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

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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

38

39 Abbreviations list: DMS, dimethylsulfide; DMSP, dimethylsulfoniopropionate; FW, fresh weight;

40 GC, gas chromatography

41 Dimethylsulfoniopropionate (DMSP) is a tertiary sulfonium compound produced intracellularly
42 mainly by micro- and macroalgae (Malin and Kirst 1997, Bullock et al. 2017). It plays a wide
43 range of physiological and ecological roles in macroalgae. Within the cells, it serves as an
44 osmolyte, and as an antioxidant (Stefels 2000, Van Alstyne 2008, Rix et al. 2012). DMSP and its
45 enzymatic breakdown product dimethylsulfide (DMS) are also known to play a key role in
46 chemical signalling, from bacteria (Barak-Gavish et al. 2018) to top predators (Nevitt 2011).
47 Recent studies showed that DMSP from macroalgae can either inhibit (Saha et al. 2012) or
48 promote (Kessler et al. 2018) bacterial activity. This production is thought to be part of an
49 activated defense system (Van Alstyne et al. 2001, Wiesemeier et al. 2007), and to induce a food-
50 source chemical signal for bacteria, that in turn allow the morphogenesis and development of the
51 DMSP-producer macroalga (Kessler et al. 2018, Wichard and Beemelmans 2018). High DMSP
52 concentrations are generally found in green macroalgae and a few red algal species, while brown
53 macroalgae only contain small amounts (Reed 1983, Van Alstyne and Puglisi 2007).
54 The most common method to measure DMSP is by GC analysis of the DMS released after 24 h
55 of NaOH hydrolysis (pH > 12; White 1982). However, DMS is a volatile compound, prone to
56 oxidation, that has been shown to decrease significantly in sealed vials after a week of storage
57 (Curran et al. 1998). Besides, other dimethylsulfonium compounds than DMSP can yield DMS
58 after prolonged NaOH hydrolysis (Howard and Russell 1996). Protocols to preserve the samples
59 of macroalgae and plants have been proposed, including drying or freezing (Karsten et al. 1994,
60 Russell and Howard 1996, McFarlin and Alber 2013, Borges and Champenois 2017). These
61 methods, however, can strongly affect DMSP contents, compared to analyses on fresh samples
62 (Bischoff et al. 1994, Karsten et al. 1994, Russell and Howard 1996). Still, storing samples over
63 more than a week can be unavoidable in case of a cruise or field sampling, or when needing to
64 analyze more than a few samples at a time. Acidification is an alternative method, which has
65 been successfully used to preserve DMSP in seawater and phytoplankton culture samples over
66 months of storage (Curran et al. 1998, del Valle et al. 2011). DMSP is highly stable in low pH
67 solutions (Dacey and Blough 1987). Furthermore, acidification lyses membranes of macroalgal
68 cells and increases their porosity (Zemke-White et al. 1999, 2000), thereby increasing the
69 potential recovery and stabilisation of intracellular DMSP in macroalgae.
70 In this study, we investigated if acidification can be a suitable method to preserve DMSP over 3
71 months in samples of macroalgae, compared to drying and freezing.

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

75 on macroalgae with high (green species) and low (red and brown species) DMSP contents (Reed
76 1983, Van Alstyne and Puglisi 2007). Our green species include the foliose *Ulva* sp. Linnaeus
77 (collected on Oct. 8th 2018; Chlorophyta, Ulvophyceae, Ulvales, Ulvaceae) and the tubular *Ulva*
78 *compressa* Linnaeus (collected on April 8th 2019; Chlorophyta, Ulvophyceae, Ulvales, Ulvaceae).
79 The brown and red species were *Bifurcaria bifurcata* (collected on Oct. 8th 2018; Ochrophyta,
80 Phaeophyceae, Fucales, Sargassaceae), and *Chondrus crispus* (collected on Nov. 7th 2018;
81 Rhodophyta, Florideophyceae, Gigartinales, Gigartinaceae), respectively. Ten different thalli
82 (i.e., individuals) per species were transported back to the laboratory in plastic bags. Shortly after
83 returning to the laboratory, each individual was carefully dried with a tissue and most of the
84 visible epiphytes (e.g., Ceramiales and Ulvales) were removed by hand. According to the DMSP
85 values expected from the literature, and to ensure that measurements using GC would be
86 detectable, a few micrograms (green macroalgae) to a few milligrams (red and brown
87 macroalgae) from each individual was sampled using a clean scissor. This was repeated 5 times
88 on each individual, so as to have 5 subsets of 10 replicates for the five different protocols. For the
89 first protocol, the samples were placed in 10 mL glass vials, 5 mL of 5 mol · L NaOH⁻¹ were
90 added and the vials were sealed. DMS resulting from DMSP degradation in these basic
91 conditions (pH > 12) was measured on the following day (Protocol P1-NaOH-T0) and this value
92 was considered as the reference value. Samples from the second subset were wrapped in
93 aluminium foil and dried in a heater at 60°C for a night (Protocol P2-Drying), and further kept at
94 room temperature for 3 months. Samples from the third subset were placed in 10 mL glass vials
95 that were sealed and stored at -20°C for 3 months (Protocol P3-Freezing). Samples from the
96 fourth and the fifth subsets were placed in 10 mL glass vials with 4 mL of 0.2 mol · L HCl⁻¹ (pH
97 < 2) and the vials were sealed and kept at room temperature. A day later, the vials of the fourth
98 subset were opened for addition of 1 mL of 5 mol · L NaOH⁻¹ (pH > 12) and the vials were sealed
99 again; DMS analyses were run on the following day (Protocol P4-HCl-T0). Vials of the fifth
100 subset were stored for 3 months (Protocol P5-HCl-Tf). After 3 months, dried samples from
101 Protocol P2-Drying were placed in 10 mL glass vials, and 5 mol · L NaOH⁻¹ was added to all
102 samples from P2-Drying (5 mL), P3-Freezing (5 mL) and P5-HCl-Tf (1 mL) so as to achieve a
103 final volume of 5 mL in the vials. Analyses for DMS were run on the following day.
104 DMSP was analyzed as DMS using a Shimadzu 2010-Plus Gas Chromatograph (GC) equipped
105 with a sulfur-selective flame photometric detector (air / H₂: 70 mL · min⁻¹ / 60 mL · min⁻¹) and an
106 Equity 1 capillary column (3.2 mm i.d., 30 m long, Supelco, T = 180°C, carrier gas: He, 6.7 mL ·
107 min⁻¹). Detector and injection port temperatures were set at 250°C. Depending on the macroalgal
108 DMSP content, 10 to 250 µL were collected from the headspace of the vials using a gas tight

109 syringe and were directly injected into the GC injector port. The same headspace volume was
110 sampled from the DMSP standards and the samples. Calibration curves for protocols P1-NaOH-
111 T0, P2-Drying and P3-Freezing consisted in increasing additions of 10^{-3} mol · L DMSP⁻¹ into 5
112 mL of 5 mol · L NaOH⁻¹ in 10 mL glass vials, which were sealed for a day before analyses.
113 Calibration curves for protocols P4-HCl-T0 and P5-HCl-Tf consisted in increasing additions of
114 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
115 day (P4-HCl-T0) or for 3 months (P5-HCl-Tf). One day before the analyses were run, the vials
116 were opened for addition of 1 mL of 5 mol · L NaOH⁻¹ and re-sealed. Five to ten standards were
117 run for calibration. The correlation coefficients R^2 of the calibration curves ranged between 0.984
118 and 0.999 (0.998 ± 0.004 , mean \pm STD, $n = 17$).

119 Statistical analyses were performed using R software (version 3.6.3; R Core Team 2020)
120 through the integrated development environment Rstudio (version 1.2.5033; Rstudio Team 2020).
121 DMSP levels measured with Protocol P1-NaOH-T0 were compared among species using a
122 Kruskal Wallis test with a confidence level of 95% as data did not meet the requirements for
123 parametric tests. Multiple comparisons were performed using the pairwise non-parametric
124 Wilcoxon test. Because the data did not follow a normal distribution (Shapiro-Wilks test, p -value
125 < 0.05 for each algal species), the differences in DMSP content among protocols for each
126 macroalgal species were tested using the non-parametric Friedman rank test for data in repeated
127 measurement designs at a confidence level of 95%. When a statistical difference among protocols
128 was detected, post-hoc pairwise comparisons using a Nemenyi-Wilcoxon test were applied. The
129 effect of epiphytes occurrence on DMSP content was tested using a Kruskal Wallis test. The
130 impact of hexane on the removal of epiphytes and therefore on DMSP content was tested using a
131 Paired t test.

132 Figure 1 displays the mean DMSP concentrations for each macroalgal species according
133 to the different protocols (in $\mu\text{mol} \cdot \text{g}^{-1}$ algal fresh weight (FW) for the two green *Ulva* spp. and
134 in $\text{nmol} \cdot \text{g FW}^{-1}$ for the brown and red species). To our knowledge, values of DMSP for
135 *Bifurcaria bifurcata* are not reported in the literature. For *Chondrus crispus* and *U. compressa*,
136 our values of DMSP measured following Protocol P1-NaOH-T0 were similar to values reported
137 previously following a similar protocol (respectively, in $\text{nmol} \cdot \text{g FW}^{-1}$: < 50 , Reed 1983; $47 \pm$
138 21 , Russell and Howard 1996; and in $\mu\text{mol} \cdot \text{g FW}^{-1}$: $25.4\text{-}39.5$, Reed 1983; 20.2 ± 7.3 , Bischoff
139 et al. 1994; ~ 20 , Steinke et al. 1996, see also Table S1 for comparison with available data from
140 the literature for the same species, including other analytical methods than GC). Acidification
141 gave the best results as a preservation method for the four studied species. Values measured after
142 24 h acidification (P4-HCl-T0) were not significantly different from values measured according

143 to P1-NaOH-T0, including for *U. compressa* (Fig. 1). This last species exhibits the same, high,
144 DMSP-lyase activity as the microalgal *Phaeocystis* sp. ($\sim 10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein;
145 Steinke et al. 1996). Del Valle et al. (2011) showed that acidification was suitable to preserve
146 DMSP for months in microalgal samples, including samples of single cell *Phaeocystis* spp., but
147 that a substantial loss of DMSP occurs within a few minutes after acid addition when *Phaeocystis*
148 spp. are present in the colonial stage. This has been attributed to possible differences in the
149 physico-chemical characteristics of the enzymes or to differences in the cellular location of
150 DMSP and the enzymes between single cell and colonial stages of *Phaeocystis* life cycle (del
151 Valle et al. 2011). In our study there was no measurable DMSP loss in *U. compressa* after
152 acidification. We cannot rule out, however, that there might be an effect of acidification in other
153 species that have also been shown to express DMSP-lyase activity (e.g., Nishiguchi and Goff
154 1995, Steinke et al. 1996, van Alstyne and Houser 2003, Garcia-Jimenez et al. 2013). The nature
155 of DMSP-lyases in macroalgae has begun to be unveiled only very recently, and their locations
156 are still unknown (De Clerck et al. 2018). Until their characteristics and functioning are better
157 constrained, we recommend to conduct preliminary tests to assess the validity of the acidification
158 protocol before storage, e.g., by comparing values from P1-NaOH-T0 and P4-HCl-T0.

159 There was no statistical difference in DMSP content between acidified samples analyzed
160 24 h after sampling (P4-HCl-T0) and after 3 months of acidified storage (P5-HCl-Tf) for any of
161 the four species (Fig. 1). On the contrary, only 2-25% of DMSP remained in all four macroalgae
162 after overnight drying at 60°C and 3 months of storage (P2-Drying; Fig. 1). Bischoff et al.
163 (1994), who oven-dried *Ulva compressa* for 12 h at 80°C, also reported that only $\sim 16\%$ of
164 DMSP remained after 3-4 months of storage. Freezing at -20°C for 3 months (P3-Freezing) also
165 decreased the initial DMSP content of the macroalgae, but to a lesser extent than P2-Drying (20-
166 63%; Fig. 1). We did not test the effects of freeze-drying of the tissues (e.g., Karsten et al. 1994),
167 or freezing at -80°C, which may improve the preservation of DMSP compared to freezing at -
168 20°C.

169 A high variability could however be noted with the acidification protocol, especially for
170 *Bifurcaria bifurcata* and *Chondrus crispus*. Removing obvious outliers for these species (Fig. 1)
171 did not change the statistical results: DMSP levels for protocols P4-HCl-T0, P5-HCl-Tf and P1-
172 NaOH-T0 remained higher than for P2-Drying and P3-Freezing (Friedman rank tests; p -
173 value = $2.286 \cdot 10^{-5}$ and $1.247 \cdot 10^{-5}$ for *B. bifurcata* and *C. crispus*, respectively). This high
174 variability may be due to intrathallus variability, and to the presence of epiphytes. In *Ulva lactuca*
175 (Van Alstyne et al. 2007) and in the brown seaweed *Taonia atomaria* (Paix et al. 2020), DMSP
176 concentrations are higher at the base of the thallus than in the middle of the thallus or at the

177 edges. For *B. bifurcata* and *C. crispus*, we had to mix several pieces of the middle part of the
178 thallus to ensure that there would be enough material to analyze (a few milligrams). This may
179 have increased DMSP variability. Epiphytes can also increase DMSP content in some plant
180 species (Dacey et al. 1994). We carefully removed epiphytes such as other algae from our
181 samples, but other epiphytes like bryozoans were still evident and difficult to remove on some
182 samples of *C. crispus*. Epiphytic microalgae and bacteria could also affect DMSP measurements
183 (Kessler et al. 2018, Paix et al. 2020). In a supplementary experiment, we collected 20
184 individuals of *C. crispus* (same site of collection, Feb. 7th 2019): 10 were colonised by epiphytes
185 (including bryozoans), and 10 were devoid of epiphytes. Each one of the 20 individuals was
186 divided in 2 subsamples. One of them was dipped in hexane for 30 s to remove microfouling
187 organisms (de Nys et al. 1998) before being processed for DMSP measurements. As a result, we
188 could compare DMSP contents (following Protocol P1-NaOH-T0) between populations with and
189 without visible epiphytes ($32 \pm 42 \text{ nmol} \cdot \text{g FW}^{-1}$, mean \pm STD, $n=10$, and $7 \pm 6 \text{ nmol} \cdot \text{g FW}^{-1}$,
190 mean \pm STD, $n=10$, respectively), and when populations with and without visible epiphytes were
191 dipped in hexane ($53 \pm 94 \text{ nmol} \cdot \text{g FW}^{-1}$, mean \pm STD, $n=10$, and $9 \pm 11 \text{ nmol} \cdot \text{g FW}^{-1}$, mean \pm
192 STD, $n=10$, respectively). The removal of fouling organisms by hexane dipping did not change
193 the DMSP measurements, neither for the epiphyte colonised samples (Paired-t-test, $t_9 = -1.221$, p
194 $= 0.253$) nor for the others (Paired-t-test, $t_9 = -0.684$, $p = 0.511$). DMSP contents on the contrary
195 were significantly higher in epiphyte colonised samples than in the others (Kruskal-Wallis, Chi-
196 Square₁ = 5.491, $p = 0.019$). The presence of epiphytes such as bryozoans may thus explain at
197 least part of the observed DMSP variability in *C. crispus*.

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

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352 Figure 1: DMSP concentrations in the four macroalgal species according to the five different
353 protocols (in μmol or $\text{nmol} \cdot \text{g}^{-1}$ algal fresh weight depending on the algal species). Significant
354 differences among protocols for each macroalgal species are marked with different letters (n =
355 10; Friedman rank test for data in repeated measurement designs with post-hoc Nemenyi-
356 Wilcoxon tests; p -value < 0.05).

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

