Symbiosis induces unique volatile profiles in the model cnidarian Aiptasia

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Summary statement

The volatilome of Aiptasia is described, showing that symbiosis affects the diversity of biogenic volatile organic compounds (BVOCs) produced in this model system.

Abstract

The establishment and maintenance of the symbiosis between a cnidarian host and its dinoflagellate symbionts is central to the success of coral reefs. To explore the metabolite production underlying this symbiosis, we focused on a group of low weight secondary metabolites, biogenic volatile organic compounds (BVOCs). BVOCs are released from an organism or environment, and can be collected in the gas phase, allowing non-invasive analysis of an organism's metabolism (i.e. 'volatilomics'). We

characterised volatile profiles of the sea anemone *Exaiptasia diaphana* (‘Aiptasia’), a model system for cnidarian–dinoflagellate symbiosis, using comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry. We compared volatile profiles between: 1) symbiotic anemones containing their native symbiont, *Breviolum minutum*; 2) aposymbiotic anemones; and 3) cultured isolates of *B. minutum*. Overall, 152 BVOCs were detected, and classified into 14 groups based on their chemical structure, the most numerous groups being alkanes and aromatic compounds. A total of 53 BVOCs were differentially abundant between aposymbiotic anemones and *B. minutum* cultures; 13 between aposymbiotic and symbiotic anemones; and 60 between symbiotic anemones and cultures of *B. minutum*. More BVOCs were differentially abundant between cultured and symbiotic dinoflagellates than between aposymbiotic and symbiotic anemones, suggesting that symbiosis may modify symbiont physiology more than host physiology. This is the first volatilome analysis of the Aiptasia model system and provides a foundation from which to explore how BVOC production is perturbed under environmental stress, and ultimately the role they play in this important symbiosis.

Introduction

All organisms synthesise a unique chemical composition of metabolites which reflects their underlying metabolic processes and relationship to the environment. A subset of these metabolites are termed biogenic volatile organic compounds (BVOCs) that are often synthesised as a by-product of central metabolism and emitted directly into the environment (Sharkey et al., 2008; Schmidt et al., 2016). BVOCs are a functionally diverse group of chemicals characterised by their low molecular weight (< 200 Da), low boiling point and high vapour pressure (Mansurova et al., 2018). In contrast to other metabolites, which must be extracted from tissues and fluids, BVOCs diffuse into the external environment, thereby allowing their detection without disruption of the producing organism and hence non-invasive analysis of metabolic state (Mansurova et al., 2018).

Terrestrial ecosystems are known to be prolific producers of BVOCs (Guenther, 2013), many of which play important physiological and ecological roles, such as pollinator attraction (Schiestl, 2015), pathogen defence (Huang et al., 2012), plant growth promotion (Bailly et al., 2014) and antioxidant production (Stashenko et al., 2004). BVOCs also function as agents of rapid communication in intra- and inter-species communication (Briard et al., 2016; Mansurova et al., 2018). The BVOC produced in highest quantities by terrestrial ecosystems is isoprene, which functions in thermal tolerance in plant cell walls (Behnke et al., 2007), predator defence (Laothawornkitkul et al., 2008) and as an antioxidant (Loreto and Velikova, 2001). The dominant BVOC produced by marine ecosystems is dimethyl sulphide (DMS) (Kettle et al., 1999; Jackson et al., 2021), which functions as an antioxidant (Sunda et al., 2002), attractant for macrofauna to a productive marine environment (Nevitt et al., 1995; Wright et al., 2011), and local climate regulator

(Park et al., 2021). Notably, coral reefs have been identified as marine 'hotspots' of BVOC emissions (Exton et al., 2014), with corals producing highly diverse BVOC cocktails – characterised as "volatilomes" (Steinke et al., 2018) – that vary by species and environmental conditions (Lawson et al., 2019; Lawson et al., 2021). Despite the diversity and abundance of BVOCs in the marine environment, resolving BVOC form and function in marine ecosystems is a comparatively new area of focus (Saha et al., 2021).

Metabolite exchange is critical for sustaining a stable and healthy symbiosis between reef-building coral hosts and their microalgal dinoflagellate partners (family: Symbiodiniaceae) (Matthews et al., 2017; Rosset et al., 2021). Dinoflagellate symbionts reside within membrane-bound vesicles in the host gastrodermis (Wakefield and Kempf, 2001), meaning that inter-partner signalling across this host-symbiont interface, including the exchange of metabolites, is crucial for maintaining the symbiosis (Rosset et al., 2021). Diverse molecules such as glycans (Markell and Wood-Charlson, 2010), lipids (Kitchen et al., 2017) and noncoding RNA (Baumgarten et al., 2017) have been identified as signalling molecules in the cnidarian–dinoflagellate symbiosis. BVOCs have likewise been identified as agents of inter-kingdom signalling molecules in other systems (Schmidt et al., 2016; Netzker et al., 2020). For example, p-anisaldehyde and phenylacetaldehyde production attracts pollinators to flowers (Theis, 2006), herbivore-damaged *Nicotiana attenuate* produce linalool to stimulate herbivore predation (Kessler and Baldwin, 2001), and *Laccaria bicolor* stimulate root formation in *Arabidopsis* and *Populus* through sesquiterpene production (Ditengou et al., 2015). Given their importance as infochemicals in other systems, BVOCs may therefore also be important for regulating the cnidarian–dinoflagellate symbiosis.

The metabolism of both cnidarian and dinoflagellate partners is altered in response to symbiotic state (Rodriguez-Lanetty et al., 2006; Yuyama et al., 2021), and these changes may also be detectable in the volatilome. These metabolic shifts function to optimise this relationship and allow the growth of both organisms in symbiosis, allowing each partner to benefit from their close association with the other. Alterations can be reflected through gene expression (Rodriguez-Lanetty et al., 2006), nutrient exchange (Xiang et al., 2020) and assimilation (Wang and Douglas, 1998), cell cycle regulation (Tivey et al., 2020b), and the modulation of the immune response (Neubauer et al., 2016). Since other, non-BVOC molecules are known to change in response to symbiotic state (Rosset et al., 2021), we hypothesise that BVOC profiles will also differ among symbiotic states in this system, thus reflecting differing metabolic processes.

The symbiotic sea anemone *Exaiptasia diaphana* ('Aiptasia') is a model system for the study of the cnidarian–dinoflagellate symbiosis that can extend to other symbiotic cnidarians, including ecologically-important reef corals. Like corals, Aiptasia is a known producer of BVOCs such as DMS when in symbiosis (Franchini and Steinke, 2017). The Aiptasia model system has been adopted for its

ease of laboratory culture and ability to separate symbiont from host to study each organism in isolation, providing insight into the roles that the cnidarian and dinoflagellate play in the symbiosis (Weis et al., 2008; Baumgarten et al., 2015), including metabolite exchange (Matthews et al., 2017, 2018). The aim of our study was to therefore profile the BVOCs of the Aiptasia model system. We describe the BVOCs that are produced by the intact symbiosis, as well as the host anemone and homologous (i.e. native) symbiotic dinoflagellate (*Breviolum minutum*) when in isolation from one another, which may inform how symbiotic state alters the volatilome. The unique volatile signatures characterised here provide a baseline for future non-invasive analysis of how symbiotic state and environmental change influence symbiosis physiology, as well as studies of inter-partner communication and functionality of BVOCs in the cnidarian–dinoflagellate symbiosis.

Methods

Experimental Organisms

The homologous symbiont of Aiptasia, *Breviolum minutum* (ITS2 type B1, culture ID 'FLAp2'), was grown in 35 ppt 0.22 µm filtered seawater (FSW) enriched with f/2-medium and maintained at 25 °C in a climate-controlled incubator. Cultures were grown under light provided by fluorescent lamps (Osram Dulux 36/W890 fluorescent bulbs) at approximately 70 µmol photons m^{-2} s^{-1} on a 12:12 h light dark cycle. One week prior to experimentation, cultures for BVOC analysis were diluted with fresh medium to ensure that they were in exponential growth. On the day of sampling, maximum photochemical efficiency, F_v/F_m (dimensionless), was used as an indicator of culture health (Table S1; Suggett et al., 2009); cultures were dark acclimated in 125 mL serum glass bottles (Wheaton, Millville, NJ, USA) for 15 min before measurement with an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany; settings: measuring light = 4, saturation intensity = 8, saturation width = 0.8 s, gain = 3 and damping = 3).

A long-term (15+ years) clonal culture of the sea anemone Aiptasia (culture ID: NZ1) of unknown Pacific origin (Matthews et al., 2017) was maintained in the laboratory in FSW at 25 °C and approximately 70 µmol photons m^{-2} s^{-1} on a 12:12 h light dark cycle. Anemones (n = 100) were rendered aposymbiotic (i.e., symbiont-free) using menthol-induced bleaching, which was achieved by exposure to menthol (20% w/v in ethanol; Sigma-Aldrich, Auckland, NZ) at a final concentration of 0.19 mmol L^{-1} in 0.22 µm FSW (Matthews et al., 2016). Anemones were incubated in menthol for 8 h during the 12-h light period, after which photosynthesis was inhibited by replacing menthol/FSW with FSW containing 5 µmol L^{-1} 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 100mmol L^{-1} dissolved in EtOH, Sigma-Aldrich), for 16 h to prevent recolonisation by inhibiting photosynthesis of the remaining symbionts. This 24-h cycle was repeated for four consecutive days, after which anemones were maintained in 0.22 µm FSW for the three days between menthol and DCMU treatments.

Anemones were fed once weekly with *Artemia* sp. nauplii, with fresh FSW changes 8 hours post-feeding. This protocol was continued for 6 weeks, and aposymbiotic anemones were maintained in the dark at 25°C for 6 months prior to BVOC sampling.

Given that the culture of *B. minutum* did not originate from our NZ1 lab stock of Aiptasia, symbiotic anemones were generated by recombining aposymbiotic anemones with the cultured symbionts. To achieve this, aposymbiosis was confirmed through fluorescence microscopy (Olympus IX53 inverted microscope; 100× magnification), and a sub-set of aposymbiotic anemones (n = 25) were starved for seven days prior to inoculation with cultured *B. minutum*. An aliquot (~20 µL) of *B. minutum* culture, concentrated by centrifugation to a density of 3×10^6 cells mL^{-1}, was pipetted directly onto the oral disc of individual aposymbiotic anemones. *Artemia* sp. nauplii were mixed into this suspension to encourage phagocytosis of algal cells (Davy et al., 1997). Inoculated anemones were fed twice weekly with *Artemia* sp. nauplii and maintained under the same ambient temperature and light conditions as described above until a fully symbiotic state was reached after three months. Anemones were fully symbiotic for six months prior to BVOC sampling. The presence of intracellular symbionts was confirmed by fluorescence microscopy three months prior to sampling as described above.

BVOC sampling and volatilome characterisation

Volatile signatures were collected and analysed from three states: vials of aposymbiotic anemones (n = 5; ~15 anemones/vial); symbiotic anemones colonised with *B. minutum* (n = 4; ~15 anemones/vial); and free-living cultures of *B. minutum* (n = 5). The experimental setup (Fig. S1) and BVOC retrieval were performed using a modified method of Lawson et al. (2019, 2021). The night before BVOC sampling, experimental organisms were transferred into sterile 150 mL glass crimp cap vials containing 75 mL FSW, where they were retained for a minimum of 12 h under conditions identical to those used for growth, and vials were refreshed with FSW and sealed immediately prior to sampling using 20 mm PTFE/Si crimp caps (Agilent, USA). BVOCs were collected by passing instrument grade air (100 mL min^{-1}; BOC Gases, Wellington, NZ) into sampling vials for 20 min, during which time air was bubbled through the FSW, facilitating the release of BVOCs into the headspace. The outgoing air then passed through open-ended thermal desorption tubes (TDTs; Markes International Ltd, Llantrisant, UK) containing the sorbent Tenax TA, onto which BVOCs adhered (Fig. S1). After the 20 min sampling time, tubes were immediately sealed with brass storage caps and stored at 4 °C until processing. All TDTs were analysed within two weeks of sampling to minimise sample degradation using comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-ToFMS) as *per* Olander et al. (2021).

Prior to desorption, TDTs were injected with 0.2 µL of 150 ppm bromobenzene (GC grade, Sigma-Aldrich, Castle Hill, NSW, Australia) in methanol (HPLC grade, Sigma-Aldrich) using an automated eVol® XR analytical pipette (SGE Analytical Science, Wetherill Park BC, NSW, Australia) as an internal standard to ensure injection repeatability. Samples were desorbed using a Markes UNITY 2 series thermal desorber and ULTRA multi-tube autosampler (Markes International Ltd. Llantrisant, UK) for 2.5 min at 300 °C and concentrated on a Markes General Purpose Carbon C4/5-C30/32 cold trap at -30 °C. Desorbed compounds were then injected into a Pegasus 4D GC×GC-ToFMS platform (LECO Corp., St Joseph, MI, USA) *via* a linked 1 m uncoated silica transfer line at 150 °C. High purity helium with a flow rate of 1 mL min^{-1} was used as the carrier gas. The GC×GC-ToFMS was equipped with a cryogenic quad jet modulator, using two liquid-nitrogen cold jets in conjunction with two pulsed hot-air jets to trap and refocus compounds eluting between the primary (Rxi-624Sil MS column (30 m×0.25 mm inner diameter (ID), 1.40 µm film thickness); Restek Corp.) and secondary (Stabilwax column (2 m×0.25 mm ID, 0.50 µm film thickness); Restek Corp) columns. The primary and secondary columns were bridged by a SilTite µ-Union connector (SGE Analytical Science Pty., Ltd, Ringwood, VIC, Australia). The temperature program was as follows: initial temperature of 35 °C with a 5 min hold followed by an increase to 240 °C at a rate of 5 °C min^{-1} with a final hold of 5 min. Temperature offsets of + 5 °C and + 15 °C for the modulator and second dimension column, respectively, were used. The modulation period was 5 s and included a 1 s hot pulse. The ToFMS collected at a rate of 100 spectra s^{-1} with a range of 29–450 amu.

Data processing was performed using ChromaTOF® (version 4.51.6.0; LECO). A signal-to-noise ratio of 150 was used with a baseline offset of 0.8. The peak widths for the first and second dimensions were 30 s and 0.15 s, respectively. Compounds were tentatively identified by comparing mass spectra against a commercial library (NIST08 library in NIST MS Search v.2.2f; NIST, Gaithersburg, MD) where a spectral match of 80% was required. Unclassified compounds were denoted as UC, with the numbers following indicating the retention times of the compound in the first and second dimension, respectively. All samples were normalised to the internal standard. BVOCs were also collected from filtered (0.22 µm) seawater blanks (n = 6) using identical methods at the time of sample collection. All samples were aligned using the Statistical Compare feature in ChromaTOF®. For alignment, the samples were classified according to treatment method, as aposymbiotic anemones (n = 5), symbiotic anemones (n = 4), cultures of *B. minutum* (n = 5) and seawater blanks (n = 6). A requirement of the BVOCs to be present in a minimum of two samples within one treatment was used to eliminate BVOCs that were not deemed relevant. After this, average values for BVOCs present in the blanks were subtracted from all samples. Finally, peak abundance was normalised to the protein content of each replicate. Protein was released from samples by sonication (VCX500; Sonics & Materials Inc., Newtown, CT, USA) with a detergent (5% sodium deoxycholate) to lyse cells. Detergent was subsequently precipitated by acidification and removed by

centrifugation (Eppendorf 5424 Centrifuge; Thermo Fisher Scientific, Waltham, MA, USA). Protein content was measured using the fluorometric Qubit Protein Assay Kit (Vergauwen et al., 2017). Compounds identified as likely methodological artifacts e.g. silicon-containing compounds, were also removed from the dataset as they are suspected contamination from dimethylpolysiloxane (a GC column component) hydrolysis (Cella and Carpenter, 1994).

Statistical Analysis

Differential abundance of BVOCs was estimated using the limma R package (Ritchie et al., 2015); the *voom* function was used to convert counts to $\log_2$-counts-*per*-million and to assign weights to each observation based on the mean-variance trend. Functions *lmFit*, *eBayes*, and *topTable* were used to fit weighted linear regression models, calculate empirical Bayes moderated t-statistic, and calculate FDR-corrected p-values (Phipson et al., 2016). Bubble plots, bar graphs and pie charts were created with ggplot2 (Wickham, 2016) in RStudio.

Pairs of biological replicates, standardised using the *decostand* function in the vegan package (Oksanen et al., 2020) in R (version 1.2.5033), were compared using the Bray-Curtis similarity measure, and this output was subjected to non-metric multidimensional scaling (NMDS) to visualise differences among groups based on BVOCs.

Results

BVOC emissions are affected by symbiotic state

A total of 152 BVOCs were detected across the dataset of aposymbiotic anemones, symbiotic anemones and symbiont cultures (Fig. 1). Of these, 7 BVOCs were produced solely by aposymbiotic anemones, 11 by symbiotic anemones, and 29 by cultures of *B. minutum;* 42 BVOCs were detected in aposymbiotic and symbiotic anemones but were absent in cultures of *B. minutum*; 5 were detected in symbiotic anemones and cultures of *B. minutum* but were absent from aposymbiotic anemones; and 23 were detected in aposymbiotic anemones and cultures of *B. minutum* but were absent in symbiotic anemones (Fig. 1a). Only BVOCs detected in a minimum of two biological replicates in at least one symbiotic state were classed as ‘present’ in a state.

35 BVOCs were present across all samples, and so were designated as ‘core compounds’ (Fig. 1a). All BVOCs were grouped according to their chemical class (Fig. 1b). Unknown compounds represented the majority of BVOCs in all states, followed by aromatic compounds in aposymbiotic

anemones, and alkanes in symbiotic anemones and cultures of *B. minutum*. BVOCs across the three states were distinct, with clear NMDS clustering of BVOCs by aposymbiotic anemones, symbiotic anemones and cultures of *B. minutum* (Fig. 2), indicating that each state was characterised by a unique volatilome. The numbers of differentially abundant BVOCs relative to the holobiont were contrasted (Fig. 3), revealing that cultured *B. minutum* produces a more distinct volatilome than aposymbiotic anemones.

Contrasting volatilomes between symbiotic states

A total of 71 BVOCs were differentially abundant between the three symbiotic states, as determined with pairwise differential abundance testing (Table S2):

(i) Aposymbiotic anemones *versus B. minutum* cultures:

53 differentially abundant BVOCs were recorded between aposymbiotic anemones and cultures of *B. minutum*. Of these, 25 were detected in higher quantities in the volatilome of aposymbiotic anemones, including 5 halogenated hydrocarbons (bromochloromethane, bromodichloromethane, dibromochloromethane, dibromomethane, and trichloromethane), plus 1,3-octadiene and styrene. By comparison, 28 BVOCs were detected in higher quantities in the volatilome of *B. minutum*, including DMS, anisole, methylal and amylene hydrate.

(ii) Aposymbiotic anemones *versus* symbiotic anemones:

When anemones were compared between the aposymbiotic and symbiotic states (colonised with *B. minutum*), their volatilomes were very similar, with only 13 compounds being differentially abundant. Only 7 compounds were detected in higher quantities in the volatilome of aposymbiotic anemones: dimethoxy-1,3-benzene, 1,3-octadiene, amylene hydrate, 3,4-dimethyl-2-pentanone, and 2 unknown compounds. By comparison, 6 compounds were detected in higher quantities in the volatilome of symbiotic anemones: DMS, ethylidenecyclopropane and 4 unknowns.

(iii) *B. minutum* culture *versus* symbiotic anemones:

Comparison between the cultured *B. minutum* and symbiotic anemones revealed the greatest divergence in the volatilome, with 60 BVOCs being differentially abundant. Of these, 33 were more abundant in *B. minutum* cultures, including DMS, amylene hydrate, 2-pentanone, 2-butanone and isopropylsulphonyl chloride. In comparison, 27 were more abundant in symbiotic anemones, including 6 halogenated methane BVOCs (bromochloromethane, bromodichloromethane, dibromochloromethane, dibromomethane, trichloromethane and tribromomethane), butanal, styrene and propylbenzene.

Discussion

BVOC emissions by organisms provide important insight into underlying metabolic regulation *via* external environmental and biotic factors (Niinemets, 2010; Mansurova et al., 2018). While recent research has shown that – as with terrestrial organisms (Hrebien et al., 2020; Peñuelas and Llusià, 2003) – reef building corals emit highly diverse BVOC mixtures (volatilomes) (Lawson et al., 2021), how such volatilomes are influenced by symbiotic state is unexplored. Since coral metabolism is altered in response to symbiosis (Matthews et al., 2017; Sproles et al., 2019; Rosset et al., 2021), BVOC analysis can provide a non-invasive technique with which to assess the intracellular mechanisms and interactions involved in symbiosis. To date, the volatilomes of corals have only been retrieved from a single heat-stress experiment, where symbiotic state between the host coral and its endosymbiotic dinoflagellate community was largely retained, but physiological competency compromised (Lawson et al., 2021). The purpose of our study was therefore to characterise BVOC production by the Aiptasia model system and its individual constituents (aposymbiotic anemones and cultures of *B. minutum*), using non-invasive analysis. In doing so, we identified distinct volatilomes between symbiotic states. Our results show that cultured *B. minutum* produce a more distinct volatilome than that of aposymbiotic anemones or anemones symbiotic with their homologous symbiont, *B. minutum*. Aposymbiotic *versus* symbiotic anemones had fewer statistically differentially abundant BVOCs (13) and a more similar composition of BVOC diversity than cultured *B. minutum* *versus* symbiotic anemones (60), indicating that the volatilome of the host cnidarian may be less impacted by symbiotic state than that of the symbiont.

Core Compounds

Of the 152 BVOCs detected in this study, 35 were present in all three symbiotic states, and thus were classified as ‘core compounds’. Of the 35 core compounds, the most common group was unclassified BVOCs (12), followed by ketones (5). Notably, dimethyl sulphide (DMS) is a core compound; to our knowledge the production of DMS in aposymbiotic anemones has not been reported, though previous studies show that cnidarians may be a cryptic source of DMS not produced by their endosymbiotic partner (Raina et al., 2013). Alternatively, it is possible that DMS was produced by members of the associated bacterial community, as coral-associated bacteria have been shown to produce the precursor to DMS, dimethylsulphoniopropionate (DMSP; Kuek et al., 2022). Regardless of its source, this information serves to highlight the ubiquitous nature of DMS in marine systems, where it serves osmoregulatory (Wittek et al., 2020), antioxidant (Sunda et al., 2002) and chemoattractant roles (DeBose et al., 2008).

Volatilomes of Aiptasia and *B. minutum* in the absence of symbiosis

Comparing the volatilome of aposymbiotic anemones *versus* cultured symbionts is important for identifying those BVOCs that are synthesised by the algae or cnidarian (or their microbial associates) in the absence of symbiosis. For example, halogenated methane compounds (chloroform, bromodichloromethane, chlorodibromomethane, bromochloromethane and dibromomethane) were detected in significantly higher quantities in aposymbiotic anemones relative to cultured *B. minutum*. This outcome is consistent with previous research showing that halogenated hydrocarbons, including bromoform and chlorodibromomethane are likely core components of coral (*Acropora intermedia* and *Pocillopora damicornis*) volatilomes under ambient and elevated temperatures (Lawson et al., 2021), but are not detectable in the volatilomes of bacteria and dinoflagellate symbionts isolated from these corals (Lawson et al., 2019, 2020). This suggests that the cnidarian host may be the primary source of these molecules in Aiptasia, and that the production of halogenated hydrocarbons is conserved among symbiotic cnidarians.

In contrast, ethylidenecyclopropane was increased in cultures of *B. minutum* as well as symbiotic anemones relative to aposymbiotic anemones, suggesting that it originated from *B. minutum*. Previously identified as a by-product of thermal stress *via in vitro* experiments simulating thermal and light stress in artificial membranes, ethylidenecyclopropane may serve a protective role in the holobiont to stabilise symbiont lipid membranes (Halahan, 2013). It is therefore plausible that the symbionts are responsible for production of this molecule and potentially upregulate its production during periods of stress.

A well-studied BVOC in marine systems is DMS, which was likewise detected in significantly higher quantities in both cultures of *B. minutum* and symbiotic anemones relative to aposymbiotic anemones (495× and 31× greater, respectively), suggesting that *B. minutum* produces DMS in both its symbiotic and cultured states. DMS is a breakdown product of dimethylsulphoniopropionate (DMSP), and marine phytoplankton – including Symbiodiniaceae – produce it prolifically (Keller et al., 1989; Yoch, 2002; Lawson et al., 2019), as do corals in response to stress (Hopkins et al., 2016). DMS is an antioxidant molecule that can penetrate thylakoid membranes and scavenge free-radicals produced by harmful lipid peroxidation reactions (Sunda et al., 2002), and conceivably may be passed from symbiont to host to provide antioxidative protection. While the symbiont is a source of free radical production during photosynthesis (Khorobrykh et al., 2020), and especially so during periods of thermal and/or light stress (Nielsen et al., 2018), the significantly higher levels of DMS produced in the symbiotic *vs.* aposymbiotic state may indicate that the symbiont is providing a molecule important for the mitigation of oxidative damage to the host (Oakley and Davy, 2018).

BVOCs that are common between aposymbiotic anemones and cultured *B. minutum* but seemingly absent from symbiotic anemones may indicate that these BVOCs, or their upstream precursors, are consumed in symbiosis, or by bacteria specific to symbiotic anemones prior to experimental detection. Alternatively, these BVOCs could be of bacterial origin, with the holobiont providing a less ideal environment to support bacterial growth and therefore bacterial BVOC synthesis. Due to the tight nutritional/metabolite exchange between cnidarians and dinoflagellates (Davy et al. 2012) there could be fewer metabolites, which support bacterial growth, leaking out of the holobiont (Morris, 2015). For example, amylene hydrate and 3,4-dimethyl-2-pentanone were detected in higher quantities in aposymbiotic anemones and cultures of Symbiodiniaceae than in the intact symbiosis, suggesting that one of these processes affected the detection of these molecules. These BVOCs are prime candidates for future functional studies, to better understand how metabolism changes with symbiotic state.

Symbiosis affects the cnidarian volatilome

Differentially abundant BVOCs between symbiotic states could represent a true alteration in the volatilome of one or both partners in response to association with the other, underlining the foundational ways in which symbiotic partners can influence one another. It could also be the case that products are consumed by the dinoflagellate symbiont or associated bacteria, resulting in a failure of detection in symbiotic anemones. Finally, BVOCs could change configuration prior to detection through reaction with other biologically produced airborne molecules (Kai et al., 2018), that may not be present in the absence of symbiosis. With the identification of key BVOCs that differ among symbiotic states, future functional studies should focus on unravelling these complex relationships.

Comparing the BVOCs of symbiotic *versus* aposymbiotic anemones allows us to infer the influence of symbiosis on the hosts volatilome. BVOCs produced by the holobiont could be produced by either partner independent of symbiotic state. Symbiosis has previously been shown to alter the host transcriptome in the symbiotic anemone *Anthopleura elegantissima* (Rodriguez-Lanetty et al., 2006), and proteome of Aiptasia (Oakley et al., 2016). Relative to aposymbiotic anemones, symbiotic anemones upregulate genes controlling lipid degradation, preventing apoptosis, and downregulate genes involved in antioxidant response and prevention of cell proliferation (Rodriguez-Lanetty et al., 2006). Likewise, symbiotic anemones show higher expression of proteins involved in lipid, nitrogen and carbon transport, intracellular trafficking and endocytosis, processes that reflect changes in host metabolism due to nutritional exchange with algal symbionts (Oakley et al., 2016). BVOCs detected in higher abundance in symbiotic anemones could be downstream products of these processes, or be end-products in themselves, serving to aid in the exchange of nutrients between host and symbiont.

Aposymbiotic anemones have been shown to produce increased proteases and chitinases, reflective of a shift towards heterotrophy in the aposymbiotic state, in addition to proteins involved in mediating reactive oxygen stress (Oakley et al., 2016). BVOCs observed in the aposymbiotic state could be breakdown products of these proteins, function in symbiont attraction, have antibacterial properties, or be a by-product of a more active immune system not suppressed by colonisation with symbionts (Detournay et al., 2012). For example, 1,3-dimethoxybenzene, used as an alarm substance produced by the terrestrial hexapod *Neanura muscorum* (Porco & Deharveng, 2007) was detected in higher quantities in aposymbiotic anemones relative to symbiotic anemones and could similarly be a product of stress in anemones that have failed to establish symbiosis.

The relatively minimal change in the cnidarian volatilome between symbiotic states is particularly notable, perhaps reflecting host-symbiont compatibility during symbiosis establishment. Indeed, during establishment of a successful symbiosis, it has been shown that the host immune system is modulated and there are few signs of cellular stress in the host (Zamioudis and Pieterse, 2012, Matthews et al., 2017). The mechanisms underlying successful inter-partner integration and communication in the cnidarian-dinoflagellate symbiosis are still unclear (Davy et al., 2012; Poole et al., 2016; Neubauer et al., 2017, Rosset et al., 2021), but our data are consistent with the symbiont successfully evading host detection and/or a high degree of host-symbiont cellular integration. In future, it will be interesting to compare our observations with the volatilome that arises during colonisation with less compatible symbionts, which can induce cellular stress and even host mortality (Starzak et al., 2014; Matthews et al., 2017; Tortorelli et al., 2020).

Symbiosis affects the *B. minutum* volatilome

Comparing the volatilome of cultured symbionts *versus* symbiotic anemones is important for identifying the ways in which symbiosis alters metabolism of the symbiont and/or how the host metabolises compounds synthesised by the symbiont. Specifically, BVOCs in higher quantities in cultured *B. minutum* relative to the holobiont may be suppressed in symbiosis, consumed by the host, or modified prior to release into the environment. BVOCs detected in symbiotic anemones but not in cultured *B. minutum* could represent compounds produced by the host, or compounds produced by the symbiont in response to being in symbiosis. When making these comparisons, however, it is important to recognise the limitations of working with cultured dinoflagellate symbionts (Maruyama and Weis, 2021). For example, bacteria associated with algal culture medium could differ in terms of both abundance and composition from those associated with the intact symbiosis, once again impacting the volatilome, given that bacteria are also prolific producers of BVOCs (Netzker et al., 2020; Ping and Boland, 2004; Lawson et al., 2020). Furthermore, previous studies have highlighted important physiological differences that arise due to nutrient availability, independent of symbiotic

state in Symbiodiniaceae. For example, C/N ratios of Symbiodiniaceae *in hospite* (11.5) are more like Symbiodiniaceae cultured in N-deprived medium (13.5) compared to those cultured in N-replete medium (3.3) (Xiang et al., 2020). Likewise, transcripts associated with N-acquisition are upregulated in *in hospite* Symbiodiniaceae and N-deprived cultured Symbiodiniaceae, but not in Symbiodiniaceae cultured in N-replete medium (Xiang et al., 2020). Considering this, the observed differences in volatilomes between Symbiodiniaceae and Aiptasia cultures could be a product of physiological differences due to nutrient availability, rather than differences in symbiotic state alone. Nevertheless, this approach still has the potential to inform our functional understanding of the cnidarian–dinoflagellate symbiosis. For example, cyclopentanone levels increased in cultured *B. minutum* relative to symbiotic anemones. Cyclopentanone is a precursor to jasmonic acid, which plays a role in the response to environmental stresses in plants (León and Sánchez-Serrano, 1998). Should this BVOC be transferred from dinoflagellate to cnidarian host, a protective benefit could be provided to the host through symbiotic association.

Our observation that the volatilome of *B. minutum* may potentially be altered more dramatically in response to symbiosis than that of the host Aiptasia, could reflect a shift in Symbiodiniaceae metabolism between cultured and symbiotic states. As free-living cells, Symbiodiniaceae are motile with two flagella and a characteristic gymnodinioid shape (Trench and Blank, 1987), while in symbiosis, their cells are coccoid, lack flagella, and are larger, with smaller plastids and thinner cell walls (Trench and Blank, 1987; Pasaribu et al., 2015). Moreover, it is known from physiological and 'omics' studies, that symbiosis induces an increase in symbiont metabolism, and metabolite transport and release (Trench, 1971; Maor-Landaw et al., 2020), with some of these released metabolites being linked to host-symbiont recognition (Wood-Charlson et al., 2006). Recent molecular studies have highlighted how genes associated with various other processes are down-regulated in the symbiotic state, including those involved in stress responses and immune regulation (Mohamed et al., 2020; Yuyama et al., 2021); these latter observations are consistent with the more stable environment provided by the intracellular habitat. When in symbiosis, there is more tissue separating the symbiont cells from the external environment, and thus more tissue through which algal-derived BVOCs must pass before release into the environment. This may provide more opportunity for BVOC modification by other metabolites as has been observed in bacteria, where BVOCs can be synthesised through the non-enzymatic combination of volatile precursors to produce a novel BVOC (Kai et al., 2018).

Conclusion

The marine environment is known to be an important source of BVOCs, however the diversity of BVOC production, and its relationship to symbiosis, has only begun to be understood. Here we have

demonstrated not only that the Aiptasia model system produces a diverse array of BVOCs, but that these volatilomes vary with symbiotic state, providing insight into the ways in which metabolism changes in response to symbiotic state. Relative to the intact symbiosis, the volatilome of the cultured symbiont *B. minutum* was more distinct than that of the anemone host in isolation. While this may be indicative of a more dramatic adjustment of algal physiology *versus* host physiology in response to symbiosis, it is important to resolve the possible conflating role of different nutrient and bacterial loads in algal cultures relative to Aiptasia stocks. Ultimately, however, the extension of volatilomics to this model system will facilitate more powerful studies of the cnidarian–dinoflagellate mutualism using a non-invasive technique and provide a platform for studying the role of BVOCs in reef-building corals.

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Figures

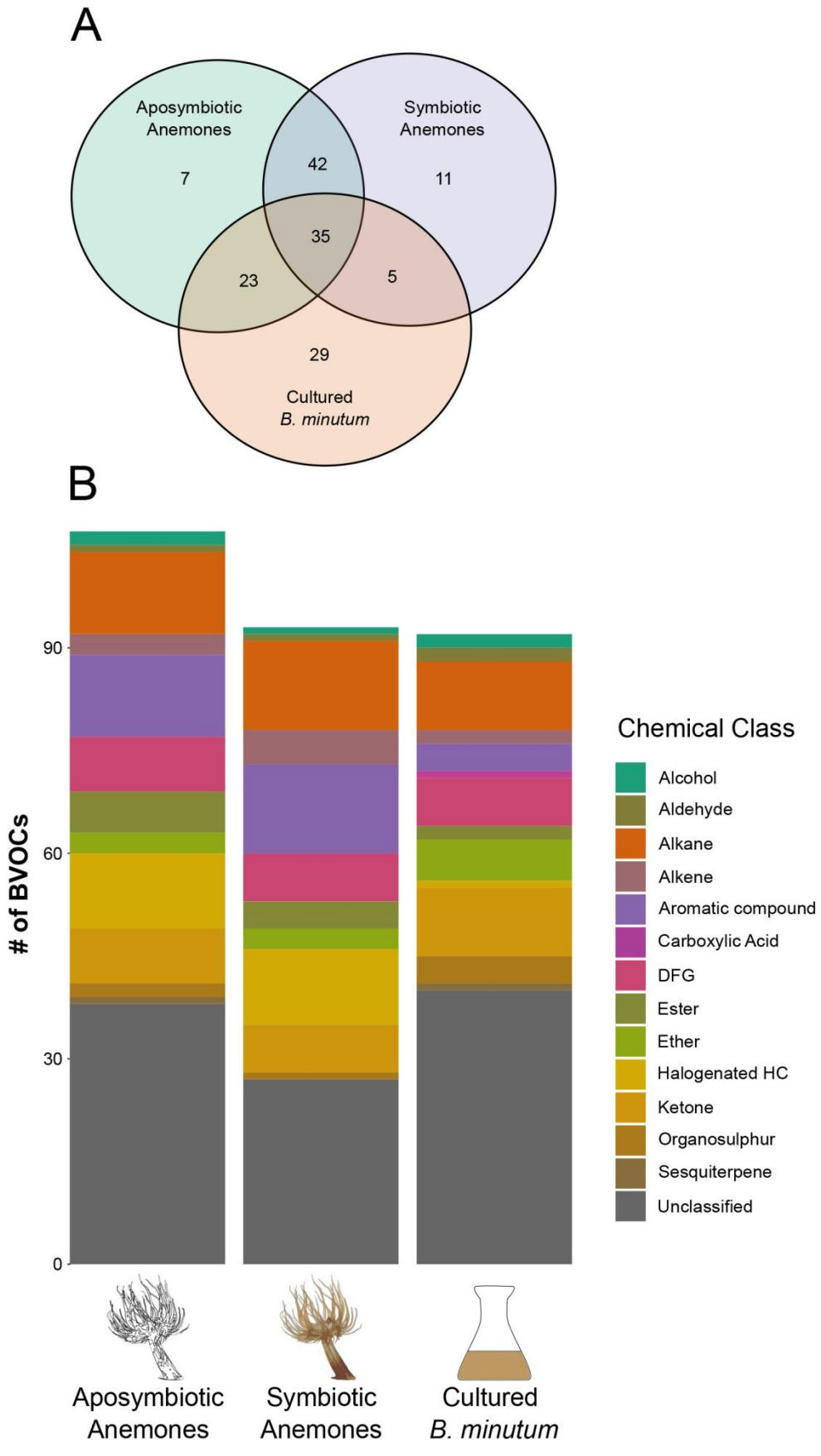

Fig. 1. Comparison of BVOC diversity among three symbiotic states. A) BVOCs detected in each symbiotic state; B) Number of BVOCs present in each state, grouped based on their chemical class. BVOCs had to be present in at least two biological replicates in at least one of three states (aposymbiotic anemones (n = 5), symbiotic anemones (n = 4), cultures of *B. minutum* (n = 5)) to be included. For differentially abundant BVOCs, see Figure 3.

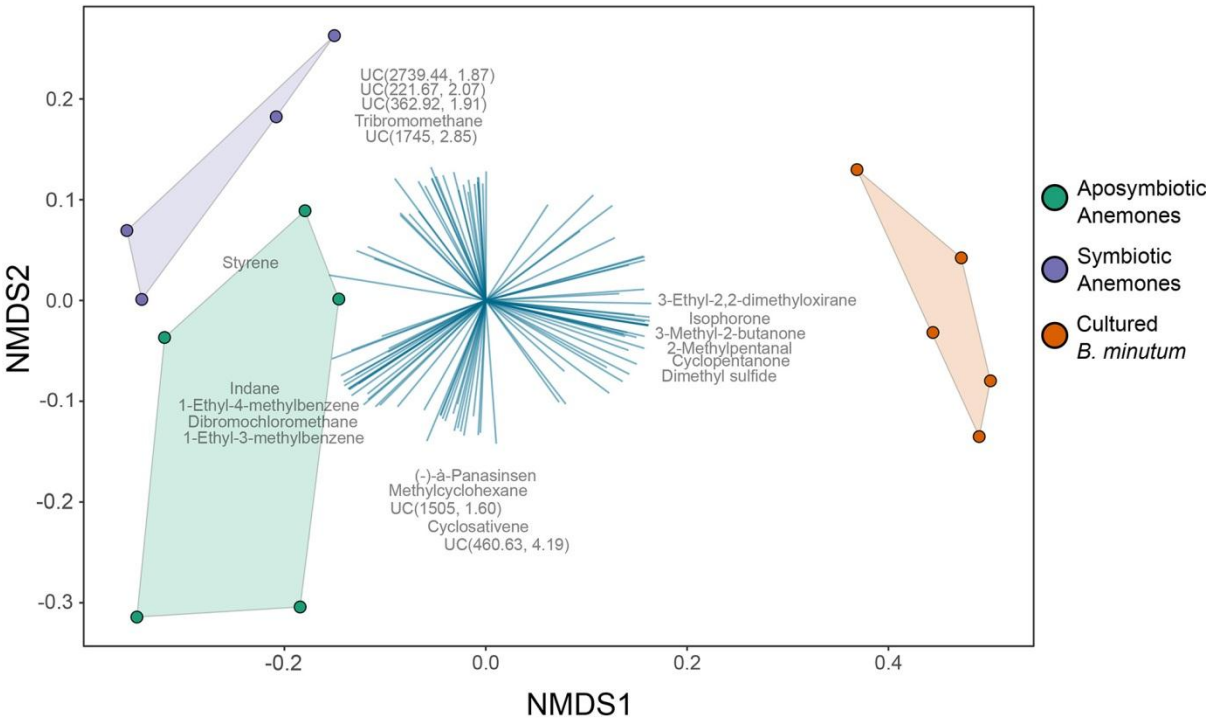

Fig. 2. Non-metric multidimensional scaling (stress = 0.058) plot of BVOCs produced by the Aiptasia model system. Plotted using the *vegan* package in R. BVOCs had to be present in at least two replicates in at least one of three states: (aposymbiotic anemones (n = 5), symbiotic anemones (n = 4) or cultures of *B. minutum* (n = 5)) to be included. Displayed BVOCs were chosen based on the top 5 loading scores for each NMDS dimension.

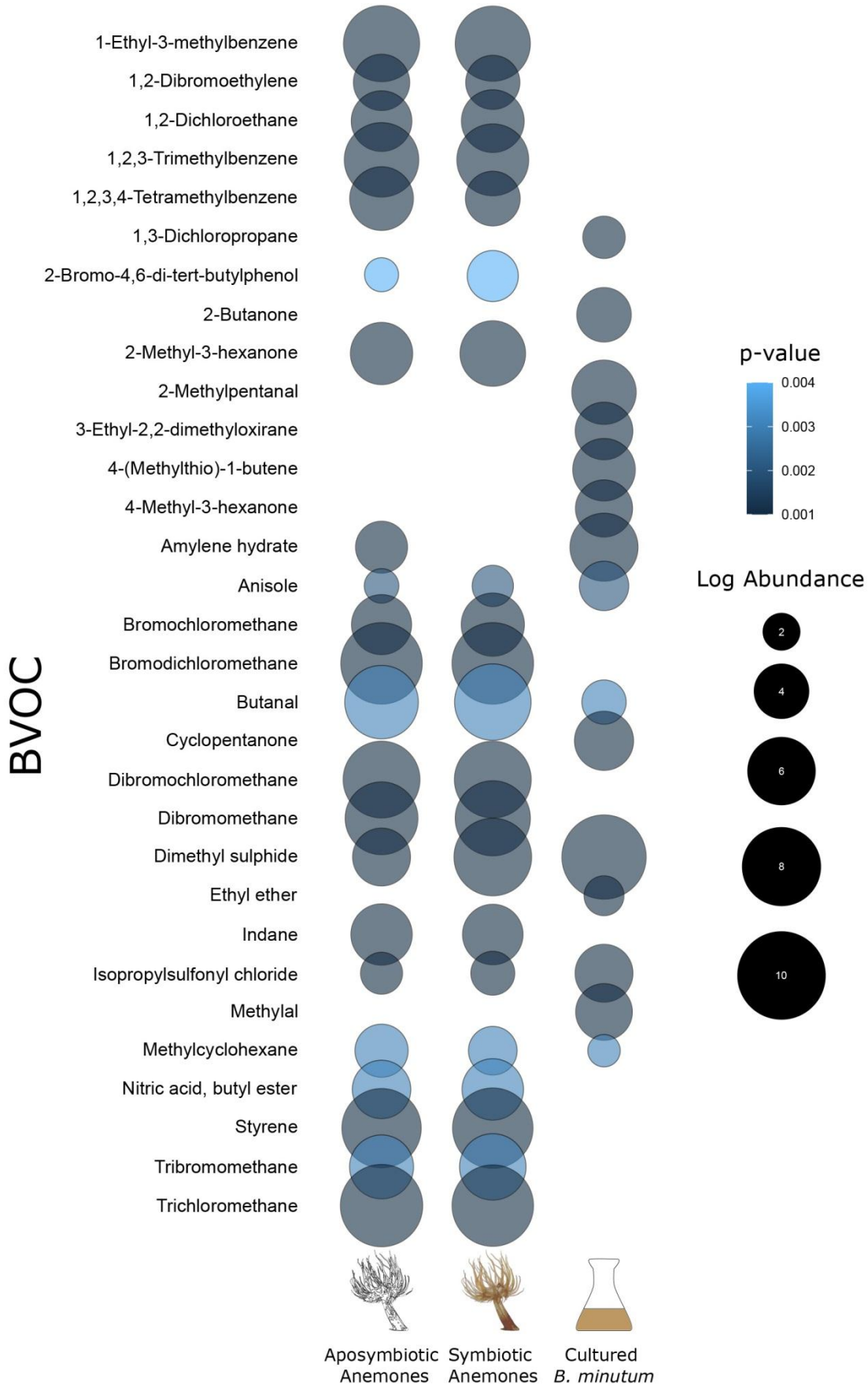

Fig. 3. Differentially abundant BVOCs detected from aposymbiotic anemones, symbiotic anemones and cultures of *B. minutum*. Bubble size is based on the log of the normalised BVOC peak area. Bubble colour is based on p-value (differential abundance testing). Only BVOCs that were tentatively identified and changed significantly among treatments are shown; unidentified BVOCs were not included in this plot. BVOCs had to be detected in at least two biological replicates in at least one of three states (aposymbiotic anemones (n = 5), symbiotic anemones (n = 4) and cultures of *B. minutum* (n = 5)) to be included.

Table S1. Fv/Fm values for *B. minutum* cultures. Cultures were dark-adapted for 15 minutes, prior to measurement. Values were collected using an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany; settings: measuring light = 4, saturation intensity = 8, saturation width = 0.8 s, gain = 3, damping = 3).

Biological replicate	Fv/Fm
1	0.563
2	0.557
3	0.580
4	0.567
5	0.597

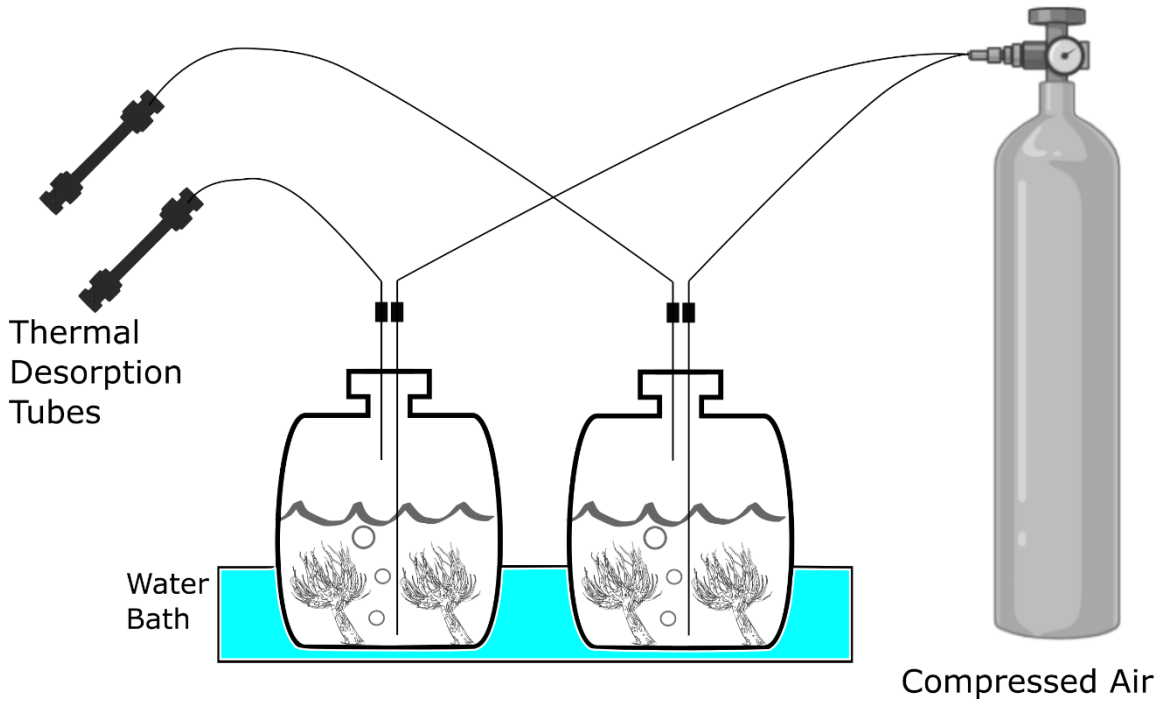

Fig. S1. BVOC sampling setup. Anemones (approx. 10 *per* vial) settled in sampling vials 24 h prior to BVOC sampling. BVOCs were collected under growth conditions in gas tight chambers over 20 min, during which time outgoing instrument-grade air (BOC Gases, Wellington, NZ) was passed through and BVOCs trapped onto the stationary phase inside Markes thermal desorption tubes (Tenax TA).

Table S2. BVOCs detected throughout dataset. All BVOCs (peak normalised to protein content) and their chemical classes that were detected in aposymbiotic anemones, symbiotic anemones and cultures of *B. minutum*. Compounds had to be detected in at least two replicates in at least one symbiotic state. Chemical class was determined based on the molecule's functional group(s). Significance was determined using differential abundance testing and the number of asterisks denotes the size of the adjusted p-value: *<0.05, **<0.01, ***<0.001.

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