

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 Introduction

³ Surfaces of marine macrophytes are inhabited by a diverse microbial community whose
⁴ structure and function are poorly understood (Egan *et al.*, 2013). As less than 1 % of all
⁵ prokaryotic species are culturable, to study these organisms, molecular methods such as 16S
⁶ rRNA sequencing, metagenomics and metaproteomics are indispensable (Amann *et al.*, 1995;
⁷ Su *et al.*, 2012). Applying these techniques requires an initial isolation step, with the purpose of
⁸ obtaining high quality DNA and proteins.

⁹ Biological material (i.e. proteins and DNA) from pelagic microbial communities is usually
¹⁰ isolated by collecting cells onto filters and subsequently isolating the aimed compound (Gilbert
¹¹ *et al.*, 2009). If a microbial size fraction is aimed sequential filtration is applied (Massana *et*
¹² *al.*, 1997; Andersson *et al.*, 2010). In contrast, obtaining biological materials from microorganism
¹³ inhabiting surfaces requires either a cell detachment procedure prior to isolation or the host material
¹⁴ is coextracted together with the targeted material. Methods for separating microbial cells from the
¹⁵ host include shaking of host tissue (Gross *et al.*, 2003; Nõges *et al.*, 2010), scraping of macrophyte
¹⁶ surfaces (Uku *et al.*, 2007) or the application of ultrasonication (Weidner *et al.*, 1996; Cai *et al.*,
¹⁷ 2014). It was shown that shaking alone is not sufficient to remove microbial cells, at least from
¹⁸ plant root surfaces (Richter-Heitmann *et al.*, 2016). Manual separation methods, such as scraping
¹⁹ and brushing, are time consuming and subjective, as the detachment efficiency depends on host
²⁰ tissue and the person performing the procedure (Cai *et al.*, 2014). Ultrasonication was proposed as
²¹ an alternative method as it is providing better results in terms of detachment efficiency (Cai *et al.*,
²² 2014; Richter-Heitmann *et al.*, 2016). The downside of this procedure is that complete cell removal
²³ was still not obtained and tissue disruption was observed especially after the application of probe
²⁴ ultrasonication (Richter-Heitmann *et al.*, 2016). An alternative to these cell detachment procedures
²⁵ is the isolation of targeted epiphytic compounds together with host materials (Staufenberger *et*
²⁶ *al.*, 2008; Jiang *et al.*, 2015). This procedure can lead to problems in the following processing
²⁷ steps such as mitochondrial and chloroplast 16S rRNA sequence contaminations from the host

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

31 An alternative to these procedures is a direct isolation of the targeted material by incubating
32 macrophyte tissues in an extraction buffer. After the incubation is done the undisrupted host tissue
33 is removed and the isolation procedure continues omitting host material contaminations. To our
34 knowledge the only procedure describing a direct and selective epiphytic DNA isolation from the
35 surfaces of marine macrophytes was provided by Burke *et al.* (2009). In contrast to previously
36 described methods this protocol enables an almost complete removal of the surface community
37 and was used for 16S rRNA gene clone libraries construction (Burke and Thomas *et al.*, 2011) and
38 metagenomes sequencing (Burke and Peter Steinberg *et al.*, 2011). This method, thought providing
39 a selective isolation procedure, is using in the extraction buffer a rapid multienzyme cleaner (3M)
40 that is not worldwide available and whose composition is not known (Burke *et al.*, 2009). Also to
41 our knowledge, no selective isolation protocol for proteins from epiphytic communities inhabiting
42 marine macrophytes was established.

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

49 **Results**

50 To assess the removal efficiency of DNA and protein isolation procedures leaves and thalli
51 were stained with SYBR Green I and examined under a confocal microscope before and after
52 treatments were performed. As it is known that marine macrophytes are harboring more algal
53 epiphytes during Autumn and Winter months (Reyes and Sansón, 2001), the sampling was
54 performed in two contrasting seasons, in December 2017 when higher abundances of algal
55 epiphytes are expected, and in June 2018, when lower abundances could be expected. In addition,
56 to further test the isolation protocols on different macrophyte species, DNA and proteins were
57 isolated from the surfaces of two macrophytes, the seagrass *C. nodosa* and the macroalga
58 *C. cylindracea*. *C. nodosa* was sampled in a monospecific meadow (Bay of Saline) and *C.*
59 *cylindracea* invaded meadow (Bay of Funtana), while samples of *C. cylindracea* originated from
60 a monospecific settlement and a *C. cylindracea* invaded *C. nodosa* meadow in the Bay of Funtana.
61 *C. nodosa* June samples from the Bay of Saline were missing as the meadow went to a decline
62 from Spring 2018 (M. Najdek, personal communication). Procedures developed for DNA and
63 protein isolation showed an almost complete removal of the surface community on both, samples
64 collected in December 2017 (Fig. 1) and June 2018 (Fig. 2). In addition, a similar removal
65 efficiency was observed for communities associated with *C. nodosa* and *C. cylindracea*. Also, no
66 effect of station, settlement or isolation procedure (DNA or protein) on the removal efficiency was
67 observed (Figs. 1 and 2).

68 To evaluate if the obtained DNA is suitable to determine the microbial community structure
69 an Illumina sequencing of the V4 16S rRNA region was performed. Sequencing yielded a total
70 of 336,937 sequences after quality curation and exclusion of eukaryotic, mitochondrial and no
71 relative sequences. The number of sequences classified as chloroplast was 97,328 (28.9 %) After
72 the exclusion of these sequences the total number of retrieved reads dropped to 239,609, ranging
73 from 22,587 and 52,958 sequences per sample (Table S1). Even when the highest sequencing effort
74 was applied the rarefaction curves did not level off that is commonly observed in high-throughput

75 16S rRNA amplicon sequencing procedures (Fig. S1). Sequence clustering at a similarity level
76 of 97 % yielded a total of 8,360 different OTUs. Taxonomic classification of reads allowed the
77 determination of the macrophyte associated epiphytic community that was mainly composed of:
78 *Alphaproteobacteria* ($14.9 \pm 3.5 \%$), *Bacteroidota* ($12.5 \pm 2.4 \%$), *Gammaproteobacteria* ($11.6 \pm$
79 4.3%), *Desulfobacterota* ($7.8 \pm 7.4 \%$), *Cyanobacteria* ($6.5 \pm 4.7 \%$) and *Planctomycetota* ($2.9 \pm$
80 1.7%) (Fig. 3).

81 Primers used in this study, as in many other 16S rRNA amplicon procedures, also amplify
82 chloroplast 16S rRNA genes. We observed a high proportion of chloroplast sequences in all
83 analyzed samples ($33.4 \pm 9.4 \%$) (Fig. 3). To determine if chloroplast sequences originate from
84 hosts or eukaryotic epiphytic organisms, we exported SILVA classified chloroplast sequences
85 and reclassified them using the RDP (Ribosomal Database Project) training set that allows for
86 a more detailed chloroplast classification. The largest proportion of sequences was classified as
87 *Bacillariophyta* ($89.7 \pm 5.7 \%$) indicating that the DNA removal procedure did not coextract larger
88 quantities of host DNA (Fig. 4).

89 **Discussion**

90 **Experimental procedures**

91 **Sampling**

92 Leaves of *C. nodosa* were sampled in a *C. nodosa* meadow in the Bay of Saline (45°7'5''
93 N, 13°37'20'' E) and in a *C. nodosa* meadow invaded by *C. cylindracea* in the proximity of the
94 village of Funtana (45°10'39'' N, 13°35'42'' E). Thalli of *C. cylindracea* were sampled in the
95 same *C. nodosa* invaded meadow in Funtana and on a locality of only *C. cylindracea* located in
96 the proximity of the invaded meadow. Leaves and thalli were collected on the same day in two
97 contrasting seasons, on 4 December 2017 and 18 June 2018. During spring 2018 the *C. nodosa*
98 meadow in the Bay of Saline decayed to an extent that no leaves could be retrieved (Najdek *et al.*,
99 unpublished data). Leaves and thalli were collected by diving and transported to the laboratory in
100 containers placed on ice and filled with site seawater. Upon arrival to the laboratory, *C. nodosa*
101 leaves were cut into sections of 1 – 2 cm, while *C. cylindracea* thalli were cut into 5 – 8 cm
102 long sections. Leaves and thalli were washed three times with sterile artificial seawater (ASW) to
103 remove loosely attached microbial cells.

104 **DNA isolation**

105 The DNA was isolated according to the protocol for isolation from filters described in
106 Massana *et al.* (1997). This protocol was modified and adapted for DNA isolation from microbial
107 communities from macrophyte surfaces as described below. 5 ml of lysis buffer (40 mM EDTA,
108 50 mM Tris-HCl, 0.75 M sucrose; pH 8.3) was added to 1 g wet weight of leaves or g wet-weight
109 of thalli. Lysozyme was added (final concentration 1 mg ml⁻¹) and the mixture was incubated at
110 37 °C for 30 min. Subsequently, proteinase K (final concentration 0.5 mg ml⁻¹) and SDS (final
111 concentration 1 %) were added and the samples were incubated at 55 °C for 2 h. Following the
112 incubation, tubes were vortexed for 10 min and the mixture containing lysed epiphytic cells was

113 separated from host leaves or thalli by transferring the solution into a clean tube. The lysate was
114 extracted twice with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1; pH 8) and once
115 with chloroform:isoamyl alcohol (24:1). After each organic solvent mixture addition tubes were
116 slightly vortexed and centrifuged at $4,500 \times g$ for 10 min. Following each centrifugation aqueous
117 phases were retrieved. After the final extraction 1/10 of chilled 3 M sodium acetate (pH 5.2) was
118 added. DNA was precipitated by adding 1 volume of chilled isopropanol, incubating the mixtures
119 overnight at -20°C and centrifuging at $16,000 \times g$ and 4°C for 20 min. The pellet was washed
120 twice with 1 ml of chilled 70 % ethanol and centrifuged after each washing step at $20,000 \times g$ and
121 4°C for 10 min. After the first washing step duplicate pellets from the same sample were pooled
122 and transferred to a clean 1.5 ml tube. The dried pellet was resuspended in 100 μl of deionized
123 water.

124 **Illumina 16S rRNA sequencing**

125 An aliquot of isolated DNA was treated with RNase A (final concentration $200 \mu\text{g ml}^{-1}$)
126 for 2 h at 37°C . The DNA concentration was determined using the Quant-iT PicoGreen
127 dsDNA Assay Kit (Invitrogen, USA) according to the manufacturer's instructions and
128 diluted to $1 \text{ ng } \mu\text{l}^{-1}$. The V4 region of the 16S rRNA gene was amplified using a two-step
129 PCR procedure. In the first PCR the 515F (5'-GTGYCAGCMGCCGCGTAA-3') and
130 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers from the Earth Microbiome Project
131 (<http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>) were used to amplify
132 the target region (Caporaso *et al.*, 2012; Apprill *et al.*, 2015; Parada *et al.*, 2016). These primers
133 contained on their 5' end a tagged sequence. Each sample was amplified in four parallel 25 μl
134 reactions of which each contained: $1 \times$ Q5 Reaction Buffer, 0.2 mM of dNTPmix, 0.7 mg ml^{-1}
135 BSA (Bovine Serum Albumin), 0.2 μM of forward and reverse primers, 0.5 U of Q5 High-Fidelity
136 DNA Polymerase (New England Biolabs, USA) and 5 ng of DNA template. Cycling conditions
137 were: initial denaturation at 94°C for 3 min, 20 cycles of denaturation at 94°C for 45 s, annealing

138 at 50 °C for 60 s and elongation at 72 °C for 90 s, finalized by an elongation step at 72 °C for 10
139 min. The four parallel reactions volumes were pooled and PCR products were purified using the
140 GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's
141 instructions and following the protocol that included isopropanol addition for better small DNA
142 fragment yield. The column was eluted in 30 µl of deionized water. Purified PCR products were
143 sent for Illumina MiSeq sequencing (2 × 250 bp) at IMGM Laboratories, Martinsried, Germany.
144 Before sequencing, at IMGM the second PCR amplification of the two-step PCR procedure was
145 performed using primers targeting the tagged region incorporated in the first PCR. In addition,
146 these primers contained adapter and sample-specific index sequences. The second PCR was
147 carried out for 8 cycles. Beside samples, a positive and negative control were sequenced. A
148 negative control was comprised of four parallel PCR reactions without DNA template, while
149 for a positive control a mock community composed of evenly mixed DNA material originating
150 from 20 bacterial strains (ATCC MSA-1002, ATCC, USA) was used. Sequences obtained in
151 this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under
152 accession numbers SAMEA6786270, SAMEA6648792 – SAMEA6648794, SAMEA6648809 –
153 SAMEA6648811.

154 **Sequence analysis**

155 Obtained sequences were analyzed on the computer cluster Isabella (University Computing
156 Center, University of Zagreb) using mothur (version 1.43.0) (Schloss *et al.*, 2009) according
157 to the MiSeq Standard Operating Procedure (MiSeq SOP; https://mothur.org/wiki/MiSeq_SOP)
158 (Kozich *et al.*, 2013) and recommendations given from the Riffomonas project to enhance data
159 reproducibility (<http://www.riffomonas.org/>). For alignment and classification of sequences the
160 SILVA SSU Ref NR 99 database (release 138; <http://www.arb-silva.de>) was used (Quast *et al.*,
161 2013; Yilmaz *et al.*, 2014). Sequences classified as chloroplasts by SILVA were exported and
162 reclassified using mothur and the RDP (<http://rdp.cme.msu.edu/>) training set (version 16) reference

163 files adapted for mothur (Cole *et al.*, 2014). In comparison to SILVA, RDP allows a more
164 detailed classification of chloroplast sequences. Pipeline data processing and visualization was
165 done using R (version 3.6.0) (R Core Team, 2019), package tidyverse (version 1.3.0) (Wickham
166 *et al.*, 2019) and multiple other packages (Xie, 2014, 2015, 2019b, 2019a, 2020; Neuwirth,
167 2014; Xie *et al.*, 2018; Allaire *et al.*, 2019; Zhu, 2019; Bengtsson, 2020). The detailed analysis
168 procedure including the R Markdown file for this paper are available as a GitHub repository
169 (https://github.com/mkorlevic/Korlevic_SelectiveRemoval_EnvironMicrobiol_2020). Based on
170 the ATCC MSA-1002 mock community included in the analysis a sequencing error rate of 0.009
171 % was determined, which is in line with previously reported values for next-generation sequencing
172 data (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative control processed together
173 with the samples yielded only 2 sequences after sequence quality curation.

174 **Protein isolation**

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

188 **Metaproteomics**

189 Isolated proteins were whole trypsin digested using the FASP (filter-aided sample preparation)
190 Protein Digestion Kit (Expedeon, UK) according to the manufacturer's instructions (Wiśniewski
191 *et al.*, 2009) with small modifications. Before loading the solution to the column, protein pellets
192 were solubilized in a urea sample buffer included in the kit amended with DTT (final concentration
193 100 mM) for 45 min at room temperature and centrifuged at 20,000 × g for 2 – 5 min at room
194 temperature to remove larger particles. The first washing step after protein solution loading was
195 repeated twice. In addition, centrifugation steps were prolonged if the column was clogged.
196 Trypsin digestion was performed on column filters at 37 °C overnight for 18 h. The final filtrate
197 containing digested proteins was acidified with 1 % (final concentration) trifluoroacetic acid,
198 freezed at –80 °C for 15 min, lyophilized and sent to the Vienna Metabolomics Center (University
199 of Vienna) for metaproteomic analysis. Peptides were resuspended in 1 % (final concentration)
200 trifluoroacetic acid, desalted using the Pierce C18 Tips (Thermo Fisher Scientific, USA) according
201 to the manufacturer's instructions and sequenced on a Q Exactive Hybrid Quadrupole-Orbitrap
202 Mass Spectrometer (Thermo Fisher Scientific, USA). Obtained MS/MS spectra were searched
203 against a protein database from metagenomic assembly published in Burke and Peter Steinberg *et*
204 *al.* (2011) using SEQUEST-HT engines and validated with Percolator in Proteome Discoverer 2.1
205 (Thermo Fisher Scientific, USA). The target-decoy approach was used to reduce the probability
206 of false peptide identification. Results whose false discovery rate at the peptide level was <1 %
207 were kept. For protein identification a minimum of of two peptides and one unique peptide were
208 required. For protein quantification, a chromatographic peak area-based free quantitative method
209 was applied.

210 **Confocal microscopy**

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

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328 **Figure Captions**

329 **Fig. 1.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of
330 Saline and the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 4 December
331 2017 and stained with SYBR Green I. Scale bar in all images is 60 μ m.

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

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

339 **Fig. 4.** Taxonomic classification and relative contribution of chloroplast sequences from surfaces
340 of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the
341 Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting seasons (4 December
342 2017 and 19 June 2018).

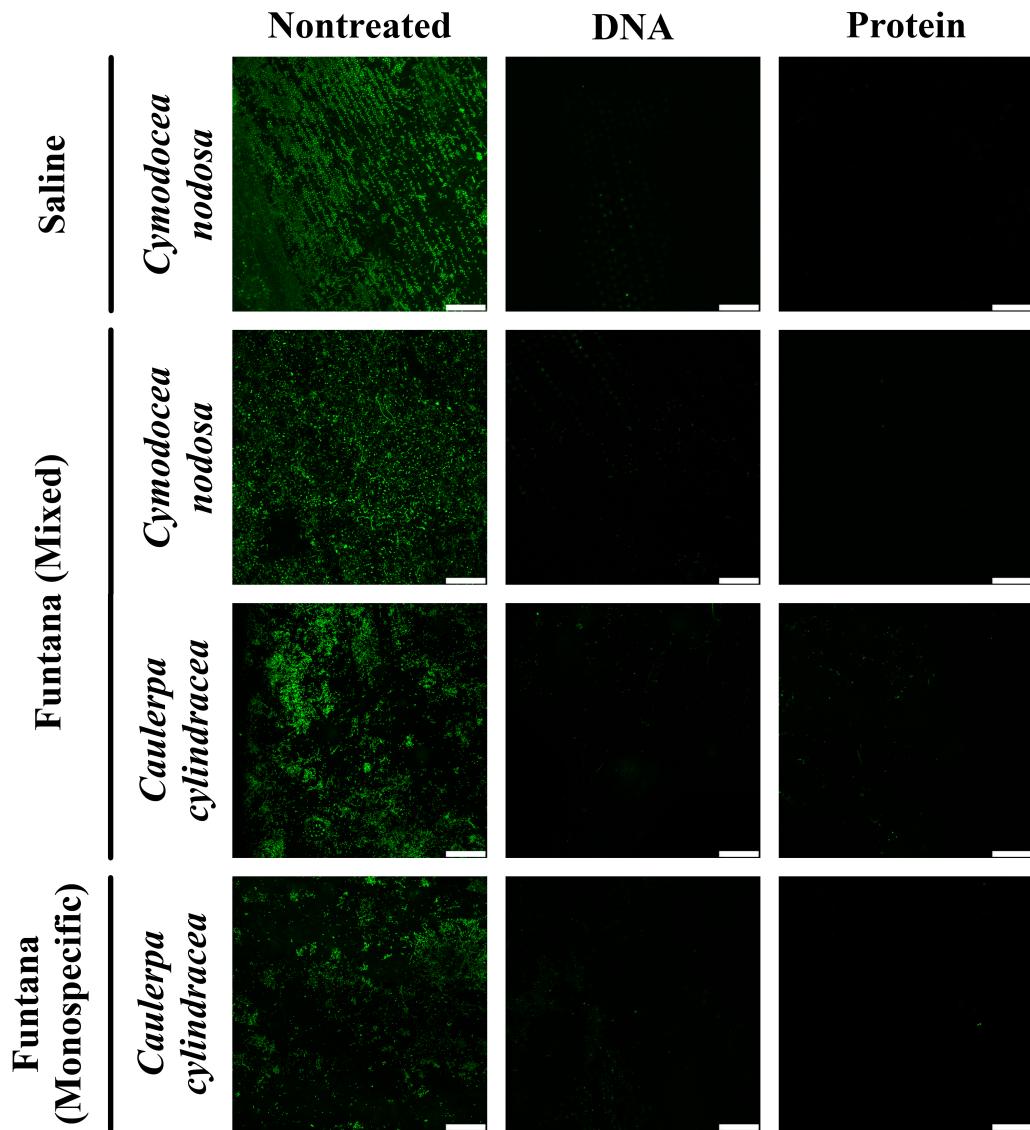


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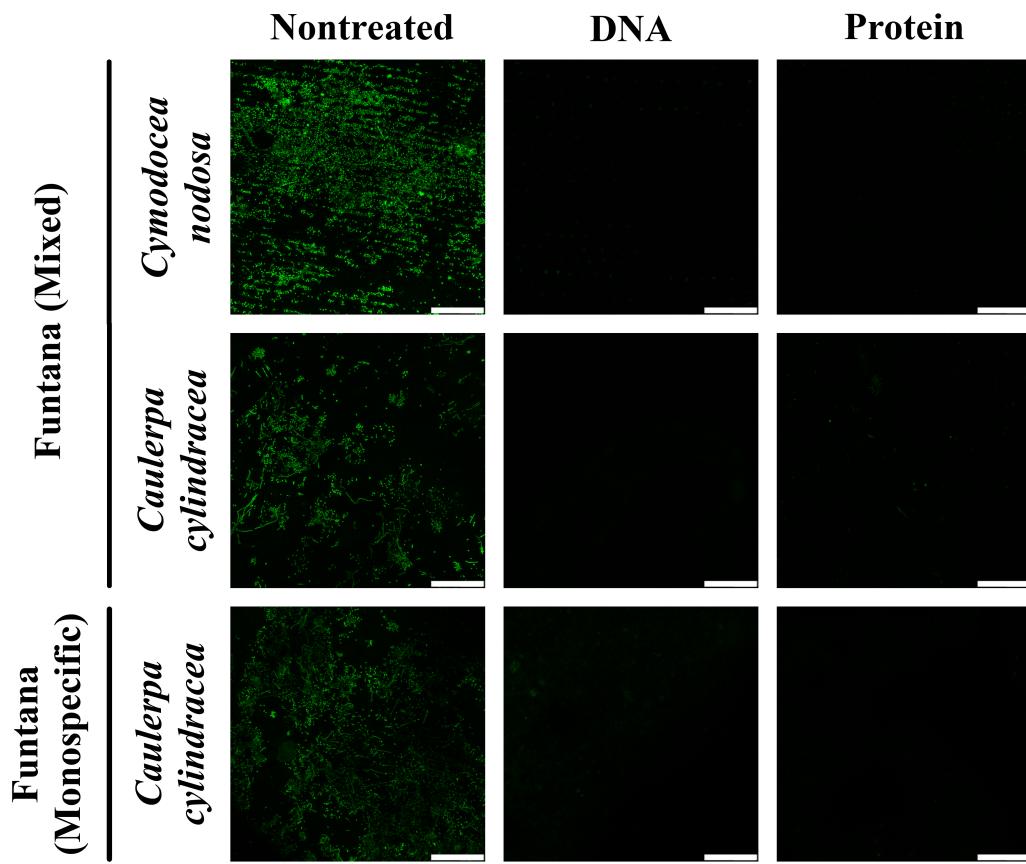


Fig. 2. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 19 June 2018 and stained with SYBR Green I. Scale bar in all images is 60 μ m.

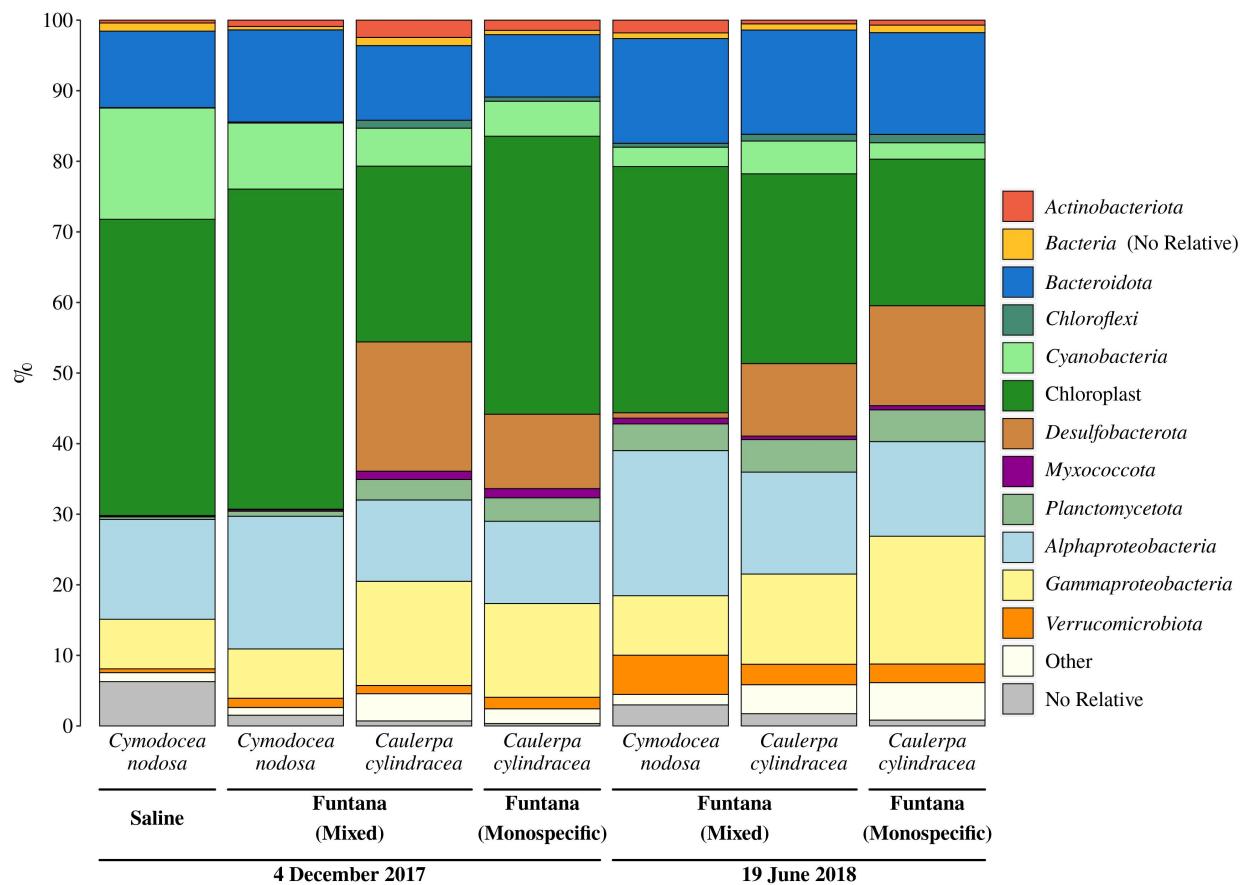


Fig. 3. Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial and archaeal sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting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

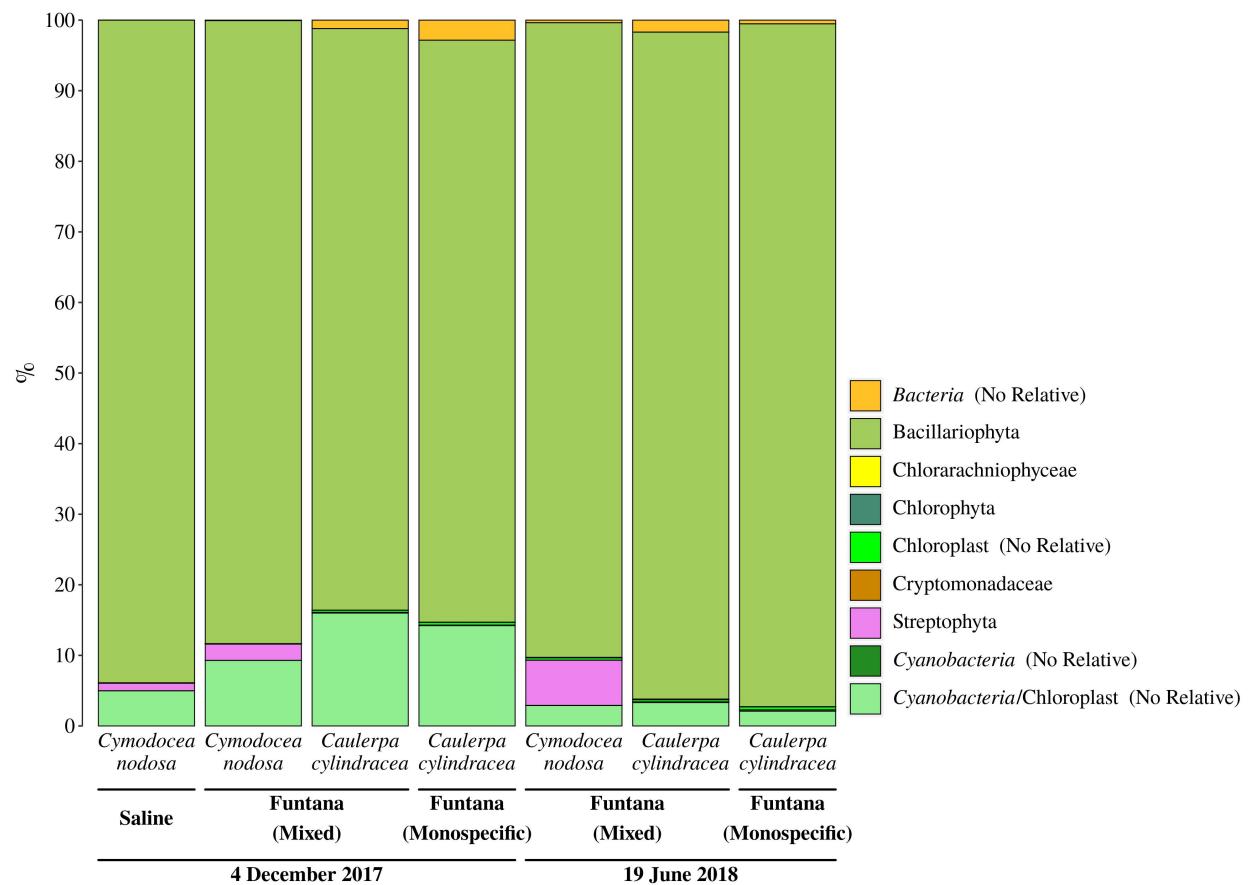


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