

# **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 al.*, 1997; Andersson *et al.*, 2010). In contrast, obtaining biological materials from microorganism  
12 inhabiting surfaces requires either a cell detachment procedure prior to isolation or the host material  
13 is coextracted together with the targeted material. Methods for separating microbial cells from the  
14 host include shaking of host tissue (Gross *et al.*, 2003; Nöges *et al.*, 2010), scraping of macrophyte  
15 surfaces (Uku *et al.*, 2007) or the application of ultrasonication (Weidner *et al.*, 1996; Cai *et al.*,  
16 2014). It was shown that shaking alone is not sufficient to remove microbial cells, at least from  
17 plant root surfaces (Richter-Heitmann *et al.*, 2016). Manual separation methods, such as scraping  
18 and brushing, are time consuming and subjective, as the detachment efficiency depends on host  
19 tissue and the person performing the procedure (Cai *et al.*, 2014). Ultrasonication was proposed as  
20 an alternative method as it is providing better results in terms of detachment efficiency (Cai *et al.*,  
21 2014; Richter-Heitmann *et al.*, 2016). The downside of this procedure is that complete cell removal  
22 was still not obtained and tissue disruption was observed especially after the application of probe  
23 ultrasonication (Richter-Heitmann *et al.*, 2016). An alternative to these cell detachment procedures  
24 is the isolation of targeted epiphytic compounds together with host materials (Staufenberger *et al.*,  
25 2008; Jiang *et al.*, 2015). This procedure can lead to problems in the following processing  
26 steps such as mitochondrial and chloroplast 16S rRNA sequence contaminations from the host  
27

(Longford *et al.*, 2007; Staufenberger *et al.*, 2008). In addition, when performing metagenomics and metaproteomics host material can cause biased results towards more abundant host DNA and proteins.

An alternative to these procedures is a direct isolation of the targeted material by incubating macrophyte tissues in an extraction buffer. After the incubation is done the undisrupted host tissue is removed and the isolation procedure continues omitting host material contaminations. To our knowledge the only procedure describing a direct and selective epiphytic DNA isolation from the surfaces of marine macrophytes was provided by Burke *et al.* (2009). In contrast to previously described methods this protocol enables an almost complete removal of the surface community and was used for 16S rRNA gene clone libraries construction (Burke *et al.*, 2011b) and metagenomes sequencing (Burke *et al.*, 2011a). This method, thought providing a selective isolation procedure, is using in the extraction buffer a rapid multienzyme cleaner (3M) that is not worldwide available and whose composition is not known (Burke *et al.*, 2009). Also to our knowledge, no selective isolation protocol for proteins from epiphytic communities inhabiting marine macrophytes was established.

In the present study, we adapted a protocol widely used for DNA isolation from filters (Massana *et al.*, 1997) and a protocol used for protein isolation from sediments (Chourey *et al.*, 2010; Hultman *et al.*, 2015) for selective extractions of DNA and proteins from epiphytic communities inhabiting the surfaces of two marine macrophytes: the seagrass *Cymodocea nodosa* and the alga *Caulerpa cylindracea*. In addition, we tested the removal efficiency of the protocol and the suitability of obtained DNA and proteins for 16S rRNA sequencing and metaproteomics.

## Materials and Methods

### Sampling

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

### DNA Isolation, Illumina 16S rRNA Sequencing and Sequences Analysis

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

incubation, tubes were vortexed for 10 min and the mixture containing lysed epiphytic cells was separated from host leaves or thalli by transferring the solution into a clean tube. The lysate was extracted twice with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1; pH 8) and once with chloroform:isoamyl alcohol (24:1). After each organic solvent mixture addition tubes were slightly vortexed and centrifuged at  $4,500 \times g$  for 10 min. Following each centrifugation aqueous phases were retrieved. After the final extraction 1/10 of chilled 3 M sodium acetate (pH 5.2) was added. DNA was precipitated by adding 1 volume of chilled isopropanol, incubating the mixtures overnight at  $-20\text{ }^{\circ}\text{C}$  and centrifuging at  $16,000 \times g$  and  $4\text{ }^{\circ}\text{C}$  for 20 min. The pellet was washed twice with 1 ml of chilled isopropanol and centrifuged after each washing step at  $20,000 \times g$  and  $4\text{ }^{\circ}\text{C}$  for 10 min. After the first washing step duplicate pellets from the same sample were pooled and transferred to a clean 1.5 ml tube. The dried pellet was resuspended in 100  $\mu\text{l}$  of deionized water.

An aliquot of isolated DNA was treated with RNase A (final concentration  $200\text{ }\mu\text{g ml}^{-1}$ ) for 2 h at  $37\text{ }^{\circ}\text{C}$ . The DNA concentration was determined using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, USA) according to the manufacturer's instructions and diluted to  $1\text{ ng}\mu\text{l}^{-1}$ . The V4 region of the 16S rRNA gene was amplified using a two-step PCR procedure. In the first PCR the 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers from the Earth Microbiome Project (<http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>) were used to amplify the target region (Caporaso *et al.*, 2012; Apprill *et al.*, 2015; Parada *et al.*, 2016). These primers contained on their 5' end a tagged sequence. Each sample was amplified in four parallel 25  $\mu\text{l}$  reactions of which each contained: 1  $\times$  Q5 Reaction Buffer, 0.2 mM of dNTPmix,  $0.7\text{ mg ml}^{-1}$  BSA (Bovine Serum Albumin), 0.2  $\mu\text{M}$  of forward and reverse primers, 0.5 U of Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) and 5 ng of DNA template. Cycling conditions were: initial denaturation at  $94\text{ }^{\circ}\text{C}$  for 3 min, 20 cycles of denaturation at  $94\text{ }^{\circ}\text{C}$  for 45 s, annealing at  $50\text{ }^{\circ}\text{C}$  for 60 s and elongation at  $72\text{ }^{\circ}\text{C}$  for 90 s, finalized by an elongation step at  $72\text{ }^{\circ}\text{C}$  for 10 min. The four parallel reactions volumes were pooled and PCR products were purified using the

99 GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's  
100 instructions and following the protocol that included isopropanol addition for better small DNA  
101 fragment yield. The column was eluted in 30 µl of deionized water. Purified PCR products were  
102 sent for Illumina MiSeq sequencing (2 × 250 bp) at IMG/M Laboratories, Martinsried, Germany.  
103 Before sequencing, at IMG/M the second PCR amplification of the two-step PCR procedure was  
104 performed using primers targeting the tagged region incorporated in the first PCR. In addition,  
105 these primers contained adapter and sample-specific index sequences. The second PCR was  
106 carried out for 8 cycles. Beside samples, a positive and negative control were sequenced. A  
107 negative control was comprised of four parallel PCR reactions without DNA template, while for a  
108 positive control a mock community composed of evenly mixed DNA material originating from 20  
109 bacterial strains (ATCC MSA-1002, ATCC, USA) was used. The sequences obtained in this study  
110 have been submitted to the European Nucleotide Archive (ENA) under accession numbers ?????.

```
111 ## Parsed with column specification:
112 ## cols(
113 ##   .default = col_double(),
114 ##   query = col_character(),
115 ##   reference = col_character()
116 ## )

117 ## See spec(...) for full column specifications.

118 ## Parsed with column specification:
119 ## cols(
120 ##   taxlevel = col_double(),
121 ##   rankID = col_character(),
122 ##   taxon = col_character(),
123 ##   daughterlevels = col_double(),
```

```

124 ## total = col_double(),
125 ## `40` = col_double(),
126 ## `41` = col_double(),
127 ## `42` = col_double(),
128 ## `43` = col_double(),
129 ## `61` = col_double(),
130 ## `62` = col_double(),
131 ## `63` = col_double(),
132 ## ATCC_1 = col_double(),
133 ## NC_1 = col_double()
134 ## )

```

135 Obtained sequences were analyzed on the computer cluster Isabella (University Computing  
136 Center, University of Zagreb) using mothur (version 1.43.0) (Schloss *et al.*, 2009) according  
137 to the MiSeq Standard Operating Procedure (MiSeq SOP; [https://mothur.org/wiki/MiSeq\\_SOP](https://mothur.org/wiki/MiSeq_SOP))  
138 (Kozich *et al.*, 2013) and recommendations given from the Riffomonas project to enhance data  
139 reproducibility (<http://www.riffomonas.org/>). For alignment and classification of sequences the  
140 SILVA SSU Ref NR 99 database (release 132; <http://www.arb-silva.de>) was used (Quast *et al.*,  
141 2013; Yilmaz *et al.*, 2014). Sequences classified as chloroplasts by mothur were exported, aligned  
142 using SILVA incremental aligner (SINA, version 1.6.0) (Pruesse *et al.*, 2012) against the same  
143 SILVA SSU Ref NR 99 database (release 132) and imported into ARB (version 6.0.6) (Ludwig  
144 *et al.*, 2004) for further phylogenetic analysis using the same database. Reference sequences close  
145 to imported ones were selected and used to calculate a phylogenetic tree using the Maximum  
146 Likelihood algorithm RAxML (version 7.0.3) with 1000 tree replicates (Stamatakis, 2006).  
147 Imported partial chloroplast sequences were added to the tree using the maximum parsimony  
148 criteria and not allowing changes to tree topology. Pipeline data processing and visualization was  
149 done using R (version 3.6.0) (R Core Team, 2019), package tidyverse (version 1.2.1) (Wickham



*et al.*, 2019) and multiple other packages (Xie, 2014, 2015, 2019a, 2019b; Xie *et al.*, 2018; Zhu, 2019; Allaire *et al.*, 2019). The detailed analysis procedure including the R Markdown file for this paper are available as a GitHub repository (????). Based on the ATCC MSA-1002 mock community included in the analysis a sequencing error rate of 0.009 % was determined that is in line with previously reported values for next-generation sequencing data (Kozich *et al.*, 2013; Schloss *et al.*, 2016). In addition, the negative control processed together with the samples yielded only 44,050 sequences.

## **Protein Isolation and Metaproteomics**

Proteins were isolated according to the protocol for isolation from soil described in Chourey *et al.* (2010) and modified by Hultman *et al.* (2015). These protocols were further modified and adapted for protein isolation from microbial communities from macrophyte surfaces as described below. 20 ml of protein extraction buffer (4 % SDS, 100 mM Tris-HCl [pH 8.0]) was added to 5 g wet weight of leaves or 10 g wet weight of thalli. The mixture was incubated in boiling water for 5 min, vortexed for 10 min and incubated again in boiling water for 5 min. After a brief vortex the lysate was transferred to a clean tube separating the host leaves or thalli from the mixture containing lysed epiphytic cells. Dithiothreitol (DTT; final concentration 24 mM) was added and proteins were precipitated with chilled 100 % trichloroacetic acid (TCA; final concentration 20 %) overnight at –20 °C. Precipitated proteins were centrifuged at 10,000 × g and 4 °C for 40 min. The obtained protein pellet was washed three times with chilled acetone. During the first washing step the pellet was transferred to a clean 1.5 ml tube. After each washing step the samples were centrifuged at 20,000 × g and 4 °C for 5 min. Dried pellets were stored at –80 °C until further analysis.

172 **Confocal Microscopy**

173 **Results and Discussion**

174 **Conclusions**

175 **Materials and Methods**

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