

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 form 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 **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⁻¹) and the mixture was incubated at
72 37 °C for 30 min. Subsequently, proteinase K (final concentration 0.5 mg ml⁻¹) 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°C and centrifuging at $16,000 \times g$ and 4°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°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 μl 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°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 μl
96 reactions of which each contained: 1 \times Q5 Reaction Buffer , 0.2 mm of dNTPmix, 0.7 mg ml^{-1}
97 BSA (Bovine Serum Albumin), 0.2 μM 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
111 for a positive control a mock community composed of evenly mixed DNA material originating
112 from 20 bacterial strains (ATCC MSA-1002, ATCC, USA) was used. Sequences obtained in
113 this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under
114 accession numbers SAMEA6786270, SAMEA6648792 – SAMEA6648794, SAMEA6648809 –
115 SAMEA6648811.

116 Sequence analysis

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

¹²⁴ reclassified using mothur and the RDP (Ribosomal Database Project; <http://rdp.cme.msu.edu/>)
¹²⁵ training set (version 16) reference files adapted for mothur (Cole *et al.*, 2014). In comparison
¹²⁶ to SILVA, RDP allows a more detailed classification of chloroplast sequences. Pipeline data
¹²⁷ processing and visualization was done using R (version 3.6.0) (R Core Team, 2019), package
¹²⁸ tidyverse (version 1.3.0) (Wickham *et al.*, 2019) and multiple other packages (Xie, 2014, 2015,
¹²⁹ 2019b, 2019a, 2020; Neuwirth, 2014; Xie *et al.*, 2018; Allaire *et al.*, 2019; Zhu, 2019). The
¹³⁰ detailed analysis procedure including the R Markdown file for this paper are available as a GitHub
¹³¹ repository (https://github.com/mkorlevic/Korlevic_SelectiveRemoval_EnvironMicrobiol_2020).
¹³² Based on the ATCC MSA-1002 mock community included in the analysis a sequencing error rate
¹³³ of 0.009 % was determined, which is in line with previously reported values for next-generation
¹³⁴ sequencing data (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative control
¹³⁵ processed together with the samples yielded only 2 sequences after sequence quality curation.

¹³⁶ Protein isolation

¹³⁷ Proteins were isolated according to the protocol for isolation from soil described in Chourey
¹³⁸ *et al.* (2010) and modified by Hultman *et al.* (2015). These protocols were further modified and
¹³⁹ adapted for protein isolation from microbial communities form macrophyte surfaces as described
¹⁴⁰ below. 20 ml of protein extraction buffer (4 % SDS, 100 mm Tris-HCl [pH 8.0]) was added to 5
¹⁴¹ g wet weight of leaves or 10 g wet weight of thalli. The mixture was incubated in boiling water
¹⁴² for 5 min, vortexed for 10 min and incubated again in boiling water for 5 min. After a brief vortex
¹⁴³ the lysate was transferred to a clean tube separating the host leaves or thalli from the mixture
¹⁴⁴ containing lyzed epiphytic cells. Dithiothreitol (DTT; final concentration 24 mM) was added and
¹⁴⁵ proteins were precipitated with chilled 100 % trichloroacetic acid (TCA; final concentration 20 %)
¹⁴⁶ overnight at -20 °C. Precipitated proteins were centrifuged at 10,000 × g and 4 °C for 40 min. The
¹⁴⁷ obtained protein pellet was washed three times with chilled acetone. During the first washing step
¹⁴⁸ the pellet was transferred to a clean 1.5 ml tube. After each washing step samples were centrifuged

¹⁴⁹ at 20,000 × g and 4 °C for 5 min. Dried pellets were stored at –80 °C until further analysis.

¹⁵⁰ **Metaproteomics**

¹⁵¹ Isolated proteins were whole trypsin digested using the FASP (filter-aided sample preparation)
¹⁵² Protein Digestion Kit (Expedeon, UK) according to the manufacturer's instructions (Wiśniewski
¹⁵³ *et al.*, 2009) with small modifications. Before loading the solution to the column, protein pellets
¹⁵⁴ were solubilized in a urea sample buffer included in the kit amended with DTT (final concentration
¹⁵⁵ 100 mM) for 45 min at room temperature and centrifuged at 20,000 × g for 2 – 5 min at room
¹⁵⁶ temperature to remove larger particles. The first washing step after protein solution loading was
¹⁵⁷ repeated twice. In addition, centrifugation steps were prolonged if the column was clogged.
¹⁵⁸ Trypsin digestion was performed on column filters at 37 °C overnight for 18 h. The final filtrate
¹⁵⁹ containing digested proteins was acidified with 1 % (final concentration) trifluoroacetic acid,
¹⁶⁰ freezed at –80 °C for 15 min, lyophilized and sent to the Vienna Metabolomics Center (University
¹⁶¹ of Vienna) for metaproteomic analysis. Peptides were resuspended in 1 % (final concentration)
¹⁶² trifluoroacetic acid, desalted using the Pierce C18 Tips (Thermo Fisher Scientific, USA) according
¹⁶³ to the manufacturer's instructions and sequenced on a Q Exactive Hybrid Quadrupole-Orbitrap
¹⁶⁴ Mass Spectrometer (Thermo Fisher Scientific, USA). Obtained MS/MS spectra were searched
¹⁶⁵ against a protein database from metagenomic assembly published in Burke and Peter Steinberg *et*
¹⁶⁶ *al.* (2011) using SEQUEST-HT engines and validated with Percolator in Proteome Discoverer 2.1
¹⁶⁷ (Thermo Fisher Scientific, USA). The target-decoy approach was used to reduce the probability
¹⁶⁸ of false peptide identification. Results whose false discovery rate at the peptide level was <1 %
¹⁶⁹ were kept. For protein identification a minimum of two peptides and one unique peptide were
¹⁷⁰ required. For protein quantification, a chromatographic peak area-based free quantitative method
¹⁷¹ was applied.

¹⁷² **Confocal microscopy**

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

¹⁸¹ **Acknowledgements**

182 **References**

- 183 Allaire, J.J., Xie, Y., McPherson, J., Luraschi, J., Ushey, K., Atkins, A., et al. (2019)
- 184 Rmarkdown: Dynamic documents for R.
- 185 Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and *in situ*
186 detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143–169.
- 187 Andersson, A.F., Riemann, L., and Bertilsson, S. (2010) Pyrosequencing reveals contrasting
188 seasonal dynamics of taxa within Baltic Sea bacterioplankton communities. *ISME J* **4**: 171–181.
- 189 Apprill, A., McNally, S., Parsons, R., and Weber, L. (2015) Minor revision to V4 region SSU
190 rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb*
191 *Ecol* **75**: 129–137.
- 192 Burke, C., Kjelleberg, S., and Thomas, T. (2009) Selective extraction of bacterial DNA from
193 the surfaces of macroalgae. *Appl Environ Microbiol* **75**: 252–256.
- 194 Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., and Thomas, T. (2011) Bacterial
195 community assembly based on functional genes rather than species. *Proc Natl Acad Sci U S A*
196 **108**: 14288–14293.
- 197 Burke, C., Thomas, T., Lewis, M., Steinberg, P., and Kjelleberg, S. (2011) Composition,
198 uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*.
199 *ISME J* **5**: 590–600.
- 200 Cai, X., Gao, G., Yang, J., Tang, X., Dai, J., Chen, D., and Song, Y. (2014) An ultrasonic
201 method for separation of epiphytic microbes from freshwater submerged macrophytes. *J Basic*
202 *Microbiol* **54**: 758–761.
- 203 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al.

204 (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq
205 platforms. *ISME J* **6**: 1621–1624.

206 Chourey, K., Jansson, J., VerBerkmoes, N., Shah, M., Chavarria, K.L., Tom, L.M., et al.
207 (2010) Direct cellular lysis/protein extraction protocol for soil metaproteomics. *J Proteome Res* **9**:
208 6615–6622.

209 Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., et al. (2014) Ribosomal
210 Database Project: Data and tools for high throughput rRNA analysis. *Nucleic Acids Res* **42**:
211 D633–D642.

212 Egan, S., Harder, T., Burke, C., Steinberg, P., Kjelleberg, S., and Thomas, T. (2013) The
213 seaweed holobiont: Understanding seaweed-bacteria interactions. *FEMS Microbiol Rev* **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. *Environ Microbiol*
217 **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* **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. *Appl Environ Microbiol* **79**:
²²⁹ 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 *Aquat Microb Ecol* **48**: 217–229.

²³³ Massana, R., Murray, A.E., Preston, C.M., and DeLong, E.F. (1997) Vertical distribution and
²³⁴ phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara Channel. *Appl*
²³⁵ *Environ Microbiol* **63**: 50–56.

²³⁶ Neuwirth, E. (2014) RColorBrewer: ColorBrewer palettes.

²³⁷ Nõges, T., Luup, H., and Feldmann, T. (2010) Primary production of aquatic macrophytes
²³⁸ and their epiphytes in two shallow lakes (Peipsi and Võrtsjärv) in Estonia. *Aquat Ecol* **44**: 83–92.

²³⁹ Parada, A.E., Needham, D.M., and Fuhrman, J.A. (2016) Every base matters: Assessing
²⁴⁰ small subunit rRNA primers for marine microbiomes with mock communities, time series and
²⁴¹ global field samples. *Environ Microbiol* **18**: 1403–1414.

²⁴² Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013) The SILVA
²⁴³ ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic*
²⁴⁴ *Acids Res* **41**: D590–D596.

²⁴⁵ R Core Team (2019) A language and environment for statistical computing, Vienna, Austria:
²⁴⁶ R Foundation for Statistical Computing.

²⁴⁷ Richter-Heitmann, T., Eickhorst, T., Knauth, S., Friedrich, M.W., and Schmidt, H. (2016)
²⁴⁸ Evaluation of strategies to separate root-associated microbial communities: A crucial choice in
²⁴⁹ rhizobiome research. *Front Microbiol* **7**: 773.

- 250 Schloss, P.D., Jenior, M.L., Koumpouras, C.C., Westcott, S.L., and Highlander, S.K. (2016)
- 251 Sequencing 16S rRNA gene fragments using the PacBio SMRT DNA sequencing system. *PeerJ*
- 252 **4:** e1869.
- 253 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al.
- 254 (2009) Introducing mothur: Open-source, platform-independent, community-supported software
- 255 for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537–7541.
- 256 Staufenberger, T., Thiel, V., Wiese, J., and Imhoff, J.F. (2008) Phylogenetic analysis of
- 257 bacteria associated with *Laminaria saccharina*. *FEMS Microbiol Ecol* **64:** 65–77.
- 258 Su, C., Lei, L., Duan, Y., Zhang, K.-Q., and Yang, J. (2012) Culture-independent methods for
- 259 studying environmental microorganisms: Methods, application, and perspective. *Appl Microbiol*
- 260 *Biotechnol* **93:** 993–1003.
- 261 Uku, J., Björk, M., Bergman, B., and Díez, B. (2007) Characterization and comparison of
- 262 prokaryotic epiphytes associated with three East African seagrasses. *J Phycol* **43:** 768–779.
- 263 Weidner, S., Arnold, W., and Puhler, A. (1996) Diversity of uncultured microorganisms
- 264 associated with the seagrass *Halophila stipulacea* estimated by restriction fragment length
- 265 polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl Environ Microbiol* **62:** 766–771.
- 266 Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D., François, R., et al. (2019)
- 267 Welcome to the tidyverse. *J Open Source Softw* **4:** 1686.
- 268 Wiśniewski, J.R., Zougman, A., Nagaraj, N., and Mann, M. (2009) Universal sample
- 269 preparation method for proteome analysis. *Nat Methods* **6:** 359–362.
- 270 Xie, Y. (2015) Dynamic Documents with R and knitr, 2nd ed. Boca Raton, Florida: Chapman
- 271 and Hall/CRC.
- 272 Xie, Y. (2014) Knitr: A comprehensive tool for reproducible research in R. In *Implementing*

²⁷³ *Reproducible Computational Research*. Stodden, V., Leisch, F., and Peng, R.D. (eds). New York:

²⁷⁴ Chapman and Hall/CRC, pp. 3–32.

²⁷⁵ Xie, Y. (2019a) Knitr: A general-purpose package for dynamic report generation in R.

²⁷⁶ Xie, Y. (2019b) TinyTeX: A lightweight, cross-platform, and easy-to-maintain LaTeX

²⁷⁷ distribution based on TeX Live. *TUGboat* **40**: 30–32.

²⁷⁸ Xie, Y. (2020) TinyTex: Helper functions to install and maintain 'TeX Live', and compile

²⁷⁹ 'LaTeX' documents.

²⁸⁰ Xie, Y., Allaire, J.J., and Grolemund, G. (2018) R Markdown: The Definitive Guide, 1st ed.

²⁸¹ Boca Raton, Florida: Chapman and Hall/CRC.

²⁸² Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014) The

²⁸³ SILVA and "All-Species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Res*

²⁸⁴ **42**: D643–D648.

²⁸⁵ Zhu, H. (2019) KableExtra: Construct complex table with 'kable' and pipe syntax.

286 **Figure Captions**

287 **Fig. 1.** Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of
288 Saline and the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 4 December
289 2017 and stained with SYBR Green I. Scale bar in all images is 60 µm.

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

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

297 **Fig. 4.** Taxonomic classification and relative contribution of chloroplast sequences from surfaces
298 of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the
299 Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting seasons (4 December
300 2017 and 19 June 2018).

301 **Figures**

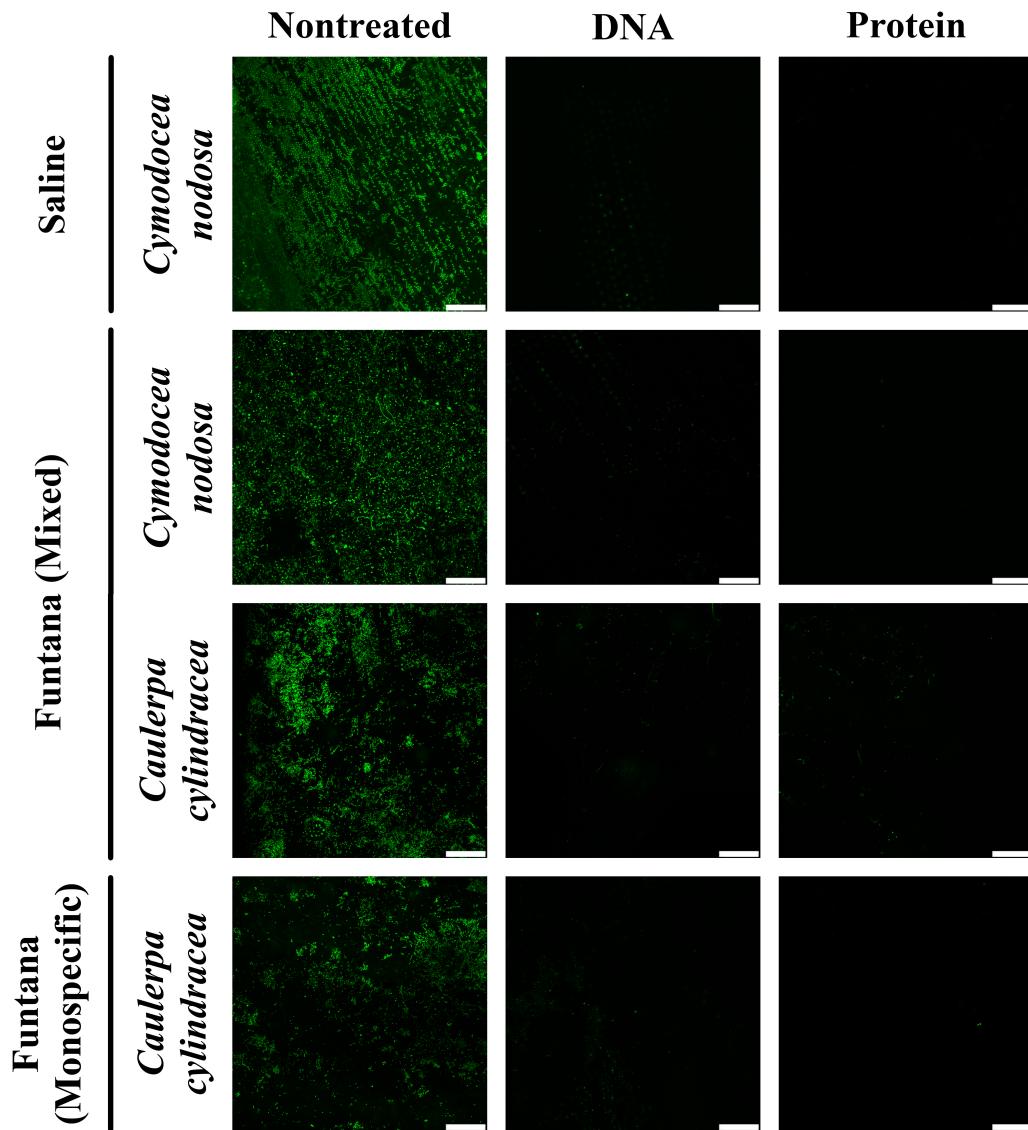


Fig. 1. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 4 December 2017 and stained with SYBR Green I. Scale bar in all images is 60 µm.

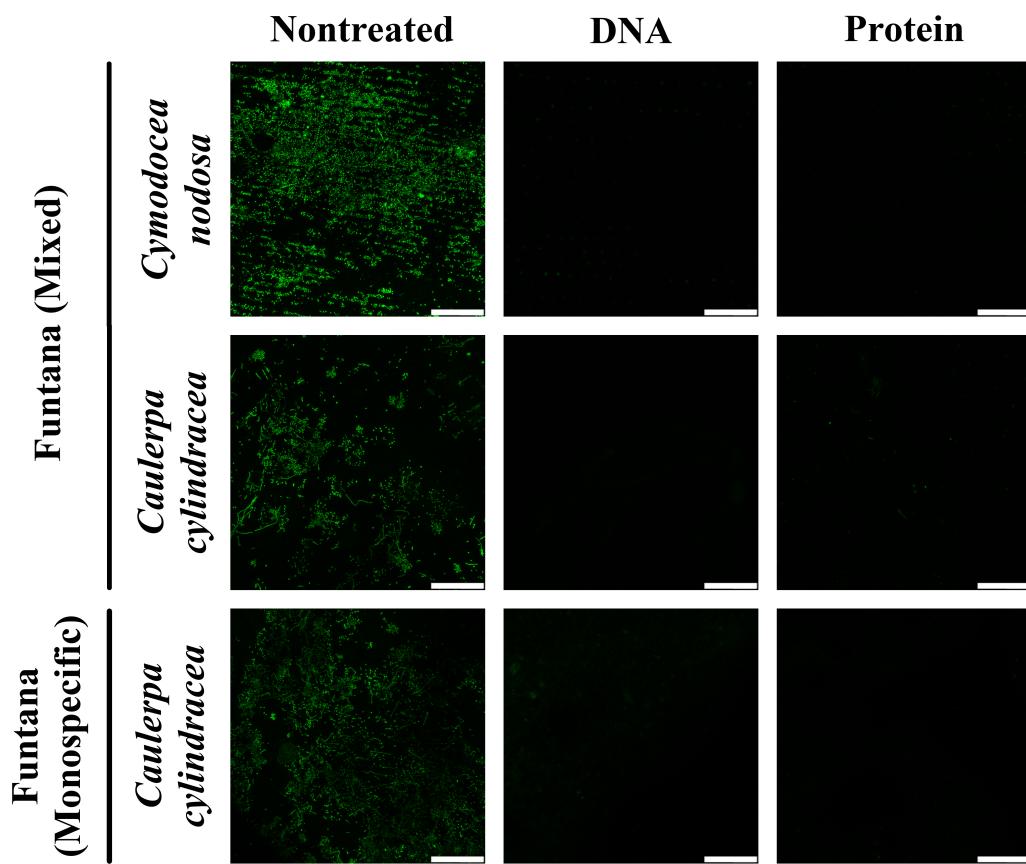


Fig. 2. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 19 June 2018 and stained with SYBR Green I. Scale bar in all images is 60 µm.

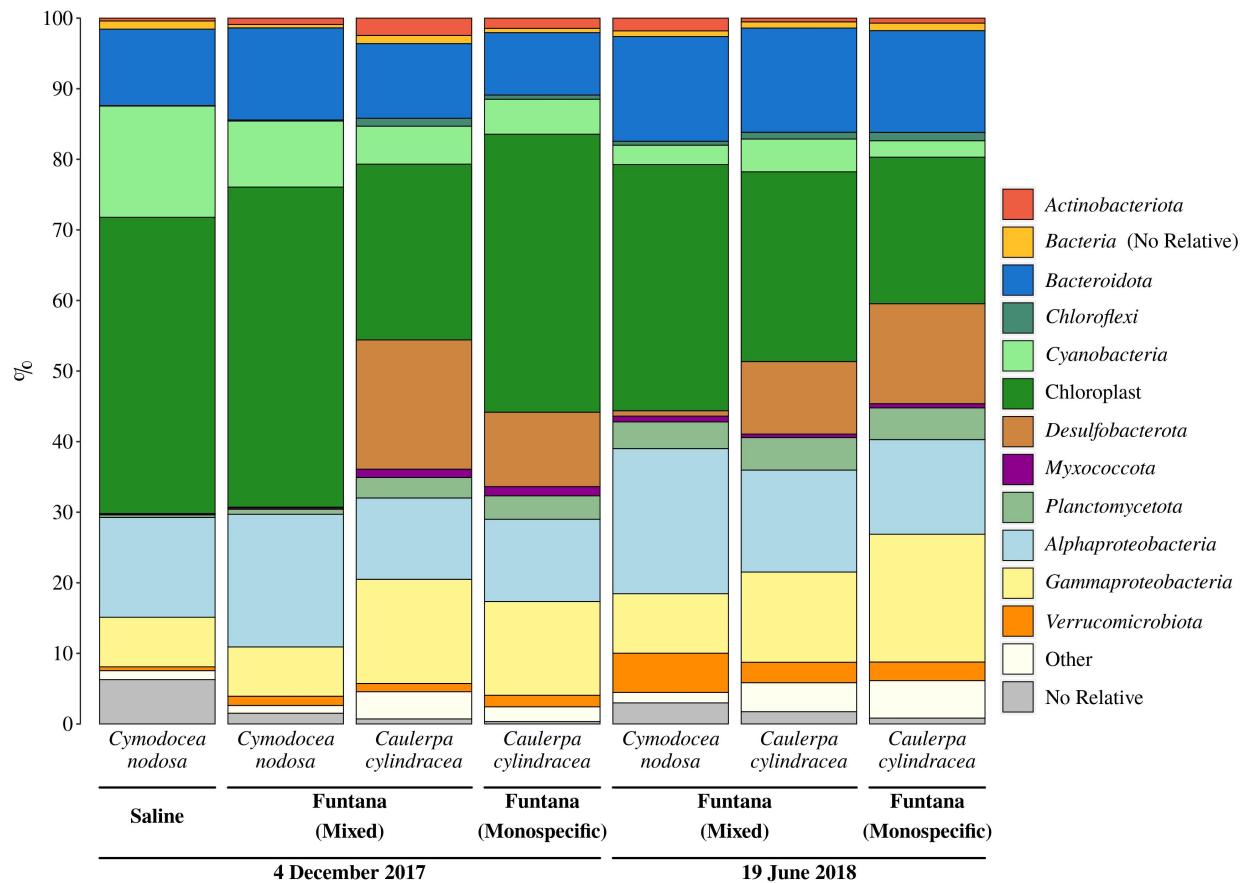


Fig. 3. Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial and archaeal sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting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

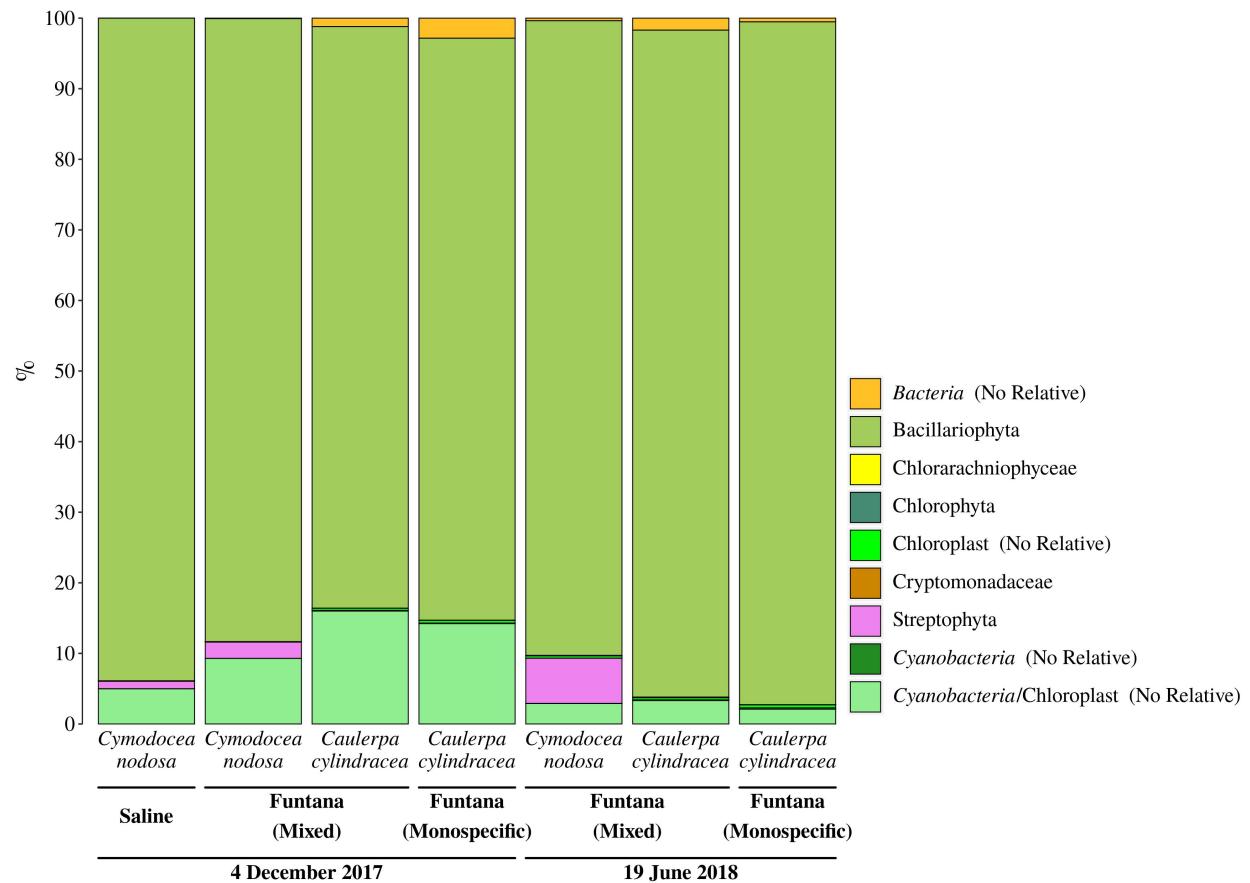


Fig. 4. Taxonomic classification and relative contribution of chloroplast sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting seasons (4 December 2017 and 19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