Preservation protocol for dimethylsulfoniopropionate and dimethylsulfoxide analysis in plant material of the Mediterranean seagrass *Posidonia oceanica*, and re-evaluation of dimethylsulfoniopropionate leaf content

A.V. Borges*†, W. Champenois†

*Corresponding author.

E-mail address: alberto.borges@ulg.ac.be (A.V. Borges).

† Both authors equally contributed to this study.

http://dx.doi.org/10.1016/j.aquabot.2017.08.004

Received 2 June 2017; Received in revised form 15 August 2017; Accepted 16 August 2017

Short communication

**Abstract**

We tested three treatments to preserve *Posidonia oceanica* leaves for the analysis of dimethylsulfoniopropionate (DMSP) and dimethylsulfoxide (DMSO): oven dried at 60 °C for 24 h, frozen at −20 °C, and frozen in ice and kept at −20 °C. The DMSP content was analyzed by proxy as dimethylsulfide (DMS) by gas chromatography after alkaline cleavage at room temperature. The DMSP leaf content of *P. oceanica* in samples that were oven dried at 60 °C for 24 h, then stored at room temperature decreased by 87% over 80 days of storage and then remained stable for about 88 additional days compared to the control. The DMSO leaf content of *P. oceanica* in samples that were oven dried increased nine-fold after 198 days of storage following drying compared to the control. Both the DMSP and DMSO leaf content of *P. oceanica* remained stable for 198 days compared to the control with frozen and frozen in ice treatments, which we both recommend as adequate protocols to preserve *P. oceanica* tissues for DMSP(O) analysis. The annual average DMSP leaf content of *P. oceanica* at 10 m in the Bay of Calvi (Corsica, France) was 205 ± 58 μmol g⁻¹ (fresh weight) based on samples preserved frozen, two orders of magnitude higher than the value we previously reported based on samples that were oven dried. The newly determined DMSP leaf content allows ranking *P. oceanica* as the highest DMSP producer reported to date among marine and inter-tidal autotrophs.

1. Introduction

Dimethylsulfoniopropionate (DMSP) is produced in variable amounts by numerous marine organisms including phytoplankton (Stefels et al., 2007), macroalgae (Van Alstyne and Puglisi, 2007), seagrasses (Dacey et al., 1994; Borges and Champenois 2015), as well as terrestrial angiosperms (Paquet et al., 1995), in particular some inter-tidal saltmarsh plants such as some species of *Spartina* (Otte et al., 2004; Dacey et al., 1987). Several physiological and ecological roles have been attributed to DMSP such as osmolyte, osmoregulator, herbivore deterrent, cryoprotectant, antioxidant against reactive oxygen species (ROS), overflow metabolite for carbon, antimicrobial against viruses or bacteria, as reviewed for marine phytoplankton by Stefels et al. (2007), for marine macroalgae by Van Alstyne and Puglisi (2007), and for higher plants by Otte et al. (2004). Dimethylsulfoxide (DMSO) is present dissolved in seawater, as well as intracellularly in phytoplankton (Simó and Vila-Costa 2006) and in inter-tidal macrophytes such as *Spartina* (Husband et al., 2012; McFarlin and Alber 2013). DMSO is formed from the oxidation of dimethylsulfide (DMS) in seawater by photo-oxidation, and in cells presumably by scavenging ROS. We recently showed the occurrence of DMSP in the marine planerogam *Posidonia oceanica*, endemic to the Mediterranean Sea, and the variations of DMSP leaf content as a function of season and of depth were attributed to the response to light availability changes. We concluded that the physiological role of DMSP in *P. oceanica* was to act as antioxidant to ROS related to high light intensity, by analogy with *Spartina alterniflora* (Husband et al., 2012; McFarlin and Alber 2013), and as also shown for phytoplankton (e.g. Sunda et al., 2002), and macroalgae (e.g. Rix et al., 2012).

The DMSP analysis in *P. oceanica* material from the Borges and Champenois (2015) study was carried out in the home laboratory in Belgium, due to logistical constraints such as the transportation of a relatively fragile and heavy gas chromatograph (GC) and several gas cylinders, the difficulty in obtaining liquid N₂ in Corsica where our study site was located, as well as occasional unstable power supply at the field research station. We then needed to preserve the samples of *P. oceanica* tissues, and we adopted the protocol recommended for the analysis of the DMSP content in macroalgae, consisting in oven drying...
the samples for 24 h at 60 °C (Karsten et al., 1994). Samples for DMS (P,O) analysis are typically preserved in an acid solution for phytoplankton (Stefels 2009) or frozen at −80 °C for Spartina (e.g. McFarlin and Alber 2013) and for corals (e.g. Broadbent et al., 2002). We chose at the time of our previous study drying over freezing because of the ease of transportation back to the home laboratory of dry samples over frozen samples, and providing data by mass of dry weight.

The present short note reports the test if drying the P. oceanica leaves leads to a substantial change of DMSP and DMSO content, and if this preservation protocol is adequate compared to freezing.

2. Material and methods

2.1. Sample collection and preparation

Since we were confronted with the same logistical problems, we brought freshly collected P. oceanica leaves to the home laboratory in Belgium, where the material was further processed. P. oceanica leaves (n = 30) were collected by SCUBA dive on 11/02/2016 in the meadow in the Bay of Revellata (Corsica, France) at 10 m depth close to the STARESO research station, at the same location as the Borges and Champenoi (2015) study. The leaves were scrapped with a razor blade to remove epiphytes. This technique was shown in a previous dedicated study (Dauby and Poulicek 1995). We only used young sections of leaves (close to the base) that are typically devoid of epiphytes that mainly colonize the older sections of leaves (close to the apex) (Cébrán et al., 1999). Furthermore, epiphyte flora growing on P. oceanica in the Bay of Revellata is dominated in terms of biomass by red coralline algae (Jacquemart and Demoulin 2006) that have a very low DMSP content < 0.1 μmol g⁻¹ (Kamenos et al., 2008), so it is unlikely that the DMSP we measured in P. oceanica material can be attributed to contamination by epiphytes, as assumed by Dacey et al. (1994) for some tropical seagrasses.

After removal of epiphytes, the leaves were stored in a plastic bag with some seawater to avoid desiccation, and stored in an isotherm container with water bottles filled with water at 4 °C to limit warming during transportation. On arrival at the home laboratory, 12 h after sample collection, the leaves were cut into small square pieces (3 × 3 mm) that were pooled together. From this pooled stock of material about 10 pieces of leaf were transferred to 20 ml pre-weighted (3 × 3 mm) that were pooled together. From this pooled stock of material about 10 pieces of leaf were transferred to 20 ml pre-weighted borosilicate vials, that were weighed to determine the fresh weight (fw) that was on average 30 mg. The vials were further processed with four treatments:

– by immediate addition of 2.5 ml of ultrapure (Type 1) Milli-Q (Millipore) water and 2.5 ml of NaOH solution (12N), and sealed with a polytetrafluoroethylene (PTFE) coated silicone septa stopper; this corresponds to the T0; DMSP measurements were made by GC the next day (see below),
– by drying for 24 h at 60 °C, after which the vial was sealed with a PTFE coated silicone septa stopper and stored at room temperature in the dark (hereafter dried treatment),
– by sealing with a PTFE coated silicone septa stopper and freezing at −20 °C (hereafter frozen treatment),
– by adding 2.5 ml of ultrapure (Type 1) Milli-Q water, sealing with a PTFE coated silicone septa stopper, and freezing at −20 °C (hereafter frozen-in-ice treatment).

The frozen-in-ice treatment was to check for DMS loss from the frozen leaves to the air trapped in the vial, that would be lost when opening the vial for further processing. DMSP and DMSO contents were measured for the three preservation protocols (dried, frozen, frozen-in-ice) on five occasions: 4, 17, 45, 80 and 198 days after the T0. Each sampling is the average of measurements in 5 replicate vials.

2.2. Analysis of the DMS(P,O) content

We used the protocol of DMSP analysis by DMS measurement in the headspace with a GC given in detail by Borges and Champenois (2015). In brief, 2.5 ml of ultrapure (Type 1) Milli-Q water and 2.5 ml of NaOH solution (12N) were added to vials containing the P. oceanica leaves. After a digestion overnight (~18 h at room temperature, under gentle agitation (rotating table)), a volume of 25 μl of gas was sampled from the headspace with a gas-tight syringe and injected directly to the head of the chromatographic column of an Agilent 7890A GC fitted with a flame photometric detector (FPD) and a flame ionization detector (only the signals of the FPD were used for this study). For each vial, headspace injections were made in triplicate. The GC was calibrated from a set of standards prepared from a DMSP standard (Research Plus Inc) dissolved in ultrapure (Type 1) Milli-Q water. The standards were treated simultaneously and the same way as the samples.

After the DMSP measurements, the DMSO content was measured on the same samples with the TiCl₃ reduction technique (Stefels 2009). Before reduction of DMSP with TiCl₃ any preexisting DMS in the solutions and vials that resulted from DMSP cleavage was removed by bubbling with ambient air for 20 min. Thereafter, 2.5 ml of H₂SO₄ 12N was added to attain acid pH, and then 1 ml of a TiCl₃ solution (30%) was added, and the reduction was done during ~48 h at room temperature, under gentle agitation (rotating table). The efficiency of the DMSO to DMS conversion was tested with solutions of pure DMSO with a known concentration and was found to be close to 100% (not shown). Prior to analysis, 1 ml of NaOH solution (12 N) was added to the vial to neutralize the solution, in order to avoid the injection of acid fumes into the chromatographic column.

3. Results and discussion

Initial DMSP and DMSO leaf content of P. oceanica was 75 ± 14 and 1.8 ± 0.5 μmol g⁻¹, respectively (Fig. 1). During the 198 days time course, for the frozen treatment, the P. oceanica leaf content ranged between 73 ± 9 and 86 ± 13 μmol g⁻¹ for DMSP, and between 1.5 ± 0.3 and 2.6 ± 0.8 μmol g⁻¹ for DMSO. During the 198 days time course, for the frozen-in-ice treatment, the P. oceanica leaf content ranged between 74 ± 11 and 82 ± 14 μmol g⁻¹ for DMSP, and between 1.3 ± 0.3 and 2.6 ± 0.6 μmol g⁻¹ for DMSO. The DMSP and DMSO leaf content of P. oceanica was not statistically different for the frozen and the frozen-in-ice treatments (paired t-test p = 0.2136, p = 0.8538, respectively). The DMSP and DMSO leaf content of P. oceanica of samples from the frozen and frozen-in-ice treatments remained very close to the T0 values and stable during 198 days (Fig. 1) (two-way analysis of variance (ANOVA) gave non-significant change with time for both variables and both treatments).

The DMSP leaf content of P. oceanica in samples from the dried treatment strongly decreased in time, with a value of 10 ± 2 μmol g⁻¹ at the end of the 198 time course, and a decrease of about half of the DMSP content after 4 days (Fig. 1). The decrease of the DMSP leaf content of P. oceanica in samples for the dried treatment was non linear, and values stabilised around 10 μmol g⁻¹ after 80 days until the end of the time course. The DMSP leaf content of P. oceanica in samples from the dried treatment increased 9-fold over time, with a value of 16.2 ± 0.9 μmol g⁻¹ at the end of the 198 days time course. Part of the DMSP seems to have been converted into DMSO during the 198 days time course for the dried treatment, however DMSP was also lost in another way since the DMSP and DMSO sum also decreased (Fig. 1). It is possible that part of the DMSP was lost to the atmosphere as DMS, since the characteristic odor of this compound was apparent during the drying process in the oven.

4. Conclusions

We conclude that drying material is not an adequate protocol to
preserve DMSP and DMSO in *P. oceanica* leaves, leading within a few days to significant decrease and increase, respectively. Our experiment was not designed to check if DMSP loss mainly occurred during the drying in the oven process itself, which would have required to analyze a sample immediately at the end of the drying, while we did the first sampling after four days, when the DMSP content had already decreased by about half. Freezing at −20 °C the material alone or in ice stabilizes adequately the DMSP and DMSO content of *P. oceanica* leaves for at least 6 months. The aim of this study was to determine the preservation protocols of *P. oceanica* tissues for the determination of DMSP and DMSO content, and not to evaluate the absolute contents. The DMSP and DMSO leaf content of *P. oceanica* at 10 m depth sampled on 05/02/2016 that were frozen on-site were 186 ± 8 and 6.4 ± 0.3 μmol gfw⁻¹, respectively. These values were higher than the T0 values of our experiment, so that DMSP and DMSO were lost during the drying in the oven process itself, which would have required to analyze a sample immediately at the end of the drying, while we did the first sampling after four days, when the DMSP content had already decreased by about half. Freezing at −20 °C the material alone or in ice stabilizes adequately the DMSP and DMSO content of *P. oceanica* leaves for at least 6 months. The aim of this study was to determine the preservation protocols of *P. oceanica* tissues for the determination of DMSP and DMSO content, and not to evaluate the absolute contents. The DMSP and DMSO leaf content of *P. oceanica* at 10 m depth sampled on 05/02/2016 that were frozen on-site were 186 ± 8 and 6.4 ± 0.3 μmol gfw⁻¹, respectively. These values were higher than the T0 values of our experiment, so that DMSP and DMSO were lost during the transportation from Corsica to the home laboratory, decreasing by approximately 111 and 4.6 μmol gfw⁻¹, respectively.

The DMSP content we previously reported in *P. oceanica* leaves (Borges and Champenois 2015) was probably under-estimated, because the samples were preserved by drying and analyzed in the home laboratory several months after collection. We obtained a new data-set of DMSP leaf content of *P. oceanica* at 10 m depth from April 2015 to July 2016 (weekly sampling) that were preserved frozen. The corresponding average DMSP leaf content of *P. oceanica* is 205 ± 58 μmol gfw⁻¹, two orders of magnitude higher than the average of 1 ± 1 μmol gfw⁻¹ at the same site and depth we previously reported based on dried material. This implies that *P. oceanica* is the largest reported producer of DMSP among marine and inter-tidal autotrophs, since dinoflagellates (the highest DMSP producing phytoplankton group reported so far) have a maximal reported DMSP content of ~180 μmol gfw⁻¹ (based on data from Stefels et al., 2007), while *Spartina alterniflora* (the highest DMSP producing angiosperm reported so far) has a maximal DMSP content of ~70 μmol gfw⁻¹ (Otte et al., 2004).

Acknowledgements

We thank Pierre Lejeune and the Stareso team for logistical support, Jonathan Richir and Arnaud Abadie for collection of samples of the 2015–2016 DMSP time-series, Guyliam Engels for GC measurements of the 2015–2016 DMSP time-series, and three anonymous reviewers for helpful suggestions on the previous version of the manuscript. This work was funded by the Fonds National de la Recherche Scientifique (FNRS) (contract 2.4.637.10) and the Territorial Collectivity of Corsica and the Rhone-Mediterranean and Corsica Water Agency (STARE-CAPMED – Station of reference and research on change of local and global anthropogenic pressures on Mediterranean ecosystem drifts). AVB is a senior research associate at the FNRS.

References


