

# Selective DNA and Protein Isolation from Marine Macrophyte Surfaces

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Running title: DNA and protein isolation from macrophyte surfaces

## **1 Summary**

## <sup>2</sup> Introduction

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

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

51 **Experimental procedures**

52 **Sampling**

53 Leaves of *Cymodocea nodosa* were sampled in a *Cymodocea nodosa* meadow in the Bay  
54 of Saline (45°7'5" N, 13°37'20" E) and in a *Cymodocea nodosa* meadow invaded by *Caulerpa*  
55 *cylindracea* in the proximity of the village of Funtana (45°10'39" N, 13°35'42" E). Thalli of  
56 *Caulerpa cylindracea* were sampled in the same *Cymodocea nodosa* invaded meadow in Funtana  
57 and on a locality of only *Caulerpa cylindracea* located in the proximity of the invaded meadow.  
58 Leaves and thalli were collected on the same day in two contrasting seasons, on 4 December 2017  
59 and 18 June 2018. During spring 2018 the *Cymodocea nodosa* meadow in the Bay of Saline  
60 decayed to an extent that no leaves could be retrieved (Najdek *et al.*, unpublished data). Leaves  
61 and thalli were collected by diving and transported to the laboratory in containers placed on ice  
62 and filled with site seawater. Upon arrival to the laboratory, *Cymodocea nodosa* leaves were cut  
63 into sections of 1 – 2 cm, while *Caulerpa cylindracea* thalli were cut into 5 – 8 cm long sections.  
64 Leaves and thalli were washed three times with sterile artificial seawater (ASW) to remove loosely  
65 attached microbial cells.

66 **DNA isolation**

67 The DNA was isolated according to the protocol for isolation from filters described in  
68 Massana *et al.* (1997). This protocol was modified and adapted for DNA isolation from microbial  
69 communities from macrophyte surfaces as described below. 5 ml of lysis buffer (40 mM EDTA,  
70 50 mM Tris-HCl, 0.75 M sucrose; pH 8.3) was added to 1 g wet weight of leaves or g wet-weight  
71 of thalli. Lysozyme was added (final concentration 1 mg ml<sup>-1</sup>) and the mixture was incubated at  
72 37 °C for 30 min. Subsequently, proteinase K (final concentration 0.5 mg ml<sup>-1</sup>) and SDS (final  
73 concentration 1 %) were added and the samples were incubated at 55 °C for 2 h. Following the

74 incubation, tubes were vortexed for 10 min and the mixture containing lysed epiphytic cells was  
75 separated from host leaves or thalli by transferring the solution into a clean tube. The lysate was  
76 extracted twice with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1; pH 8) and once  
77 with chloroform:isoamyl alcohol (24:1). After each organic solvent mixture addition tubes were  
78 slightly vortexed and centrifuged at  $4,500 \times g$  for 10 min. Following each centrifugation aqueous  
79 phases were retrieved. After the final extraction 1/10 of chilled 3 M sodium acetate (pH 5.2) was  
80 added. DNA was precipitated by adding 1 volume of chilled isopropanol, incubating the mixtures  
81 overnight at  $-20^{\circ}\text{C}$  and centrifuging at  $16,000 \times g$  and  $4^{\circ}\text{C}$  for 20 min. The pellet was washed  
82 twice with 1 ml of chilled 70 % ethanol and centrifuged after each washing step at  $20,000 \times g$  and  
83  $4^{\circ}\text{C}$  for 10 min. After the first washing step duplicate pellets from the same sample were pooled  
84 and transferred to a clean 1.5 ml tube. The dried pellet was resuspended in 100  $\mu\text{l}$  of deionized  
85 water.

## 86 Illumina 16S rRNA sequencing

87 An aliquot of isolated DNA was treated with RNase A (final concentration  $200 \mu\text{g ml}^{-1}$ )  
88 for 2 h at  $37^{\circ}\text{C}$ . The DNA concentration was determined using the Quant-iT PicoGreen  
89 dsDNA Assay Kit (Invitrogen, USA) according to the manufacturer's instructions and  
90 diluted to  $1 \text{ ng } \mu\text{l}^{-1}$ . The V4 region of the 16S rRNA gene was amplified using a two-step  
91 PCR procedure. In the first PCR the 515F (5'-GTGYCAGCMGCCGCGTAA-3') and  
92 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers from the Earth Microbiome Project  
93 (<http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>) were used to amplify  
94 the target region (Caporaso *et al.*, 2012; Apprill *et al.*, 2015; Parada *et al.*, 2016). These primers  
95 contained on their 5' end a tagged sequence. Each sample was amplified in four parallel 25  $\mu\text{l}$   
96 reactions of which each contained: 1  $\times$  Q5 Reaction Buffer, 0.2 mm of dNTPmix, 0.7  $\text{mg ml}^{-1}$   
97 BSA (Bovine Serum Albumin), 0.2  $\mu\text{M}$  of forward and reverse primers, 0.5 U of Q5 High-Fidelity  
98 DNA Polymerase (New England Biolabs, USA) and 5 ng of DNA template. Cycling conditions

99 were: initial denaturation at 94 °C for 3 min, 20 cycles of denaturation at 94 °C for 45 s, annealing  
100 at 50 °C for 60 s and elongation at 72 °C for 90 s, finalized by an elongation step at 72 °C for 10  
101 min. The four parallel reactions volumes were pooled and PCR products were purified using the  
102 GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's  
103 instructions and following the protocol that included isopropanol addition for better small DNA  
104 fragment yield. The column was eluted in 30 µl of deionized water. Purified PCR products were  
105 sent for Illumina MiSeq sequencing (2 × 250 bp) at IMGM Laboratories, Martinsried, Germany.  
106 Before sequencing, at IMGM the second PCR amplification of the two-step PCR procedure was  
107 performed using primers targeting the tagged region incorporated in the first PCR. In addition,  
108 these primers contained adapter and sample-specific index sequences. The second PCR was  
109 carried out for 8 cycles. Beside samples, a positive and negative control were sequenced. A  
110 negative control was comprised of four parallel PCR reactions without DNA template, while for a  
111 positive control a mock community composed of evenly mixed DNA material originating from 20  
112 bacterial strains (ATCC MSA-1002, ATCC, USA) was used. The sequences obtained in this study  
113 have been submitted to the European Nucleotide Archive (ENA) under accession numbers **TO BE**  
114 **ADDED LATER!**.

## 115 **Sequence analysis**

116 Obtained sequences were analyzed on the computer cluster Isabella (University Computing  
117 Center, University of Zagreb) using mothur (version 1.43.0) (Schloss *et al.*, 2009) according  
118 to the MiSeq Standard Operating Procedure (MiSeq SOP; [https://mothur.org/wiki/MiSeq\\_SOP](https://mothur.org/wiki/MiSeq_SOP))  
119 (Kozich *et al.*, 2013) and recommendations given from the Riffomonas project to enhance data  
120 reproducibility (<http://www.riffomonas.org/>). For alignment and classification of sequences the  
121 SILVA SSU Ref NR 99 database (release 138; <http://www.arb-silva.de>) was used (Quast *et al.*,  
122 2013; Yilmaz *et al.*, 2014). Sequences classified as chloroplasts by mothur were exported, aligned  
123 using SILVA incremental aligner (SINA, version 1.6.0) (Pruesse *et al.*, 2012) against the same

<sup>124</sup> SILVA SSU Ref NR 99 database (release 138) and imported into ARB (version 6.0.6) (Ludwig *et*  
<sup>125</sup> *al.*, 2004) for further phylogenetic analysis using the same database. Reference sequences close  
<sup>126</sup> to imported ones were selected and used to calculate a phylogenetic tree using the Maximum  
<sup>127</sup> Likelihood algorithm RAxML (version 7.0.3) with 1000 tree replicates (Stamatakis, 2006).  
<sup>128</sup> Imported partial chloroplast sequences were added to the tree using the maximum parsimony  
<sup>129</sup> criteria and not allowing changes to tree topology. Pipeline data processing and visualization was  
<sup>130</sup> done using R (version 3.6.0) (R Core Team, 2019), package tidyverse (version 1.2.1) (Wickham  
<sup>131</sup> *et al.*, 2019) and multiple other packages (Xie, 2014, 2015, 2019a, 2019b; Xie *et al.*, 2018; Zhu,  
<sup>132</sup> 2019; Allaire *et al.*, 2019). The detailed analysis procedure including the R Markdown file for  
<sup>133</sup> this paper are available as a GitHub repository (**TO BE ADDED LATER!**). Based on the ATCC  
<sup>134</sup> MSA-1002 mock community included in the analysis a sequencing error rate of 0.009 % was  
<sup>135</sup> determined, which is in line with previously reported values for next-generation sequencing data  
<sup>136</sup> (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative control processed together  
<sup>137</sup> with the samples yielded only 2 sequences after sequence quality curation.

### <sup>138</sup> Protein isolation

<sup>139</sup> Proteins were isolated according to the protocol for isolation from soil described in Chourey  
<sup>140</sup> *et al.* (2010) and modified by Hultman *et al.* (2015). These protocols were further modified and  
<sup>141</sup> adapted for protein isolation from microbial communities from macrophyte surfaces as described  
<sup>142</sup> below. 20 ml of protein extraction buffer (4 % SDS, 100 mM Tris-HCl [pH 8.0]) was added to 5  
<sup>143</sup> g wet weight of leaves or 10 g wet weight of thalli. The mixture was incubated in boiling water  
<sup>144</sup> for 5 min, vortexed for 10 min and incubated again in boiling water for 5 min. After a brief vortex  
<sup>145</sup> the lysate was transferred to a clean tube separating the host leaves or thalli from the mixture  
<sup>146</sup> containing lysed epiphytic cells. Dithiothreitol (DTT; final concentration 24 mM) was added and  
<sup>147</sup> proteins were precipitated with chilled 100 % trichloroacetic acid (TCA; final concentration 20 %)  
<sup>148</sup> overnight at -20 °C. Precipitated proteins were centrifuged at 10,000 × g and 4 °C for 40 min. The

149 obtained protein pellet was washed three times with chilled acetone. During the first washing step  
150 the pellet was transferred to a clean 1.5 ml tube. After each washing step samples were centrifuged  
151 at 20,000 × g and 4 °C for 5 min. Dried pellets were stored at –80 °C until further analysis.

152 **Metaproteomics**

153 Isolated proteins were whole trypsin digested using the FASP (filter-aided sample preparation)  
154 Protein Digestion Kit (Expedeon, UK) according to the manufacturer's instructions (Wiśniewski  
155 *et al.*, 2009) with small modifications. Before loading the solution to the column, protein pellets  
156 were solubilized in a urea sample buffer included in the kit amended with DTT (final concentration  
157 100 mM) for 45 min at room temperature and centrifuged at 20,000 × g for 2 – 5 min at room  
158 temperature to remove larger particles. The first washing step after protein solution loading was  
159 repeated twice. In addition, centrifugation steps were prolonged if the column was clogged.  
160 Trypsin digestion was performed on column filters at 37 °C overnight for 18 h. The final filtrate  
161 containing digested proteins was acidified with 1 % (final concentration) trifluoroacetic acid,  
162 freezed at –80 °C for 15 min, lyophilized and sent to the Vienna Metabolomics Center (University  
163 of Vienna) for metaproteomic analysis. Peptides were resuspended in 1 % (final concentration)  
164 trifluoroacetic acid, desalted using the Pierce C18 Tips (Thermo Fisher Scientific, USA) according  
165 to the manufacturer's instructions and sequenced on a Q Exactive Hybrid Quadrupole-Orbitrap  
166 Mass Spectrometer (Thermo Fisher Scientific, USA). Obtained MS/MS spectra were searched  
167 against a protein database from metagenomic assembly published in Burke and Peter Steinberg *et*  
168 *al.* (2011) using SEQUEST-HIT engines and validated with Percolator in Proteome Discoverer 2.1  
169 (Thermo Fisher Scientific, USA). The target-decoy approach was used to reduce the probability  
170 of false peptide identification. Results whose false discovery rate at the peptide level was <1 %  
171 were kept. For protein identification a minimum of of two peptides and one unique peptide were  
172 required. For protein quantification, a chromatographic peak area-based free quantitative method  
173 was applied.

<sup>174</sup> **Confocal microscopy**

<sup>175</sup> Host leaves and thalli from DNA and protein isolation steps were washed seven times in  
<sup>176</sup> deionized water and fixed with formaldehyde (final concentration ~ 3 %). In addition, nontreated  
<sup>177</sup> leaves and thalli, washed three times in ASW to remove loosely attached microbial cells, were  
<sup>178</sup> fixed in the same concentration of formaldehyde and used as a positive control. For long therm  
<sup>179</sup> storage, fixed leaves and thalli were immersed in a mixture of phosphate-buffered saline (PBS)  
<sup>180</sup> and ethanol (1:1) and stored at -20 °C. Treated and untreated leaves and thalli segments were  
<sup>181</sup> stained in a 2 × solution of SYBR Green I and examined under a Leica TCS SP8 X FLIM confocal  
<sup>182</sup> microscope (Leica Microsystems, Germany).

<sup>183</sup> **Acknowledgements**

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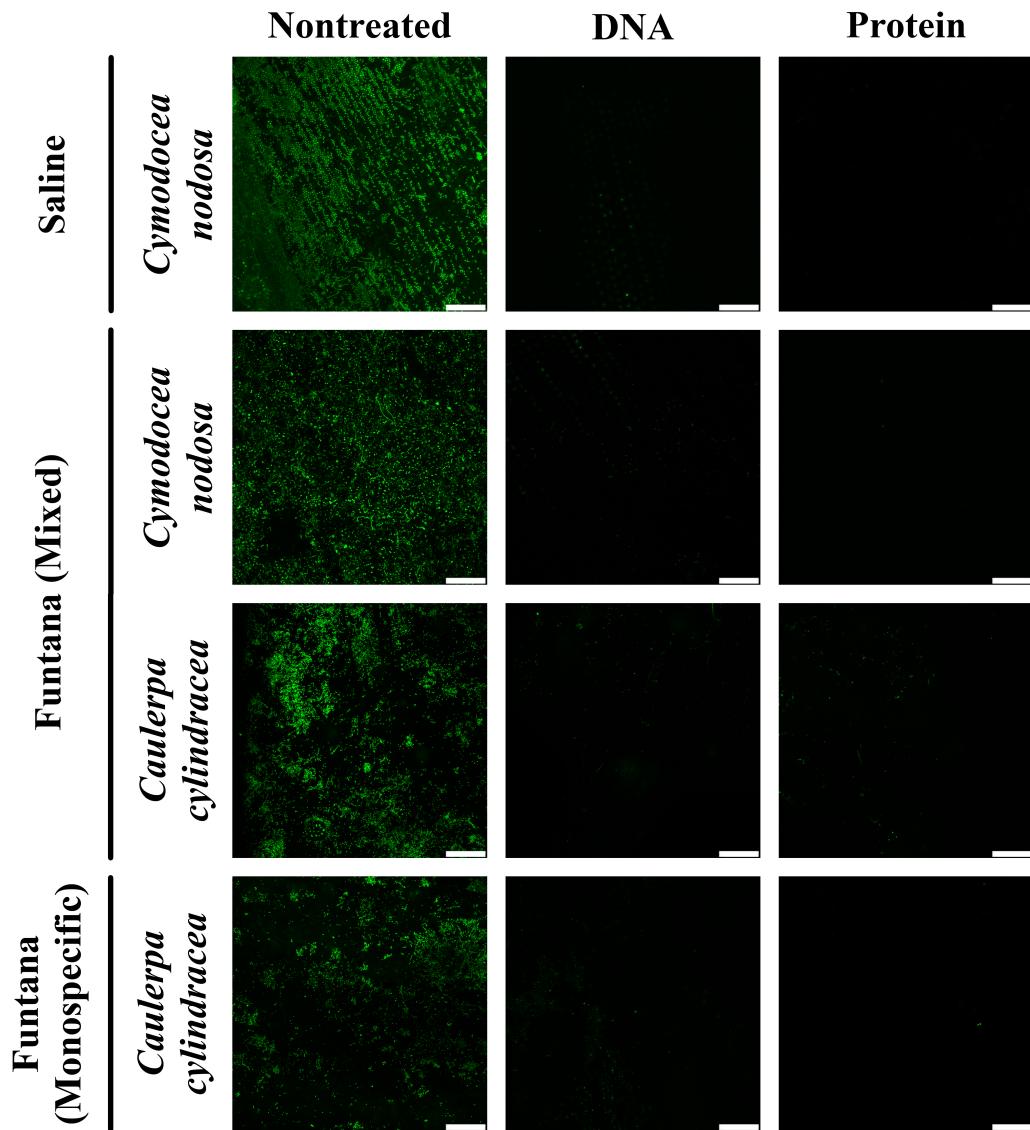
292 **Figure Captions**

293 **Fig. 1.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of  
294 Saline and the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 4 December  
295 2017 and stained with SYBR Green I. Scale bar in all images is 60  $\mu$ m.

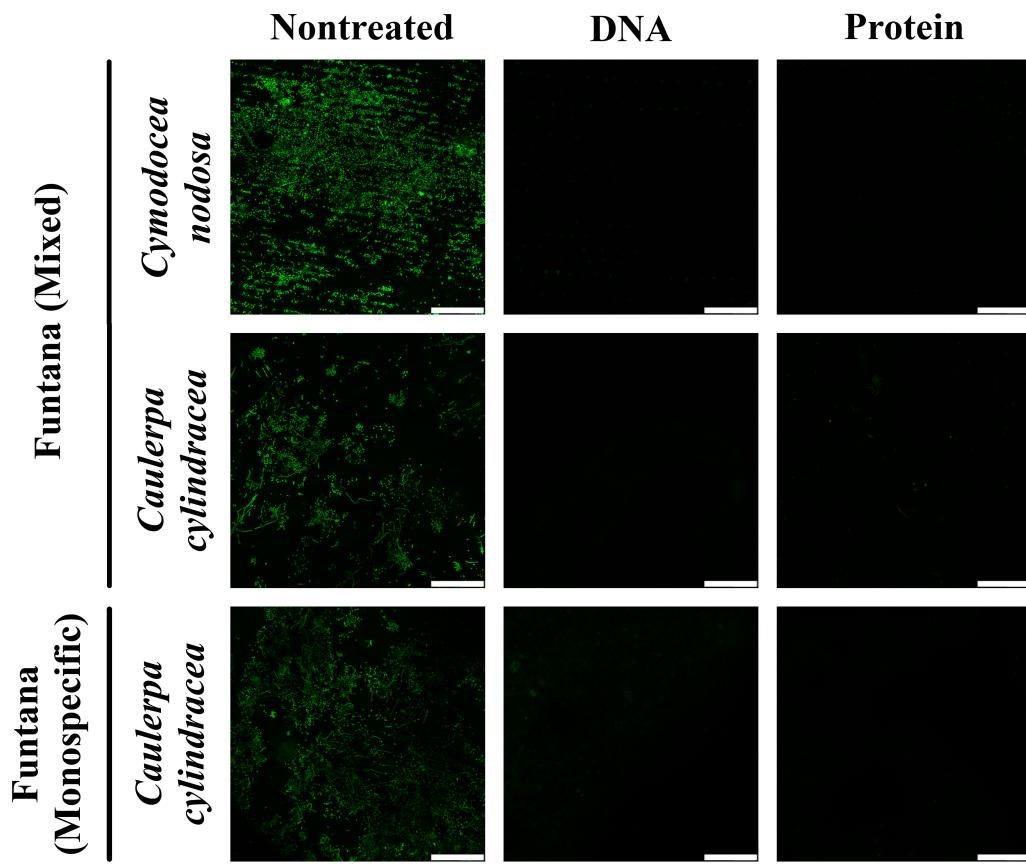
296 **Fig. 2.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of  
297 Funtana (Mixed and Monospecific Settlements) sampled on 19 June 2018 and stained with SYBR  
298 Green I. Scale bar in all images is 60  $\mu$ m.

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

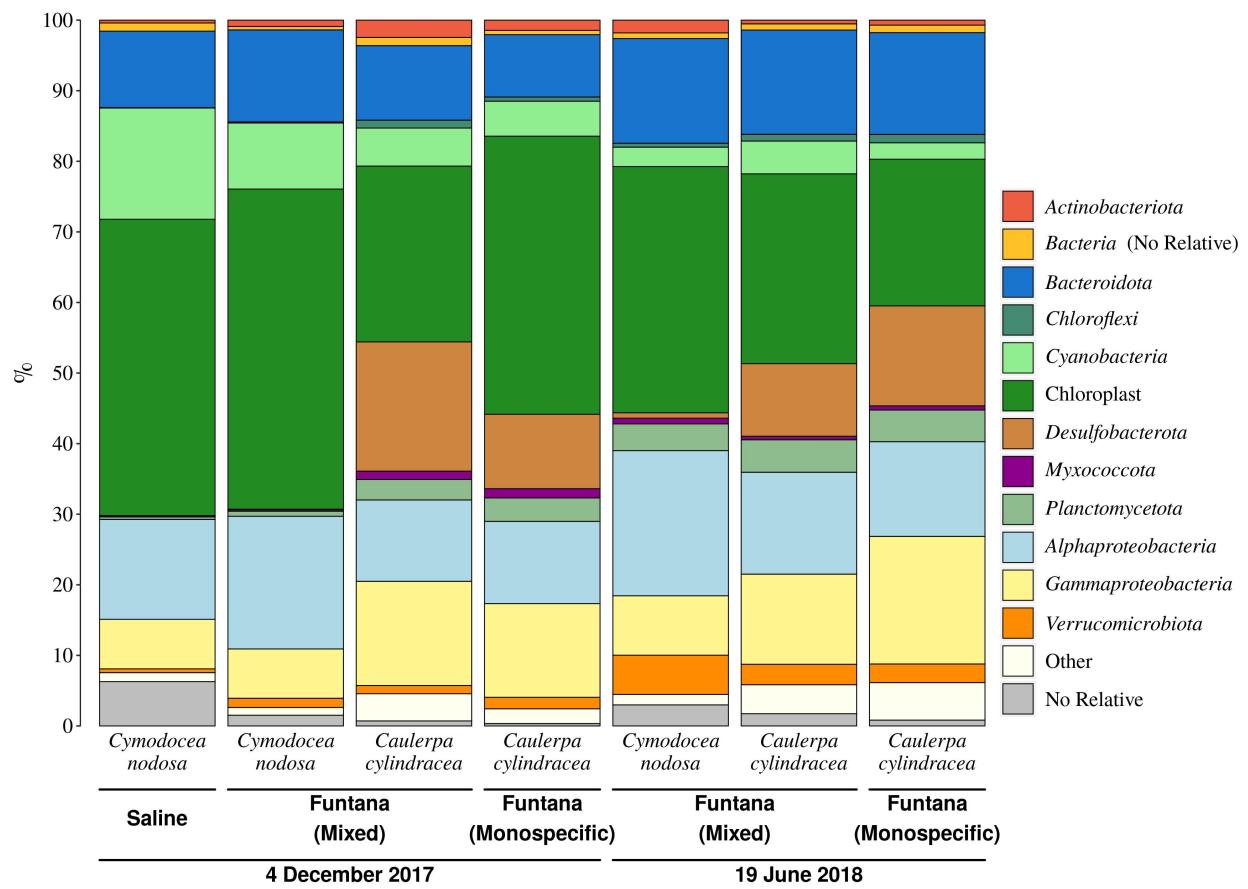
303 **Figures**



**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  $\mu$ m.



**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  $\mu$ 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).