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METHODS
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Studies With Soft Corals – Recommendations on Sample Processing and Normalization Metrics

Chloé A. Pupier[1,2], Vanessa N. Bednarz[1] and Christine Ferrier-Pagès[1]*

[1] Marine Department, Ecophysiology, Centre Scientifique de Monaco, Monaco City, Monaco, [2] Collège Doctoral, Sorbonne Université, Paris, France

Soft corals (Octocorallia) often constitute the second most abundant macrobenthic group on many tropical and temperate reefs. However, the gelatinous and leather-like nature of their tissue and their variable hydroskeleton entails a number of problems for tissue homogenization and data normalization. An easy and fast protocol for tissue homogenization, as well as a normalization metric that can be used to perform inter-studies or inter-species comparisons, are thus needed. In this study, we tested whether the tissue sample state before processing (frozen vs. freeze-dried samples) and the media used for tissue homogenization (0.2 μm filtered seawater; FSW vs. Milli-Q water; DI) affect the quantitative measurements of tissue descriptors (chlorophyll, protein, and *Symbiodinium* concentrations) in the model species *Heteroxenia fuscescens*. Furthermore, the suitability of dry weight (DW) and ash-free dry weight (AFDW) as size-normalizing metric was investigated across different soft coral species. Our results reveal that freeze-drying the samples and homogenizing them in DI water exhibited several benefits, namely enhancing chlorophyll and protein concentrations up to 50%, saving processing time and providing a more accurate determination of DW and AFDW. Overall, this optimized tissue processing protocol offers a more reliable quantification of tissue descriptors and reduces the chance of underestimating these parameters in soft corals. Finally, since the contribution of sclerites to the total DW of the colony can highly differ between species, we demonstrate that AFDW is a reliable metric for normalizing soft coral data, particularly when inter-species comparisons are made.

Keywords: soft corals, tissue descriptors, normalization, ash-free dry weight, freeze-drying

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****Correspondence:***
Chloé A. Pupier
cpupier@centrescientifique.mc;
chloe.pupier@gmail.com

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INTRODUCTION

Soft corals (Octocorallia: Alcyonacea) represent, after reef-building scleractinian corals, the second most abundant macrobenthic group on many tropical and temperate reefs (Fabricius and Alderslade, 2001). They notably become often dominant within disturbed reef systems (Inoue et al., 2013). High abundances of octocorals (soft corals or gorgonians) have thus been observed in Caribbean and Indo-Pacific eutrophicated reefs, where a reduced water quality does not favor the growth of scleractinian corals (Fabricius et al., 2005; Baum et al., 2016). Despite the fact that soft corals are increasingly recognized as key taxa on reefs, two major issues regarding physiological and ecological studies remain, however, to be clarified.

First, the coenenchyme (colonial tissue) of soft corals consists of thick gelatinous material containing fibers, amoeboid cells, scleroblast cells, and calcareous sclerites (Fabricius and Alderslade, 2001). Altogether, these leather-like tissue elements make cell layers difficult to break apart and tissue homogenization challenging. Therefore, measurements of tissue descriptors (*Symbiodinium*, chlorophyll, and protein concentrations) are difficult to obtain from frozen or fresh soft coral samples, and a reliable and easy tissue homogenization protocol remains to be implemented.

Second, a common normalization metric to perform interspecies comparisons of physiological and/or ecological processes is required. For example, apprehending functional processes that occur at the reef-ecosystem level (e.g., primary production) implies to understand how soft corals operate over space and time compared to scleractinian corals. Comparative measurements related to these sub-classes are therefore necessary, but are only enabled if they are normalized to the same metric. Normalization of tissue parameters or physiological measurements requires a metric that reflects the size of the organisms and is stable across variable environmental conditions. In scleractinian corals, skeletal surface area is the most commonly used metric for normalizing data to the size of the colony (Edmunds and Gates, 2002). However, soft corals lack a calcium carbonate skeleton and rely on hydrostatic pressure for body shape maintenance (Fabricius and Alderslade, 2001). Although this hydroskeleton can vary over time depending on environmental fluctuations (Fabricius, 1995; Hellström and Benzie, 2011), few studies have approximated soft coral surface area based on geometric body measurements (e.g., Bednarz et al., 2012, 2015; Kremien et al., 2013) or linear projections (Fabricius and Dommisse, 2000). Such normalization metric is critical when studying processes (e.g., photosynthesis) that strongly depend on a vectorial parameter (e.g., light) as it allows understanding the effectiveness of these processes (Kremien et al., 2013). For other measurements, such as the quantification of structural tissue descriptors or the production of metabolites, the variable size of the hydroskeleton excludes the possibility to accurately normalize to surface area at different time intervals (Haydon et al., 2018). Several other normalization metrics have thus been applied to soft corals and are summarized in **Figure 1**, **Table 1**, and **Supplementary Table S1**. Among these metrics, those involving wet weight and buoyant weight may also be sources of error as soft coral tissue withholds a lot of water and the density of a colony may be similar to that of seawater (Davies, 1989). For example, normalization of dimethylsulfoniopropionate (DMSP) production to soft coral fresh weight (Van Alstyne et al., 2006) led to significant lower values compared to the normalization to protein content, underestimating the functional role of soft compared to hard corals (Haydon et al., 2018). Coral biomass may therefore offer a greater potential to accurately reflect the size of a soft coral colony. Protein content has been widely used to normalize physiological data in corals. However, it can encounter great variability across environmental conditions, and may thus interfere with the intrinsic pattern of the parameter of interest (Edmunds and Gates, 2002). Ash-free dry weight (AFDW) is another common proxy for tissue biomass of many benthic reef organisms such as scleractinian corals, coralline algae, sponges (Kötter and Pernthaler, 2002; Schoepf et al., 2013; Comeau et al., 2014) and soft corals (**Figure 1**). AFDW quantification relies on a completely destructive method, whereas dry weight (DW) allows for potential analysis of tissue parameters before burning the sample. Although tissue energy reserves (i.e., lipid, protein, and carbohydrate) of soft corals have been determined from dried material (Ben-David-Zaslow and Benayahu, 1999), the possibility to obtain reliable measurements involving *Symbiodinium* (cell density and chlorophyll content) still needs to be tested.

Based on these issues the present study aimed (1) to optimize a protocol for soft coral tissue processing and (2) to find the normalization metric most relevant for physiological experiments involving interspecies comparisons. We tested whether the tissue sample state before processing (frozen vs. freeze-dried samples) and the media used for tissue homogenization (0.2 μm filtered seawater; FSW vs. Milli-Q water; DI) affect the quantitative measurements of tissue descriptors in the model species *Heteroxenia fuscescens* (Ehrenberg, 1834). Furthermore, the suitability of DW and AFDW as size-normalizing metric was investigated across different soft coral species (*H. fuscescens*, *Dendronephthya* sp. Kuekenthal, 1905), *Litophyton arboreum* (Forskål, 1775), and *Rhytisma fulvum* (Forskål, 1775), which are key components of Red Sea reefs. These species particularly exhibit different growth forms ranging from arborescent (*Dendronephthya* sp. and *L. arboreum*) to capitate (*H. fuscescens*) and encrusting (*R. fulvum*).

MATERIALS AND METHODS

Biological Material

Nineteen colonies of *H. fuscescens* with 20 polyps and four colonies of *Dendronephthya* sp., *L. arboreum,* and *R. fulvum*,

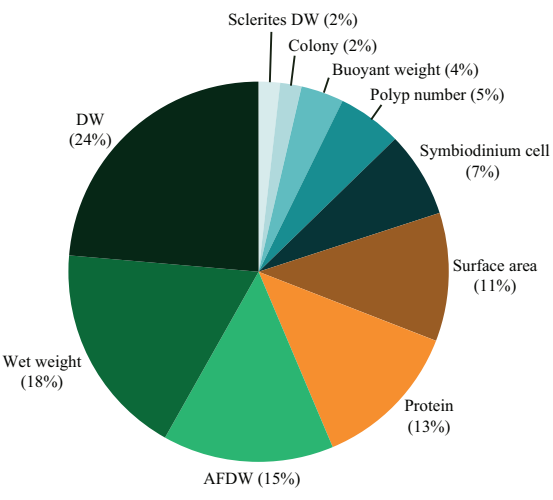

FIGURE 1 | Normalization metrics used in physiological studies on soft corals. Percentages are based on the occurrence of the studies using the metric of interest. DW, dry weight; AFDW, ash-free dry weight.

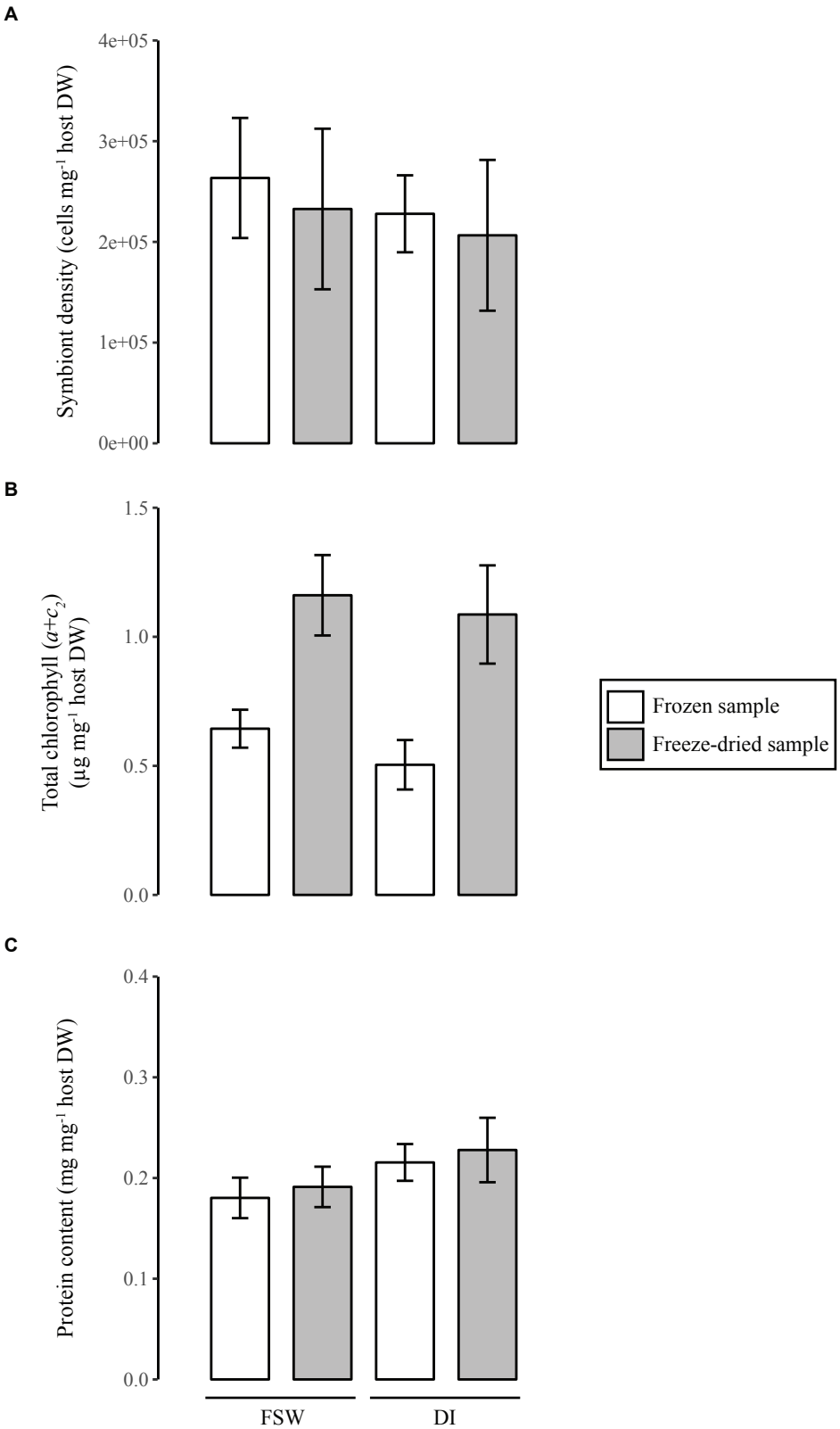

FIGURE 2 | Tissue parameter quantification in soft corals using different sample processing techniques. Samples were either processed frozen or after freeze-drying and homogenized in filtered seawater (FSW) or Milli-Q water (DI). All tissue parameters (*Symbiodinium* density, **A**; chlorophyll, **B**; and protein content, **C**), are normalized to host dry weight. Error bars represent standard deviation of five samples.

TABLE 1 | Summary about the range of metrics used in previous studies to normalize physiological processes in soft corals.

Normalization metric	Family	Measured parameter	Reference
AFDW	Alcyoniidae	Biochemical composition of tissues, DMSP concentration, feeding rates, mucus production, nutrient flux, oxygen flux	Fabricius and Klumpp, 1995; Riegl and Branch, 1995*; Migné, 1997a,b, 2002; Van Alstyne et al., 2006; Slattery et al., 2013
	Briareidae	Oxygen flux	Fabricius and Klumpp, 1995
	Nephtheidae	Feeding rates, oxygen flux	Fabricius and Klumpp, 1995; Fabricius et al., 1995b, 1998
	Xeniidae	Oxygen flux	Fabricius and Klumpp, 1995
DW	Alcyoniidae	Biochemical composition of tissues, caloric content, metabolite concentration, P/A pigments, *Symbiodinium* density	Slattery and McClintock, 1995; Ben-David-Zaslow and Benayahu, 1998; Khalesi et al., 2007, 2009, Rocha et al., 2013a,b; Leal et al., 2015; Costa et al., 2016
	Clavulariidae	Biochemical composition of tissues	Slattery and McClintock, 1995
	Nephtheidae	Biochemical composition of tissues, inorganic carbon, oxygen flux, P/A pigments, *Symbiodinium* density	Farrant et al., 1987; Slattery and McClintock, 1995
	Xeniidae	Biochemical composition of tissues, caloric content, inorganic carbon, P/A pigments	Schlichter et al., 1983, 1984; Ben-David-Zaslow and Benayahu, 1998, 1999; Bednarz et al., 2012
Sclerites DW	Alcyoniidae	Calcification rate	Tentori and Allemand, 2006
Wet weight	Alcyoniidae	Biochemical composition of tissues, calcification rate, DMSP concentration, ETS activity, P/A pigments, *Symbiodinium* density	Michalek-Wagner and Willis, 2001; Tentori and Allemand, 2006; Van Alstyne et al., 2006; Khalesi et al., 2007; Baum et al., 2016; Imbs et al., 2016; Haydon et al., 2018
	Anthothelidae	DMSP concentration	Haydon et al., 2018
	Nephtheidae	Biochemical composition of tissues, calcification rate, cell growth, DMSP concentration, ETS activity, inorganic carbon, oxygen flux, P/A pigments, *Symbiodinium* density	Farrant et al., 1987; Tentori et al., 2004; Baum et al., 2016; Haydon et al., 2018
	Xeniidae	Biochemical composition of tissues, DOM flux, inorganic carbon	Schlichter, 1982
BW	Alcyoniidae	Budding rate, energy expenditure, oxygen flux	Khalesi et al., 2009, 2011
Colony	Xeniidae	Oxygen flux	Kremien et al., 2013
Polyp number	Nephtheidae	Feeding rates, oxygen flux, P/A pigments	Fabricius et al., 1995a,b, 1998
Protein	Alcyoniidae	Calcification rate, DMSP concentration, P/A pigments, *Symbiodinium* density	Tentori and Allemand, 2006; Khalesi et al., 2009; Haydon et al., 2018
	Anthothelidae	DMSP concentration	Haydon et al., 2018
	Nephtheidae	Calcification rate, cell growth, DMSP concentration	Tentori et al., 2004; Haydon et al., 2018
	Xeniidae	Metabolite concentration, oxygen flux, P/A pigments, *symbiodinium* density, uptakes of nutrients	Zeevi Ben-Yosef et al., 2006; Gabay et al., 2013; Ezzat et al., 2016
Surface area	Alcyoniidae	Oxygen flux, *Symbiodinium* density	Drew, 1972; Bednarz et al., 2015; Cardini et al., 2016
	Nephtheidae	Dinitrogen fixation, inorganic carbon, oxygen flux, P/A pigments, *Symbiodinium* density	Farrant et al., 1987; Bednarz et al., 2015; Cardini et al., 2016
	Xeniidae	Dinitrogen fixation, OM flux, oxygen flux	Bednarz et al., 2012, 2015; Kremien et al., 2013; Cardini et al., 2016
Symbiodinium cell	Alcyoniidae	DMSP concentration, P/A pigments	Van Alstyne et al., 2006; Gabay et al., 2013; Rocha et al., 2013a,b
	Xeniidae	P/A pigments	Gabay et al., 2013

AFDW, ash-free dry weight; DW, total dry weight; BW, buoyant weight; DMSP, dimethylsulfoniopropionate; P/A pigments, photosynthetic, and accessory pigments; ETS, electron transport system; DOM, dissolved organic matter. The group "biochemical composition of tissues" gathers measured parameters such as amino acid, protein, carbohydrate, and/or lipid content. The asterisk notifies a study for which the normalizing metric (i.e., dry weight of decalcified samples), has been considered as ash-free dry weight by the authors.

collected in the Red Sea were used for the experiments. They were equally allocated to five 20 L tanks and maintained under the same conditions for 8 weeks prior to starting the experiments. Tanks were continuously supplied with seawater, at a renewal rate of 10 Lh^{-1}. HQI lamps (Tiger E40, Faeber, Italy) above the tanks provided photosynthetically active radiation (PAR) of 200 μmol photons $m^{-2}s^{-1}$.

Optimized Protocol for *Symbiodinium*, Chlorophyll, and Protein Determination

A first experiment, performed with *H. fuscescens*, aimed to compare *Symbiodinium*, chlorophyll, and protein concentrations in frozen or freeze-dried samples, homogenized in FSW or DI. Four combinations were thus considered, with five replicates per

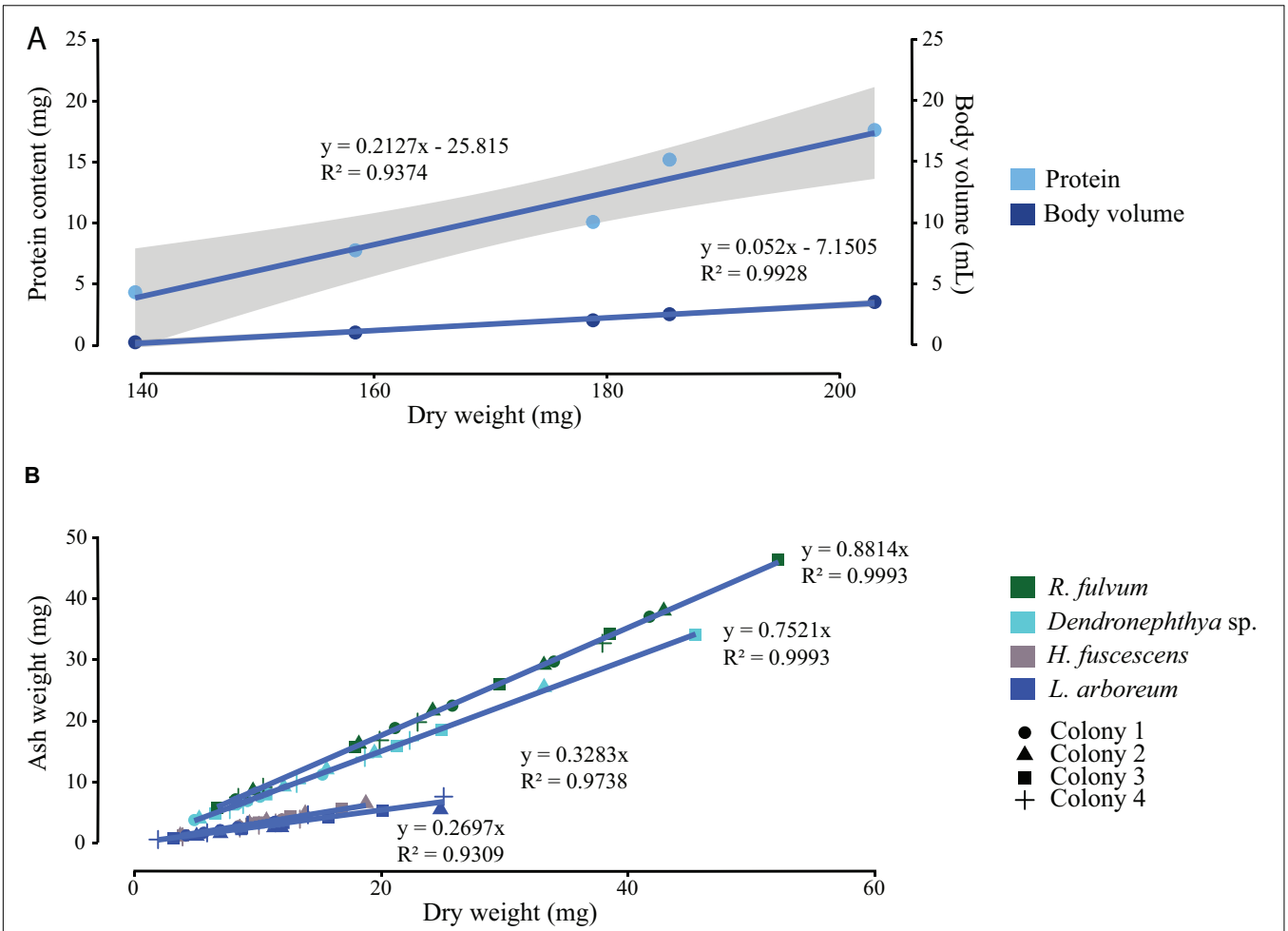

FIGURE 3 | Correlations between the normalizing metrics. **(A)** Total dry weight with protein content and body volume of five *Heteroxenia fuscescens* colonies. **(B)** Linear regressions between total dry weight and ash weight of four colonies of *Rhytisma fulvum*, *Dendronephthya* sp., *H. fuscescens,* and *Litophyton arboreum*. Gray areas display confidence interval of 0.95 around the regression lines.

TABLE 2 | Statistical results about the effect of sample state, and homogenization media on *Symbiodinium* density, total chlorophyll, and protein concentrations measured in *Heteroxenia fuscescens*.

Factor	*df*	*F*-values	*p*-values
***Symbiodinium* density**			
Sample state	1	0.801	0.384
Homogenization media	1	1.121	0.305
Sample state × homogenization media	1	0.026	0.873
Residuals	16		
Total chlorophyll concentration			
Sample state	1	80.478	<0.01
Homogenization media	1	3.058	0.100
Sample state × homogenization media	1	0.284	0.602
Residuals	16		
Protein concentration			
Sample state	1	1.251	0.280
Homogenization media	1	11.925	<0.01
Sample state × homogenization media	1	0.005	0.947
Residuals	16		

TABLE 3 | Significant correlations between dry weight (DW), protein content, body volume, and ash weight (AW), of *Heteroxenia fuscescens*, *Rhytisma fulvum*, *Dendronephthya* sp., and *Litophyton arboreum*.

Species	Tested correlation	*R*	*t*	*df*	*p*-values
H. fuscescens	DW × protein content	0.9682	6.7005	3	<0.01
	DW × body volume	0.9964	20.341	3	<0.01
R. fulvum	DW × AW	0.9997	161.28	18	<0.01
Dendronephthya sp.	DW × AW	0.9996	157.53	18	<0.01
H. fuscescens	DW × AW	0.9868	25.866	18	<0.01
L. arboreum	DW × AW	0.9648	15.575	18	<0.01

condition: (i) freeze-dried samples homogenized in FSW, (ii) freeze-dried samples homogenized in DI, (iii) frozen samples homogenized in FSW, and (iv) frozen samples homogenized in DI. For this purpose, 20 tubes were prepared, each containing one freshly cut polyp of 10 colonies of *H. fuscescens* randomly chosen in the tanks. All tubes were flash-frozen in liquid nitrogen. Half of them were processed directly whereas the other half

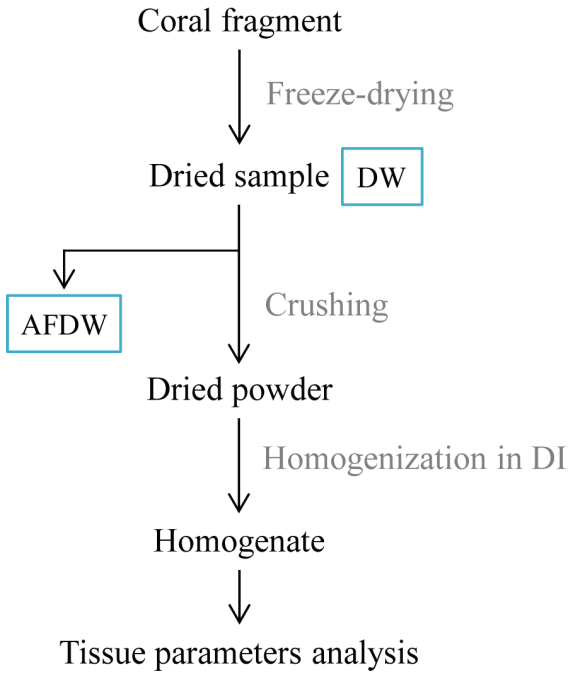

FIGURE 4 | Optimized workflow for processing soft coral tissue samples. DW, dry weight; AFDW, ash-free dry weight; DI, Milli-Q water.

was freeze-dried (Alpha 2-4 LDplus freeze-dryer, Martin Christ, Germany), until obtaining a dried powder. Frozen and freeze-dried samples were ground with a potter tissue grinder and solubilized in 10 mL FSW or DI. This procedure was rapid for the freeze-dried samples (5 min) but took more than 30 min for the frozen samples. From each homogenate, 500 µL was collected for total protein measurement (see following section). The remaining homogenate was centrifuged for 10 min (at 11000 *g* at 4°C) to pellet the *Symbiodinium*. Light microscopy confirmed the total removal of *Symbiodinium* from the supernatant (i.e., host fraction), which was freeze-dried again. This was done to determine the host DW and to subsequently compare the tissue parameters of frozen and freeze-dried samples normalized to the same metric. *Symbiodinium* pellets were rinsed twice to eliminate any remaining host cells (Tremblay et al., 2012), and re-suspended in 10 mL FSW or DI. After mixing, 100 µL and 0.5 mL were subsampled for the determination of *Symbiodinium* density and total chlorophyll concentration, respectively (see following section).

Tissue Parameter Measurements

Proteins were extracted from the 500 µL subsamples in a sodium hydroxide solution (0.5 M) for 5 h at 60°C. Thereafter, protein content was measured using a BC assay kit (Interchim, France) (Smith et al., 1985), and protein standards were prepared using bovine serum albumin (Interchim, France). Absorbance of subsamples was measured at 562 nm by an UVmc^2 spectrophotometer (SAFAS, Monaco). *Symbiodinium* density was quantified microscopically via eight replicate haemocytometer counts in the 100 µL subsamples (Neubauer haemocytometer, Marienfeld, Germany). For chlorophyll analysis, the 5 mL subsamples were centrifuged for 10 min (at 8000 *g* at 4°C). The supernatant was discarded and 5 mL of acetone (100%) amended with magnesium chloride (Sigma-Aldrich, Germany) were added to the *Symbiodinium* pellet to extract the chlorophyll over 24 h in the dark (at 4°C). After centrifugation at 11000 *g* for 15 min at 4°C, absorbances were measured at 750, 663, and 630 nm using a Xenius spectrophotometer (SAFAS, Monaco) and chlorophyll concentrations calculated according to Jeffrey and Humphrey (1975).

Normalization Parameters

An experiment was performed with *H. fuscescens* to highlight any correlation between biomass (DW), protein content, and colony's body volume. The body volume of five colonies with different polyp numbers was estimated using the water displacement technique (Benayahu and Loya, 1986), before colonies were freeze-dried and homogenized in 10 mL DI. A subsample of 500 µL was used for protein content determination. We also compared the DW and ash weight (AW) of the four different coral species listed above. For this purpose, colonies of each species were freeze-dried, crushed, and split in five powder heaps of different weights (DW). They were combusted at 450°C for 4 h in a muffle furnace (Thermolyne 62700, Thermo Fisher Scientific, United States). Such combustion process avoids losses of sclerites (Harvell and Fenical, 1989) and allows estimating the DW of sclerites.

Statistical Analyses

Analyses were performed using R software (R Foundation for Statistical Computing) and assumptions of normality and homoscedasticity of variance were evaluated through Shapiro's and Bartlett's tests along with graphical analyses of residuals. Protein, *Symbiodinium,* and chlorophyll concentrations were analyzed using a two-way ANOVA, in which the sample state (frozen vs. freeze-dried) and type of water (FSW vs. DI) were fixed effects. Regression equations were established between colony DW and protein content, volume or AW. Pearson's product moment correlation coefficients were calculated to test the strength of the linear associations.

RESULTS AND DISCUSSION

This study shows that both the sample state (i.e., frozen, freeze-dried) and the homogenization media (i.e., DI and FSW) significantly influence the quantification of tissue parameters in soft corals, while no effect was observed for *Symbiodinium* counts (**Figure 2** and **Table 2**). Sample state mainly affects chlorophyll determinations with twofold higher concentrations in freeze-dried as compared to frozen samples (**Figure 2B**). A previous work on reef sands also demonstrated a 27 to 39% increased chlorophyll concentration in freeze-dried over frozen samples, likely due to the removal of mucous matrices surrounding the cells and a better exposure of pigments to the solvent in the former (Hannides et al., 2014). Here, the reduced

chlorophyll concentration in frozen samples likely results from pigment damage due to the longer homogenization period of frozen fragments (30 min) as compared to the freeze-dried fragments (5 min). Although samples were kept on ice during homogenization, this caution did not prevent tissue slurry from mechanic heating with the Potter grinder. Similarly, Hannides et al. (2014) highlighted that longer sonication periods caused a significant decline in chlorophyll content, due to pigment degradation (Metaxatos and Ignatiades, 2002). Variations in the length of the homogenization procedure can occur for coral samples with different tissue thickness, and may amplify variance in the chlorophyll measurements. Future studies on corals should thus take into account such tissue processing flaws and compare the efficacy of the usual tissue extraction procedure with the freeze-drying one. Care must also be taken when comparing studies that use photosynthetic pigments as normalization metric for other parameters (e.g., coral productivity). As highlighted in this study, a 50% reduced photosynthetic pigment extraction in frozen as compared to freeze-dried samples can lead to a significant overestimation of the productivity in the former, an issue that can be extended to scleractinian corals too. Finally, saving time is another benefit when processing freeze-dried over frozen samples, which is important in experiments involving numerous samples. Although the homogenization media has an overall low effect on tissue processing, the use of DI compared to FSW significantly increases the protein content measurable in tissue samples (**Figure 2C** and **Table 2**). This could have important implications when comparing physiological processes across different coral species and environmental conditions. The use of DI also reduces the amount of salts in homogenates, thereby providing a higher accuracy for the determination of absolute host and *Symbiodinium* DW as well as avoiding salt crystals entering instruments such as mass spectrometers (Baker et al., 2015).

As highlighted in the introduction, a literature review identified 36 physiological studies on soft corals, which display a wide variety of normalization metrics (**Figure 1**, **Table 1**, and **Supplementary Table S1**). Although the choice of the metrics certainly depends on the process under study and the information provided, a consensual normalization metric is important to allow better comparability of results among studies. For a given soft coral species maintained under a constant environmental condition, all metrics can be proxies for biomass and colony size. Results obtained with *H. fuscescens* indeed exhibit significant linear relationships between total DW and body volume or protein concentration (**Figure 3A** and **Table 3**). Other studies have also shown significant correlations between AFDW or DW and polyp number, colony height, or carbon and nitrogen content (Migné, 1993; Fabricius et al., 1995b; Migné et al., 1996). However, the use of several metrics listed above can be unsatisfactory when comparing processes between different species or different environmental conditions. We demonstrate that DW does not provide a reliable normalization metric to compare tissue parameters across coral species (**Figure 3B** and **Table 3**), although dried samples enable better tissue processing in soft corals. Linear regression analyses between DW and AW of different soft coral species show species-specific linear relationships; this is due to the presence of sclerites, which can differ in size, shape, and abundance within a colony and between species (Van Alstyne et al., 1992). Sclerites are frequently used as taxonomic indicator, and species-specific differences likely depend on the necessity to accumulate sclerites for structural defense against predators and/or skeletal support against wave action (Van Alstyne et al., 1992). Overall, our results show that the contribution of sclerites to the total DW of the colony can highly differ between species, and thus, we recommend the use of AFDW over DW for normalizing physiological processes in soft corals, particularly when inter-species comparisons are made.

CONCLUSION

In conclusion, we recommend to dry the biological material for biochemical and tissue parameter analyses and to use a subset of the dried powder for AFDW determination as final normalization metric (**Figure 4**). AFDW stands for a useful metric to normalize tissue parameters in soft corals, benefiting of the aforementioned advantages owed from its freeze-dried state and allowing best comparability among species. Other non-destructive normalization metrics, such as surface area, can, however, be used in parallel to provide a different information on the physiological processes studied.

ETHICS STATEMENT

Animals involved in the subject are not listed in CITES and were cultured in the laboratory for experimental purpose.

AUTHOR CONTRIBUTIONS

CP, VB, and CF-P designed the study, wrote and reviewed the manuscript. CP and VB performed the experimental works. CP analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00348/full#supplementary-material

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