



OPEN

Dimethylsulfoniopropionate concentration in coral reef invertebrates varies according to species assemblages

Isis Guibert^{1,2,3} , Flavien Bourdreux^{4,5}, Isabelle Bonnard⁶, Xavier Pochon^{7,8}, Vaimiti Dubousquet⁹, Phila Raharivelomanana¹⁰, Véronique Berteaux-Lecellier^{11,3} & Gael Lecellier^{11,4}

Dimethylsulfoniopropionate (DMSP) is a key compound in the marine sulfur cycle, and is produced in large quantities in coral reefs. In addition to Symbiodiniaceae, corals and associated bacteria have recently been shown to play a role in DMSP metabolism. Numerous ecological studies have focused on DMSP concentrations in corals, which led to the hypothesis that increases in DMSP levels might be a general response to stress. Here we used multiple species assemblages of three common Indo-Pacific holobionts, the scleractinian corals *Pocillopora damicornis* and *Acropora cytherea*, and the giant clam *Tridacna maxima* and examined the DMSP concentrations associated with each species within different assemblages and thermal conditions. Results showed that the concentration of DMSP in *A. cytherea* and *T. maxima* is modulated according to the complexity of species assemblages. To determine the potential importance of symbiotic dinoflagellates in DMSP production, we then explored the relative abundance of Symbiodiniaceae clades in relation to DMSP levels using metabarcoding, and found no significant correlation between these factors. Finally, this study also revealed the existence of homologs involved in DMSP production in giant clams, suggesting for the first time that, like corals, they may also contribute to DMSP production. Taken together, our results demonstrated that corals and giant clams play important roles in the sulfur cycle. Because DMSP production varies in response to specific species-environment interactions, this study offers new perspectives for future global sulfur cycling research.

Coral reefs have been described as dimethylsulfoniopropionate (DMSP) hotspots^{1,2}. This compound is an important metabolite that plays a central role in the marine sulfur cycle³. It is involved in numerous cellular and ecological processes. Among several known functions, DMSP possesses antioxidant properties^{4,5} as evidenced by cellular increase of DMSP under CO₂ depletion⁶. Dimethylsulfoniopropionate has other protective physiological functions, notably serving as an osmolyte and cryoprotectant in marine algae^{7,8}. Acting as a signalling molecule, DMSP is also involved in antiviral defense mechanisms and sulfide detoxification^{9–11}. Numerous studies have recently drawn attention to variations of DMSP concentration in organisms subjected to environmental changes. Marine algae and/or coral studies have demonstrated that DMSP concentration changes with light intensity and

¹Swire Institute of Marine Science, The University of Hong Kong, Hong Kong S.A.R, China. ²Sorbonne Université, UMR250/9220 ENTROPIE IRD-CNRS-UR-IFREMER-UNC, Promenade Roger-Laroque, Noumea cedex, New Caledonia, France. ³USR3278 PSL CRILOBE CNRS-EPHE-UPVD, LabEx CORAIL, Papetoai, Moorea, French Polynesia. ⁴Université de Paris-Saclay, UVSQ, 45 avenue des Etats-Unis, Versailles Cedex, France. ⁵Institut Lavoisier de Versailles, UMR CNRS 8180, 45 avenue des Etats-Unis, Versailles Cedex, France. ⁶USR3278 PSL CRILOBE CNRS-EPHE-UPVD, LabEx CORAIL, Université de Perpignan, 58 avenue Paul Alduy, 66860, Perpignan, France. ⁷Coastal and Freshwater Group, Cawthron Institute, Private Bag 2, Nelson, 7042, New Zealand. ⁸Institute of Marine Science, University of Auckland, Private Bag 349, Warkworth, 0941, New Zealand. ⁹Délégation à la recherche, Government of French Polynesia BP 20981, 98713, Papeete, Tahiti, French Polynesia. ¹⁰UMR 241 EIO, Université de la Polynésie Française, BP 6570 Faaa, 98702, Faaa, Tahiti, French Polynesia. ¹¹UMR250/9220 ENTROPIE IRD-CNRS-UR-IFREMER-UNC, Promenade Roger-Laroque, Noumea cedex, New Caledonia, France. ✉e-mail: guibert.isis@orange.fr

salinity, as well as in response to oxidative stressors^{6,12–14}. DMSP is therefore believed to be involved in organisms' stress response, especially in coral species^{14,15}. Dimethylsulfoniopropionate has also been extensively studied for its role in climate regulation¹⁶, because it can be converted into dimethylsulfide (DMS), a trace gas that is a source of reduced sulfur and which plays a role in cloud formation^{17,18}. Consequently, precise methodologies such as nuclear magnetic resonance (NMR) spectroscopy have been developed for accurate detection and quantification of DMSP^{16,19}.

Dimethylsulfoniopropionate is found only in certain terrestrial or marine organisms. It has been recorded in graminoids, rhodophytes, mussels and also in benthic flatworms^{20–23}. However, only a few species are able to produce DMSP, and the majority of organisms are believed to accumulate this molecule through their diets or phototrophic symbionts²⁴. To date, pathways of DMSP biosynthesis have been characterized in higher plants^{22,25}, marine algae such as Symbiodiniaceae^{26,27} and more recently in corals, with methionine as a common precursor¹⁵. Higher plants and marine algae use two different DMSP biosynthetic pathways, while corals harbor an algal-like pathway^{15,26}. Similarly to marine algae, coral species such as *Acropora* sp. likely encode the enzymatic machinery required for biosynthesis of DMSP (specific aminotransferase, reductase, methyltransferase and decarboxylase enzymes)^{3,15}. Even though the specific biosynthesis pathway has not yet been studied in other marine organisms, DMSP has been found in other coral taxa (e.g. Pocilloporidae, Poritidae) and sessile organisms such as giant clams and anemones^{20,28}. The concentrations of DMSP were higher in corals (*Acropora* sp., *Heliopora* sp., *Pavona* sp.) and giant clams (*Tridacna maxima* and *Tridacna squamosa*) than in other animals^{20,28}. Dinoflagellates of the family Symbiodiniaceae account for most of the DMSP production in coral reefs^{24,29}. This family is currently classified into nine divergent lineages, hereafter referred to as clades A to I³⁰, of which seven have recently been elevated to genus level³¹, with clades A–D commonly found in symbiosis with scleractinian corals and giant clams³². Symbiodiniaceae can possess a DMSP-lyase activity leading to the conversion of DMSP into DMS that differs among clades³³. Thus, the presence or proportion of different Symbiodiniaceae clades within a host may influence the variation of DMSP concentration often observed between coral species³⁴. Additionally, the differences observed in coral studies could also be the result of other symbionts hosted by corals. Certain bacteria possess crucial genes for DMSP degradation such as homologs of the *dmdA* gene that are involved in the demethylation pathway or of the *dddD* gene known to be involved in the degradation of DMSP into DMS^{35–38}. These homologs have been found in Proteobacteria that are common in coral species. Additionally, the occurrence of bacteria involved in DMSP production in coral is possible³⁹, further highlighting the complexity of DMSP-cycling processes in benthic holobionts. Most of these bacteria have been recently described in microbiomes of giant clams⁴⁰. Further, DMSP production in heterotrophic bacteria has also been recently highlighted⁴¹.

It is well known that coral symbionts such as dinoflagellates (Symbiodiniaceae) and bacteria vary according to environmental conditions^{42–46}, and are therefore assumed to play a key role in their host fitness⁴⁷. In addition to symbionts, interaction between holobionts could also impact the host fitness. While it is well known that marine sessile organisms interact, either directly or indirectly^{48–50}, only a few studies have underlined the role of benthic species assemblages on coral reef dynamics^{51,52}, as well as on giant clams' fitness⁴⁰. To better understand how benthic species assemblages could influence their respective fitness, we artificially combined three coral reef builders, including two coral species (*Pocillopora damicornis*; Linnaeus, 1758 and *Acropora cytherea*; Dana, 1846) and one giant clam species (*Tridacna maxima*; Röding, 1798), and measured DMSP concentration in each species using NMR spectroscopy. Metabarcoding was used to characterize the Symbiodiniaceae composition within the three studied species and determine the putative role of Symbiodiniaceae in DMSP production. We also examined whether giant clams contribute to DMSP production by screening our previously described transcriptomes of *T. maxima*⁵³ for enzymes involved in the DMSP biosynthesis. Finally, because DMSP is a stress biomarker, the experiments were performed either at lagoon temperature or under thermal stress.

Material and Methods

Coral and giant clam collection and experimental design. The coral and giant clam samples as well as the experimental design used in this study were described previously⁵². Briefly, coral species of *A. cytherea* and *P. damicornis* were collected in Moorea lagoon, French Polynesia (17°30'S, 149°50'W, Linareva fringing reef)⁵⁴. For each species, 4 colonies were sampled and nubbinized into 45 small fragments. Giant clams of the species *T. maxima* were purchased from a French Polynesian nursery on Reao Island (18°28'S, 136°25'W; N°Tahiti: 139 519). Corals nubbins and giant clams were reared in a common garden. A CITES permit was obtained to allow specimen exports (CITES – FR1698700087 – E).

Experiments were conducted in open-circuit aquariums (20 L/h), using distinct assemblages of either one species *P. damicornis* (P, n = 4 aquariums) or *T. maxima* (T, n = 4 aquariums), two species *P. damicornis* + *A. cytherea* (PA, n = 4 aquariums) or *A. cytherea* + *T. maxima* (AT, n = 4 aquariums), or three species *P. damicornis* + *A. cytherea* + *T. maxima* (PAT, n = 4 aquariums; Figure S1a). Three nubbins per coral colony (n = 3 nubbins x 4 colonies per species) and 12 giant clams were used when required in an assemblage. Coral fragments (5–8 cm in height and 2 cm in diameter) and giant clams (4–8 cm in size) were spaced 5 to 10 cm apart to avoid contact. Assemblages were either under thermal stress (32 °C, S, n = 10 aquariums) or at lagoon temperature (27 °C, L, n = 10 aquariums). After 12 days of acclimation at 27 °C (time 0), half of the aquariums (n = 10) were placed under thermal stress by increasing the temperature by 1 °C per day until it reached 32 °C on day 17 (Fig. S1b). At each sampling time (day 12 and 17), half of the sampled giant clam mantles (4 cm²) and 80% of the sampled coral fragments were immediately snap-frozen in liquid nitrogen and stored at –80 °C until further analysis. The remaining portions of the sampled giant clams and corals were stored in 70% ethanol for DNA analysis. Seawater temperature data were recorded every 10 minutes with Temperature/Light Data Loggers (P/N U22–001, Onset, Bourne, Massachusetts; or Ruskin, Ottawa, Canada; Fig. S1b). Seawater temperature was controlled with the Biotherm pro system (Hobby, Stukenbrock, Germany). Health status checks on the coral nubbins were carried out

using the standardized coral coloration scale as a proxy of symbiont density and chlorophyll a content variation (Coral Health Chart, www.CoralWatch.org)^{55,56}, and through daily visual observation for giant clams.

Metabolite extraction for dimethylsulfoniopropionate quantification. Coral samples were lyophilized overnight, and 1 to 1.5 g of powder was obtained for extraction. The extraction protocol was adapted from Tapiolas and collaborators¹⁹. In total, 51 *A. cytherea* and 54 *P. damicornis* samples were extracted with 3 mL of HPLC-grade methanol (CH₃OH). After 5 minutes of sonication (35 kHz Transsonic 950/H, Elma, Germany) at room temperature, the extracts were shaken for 3 hours. A second extraction with an additional 2 mL of CH₃OH was performed using 5 minutes of sonication and 5 minutes of shaking. The two extracts were then pooled and dried using a vacuum centrifuge drier (Genevac EC-2 plus, Genevac, UK) before being stored at −20 °C until NMR analysis. Samples were lyophilized overnight and weighed to measure the quantity of extracts.

Giant clam samples (n = 42) were lyophilized overnight and 0.1 to 0.5 g of powder was obtained for extraction. Protocol extraction was adapted from Mohamadi⁵⁷. Solid-liquid extractions were performed using 2 mL of H₂O and 0.5 mL of HPLC-grade methanol (CH₃OH) prior to vortexing. After adding 1.5 mL of CH₃OH and 2 mL of dichloromethane (CH₂Cl₂), the solutions were mixed using a vortex and sonicated for 10 minutes. The solid-liquid extraction was carried out three times and the three extracts were pooled and centrifuged at 2050 g for 20 min in order to separate the polar and apolar phases. The apolar extracts were pooled as per the polar extracts and lyophilized. In this study, only the hydroalcoholic (polar) extracts of the giant clam samples were analyzed.

All extracts were resolubilized in 500 µL of deuterium oxide (D₂O), vortex mixed and transferred into a 5 mm NMR tube, then analyzed immediately by ¹H-NMR spectroscopy.

Nuclear magnetic resonance data acquisition and dimethylsulfoniopropionate quantification.

All ¹H spectra were recorded on a Bruker Avance 1 spectrometer (Bruker, Germany) at 300 MHz in the same conditions with 128 transients; chemical shifts were reported in ppm from tetramethylsilane (TMS). Absolute Area was determined by NMR analysis on the topspin 2.1 Bruker software.

Two external calibration curves of DMSP were performed under the same conditions as the samples with 128 transients (Figure S1c,d). Integration zone A (3.41 ppm to 3.34 ppm) was used for *A. cytherea* and *P. damicornis* samples because there were no overlapping signals. Integration zone B was used for *T. maxima* samples because of overlapping signals on zone A and no overlapping signal on zone B (Fig. S1e–h). The concentrations of DMSP calculated with the calibration curve (in mg per mL) were normalized with the mass ratio corresponding to the dry weight of the extract. Analysis of variance (ANOVA) and *ad hoc* pairwise comparisons (Tukey test) were performed using R software⁵⁸.

DNA extraction; PCR amplification and sequencing of Symbiodiniaceae communities.

Coral DNA extractions (n = 2 to 9 per assemblage) were performed following a Cetyl Trimethylammonium Bromide (CTAB) based protocol⁵⁹. Symbiodiniaceae diversity was studied using the internal transcribed spacer 2 (ITS2) marker. The ITS2 region of the nuclear ribosomal array was amplified using the forward primer “its-Dino” (5′-GTGAATTGCAGAACTCCGTG-3′) and the reverse primer “ITS2-Rev2” (5′-GCCTCCGCTTACTTATATGCT-3′)^{60,61}. Illumina™ overhang adaptors were included in the primers as described in Kozich *et al.*⁶². PCR amplification and sequencing were performed as described in Guibert *et al.*⁵².

Symbiodiniaceae composition analysis of *A. cytherea* and *P. damicornis* samples was adapted from the Arif *et al.* (2014) pipeline in mothur v1.39⁶³. An ITS2 database⁶⁴ was used to annotate the sequences via Basic Local Alignment Search Tool (BLASTN). The relative abundance of Symbiodiniaceae subclades was represented using R (Reshape2 and ggplot2 packages).

Symbiodiniaceae composition analysis of *T. maxima* samples was processed using the same methodology as described in Guibert *et al.*⁴⁰.

Identification of candidate genes. A previous predicted peptide data set of *T. maxima* metatranscriptome⁵³ was blasted against a custom database at a threshold of 10^{−3}. This database contained molluscan and cnidarian sequences extracted from the Protein database of the National Center for Biotechnology Information (NCBI) filtered with the DMSP enzyme names or EC numbers and from the DMSP candidate genes previously identified in *Acropora millepora*¹⁵ (Table S2, sheet 2). The selected sequences were blasted against the whole NCBI Protein database and any sequence with a top hit with a plant, algae or bacterial taxonomy was considered a false positive and removed (Table S2, sheet 1).

A differential gene expression analysis was then performed from previous RNAseq results of *T. maxima* under thermal stress at 32 °C for 5 days (Bioproject accession number: PRJNA309928). Transcript quantification of the above selected sequences was performed with RNA-Seq by Expectation Maximization (RSEM)⁶⁵ across 14 samples of the Bioproject (8 biological samples: B0Tx and B0 as the control group and 6 BTx biological samples as the Heating group⁵³). The differential expression between the 2 groups was performed with the edgeRun package⁶⁶ with 50,000 iterations.

Results

Analyses of dimethylsulfoniopropionate concentrations according to thermal stress, species and assemblages. Dimethylsulfoniopropionate concentration was evaluated by ¹H-NMR spectroscopy and standardized with the mass ratio in the three species: *P. damicornis*, *A. cytherea* and *T. maxima* (Table S3). An ANOVA test (Table 1) revealed significant differences among assemblages (p = 1.01e-11) or species (p < 2e-16) with a significant interaction among them (p = 3.90e-08). No significant differences between assemblages and temperatures were found. Thus, the DMSP data were pooled by assemblages for each species.

	Df	Sum Sq	Mean Sq	F value	P value
Assemblage	5	184.3	36.86	15.274	1.01e-11***
Temperature	1	2.7	2.67	1.107	0.295
Species	2	339.5	169.75	70.343	<2e-16***
Assemblage: Temperature	4	11.3	2.81	1.166	0.329
Assemblage: Species	2	94.6	47.28	19.594	3.90e-08***
Temperature: Species	2	4.1	2.05	0.848	0.431
Assemblage: Temperature: Species	2	0.3	0.14	0.060	0.942
Residuals	126	304.1	2.41		

Table 1. Results of ANOVA test on DMSP concentration (mass ratio) by assemblage, species and temperature. Df: degrees of freedom; SumSq: Sum of squares; Mean Sq: mean sum of squares, F value: F statistic.

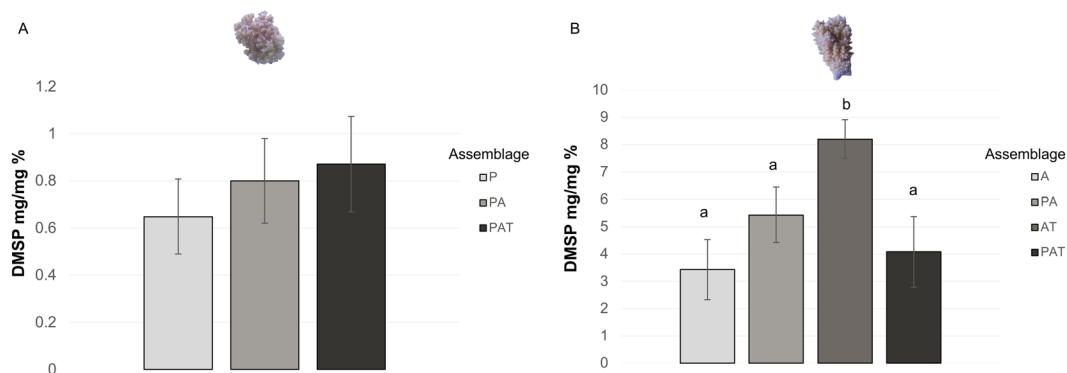


Figure 1. Concentration of dimethylsulfoniopropionate (Mass ratio: mg/mg %) of *Pocillopora damicornis* (A) and *Acropora cytherea* (B) by assemblage. Assemblages: P: *P. damicornis* (nA = 25); A: *A. cytherea* (nB = 12); PA: *P. damicornis* and *A. cytherea* (nA = 16, nB = 16); AT: *A. cytherea* and *T. maxima* (nB = 8); PAT: *P. damicornis*, *A. cytherea* and *T. maxima* (nA = 13, nB = 15). Letters in lower case indicate significant differences between means (Tukey, $p < 0.05$). Photographs: Isis Guibert.

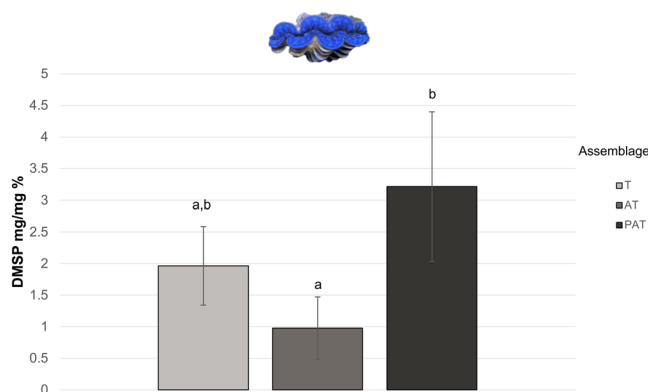


Figure 2. Concentration of dimethylsulfoniopropionate (Mass ratio: mg/mg %) of *Tridacna maxima* by assemblage. Assemblages: T: *T. maxima* (n = 19); AT: *A. cytherea* and *T. maxima* (n = 9); PAT: *P. damicornis*, *A. cytherea* and *T. maxima* (n = 14). Letters in lower case indicate significant differences between means (Tukey, $p < 0.05$). Photograph: Isis Guibert.

In corals, the concentrations of DMSP were from 5 to 10-fold lower in *P. damicornis* than in *A. cytherea* (Tukey test, $p < 0.001$), with means ranging between 0.65 and 0.87% ($\% = \text{mg DMSP}/100 \text{ mg extract}$) for *P. damicornis* and between 3.43 and 8.20% for *A. cytherea* (Fig. 1). In *P. damicornis*, while mean DMSP concentrations increased with the increasing complexity of assemblages, no significant differences were found (Fig. 1A). However, in *A. cytherea*, the DMSP concentration increased for the two-species assemblages (PA and AT) but decreased in PAT assemblages. A significant difference was found between AT and the other assemblages (A, $p < 0.0001$; PA, $p < 0.05$; PAT, $p < 0.001$; Fig. 1B).

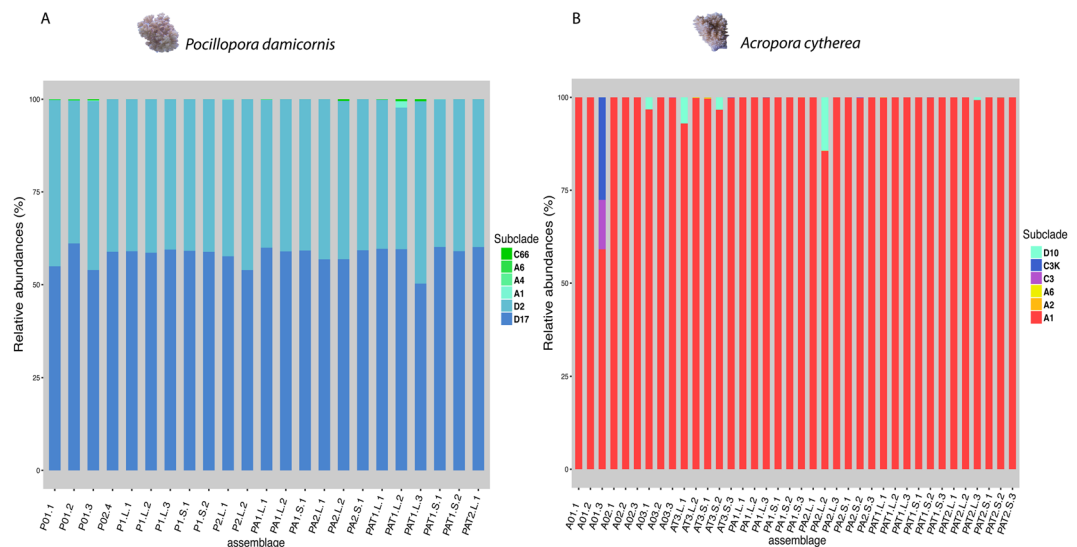


Figure 3. Relative abundance of Symbiodiniaceae subclades in *Pocillopora damicornis* (A) and *Acropora cytherea* (B). All samples were collected at day 17 except those with a 0 in their name that were collected at day 12. PAT: *P. damicornis*, *A. cytherea* and *T. maxima*; AT: *A. cytherea* and *T. maxima*; A: *A. cytherea*; P: *P. damicornis*; 0: time 0 - day 12; (1–3): number of the experiment; L: lagoon temperature; S: thermal stress; (1–4): number of sample. Photograph: Isis Guibert.

Interestingly, in *T. maxima*, mean DMSP concentrations decreased when exclusively associated with *A. cytherea* (from 1.97% to 0.98%) but increased in the PAT assemblage (3.22%, Fig. 2). *Tridacna maxima* exhibited a significantly higher DMSP concentration in the PAT assemblages than in the AT assemblages ($p < 0.5$).

Symbiodiniaceae composition by assemblage. The relative abundances of Symbiodiniaceae genotypes associated with the different assemblages and thermal stress conditions were measured at the clade and subclade levels (Fig. 3 and Table S4). Among the three Symbiodiniaceae clades (A, C and D) detected in the samples, clade D was the dominant lineage in *P. damicornis* nubbins ($> 90\%$; Fig. 3a). Two D subclades were systematically detected in all nubbins. Subclade D17 represented more than half of the relative abundance (58–60%) and subclade D2 ranged from 39 to 44%. Clades A and C were only detected as background clades with subclades A1, A4, A6 and C66 sporadically present in the nubbins, either alone or associated, regardless of temperature levels and/or assemblages.

Three clades (A, C and D) were also detected in *A. cytherea* (Fig. 3b), although clade A largely dominated in all nubbins ($n = 38$), 24 of which harbored only this clade. Subclade A1 was dominant in the Symbiodiniaceae composition of all *A. cytherea* nubbins, with the presence of A2 subclade only once, and A6, 5 times, with both subclades occurring at a background level ($< 1\%$). Clade C was detected in 9 nubbins at the background level ($< 0.2\%$) except for one nubbin (A01.3 composed of 59% A and 41% C). Two C subclades, C3 and C3K were found concomitantly (in 4 nubbins) or separately (in 2 and 3 nubbins, respectively). Finally, clade D (subclade D10) was found in 5 nubbins, two of which contained D10 above the background level (7% and 14%). No significant correlation between Symbiodiniaceae clade/subclade and host assemblages or temperature was found. Moreover, no variation of nubbin color, was recorded according to temperature levels and/or assemblages meaning that there was no drastic variation in Symbiodiniaceae densities.

Identification of candidate genes for dimethylsulfoniopropionate biosynthesis in *Tridacna maxima*. The blasting of the predicted peptide *T. maxima* dataset against a DMSP enzyme custom database allowed the selection of 475 predicted peptides potentially involved in pathways of DMSP biosynthesis (Table S2 and S5, sheet 2). After functional clustering, all identified peptides with potential roles in DMSP synthesis were found to exhibit at least 1 homolog per step. Among them, 69 were significantly (False Discovery Rate < 0.05) up-regulated during a thermal stress event (Table S5, sheet 1). More precisely, 29 peptides were identified as homologs to those of the coral pathway: 6 homologs of AT1 and AT5 for transamination step, 16 for the reduction step, 6 homologs of REDOX2,3,4,6 and 10, for the methylation step of METHYL 1 and 2, and one homolog of DECARB3 for the decarboxylation step (Fig. 4).

Discussion

Dimethylsulfoniopropionate concentrations differ according to species and assemblage.

Dimethylsulfoniopropionate concentrations varied among the three invertebrate species analyzed. In *A. cytherea*, DMSP concentrations were 5 to 10-fold higher than in *P. damicornis*. A previous study performed on Pocilloporidae and Acroporidae, measured DMSP concentrations of, respectively, 0.333 nmol/mm² and 2.473 nmol/mm² in *P. damicornis* and *A. millepora*¹⁹, which is consistent with our results. Also, similar DMSP concentrations were found in three different species from the Pocilloporidae family, while they were highly variable in

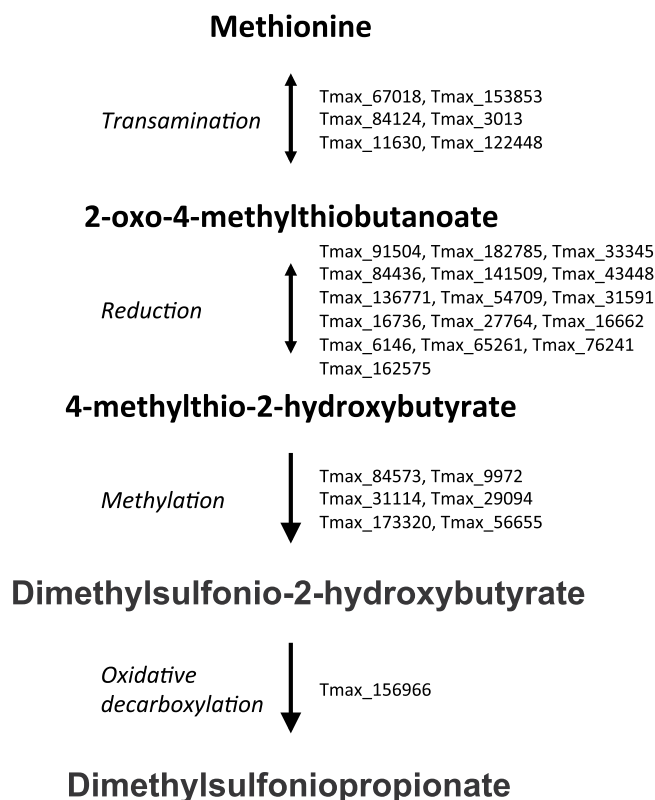


Figure 4. Putative pathway of dimethylsulfoniopropionate biosynthesis in *Tridacna maxima*. ID numbers of *T. maxima* peptides are indicated for each step.

Acroporidae¹⁹. As we used different NMR quantification approaches for corals and giant clams, we were not able to directly compare concentrations between them. Nevertheless, beyond differences between species, our results revealed that the DMSP concentrations might vary in a species according to its close environment, namely interspecies assemblages.

In corals, increases in DMSP have been associated with direct sunlight, thermal stress, air exposure or oxidative stressors^{3,5,14}. Measurements of DMSP concentration in *Acropora* species (*A. aspera*, *A. tenuis* and *A. millepora*) after 4⁶⁷ and 5³ days at 31 °C revealed an increase of DMSP concentration in bleached corals. Here we show that after 2 days at >31 °C the DMSP production, in still unbleached *A. Cytherea*, *P. damicornis* and *T. maxima*, is not sufficiently enhanced to detect a significant difference in DMSP concentrations between stressed and unstressed corals and giant clams. This indicates that at least three days of temperature stress and/or the concurrent loss/gain of a certain quantity of Symbiodiniaceae/bacteria might be required for enhancing DMSP production. However, significant variations of DMSP concentrations were observed among the three species. These variations occurred in different environmental contexts. For *A. cytherea*, DMSP concentration increased significantly in AT assemblages but not in PA and PAT assemblages, whereas for *T. maxima*, it increased in PAT assemblages but decreased in association with *A. cytherea*. These previous results suggest that DMSP concentration in coral reef holobionts is dependent on neighboring species, possibly acting as a stressor¹⁴ or an activator of the sulfur metabolism. For *P. damicornis*, the association with *A. cytherea* alone or with *T. maxima* does not lead to a highly significant increase in DMSP. For *A. cytherea*, an increase of DMSP is observed in the AT assemblage. However, when *P. damicornis* is also present in the assemblage (PA or PAT), this effect disappeared. Therefore, the presence of *P. damicornis* prevents the clams effect, highlighting possible yet uncharacterized interspecies communication responses. It is also known that, depending on the species, the variation in DMSP is not always observed under the same environmental factor. For example, a decrease in salinity leads to a decrease of DMSP in *A. millepora*, but such an effect had not previously been observed in *Stylophora pistillata* and *P. damicornis*⁶⁸. In the present study, contrary to *A. cytherea*, the DMSP concentration in giant clams was increased in PAT assemblages rather than in AT assemblages. For giant clams, the presence of the two coral species seems to have a stronger effect than the sole presence of *A. cytherea*. In the case of DMSP as an indicator of stress, we have previously shown that giant clam mortality occurred in presence of *A. cytherea*, in both AT and PAT assemblages⁴⁰. However, giant clam DMSP increases occurred only in PAT assemblages and we did not find any correlation between health status and DMSP levels in giant clams. Thus, the “health status” (i.e. declining clams) does not appear to systematically lead to a significant change in DMSP level. Taken together, our results suggest that DMSP concentration in the holobionts is influenced by their neighboring species, modifying the metabolism of the sulfur pathway.

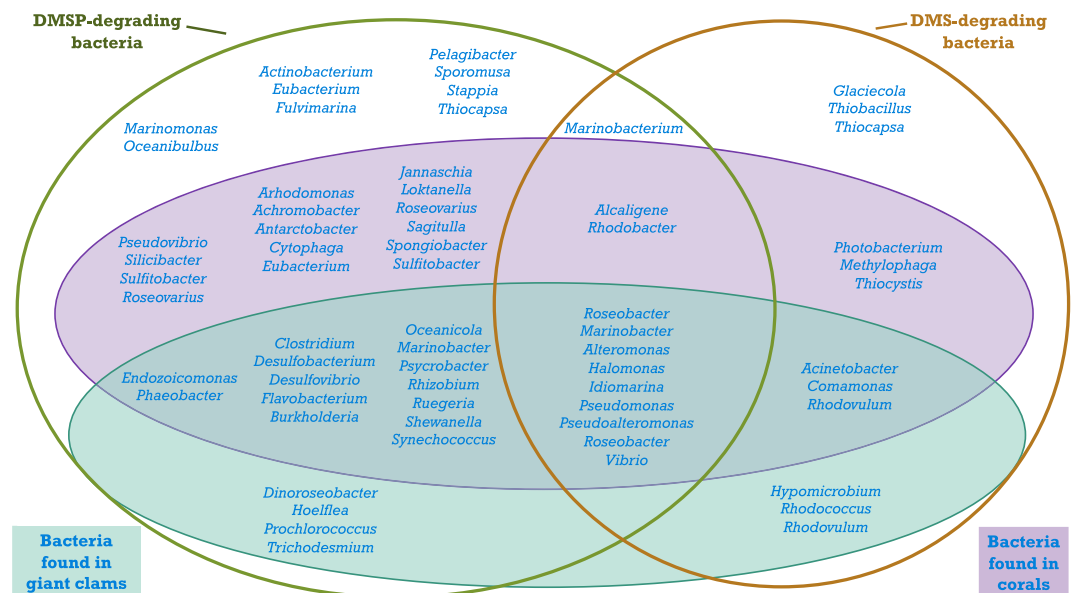


Figure 5. Marine bacterial genera degrading DMSP or DMS in the water column, associated or not with corals and/or giant clams. DMSP-degrading (green circle) and DMS-degrading (brown circle) found in the water column, bacterial genera found in corals (blue oval) and in giant clams (green oval) (adapted from^{37,40}; and further developed from^{71–77}).

Dimethylsulfoniopropionate production. The variations in DMSP concentrations among invertebrates harboring Symbiodiniaceae have been essentially attributed to the types and densities of their symbiotic dinoflagellates²⁰. Significant DMSP variation also exists between Symbiodiniaceae clades^{5,20,33,67}. Consequently, the types and densities of Symbiodiniaceae possibly impact the DMSP concentrations in holobionts, by translocating DMSP to their hosts^{69,70}. Nevertheless, DMSP concentrations and the types of clades in our samples were not correlated. While the DMSP concentration of *A. cytherea* was high in AT assemblages and low in PAT assemblages, the relative abundance of clade A and D were similar in both assemblages. If Symbiodiniaceae are indeed responsible for the DMSP concentrations, we have to assume that their density should be higher in *A. cytherea* nubbins in AT than in other assemblages. Moreover, as DMSP concentrations in *P. damicornis* nubbins are between 5 and 10-fold lower than in *A. cytherea* nubbins, and since clade D has been described as a lower DMSP producer than clade C⁶⁷, we can also hypothesize that clade D is a lower DMSP producer than clade A, a permanent clade in *A. cytherea*. Another non-exclusive alternative hypothesis for explaining the difference in DMSP production between *A. cytherea* and *P. damicornis* relies on a difference in Symbiodiniaceae densities between these two coral species, as observed in our previous work, *A. cytherea* harboring more Symbiodiniaceae than *P. damicornis* in Moorea⁵⁹. Regarding giant clams, as for corals, Symbiodiniaceae types are not sufficient to account for DMSP concentrations variations between assemblages. At the individual level, giant clams in the same assemblage, with either 99% of subclade C66 or 99% of subclade A6 did not exhibit any difference in DMSP concentration⁴⁰. Thus, even if the Symbiodiniaceae are well known DMSP producers, our results suggested that Symbiodiniaceae genotypes did not account for the DMSP variations observed in our study.

In addition to Symbiodiniaceae, corals and giant clams harbor numerous other associated organisms, including various families of bacteria. Raina and collaborators³⁷ reported that more than 65% of the bacteria genera playing a role in DMSP/DMS metabolism can be found in association with coral species. The bacterial communities associated within the giant clams of this experiment have already been characterized⁴⁰. By overlapping the data from both studies, we demonstrated that several bacterial genera involved in the degradation of DMSP or DMS in corals were also associated with *T. maxima* (Fig. 5). This suggests that the microbial communities of giant clams are also structured by the presence of DMSP and could indicate that bacteria influence the composition and variation in DMSP concentration between assemblages. However, as for Symbiodiniaceae, no link was found between bacterial composition and assemblages in *T. maxima*. Altogether our results suggested that the variation in DMSP concentration observed here did not result from Symbiodiniaceae or bacterial genera present in the sample, but might result from differences in their relative densities.

Recent studies have highlighted the existence of DMSP biosynthesis pathways in animals, and in corals notably^{3,15}. The increase in DMSP concentration measured in some assemblages might also be due to an enhancement in DMSP biosynthesis by the hosts, including giant clams. Of relevance, we showed for the first time that, similarly to corals, the giant clam *T. maxima* possesses algal gene homologs putatively involved in DMSP biosynthesis pathways. Candidate genes were not only homologous to DMSP alga-like pathway genes (top blast hit), but were also overexpressed during a thermal stress event. As at least one candidate gene for each step of the alga-like pathway has been found, we assume that the whole pathway is present in *T. maxima*. Future studies on complete holobiont communities and gene expression by assemblage will be required to decipher the involvement of each holobiont partner in DMSP biosynthesis.

This study explored the influence of interspecies assemblages on DMSP concentrations in corals and giant clams. We showed that DMSP concentration is higher in *A. cytherea* when associated with *T. maxima* and higher in *T. maxima* when they are in a three species assemblage. Because the balance between Symbiodiniaceae genera was maintained regardless of host assemblages, our results also show that microbial communities cannot solely account for the DMSP differences observed within and among assemblages. Further studies are required to decipher if symbiont densities and/or the host alga-like DMSP biosynthesis pathway contribute to the differences in DMSP production between assemblages.

Data availability

Supplementary data for this article can be found at Aquatic Sciences Website. All data are available upon request.

Received: 28 January 2019; Accepted: 18 May 2020;

Published online: 18 June 2020

References

- Broadbent, A. D., Jones, G. B. & Jones, R. J. DMSP in corals and benthic algae from the Great Barrier Reef. *Estuar. Coast. Shelf Sci.* **55**, 547–555 (2002).
- Hill, R. W., Dacey, J. W. H. & Krupp, D. A. Dimethylsulfoniopropionate in reef corals. *Bull. Mar. Sci.* **57**, 489–494 (1995).
- Raina, J. B. *et al.* DMSP biosynthesis by an animal and its role in coral thermal stress response. *Nature* **502**, 677–80 (2013).
- Downs, C. A. *et al.* Oxidative stress and seasonal coral bleaching. *Free Radic. Biol. Med.* **33**, 533–543 (2002).
- Deschaseaux, E. S. M. *et al.* Effects of environmental factors on dimethylated sulfur compounds and their potential role in the antioxidant system of the coral holobiont. *Limnol. Oceanogr.* **59**, 758–768 (2014).
- Sunda, W., Kieber, D. J., Kiene, R. P. & Huntsman, S. An antioxidant function for DMSP and DMS in marine algae. *Nature* **418**, 317–320 (2002).
- Kirst, G. O. *et al.* Dimethylsulfoniopropionate (DMSP) in ice-algae and its possible biological role. *Mar. Chem.* **35**, 381–388 (1991).
- Trevena, A. J., Jones, G. B., Wright, S. W. & Van Den Enden, R. L. Profiles of DMSP, algal pigments, nutrients and salinity in pack ice from eastern Antarctica. *J. Sea Res.* **43**, 265–273 (2000).
- Evans, C., Malin, G., Wilson, W. H. & Liss, P. S. Infectious titers of *Emiliania huxleyi* virus 86 are reduced by exposure to millimolar dimethyl sulfide and acrylic acid. *Limnol. Oceanogr.* **51**, 2468–2471 (2006).
- Havill, D. C., Ingold, A. & Pearson, J. Sulphide tolerance in coastal halophytes. *Vegetatio* **62**, 279–285 (1985).
- Seymour, J. R., Simó, R., Ahmed, T. & Stocker, R. Chemoattraction to dimethylsulfoniopropionate throughout the marine microbial food web. *Science* **329**, 342–346 (2010).
- Van Rijssel, M. & Gieskes, W. W. C. Temperature, light, and the dimethylsulfoniopropionate (DMSP) content of *Emiliania huxleyi* (Prymnesiophyceae). *J. Sea Res.* **48**, 17–27 (2002).
- Van Alstyne, K. L., Gifford, S. A., Dohman, J. M. & Savedo, M. M. Effects of environmental changes, tissue types and reproduction on the emissions of dimethyl sulfide from seaweeds that form green tides. *Environ. Chem.* **13**, 220–230 (2016).
- Yost, D. M., Jones, R. J. & Mitchelmore, C. L. Alterations in dimethylsulfoniopropionate (DMSP) levels in the coral *Montastraea franksi* in response to copper exposure. *Aquat. Toxicol.* **98**, 367–373 (2010).
- Aguilar, C. *et al.* Transcriptomic analysis of the response of *Acropora millepora* to hypo-osmotic stress provides insights into DMSP biosynthesis by corals. *BMC Genomics* **18**, 612–626 (2017).
- Rap, A. *et al.* Natural aerosol direct and indirect radiative effects. *Geophys. Res. Lett.* **40**, 3297–3301 (2013).
- Nunes, J. *et al.* Two intertidal, non-calcifying macroalgae (*Palmaria palmata* and *Saccharina latissima*) show complex and variable responses to short-term CO₂ acidification. *ICES J. Mar. Sci.* **73**, 887–896 (2016).
- Simó, R. Production of atmospheric sulfur by oceanic plankton: Biogeochemical, ecological and evolutionary links. *Trends Ecol. Evol.* **16**, 287–294 (2001).
- Tapiolas, D. M., Raina, J. B., Lutz, A., Willis, B. L. & Motti, C. A. Direct measurement of dimethylsulfoniopropionate (DMSP) in reef-building corals using quantitative nuclear magnetic resonance (qNMR). *spectroscopy: J. Exp. Mar. Bio. Ecol.* **443**, 85–89 (2013).
- Van Alstyne, K. L., Schupp, P. & Slattery, M. The distribution of dimethylsulfoniopropionate in tropical Pacific coral reef invertebrates. *Coral Reefs* **25**, 321–327 (2006).
- Becker, S. *et al.* The biology of an Antarctic rhodophyte, *Palmaria decipiens*: recent advances. *Antarct. Sci.* **23**, 419–430 (2011).
- Kocsis, M. G. *et al.* Dimethylsulfoniopropionate biosynthesis in *Spartina alterniflora*. Evidence that S-methylmethionine and dimethylsulfoniopropylamine are intermediates. *Plant Physiol.* **117**, 273–281 (1998).
- White, B. A., Lansing, E., Hill, R. W. & Dacey, J. W. H. Accumulation of Dimethylsulfoniopropionate in *Geukensia demissa* Depends on trophic Interactions. *Nature* **189**, 235–236 (1995).
- Van Alstyne, K. L., Dominique, V. J. & Muller-Parker, G. Is dimethylsulfoniopropionate (DMSP) produced by the symbionts or the host in an anemone-zooxanthella symbiosis? *Coral Reefs* **28**, 167–176 (2009).
- Hanson, A. D., Rivoal, J., Paquet, L. & Cage, D. A. Biosynthesis of 3-Dimethylsulfoniopropionate in *Wollastonia biflora* (L.) DC. *Plant Physiol.* **105**, 103–110 (1994).
- Stefels, J. Physiological aspects of the production and conversion of DMSP in marine algae and higher plants. *J. Sea Res.* **43**, 183–197 (2000).
- Keller, M. D., Bellows, W. K. & Guillard, R. R. L. Dimethyl sulfide production in marine phytoplankton. *Biog. sulfur Environ.* **393**, 167–182 (1989).
- Hill, R. W., Dacey, J. W. H. & Edward, A. Dimethylsulfoniopropionate in giant clams (Tridacnidae). *Biol. Bull.* **199**, 108–115 (2000).
- Steinke, M., Brading, P., Kerrison, P., Warner, M. E. & Suggett, D. J. Concentrations of dimethylsulfoniopropionate and dimethyl sulfide are strain-specific in symbiotic dinoflagellates (*Symbiodinium* sp., dinophyceae). *J. Phycol.* **47**, 775–783 (2011).
- Pochon, X. & Gates, R. D. A new *Symbiodinium* clade (Dinophyceae) from soritid foraminifera in Hawai'i. *Mol. Phylogenet. Evol.* **56**, 492–497 (2010).
- LaJeunesse, T. C. *et al.* Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Curr. Biol.* **28**, 1–11 (2018).
- Neo, M. L., Eckman, W., Vicentuan, K., Teo, S. L. M. & Todd, P. A. The ecological significance of giant clams in coral reef ecosystems. *Biol. Conserv.* **181**, 111–123 (2015).
- Yost, D. M. & Mitchelmore, C. L. Dimethylsulfoniopropionate (DMSP) lyase activity in different strains of the symbiotic alga *Symbiodinium microadriaticum*. *Mar. Ecol. Prog. Ser.* **386**, 61–70 (2009).
- Van Alstyne, K. L. & Puglisi, M. P. DMSP in marine macroalgae and macroinvertebrates: Distribution, function, and ecological impacts. *Aquat. Sci.* **69**, 394–402 (2007).
- Howard, E. C. *et al.* Bacterial Taxa That Limit Sulfur Flux from the Ocean. *Science* **314**, 649–652 (2006).
- Littman, R. A., Willis, B. L., Pfeffer, C. & Bourne, D. G. Diversities of coral-associated bacteria differ with location, but not species, for three acroporid corals on the Great Barrier Reef. *FEMS Microbiol. Ecol.* **68**, 152–163 (2009).

37. Raina, J. B., Dinsdale, E. A., Willis, B. L. & Bourne, D. G. Do the organic sulfur compounds DMSP and DMS drive coral microbial associations? *Trends Microbiol.* **18**, 101–108 (2010).
38. Todd, J. D. *et al.* Structural and regulatory genes required to make the gas dimethyl sulfide in bacteria. *Science*. **315**, 666–669 (2007).
39. Raina, J. B., Tapiolas, D., Willis, B. L. & Bourne, D. G. Coral-associated bacteria and their role in the biogeochemical cycling of sulfur. *Appl. Environ. Microbiol.* **75**, 3492–3501 (2009).
40. Guibert, I., Pochon, X., Lecellier, G. & Berteaux-lecellier, V. Metabarcoding reveals distinct microbiotypes in the giant clam *Tridacna maxima*. *Microbiome* **8**, 57 (2020).
41. Curson, A. R. J. *et al.* Dimethylsulphoniopropionate biosynthesis in marine bacteria and identification of the key gene in this process. *Nat. Microbiol.* **2**, 1–35 (2017).
42. Silverstein, R. N., Cunnig, R. & Baker, A. C. Change in algal symbiont communities after bleaching, not prior heat exposure, increases heat tolerance of reef corals. *Glob. Chang. Biol.* **21**, 236–249 (2015).
43. Cunnig, R., Yost, D. M., Guarinello, M. L., Putnam, H. M. & Gates, R. D. Variability of *Symbiodinium* communities in waters, sediments, and corals of thermally distinct reef pools in American Samoa. *PLoS One* **10**, 1–17 (2015).
44. Ziegler, M., Seneca, F. O., Yum, L. K., Palumbi, S. R. & Voolstra, C. R. Bacterial community dynamics are linked to patterns of coral heat tolerance. *Nat. Commun.* **8**, 14213 (2017).
45. Sharp, K. H., Pratte, Z. A., Kerwin, A. H., Rotjan, R. D. & Stewart, F. J. Season, but not symbiont state, drives microbiome structure in the temperate coral *Astrangia poculata*. *Microbiome* **5**, 120 (2017).
46. Camp, E. F. *et al.* Corals exhibit distinct patterns of microbial reorganisation to thrive in an extreme inshore environment. *Coral Reefs* **3**, 1–16 (2020).
47. Rosenberg, E. & Zilber-Rosenberg, I. Symbiosis and development: The hologenome concept. *Birth Defects Res. Part C - Embryo Today Rev.* **93**, 56–66 (2011).
48. Chornesky, E. Induced development of sweeper tentacles on the reef coral *Agaricia agaricites*: A response to direct competition. *Biol. Bull.* **165**, 569–581 (1983).
49. Connell, J. H. *et al.* A long-term study of competition and diversity of corals. *Ecol. Monogr.* **74**, 179–210 (2004).
50. Lang, J. Interspecific aggression by scleractinian corals. 2. why the race is not only to the swift. *Bull. Mar. Sci.* **23**, 260–279 (1973).
51. Adjerdoud, M., Briand, M. J., Kayal, M. & Dumas, P. Coral assemblages in Tonga: Spatial patterns, replenishment capacities, and implications for conservation strategies. *Environ. Monit. Assess.* **185**, 5763–5773 (2013).
52. Guibert, I. *et al.* Differential effects of coral-giant clam assemblages on biofouling formation. *Sci. Rep.* **9**, 1–12 (2019).
53. Dubousquet, V. *et al.* Changes in fatty acid composition in the giant clam *Tridacna maxima* in response to thermal stress. *Biol. Open* **5**, 1400–1407 (2016).
54. Rouzé, H., Lecellier, G., Langlade, M., Planes, S. & Berteaux-Lecellier, V. Fringing reefs exposed to different levels of eutrophication and sedimentation can support the same benthic communities. *Mar. Pollut. Bull.* **92**, 212–221 (2015).
55. Siebeck, U. E., Marshall, N. J., Klüter, A. & Hoegh-Guldberg, O. Monitoring coral bleaching using a colour reference card. *Coral Reefs* **25**, 453–460 (2006).
56. Siebeck, U. E., Logan, D. & Marshall, N. J. CoralWatch - a flexible coral bleaching monitoring tool for you and your group. *Proc. 11th Int. Coral Reef Symp.* 549–553 (2008).
57. Mohamadi, F. La métabolomique appliquée à l'étude de l'impact de stress environnementaux sur les coraux scléractiniaux (2014).
58. R core team. A language and environment for statistical computing (2014).
59. Rouzé, H. *et al.* An updated assessment of *Symbiodinium* spp. that associate with common scleractinian corals from Moorea (French Polynesia) reveals high diversity among background symbionts and a novel finding of clade B. *PeerJ* **5**, 1–22 (2017).
60. Pochon, X., Pawlowski, J., Zaninetti, L. & Rowan, R. High genetic diversity and relative specificity among *Symbiodinium*-like endosymbiotic dinoflagellates in soritid foraminiferans. *Mar. Biol.* **139**, 1069–1078 (2001).
61. Stat, M., Pochon, X., Cowie, R. O. M. & Gates, R. D. Specificity in communities of *Symbiodinium* in corals from Johnston Atoll. *Mar. Ecol. Prog. Ser.* **386**, 83–96 (2009).
62. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ. Microbiol.* **79**, 5112–5120 (2013).
63. Schloss, P. D. *et al.* Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75**, 7537–7541 (2009).
64. Arif, C. *et al.* Assessing *Symbiodinium* diversity in scleractinian corals via next-generation sequencing-based genotyping of the ITS2 rDNA region. *Mol. Ecol.* **23**, 4418–4433 (2014).
65. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).
66. Dimont, E., Shi, J., Kirchner, R. & Hide, W. edgeRun: An R package for sensitive, functionally relevant differential expression discovery using an unconditional exact test. *Bioinformatics* **31**, 2589–90 (2015).
67. Deschaseaux, E. S. M. *et al.* Comparative response of DMS and DMSP concentrations in *Symbiodinium* clades C1 and D1 under thermal stress. *J. Exp. Mar. Bio. Ecol.* **459**, 181–189 (2014).
68. Gardner, S. G. *et al.* Dimethylsulfonylpropionate, superoxide dismutase and glutathione as stress response indicators in three corals under short-term hyposalinity stress. *Proc. R. Soc. B Biol. Sci.* **283**, 1–9 (2016).
69. Hill, R. W., Dacey, J. W., Hill, S. D., Edward, A. & Hicks, W. A. Dimethylsulfonylpropionate in six species of giant clams and the evolution of dimethylsulfide after death. *Can. J. Fish. Aquat. Sci.* **61**, 758–764 (2004).
70. Yost, D. M. & Mitchelmore, C. L. Determination of total and particulate dimethylsulfonylpropionate (DMSP) concentrations in four scleractinian coral species: A comparison of methods. *J. Exp. Mar. Bio. Ecol.* **395**, 72–79 (2010).
71. Alex, A. & Antunes, A. Genus-wide comparison of *Pseudovibrio* bacterial genomes reveal diverse adaptations to different marine invertebrate hosts. *PLoS One* **13**, 1–25 (2018).
72. Zeng, Y. X., Qiao, Z. Y., Yu, Y., Li, H. R. & Luo, W. Diversity of bacterial dimethylsulfonylpropionate degradation genes in surface seawater of Arctic Kongsfjorden. *Sci. Rep.* **6**, 1–10 (2016).
73. González, J. M. *et al.* *Silicibacter pomeroyi* sp. nov. and *Roseovarius nubinihibens* sp. nov., dimethylsulfonylpropionate-demethylating bacteria from marine environments. *Int. J. Syst. Evol. Microbiol.* **53**, 1261–1269 (2003).
74. Rossbach, S., Cardenas, A., Perna, G., Duarte, C. M. & Voolstra, C. R. Tissue-specific microbiomes of the Red Sea giant clam *Tridacna maxima* highlight differential abundance of Endozoicomonadaceae. *Front. Microbiol.* **10** (2019).
75. Shiu, J. H. *et al.* A newly designed primer revealed high phylogenetic diversity of endozoicomonas in coral reefs. *Microbes Environ.* **33**, 172–185 (2018).
76. Schäfer, H. Isolation of Methylophaga spp. from marine dimethylsulfide-degrading enrichment cultures and identification of polypeptides induced during growth on dimethylsulfide. *Appl. Environ. Microbiol.* **73**, 2580–2591 (2007).
77. Cai, L. *et al.* Season structures prokaryotic partners but not algal symbionts in subtropical hard corals. *Appl. Microbiol. Biotechnol.* **102**, 4963–4973 (2018).

Acknowledgements

This work was supported by LabexCorail “SynTox”. I. Guibert was a fellow of Sorbonne University – Doctoral School 129. We acknowledge the InterContinental Resort & Spa Moorea and the Moorea Dolphin Center for providing a coral garden protected area. We also thank Franck Lerouvreur, Pascal Ung, Ewen Morin and all the students from CRILOBE who helped us in the field. We are much indebted to Roy Benyon for English improvement of the manuscript.

Author contributions

Conceived and designed the experiments: I.G., V.B., P.R., G.L., Performed the experiments: I.G., V.D., V.B., Collected the samples: I.G., Processed the samples: I.G., F.B., G.L., I.B., Generated the data: F.B., V.D., X.P., G.L., Analysed the data: I.G., G.L., Wrote the manuscript: I.G., V.B., G.L., I.B., X.P.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41598-020-66290-5>.

Correspondence and requests for materials should be addressed to I.G.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Summary statement Various assemblages of corals and giant clams modulate dimethylsulfoniopropionate concentration, a key compound in the marine sulfur cycle.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2020