

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 such as 16S rRNA sequencing,
3 metagenomics and metaproteomics. To apply these techniques to the microbial community
4 inhabiting the surfaces of marine macrophytes it is advisable to perform a selective DNA and
5 protein isolation prior to the analysis to avoid biases due to the host material being present in high
6 quantities. Two protocols for DNA and protein isolation were adapted for selective extractions of
7 DNA and proteins from epiphytic communities inhabiting the surfaces of two marine macrophytes,
8 the seagrass *Cymodocea nodosa* and the macroalga *Caulerpa cylindracea*. Protocols showed an
9 almost complete removal of the epiphytic community regardless of the sampling season, station,
10 settlement or host species. The obtained DNA was suitable for metagenomic and 16S rRNA
11 sequencing, while isolated proteins could be identified by mass spectrometry. Low presence of host
12 DNA and proteins in the samples indicated a high specificity of the protocols. The procedures are
13 based on universally available laboratory chemicals making the protocols widely applicable. Taken
14 together, the adapted protocols ensure an almost complete removal of the macrophyte epiphytic
15 community. The procedures are selective for microbes inhabiting macrophyte surfaces and provide
16 DNA and proteins applicable in 16S rRNA sequencing, metagenomics and metaproteomics.

17 **Introduction**

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

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

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

46 An alternative to these procedures is a direct isolation of the target material by incubating
47 macrophyte tissues in an extraction buffer. After the incubation, the undisrupted host tissue is
48 removed followed by the isolation procedure, omitting host material contaminations. To our
49 knowledge, the only procedure describing a direct and selective epiphytic DNA isolation from the
50 surfaces of marine macrophytes was described by Burke et al. (2009). In contrast to previously
51 described methods, this protocol enables an almost complete removal of the surface community.
52 It was used for 16S rRNA gene clone library construction (Burke et al., 2011b) and metagenome
53 sequencing (Burke et al., 2011a). This method, although providing a selective isolation procedure,
54 uses a rapid multi-enzyme cleaner (3M) that is not available worldwide and the chemical constituents
55 are unknown (Burke et al., 2009). Also to our knowledge, no selective isolation protocol to perform
56 (meta)proteomics of epiphytic communities associated with marine macrophytes has been developed
57 yet.

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

65 **Materials and methods**

66 **Sampling**

67 Leaves of *C. nodosa* were sampled in a *C. nodosa* meadow in the Bay of Saline, northern
68 Adriatic Sea ($45^{\circ}7'5''$ N, $13^{\circ}37'20''$ E) and in a *C. nodosa* meadow invaded by *C. cylindracea* in
69 the Bay of Funtana, northern Adriatic Sea ($45^{\circ}10'39''$ N, $13^{\circ}35'42''$ E). Thalli of *C. cylindracea*
70 were sampled in the same *C. nodosa* invaded meadow in the Bay of Funtana and at a locality of
71 only *C. cylindracea* located in the proximity of the invaded meadow. Leaves and thalli for 16S
72 rRNA analysis, metagenomics and metaproteomics were collected in two contrasting seasons, on 4
73 December 2017 (16S rRNA analysis and metaproteomics), 14 December 2017 (metagenomics) and
74 18 June 2018 (16S rRNA analysis, metagenomics and metaproteomics). During spring 2018, the *C.*
75 *nodosa* meadow in the Bay of Saline decayed to an extent that no leaves could be retrieved (Najdek
76 et al., 2020). In addition, as not enough DNA for both metagenomic and 16S RNA analysis were
77 obtained during the sampling on 4 December 2017, an additional sampling on 14 December 2017
78 was carried out in the Bay of Funtana. Leaves and thalli were collected by diving and transported to
79 the laboratory in containers placed on ice and filled with seawater from this site. Upon arrival to the
80 laboratory, *C. nodosa* leaves were cut into sections of 1 – 2 cm, while *C. cylindracea* thalli were
81 cut into 5 – 8 cm long sections. Leaves and thalli were washed three times with sterile artificial
82 seawater (ASW) to remove loosely attached microbial cells.

83 **DNA isolation**

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

88 duplicate isolations were performed. Lysozyme was added (final concentration 1 mg ml⁻¹) and the
89 mixture was incubated at 37 °C for 30 min. Subsequently, proteinase K (final concentration 0.5
90 mg ml⁻¹) and SDS (final concentration 1 %) were added and the samples were incubated at 55 °C
91 for 2 h. Following the incubation, tubes were vortexed for 10 min and the mixture containing lysed
92 epiphytic cells was separated from host leaves or thalli by transferring the solution into a clean tube.
93 The lysate was extracted twice with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1; pH 8)
94 and once with chloroform:isoamyl alcohol (24:1). After each addition of an organic solvent mixture,
95 tubes were slightly vortexed and centrifuged at 4,500 × g for 10 min. Following each centrifugation
96 the aqueous phases were retrieved. After the final extraction 1/10 of chilled 3 M sodium acetate
97 (pH 5.2) was added. DNA was precipitated by adding 1 volume of chilled isopropanol, incubating
98 the mixtures overnight at -20 °C and centrifuging at 16,000 × g and 4 °C for 20 min. The pellet
99 was washed twice with 1 ml of chilled 70 % ethanol and centrifuged after each washing step at
100 20,000 × g and 4 °C for 10 min. After the first washing step duplicate pellets from the same sample
101 were pooled and transferred to a clean 1.5 ml tube. The dried pellet was re-suspended in 100 µl of
102 deionized water.

103 Illumina 16S rRNA sequencing

104 An aliquot of isolated DNA was treated with RNase A (final concentration 200 µg ml⁻¹)
105 for 2 h at 37 °C. The DNA concentration was determined using the Quant-iT PicoGreen
106 dsDNA Assay Kit (Invitrogen, USA) according to the manufacturer's instructions and
107 diluted to 1 ng µl⁻¹. The V4 region of the 16S rRNA gene was amplified using a two-step
108 PCR procedure. In the first PCR the 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and
109 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers from the Earth Microbiome Project
110 (<http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>) were used to amplify
111 the target region (Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016). These primers
112 contained on their 5' end a tagged sequence. Each sample was amplified in four parallel 25 µl

reactions of which each contained 1 × Q5 Reaction Buffer, 0.2 mM of dNTPmix, 0.7 mg ml⁻¹ BSA (Bovine Serum Albumin), 0.2 µM of forward and reverse primers, 0.5 U of Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) and 5 ng of DNA template. Cycling conditions were: initial denaturation at 94 °C for 3 min, 20 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s and elongation at 72 °C for 90 s, finalized by an elongation step at 72 °C for 10 min. The four parallel reactions volumes were pooled and PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions and following the protocol that included isopropanol addition for better small DNA fragment yield. The column was eluted in 30 µl of deionized water. Purified PCR products were sent for Illumina MiSeq sequencing (2 × 250 bp) at IMGM Laboratories, Martinsried, Germany. Before sequencing at IMGM, the second PCR amplification of the two-step PCR procedure was performed using primers targeting the tagged region incorporated in the first PCR. In addition, these primers contained adapter and sample-specific index sequences. The second PCR was carried out for 8 cycles. Beside samples, a positive and negative control were sequenced. A negative control was comprised of four parallel PCR reactions without DNA template, while for a positive control a mock community composed of evenly mixed DNA material originating from 20 bacterial strains (ATCC MSA-1002, ATCC, USA) was used. Partial 16S rRNA sequences obtained in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession numbers SAMEA6786270, SAMEA6648792 – SAMEA6648794, SAMEA6648809 – SAMEA6648811 and SAMEA6648824.

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

¹⁴⁰ mothur and the RDP (<http://rdp.cme.msu.edu/>) training set (version 16) reference files adapted for
¹⁴¹ mothur (Cole et al., 2014). In comparison to SILVA, RDP allows a more detailed classification of
¹⁴² chloroplast sequences. Based on the ATCC MSA-1002 mock community included in the analysis a
¹⁴³ sequencing error rate of 0.009 % was determined, which is in line with previously reported values
¹⁴⁴ for next-generation sequencing data (Kozich et al., 2013; Schloss et al., 2016). In addition, the
¹⁴⁵ negative control processed together with the samples yielded only 2 sequences after sequence
¹⁴⁶ quality curation.

¹⁴⁷ **Metagenomics**

¹⁴⁸ Four DNA samples were selected and sent on dry ice to IMGM Laboratories, Martinsried,
¹⁴⁹ Germany for metagenomic sequencing. DNA was purified using AMPure XP Beads (Beckman
¹⁵⁰ Coulter, USA) applying a bead:DNA ratio of 1:1 (v/v), quantified with a Qubit dsDNA HS Assay
¹⁵¹ Kit (Thermo Fisher Scientific, USA) and check for integrity on a 1 % agarose gel. Metagenomic
¹⁵² sequencing libraries were generated from 300 ng of input DNA using a NEBNext Ultra II FS
¹⁵³ DNA Library Prep Kit for Illumina (New England Biolabs, USA) according to the manufacturer's
¹⁵⁴ instructions. Fragments were selected (500 – 700 bp) using AMPure XP Beads, PCR enriched for 3
¹⁵⁵ – 5 cycles and quality controlled. Libraries generated from different DNA samples were pooled and
¹⁵⁶ sequenced on an Illumina NovaSeq 6000 sequencing system (2 × 250 bp).

¹⁵⁷ Obtained sequences were analysed on the Life Science Compute Cluster (LiSC) (CUBE –
¹⁵⁸ Computational Systems Biology, University of Vienna). Individual sequences were assembled using
¹⁵⁹ MEGAHIT (version 1.1.2) (Li et al., 2015) under default settings. Putative genes were predicted
¹⁶⁰ from contigs longer than 200 bp using Prodigal (version 2.6.3) (Hyatt et al., 2010) in metagenome
¹⁶¹ mode (-p meta). Abundances of predicted genes were expressed as Reads Per Kilobase Million
¹⁶² (RPKM) and calculated using the BWA algorithm (version 0.7.16a) (Li and Durbin, 2009). All
¹⁶³ predicted genes were functionally annotated using the eggNOG-mapper (Huerta-Cepas et al., 2017)
¹⁶⁴ and eggNOG database (version 5.0) (Huerta-Cepas et al., 2019). Sequence taxonomy classification

165 was determined using the lowest common ancestor algorithm adapted from DIAMOND (version
166 0.8.36) (Buchfink et al., 2015) and by searching against the NCBI non-redundant database (NR). To
167 determine the phylogeny, the top 10 % hits with an e-value $< 1 \times 10^{-5}$ were used (--top 10). Sequence
168 renaming, coverage information computing and metagenomic statistics calculations were performed
169 using software tools from BBTools (<https://jgi.doe.gov/data-and-tools/bbtools>). Metagenomic
170 sequences obtained in this study have been deposited in the European Nucleotide Archive (ENA) at
171 EMBL-EBI under accession numbers SAMEA6648795, SAMEA6648797, SAMEA6648809 and
172 SAMEA6648811.

173 **Protein isolation**

174 Proteins were isolated according to the protocol for protein isolation from soil described in
175 Chourey et al. (2010) and modified by Hultman et al. (2015). This protocol was further modified
176 and adapted for microbial protein isolation from macrophyte surfaces as described below. Twenty
177 (20) ml of protein extraction buffer (4 % SDS, 100 mm Tris-HCl [pH 8.0]) was added to 5 g wet
178 weight of leaves or 10 g wet weight of thalli. The mixture was incubated in boiling water for 5
179 min, vortexed for 10 min and incubated again in boiling water for 5 min. After a brief vortex, the
180 lysate was transferred to a clean tube separating the host leaves or thalli from the mixture containing
181 lysed epiphytic cells. Dithiothreitol (DTT; final concentration 24 mm) was added and proteins were
182 precipitated with chilled 100 % trichloroacetic acid (TCA; final concentration 20 %) overnight at
183 -20°C . Precipitated proteins were centrifuged at $10,000 \times g$ and 4°C for 40 min. The obtained
184 protein pellet was washed three times with chilled acetone. During the first washing step the pellet
185 was transferred to a clean 1.5 ml tube. After each washing step, samples were centrifuged at $20,000$
186 $\times g$ and 4°C for 5 min. Dried pellets were stored at -80°C until further analysis.

187 **Metaproteomics**

188 Isolated proteins were whole trypsin digested using the FASP (filter-aided sample preparation)
189 Protein Digestion Kit (Expedeon, UK) according to the manufacturer's instructions with small
190 modifications (Wiśniewski et al., 2009). Prior to loading the solution onto the column, protein
191 pellets were solubilized in a urea sample buffer included in the kit amended with DTT (final
192 concentration 100 mM) for 45 min at room temperature and centrifuged at 20,000 × g for 2 – 5
193 min at room temperature to remove larger particles. The first washing step after protein solution
194 loading was repeated twice. In addition, the centrifugation steps were prolonged if the column
195 was clogged. Trypsin digestion was performed on column filters at 37 °C overnight for 18 h.
196 The final filtrate containing peptides was acidified with 1 % (final concentration) trifluoroacetic
197 acid (TFA), freezed at –80 °C, lyophilized and sent to VIME – Vienna Metabolomics Center
198 (University of Vienna) for metaproteomic analysis. Peptides were re-suspended in 1 % (final
199 concentration) TFA, desalting using the Pierce C18 Tips (Thermo Fisher Scientific, USA) according
200 to the manufacturer's instructions and sequenced on a Q Exactive Hybrid Quadrupole-Orbitrap
201 Mass Spectrometer (Thermo Fisher Scientific, USA). Obtained MS/MS spectra were searched
202 against a protein database composed of combined sequenced metagenomes using SEQUEST-HT
203 engines and validated with Percolator in Proteome Discoverer 2.1 (Thermo Fisher Scientific, USA).
204 The target-decoy approach was used to reduce the probability of false peptide identification. Results
205 whose false discovery rate at the peptide level was < 1 % were kept. For protein identification
206 a minimum of two peptides and one unique peptide were required. For protein quantification, a
207 chromatographic peak area-based free quantitative method was applied.

208 **Data processing and visualization**

209 Processing and visualization of 16S rRNA, metagenomic and metaproteomic data were done
210 using R (version 3.6.0) (R Core Team, 2019), package tidyverse (version 1.3.0) (Wickham et

211 al., 2019) and multiple other packages (Neuwirth, 2014; Xie, 2014, 2015, 2019, 2020; Wilke,
212 2018; Xie et al., 2018; Allaire et al., 2019; Zhu, 2019; Bengtsson, 2020). The detailed analysis
213 procedure including the R Markdown file for this paper are available as a GitHub repository
214 (https://github.com/MicrobesRovinj/Korlevic_SelectiveRemoval_FrontMicrobiol_2021).

215 **Confocal microscopy**

216 Host leaves and thalli from DNA and protein isolation steps were washed seven times in
217 deionized water and fixed with formaldehyde (final concentration ~3 %). In addition, non-treated
218 leaves and thalli, washed three times in ASW to remove loosely attached microbial cells, were fixed
219 in the same concentration of formaldehyde and used as a positive control. For long term storage,
220 fixed leaves and thalli were immersed in a mixture of phosphate-buffered saline (PBS) and ethanol
221 (1:1) and stored at -20 °C. Treated and untreated segments of leaves and thalli were stained in a 2
222 × solution of SYBR Green I and examined under a Leica TCS SP8 X FLIM confocal microscope
223 (Leica Microsystems, Germany).

224 **Results**

225 To assess the removal efficiency of the DNA and protein isolation procedures, leaves and thalli
226 were examined under a confocal microscope before and after treatments were performed. The
227 modified procedures resulted in an almost complete removal of the surface community of both, *C.*
228 *nodosa* and *C. cylindracea*. In addition, a similar removal efficiency was observed for communities
229 sampled in contrasting months, December 2017 (Fig. 1) and June 2018 (Fig. 2). Also, no effect of
230 station, settlement or isolation procedure (DNA or protein) on the removal efficiency was observed
231 (Figs. 1 and 2).

232 To evaluate whether the obtained DNA is suitable to determine the composition of the microbial
233 community Illumina sequencing of the V4 region of the 16S rRNA was performed. Sequencing
234 yielded a total of 336,944 sequences after quality curation and exclusion of eukaryotic, mitochondrial
235 and no relative sequences. The number of sequences classified as chloroplasts was 97,334. After
236 excluding these sequences, the total number of retrieved reads was 239,610, ranging from 22,596
237 to 52,930 sequences per sample (Table S1). Even when the highest sequencing effort was applied,
238 the rarefaction curves did not level off which is commonly observed in high-throughput 16S rRNA
239 amplicon sequencing (Fig. S1). Sequences clustering at a similarity level of 97 % yielded a total of
240 8,355 different OTUs. Taxonomic classification of reads revealed a macrophyte-associated epiphytic
241 community that mainly composed of *Alphaproteobacteria* ($14.9 \pm 3.5\%$), *Bacteroidota* ($12.5 \pm 2.4\%$),
242 *Gammaproteobacteria* ($11.6 \pm 4.3\%$), *Desulfobacterota* ($7.8 \pm 7.5\%$), *Cyanobacteria* ($6.5 \pm$
243 4.7%) and *Planctomycetota* ($2.9 \pm 1.7\%$) (Fig. 3).

244 Primers used in this study, as in many other 16S rRNA amplicon procedures, also amplified
245 chloroplast 16S rRNA genes. We observed a high proportion of chloroplast sequences in all analysed
246 samples ($33.4 \pm 9.4\%$) (Fig. 3). To determine whether chloroplast sequences originate from
247 the host or eukaryotic epiphytic organisms, we exported SILVA-classified chloroplast sequences
248 and reclassified them using the RDP (Ribosomal Database Project) training set that allows for

249 a more detailed chloroplast classification. The largest proportion of sequences was classified as
250 Bacillariophyta ($89.7 \pm 5.7\%$) indicating that the DNA removal procedure resulted in only minor
251 co-extracted quantities of host DNA (Fig. 4). Chloroplast sequences classified as Streptophyta
252 constituted $3.3 \pm 2.8\%$ of all chloroplast sequences originating from *C. nodosa* samples, while
253 sequences classified as Chlorophyta comprised only $0.02 \pm 0.01\%$ of all chloroplast sequences
254 associated with *C. cylindracea* samples.

255 To determine whether the extracted DNA can be used for metagenomic sequencing, four
256 samples containing epiphytic DNA were selected and shotgun sequenced using an Illumina platform.
257 Metagenomic sequencing yielded between 207,149,524 and 624,029,930 sequence pairs (Table S2).
258 Obtained sequences were successfully assembled into contigs whose L50 ranged from 654 to 1,011
259 bp. In addition, predicted coding sequences were functionally annotated (9,066,667 – 20,256,215
260 annotated sequences; Fig. 5a) and taxonomically classified. Functional annotation allowed for an
261 assessment of the relative contribution of each COG (Clusters of Orthologous Groups) functional
262 category to the total number of annotated coding sequences (Fig. 5a). Functional categories
263 containing the highest number of sequences were C (Energy production and conversion), E (Amino
264 acid transport and metabolism), M (Cell wall/membrane/envelope biogenesis), L (Replication,
265 recombination and repair) and P (Inorganic ion transport and metabolism). If host DNA is
266 co-extracted with epiphytes it should be detected in large proportions in sequenced metagenomes.
267 However, no large proportions of coding sequences classified as Streptophyta and Chlorophyta
268 were detected (Table S3). Sequenced metagenomic DNA originating from the surface of *C. nodosa*
269 contained 1.3 % of coding sequences classified as Streptophyta in December 2017 and 0.7 % in June
270 2018. Furthermore, the summed RPKM (Reads Per Kilobase Million) of these sequences constituted
271 1.7 % of total RPKM of all successfully classified sequences in December 2017 and 1.1 % in June
272 2018. Similar low proportions of host coding sequences were detected in metagenomic samples
273 originating from the surfaces of *C. cylindracea*. Of all successfully classified coding sequences 0.2
274 % were classified as Chlorophyta in December 2017 and 0.1 % in June 2018. A relatively higher
275 proportion of RPKM of these sequences than in the case of *C. nodosa* was observed, indicating

²⁷⁶ a higher co-extraction of host DNA in *C. cylindracea*. In December, the proportion of RPKM of
²⁷⁷ sequences classified as Chlorophyta was 8.2 %, while in June 2018 it reached 13.6 %.

²⁷⁸ To evaluate whether the procedure for protein extraction is suitable for metaproteomic analysis,
²⁷⁹ obtained proteins were trypsin digested and sequenced using a mass spectrometer. Obtained
²⁸⁰ MS/MS spectra were searched against a protein database from sequenced metagenomes. From
²⁸¹ 14,219 to 16,449 proteins were identified in isolated protein samples (Fig. 5b). In addition,
²⁸² successful identification of proteins allowed for an assessment of the relative contribution of
²⁸³ each COG functional category to the total number of identified proteins (Fig. 5b). Functional
²⁸⁴ categories containing the highest number of identified proteins were C (Energy production and
²⁸⁵ conversion), G (Carbohydrate transport and metabolism), P (Inorganic ion transport and metabolism),
²⁸⁶ O (Posttranslational modification, protein turnover, chaperones) and E (Amino acid transport and
²⁸⁷ metabolism). Isolated proteins could originate from epiphytic organisms inhabiting the macrophyte
²⁸⁸ surface and/or from macrophyte tissue underlying them. The contribution of proteins originating
²⁸⁹ from host tissues was evaluated by identifying all the proteins predicted to belong to any taxonomic
²⁹⁰ group within the phyla Streptophyta and Chlorophyta and by calculating their contribution to the
²⁹¹ number and abundance (NAAF – Normalized Abundance Area Factor) of all identified proteins.
²⁹² On average, proteins isolated from the surface of *C. nodosa* contained 1.8 ± 0.06 % of proteins
²⁹³ associated with Streptophyta, contributing to 2.2 ± 0.8 % of total proteins. Similar to metagenomes,
²⁹⁴ proteins associated with Chlorophyta contributed more to *C. cylindracea* than proteins associated
²⁹⁵ with Streptophyta to *C. nodosa*. Chlorophyta associated proteins comprised 5.2 ± 0.06 % of all
²⁹⁶ identified proteins in *C. cylindracea*, contributing 19.2 ± 1.5 % to the total protein abundance.

297 **Discussion**

298 To test whether the developed DNA and protein isolation protocols efficiently detach microbes
299 from the macrophyte surface we selected *C. nodosa* and *C. cylindracea* as representatives of seagrass
300 and macroalgal species. These species differ morphologically. While *C. nodosa* leaves are flat, *C.*
301 *cylindracea* thalli are characterized by an uneven surface (Kuo and den Hartog, 2001; Verlaque et
302 al., 2003). The developed protocol led to an almost complete removal of epiphytic cells from the
303 surfaces of both species comparable to the result of Burke et al. (2009), indicating that structural
304 differences do not impact the removal efficiency. In addition, isolation protocols were tested in two
305 contrasting seasons, as it is known that macrophytes are harbouring more algal epiphytes during
306 autumn and winter (Reyes and Sansón, 2001). No differences in the removal efficiency was observed
307 between seasons suggesting that these protocols can be used on macrophyte samples retrieved
308 throughout the year. Also, no removal differences were observed on samples derived from the same
309 host but from different locations.

310 Successful amplification and sequencing of the V4 region of the 16S rRNA gene proved that the
311 isolated DNA can be used to estimate the microbial epiphytic diversity. Taxonomic groups detected
312 in this step can also be often found in epiphytic communities associated with other macrophytes
313 (Burke et al., 2011b; Morrissey et al., 2019). A problem often encountered in studies focusing on
314 epiphytic communities is the presence of large proportions of chloroplast 16S rRNA sequences in
315 the pool of amplified molecules, especially if the epiphytic DNA was isolated without prior selection
316 (Staufenberger et al., 2008). These sequences can derive from host chloroplasts or from eukaryotic
317 epiphytic chloroplast DNA. Although the proportion of obtained chloroplast 16S rRNA sequences
318 in our samples was substantial, they derived almost exclusively from eukaryotic epiphytes. High
319 proportion of chloroplast 16S rRNA sequences in studies applying selective procedures that include
320 direct cellular lysis on host surfaces were observed before (Michelou et al., 2013). It is possible that
321 chloroplast-specific sequences even in these studies originated from eukaryotic epiphytic cells and
322 not from host chloroplasts. Indeed, it is common during 16S rRNA profiling of pelagic microbial

323 communities to observe high proportions of chloroplast sequences (Gilbert et al., 2009; Korlević
324 et al., 2016). In addition, a very low proportion of chloroplast 16S rRNA sequences in samples
325 originating from *C. cylindracea* in comparison to *C. nodosa* could be explained by the presence of
326 three introns in the gene for 16S rRNA in some members of the genus *Caulerpa* that could hamper
327 the amplification process (Lam and Lopez-Bautista, 2016).

328 High quality DNA is also needed for metagenomics. The obtained number of metagenomic
329 sequences and assembly statistics were comparable to metagenomes and metatranscriptomes derived
330 from similar surface associated communities (Crump et al., 2018; Cúcio et al., 2018). In addition,
331 functional annotation of predicted coding sequences to COG functional categories showed that
332 the obtained metagenomes can be used to determine the metabolic capacity of surface associated
333 communities (Leary et al., 2014; Cúcio et al., 2018). The proportion of coding sequences, including
334 their RPKM, originating from *C. nodosa* metagenomes and classified as Streptophyta was low
335 indicating that the isolation procedure was specific for epiphytic cells. DNA samples isolated from
336 the surface of *C. cylindracea* exhibited a low proportion of Chlorophyta coding sequences, however,
337 their RPKM was higher than in the samples originating from *C. nodosa*. One of the reasons for this
338 elevated RPKM of Chlorophyta sequences in *C. cylindracea* could be the differences in the tissue
339 structure between these two host species. While *C. nodosa* leaves are composed of individual cells,
340 the thallus of *C. cylindracea* is, like in other siphonous algal species, composed of a single large
341 multinucleate cell (Coneva and Chitwood, 2015). The absence of individual cells in *C. cylindracea*
342 could cause a leakage of genetic material into the extraction buffer causing an elevated presence of
343 host sequences in the samples for metagenome analyses.

344 To obtain insight into the metabolic status of uncultivated prokaryotes, a metaproteomic
345 approach is required (Saito et al., 2019). The applied protocol for epiphytic protein isolation
346 followed by a metaproteomic analysis identified between 14,219 and 16,449 proteins, which is
347 higher than previously reported for e.g. soils (Chourey et al., 2010; Hultman et al., 2015), seawater
348 (Williams et al., 2012) and biofilms (Leary et al., 2014). The functional annotation of identified

349 proteins into COG functional categories showed that the protein isolation protocol can be used to
350 assess the metabolic status of the epiphytic community (Leary et al., 2014). Similar to the results of
351 the metagenomic analysis, the number and abundance of identified proteins affiliated to Streptophyta
352 in *C. nodosa* samples were low, indicating that the procedure is selective for epiphytic cell proteins.
353 In addition, a higher number and abundance of identified proteins associated with Chlorophyta were
354 observed in *C. cylindracea* samples. The cause of this elevated presence of Chlorophyta-associated
355 proteins can be, similar to the DNA isolation protocol, explained by the absence of individual cells
356 in this siphonous alga (Coneva and Chitwood, 2015).

357 In conclusion, the developed protocols for DNA and protein isolation from macrophyte surfaces
358 almost completely remove the epiphytic community from both, *C. nodosa* and *C. cylindracea*, in
359 different seasons. Also, the obtained DNA and proteins are suitable for 16S rRNA sequencing,
360 metagenomics and metaproteomics analyses while the obtained material contains low quantities of
361 host DNA and proteins making the protocols specific for epiphytes. Furthermore, the protocols are
362 based on universally available laboratory chemicals hence, making them widely applicable.

363 Acknowledgements

364 This work was funded by the Croatian Science Foundation through the MICRO-SEAGRASS
365 project (project number IP-2016-06-7118). ZZ and GJH were supported by the Austrian Science
366 Fund (FWF) project ARTEMIS (project number P28781-B21). We would like to thank Margareta
367 Buterer for technical support, Paolo Paliaga for help during sampling, Lucija Horvat for technical
368 support in confocal microscopy, Dušica Vujaklija for help in accessing the confocal microscope
369 and Sonja Tischler for technical support in mass spectrometry. In addition, we would like to thank
370 the University Computing Center of the University of Zagreb for access to the computer cluster
371 Isabella and CUBE – Computational Systems Biology of the University of Vienna for access to the
372 Life Science Compute Cluster (LiSC).

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544 **Figure legends**

545 **Fig. 1.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of
546 Saline and the Bay of Funtana (mixed and monospecific settlements) sampled on 4 December 2017
547 and stained with SYBR Green I. Scale bar at all images is 60 µm.

548 **Fig. 2.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of
549 Funtana (mixed and monospecific settlements) sampled on 19 June 2018 and stained with SYBR
550 Green I. Scale bar at all images is 60 µm.

551 **Fig. 3.** Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) bacterial
552 and archaeal sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*)
553 sampled in the Bay of Saline and the Bay of Funtana (mixed and monospecific settlements) and in
554 two contrasting seasons (4 December 2017 and 19 June 2018).

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

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

565 **Figures**

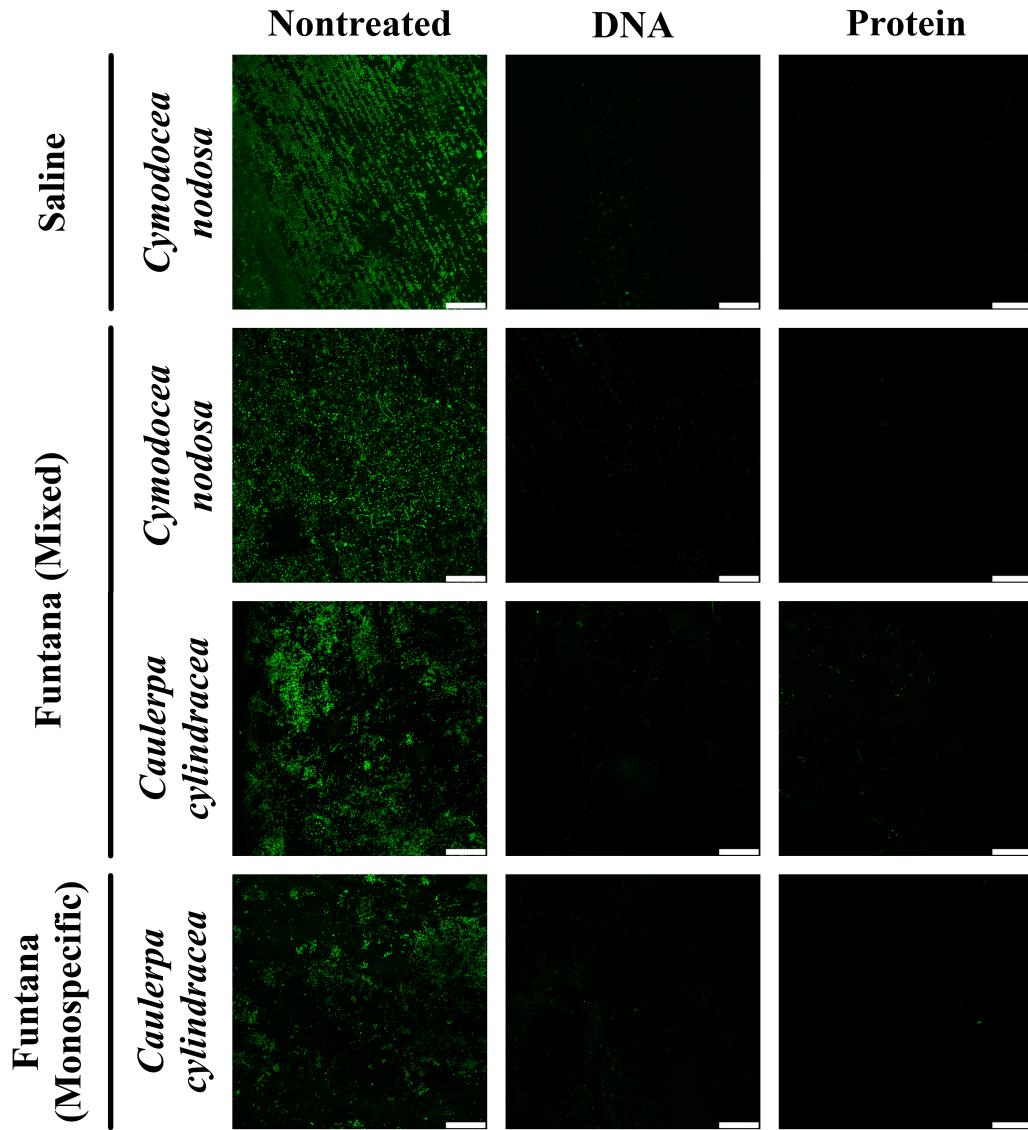


Fig. 1. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Saline and the Bay of Funtana (mixed and monospecific settlements) sampled on 4 December 2017 and stained with SYBR Green I. Scale bar at all images is 60 µm.

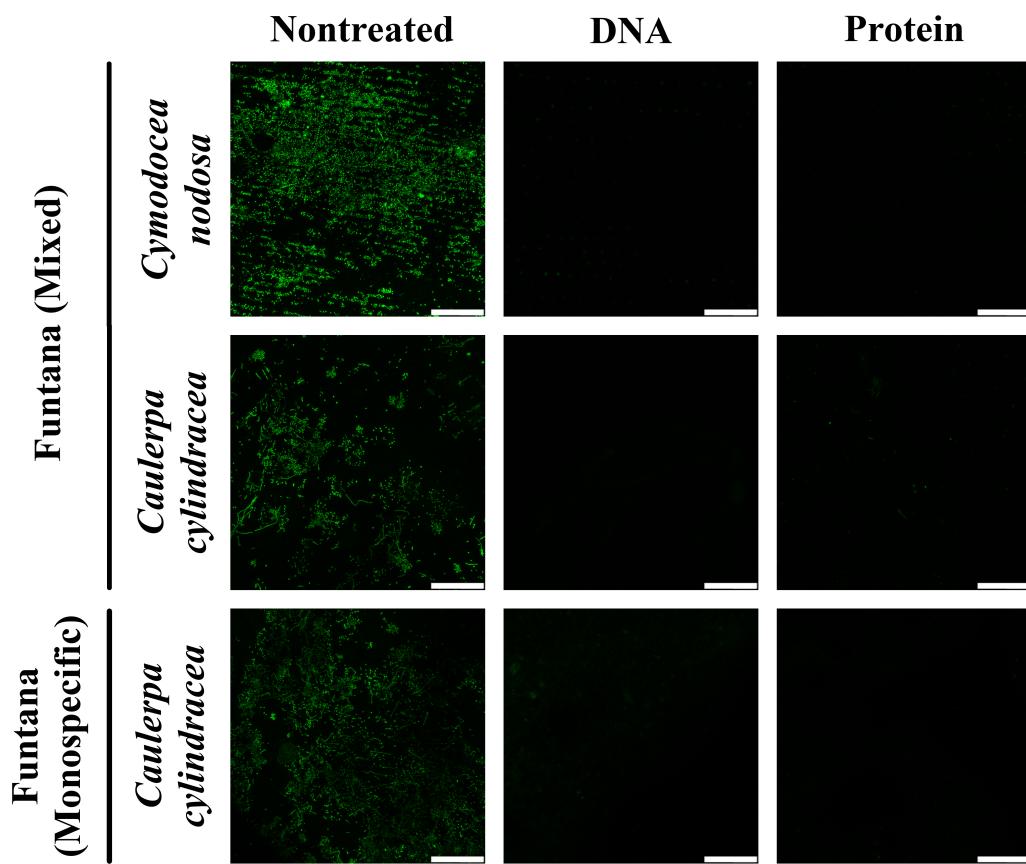


Fig. 2. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Funtana (mixed and monospecific settlements) sampled on 19 June 2018 and stained with SYBR Green I. Scale bar at all images is 60 μ m.

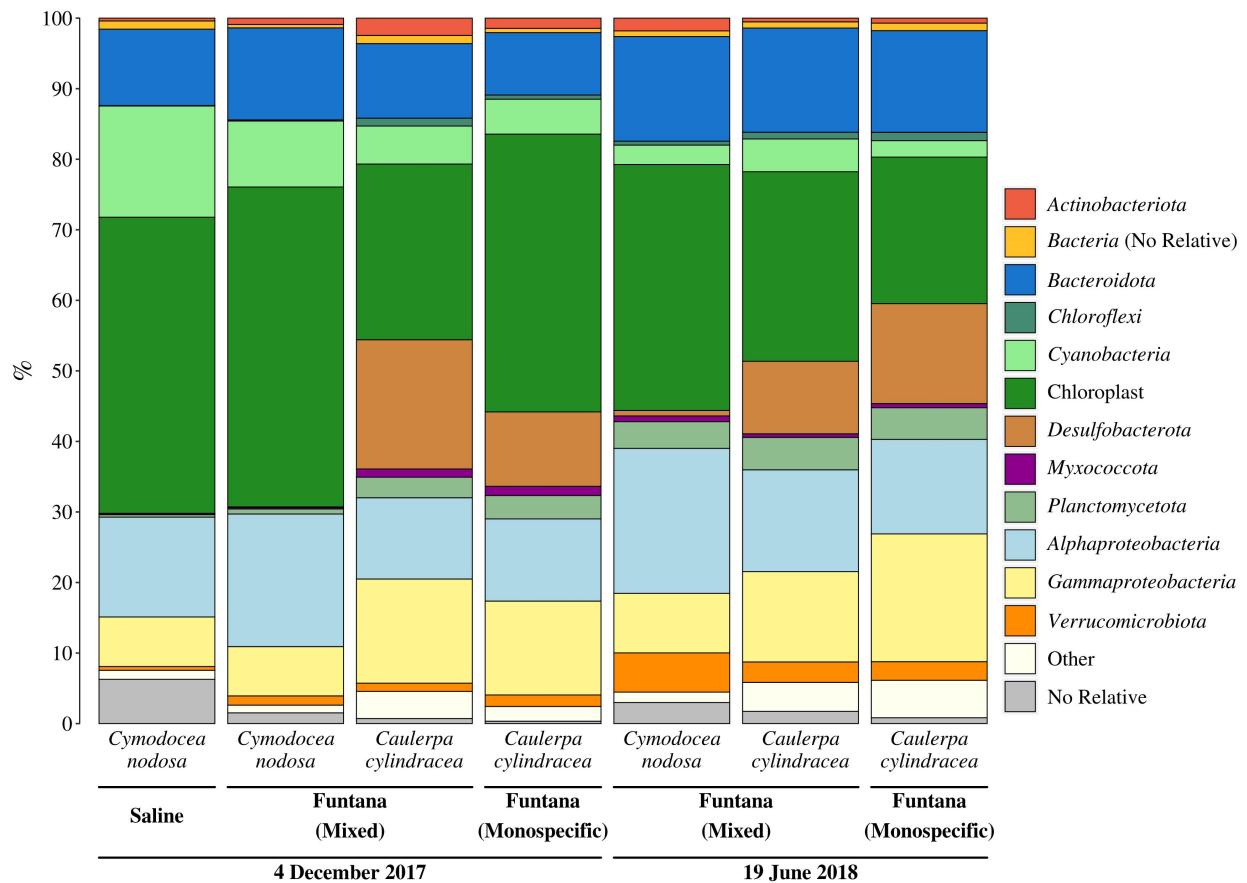


Fig. 3. Taxonomic classification and relative contribution of the most abundant ($\geq 1\%$) bacterial and archaeal sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (mixed and monospecific settlements) and in two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

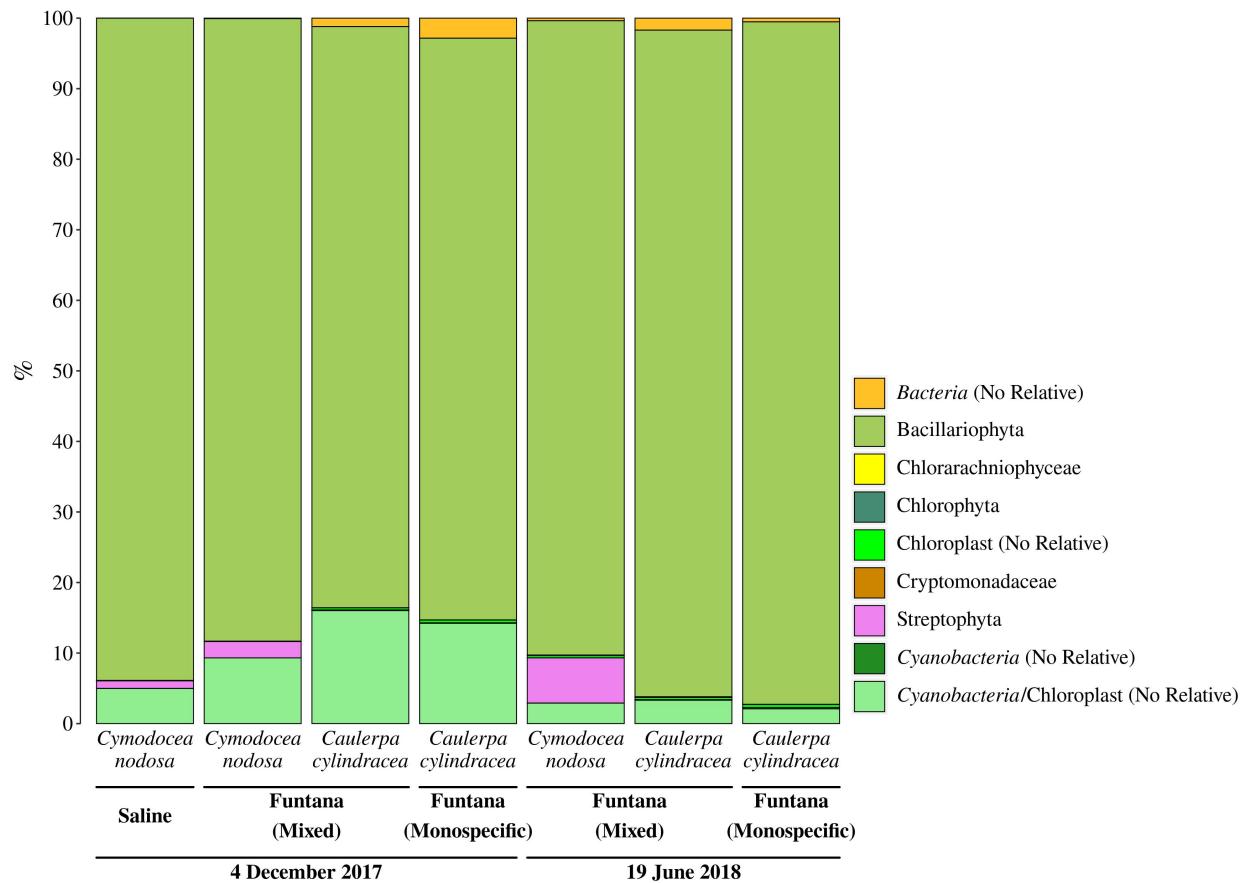


Fig. 4. Taxonomic classification and relative contribution of chloroplast sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (mixed and monospecific settlements) and in two contrasting seasons (4 December 2017 and 19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

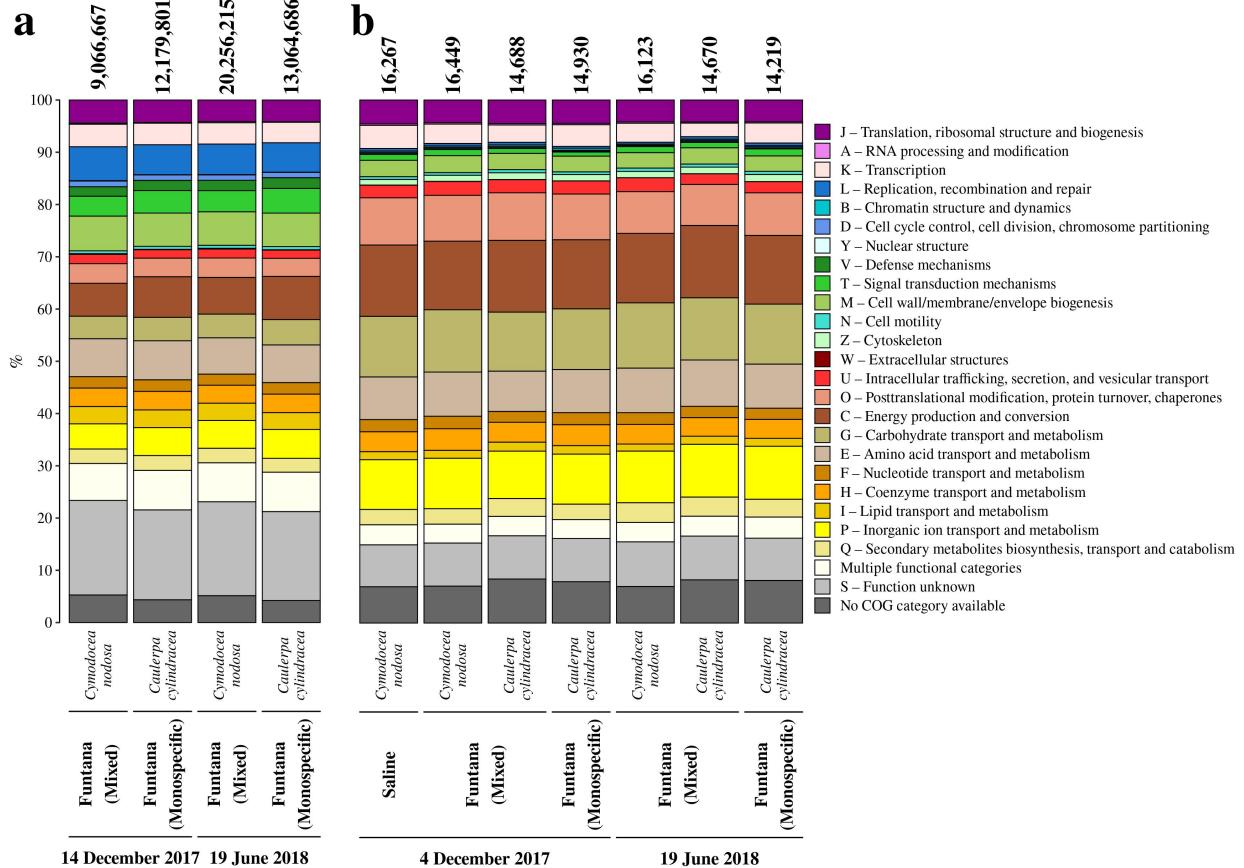


Fig. 5. Relative contribution of each COG category to the total number of annotated coding sequences (A) from metagenomes and identified proteins (B) from metaproteomes associated with surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (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.