

# Selective DNA and Protein Isolation from Marine Macrophyte Surfaces

1†

† To whom correspondence should be addressed: marino.korlevic@irb.hr

1. Ruđer Bošković Institute, Center for Marine Research, G. Paliaga 5, Rovinj, Croatia
2. University of Vienna, Department of Limnology and Bio-Oceanography, Althanstraße 14, Vienna, Austria

## **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  
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.

<sup>49</sup> **Materials and Methods**

<sup>50</sup> **Sampling**

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 decayed to an extent that no leaves could be retrieved (Najdek *et al.*, unpublished data).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

<sup>64</sup> **DNA Isolation**

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

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. The sequences obtained in this study have been submitted to the European Nucleotide Archive (ENA) under accession numbers **TO BE ADDED LATER!**.

### Sequence Analysis

Obtained sequences were analyzed 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](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 mothur were exported, aligned 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 and Peter Steinberg *et*  
<sup>166</sup> *al.* (2011) using SEQUEST-HIT engines and validated with Percolator in Proteome Discoverer 2.1  
<sup>167</sup> (Thermo Fisher Scientific, USA). The target-decoy approach was used to reduce the probability  
<sup>168</sup> of false peptide identification. Results whose false discovery rate at the peptide level was <1 %  
<sup>169</sup> were kept. For protein identification a minimum of two peptides and one unique peptide were  
<sup>170</sup> required. For protein quantification, a chromatographic peak area-based free quantitative method  
<sup>171</sup> was applied.

<sup>172</sup> **Confocal Microscopy**

<sup>173</sup> Host leaves and thalli from DNA and protein isolation steps were washed seven times in  
<sup>174</sup> deionized water and fixed with formaldehyde (final concentration ~ 3 %). In addition, nontreated  
<sup>175</sup> leaves and thalli, washed three times in ASW to remove loosely attached microbial cells, were  
<sup>176</sup> fixed in the same concentration of formaldehyde and used as a positive control. For long therm  
<sup>177</sup> storage, fixed leaves and thalli were immersed in a mixture of phosphate-buffered saline (PBS)  
<sup>178</sup> and ethanol (1:1) and stored at -20 °C. Treated and untreated leaves and thalli segments were  
<sup>179</sup> stained in a 2 × solution of SYBR Green I and examined under a Leica TCS SP8 X FLIM confocal  
<sup>180</sup> microscope (Leica Microsystems, Germany).

<sup>181</sup> **Results**

<sup>182</sup> **Discussion**

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

184 **References**

- 185 Allaire, J.J., Xie, Y., McPherson, J., Luraschi, J., Ushey, K., Atkins, A., et al. (2019)
- 186 rmarkdown: Dynamic Documents for R.
- 187 Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and in situ
- 188 detection of individual microbial cells without cultivation. *Microbiological reviews* **59**: 143–169.
- 189 Andersson, A.F., Riemann, L., and Bertilsson, S. (2010) Pyrosequencing reveals contrasting
- 190 seasonal dynamics of taxa within Baltic Sea bacterioplankton communities. *The ISME journal* **4**:
- 191 171–181.
- 192 Apprill, A., McNally, S., Parsons, R., and Weber, L. (2015) Minor revision to V4 region
- 193 SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic*
- 194 *Microbial Ecology* **75**: 129–137.
- 195 Burke, C., Kjelleberg, S., and Thomas, T. (2009) Selective extraction of bacterial DNA from
- 196 the surfaces of macroalgae. *Applied and environmental microbiology* **75**: 252–256.
- 197 Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., and Thomas, T. (2011) Bacterial
- 198 community assembly based on functional genes rather than species. *Proceedings of the National*
- 199 *Academy of Sciences of the United States of America* **108**: 14288–14293.
- 200 Burke, C., Thomas, T., Lewis, M., Steinberg, P., and Kjelleberg, S. (2011) Composition,
- 201 uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*.
- 202 *The ISME journal* **5**: 590–600.
- 203 Cai, X., Gao, G., Yang, J., Tang, X., Dai, J., Chen, D., and Song, Y. (2014) An ultrasonic
- 204 method for separation of epiphytic microbes from freshwater submerged macrophytes. *Journal of*
- 205 *basic microbiology* **54**: 758–761.

206 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al.  
207 (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq  
208 platforms. *The ISME Journal* **6**: 1621–1624.

209 Chourey, K., Jansson, J., VerBerkmoes, N., Shah, M., Chavarria, K.L., Tom, L.M., et al.  
210 (2010) Direct Cellular Lysis/Protein Extraction Protocol for Soil Metaproteomics. *Journal of*  
211 *Proteome Research* **9**: 6615–6622.

212 Egan, S., Harder, T., Burke, C., Steinberg, P., Kjelleberg, S., and Thomas, T. (2013) The  
213 seaweed holobiont: understanding seaweed-bacteria interactions. *FEMS microbiology reviews* **37**:  
214 462–476.

215 Gilbert, J.A., Field, D., Swift, P., Newbold, L., Oliver, A., Smyth, T., et al. (2009) The  
216 seasonal structure of microbial communities in the Western English Channel. *Environmental*  
217 *Microbiology* **11**: 3132–3139.

218 Gross, E.M., Feldbaum, C., and Graf, A. (2003) Epiphyte biomass and elemental composition  
219 on submersed macrophytes in shallow eutrophic lakes. *Hydrobiologia* **506-509**: 559–565.

220 Hultman, J., Waldrop, M.P., Mackelprang, R., David, M.M., McFarland, J., Blazewicz, S.J., et  
221 al. (2015) Multi-omics of permafrost, active layer and thermokarst bog soil microbiomes. *Nature*  
222 **521**: 208–212.

223 Jiang, Y.-F., Ling, J., Dong, J.-D., Chen, B., Zhang, Y.-Y., Zhang, Y.-Z., and Wang, Y.-S.  
224 (2015) Illumina-based analysis the microbial diversity associated with Thalassia hemprichii in  
225 Xincun Bay, South China Sea. *Ecotoxicology (London, England)* **24**: 1548–56.

226 Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013)  
227 Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon  
228 sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental*  
229 *microbiology* **79**: 5112–5120.

- 230 Longford, S., Tujula, N., Crocetti, G., Holmes, A., Holmström, C., Kjelleberg, S., et al. (2007)
- 231 Comparisons of diversity of bacterial communities associated with three sessile marine eukaryotes.
- 232 *Aquatic Microbial Ecology* **48**: 217–229.
- 233 Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, et al. (2004) ARB:
- 234 a software environment for sequence data. *Nucleic Acids Research* **32**: 1363–1371.
- 235 Massana, R., Murray, A.E., Preston, C.M., and DeLong, E.F. (1997) Vertical distribution and
- 236 phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. *Applied*
- 237 *and environmental microbiology* **63**: 50–56.
- 238 Nõges, T., Luup, H., and Feldmann, T. (2010) Primary production of aquatic macrophytes
- 239 and their epiphytes in two shallow lakes (Peipsi and Võrtsjärv) in Estonia. *Aquatic Ecology* **44**:
- 240 83–92.
- 241 Parada, A.E., Needham, D.M., and Fuhrman, J.A. (2016) Every base matters: assessing small
- 242 subunit rRNA primers for marine microbiomes with mock communities, time series and global
- 243 field samples. *Environmental Microbiology* **18**: 1403–1414.
- 244 Pruesse, E., Peplies, J., and Glöckner, F.O. (2012) SINA: accurate high-throughput multiple
- 245 sequence alignment of ribosomal RNA genes. *Bioinformatics (Oxford, England)* **28**: 1823–1829.
- 246 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013) The SILVA
- 247 ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic*
- 248 *acids research* **41**: D590–6.
- 249 R Core Team (2019) R: A Language and Environment for Statistical Computing, Vienna,
- 250 Austria: R Foundation for Statistical Computing.
- 251 Richter-Heitmann, T., Eickhorst, T., Knauth, S., Friedrich, M.W., and Schmidt, H. (2016)
- 252 Evaluation of Strategies to Separate Root-Associated Microbial Communities: A Crucial Choice

- 253 in Rhizobiome Research. *Frontiers in Microbiology* **7**: 773.
- 254 Schloss, P.D., Girard, R.A., Martin, T., Edwards, J., and Thrash, J.C. (2016) Status of the  
255 Archaeal and Bacterial Census: an Update. *mBio* **7**: e00201–16.
- 256 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al.  
257 (2009) Introducing mothur: open-source, platform-independent, community-supported software  
258 for describing and comparing microbial communities. *Applied and environmental microbiology*  
259 **75**: 7537–7541.
- 260 Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses  
261 with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.
- 262 Staufenberger, T., Thiel, V., Wiese, J., and Imhoff, J.F. (2008) Phylogenetic analysis of  
263 bacteria associated with *Laminaria saccharina*. *FEMS Microbiology Ecology* **64**: 65–77.
- 264 Su, C., Lei, L., Duan, Y., Zhang, K.-Q., and Yang, J. (2012) Culture-independent methods  
265 for studying environmental microorganisms: methods, application, and perspective. *Applied*  
266 *microbiology and biotechnology* **93**: 993–1003.
- 267 Uku, J., Björk, M., Bergman, B., and Díez, B. (2007) Characterization and comparison of  
268 prokaryotic epiphytes associated with three East African seagrasses. *Journal of Phycology* **43**:  
269 768–779.
- 270 Weidner, S., Arnold, W., and Puhler, A. (1996) Diversity of uncultured microorganisms  
271 associated with the seagrass *Halophila stipulacea* estimated by restriction fragment length  
272 polymorphism analysis of PCR-amplified 16S rRNA genes. *Applied and environmental*  
273 *microbiology* **62**: 766–771.
- 274 Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D., François, R., et al. (2019)  
275 Welcome to the tidyverse. *Journal of Open Source Software* **4**: 1686.

- 276 Wiśniewski, J.R., Zougman, A., Nagaraj, N., and Mann, M. (2009) Universal sample  
277 preparation method for proteome analysis. *Nature Methods* **6**: 359–362.
- 278 Xie, Y. (2015) Dynamic Documents with {R} and knitr, 2nd ed. Boca Raton, Florida:  
279 Chapman; Hall/CRC.
- 280 Xie, Y. (2014) knitr: A Comprehensive Tool for Reproducible Research in {R}. In  
281 *Implementing reproducible computational research*. Stodden, V., Leisch, F., and Peng, R.D. (eds).  
282 Chapman; Hall/CRC.
- 283 Xie, Y. (2019a) knitr: A General-Purpose Package for Dynamic Report Generation in R.
- 284 Xie, Y. (2019b) tinytex: Helper Functions to Install and Maintain 'TeX Live', and Compile  
285 'LaTeX' Documents.
- 286 Xie, Y., Allaire, J.J., and Grolemund, G. (2018) R Markdown: The Definitive Guide, Boca  
287 Raton, Florida: Chapman; Hall/CRC.
- 288 Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014) The SILVA  
289 and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic acids research* **42**:  
290 D643–8.
- 291 Zhu, H. (2019) kableExtra: Construct Complex Table with 'kable' and Pipe Syntax.

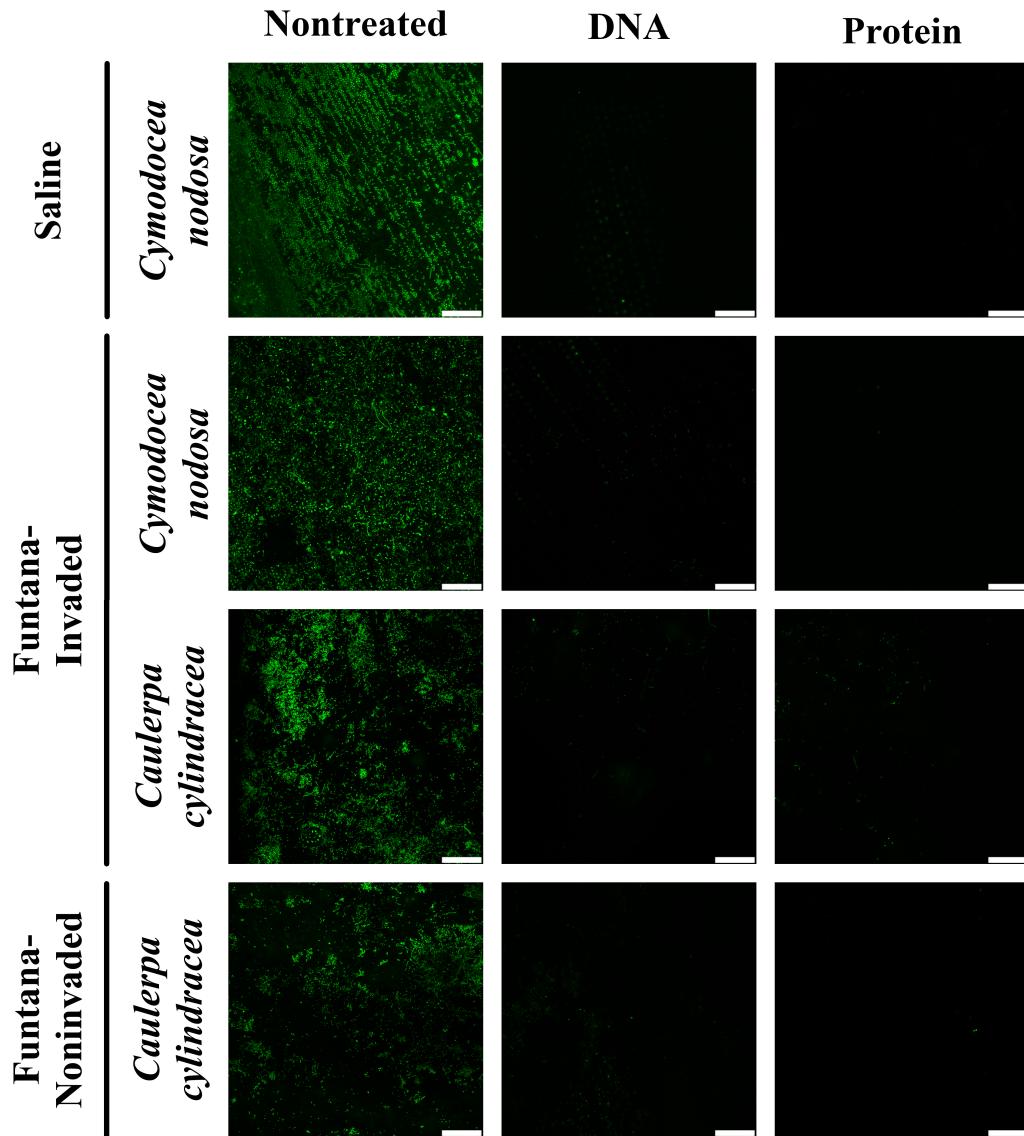
292 **Figure Captions**

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

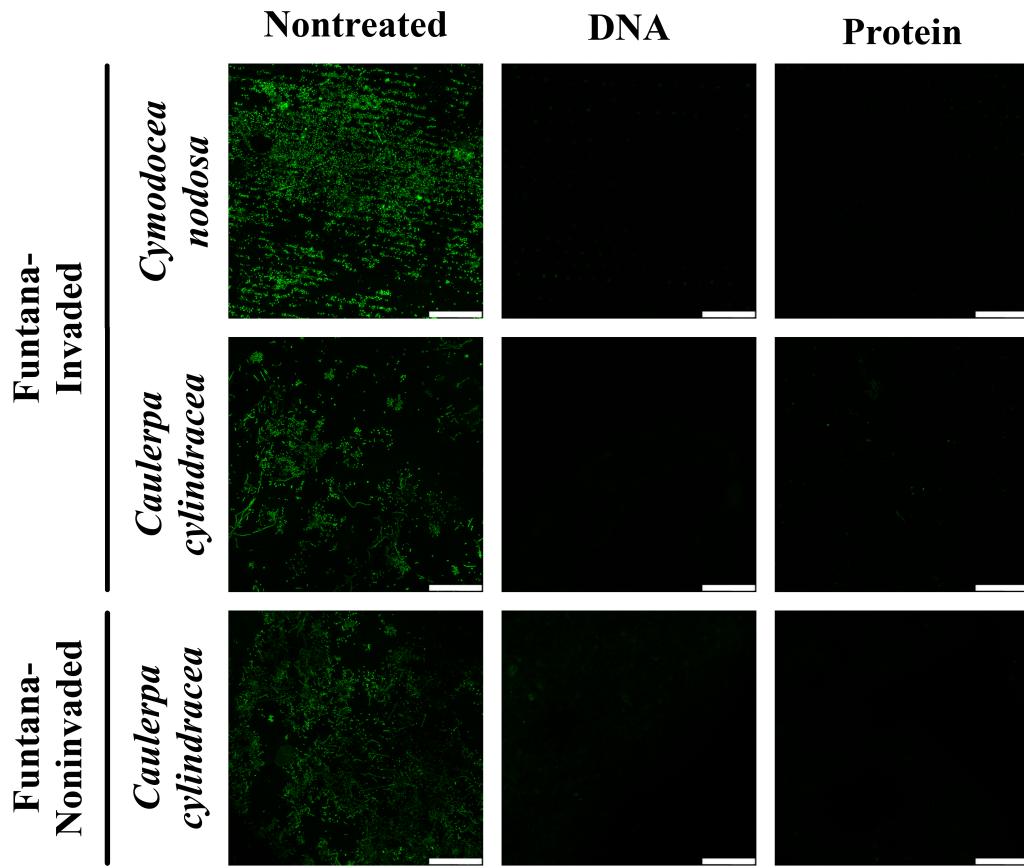
296 **Fig. 2.** Confocal microscope images of *Cymodocea nodosa* and *Caulerpa cylindracea* surfaces  
297 from different locations (Funtana-Invaded and Funtana-Noninvaded) sampled on 19 June 2018  
298 and stained with SYBR Green I. Scale bar in all images is 60 µm.

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

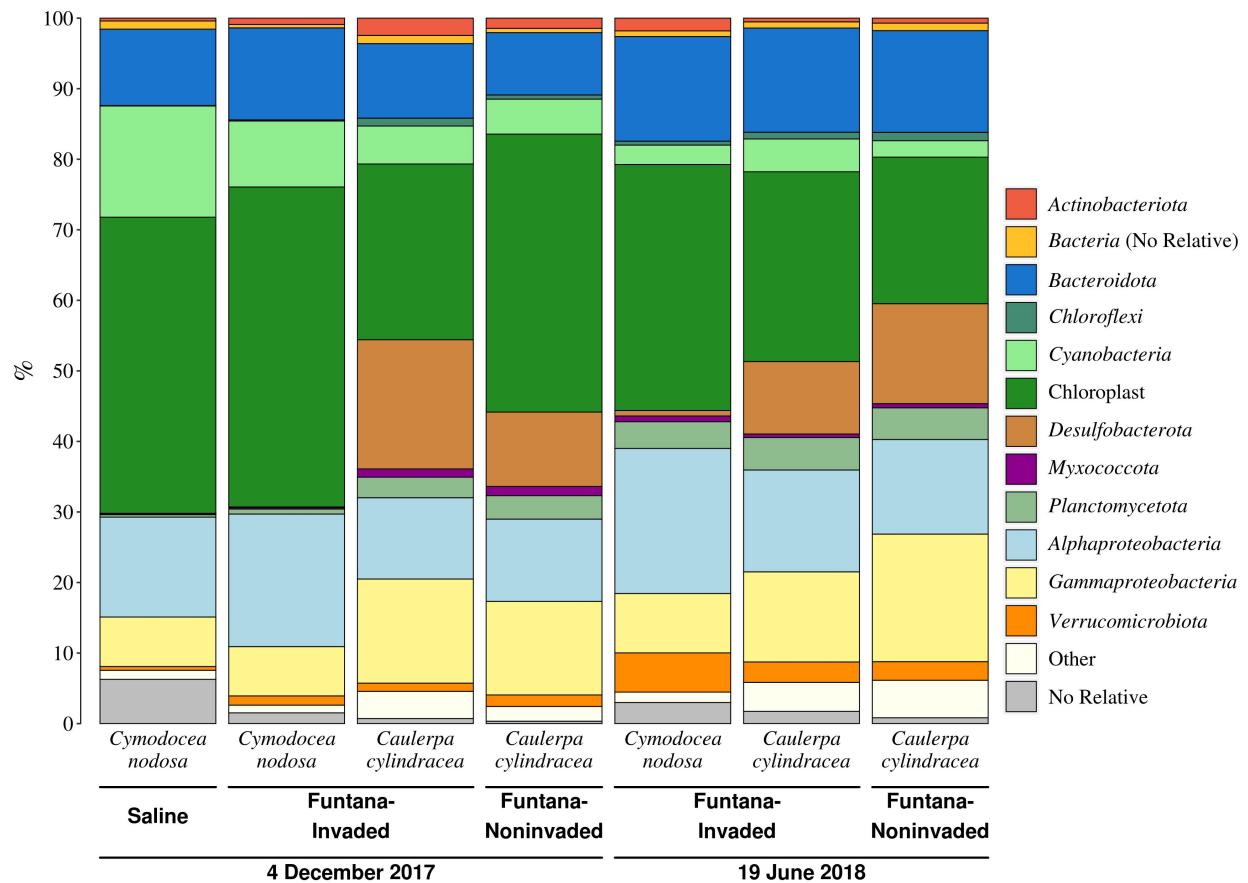
303 **Figures**



**Fig. 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 µm.



**Fig. 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.



**Fig. 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).