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Effects of sampling and storage procedures on 16S rDNA amplicon sequencing results of kelp microbiomes

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11 Abstract

Brown macroalgae, including the kelp Saccharina latissima, are of both ecological and increasing 12 13 economic interest. Together with their microbiota, these organisms form a singular entity, the 14 holobiont. Sampling campaigns are required to study the microbiome of algae in natural populations, 15 but freezing samples in liquid nitrogen is complex in the field, particularly at remote locations. Here 16 we tested two simple alternative methods for sampling the microbial diversity associated with the kelp S. latissima: silica gel conservation of tissue and swab samples preserved in DNA/RNA shield solution. 17 18 We used these techniques to compare apex and meristem samples from Roscoff (Brittany, France) and 19 evaluated their impact on the results of 16S rDNA metabarcoding experiments. Both methods were 20 able to separate apex and meristem microbiomes, and the results were concordant with results 21 obtained for flash-frozen samples. However, differences were observed for several rare genera and 22 ASVs, and the detection of contaminant sequences in the silica gel-preserved samples underline the

- 23 importance of including blank samples for this method. Globally, our results confirm that the silica gel 24 technique and swabbing combined with DNA/RNA shield preservation are valid alternatives to liquid 25 nitrogen preservation when sampling brown macroalgae in the field. However, they also underline 26 that, regardless of the method, caution should be taken when interpreting data on rare sequences.
- 27
- 28 *Keywords*: Silica gel, swab, liquid nitrogen, brown algae, microbiome, metabarcoding, holobiont.

29 1. Introduction

Brown macroalgae, particularly kelps (Laminariales), play essential ecosystem engineering roles in
coastal temperate marine environments. They contribute to primary productivity and are habitat
engineers providing food and shelter to the local biodiversity (Schiel and Lilley, 2007; Schiel and Foster,
2006). In addition, kelp species are important in many industries to produce alginates (Peteiro, 2018),
human food, medicine (Smit, 2004), or food for abalone aquaculture (Roussel et al., 2019).

Macroalgal functioning has to be seen as the result of the interactions between the algal host and its associated microbiota, constituting a complex system termed the algal holobiont (Egan et al., 2013). It has been shown that macroalgal health, fitness, pathogen resistance (Wiese et al., 2009), acclimation to a changing environment (Dittami et al., 2016), and metabolism (Burgunter-Delamare et al., 2020) are regulated and supported by bacterial partners (Goecke et al., 2010). Considering the biofilm composition and deciphering the interactions within the holobiont is thus essential to fully understand the biology of algae.

To study the microbiota of natural populations, especially in remote regions, we need simple 42 43 sampling protocols and storage methods. Methods available involve flash-freezing in liquid nitrogen 44 (Tourneroche et al., 2020; van der Meer and Simpson, 1984), ethanol (Hammer et al., 2015; Song et 45 al., 2016), various preserving reagents (Hammer et al., 2015; Song et al., 2016), and silica gel (Esteban 46 et al., 2009; Hoarau et al., 2007; Phillips et al., 2001; Toishi, 1959). These methods can be applied to 47 both algal tissue and surface swabs (Lachnit et al., 2011; Parrot et al., 2019; Qiu et al., 2019). A few 48 comparative studies of conservation methods in insect-, soil-, and human microbiota have established 49 that differences introduced by storage techniques, while perceptible, did not outweigh differences 50 classically found in the bacterial communities between species, individuals, or sample types (Hammer 51 et al., 2015; Lauber et al., 2010; Song et al., 2016). Furthermore, a study on the red alga Porphyra 52 umbilicals has shown that silica gel was as effective as flash-freezing/lyophilisation (Quigley et al., 53 2018) to preserve the core microbiome. However, as red and brown algae belong to different lineages, display different metabolic pathways, and are at least partially in interaction with different bacteria
(Hollants et al., 2013), caution needs to be taken when transferring results from one group of algae to
another. Here we examine if these results were transferable also to the kelp *S. latissim*a.

57 The sugar kelp or sea belt Saccharina latissima (L.) (Phaeophyceae, Laminariales) is one of the dominant kelp-forming species of brown macroalgae in Europe and is becoming a research model for 58 59 holobiont studies and others (Staufenberger et al., 2008; Tourneroche et al., 2020; Wiese et al., 2009). 60 We compared the impact of flash-freezing of tissue in liquid nitrogen, desiccation of tissue in silica gel, and swab sampling followed by preservation in DNA/RNA shield solution on DNA metabarcoding 61 results of algal apex and meristem samples. Our data show that all three methods yield similar results 62 for the vast majority of genera and that both swabs and silica gel are viable alternatives to flash-63 64 freezing of tissues in the field.

65 2. Material & Methods

66 2.1 Biological material

Saccharina latissima (Phaeophyceae) samples were collected by hand at low tide on 22 March
2019, at Perharidy (48°43′47.0 "N 4°00′17.1 "W), Roscoff (France). Among young individuals (<1 m
length), ten algae were randomly selected. The algal material was immediately placed in sterile plastic
bags and rapidly transported to the laboratory in an icebox at ca. 4°C.

71 2.2 Sample preparation technics

All three techniques were carried out under a sterile hood and for each individual. Two areas of the blades were sampled: the basal meristem part and the tip (**Figure 1**). Two discs (\emptyset 2 cm) were punched out in immediate proximity for each part of the blade, ensuring that no epiphytes or animal colonizers were in the sampled area. One of the discs was placed in a 15 ml Falcon tube containing 5ml of clean silica gel (2-6 mm; VWR) and stored at 4°C for ca two weeks before use. The other disc was

- placed in a 2 ml cryotube, flash-frozen in liquid nitrogen, and stored at -80°C until use. For the swab samples, an area of 2 cm² was swabbed (Swab collection kit, Zymo Research) until a brown colouration was reached (30 s - 1 min), and the swab was placed in a collection tube filled with 1 ml of DNA/RNA
- 80 Shield (Zymo Research) and stored at -20°C until use.



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Figure 1 - Sampled parts of the thallus of *S. latissima*. Two discs (Ø 2 cm) were punched out in immediate
 proximity for each part of the blade, and an area of 2cm² was swabbed.

84 2.3 DNA extraction

85 DNA extraction was carried out according to Bernard et al. (2017) for samples stored in silica gel 86 and samples flash-frozen in liquid nitrogen. Briefly, samples were freeze-dried, and ½ of a disk was 87 ground using a Qiagen TissueLyser II bead beater (3 sessions, 45 s, 30 Hz, 3 mm stainless steel beads). 88 Nucleic acids were then extracted using a 2% CTAB extraction buffer (100 mM Tris-HCl [pH 7.5], 1.5 M 89 NaCl, 2% CTAB, 50 mM EDTA [pH 8], 50 mM DTT; shaker 250 rpm at room temperature). Supernatants 90 were purified twice with chloroform/isoamyl alcohol (24:1; centrifugation 15 min, 10 000 rpm, 16°C) and then bound to the Nucleospin plant II DNA columns (Macherey-Nagel, Germany). After a wash 91 92 with the PW1 and two with PW2 (Macherey-Nagel), DNA was eluted in 50µl of elution buffer

93 (Macherey-Nagel). For the swab samples, DNA extraction was carried out with a ZymoBIOMICS[™] DNA 94 Miniprep Kit following the manufacturer's protocol (https://files.zymoresearch.com/protocols/ d4300t d4300 d4304 zymobiomics dna miniprep kit. 95 96 pdf). One hundred μ l of DNA extract was obtained. Blank extractions were also performed for each 97 technique. While swab and liquid nitrogen samples yielded no or only a few reads in the blank samples 98 (no DNA detected in extraction and no visible PCR products), the blanks for the silica gel samples were 99 dominated by unclassified Saprospiraceae, Achromobacter sp., and Alteromonas sp. (Supplementary 100 Table S1). As these ASVs corresponded to potential contaminations in the silica gel, they were removed 101 from the dataset prior to further processing.

102 2.4 16S rDNA metabarcoding

103 The bacterial community composition associated with algal cultures was determined by 16S rDNA 104 metabarcoding. A mock community comprising a mix of DNA from 26 cultivated bacterial strains 105 (Thomas et al., 2019) was run parallel to the DNA extracts. For all of these samples, the V3 and V4 106 regions of the 16S rDNA gene were amplified using the NOCHL primers including Illumina adapters (Thomas et al., 2019), to avoid plastid DNA amplification. Then the adapted Illumina protocol for 107 108 metabarcoding (Illumina, 2013) was run using the Q5® High-Fidelity 2X Master mix (New England 109 BioLabs, MA, USA), the AMPure XP for PCR Purification Kit (Beckman Coulter, Brea, CA, USA), and the 110 Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Libraries were quantified with 111 a Quantifluor[®] ds DNA System (Promega, WI, USA), and the mean fragment size was determined using a LabChip[®] GX Touch[™] (Perkin Elmer, MA, USA). An equimolar pool of all samples was generated at a 112 113 concentration of 4 nM, diluted to 3 pM, spiked with 10% PhiX (Illumina), and sequenced on an Illumina MiSeq sequencer at the Genomer platform (Station Biologique de Roscoff) using a MiSeq v3 kit 114 115 (2x300bp, paired-end).

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116 2.5 Analyses

117 Sequence analysis was performed using the DADA2 version 1.14.0 package (Callahan et al., 2016) 118 on R 3.6.2. Following the protocol by Benjamin Callahan 119 (https://benjineb.github.io/dada2/tutorial.html), sequences were filtered allowing for a maximum of 120 two expected errors and reducing the read length to 291 bp for forward reads and 265 bp for reverse 121 reads. An amplicon sequence variant (ASV) table was constructed, and chimaeras were removed. The 122 taxonomy of the remaining ASVs was assigned using the Silva SEED 138 database. The resulting 123 abundance table and taxonomic classification were analysed using Phyloseq version 1.30.0 (McMurdie 124 and Holmes, 2013). Organellar and eukaryote reads, rare ASVs (<0.01% of total reads) and ASV that 125 were more abundant in the blank samples than in all other samples, as well as samples with less than 126 7000 total reads were removed, leading to a final number of 3 to 5 replicates per condition. Non-Metric 127 Multidimensional Scaling analyses (NMDS) were carried out using the Bray-Curtis distances derived from the ASV table using the vegan R package version 2.5-6. The Shannon H diversity index was also 128 129 calculated based on the ASV table using Past version 4.02 (Hammer et al., 2001). Statistical analysis of 130 differential abundance was carried out at the genus level using ANCOM-BC version 1.4.0 (Lin and Peddada, 2020) with default parameters. A joint analysis was performed with both methods and 131 132 thallus part as factors to identify ASVs specifically impacted by the storage methods. Venn diagrams 133 were generated using BioVenn (Hulsen et al., 2008), and the mean abundance of genera across storage methods was compared using linear regression on log10-transformed data in Past version 4.02. The 134 residuals of the linear regressions were subjected to a Shapiro-Wilk test to confirm that they did not 135 136 deviate significantly from a normal distribution (p>0.05).

137 **3. Results**

A total of 3,935,663 raw sequences were generated and, after filtering, assembled into 1,743,565
 contigs. The taxonomic assignation of mock samples was consistent with the mock composition. A total

of 11,106 ASVs were identified in the dataset corresponding to 572 genera. The final ASV matrix is
 provided as Supplementary Table S1.

142 3.1 Comparison of apex and meristem samples with the three storage methods

143 Regardless of the storage method used, the sequences followed the same general patterns. In all 144 samples, they corresponded predominantly to Alphaproteobacteria (34.8% of reads, on average), 145 followed by Gammaproteobacteria (31.5% of reads) and Bacteroidota (21.1% of reads), although their 146 exact proportion varied slightly (Figure 2). Planctomycetota were significantly more abundant (t-test, 147 p < 0.001) in the apex samples (5 to 8% of reads) than in the meristem ones (0.76% to 1.95% of reads). Actinobacteriota were less detected after desiccation in silica gel (meristem: 0.08% and apex: 0.7% of 148 149 reads) compared to the samples treated with liquid nitrogen (meristem: 1.85% and apex: 1.93% of 150 reads); and Acidobacteriota were almost exclusively found in the swab samples (meristem: 0.35% and 151 apex: 0.16% of reads; <0.04% in others, t-test *p*=0.049). Overall, as indicated by the Shannon H index 152 in Figure 3, the alpha diversity was higher in apex samples than meristem samples, although this 153 difference was statistically significant only for the liquid nitrogen and the silica gel samples. Finally, 154 NMDS analyses confirmed a clear separation of apex and meristem samples regardless of the sampling 155 and storage method (Figure 4A-C). This separation was also observed in a combined NMDS plot (Figure 156 4D). Here, separation according to the storage method was only detected at a smaller scale in the 157 meristem samples.



159 Figure 2 - Distribution of 16S rRNA gene metabarcoding sequences per phylum.

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161 Figure 3 - Box plot of alpha-diversity (Shannon H index) across sample types. P-values correspond to the results

¹⁶² of a two-sided t-test; n.s. = not significant (p > 0.05).



Figure 4 - NMDS analysis of the microbiome composition. Results show a clear separation of the apex and meristem samples for the (A) liquid nitrogen, (B) silica gel, and (C) swab method, as well as (D) all methods combined. The points of the same colours and connected by lines correspond to the same "parent" sample.

167 3.2 Direct comparison of storage methods

168 Differences were observed primarily regarding the detection of different genera depending on the 169 storage and sampling methods (Figure 5). Among the 572 genera in our dataset 158 were detected in 170 at least one sample with all storage methods. 143 genera were detected in samples from two different storage/sampling methods, with the highest overlap occurring between liquid nitrogen and swab 171 172 samples. However, most genera (271) were found only with one of the tested methods, and most of 173 these with the swab samples. Please note, however, that as shown in Figure 5, these method-specific 174 genera correspond only to a small percentage of the total reads. 96.7% of all reads were covered by the genera detected with all methods. Moreover, among these shared genera as well the genera 175 176 present in samples from two sampling methods, total read counts were strongly correlated across 177 methods (Figure 6). This confirms that similar read abundances were observed across the different 178 methods for >96% of the reads. As for the method-specific ASVs, global ANCOM-BC analyses of the 179 abundance of all ASVs across methods and sample types confirmed several significant differences. 180 Thirty-one ASVs (corresponding to 1.79% of total reads) were significantly differentially abundant between the liquid nitrogen and silica methods, 52 ASVs (3.48% of total reads) between liquid nitrogen 181 and swabs methods, and 55 ASVs (3.10% of total reads) between the liquid nitrogen methods and the 182 183 other two methods (Supplementary Table S2). Four ASVs were only detected by the swab method (a 184 Granulosicoccus sp., a Bacillus sp., a Robiginitomaculum sp., and a Thalassotalea sp. ASV; 0.54% of 185 total reads), and two ASVs only by the silica method (an unclassified Saprospiraceae and another 186 Granulosicoccus sp. ASV; 0.162% of total reads). No ASVs were detected specifically with the liquid 187 nitrogen method. However, most of the differentially abundant ASVs were detected at similar levels 188 with two methods but absent in the third. The 55 ASVs undetected in the liquid nitrogen dataset, the 48 ASVs undetected in the swab dataset, and the 29 ASVs undetected in the silica dataset are listed in 189 190 Supplementary Table S2. They belong mainly to the Gammaproteobacteria and Alphaproteobacteria, 191 but also Bacteroidota, Planctomycetota, and Firmicutes. We did not find any apparent link between the method-specific ASVs and the characteristics methods employed. 192



193

195 swab (green) datasets. Numbers in parentheses indicate the percentage of total reads represented by the genera

in each section.

¹⁹⁴ Figure 5 - Venn diagram illustrating shared genera between the liquid nitrogen (pink), silica gel (blue), and



Figure 6 - Correlation of log10-transformed mean sequence abundance across the sampling protocols. Only
 genera shared by at least two of the protocols were considered. R=Pearson correlation coefficient, red line =
 linear regression, p < 0.001.

201 4. Discussion & Conclusion

202 Simple sampling protocols are required to study bacterial partners in natural populations of macroalgae. In this study, we wanted to test if using silica gel and swab techniques, both of which are 203 204 more convenient to put into place during field sampling campaigns, would introduce a bias in the 205 results compared to flash-freezing in liquid nitrogen. Unlike similar studies carried out on insect- soil-206 and human microbiota (Hammer et al., 2015; Lauber et al., 2010; Song et al., 2016), we use more subtle 207 differences as a benchmark for our comparisons, i.e. different thallus parts of the same alga rather 208 than different species (butterfly vs bee vs grasshopper vs beetle or human vs dog) or different sample 209 types (faeces vs skin vs soil). Furthermore, unlike the former two studies, we examined the results not 210 only at the family or class level but also at the genus and ASV level. These differences render our 211 analyses more sensitive to small biases introduced by the sampling method.

Our results demonstrate that, regardless of the sampling and storage method, coherent results were obtained. The global bacterial composition dominated by *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidota* was obtained regardless of the method employed and is typical for brown algae-associated microbiomes (Hollants et al., 2013; KleinJan et al., 2017; Parrot et al., 2019; Tourneroche et al., 2020). In the same vein, global differences in the community composition between apex and meristem samples persisted regardless of the sampling methods. These differences
were expected, as *S. latissima* is a perennial species, and growth occurs mainly in the meristem region.
Hence, the younger meristem tissues thus are typically less colonised by bacteria and, as confirmed in
our study, exhibit lower bacterial diversity (Goecke et al., 2010; Ihua et al., 2020; Staufenberger et al.,
2008). *Planctomycetes*, which we detected predominantly in the apex samples, are typical components
of algal biofilms (Lage and Bondoso, 2014) and are, in agreement with our results, more abundant also
in apices of the brown alga *Fucus vesiculosus* (Parrot et al., 2019).

224 The main differences between the examined sampling methods were observed at the genus and 225 ASV levels, where our analyses show that numerous genera and ASVs were not detected with all 226 sampling methods. This observation may seem disconcerting at first, but these differences were driven 227 by rare genera and ASVs, which were usually detected in one or a few replicates. Our data are thus in 228 line with the results obtained by Quigley et al. (2018) on the red alga P. umbilicalis, who found silica 229 gel and flash freezing to yield similar patterns for the abundant core taxa that constituted >0.1% of 230 sequences. Furthermore, an important observation was that significant ASVs were absent in one 231 method compared to the other two, even in the liquid nitrogen subset. This suggests that each method 232 has its own biases regarding rare genera, and there is no one method superior to the others.

233 We can currently only speculate why the phenomenon of method-specific detection of genera was 234 more pronounced in the swab samples, although this was not the case at the ASV level. The swab protocol is different from the two other protocols because of the storage method (DNA/RNA Shield 235 236 solution, vs silica gel, vs flash-freezing) and the DNA extraction protocol. The exact composition of the 237 DNA/RNA Shield solution has not been published, so it is difficult to evaluate if some microbes may 238 still develop after fixation. Furthermore, it is known that different extraction methods may impact the 239 recovery of microbial reads, and this effect is strongest for rare species (Liu et al., 2019). Lastly, swabs 240 sample only detect microbes at the algal surface, unlike the other methods. All of these factors likely 241 contribute to the observed differences. However, the fact that these differences were not also

observed at the ASV level after statistical testing using ANCOM suggests that a fourth factor may be important: variability. Although efforts were made to standardize the swabbing procedure, differences in the applied pressure or the target area's swabbing precision may occur. This constitutes an additional source of variability, and higher variability could explain why more (rare) genera were detected overall, while the number of statistically significant ASVs stayed comparable.

247 Based on our data, we conclude that caution needs to be taken when interpreting data on rare 248 species or genera, as these may vary according to the sampling method. As suggested by the presence 249 or absence of genera in the swab samples, random processes may enhance these differences if no 250 statistical evaluation is applied. For silica-gel preserved samples, we observed more contaminants 251 sequences in the blank samples emphasising the importance of this latter control. When examining 252 the global patterns, however, only slight biases are introduced by the tested methods, and all three 253 methods, flash-freezing in liquid nitrogen, drying with silica gel followed by tissue grinding, and 254 swabbing followed by preservation in DNA/RNA shield solution, are suitable to assess the microbiome 255 of S. latissima.

256 **5.** Declarations

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- 261 **Competing interests**: The authors declare that they have no competing interests.
- 262 Data availability: Raw sequence data were deposited at the European Nucleotide Archive under
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- 264 **Authors' contributions**: Designed study: BBD, SD; Performed experiments: BBD, EL, GT; Analyzed data:
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- 383

384 8. Supplementary Data

- 385 Table S1 ASV matrix
- 386 Table S2 ANCOM-BC results

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