

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'-GGACTACNVGGTWTCTAAT-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

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) (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

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

¹³⁸ Protein isolation

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

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

¹⁵² **Metaproteomics**

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

¹⁷⁴ **Confocal microscopy**

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

¹⁸³ **Acknowledgements**

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292 **Figure Captions**

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

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

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

303 **Figures**

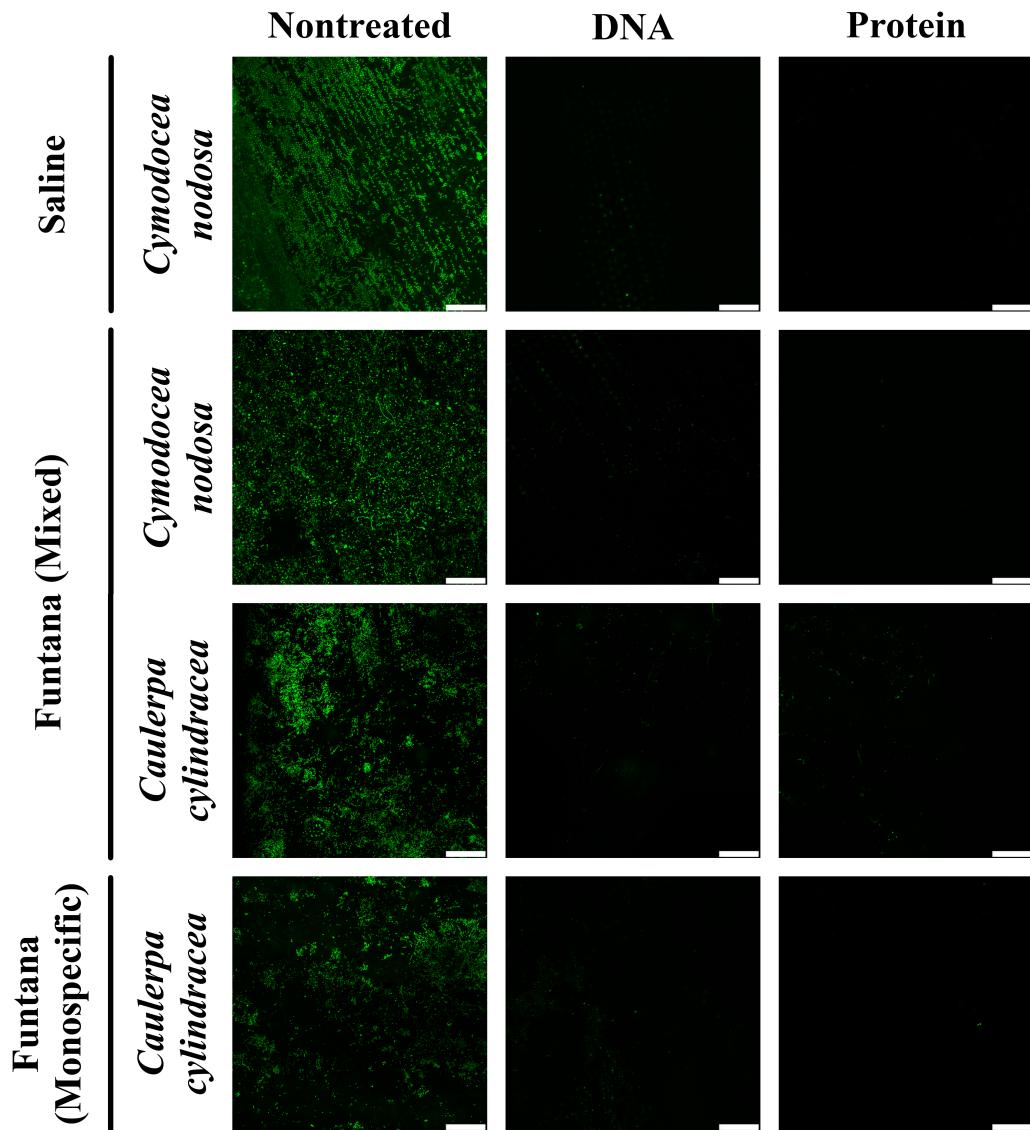


Fig. 1. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 4 December 2017 and stained with SYBR Green I. Scale bar in all images is 60 µm.

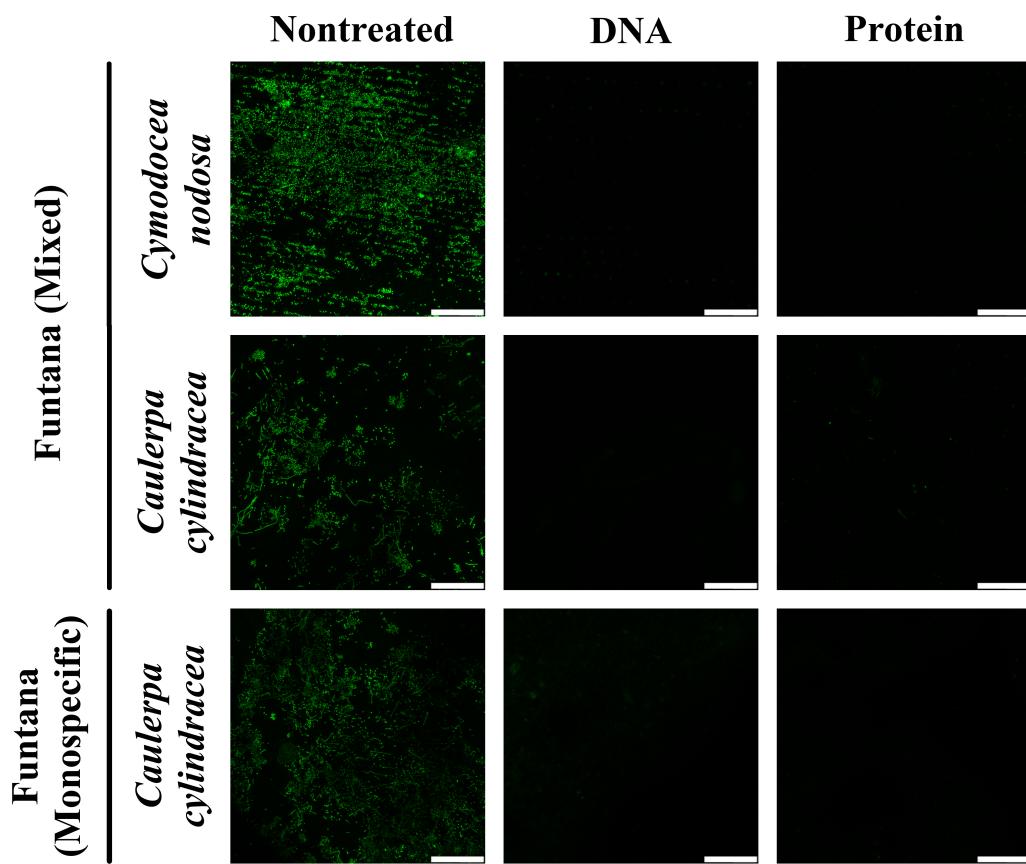


Fig. 2. Confocal microscope images of *C. nodosa* and *C. cylindracea* surfaces from the Bay of Funtana (Mixed and Monospecific Settlements) sampled on 19 June 2018 and stained with SYBR Green I. Scale bar in all images is 60 µm.

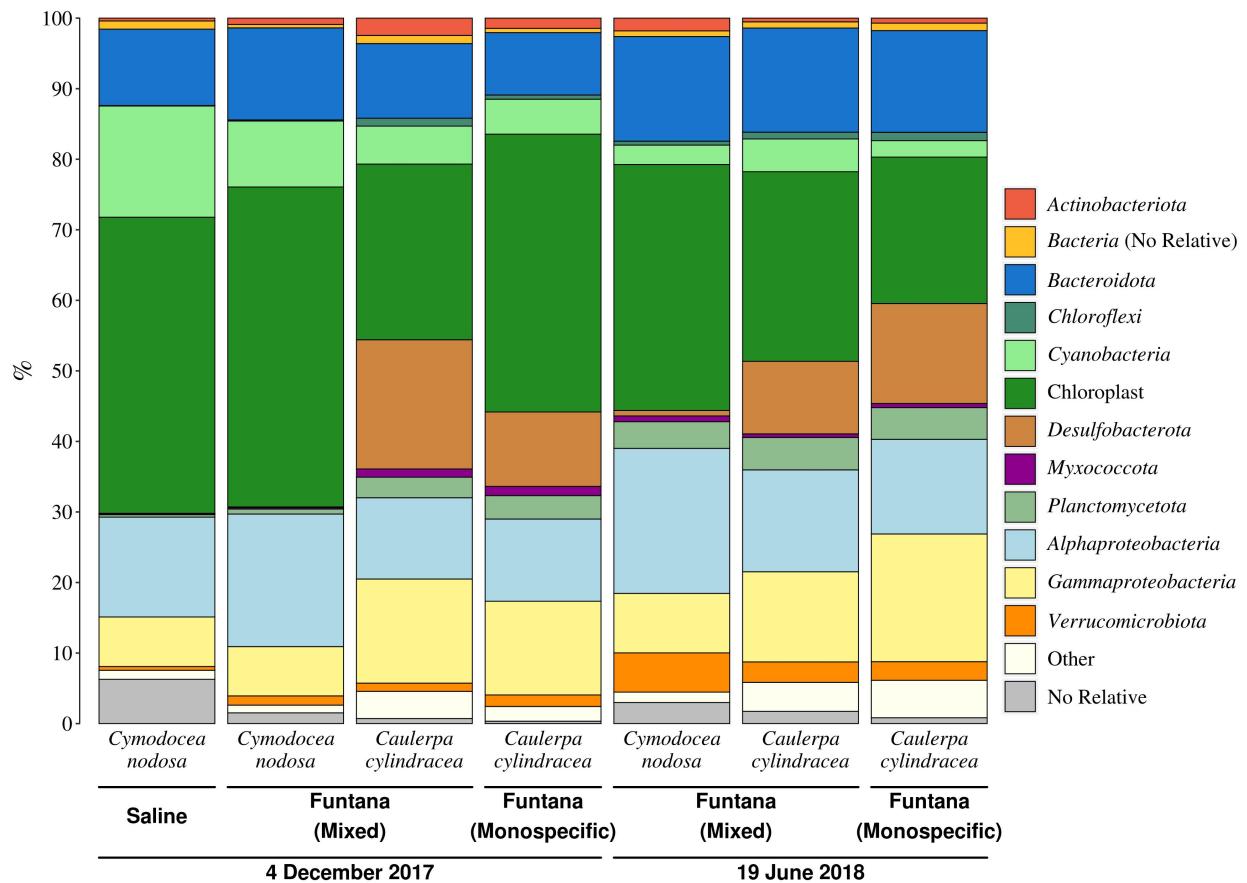


Fig. 3. Taxonomic classification and relative contribution of the most abundant (> 1 %) bacterial and archaeal sequences from surfaces of two marine macrophytes (*C. nodosa* and *C. cylindracea*) sampled in the Bay of Saline and the Bay of Funtana (Mixed and Monospecific Settlements) and in two contrasting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