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Amphipod diversity and metabolomics of the Antarctic sponge *Dendrilla antarctica*

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Abstract: The western Antarctic Peninsula harbours a diverse benthic marine community where dense canopies of macroalgae can dominate the shallow subtidal zone (0–40 m or greater). In the lower portion of this range (below 25–35 m depending on topography), invertebrates such as sponges and echinoderms can be found in greater abundance due to reduced competition for space from the algal species. *Dendrilla antarctica* (previously *Dendrilla membranosa*) is a common demosponge that thrives in both communities and is known for producing diterpene secondary metabolites as a defence against sympatric sea star and amphipod predators. Omnivorous mesograzers such as amphipods inhabit both communities; however, they are in greatest abundance within the macroalgal canopy. Due to the differences between habitats, it was hypothesized that specific amphipod species not susceptible to the defensive metabolites of *D. antarctica* would take refuge from predators in the chemically defended sponge. Analysis of the metabolome and amphipod communities from sponges in both habitats found correlations of metabolic profile to both abundance and habitat. These studies serve to inform our understanding of the complex ecosystem of the Antarctic benthos that stands to be dramatically altered by the rapidly changing climate in the years to come.

Introduction

Antarctic marine invertebrates produce a wide range of secondary metabolites, consistent with the hypothesis that their ecology is driven as much by interspecific interaction as it is by the harsh polar environment (McClintock *et al.* 2010, von Salm *et al.* 2019). The ecological consequences of secondary metabolites on, for example, predation pressure (Wilson *et al.* 2013) and spatial variability (Young *et al.* 2013) have been described in recent studies on the western Antarctic Peninsula. Such studies in other systems demonstrate that ecological pressures can transform the metabolism of chemically defended marine organisms including algae (Paul & Vanalstyne 1988, Van Alstyne 1988, Amade & Lemee 1998, Matlock *et al.* 1999, Wright *et al.* 2000), bryozoans (Mendola 2003, Marti *et al.* 2005), cnidarians (Harvell *et al.* 1993, Maida *et al.* 1993, Kelman *et al.* 2000, Slattery *et al.* 2001), tunicates (Lopez-Legentil *et al.* 2005, Marti *et al.* 2005) and

sponges (Thompson *et al.* 1987, Page *et al.* 2005, Rohde *et al.* 2012, Pawlik *et al.* 2013). For example, transplanting the sponge *Rhopaloeides odorabile* to various depths and locations on the Great Barrier Reef demonstrated higher diterpene content only in shallow habitats (Thompson *et al.* 1987). *Stylissa massa*, a chemically defended Indo-Pacific sponge, exhibited intraspecific chemical diversity due to temporal and spatial criteria, though predation had no influence on secondary metabolite concentrations (Rohde *et al.* 2012). The Antarctic sponge *Dendrilla antarctica* (family Darwinellidae, order Dendroceratida; previously *Dendrilla membranosa*) is chemically rich and widely distributed on the western Antarctic Peninsula (Shilling *et al.* 2020), raising questions regarding how variable amphipod predation pressure might structure the secondary metabolome. Signs of predation towards *D. antarctica* are rare (Dayton *et al.* 1974), and collections from around the continent have afforded multiple diterpenoids (Fig. 1; Molinski & Faulkner 1989, Baker *et al.* 1995, Fontana
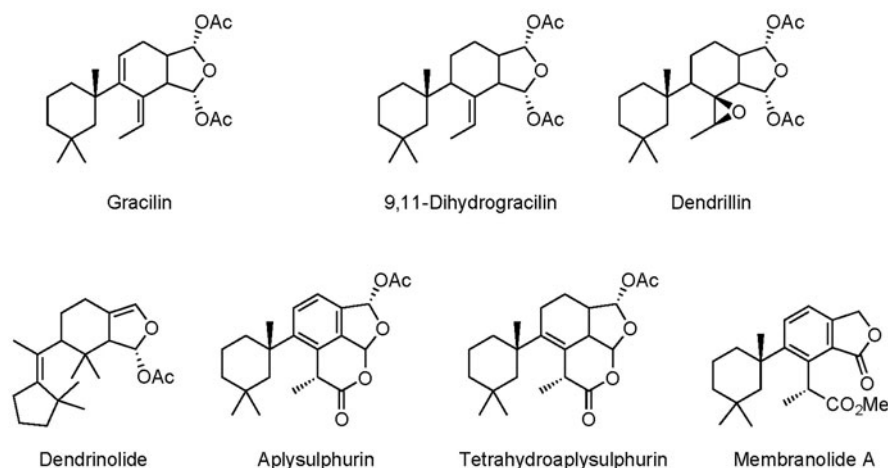
Fig. 1. Diterpenes reported from *Dendrilla antarctica.*
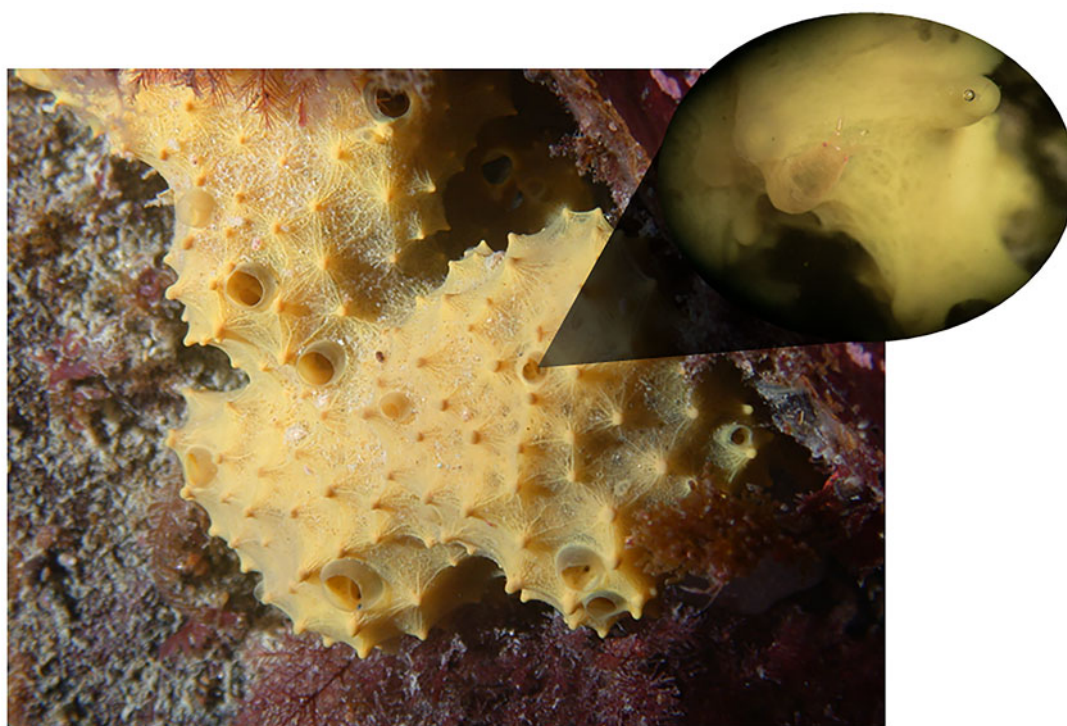
Fig. 2. Image of *Dendrilla antarctica* and *Colomastix fissilingua*. *D. antarctica* (left) has a highly porous inner structure, which allows high densities of amphipods (top right) to inhabit the sponge.

et al. 1997, Ankisetty *et al.* 2004). Methanolic extracts of *D. antarctica* containing 1-methyladenine and 3-methyladenine showed tube-foot retraction activity towards the spongivorous sea star *Perknaster fuscus* (Baker *et al.* 1995), and lipophilic extracts of *D. antarctica* displayed significant feeding deterrence towards the omnivorous amphipod *Gondogeneia antarctica* (Amsler *et al.* 2009). In addition, broad-spectrum antibiotic activity was noted for various membranoids against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (Molinski & Faulkner 1989, Ankisetty *et al.* 2004, von Salm *et al.* 2016, Bory *et al.* 2020, Shilling *et al.* 2020). The defensive nature of these secondary metabolites constitutes an ecological advantage for *D. antarctica* and the potential for environmental changes to alter their production to what may be optimal in specific conditions.

Along the western Antarctic Peninsula, a subtidal macroalgal canopy supports high densities of mesograzers engaged with the algae in a community-wide mutualism of chemical defence, refuge and ecological support

Fig. 3. Map of the Palmer Station boating area. The four collection sites shown are within a 3.5 km (2 mi) radius from the station: 1) Norsel Point, 2) Bonaparte Point, 3) Gamage Point and 4) Laggard Island all surround Palmer Station (located next to Gamage Point or site 3), Anvers Island, Antarctica.

services (Jazdzewski *et al.* 1991, Kunzmann 1996, Amsler *et al.* 2014). While the macroalgae can extend to 40 m depth and below (Wiencke *et al.* 2014), where the underwater topography becomes vertical or near-vertical, below ~25 m macroalgae are lower in abundance, with sessile invertebrates dominating. The western Antarctic Peninsula harbours amphipod mesograzers that can reach densities of 308,000 m² in near-shore communities and can be significant consumers (Huang *et al.* 2007, Amsler *et al.* 2008, 2014). The macroalgal canopy offers amphipods a site for reproduction, habitat and direct and indirect nutrition (Biernbaum 1981, Lorz & De Broyer 2004, Huang *et al.* 2008, Amsler *et al.* 2014), leading to a diverse amphipod community (Huang *et al.* 2007). Gut

content analysis performed on amphipods reveals an eclectic diet of sponges, filamentous and unicellular algae, macroalgae and bryozoans, with highly variable feeding habits from omnivory to specialization and suspension feeding to grazing (Dayton *et al.* 1974, Coleman 1991, Jazdzewski *et al.* 1991, De Broyer & Jazdzewski 1996, Kunzmann 1996, Iken *et al.* 1998, Lippert *et al.* 2001, Takeuchi & Watanabe 2002, Huang *et al.* 2006, Amsler *et al.* 2009, Aumack *et al.* 2017). In the circalittoral zone, which is below the macroalgal canopy zone, the diversity of amphipod species is lower, and a sponge-rich benthic community under biological mediation (Dayton *et al.* 1970, McClintock 1987, Cattaneo-Vietti *et al.* 1999, Cerrano *et al.* 2000, McClintock *et al.* 2005) supports

chemically rich species (Lebar *et al.* 2007, Avila *et al.* 2008, Soldatou & Baker 2017).

For omnivorous or spongivorous amphipods, the macroalgal canopy of the western Antarctic Peninsula provides refuge from fish predation (Zamzow *et al.* 2010) but at the same time exposes sponges in that habitat to higher densities of potentially predatory amphipods. The highly branched inner structure of *D. antarctica* creates a complex network for amphipods to inhabit (Fig. 2). This in turn leads to high abundances of amphipods on and within the sponge (McClintock *et al.* 2005, Amsler *et al.* 2009). Omnivorous amphipods associated with sponges occupying the more exposed habitat that exists at depths below the algal canopy might be expected to be less common than those associated with sponges found in the algal canopy due to the lack of refugia provided by macroalgal cover. We were interested to study whether sponges can modulate their chemical defences in response to predator density.

We hypothesized that *D. antarctica* found in shallower, amphipod-rich waters with increased ecological competition would produce distinct secondary metabolites from specimens found below the algal canopy zone using metabolomics techniques (Kuhlisch & Pohnert 2015). This methodology is highly useful for the study of natural products with the potential to distinguish phenotypes and provide insights into the biological processes involved in environmental responses and genetic modifications (Boccard *et al.* 2010). Our study design utilized specimens of *D. antarctica* from two distinct habitats - that within (W-habitat) the algal canopy and that at depths below (B-habitat) the canopy - from four distinct sites near Palmer Station, Antarctica. Individual specimens were subject to metabolomic profiling using liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/QToF-MS) and assessment of amphipod distribution.

We found limited correlations between habitat and the three major secondary metabolites of *D. antarctica*: aplysulphurin, tetrahydroaplysulphurin and membranolide. However, multidimensional scaling (MDS) plots used to visualize full metabolomic patterns of individual sponges, which included these three diterpenoids in addition to other terpenes and secondary metabolites produced by the sponges, were able to distinguish the two habitats. Analysis of similarities (ANOSIM), similarity percentage (SIMPER) and biota and/or environment matching (BIOENV) were used to correlate the statistical relevance of site, habitat and amphipod density based on the untargeted metabolomic fingerprints.

Materials and methods

Habitat and site description

Four sampling sites were chosen within a 3.5 km boating radius from Palmer Station, including 1) Norsel Point (64°45.674'S, 64°05.467'W), 2) Bonaparte Point (64°46.748'S, 64°02.542'W), 3) Gamage Point (64°46.345'S, 64°02.915'W) and 4) Laggard Island (64°48.568'S, 64 00.984'W) (Fig. 3). Site selection was based on the presence of *D. antarctica* specimens within the two distinct habitats of interest: the shallow, macroalgal-dominated (W-habitat) depths and at deeper depths below (B-habitat) where macroalgae provided sufficient abundance to form a canopy. Generally, the two habitats were found at depths of < 20 and > 25 m, respectively.

Biological specimens

Twenty sponge specimens with associated amphipods were collected from four sites by scuba. Specimens were taken in triplicate from both habitats from each site with the exception of Laggard Island, where only one specimen from each habitat was found. Fine-mesh collecting bags were used to capture amphipods associated with each specimen in the manner of Huang *et al.* (2008). Additional bulk *D. antarctica* samples were collected to produce chemical standards in support of quantification in study specimens. Sponge samples were frozen and transported back to the University of South Florida at -70°C, where tissues were lyophilized and stored at -80°C until further processing.

Larger amphipod species were separated from individual sponges by gentle agitation and sorted into species-specific bins. Amphipods hiding within sponge pores and canals (e.g. *Colomastix fissilingua*) were quantified under a microscope. Amphipod and algal identification were based on the methods of our previous studies of the local taxa and taxonomic keys (Huang *et al.* 2007, Amsler *et al.* 2009). Amphipods were identified to species level where possible, although in one case (Oedicerotidae) only identification to the family level was possible.

Isolation and characterization of the diterpene standards

From the bulk collection, 25.7 g of freeze-dried *D. antarctica* were extracted thrice with dichloromethane, then combined extracts were filtered and then concentrated *in vacuo*. The lipophilic extract (994 mg) was absorbed onto Waters Sep-Pak® C18 cartridges and eluted with acetonitrile. The dried material (205 mg) was separated by isocratic high-performance liquid chromatography (HPLC) using 60% acetonitrile in water on a Phenomenex Luna C18 column (250 × 10 mm, 5 μm) to afford aplysulphurin (10.2 mg), tetrahydroaplysulphurin (1.5 mg) and membranolide (8.7 mg). Compounds were analysed using ^{1}H nuclear magnetic resonance (NMR; CDCl$_3$, 500 MHz Varian DirectDrive spectrometer equipped with a cold probe) spectroscopy and electrospray ionization (ESI) mass spectrometry (Agilent 6540 LC/QToF). Structures assigned

were based on comparison to their published data (Karuso *et al.* 1986, Molinski & Faulkner 1989).

Metabolomic analysis

Following a similar procedure to that used for the isolation of natural product standards, study specimens were extracted with dichloromethane and subjected to C18 solid-phase extraction. The dried eluent was then resuspended at 1.0 mg ml^{-1} in acetonitrile for analysis on an Agilent 6540 LC/QToF-MS with ESI in positive mode. Separation was achieved on a Phenomenex Kinetex® C18 column (50 × 2.1 mm, 2.6 µm) using water with 0.1% formic acid as mobile phase (A) and acetonitrile with 0.1% formic acid as mobile phase (B). A binary gradient was employed ramping from 40% to 60% (B) over 3 min, 60% (B) isocratic for 4 min, increased to 60–100% (B) over 3 min and finally held at 100% (B) for 2 min. The source was maintained at 300°C and a capillary voltage of 3500 V. Nitrogen was used as the drying gas (8 l min^{-1}) and sheath gas (11 l min^{-1}) at temperatures of 300°C and 350°C, respectively. Aplysulphurin, tetrahydroaplysulphurin and membranolide were quantified as external standards with concentration curves (data not shown). All samples were analysed in triplicate with injection volumes of 2 µl.

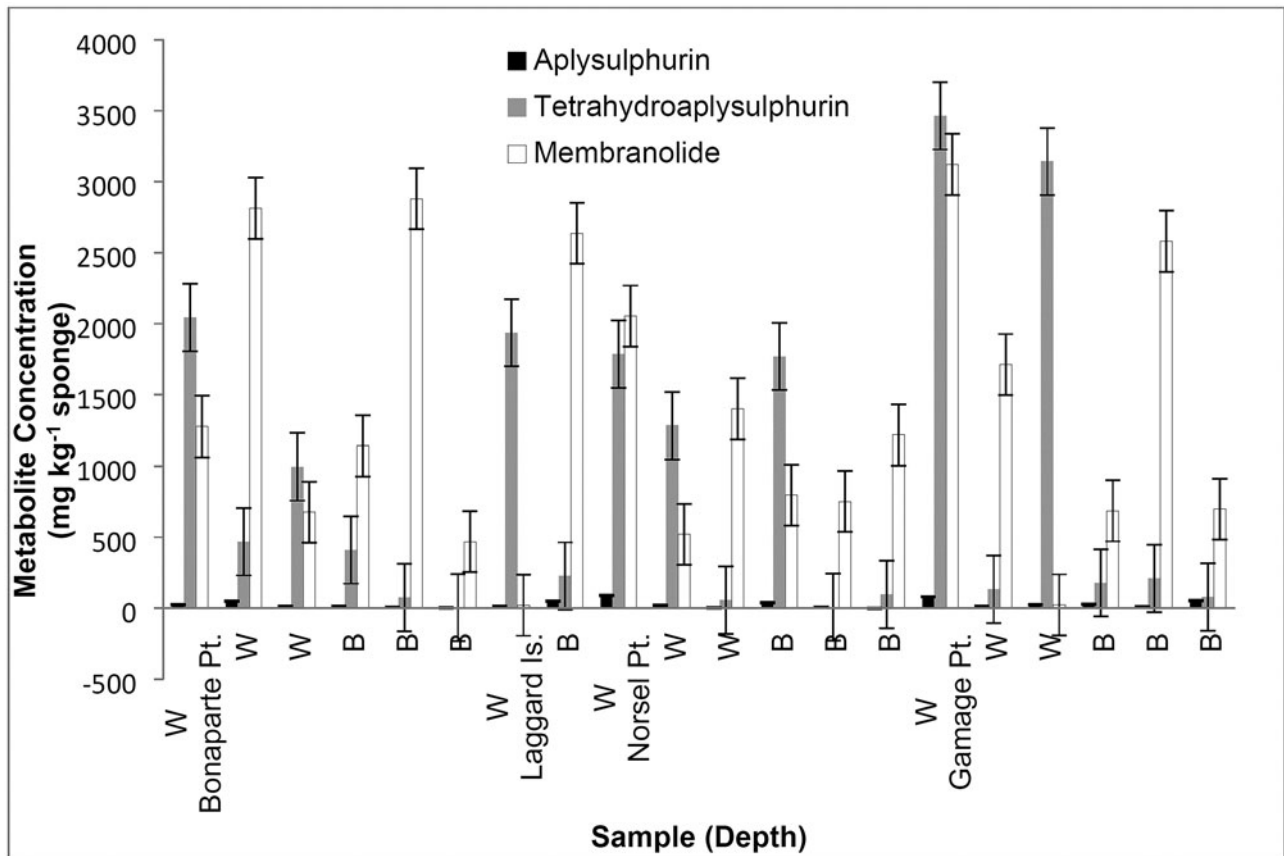## Statistical analysis of the metabolomic data

The top 100 compounds as determined by peak area (including known secondary metabolites) in each sponge extract were identified in the total ion chromatogram by molecular feature extraction using *MassHunter Qualitative Analysis 5.0*. *Mass Profiler Professional 11.0* was used to classify entities by interpretation, then each entity was normalized to the crude extract mass. Blanks consisting of dried dichloromethane and acetonitrile were used to subtract solvent impurities from sample fingerprints. Compounds that were present in two of three replicates were included in the final entity list.

The entity lists for each sample were imported into *PRIMER-E v6* following the recommendations of Clarke *et al.* (2014). Chemical data were square-root transformed along with the amphipod abundance numbers, which were standardized to sponge volume by measuring displacement in seawater. A Bray-Curtis resemblance matrix was created for the top 100 compounds. To assess the validity of the chemical data influencing habitat and site, ANOSIM and SIMPER analyses were employed. Amphipod counts included only those amphipods that were deemed to be omnivorous and therefore potential predators, as well as those shown to be statistically correlated with the chemical entity data as per the BIOENV procedure as part of the BEST routine. The analysis of that matrix

Table I. Amphipods found on sponges collected from habitats within (W; 0–20 m) the algal canopy and at depths below (B; 20–35 m) the algal canopy zone. Potential sponge-predator amphipods are shaded in grey.

Location	Depth	Sponge wet mass (g)	Antarctogeneia macrodactyla	Atyloella magellanica	Atylopsis marginatus	Atylopsis orthodactyla	Atylopsis sp.	Colomastix fissilingua	Eusirus antarcticus	Gondogeneia antarctica	Liljeborgia sp.	Leucothoe spinicarpa
Bonaparte	B	2.88						8				
	B	41.1						48				
	B	41.2			2			57		2		
	W	19.3		1				41				
	W	3.4	1					8				
	W	44.1	9	2				140		1	2	
North	B	17.5						68				
Laggard	W	55.1						61		2		
Alan's Wall	B	52.7	6					54	1			
	B	35.9						237				
	B	86.9	7	1			1	348				
	W	75.8						42				1
	W	137	3	18		1		514				
	W	155	26		9			291				
Gamage Point	B	13.5	2		7			23				
	B	32.8			5			36				
	B	2.0	2		2			4				
	W	30.7		1	4			62				
	W	17.6			7							
	W	21.2						11		1		

Fig. 4. Quantification of *Dendrilla antarctica* diterpene metabolites. Variability in the concentrations of known secondary metabolites from *D. antarctica* found within (W) and below (B) the algal canopy from the four study sites is shown.

was plotted with dendrograms and MDS; the amphipod counts were overlaid directly as 2D bubbled factors on the MDS plot.

Results

Biological specimens

A total of approximately 2700 amphipods present on the 20 sponge specimens were characterized. Qualitatively, it was very obvious that the shallower sponge specimens contained larger amphipods in higher quantities with more diversity present. A total of 22 different taxa were identified, most to species level, with six of these being statistically correlated via the BIOENV procedure and omnivorous or potential predators (Table I, shaded in grey). Ischyroceridae found on the specimens were not included in the analysis as these species are considered filter feeders and non-predatory to *Dendrilla antarctica*. Other presumptive non-predators in association with *D. antarctica* included copepods, isopods and micrograzing amphipods *Ausatelson* sp., *Gitanopsis squamosa*, *Proboloides* sp., *Probolisca ovata*, *Prothaumatelson nasutum* and *Thaumatelson hermani*.

Diterpene analysis

Three major diterpenes, membranolide, aplysulphurin and tetrahydroaplysulphurin, were identified in the sponge specimens in order to confirm their relative abundance and presence. Diterpene concentrations were highly variable among individuals, including biological replicates from the same habitat and site. The known amphipod predation deterrent membranolide varied among the 20 sponges between 0 and 3.2 mg kg^{-1} dry weight (DW) with an average concentration of 1.3 mg kg^{-1} DW (Fig. 4). Other diterpenes were generally less abundant: aplysulphurin was never present at > 0.1 mg kg^{-1} DW and tetrahydroaplysulphurin varied between 0 and 3.4 mg kg^{-1} DW but averaged only 0.9 mg kg^{-1} DW. None of the secondary metabolites had significant variance between sites; however, tetrahydroaplysulphurin was produced in significantly higher concentrations within the algal canopy than below it (unpaired *t*-test, $P < 0.05$). LC/MS data showed that sponges within the algal canopy (W-habitat) produced more of the known secondary metabolites aplysulphurin, tetrahydroaplysulphurin and membranolide. The sponges below the algal canopy (B-habitat) showed greater chemical diversity in their metabolite profile.
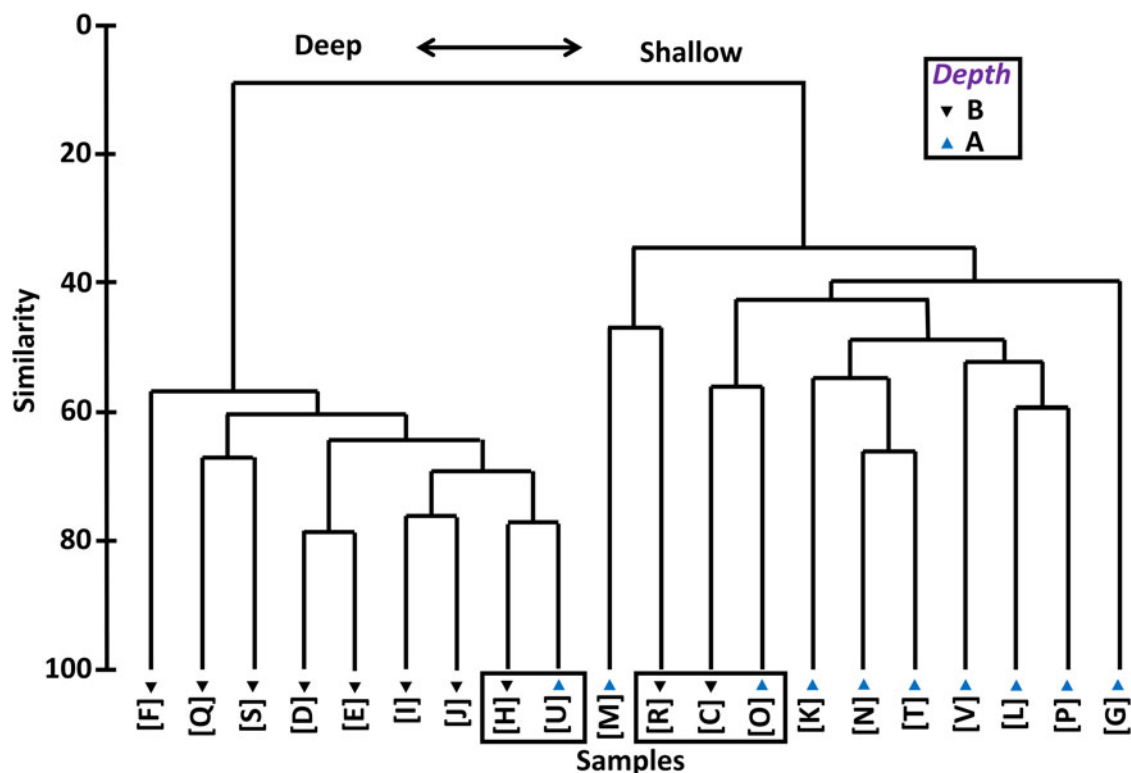
Fig. 5. Dendrogram cluster analysis of the top 100 compounds from *Dendrilla antarctica.* The samples (lettered) from depths represented as within the canopy region (blue triangles) and those from depths below the canopy region (black rotated triangles) are depicted. Similarity is measured as a percentage.

Statistical analysis

Cluster analyses of metabolomic data (Fig. 4) show a clear distinction between sponges from the two habitats under consideration. The deeper-occurring sponges (B-habitat) show a 60% similarity, while the shallower sponges in the canopy region (W-habitat) display 30% similarity according to the Bray-Curtis analysis. A SIMPER test showed an average dissimilarity of 80% between the metabolomic fingerprints of the two habitats, providing evidence of how greatly chemical profiles differ among the sponges found in these two environments. The rejection or applicability of the null hypothesis pertaining to metabolite abundance *vs* habitat, site and amphipod density was analysed via one- and two-way ANOSIM. A global R of ∼0 ($R = |0.07|$) for site specificity against secondary metabolite abundance shows that this is not a statistically significant factor. A global R of 0.57 for habitat against compound abundance displays overlap; however, it remains statistically significant and rejects the null hypothesis. A lone outlier in the shallower habitat at site 3 (U) clustered with sponges below the algal canopy (left of centre of Fig. 5); however, a leathery morphology and lack of associated amphipods could indicate a diseased

or older sponge (J.L. von Salm & M.O. Amsler, personal observations 2011). This specimen had a similar chromatographic profile and clustered well with a B-habitat sponge collected at site 1 (data point H), also displaying a similar non-porous morphology. Two sponges from site 2 (data point C from the B-habitat and data point O from the W-habitat) are correlated by similar chromatographic profiles and nearly identical concentrations of tetrahydroaplysulphurin (0.41 and 0.47 mg kg^{-1} DW for data points C and O, respectively). Similarly, a site 3 specimen (data point R) had a significantly higher concentration of membranolide than its biological replicates, grouping it with other W-habitat, chemically defended sponges (data points M and O; Fig. 5).

A two-way ANOSIM of depth and amphipod densities as compared to secondary metabolite concentrations provided a global R of ∼0 similarity ($R = 0.07$) for amphipod significance against compound abundance. The null hypothesis therefore applies, so SIMPER analysis was used to determine whether significant amphipod species variability exists between the W-habitat and B-habitat, with an average dissimilarity of 34.8% being found. As neither ANOSIM nor SIMPER
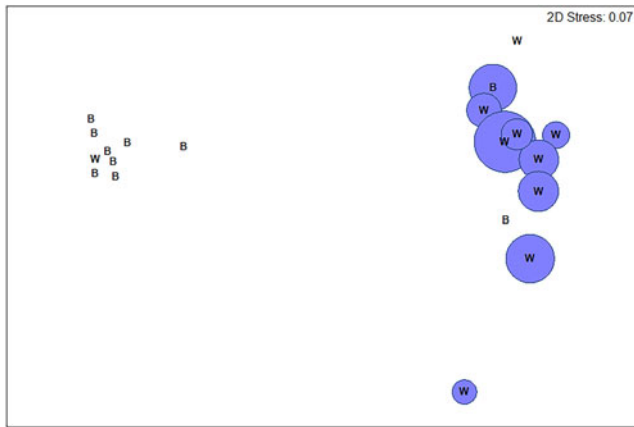
Fig. 6. 2D multidimensional scaling (MDS) ordination of the *Dendrilla antarctica* metabolomic profile. The MDS plot is overlaid with statistically correlated omnivorous amphipod densities (blue circles) showing a clear distinction between sponge specimens within (W) the algal canopy (right) and specimens found at depths below (B) the canopy zone (left).

analyses provided evidence that amphipods are a significant driving force for sponge secondary metabolism, the BIOENV procedure was performed on the whole amphipod community (amphipods, Table I) in order to assess which combination of amphipod species best describes the divergence within the chemical dataset. This test afforded seven statistically correlated amphipod taxa (*Atylopsis orthodactyla, G. antarctica, Liljeborgia* sp., Oedicerotidae, *Paradexamine fissicauda, Prostebbingia brevicornis* and *Prostebbingia gracilis*), of which all but Oedicerotidae and *P. fissicauda* were deemed potential spongivores. It was noted that the specimen from Bonaparte Point had a red alga, *Plocamium cartilagineum*, growing with the sponge tissue. *P. fissicauda* has a known preference for this algal species (Amsler *et al.* 2013); however, it is still considered a potential consumer of *D. antarctica*. These data are overlaid as bubble plots in the 2D MDS ordination (Fig. 6). Of the correlated amphipod species, those present only within the algal canopy (W-habitat) include *G. antarctica, Liljeborgia* sp. and *P. gracilis*, while *A. orthodactyla* and *P. brevicornis* were found only rarely below the canopy (B-habitat). No species were specific only to the B-habitat.

Discussion

Similarly to so many sessile organisms, sponges produce biologically active natural products in response to environmental threats such as predation and competition (Puglisi *et al.* 2014, 2019). The secondary metabolites of *D. antarctica* have been investigated for decades and, as a result, multiple oxidized diterpenes have been identified with a wide array of ecological and therapeutic

bioactivities (Molinski & Faulkner 1989, Fontana *et al.* 1997, Ankisetty *et al.* 2004, Bory *et al.* 2020, Shilling *et al.* 2020). Among the major sponge predators influencing these defences along the western Antarctic Peninsula are small arthropods known as amphipods, which have been shown to have a more prominent presence within the algal canopy (Jazdzewski *et al.* 1991, Amsler *et al.* 2009). The current study has described evidence that sponge proximity to predatory amphipod species influences the secondary metabolism in *D. antarctica*. Concomitant variability in amphipod predation pressure provides an explanation; however, other ecological factors were not investigated in this study.

Analysis of the metabolomic profiles of individual *D. antarctica* among collection sites suggested no obvious variability, thus ruling out site specificity. However, collections from the two habitats provided a clear distinction between the metabolites produced (Figs 5 & 6). The shallow region (W-habitat) contains several stressors to consider: ultraviolet (UV) light penetration, greater fouling potential, algal competition and higher amphipod densities. Some organisms produce compounds that protect the host against UV radiation (Pavia *et al.* 1997, Shick & Dunlap 2002) such as mycosporine-like amino acids (MAAs), which are found in many Antarctic invertebrates (Karentz *et al.* 1991). The presence of MAAs was not tested in this study, but previous work (McClintock & Karentz 1997) showed modest MAA concentrations (424 μg g^{-1} DW) in *D. antarctica* from McMurdo Sound. It should be noted that diterpenes from *D. antarctica* are not strong absorbers of UV light, so it is improbable that these compounds would play a role in photo-induced stress. In a region with greater algal abundance, some sponges could increase their defensive compounds to prevent fouling from algae, as was suggested for the Australian sponge *Rhopaloeides odorabile* (Thompson *et al.* 1987). Currently, the role of these secondary metabolites has not been tested as an inhibitor of algal fouling or other allelopathic interactions; therefore, the potential of such activity should not be overlooked. It has, however, been proven that extracts of *D. antarctica* deter the feeding behaviours of the sympatric spongivorous sea stars *P. fuscus* (McClintock *et al.* 1994, Baker *et al.* 1995) and *G. antarctica* (Amsler *et al.* 2009), where membranolide has been identified as a feeding deterrent of *G. antarctica*. Other diterpenoids discussed have not been specifically tested regarding their ability to deter predators; however, chemical similarities within the scaffold make this role probable.

In an effort to understand whether secondary metabolites correlate to habitat or amphipod abundance, the diterpene membranolide was quantified in individual sponges. The membranolide concentration was found to be highly variable, even among biological replicates from

the same site and habitat. This variability between individual sponges of the same species is not uncommon (Puyana *et al.* 2003, Rohde *et al.* 2012). We hypothesized that sponges within the algal canopy (W-habitat) would have higher concentrations of membranolide due to its feeding-deterrent properties; however, no distinct trend was observed. Membranolide was present in all sponges, and it is probable that *D. antarctica* produces membranolide constitutively rather than as a response to environmental pressure, such as amphipod predation. This provides evidence that other predator-specific or feeding-deterrent compounds may remain unidentified. Feeding-deterrent properties have been noted for extracts of the sponge (Amsler *et al.* 2009), but fractionation efforts have resulted in a loss of activity (Baker *et al.* 1995); therefore, mixtures of compounds could have a synergistic effect on deterrence or the limited quantity of the metabolites upon fractionation could reduce their activity. Variability in the production of tetrahydroaplysulphurin by *D. antarctica* provides evidence that this compound probably has an ecologically significant yet unknown role. Ecological factors within the algal canopy may warrant a shift in the secondary metabolism of shallower *D. antarctica* to focus more energy on the production of tetrahydroaplysulphurin.

Dendrogram cluster analysis of the chemical fingerprints using Bray-Curtis similarities showed a clear distinction between sponges in both habitats (Fig. 5). One of the most abundant amphipod species found to be associated with *D. antarctica* was *C. fissilingua* (Fig. 2 & Table I). Similarly to other amphipods and specifically *Colomastix* species (Gerovasileiou *et al.* 2016), *C. fissilingua* inhabits the sponge as a refuge from predation rather than preying on the sponge itself. However, data published from previous investigations show high associations of *C. fissilingua* with multiple chemically defended sponge species: *Artemisina* sp., *Clathria flabellata*, *Isodictya erinacea*, *Isodictya lankesteri* and *Lissodendoryx ramilobosa* (Amsler *et al.* 2009, Peters *et al.* 2010, Tripathi *et al.* 2018). Whether this is a result of physical ease of accessibility to the inner cavities of these sponges or specific chemical associations is yet to be determined.

The prominence of amphipods found on *D. antarctica* that lack directly established predatory roles meant that the BIOENV routine was required to reveal species found to correlate statistically with the secondary metabolite distribution of *D. antarctica* (Table I, shaded in grey). These species were found to be differentially distributed only on sponges from within the canopy (W-habitat). The relative densities of the six potentially spongivorous amphipods identified were overlaid (blue bubbles in Fig. 6) with the chemical fingerprints to reveal a correlation (stress = 0.07) between omnivorous amphipod predators and depth within and below the

algal canopy. Sponges associated with these six amphipod species plot in close proximity in the W-habitat (right of the MDS plot in Fig. 6), whereas the sponges from the B-habitat all cluster tightly with regards to their metabolome, and predatory amphipods are absent from these samples. Feeding-deterrent compounds probably play a role in the clustering of sponges based on their metabolite profile in the shallow, algal-dominated W-habitat, where sponges are more likely to be preyed upon; however, it should be noted that greater dissimilarity between the secondary metabolites produced is shown within the shallow W-habitat specimens compared with those from the B-habitat. The diversity and probably stressful environment in the W-habitat causes *D. antarctica* to modulate its metabolism accordingly, which is clearly established by the distinction in habitat observed in the MDS ordination (Figs 5 & 6). As the W-habitat specimens were the only sponges associated with the amphipod species identified by the BIOENV procedure, this provides further evidence that omnivorous amphipods are more prevalent within the macroalgal canopy and may influence the secondary metabolism of *D. antarctica* and other shallow invertebrates.

In similar studies near Palmer Station, the rhodophyte *P. cartilagineum* (Young *et al.* 2013, Shilling *et al.* 2021) and the nudibranch *Austrodoris kerguelenensis* (Wilson *et al.* 2013) have shown significant metabolic variations between specimens. The authors note that repeated glaciations can segregate regions, therefore causing genetic and chemical divergence among individuals of the same species (Diez-Vives *et al.* 2020). Although site specificity was not seen for this metabolomics investigation of *D. antarctica* and these findings are not believed to be associated with cryptic speciation, follow-up experiments with expanded locations show evidence of location-dependent chemodiversity for this species (Shilling *et al.* 2020). Along with the present work, this provides yet another example of metabolic divergence in the benthic community associated with the western Antarctic Peninsula. Similarly to *A. kerguelenensis*, predator or possibly allelopathic interactions seem to play a predominant role in the clustering of these organisms by metabolomics. The sponge microbiome may play a role in either the biological or metabolomic variability (Levia *et al.* 2019, Murray *et al.* 2020, Sacristan-Soriano *et al.* 2020).

Many hidden interactions regulate the ecology of competing organisms in Antarctic waters. We have now identified predation stress from amphipods as a potential factor influencing the metabolome of *D. antarctica*. The amphipod feeding deterrent membranolide is now shown to be produced only constitutively among sponge specimens and does not account for the metabolomic variability of *D. antarctica* in the habitat within and at depths below the macroalgal canopy. Tetrahydroaplysulphurin has instead

been identified as a major contributor to the metabolic variability between these depths. Competition at shallower depths due to sponge-algae interactions is also prominent in the phototropic zone; therefore, these and other interactions could be relevant to the observed chemical diversity in *D. antarctica*. It should not be overlooked that some of the most important factors for secondary metabolite production, such as temperature, salinity and pH, are not as constant in this environment as was once believed (Schram *et al.* 2015). The Antarctic Circumpolar Current provides cold, nutrient-rich waters that are essential to Antarctic marine life, and polar regions are particularly vulnerable to the effects of warming climates (Clarke *et al.* 2007). This delicate environment harbours unique species and biodiversity with > 4000 benthic macroinvertebrate species having been identified, which we have only just started to understand (Arntz *et al.* 1997, Clarke & Johnston 2003, Gutt *et al.* 2004, McClintock *et al.* 2005, 2010).

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Details of data deposit

Data are available at the United States Antarctic Program Data Center: https://www.usap-dc.org/view/project/p0010016.

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