

Selective DNA and Protein Isolation from Marine Macrophyte Surfaces

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1 Abstract

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 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 mg ml^{-1}) and the mixture was incubated at
70 37°C for 30 min. Subsequently, proteinase K (final concentration 0.5 mg ml^{-1}) and SDS (final
71 concentration 1 %) were added and the samples were incubated at 55°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°C and centrifuging at $16,000 \times g$ and 4°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°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 μl 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°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 ($5'\text{-GTGYCAGCMGCCGCGTAA-3'}$) and
90 806R ($5'\text{-GGACTACNVGGTWTCTAAT-3'}$) 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 μl
94 reactions of which each contained: $1 \times$ Q5 Reaction Buffer , 0.2 mM of dNTPmix, 0.7 mg ml^{-1}
95 BSA (Bovine Serum Albumin), 0.2 μM 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)
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 132; <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

¹²² SILVA SSU Ref NR 99 database (release 132) and imported into ARB (version 6.0.6) (Ludwig *et*
¹²³ *al.*, 2004) for further phylogenetic analysis using the same database. Reference sequences close
¹²⁴ to imported ones were selected and used to calculate a phylogenetic tree using the Maximum
¹²⁵ Likelihood algorithm RAxML (version 7.0.3) with 1000 tree replicates (Stamatakis, 2006).
¹²⁶ Imported partial chloroplast sequences were added to the tree using the maximum parsimony
¹²⁷ criteria and not allowing changes to tree topology. Pipeline data processing and visualization was
¹²⁸ done using R (version 3.6.0) (R Core Team, 2019), package tidyverse (version 1.2.1) (Wickham
¹²⁹ *et al.*, 2019) and multiple other packages (Xie, 2014, 2015, 2019a, 2019b; Xie *et al.*, 2018; Zhu,
¹³⁰ 2019; Allaire *et al.*, 2019). The detailed analysis procedure including the R Markdown file for
¹³¹ this paper are available as a GitHub repository (**TO BE ADDED LATER!**). Based on the ATCC
¹³² MSA-1002 mock community included in the analysis a sequencing error rate of 0.009 % was
¹³³ determined, which is in line with previously reported values for next-generation sequencing data
¹³⁴ (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative control processed together
¹³⁵ 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, desalting 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 *et al.* (2011a) using
¹⁶⁶ SEQUEST-HIT engines and validated with Percolator in Proteome Discoverer 2.1 (Thermo Fisher
¹⁶⁷ Scientific, USA). The target-decoy approach was used to reduce the probability of false peptide
¹⁶⁸ identification. Results whose false discovery rate at the peptide level was <1 % were kept. For
¹⁶⁹ protein identification a minimum of two peptides and one unique peptide were required. For
¹⁷⁰ protein quantification, a chromatographic peak area-based free quantitative method was applied.

¹⁷¹ **Confocal Microscopy**

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

¹⁸⁰ **Results**

¹⁸¹ **Discussion**

¹⁸² **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**

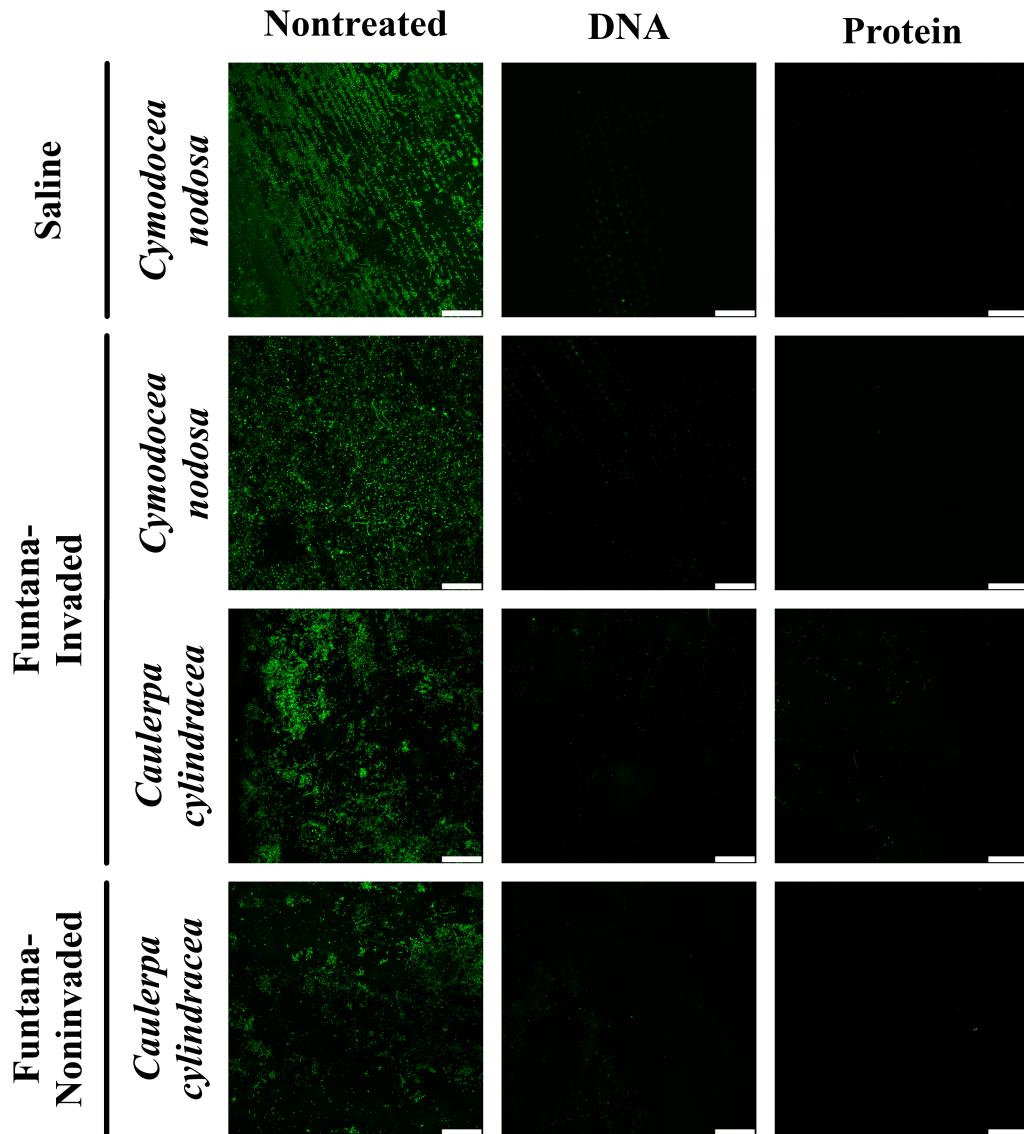


Figure 1. Confocal microscope images of *Cymodocea nodosa* and *Caulerpa cylindracea* surfaces from different locations (Saline, Funtana-Invaded and Funtana-Noninvaded) sampled on 4 December 2017 and stained with SYBR Green I. Scale bar in all images is 60 µm.

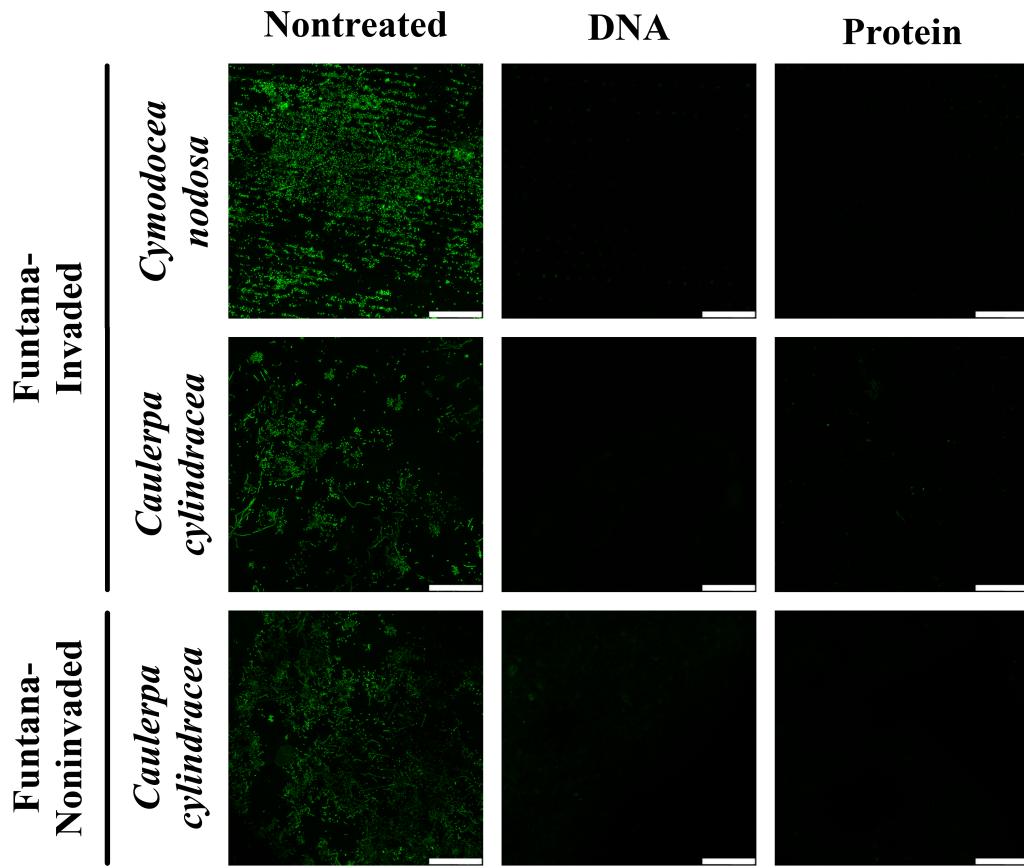


Figure 2. Confocal microscope images of *Cymodocea nodosa* and *Caulerpa cylindracea* surfaces from different locations (Funtana-Invaded and Funtana-Noninvaded) sampled on 19 June 2018 and stained with SYBR Green I. Scale bar in all images is 60 µm.

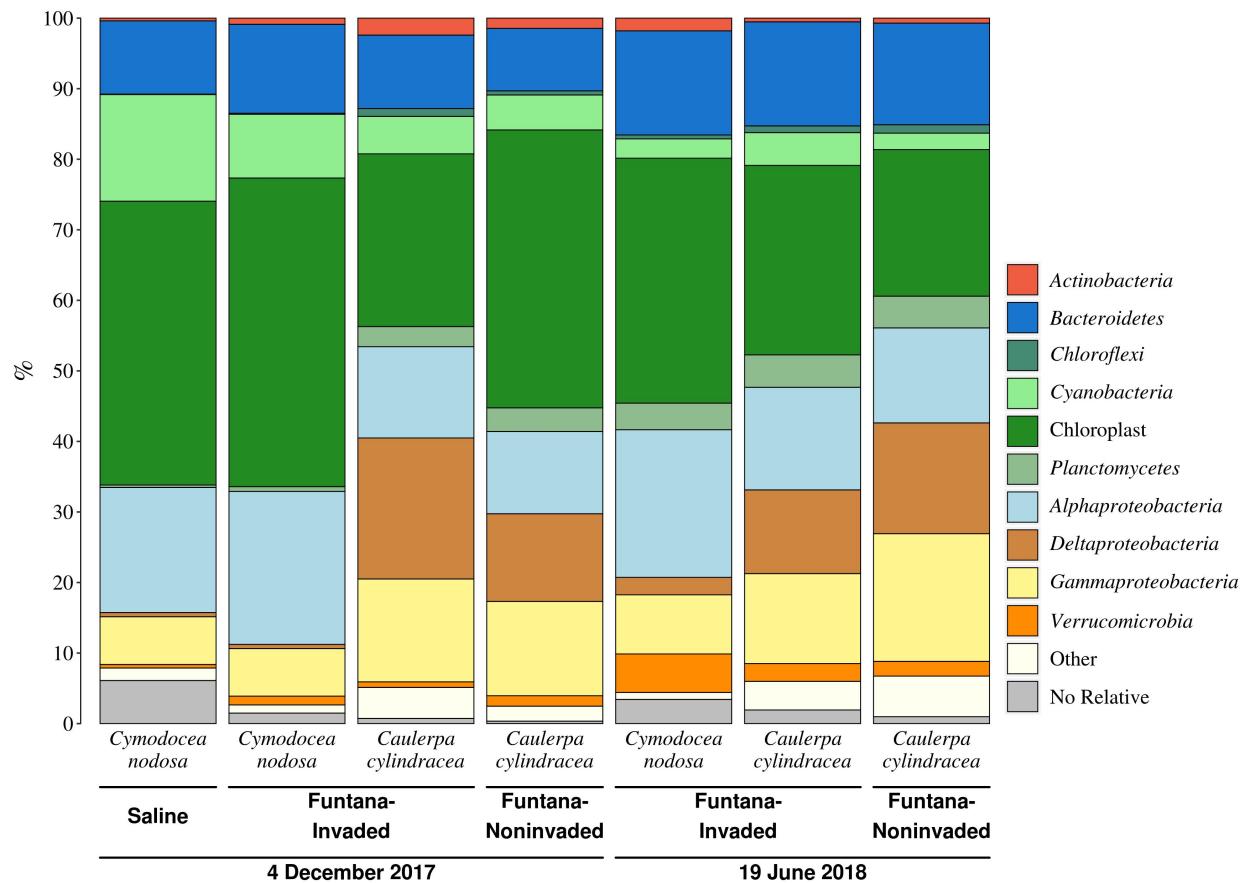


Figure 3. Taxonomic classification and relative contribution of the most abundant bacterial sequences from surfaces of two marine macrophytes (*Cymodocea nodosa* and *Caulerpa cylindracea*) sampled in different locations (Saline, Funtana-Invaded and Funtana-Noninvaded) and in two contrasting seasons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