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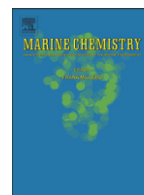

Dimethylsulphoxide (DMSO) in biological samples: A comparison of the $TiCl_3$ and $NaBH_4$ reduction methods using headspace analysis

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ABSTRACT

Dimethylsulphoxide (DMSO) is a sulphur compound that can result from the oxidation of biogenic dimethylsulphide (DMS) in marine algae and bacteria; with dimethylsulphoniopropionate (DMSP) being the main precursor of DMS. The two most commonly used methods for the analysis of DMSO in seawater and biological samples consist of its chemical reduction to DMS by either titanium trichloride ($TiCl_3$) or sodium borohydride ($NaBH_4$), with subsequent measurement of derived DMS by gas chromatography. Here, these two methods have been compared for the quantitative analysis of DMSO in the zooxanthellate coral *Acropora aspera* and in two species of marine algae (*Ulva intestinalis* and *Ulva lactuca*) using headspace analysis on DMSO-derived DMS. Reduction by $NaBH_4$ or $TiCl_3$ in biological samples yielded highly linear calibrations ($R^2 \geq 0.99$) and excellent repeatability (RSD = 6.17% and 4.32% for $TiCl_3$ and $NaBH_4$ respectively, $n = 10$). In coral samples, although a strong linear correlation was generally obtained between the two reduction methods ($R^2 = 0.8464$, $p < 0.001$, $n = 72$), the regression slope of 0.6 indicated that DMSO concentrations were either underestimated with $NaBH_4$ reduction or overestimated with $TiCl_3$. Reduction with $TiCl_3$ yielded lower values than $NaBH_4$ at DMSO concentrations <0.6 μM, whereas $TiCl_3$ gave higher values than $NaBH_4$ when DMSO was >2 μM. The reasons for these significant differences remain unclear at this stage and we therefore cannot draw conclusions on the preferential suitability of one reducing agent over the other for coral DMSO analysis. In macroalgae samples, significantly lower DMSO concentrations were obtained with $NaBH_4$ than with $TiCl_3$ for DMSO concentrations averaging 0.6 μM and 0.8 μM for *U. intestinalis* and *U. lactuca* respectively. The difference between reduction methods in the analysis of DMSO across macroalgae and coral samples was interpreted as a difference in taxa or in sample preparation, although this needs to be further investigated. Corals were found to contain more DMSO than macroalgae with similar DMSP concentrations.

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1. Introduction

Dimethylsulphoxide (DMSO) was reported for the first time in the marine environment in 1980 (Andreae, 1980) and was later identified as the most dominant dissolved dimethylated sulphur species in the Mediterranean Sea (Simó et al., 1997). Whilst several studies have reported higher concentrations of DMSO than those of both dimethylsulphide (DMS) or dimethylsulphoniopropionate (DMSP) (Hatton et al., 1996; Simó et al., 1997), other studies have shown that DMSO concentrations were lower than those of DMS and DMSP in seawater (Gibson et al., 1990). However, such differences in the proportions of these sulphur compounds could be explained by diurnal and seasonal variations of DMSO (Lee and de Mora, 1999).

Marine DMSO was initially thought to be derived mainly from the photo-oxidation of DMS in the euphotic zone of the water column (Brimblecombe and Shooter, 1986); however marine bacteria were later found to play an important role in this reaction (Hatton et al., 2012). Other studies have demonstrated the presence of particulate (cellular) DMSO in phytoplankton (Hatton and Wilson, 2007; Simó et al., 1998), indicating that this oxidised sulphur species is also

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biologically produced. Given that DMSO can easily permeate through cell membranes (Hatton and Wilson, 2007), the particulate pool should be a source for the dissolved pool, but relatively little is known about the cycling of DMSO in marine systems.

DMS oxidation to DMSO through the scavenging of reactive oxygen species (ROS) appears to be the main source of DMSO synthesis in marine algae (Sunda et al., 2002). This mechanism is expected to be enhanced under oxidative stress (Husband et al., 2012), when ROS accumulate within the organism as a consequence of stress exposure (Lesser, 2006). DMSO can also be biologically reduced to DMS through enzymatic reactions that most likely involve reductases (Spiese et al., 2009). Thus, DMSO is a key compound in the complex redox loop that is involved in the marine sulphur cycle as it can be both an end-product of DMS oxidation and a precursor of DMS, which potentially plays an important role in climate regulation (Charlson et al., 1987).

Dissolved DMSO has been found in trace concentrations (ranging from 1 nM to 70 nM) in rainwater and freshwater environments (Andreae, 1980), and at a higher concentration range (1–200 nM) in seawater (Gibson et al., 1990; Lee and Wakeham, 1988). Although particulate DMSO concentrations have been quantified on multiple occasions in seawater (Hatton, 2002; Riseman and DiTullio, 2004; Simó et al., 1998), only a few studies have measured intracellular DMSO concentrations within specific organisms (Table 1). Moreover, relative particulate concentrations across species are often difficult to compare as they are frequently normalised to different parameters among studies (fresh weight, cell density, cell volume, surface area, etc.).

Because of its importance in the algal antioxidant system and marine sulphur cycle, more research is required on DMSO and therefore the choice of analytical techniques for DMSO measurements in biological samples needs to be further examined. To date, DMSO has mainly been quantified by gas chromatography following its chemical reduction to DMS using either titanium trichloride ($TiCl_3$) (Del Valle et al., 2007; Harada et al., 2009; Husband and Kiene, 2007; Husband et al., 2012; Kiene and Gerard, 1994; Rellinger et al., 2009; Spiese et al., 2009; Vila-Costa et al., 2006) or sodium borohydride ($NaBH_4$) (Riseman and DiTullio, 2004; Sciare and Mihalopoulos, 2000; Sciare et al., 2002; Simó et al., 1996, 1997; Simó and Vila-Costa, 2006; Ui et al., 2004; Vila-Costa et al., 2008; Wang et al., 2013), with other studies using the enzyme-based reduction method developed by Hatton et al. (1994) (Hatton and Wilson, 2007; Simó et al., 1998) or the proton nuclear magnetic resonance technique (Zeyer et al., 1987). Although $TiCl_3$ and $NaBH_4$ are both in common use for the analysis of DMSO, their relative efficiencies have not been extensively assessed. Moreover, comparisons among studies that have used these two techniques independently suggested that reduction of DMSO with $NaBH_4$ could result in significantly higher DMS concentrations than with $TiCl_3$ (Spiese et al., 2009). Here we tested both reducing agents with the intention to assist future research in the selection of the most appropriate method for DMSO measurements in biological samples and to facilitate comparisons of DMSO analysis between studies that have used these reduction methods independently.

2. Material and methods

2.1. Collection, preservation and DMSP analysis of coral-zooxanthellae samples

Colonies of the zooxanthellate coral *Acropora aspera* were collected at low tide from the Heron Island reef flat (23°26′46.19″S/151°54′46.35″E), Australia. In order to obtain different levels of DMSO production, five sets of twelve random nubbins representing an even mix of colonies were assigned to experimental treatments simulating environmental stress over set periods before being snap frozen in liquid nitrogen (see details of experimental protocol in Deschaseaux et al., 2014). Another set of 12 nubbins was immediately snap frozen as controls under basal conditions (unexposed to stress) following collection. Samples were kept at −80 °C until the coral tissue (containing both coral and algal cells, as well as most likely bacteria and fungi that are often present in corals) was extracted by air blasting in 10 mL of 75 mM sodium phosphate buffer (Deschaseaux et al., 2013). This generated what is termed the "coral blastate". Although essential to provide preliminary information on how coral samples had been handled prior to analysis, the outcome of experimental treatments will not be commented on in this manuscript as it is already been discussed in another article (Deschaseaux et al., 2014). Instead, we focus on the quantification of DMSO by two different approaches using $TiCl_3$ and $NaBH_4$.

Prior to DMSO analysis all samples were analysed for DMSP. A 1 mL subsample of the coral blastate was diluted to 10 mL with MilliQ water and purged with high purity nitrogen (N_2) for 10 min at a flow rate of 100 mL min^{-1} to remove free DMS. Following addition of 1 mL of 10 M NaOH to each aliquot, vials were immediately sealed with gas-tight septa (Agilent Technology, PTFE/Silicone septa, P/N 5183-4477) and crimp capped for subsequent DMSP analysis that was conducted within two weeks of sample preservation.

Coral DMSP samples were processed by headspace analysis using a gas chromatograph (GC) (Agilent Technologies 6890 N) with a Mass Selective Detector (MSD) (Agilent Technologies 5973N) operated in scan mode coupled with a Gerstel multipurpose sampler (MPS 2XL). Headspace injection (1 mL) was performed using a 2.5 mL syringe set at 95 °C at an injection speed of 500 μL s^{-1}. The GC injector temperature was set at 280 °C and the injection was made with a split ratio of 25:1. Volatiles were separated using a capillary column (BPX, 50 m, 0.22 mm × 1 μm film thickness, SGE) with high purity helium (He) as carrier gas at a constant flow rate of 1.1 mL min^{-1}. The oven temperature was programmed from 35 °C (held for 8.0 min) to 180 °C at a temperature ramp of 80 °C per min and held at 180 °C for 2 min. Compounds were identified by reference to MS library database and data were processed using MSD ChemStation Software (Version D.02.00.275, Agilent Technologies). DMSP stock standard solution was made by diluting a known quantity of DMSP powder (DMSP.HCl reference material WR002 of certified purity 90.3 ± 1.8% w/w, National Measurement Institute, Sydney, Australia) into acidified MilliQ water (final pH < 2) and was kept at −20 °C. DMSP standards and blanks were prepared daily in the exact same proportions of phosphate buffer, MilliQ water and NaOH as the coral samples. Seven-point calibration curves and three blanks were run daily at the start and end of the sample analysis for calibration and contamination tests respectively under the exact same analytical conditions as for samples. The value of blanks was subtracted from all measurements.

Because the DMSO analysis in coral samples was conducted on a Shimadzu GC coupled with a Flame Photometric Detector (FPD) (see analytical details below), which is a more sensitive detector than the MSD used for the DMSP analysis, samples that had been treated with NaOH for DMSP analysis were diluted 10- or 100-fold into 75 mM sodium phosphate buffer, pH 7.4, following DMSP sample processing. Diluted samples were purged with N_2 for 10 min to remove the DMS that was generated during the alkaline treatment of DMSP. The absence of DMS following purging was verified on twenty random samples by purge-and-trap analysis as follows: vials were individually connected to the GC-FPD by piercing septa with (1) the outlet needle (Terumo corporation, sterile needle, 1.10 × 38 mm) that was in line with a cryogenic Teflon loop dipped into liquid nitrogen and (2) the inlet needle (Cadence Science, Septum penetration needle, 0.2 × 152.4 mm) which was delivering a high purity He flow of 100 mL min^{-1}. If DMS was present, it was purged out of the vials and cryo-trapped for 3 min at this flow rate and subsequently measured by transferring the cryo-loop into a hot water bath (~60 °C). A Nafion dryer (Perma Pure, MD-050-48 P2) was placed upstream of the cryotrap to avoid ice blockage during the purge due to moisture that could build up in the cryogenic loop (Simó et al., 1996). Once this verification step had attested of the absence of DMS following the 10 min purge, 1 mL aliquots were transferred into

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