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A new protocol using acidification for preserving DMSP in macroalgae and comparison with existing protocols

Bucciarelli Eva [1, *], Stiger-Pouvreau Valerie [1], Connan Solène [1]

[1] Univ Brest CNRS IRD, Ifremer, LEMAR F-29280 Plouzane, France

* Corresponding author : Eva Bucciarelli, email address : Eva.Bucciarelli@univ-brest.fr

Abstract :

Dimethylsulfoniopropionate (DMSP) plays many important physiological and ecological roles in macroalgae. The most common method to measure DMSP is by gas chromatography analysis of the dimethylsulfide (DMS) produced after NaOH hydrolysis (pH > 12). Storage of DMS, however, is not recommended for more than a week. We investigated if acidification can be a suitable method to preserve DMSP in macroalgal samples over three months of storage, compared to widely used protocols such as drying and freezing at -20°C. The DMSP content of green (Ulva sp. and Ulva compressa), red (Chondrus crispus) and brown (Bifurcaria bifurcata) macroalgae were analyzed 24 h after NaOH addition (control values); and after acidification (0.2 mol · L HCl-1) for 24 h of fresh material, followed by NaOH addition for 24 h. These values were compared to measurements after 3-month storage of samples that had been either dried in a heater (60°C for a night, and storage at room temperature), or frozen at -20°C, or kept in 0.2 mol · L HCl-1. There was no significant difference between DMSP measurements on freshly collected material and after acidification of the samples, whether 24 h later or after 3 months of storage. This was in contrast with 3-month storage protocols involving overnight drying at 60°C (75-98% DMSP loss), and to a lesser degree freezing at -20°C (37-80% DMSP loss). We thus advise to acidify macroalgal samples for preservation over long periods of time rather than drying or freezing, when assaying DMSP content.

Keywords : acidification, DMSP, drying, freezing, macroalgae, storage protocol

Abbreviations list: DMS, dimethylsulfide; DMSP, dimethylsulfoniopropionate; FW, fresh weight; GC, gas chromatography

Dimethylsulfoniopropionate (DMSP) is a tertiary sulfonium compound produced intracellularly mainly by micro- and macroalgae (Malin and Kirst 1997, Bullock et al. 2017). It plays a wide range of physiological and ecological roles in macroalgae. Within the cells, it serves as an osmolyte, and as an antioxidant (Stefels 2000, Van Alstyne 2008, Rix et al. 2012). DMSP and its enzymatic breakdown product dimethylsulfide (DMS) are also known to play a key role in chemical signalling, from bacteria (Barak-Gavish et al. 2018) to top predators (Nevitt 2011). Recent studies showed that DMSP from macroalgae can either inhibit (Saha et al. 2012) or promote (Kessler et al. 2018) bacterial activity. This production is thought to be part of an activated defense system (Van Alstyne et al. 2001, Wiesemeier et al. 2007), and to induce a food-source chemical signal for bacteria, that in turn allow the morphogenesis and development of the DMSP-producer macroalga (Kessler et al. 2018, Wichard and Beemelmanns 2018). High DMSP concentrations are generally found in green macroalgae and a few red algal species, while brown macroalgae only contain small amounts (Reed 1983, Van Alstyne and Puglisi 2007).

The most common method to measure DMSP is by GC analysis of the DMS released after 24 h of NaOH hydrolysis (pH> 12; White 1982). However, DMS is a volatile compound, prone to oxidation, that has been shown to decrease significantly in sealed vials after a week of storage (Curran et al. 1998). Besides, other dimethylsulfonium compounds than DMSP can yield DMS after prolonged NaOH hydrolysis (Howard and Russell 1996). Protocols to preserve the samples of macroalgae and plants have been proposed, including drying or freezing (Karsten et al. 1994, Russell and Howard 1996, McFarlin and Alber 2013, Borges and Champenois 2017). These methods, however, can strongly affect DMSP contents, compared to analyses on fresh samples (Bischoff et al. 1994, Karsten et al. 1994, Russell and Howard 1996). Still, storing samples over more than a week can be unavoidable in case of a cruise or field sampling, or when needing to analyze more than a few samples at a time. Acidification is an alternative method, which has been successfully used to preserve DMSP in seawater and phytoplankton culture samples over months of storage (Curran et al. 1998, del Valle et al. 2011). DMSP is highly stable in low pH solutions (Dacey and Blough 1987). Furthermore, acidification lyses membranes of macroalgal cells and increases their porosity (Zemke-White et al. 1999, 2000), thereby increasing the potential recovery and stabilisation of intracellular DMSP in macroalgae.

In this study, we investigated if acidification can be a suitable method to preserve DMSP over 3 months in samples of macroalgae, compared to drying and freezing.

Four species of macroalgae were collected by hand at low tide on the west coast of Brittany (Pointe du Diable, 48°28.871' N, 4°46.142' W), a field site nearby the laboratory. These macroalgae include two green, a brown and a red species, to test for the validity of our protocols

on macroalgae with high (green species) and low (red and brown species) DMSP contents (Reed 1983, Van Alstyne and Puglisi 2007). Our green species include the foliose *Ulva* sp. Linnaeus (collected on Oct. 8th 2018; Chlorophyta, Ulvophyceae, Ulvales, Ulvaceae) and the tubular *Ulva compressa* Linnaeus (collected on April 8th 2019; Chlorophyta, Ulvophyceae, Ulvales, Ulvaceae). The brown and red species were *Bifurcaria bifurcata* (collected on Oct. 8th 2018; Ochrophyta, Phaeophyceae, Fucales, Sargassaceae), and *Chondrus crispus* (collected on Nov. 7th 2018; Rhodophyta, Florideophyceae, Gigartinales, Gigartinaceae), respectively. Ten different thalli (i.e., individuals) per species were transported back to the laboratory in plastic bags. Shortly after returning to the laboratory, each individual was carefully dried with a tissue and most of the visible epiphytes (e.g., Ceramiales and Ulvales) were removed by hand. According to the DMSP values expected from the literature, and to ensure that measurements using GC would be detectable, a few micrograms (green macroalgae) to a few milligrams (red and brown macroalgae) from each individual was sampled using a clean scissor. This was repeated 5 times on each individual, so as to have 5 subsets of 10 replicates for the five different protocols. For the first protocol, the samples were placed in 10 mL glass vials, 5 mL of 5 mol · L $NaOH^{-1}$ were added and the vials were sealed. DMS resulting from DMSP degradation in these basic conditions (pH > 12) was measured on the following day (Protocol P1-NaOH-T0) and this value was considered as the reference value. Samples from the second subset were wrapped in aluminium foil and dried in a heater at 60°C for a night (Protocol P2-Drying), and further kept at room temperature for 3 months. Samples from the third subset were placed in 10 mL glass vials that were sealed and stored at -20°C for 3 months (Protocol P3-Freezing). Samples from the fourth and the fifth subsets were placed in 10 mL glass vials with 4 mL of 0.2 mol · L HCl^{-1} (pH < 2) and the vials were sealed and kept at room temperature. A day later, the vials of the fourth subset were opened for addition of 1 mL of 5 mol · L $NaOH^{-1}$ (pH > 12) and the vials were sealed again; DMS analyses were run on the following day (Protocol P4-HCl-T0). Vials of the fifth subset were stored for 3 months (Protocol P5-HCl-Tf). After 3 months, dried samples from Protocol P2-Drying were placed in 10 mL glass vials, and 5 mol · L $NaOH^{-1}$ was added to all samples from P2-Drying (5 mL), P3-Freezing (5 mL) and P5-HCl-Tf (1 mL) so as to achieve a final volume of 5 mL in the vials. Analyses for DMS were run on the following day.

DMSP was analyzed as DMS using a Shimadzu 2010-Plus Gas Chromatograph (GC) equipped with a sulfur-selective flame photometric detector (air / H_2: 70 mL · min^{-1} / 60 mL · min^{-1}) and an Equity 1 capillary column (3.2 mm i.d., 30 m long, Supelco, T = 180°C, carrier gas: He, 6.7 mL · min^{-1}). Detector and injection port temperatures were set at 250°C. Depending on the macroalgal DMSP content, 10 to 250 µL were collected from the headspace of the vials using a gas tight

syringe and were directly injected into the GC injector port. The same headspace volume was sampled from the DMSP standards and the samples. Calibration curves for protocols P1-NaOH-T0, P2-Drying and P3-Freezing consisted in increasing additions of 10^{-3} mol · L $DMSP^{-1}$ into 5 mL of 5 mol · L $NaOH^{-1}$ in 10 mL glass vials, which were sealed for a day before analyses. Calibration curves for protocols P4-HCl-T0 and P5-HCl-Tf consisted in increasing additions of 10^{-3} mol · L $DMSP^{-1}$ into 4 mL of 0.2 mol · L HCl^{-1} in 10 mL glass vials, which were sealed for a day (P4-HCl-T0) or for 3 months (P5-HCl-Tf). One day before the analyses were run, the vials were opened for addition of 1 mL of 5 mol · L $NaOH^{-1}$ and re-sealed. Five to ten standards were run for calibration. The correlation coefficients R^2 of the calibration curves ranged between 0.984 and 0.999 (0.998 ± 0.004, mean ± STD, n = 17).

Statistical analyses were performed using R software (version 3.6.3; R Core Team 2020) through the integrated development environment Rstudio (version 1.2.5033; Rstudio Team 2020). DMSP levels measured with Protocol P1-NaOH-T0 were compared among species using a Kruskal Wallis test with a confidence level of 95% as data did not meet the requirements for parametric tests. Multiple comparisons were performed using the pairwise non-parametric Wilcoxon test. Because the data did not follow a normal distribution (Shapiro-Wilks test, *p*-value < 0.05 for each algal species), the differences in DMSP content among protocols for each macroalgal species were tested using the non-parametric Friedman rank test for data in repeated measurement designs at a confidence level of 95%. When a statistical difference among protocols was detected, post-hoc pairwise comparisons using a Nemenyi-Wilcoxon test were applied. The effect of epiphytes occurrence on DMSP content was tested using a Kruskal Wallis test. The impact of hexane on the removal of epiphytes and therefore on DMSP content was tested using a Paired t test.

Figure 1 displays the mean DMSP concentrations for each macroalgal species according to the different protocols (in µmol · g^{-1} algal fresh weight (FW) for the two green *Ulva* spp. and in nmol · g FW^{-1} for the brown and red species). To our knowledge, values of DMSP for *Bifurcaria bifurcata* are not reported in the literature. For *Chondrus crispus* and *U. compressa*, our values of DMSP measured following Protocol P1-NaOH-T0 were similar to values reported previously following a similar protocol (respectively, in nmol · g FW^{-1}: < 50, Reed 1983; 47 ± 21, Russell and Howard 1996; and in µmol · g FW^{-1}: 25.4-39.5, Reed 1983; 20.2 ± 7.3, Bischoff et al. 1994; ~ 20, Steinke et al. 1996, see also Table S1 for comparison with available data from the literature for the same species, including other analytical methods than GC). Acidification gave the best results as a preservation method for the four studied species. Values measured after 24 h acidification (P4-HCl-T0) were not significantly different from values measured according

to P1-NaOH-T0, including for *U. compressa* (Fig. 1). This last species exhibits the same, high, DMSP-lyase activity as the microalgal *Phaeocystis* sp. (~ 10 nmol · min^{-1} · mg^{-1} of protein; Steinke et al. 1996). Del Valle et al. (2011) showed that acidification was suitable to preserve DMSP for months in microalgal samples, including samples of single cell *Phaeocystis* spp., but that a substantial loss of DMSP occurs within a few minutes after acid addition when *Phaeocystis* spp. are present in the colonial stage. This has been attributed to possible differences in the physico-chemical characteristics of the enzymes or to differences in the cellular location of DMSP and the enzymes between single cell and colonial stages of *Phaeocystis* life cycle (del Valle et al. 2011). In our study there was no measurable DMSP loss in *U. compressa* after acidification. We cannot rule out, however, that there might be an effect of acidification in other species that have also been shown to express DMSP-lyase activity (e.g., Nishiguchi and Goff 1995, Steinke et al. 1996, van Alstyne and Houser 2003, Garcia-Jimenez et al. 2013). The nature of DMSP-lyases in macroalgae has begun to be unveiled only very recently, and their locations are still unknown (De Clerck et al. 2018). Until their characteristics and functioning are better constrained, we recommend to conduct preliminary tests to assess the validity of the acidification protocol before storage, e.g., by comparing values from P1-NaOH-T0 and P4-HCl-T0.

There was no statistical difference in DMSP content between acidified samples analyzed 24 h after sampling (P4-HCl-T0) and after 3 months of acidified storage (P5-HCl-Tf) for any of the four species (Fig. 1). On the contrary, only 2-25% of DMSP remained in all four macroalgae after overnight drying at 60°C and 3 months of storage (P2-Drying; Fig. 1). Bischoff et al. (1994), who oven-dried *Ulva compressa* for 12 h at 80°C, also reported that only ~ 16% of DMSP remained after 3-4 months of storage. Freezing at -20°C for 3 months (P3-Freezing) also decreased the initial DMSP content of the macroalgae, but to a lesser extent than P2-Drying (20-63%; Fig. 1). We did not test the effects of freeze-drying of the tissues (e.g., Karsten et al. 1994), or freezing at -80°C, which may improve the preservation of DMSP compared to freezing at -20°C.

A high variability could however be noted with the acidification protocol, especially for *Bifurcaria bifurcata* and *Chondrus crispus*. Removing obvious outliers for these species (Fig. 1) did not change the statistical results: DMSP levels for protocols P4-HCl-T0, P5-HCl-Tf and P1-NaOH-T0 remained higher than for P2-Drying and P3-Freezing (Friedman rank tests; *p*-value = $2.286.10^{-5}$ and $1.247.10^{-5}$ for *B. bifurcata* and *C. crispus*, respectively). This high variability may be due to intrathallus variability, and to the presence of epiphytes. In *Ulva lactuca* (Van Alstyne et al. 2007) and in the brown seaweed *Taonia atomaria* (Paix et al. 2020), DMSP concentrations are higher at the base of the thallus than in the middle of the thallus or at the

edges. For *B. bifurcata* and *C. crispus*, we had to mix several pieces of the middle part of the thallus to ensure that there would be enough material to analyze (a few milligrams). This may have increased DMSP variability. Epiphytes can also increase DMSP content in some plant species (Dacey et al. 1994). We carefully removed epiphytes such as other algae from our samples, but other epiphytes like bryozoans were still evident and difficult to remove on some samples of *C. crispus*. Epiphytic microalgae and bacteria could also affect DMSP measurements (Kessler et al. 2018, Paix et al. 2020). In a supplementary experiment, we collected 20 individuals of *C. crispus* (same site of collection, Feb. 7th 2019): 10 were colonised by epiphytes (including bryozoans), and 10 were devoid of epiphytes. Each one of the 20 individuals was divided in 2 subsamples. One of them was dipped in hexane for 30 s to remove microfouling organisms (de Nys et al. 1998) before being processed for DMSP measurements. As a result, we could compare DMSP contents (following Protocol P1-NaOH-T0) between populations with and without visible epiphytes (32 ± 42 nmol · g FW^{-1}, mean ± STD, n=10, and 7 ± 6 nmol · g FW^{-1}, mean ± STD, n=10, respectively), and when populations with and without visible epiphytes were dipped in hexane (53 ± 94 nmol · g FW^{-1}, mean ± STD, n=10, and 9 ± 11 nmol · g FW^{-1}, mean ± STD, n=10, respectively). The removal of fouling organisms by hexane dipping did not change the DMSP measurements, neither for the epiphyte colonised samples (Paired-t-test, $t_9 = -1.221$, $p = 0.253$) nor for the others (Paired-t-test, $t_9 = -0.684$, $p = 0.511$). DMSP contents on the contrary were significantly higher in epiphyte colonised samples than in the others (Kruskal-Wallis, Chi-Square$_1 = 5.491$, $p = 0.019$). The presence of epiphytes such as bryozoans may thus explain at least part of the observed DMSP variability in *C. crispus*.

In conclusion, there was no significant difference between DMSP measurements on freshly collected material (P1-NaOH-T0) and after acidification of the samples, whether 24 h later (P4-HCl-T0) or after 3 months of storage (P5-HCl-Tf), for any of the species that we considered. This is in contrast with 3-month storage protocols involving overnight drying at 60°C (P2-Drying), and to a lesser degree freezing at -20°C (P3-Freezing). We thus advise to use acidification (0.2 mol · L HCl^{-1}, pH < 2) for preservation of macroalgal samples over long periods of time rather than drying or freezing at -20°C, when assaying DMSP content.

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Figure 1: DMSP concentrations in the four macroalgal species according to the five different protocols (in µmol or nmol · g^{-1} algal fresh weight depending on the algal species). Significant differences among protocols for each macroalgal species are marked with different letters (n = 10; Friedman rank test for data in repeated measurement designs with post-hoc Nemenyi-Wilcoxon tests; *p*-value < 0.05).

Table S1: DMSP content (in µmol or nmol DMSP · g of algal FW^{-1} depending on the algal species) for the four species of macroalgae according to the five different protocols of preservation in our study (see text for detail), and comparison with available data from the literature for the same species (or *Ulva lactuca* as a foliose *Ulva* sp.). For this study: mean ± standard deviation; n = 10; for literature data, depending on the available data: range, mean or mean ± standard deviation. $^{\#}$ = *Ulva lactuca* as a foliose *Ulva* sp. * = *Ulva mutabilis* conspecific with *Ulva compressa* (Steinhagen et al. 2019).