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

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The final publication in *Marine Genomics* is available via https://doi.org/10.1016/j.margen.2022.100944

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

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Abstract

Brown macroalgae, including the kelp *Saccharina latissima,* are of both ecological and increasing economic interest. Together with their microbiota, these organisms form a singular entity, the holobiont. Sampling campaigns are required to study the microbiome of algae in natural populations, but freezing samples in liquid nitrogen is complex in the field, particularly at remote locations. Here 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. We used these techniques to compare apex and meristem samples from Roscoff (Brittany, France) and evaluated their impact on the results of 16S rDNA metabarcoding experiments. Both methods were able to separate apex and meristem microbiomes, and the results were concordant with results obtained for flash-frozen samples. However, differences were observed for several rare genera and ASVs, and the detection of contaminant sequences in the silica gel-preserved samples underline the

importance of including blank samples for this method. Globally, our results confirm that the silica gel technique and swabbing combined with DNA/RNA shield preservation are valid alternatives to liquid nitrogen preservation when sampling brown macroalgae in the field. However, they also underline that, regardless of the method, caution should be taken when interpreting data on rare sequences.

Keywords: Silica gel, swab, liquid nitrogen, brown algae, microbiome, metabarcoding, holobiont.

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 sampling protocols and storage methods. Methods available involve flash-freezing in liquid nitrogen (Tourneroche et al., 2020; van der Meer and Simpson, 1984), ethanol (Hammer et al., 2015; Song et al., 2016), various preserving reagents (Hammer et al., 2015; Song et al., 2016), and silica gel (Esteban et al., 2009; Hoarau et al., 2007; Phillips et al., 2001; Toishi, 1959). These methods can be applied to both algal tissue and surface swabs (Lachnit et al., 2011; Parrot et al., 2019; Qiu et al., 2019). A few comparative studies of conservation methods in insect-, soil-, and human microbiota have established that differences introduced by storage techniques, while perceptible, did not outweigh differences classically found in the bacterial communities between species, individuals, or sample types (Hammer et al., 2015; Lauber et al., 2010; Song et al., 2016). Furthermore, a study on the red alga *Porphyra umbilicals* has shown that silica gel was as effective as flash-freezing/lyophilisation (Quigley et al., 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.

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 holobiont studies and others (Staufenberger et al., 2008; Tourneroche et al., 2020; Wiese et al., 2009). 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 results of algal apex and meristem samples. Our data show that all three methods yield similar results for the vast majority of genera and that both swabs and silica gel are viable alternatives to flash-freezing of tissues in the field.

2. Material & Methods

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.

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 (Ø 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 Shield (Zymo Research) and stored at -20°C until use.

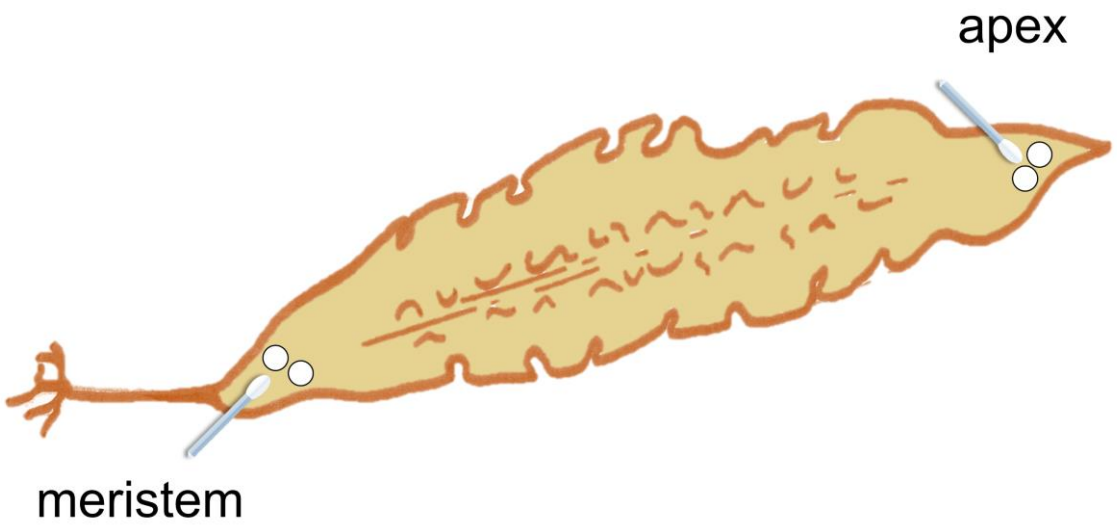


Figure 1 - Sampled parts of the thallus of *S. latissima*. Two discs (Ø 2 cm) were punched out in immediate proximity for each part of the blade, and an area of 2cm² was swabbed.

2.3 DNA extraction

DNA extraction was carried out according to Bernard et al. (2017) for samples stored in silica gel and samples flash-frozen in liquid nitrogen. Briefly, samples were freeze-dried, and ½ of a disk was ground using a Qiagen TissueLyser II bead beater (3 sessions, 45 s, 30 Hz, 3 mm stainless steel beads). Nucleic acids were then extracted using a 2% CTAB extraction buffer (100 mM Tris-HCl [pH 7.5], 1.5 M NaCl, 2% CTAB, 50 mM EDTA [pH 8], 50 mM DTT; shaker 250 rpm at room temperature). Supernatants 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 with the PW1 and two with PW2 (Macherey-Nagel), DNA was eluted in 50µl of elution buffer

(Macherey-Nagel). For the swab samples, DNA extraction was carried out with a ZymoBIOMICS™ DNA Miniprep Kit following the manufacturer's protocol (https://files.zymoresearch.com/protocols/_d4300t_d4300_d4304_zymobiomics_dna_miniprep_kit.pdf). One hundred µl of DNA extract was obtained. Blank extractions were also performed for each technique. While swab and liquid nitrogen samples yielded no or only a few reads in the blank samples (no DNA detected in extraction and no visible PCR products), the blanks for the silica gel samples were dominated by unclassified *Saprospiraceae*, *Achromobacter* sp., and *Alteromonas* sp. (**Supplementary Table S1)**. As these ASVs corresponded to potential contaminations in the silica gel, they were removed from the dataset prior to further processing.

2.4 16S rDNA metabarcoding

The bacterial community composition associated with algal cultures was determined by 16S rDNA metabarcoding. A mock community comprising a mix of DNA from 26 cultivated bacterial strains (Thomas et al., 2019) was run parallel to the DNA extracts. For all of these samples, the V3 and V4 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 metabarcoding (Illumina, 2013) was run using the Q5® High-Fidelity 2X Master mix (New England BioLabs, MA, USA), the AMPure XP for PCR Purification Kit (Beckman Coulter, Brea, CA, USA), and the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Libraries were quantified with 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 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 (2x300bp, paired-end).

2.5 Analyses

Sequence analysis was performed using the DADA2 version 1.14.0 package (Callahan et al., 2016) on R 3.6.2. Following the protocol by Benjamin Callahan (https://benjjneb.github.io/dada2/tutorial.html), sequences were filtered allowing for a maximum of two expected errors and reducing the read length to 291 bp for forward reads and 265 bp for reverse reads. An amplicon sequence variant (ASV) table was constructed, and chimaeras were removed. The taxonomy of the remaining ASVs was assigned using the Silva_SEED 138 database. The resulting abundance table and taxonomic classification were analysed using Phyloseq version 1.30.0 (McMurdie and Holmes, 2013). Organellar and eukaryote reads, rare ASVs (<0.01% of total reads) and ASV that were more abundant in the blank samples than in all other samples, as well as samples with less than 7000 total reads were removed, leading to a final number of 3 to 5 replicates per condition. Non-Metric 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 calculated based on the ASV table using Past version 4.02 (Hammer et al., 2001). Statistical analysis of 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 thallus part as factors to identify ASVs specifically impacted by the storage methods. Venn diagrams 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 residuals of the linear regressions were subjected to a Shapiro-Wilk test to confirm that they did not deviate significantly from a normal distribution ($p>0.05$).

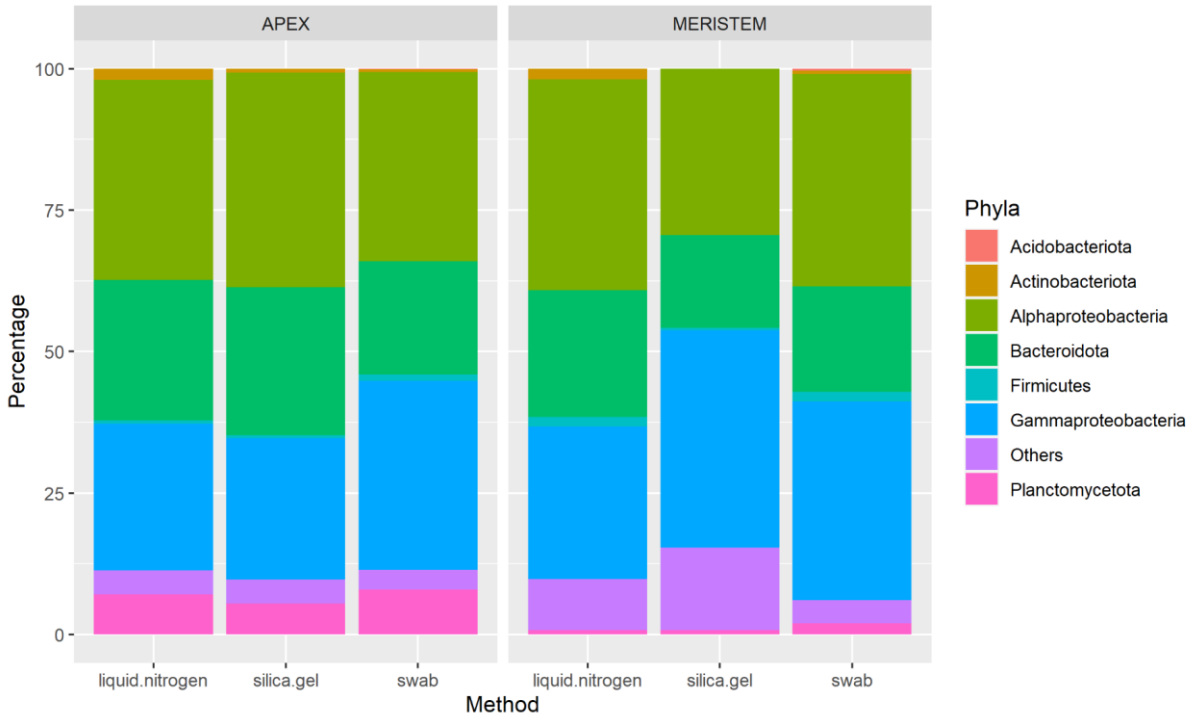
3. Results

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**.

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

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



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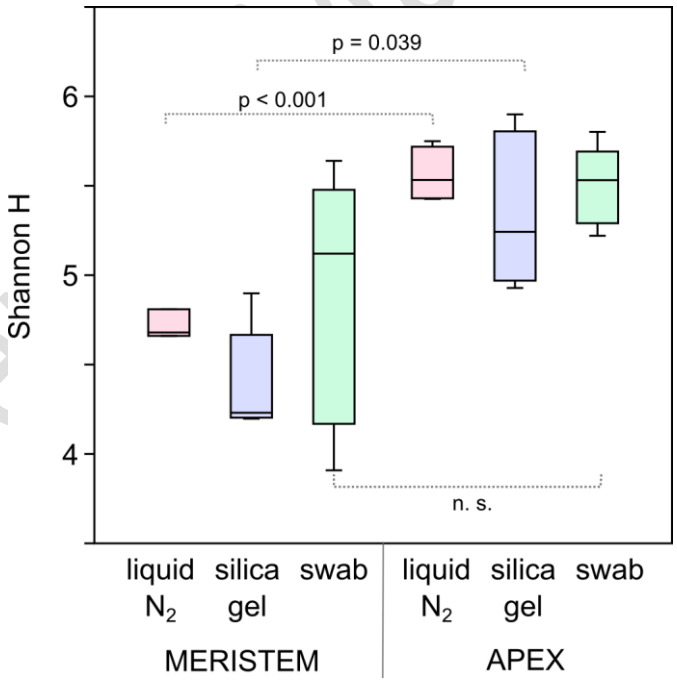
159 **Figure 2 - Distribution of 16S rRNA gene metabarcoding sequences per phylum.**



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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 ($p > 0.05$).

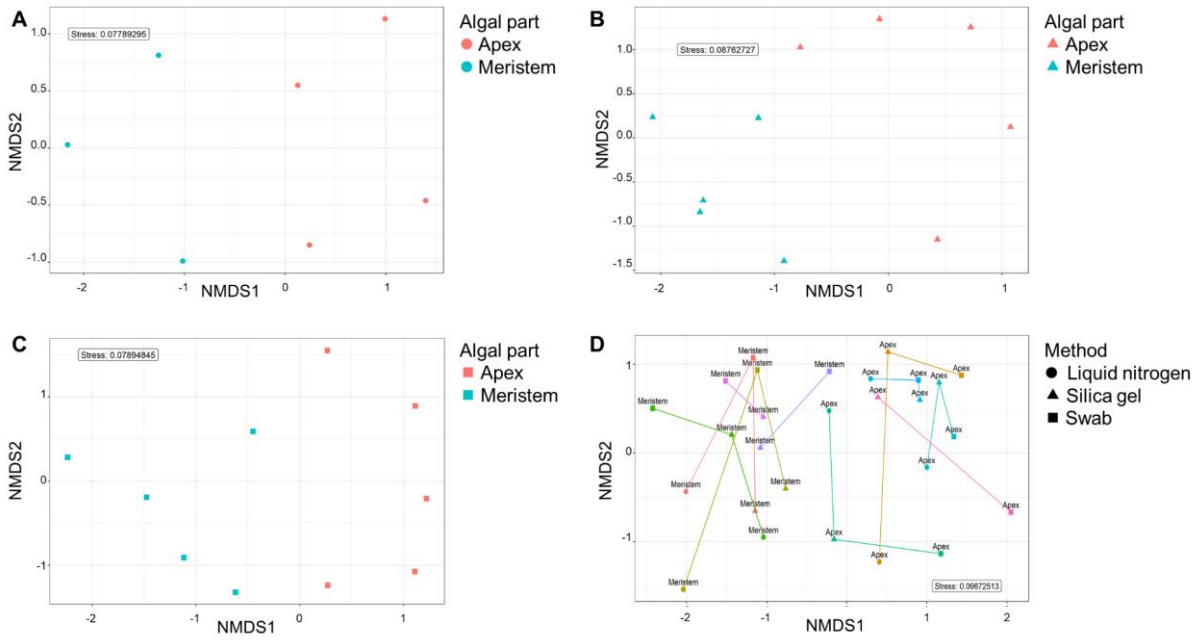

Figure 4 - NMDS analysis of the microbiome composition. Results show a clear separation of the apex and meristem samples for the (A) liquid nitrogen, (B) silica gel, and (C) swab method, as well as (D) all methods combined. The points of the same colours and connected by lines correspond to the same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

3.2 Direct comparison of storage methods

Differences were observed primarily regarding the detection of different genera depending on the storage and sampling methods (**Figure 5**). Among the 572 genera in our dataset 158 were detected in 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 samples. However, most genera (271) were found only with one of the tested methods, and most of these with the swab samples. Please note, however, that as shown in **Figure 5,** these method-specific 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 present in samples from two sampling methods, total read counts were strongly correlated across methods (**Figure 6**). This confirms that similar read abundances were observed across the different methods for >96% of the reads. As for the method-specific ASVs, global ANCOM-BC analyses of the abundance of all ASVs across methods and sample types confirmed several significant differences.

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 and swabs methods, and 55 ASVs (3.10% of total reads) between the liquid nitrogen methods and the other two methods (**Supplementary Table S2)**. Four ASVs were only detected by the swab method (a *Granulosicoccus* sp., a *Bacillus* sp., a *Robiginitomaculum* sp., and a *Thalassotalea* sp. ASV; 0.54% of total reads), and two ASVs only by the silica method (an unclassified *Saprospiraceae* and another *Granulosicoccus* sp. ASV; 0.162% of total reads). No ASVs were detected specifically with the liquid nitrogen method. However, most of the differentially abundant ASVs were detected at similar levels 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 **Supplementary Table S2.** They belong mainly to the *Gammaproteobacteria* and *Alphaproteobacteria*, but also *Bacteroidota*, *Planctomycetota*, and *Firmicutes*. We did not find any apparent link between the method-specific ASVs and the characteristics methods employed.

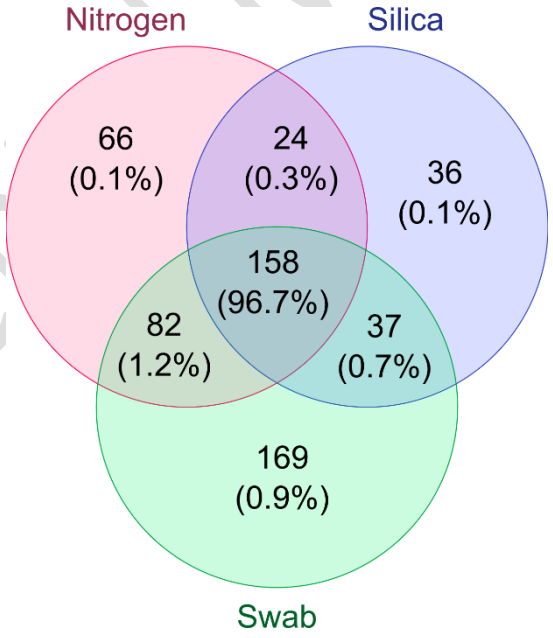

Figure 5 - Venn diagram illustrating shared genera between the liquid nitrogen (pink), silica gel (blue), and swab (green) datasets. Numbers in parentheses indicate the percentage of total reads represented by the genera in each section.

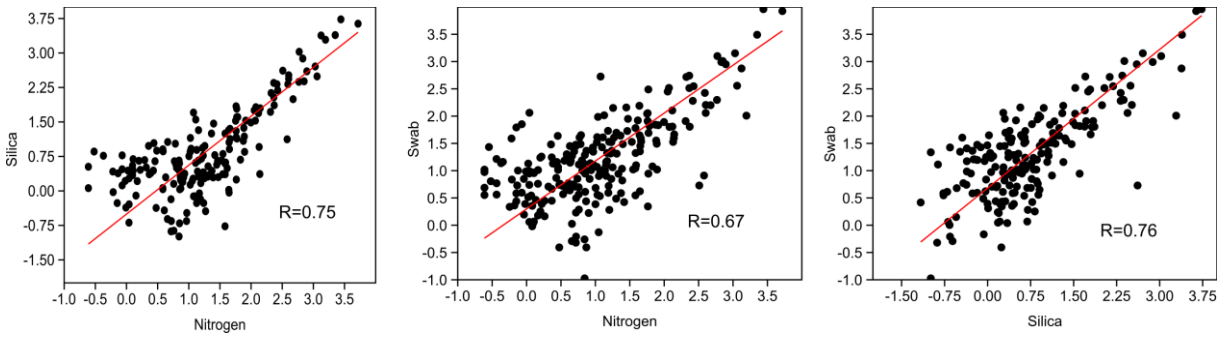

Figure 6 - Correlation of log10-transformed mean sequence abundance across the sampling protocols. Only genera shared by at least two of the protocols were considered. R=Pearson correlation coefficient, red line = linear regression, $p < 0.001$.

4. Discussion & Conclusion

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 more convenient to put into place during field sampling campaigns, would introduce a bias in the results compared to flash-freezing in liquid nitrogen. Unlike similar studies carried out on insect- soil- and human microbiota (Hammer et al., 2015; Lauber et al., 2010; Song et al., 2016), we use more subtle differences as a benchmark for our comparisons, i.e. different thallus parts of the same alga rather than different species (butterfly vs bee vs grasshopper vs beetle or human vs dog) or different sample types (faeces vs skin vs soil). Furthermore, unlike the former two studies, we examined the results not only at the family or class level but also at the genus and ASV level. These differences render our 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).

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

We can currently only speculate why the phenomenon of method-specific detection of genera was 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 solution, vs silica gel, vs flash-freezing) and the DNA extraction protocol. The exact composition of the DNA/RNA Shield solution has not been published, so it is difficult to evaluate if some microbes may still develop after fixation. Furthermore, it is known that different extraction methods may impact the recovery of microbial reads, and this effect is strongest for rare species (Liu et al., 2019). Lastly, swabs sample only detect microbes at the algal surface, unlike the other methods. All of these factors likely 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.

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

5. Declarations

Funding: This work was funded partially by the ANR project IDEALG (ANR-10-BTBR-04) "Investissements d'Avenir, Biotechnologies-Bioressources" and the CNRS momentum call (2017). BBD was funded by a joint PhD scholarship from the Brittany region (Project HOSALA) and Sorbonne University (ED227).

Competing interests: The authors declare that they have no competing interests.

Data availability: Raw sequence data were deposited at the European Nucleotide Archive under project accession number ENA: PRJEB37561.

Authors' contributions: Designed study: BBD, SD; Performed experiments: BBD, EL, GT; Analyzed data: BBD, SD; Wrote the manuscript: BBD, SD; Provided valuable input and corrected the manuscript: CB.

6. Acknowledgements

We thank François Thomas, Maéva Brunet, and Nolwen Le Duff for sharing primers and providing the mock community. This work benefited from access to the Station Biologique de Roscoff, an EMBRC-France and EMBRC-ERIC Site.

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8. Supplementary Data

Table S1 – ASV matrix

Table S2 – ANCOM-BC results