Optimization of preservation and processing of sea anemones for microbial community analysis using molecular tools

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For several years, knowledge on the microbiome associated with marine invertebrates was impaired by the challenges associated with the characterization of bacterial communities. With the advent of culture independent molecular tools it is possible to gain new insights on the diversity and richness of microorganisms associated with marine invertebrates. In the present study, we evaluated if different preservation and processing methodologies (prior to DNA extraction) can affect the bacterial diversity retrieved from snakelocks anemone Anemonia viridis. Denaturing gradient gel electrophoresis (DGGE) community fingerprints were used as proxy to determine the bacterial diversity retrieved (H'). Statistical analyses indicated that preservation significantly affects H'. The best approach to preserve and process A. viridis biomass for bacterial community fingerprint analysis was flash freezing in liquid nitrogen (preservation) followed by the use of a mechanical homogenizer (process), as it consistently yielded higher H'. Alternatively, biomass samples can be processed fresh followed by cell lyses using a mechanical homogenizer or mortar & pestle. The suitability of employing these two alternative procedures was further reinforced by the quantification of the 16S rRNA gene; no significant differences were recorded when comparing these two approaches and the use of liquid nitrogen followed by processing with a mechanical homogenizer.

Phylum Cnidaria is a large, diverse and ecologically important group of relatively simple organisms that is widely distributed in marine environments5. Research on marine cnidarians experienced a significant advance over the last decades with the growing awareness on the vulnerability of certain key ecosystems (e.g. coral reefs) driven by direct or indirect anthropogenic actions2–4. Additionally, with the intensification of bioprospecting of marine invertebrates for drug discovery, as well as other biotechnological applications, researchers have started to target cnidarians in their quest for marine bioactive compounds5–6. Alongside with this new trend, there are growing evidences that microbes associated with marine invertebrates may be the true producers of such bioactive compounds or, at least, partially involved in the process of biosynthesis of some of these molecules7. Several of these compounds are secondary metabolites produced by symbiotic microorganisms in chemical mediation and/or defense of interaction among marine microorganisms4. The microbiome of certain marine invertebrates may represent a remarkable proportion of the holobiont biomass, with anthozoan cnidarians being no exception and hosting abundant and diverse communities of bacteria5. Certain species able to secrete mucus may reach microbial concentrations up to 1000-fold higher than those observed in seawater8. While microbial communities associated with tropical reef building corals are already starting to be unraveled, those colonizing other groups of anthozoans are still largely unknown11. For several years, this gap of knowledge has been mainly due to the challenges associated with the characterization of bacterial communities using culture dependent approaches12. Only a small fraction of microbial symbionts can be cultured outside its cnidarian host using conventional culture media. The advent of culture independent molecular technologies [e.g. Denaturing Gradient Gel Electrophoresis (DGGE) and high–throughput DNA sequencing], made possible to overcome these bottlenecks and reveal the diversity and richness of microorganisms associated with marine invertebrates in...
Anthozoan cnidarians are no exception to this breakthrough. Regardless of the potential associated with the use of high-throughput DNA sequencing to profile the microbiome of marine invertebrates, the final results achieved are still largely dependent on the quality and quantity of DNA extracted from collected samples. DNA quality and quantity is known to vary with the procedures employed for preserving and processing samples, as well as the reliability of the DNA extraction method.

Sea anemones, namely those hosting endosymbiotic photosynthetic dinoflagellates, are recognized to be important sentinel species. These organisms may help researchers to monitor potential environmental shifts in temperate coastal waters triggered by global climate changes. Extreme bleaching events of *Anemone* in the Mediterranean under abnormally warm water conditions are a good example on the suitability of these anthozoans as sentinel species. In light of the holobiont concept, these anemones should be considered as holobionts, a complex symbiosis between the cnidarian animal, its photosynthetic microalgae (e.g., zooxanthellae) and its complex community of associated microorganisms that play a key role on the overall health of the cnidarian host. Therefore, it is important to monitor potential shifts in the microbiota associated with these sea anemones to understand how environmental disturbances may shape anemone individuals and populations.

Despite the existence of reports on the suitability of processing techniques to preserve samples and extract microbial DNA from marine invertebrates (e.g., sponges), only a few studies are currently available on sea anemones. Given the current state of the art on this topic and the complexity/specificity of this biological matrix, we consider that it is relevant to standardize a protocol that can allow researchers to extract good-quality DNA in order to perform a reliable analysis of the bacterial communities associated with sea anemones. In line with this goal, we used the snakelocks anemone *Anemonia viridis* (Forskal, 1775) as a model species to evaluate how different preservation and processing approaches could affect the quality of extracted DNA and the molecular profiles of bacterial communities retrieved from these organisms.

### Results

The two-way ANOVA revealed that there was no significant interaction between the processing and preserving procedures that were tested and that the categorical factor processing did not significantly affect the Shannon’s index of diversity (H’) calculated from the bacterial fingerprints generated from DGGE. However, the categorical factor preserving significantly affected H’ values. Experimental treatments employing freezing at −80°C differed significantly from those processing fresh samples or samples flash frozen with liquid nitrogen (P = 0.017 and P = 0.025, respectively). The highest average H’ value (±s.d.) was displayed by LN_H (H’ = 2.72 ± 0.17), while the lowest was that of F-80_H (H’ = 1.68 ± 0.79) (Figure 1).

The bacterial fingerprints recorded in the DGGE of the three experimental treatments promoting the highest H’ (in descending order, LN_H, Fr_H and Fr_MP) are illustrated in Figure 2.

The first two axis of the PCO explained 65.1% of the variability recorded in the bacterial fingerprints of the three experimental treatments yielding the highest H’ (Figure 3). Samples from treatment LN_H are clearly clustered apart from those where samples were processed fresh (Fr).

Real-time PCR quantification of 16S rRNA gene did not reveal the existence of any significant differences between the three procedures yielding the highest H’ (P = 0.008).

### Discussion

The present study reveals that the use of community fingerprinting approaches such as PCR-DGGE is a robust technique to assess and/or optimize processing and preservation methodologies of biological samples destined for microbial communities analysis using molecular tools. While it is true that PCR-DGGE only detects the more abundant taxa present in the sample being analyzed it also provides an excellent high-throughput tool for comparative community structure analysis, it allows researchers to determine and compare the relative abundance of different bacterial populations, and therefore compare procedures, without the need to use more expensive and labor intensive techniques. Indeed, by using this approach it was possible to verify that there are no significant interactions between preservation and processing procedures employed for samples of *A. viridis* meant to be used in bacterial diversity analysis using molecular techniques. Preservation was recorded to significantly affect H’ of bacterial communities retrieved from sea anemones, as already recorded for sponges. It is now recognized by researchers that the preservation technique employed for marine invertebrate samples is a key point for molecular analysis of microbial communities. In the present study it was possible to show that flash freezing and homogenizing (LN_H) collected samples consistently yielded the highest bacterial diversity from snakelocks anemones (Figure 1). It was also possible to verify that sea anemones tissue can also be processed fresh (e.g. Fr_H and Fr_MP) with satisfactory results if researchers have the constraint of not being able to flash freeze samples with liquid nitrogen.

![Figure 1](https://example.com/image1.png)

**Figure 1** | Shannon’s index of diversity (H’) calculated from DGGE community profiles of Bacteria detected on snakelocks anemone *Anemonia viridis* from each experimental treatment. Values presented are means (±s.d.) of five independent replicates. Fr – Fresh (blue); NH – non-homogenized (full colored); H – maceration with homogenizer (pinstripe right); MP – maceration with mortar & pestle (pinstripe left); LN – freezing with liquid nitrogen followed by preservation at −80°C (green); F-80 – freezing and preservation at −80°C (red). Different letters represent significant differences (Tukey’s test, P < 0.05).
According to the 16S rRNA gene quantification results from Real-time PCR, any of the three procedures was considered a suitable option to obtain bacterial DNA for molecular studies of bacterial communities from sea anemones.

The best results achieved in our study through the flash freezing of collected samples are in line with the fact that at such extremely low temperatures no DNA degradation occurs through enzymatic activity; in this way the bacterial diversity retained is close to that present at sampling time.

Liquid nitrogen can be difficult to obtain and transport in remote locations and keeping samples frozen while in transit can at times be a challenging task. However, our results support the fact that flash freezing is indeed the most efficient approach when aiming to preserve biological samples from invertebrates for molecular analysis of their microbial communities. Successfully retrieving microbial communities associated with these marine animals can be of paramount importance for biotechnological and/or ecological purposes. The extraction method can affect the diversity of microorganisms retrieved from sea anemones. Nonetheless, as the extraction method employed in the present study displays a good compromise between the quantity and quality of extracted DNA, processing costs and processing time per sample, we recommend researchers to use our methodology. In the future, the use of standardized procedures for processing and preserving collected samples of sea anemones will allow researchers to perform reliable comparisons by ensuring homogeneity between studies. Moreover, it also makes possible the use of less expensive approaches (e.g. DGGE) to compare shifts in the relative abundance of the microbiome associated with these marine invertebrates.

**Methods**

Sample collection, preservation and processing. Five snakelocks anemones *A. viridis* were collected at low tide, in the intertidal region of Praia da Aguda (41°02’51.06″N; 8°39’14.20″W), Arcozelo, Portugal, in November 2011 and individually stocked in sterile plastic bags for immediate transportation to the laboratory.

Each of the five sea anemones collected was fragmented into 9 similar sized pieces using sterile scalpels blades along their radial axis; each piece included similar amounts of anemones body and tentacles, as well as a similar wet weight. A factorial experimental design employing three levels of preservation (samples used fresh, frozen at −80°C and flash frozen with liquid nitrogen) and three levels of processing (non-homogenized samples, samples homogenized with mortar and pestle and samples homogenized with a mechanical tissue homogenizer) was tested prior to DNA extraction. Briefly, this factorial design allowed us to evaluate 9 different experimental treatments, each with five independent replicates: Fr_NH (fresh samples non homogenized, where fresh samples were used directly for extraction of nucleic acids without any further processing or preservation); Fr_H (fresh samples were processed with the Omni Tissue Homogenizer (Omni International, Kennesaw, Georgia, USA) and used for DNA extraction without any further treatment); Fr_MP (fresh samples were processed with the mortar & pestle and used for DNA extraction without any further treatment); LN_H and LN_MP (samples were first preserved by flash freezing in liquid nitrogen and kept at −80°C and then homogenized with the...
Cycling conditions were of 4 min at 94 °C and then homogenized with the Omnis Tissue Homogenizer or mortar & pestle, respectively, prior to DNA extraction; F-80_H and F-80_MP (samples were first frozen and kept at −80 °C and then homogenized with the Omnis Tissue Homogenizer or mortar & pestle, respectively, prior to DNA extraction); LN_NH (samples were flash frozen in liquid nitrogen and kept at −80 °C and used for DNA extraction without any further processing); and F-80_NH (samples were frozen and kept at −80 °C and used for DNA extraction without any further processing) (see Figure 4 for a schematic representation of the experimental design).

**Extraction of nucleic acid.** Nucleic acids were extracted from 0.5 g of sea anemone samples from each experimental treatment described above. All samples were homogenized using FastPrep® (QiBiogene Inc., USA) bead-beating system in combination with a mixture of beads (0.10 g Zirconia beads (0.1 mm) + 0.20 g glass beads (0.25–0.5 mm) + 0.20 g glass beads (0.75–1.0 mm) + 2 glass beads (2.85–3.45 mm)) (ROTH, DE) and Buffer SLX Mlus from E.Z.N.A.® Soil DNA Kit (Omega Bio-TeK Inc., USA). Extraction was performed according to the instructions provided by the manufacturer. DNA was determined using Qubit™ dsDNA HS Assay Kits for Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies Corporation) (see Supplementary Table ST1).

**Bacterial community diversity.** Bacterial community composition was evaluated by performing a DGGE based on DNA (16S rRNA gene). The bacterial fingerprints yielded by the DGGE were used as a proxy to evaluate the diversity of the bacterial community retrieved from sea anemones handled according to each of the preservation and processing combinations described above. NESTED PCR was used for a more efficient amplification of 16S rRNA gene fragments of bacterial genomic DNA extracted from sea anemone. In the first PCR, the universal bacterial primers F-27 (5′-AGAGTTTGATCCTTGAGCTC-3′) and R-1492 (5′-TACGGA(T/C)YTACCTGTAGCTAC-3′) were used to amplify c. 1500 bp of the 16S rRNA gene31,32. The PCR reaction mixtures (25 µL) consisted of DNA template (1 µL), DreamTaq PCR Master Mix (2.5 µL) (Fermentas, Thermo Fisher Scientific Inc., USA), bovine serum albumin (BSA; 2.0 mg/mL) and forward and reverse primers (0.1 µM) plus 0.5% acetic acid for fixation, 0.1% (wt/vol) silver nitrate for staining, freshly prepared solutions containing 0.2% (wt/vol) sodium hydroxide, 0.15% formaldehyde, 1.5% (wt/vol) NaOH, and, finally, 0.75% (wt/vol) sodium carbonate solution to stop the development. Gels were documented with a Molecular Imager chemiDoc XR+ digital system (Bio-Rad). A total of five DGGEs were performed and analyzed: four DGGEs to cover all the samples of the nine experimental treatments along the first two axes. The raw data matrix was log (x + 1) transformed prior to the statistical analysis in order to place more emphasis on compositional differences among samples rather than on quantitative differences. A similarity/difference matrix was later constructed using the Euclidean distance. This multivariate statistical test was performed using Primer 6.1 with the Qubit fluorometer (Invitrogen, USA). The gene copy number in the initial standard curve was calculated considering the DNA content, the length of the fragment and the average weight of a base pair (650 Da). A standard curve was constructed by producing a ten times dilution series from 108 to 101 target gene copies per µL. Sample copy numbers were log transformed and normalize to DNA input.

**Statistical analysis.** Bacterial fingerprints of each denaturing gradient gel were normalized using the GelCompar 4.0 software (Applied Maths, Belgium), as described by Smalla et al. (2001). Shannon’s index of diversity (H’), was determined as H’ = −∑ pi ln pi, where pi is the often the proportion of individuals belonging to the each species in the dataset of interest. The existence of significant differences in Shannon’s index of diversity (H’) values calculated from each experimental treatment was investigated by using a two-way ANOVA (with processing and preserving treatment and the average weight of a base pair (650 Da). A standard curve was constructed by producing a ten times dilution series from 108 to 101 target gene copies per µL. Sample copy numbers were log transformed and normalize to DNA input.

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