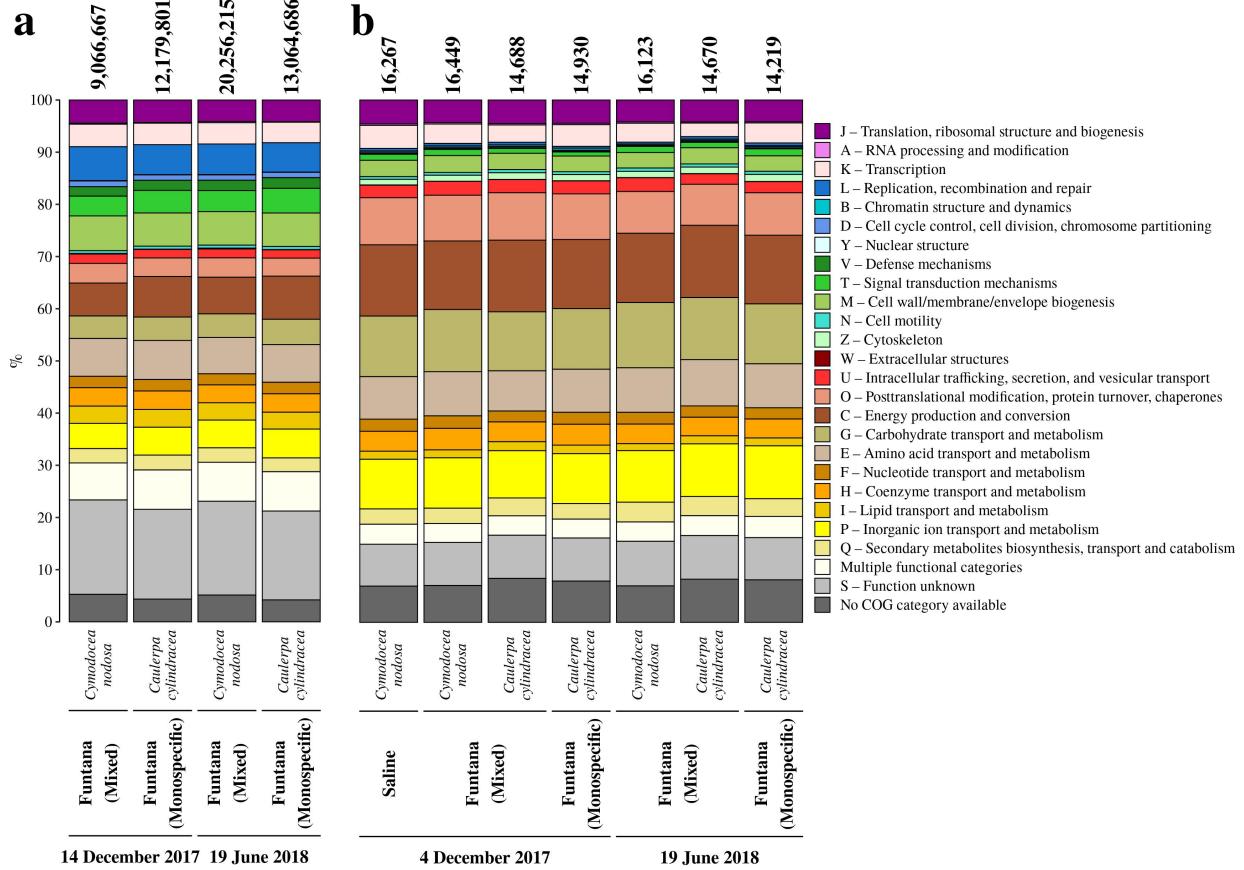


Fig. 5. Relative contribution of each COG category to the total number of annotated coding sequences (A) from metagenomes and identified proteins (B) from metaproteomes associated with surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting seasons (4/14 December 2017 and 19 June 2018). Total number of annotated coding sequences and identified proteins is given above the corresponding bar.