

# Selective DNA and Protein Isolation from Marine Macrophyte Surfaces

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## **1 Abstract**

## <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 form 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 and  
37 was used for 16S rRNA gene clone libraries construction (Burke *et al.*, 2011b) and metagenomes  
38 sequencing (Burke *et al.*, 2011a). This method, thought providing a selective isolation procedure,  
39 is using in the extraction buffer a rapid multienzyme cleaner (3M) that is not worldwide available  
40 and whose composition is not known (Burke *et al.*, 2009). Also to our knowledge, no selective  
41 isolation protocol for proteins from epiphytic communities inhabiting marine macrophytes was  
42 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 **Materials and Methods**

50 **Sampling**

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58 decayed to an extent that no leaves could be retrieved (Najdek *et al.*, unpublished data).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

64 **DNA Isolation**

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

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

#### 84 Illumina 16S rRNA Sequencing

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

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

### 113 Sequence Analysis

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

<sup>122</sup> SILVA SSU Ref NR 99 database (release 138) and imported into ARB (version 6.0.6) (Ludwig *et*  
<sup>123</sup> *al.*, 2004) for further phylogenetic analysis using the same database. Reference sequences close  
<sup>124</sup> to imported ones were selected and used to calculate a phylogenetic tree using the Maximum  
<sup>125</sup> Likelihood algorithm RAxML (version 7.0.3) with 1000 tree replicates (Stamatakis, 2006).  
<sup>126</sup> Imported partial chloroplast sequences were added to the tree using the maximum parsimony  
<sup>127</sup> criteria and not allowing changes to tree topology. Pipeline data processing and visualization was  
<sup>128</sup> done using R (version 3.6.0) (R Core Team, 2019), package tidyverse (version 1.2.1) (Wickham  
<sup>129</sup> *et al.*, 2019) and multiple other packages (Xie, 2014, 2015, 2019a, 2019b; Xie *et al.*, 2018; Zhu,  
<sup>130</sup> 2019; Allaire *et al.*, 2019). The detailed analysis procedure including the R Markdown file for  
<sup>131</sup> this paper are available as a GitHub repository (**TO BE ADDED LATER!**). Based on the ATCC  
<sup>132</sup> MSA-1002 mock community included in the analysis a sequencing error rate of 0.009 % was  
<sup>133</sup> determined, which is in line with previously reported values for next-generation sequencing data  
<sup>134</sup> (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative control processed together  
<sup>135</sup> with the samples yielded only 2 sequences after sequence quality curation.

## <sup>136</sup> Protein Isolation

<sup>137</sup> Proteins were isolated according to the protocol for isolation from soil described in Chourey  
<sup>138</sup> *et al.* (2010) and modified by Hultman *et al.* (2015). These protocols were further modified and  
<sup>139</sup> adapted for protein isolation from microbial communities form macrophyte surfaces as described  
<sup>140</sup> below. 20 ml of protein extraction buffer (4 % SDS, 100 mM Tris-HCl [pH 8.0]) was added to 5  
<sup>141</sup> g wet weight of leaves or 10 g wet weight of thalli. The mixture was incubated in boiling water  
<sup>142</sup> for 5 min, vortexed for 10 min and incubated again in boiling water for 5 min. After a brief vortex  
<sup>143</sup> the lysate was transferred to a clean tube separating the host leaves or thalli from the mixture  
<sup>144</sup> containing lyzed epiphytic cells. Dithiothreitol (DTT; final concentration 24 mM) was added and  
<sup>145</sup> proteins were precipitated with chilled 100 % trichloroacetic acid (TCA; final concentration 20 %)  
<sup>146</sup> overnight at -20 °C. Precipitated proteins were centrifuged at 10,000 × g and 4 °C for 40 min. The

<sup>147</sup> obtained protein pellet was washed three times with chilled acetone. During the first washing step  
<sup>148</sup> the pellet was transferred to a clean 1.5 ml tube. After each washing step samples were centrifuged  
<sup>149</sup> at 20,000 × g and 4 °C for 5 min. Dried pellets were stored at –80 °C until further analysis.

<sup>150</sup> **Metaproteomics**

<sup>151</sup> Isolated proteins were whole trypsin digested using the FASP (filter-aided sample preparation)  
<sup>152</sup> Protein Digestion Kit (Expedeon, UK) according to the manufacturer's instructions (Wiśniewski  
<sup>153</sup> *et al.*, 2009) with small modifications. Before loading the solution to the column, protein pellets  
<sup>154</sup> were solubilized in a urea sample buffer included in the kit amended with DTT (final concentration  
<sup>155</sup> 100 mM) for 45 min at room temperature and centrifuged at 20,000 × g for 2 – 5 min at room  
<sup>156</sup> temperature to remove larger particles. The first washing step after protein solution loading was  
<sup>157</sup> repeated twice. In addition, centrifugation steps were prolonged if the column was clogged.  
<sup>158</sup> Trypsin digestion was performed on column filters at 37 °C overnight for 18 h. The final filtrate  
<sup>159</sup> containing digested proteins was acidified with 1 % (final concentration) trifluoroacetic acid,  
<sup>160</sup> freezed at –80 °C for 15 min, lyophilized and sent to the Vienna Metabolomics Center (University  
<sup>161</sup> of Vienna) for metaproteomic analysis. Peptides were resuspended in 1 % (final concentration)  
<sup>162</sup> trifluoroacetic acid, desalting using the Pierce C18 Tips (Thermo Fisher Scientific, USA) according  
<sup>163</sup> to the manufacturer's instructions and sequenced on a Q Exactive Hybrid Quadrupole-Orbitrap  
<sup>164</sup> Mass Spectrometer (Thermo Fisher Scientific, USA). Obtained MS/MS spectra were searched  
<sup>165</sup> against a protein database from metagenomic assembly published in Burke *et al.* (2011a) using  
<sup>166</sup> SEQUEST-HIT engines and validated with Percolator in Proteome Discoverer 2.1 (Thermo Fisher  
<sup>167</sup> Scientific, USA). The target-decoy approach was used to reduce the probability of false peptide  
<sup>168</sup> identification. Results whose false discovery rate at the peptide level was <1 % were kept. For  
<sup>169</sup> protein identification a minimum of two peptides and one unique peptide were required. For  
<sup>170</sup> protein quantification, a chromatographic peak area-based free quantitative method was applied.

<sup>171</sup> **Confocal Microscopy**

<sup>172</sup> Host leaves and thalli from DNA and protein isolation steps were washed seven times in  
<sup>173</sup> deionized water and fixed with formaldehyde (final concentration ~ 3 %). In addition, nontreated  
<sup>174</sup> leaves and thalli, washed three times in ASW to remove loosely attached microbial cells, were  
<sup>175</sup> fixed in the same concentration of formaldehyde and used as a positive control. For long therm  
<sup>176</sup> storage, fixed leaves and thalli were immersed in a mixture of phosphate-buffered saline (PBS)  
<sup>177</sup> and ethanol (1:1) and stored at -20 °C. Treated and untreated leaves and thalli segments were  
<sup>178</sup> stained in a 2 × solution of SYBR Green I and examined under a Leica TCS SP8 X FLIM confocal  
<sup>179</sup> microscope (Leica Microsystems, Germany).

<sup>180</sup> **Results**

<sup>181</sup> **Discussion**

<sup>182</sup> **Acknowledgements**

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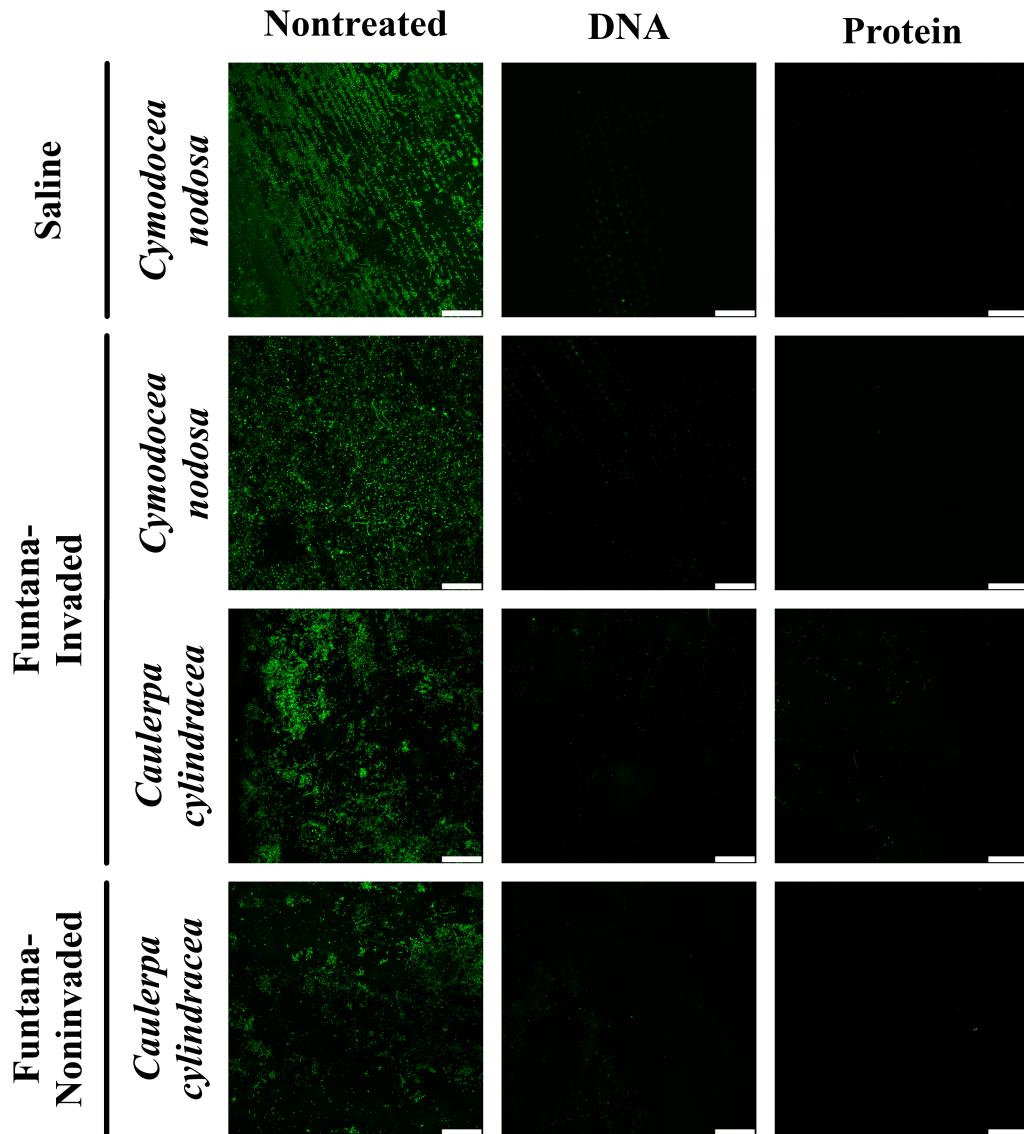
291 **Figure Captions**

292 **Figure 1.** Confocal microscope images of *Cymodocea nodosa* and *Caulerpa cylindracea* surfaces  
293 from different locations (Saline, Funtana-Invaded and Funtana-Noninvaded) sampled on 4  
294 December 2017 and stained with SYBR Green I. Scale bar in all images is 60 µm.

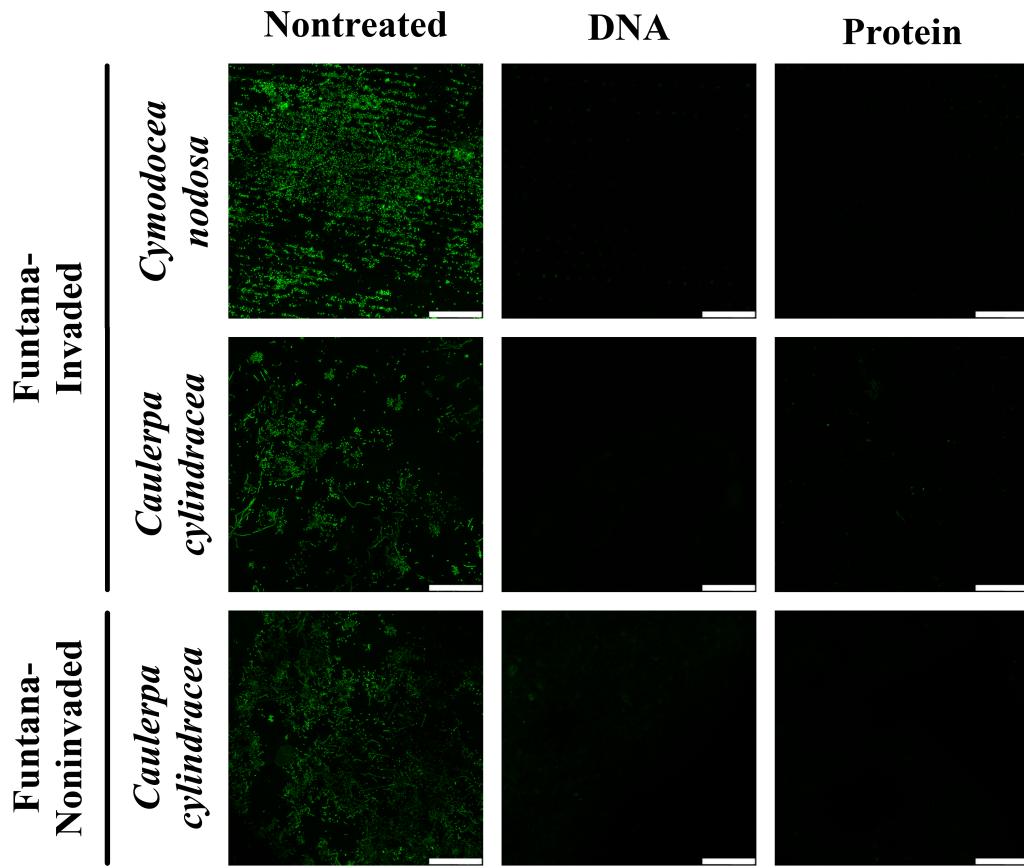
295 **Figure 2.** Confocal microscope images of *Cymodocea nodosa* and *Caulerpa cylindracea* surfaces  
296 from different locations (Funtana-Invaded and Funtana-Noninvaded) sampled on 19 June 2018 and  
297 stained with SYBR Green I. Scale bar in all images is 60 µm.

298 **Figure 3.** Taxonomic classification and relative contribution of the most abundant bacterial  
299 sequences from surfaces of two marine macrophytes (*Cymodocea nodosa* and *Caulerpa*  
300 *cylindracea*) sampled in different locations (Saline, Funtana-Invaded and Funtana-Noninvaded)  
301 and in two contrasting seasons (4 December 2017 and 19 June 2018).

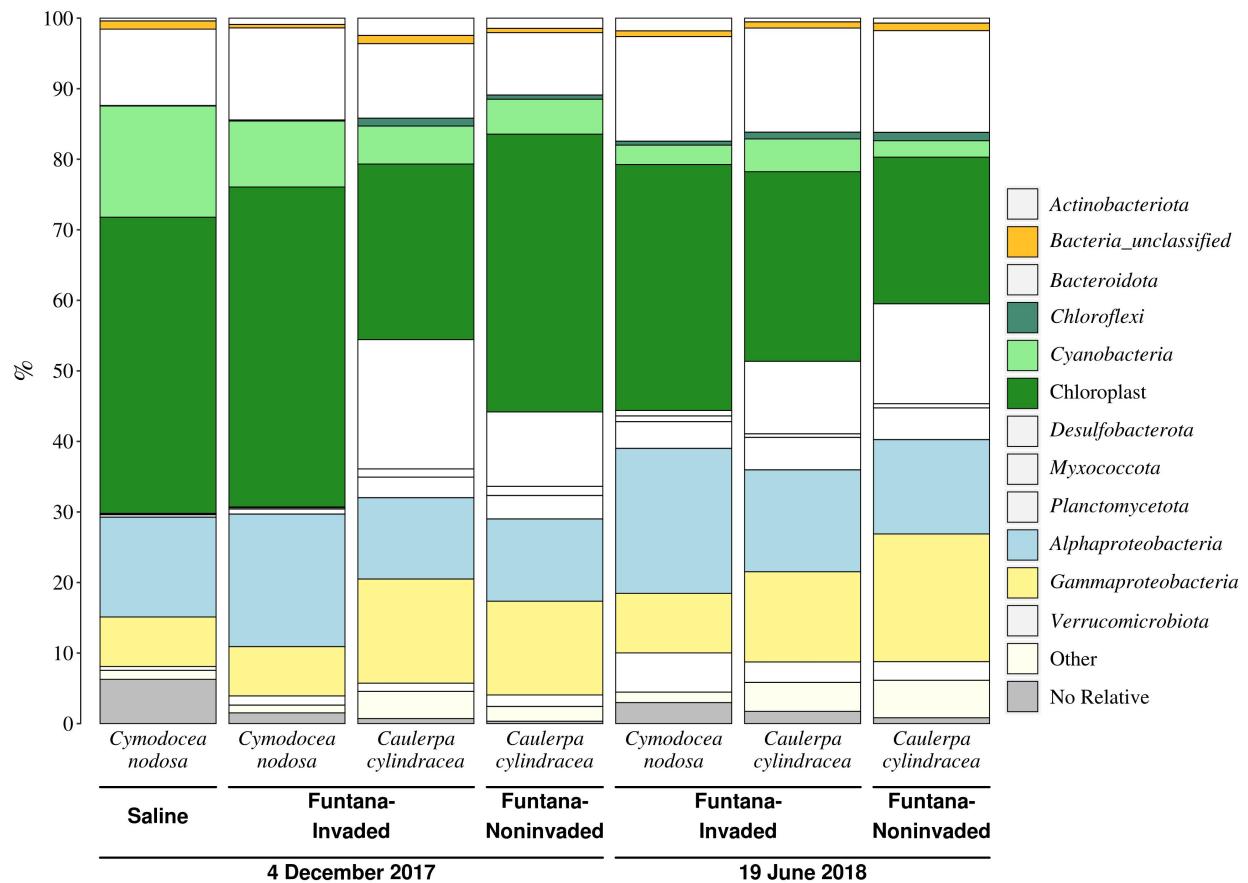
302 **Figures**



**Figure 1.** Confocal microscope images of *Cymodocea nodosa* and *Caulerpa cylindracea* surfaces from different locations (Saline, Funtana-Invaded and Funtana-Noninvaded) sampled on 4 December 2017 and stained with SYBR Green I. Scale bar in all images is 60  $\mu$ m.



**Figure 2.** Confocal microscope images of *Cymodocea nodosa* and *Caulerpa cylindracea* surfaces from different locations (Funtana-Invaded and Funtana-Noninvaded) sampled on 19 June 2018 and stained with SYBR Green I. Scale bar in all images is 60 µm.



**Figure 3.** Taxonomic classification and relative contribution of the most abundant bacterial sequences from surfaces of two marine macrophytes (*Cymodocea nodosa* and *Caulerpa cylindracea*) sampled in different locations (Saline, Funtana-Invaded and Funtana-Noninvaded) and in two contrasting seasons (4 December 2017 and 19 June 2018).