REEF RESTORATION & ADAPTATION PROGRAM

Final report - metabolomic data on adult coral x Symbiodiniaceae pairings analysed and interpreted

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We specifically acknowledge and thank the following Traditional Owners of sea Country that this report relates to:

Location	**Traditional Owner Group**
Falcon Reef	Manbarra Sea Country

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1 Executive Summary

To assess if heat-evolved algal symbionts can improve coral thermotolerance, the coral *Platygyra daedalea* was chemically bleached and inoculated with heat-evolved algal symbionts (*Cladocopium proliferum* SS1, SS8) and wild-type algal symbionts (*C. proliferum* WT10) in a previous experiment and the performance of the experimental corals was tested under heat stress. To examine the biochemical consequence of symbiont association, the coral host and symbiont metabolite profiles were examined with mass spectrometry imaging. The analysis will help identify biomarkers and pathways that could be linked to coral resilience or vulnerability, which could be used for screening beneficial algal symbionts for reef restoration works. The coral host profiles showed little separation between experimental groups (i.e., corals inoculated with different symbionts). However, a temperature treatment effect was observed. The symbiont profiles showed a clearer separation between experimental groups, however, when the inoculum was used as a factor there was little group separation, likely because of varying abundances of the inoculum within individual corals. An analysis that characterised corals by their dominant symbiont (instead of presence of certain symbionts) confirmed this notion, as the corals dominated by native symbionts (C21) had significantly different metabolite profiles from those inoculated with *C. proliferum*. Nevertheless, the presence of secondary symbionts of lower abundance also affected the clustering, with SS8 clearly separating from WT10 in the under elevated temperature. Our findings indicate that a symbiont species may still influence the holobiont thermal stress response even when it co-occurs with other symbionts and when it is not dominant. This indicates that heat-tolerant symbionts introduced into corals to enhance coral holobiont bleaching tolerance do not necessarily need to achieve 100% abundance in corals to achieve this goal.

1.1 Background

Climate change is contributing to more frequent marine heatwaves, which present a growing challenge to reef-building corals by disrupting their relationship with algal symbionts. Rising seawater temperatures cause coral bleaching, where the breakdown of this symbiosis weakens coral health and survival. One promising approach to address this is to experimentally evolve coral algal symbionts under elevated temperature. At the Australian Institute of Marine Science (AIMS) this approach has been used to develop heat-evolved algal symbionts, which have been demonstrated to improve coral resilience under temperature stress without compromising growth. In a previous experiment, we used heat-evolved symbionts to inoculate chemically bleached fragments of the coral *Platygyra daedalea* collected from Falcon Reef in Manbarra Sea Country, Central Great Barrier Reef (permit number G12/35236.1). Briefly, four coral colonies were transferred to the National Sea Simulator at AIMS, split into fragments, chemically bleached using a menthol-diuron approach (described elsewhere, (Chan et al. 2023a)) to remove native symbionts, and then inoculated with cultured symbionts. The cultured symbionts were heat-evolved strains of either SCF055.01.01 (SS1), SCF055.01.08 (SS8), or a wildtype strain SCF055.01.10 (WT10) (Figure 1). Control fragments that were not subjected to chemical bleaching or inoculation were also included. Corals recovered from chemical bleaching for approximately three months, after which they were subjected to an approximately 4-degree heating week simulated stress event (ramping from 27 °C to 32.25 °C at a rate of 0.25 °C per day and holding at maximum temperature for 10 days (Figure 1), at which point corals were sampled for spatial metabolomic analysis. Corals retained at ambient temperatures (27 °C) for the duration of the experiment were also sampled as temperature controls.

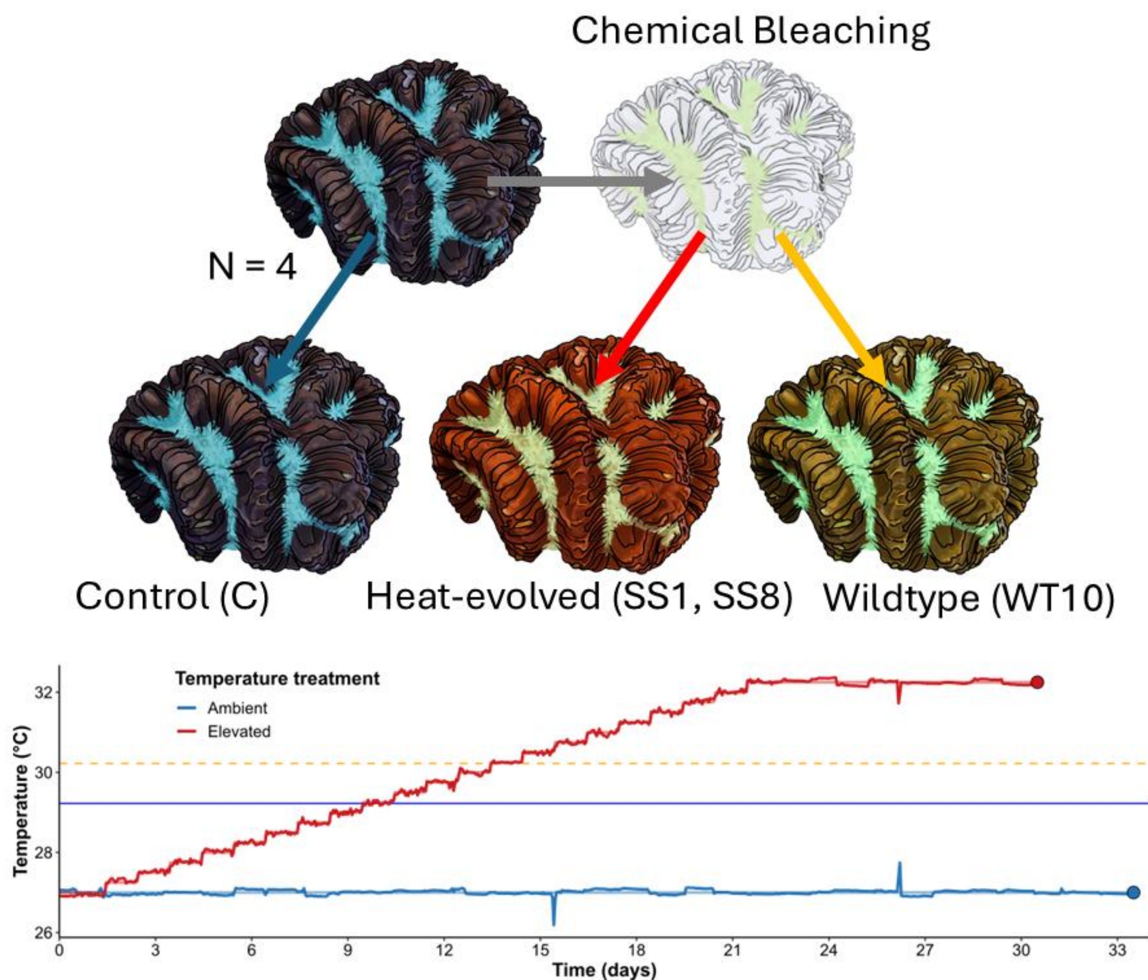

Figure 1. Experimental design. Four genotypes of Platygyra daedalea were collected, fragmented, and subjected to chemical bleaching to reduce the density of symbionts. Fragments were then inoculated with cultures of heat-evolved (SS1, SS8) or wildtype (WT10) Cladocopium proliferum symbionts. Control (C) were not chemically bleached or inoculated. A fourth treatment (re-inoculation control) was present in this experiment but is omitted from the above figure as it was not included in the metabolomic imaging (see below). Three months after inoculation, treatment corals were subjected to a simulated heatwave (bottom) either 'ambient' temperatures (27 °C, dark blue line) or elevated temperatures (32.25 °C, red line). The maximum monthly mean (29.25 °C) is represented by the light blue line and maximum monthly mean + 1 C the dashed orange line. Sampling for metabolomics of elevated treatment is indicated by the red dot and for ambient the blue dot, respectively.

1.2 Sample collection, matrix application and mass spectrometry imaging

Four *Platygyra daedalea* groups (i.e., control, coral inoculated with SS1, SS8 and WT10, hereafter refer to as "coral groups") were selected for mass spectrometry imaging (MSI). A coral sample group was only included if 1) the sample group contained the inoculum SS1, SS8 or WT10, or 2) the control samples had at least 3 replicates of identical dominant ITS2 profiles. The reinoculation control (chemically bleached and reinoculated with symbionts freshly isolated from the same *P. daedalea* colony; RIC) did not meet the requirements and was excluded. A total of 22 samples were imaged (control n = 6, SS1 n = 5, SS8 n = 5, and WT10 n = 6). Two technical replicates were imaged for each sample (total n = 44 sections). Coral samples (~1 cm^3 each) were collected on Day 10 of exposure to peak-temperature (32.25 °C), embedded and snap frozen following the protocol in (Chan et al. 2023b) and cryosectioned to 12 µm thickness sections according to (Chan et al. 2023a). Coral sections were then freeze-dried and mounted on an indium tin oxide coated slide. For matrix deposition, 300 mg of α-Cyano-4-hydroxycinnamic acid (HCCA) was sublimated at 250°C under vacuum (5 x 10^{-2} Pa) in the Shimadzu inlayer to achieve a matrix thickness of 1.5 µm. To enhance extraction efficiency, 5% methanol was vaporized at 60°C for 90 s for recrystallization following (Morikawa-Ichinose et

al. 2019), and the slides were desiccated in the desiccator prior to imaging. Matrix-assisted laser desorption/ionization MSI (MALDI-MSI) was conducted on the Bruker tipstaff fleX MALDI-2 and samples were randomized between runs. Note that ion mobility and post-ionisation functions were not employed in this study. Sample imaging was conducted on the positive ion mode for the mass range of 300- 1300 m/z and a spatial resolution of 50 me. The laser parameters were set as a single burst of 500 shots, at a frequency of 10,000 Hz.

1.3 Data curation and statistical analysis

Noise removal and peak picking was conducted in SCiLS. Co-localization analysis of metabolites with coral versus photosymbiont tissue was conducted to identify peaks associated with the symbiont and host. Features co-localized to m/z 800.603 (diacylglyceryl carboxyhydroxymethylcholine, DGCC, a betaine lipid known to occur in Symbiodiniaceae (Roach et al. 2021; Chan et al. 2023a)) were categorized as symbiont peaks; whereas features co-localized with m/z 790.571 (a feature co-localized with m/z 792.590, a glycerophosphocholine, PC, known to be found in cnidarian host (Chan et al. 2023b)) were categorized as host peaks (Figure 2). Each peak was visually inspected to ensure that 1) their localization (i.e., as symbiont or host) was correct, 2) the bin size (± 10 ppm) covered the entire peak area and did not overlap with another peak, and 3) the peak was sufficiently above background noise. A total of 319 peaks (163 symbiont peaks, 156 host peaks) were curated in the final peak list; their intensities (average intensity of peak maximum, within ± 10 ppm) were normalized by total ion count and by the surface area of each section. Section surface area was calculated based on (Chan et al. 2023b). The normalized data were exported to R studio and MetaboAnalyst 6.0 (Pang et al. 2024) for statistical analyses. Data analyzed in MetaboAnalyst were log transformed and visually inspected to confirm normality and homogeneity. Principal component analyses (PCA) were conducted using all samples, and heatmaps were generated using Euclidean distance and Ward clustering algorithm, based on peaks that were significantly different in intensity identified in an ANOVA (FDR < 0.05).

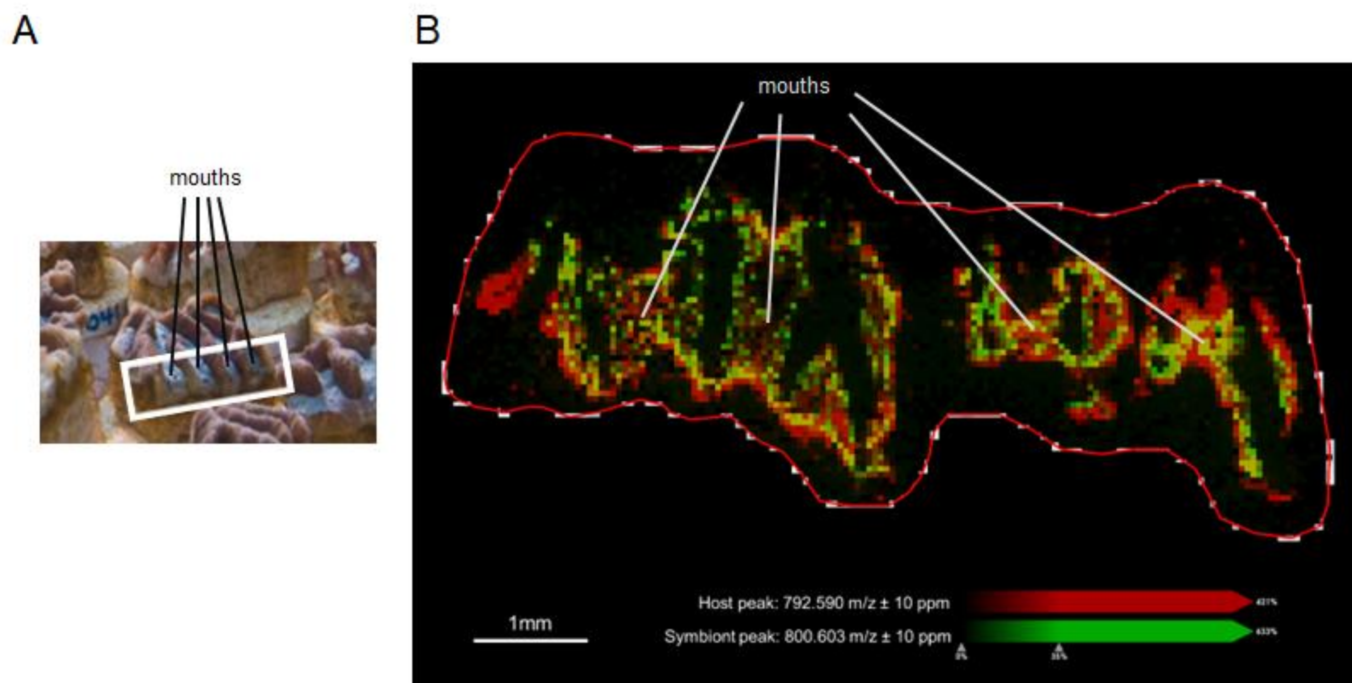

Figure 2 A) A Platygyra daedalea sample. The white square indicates the orientation where the coral section was taken from for mass spectrometry imaging (MSI). B) MSI image of a P.daedalea section. Localization of the symbiont peak (diacylglyceryl carboxyhydroxymethylcholine, DGCC) and host peak (glycerophosphocholine, PC) that were used for co-localized analyses to identify symbiont- or host-associated peaks. The image represents a coral inoculated with SS8 under elevated temperature (sample SS8E_1109R1).

2 Results and Discussion

2.1 Symbiont community composition

The community composition of symbionts within corals was confirmed using ITS2 metabarcoding (Hume et al. 2019) prior to sample processing and mass spectrometry imaging. Three symbionts were present: C21 (a wild symbiont often found in *P. daedalea*); *Durusdinium* (a temperature tolerant symbiont often found in corals following bleaching events (Boulotte et al. 2016; Quigley et al. 2022; Palacio-Castro et al. 2023), and the inoculum (SS1, SS8, and WT10 which are all strains of *C. proliferum*) (Figure 3). Mixtures of C21 and *C. proliferum* were detected, requiring that analyses must be conducted not only at the level of the treatment (i.e., using inoculum as a factor), but also according the 'dominant' symbiont (based on relative abundance) recovered using metabarcoding (Figure 3).

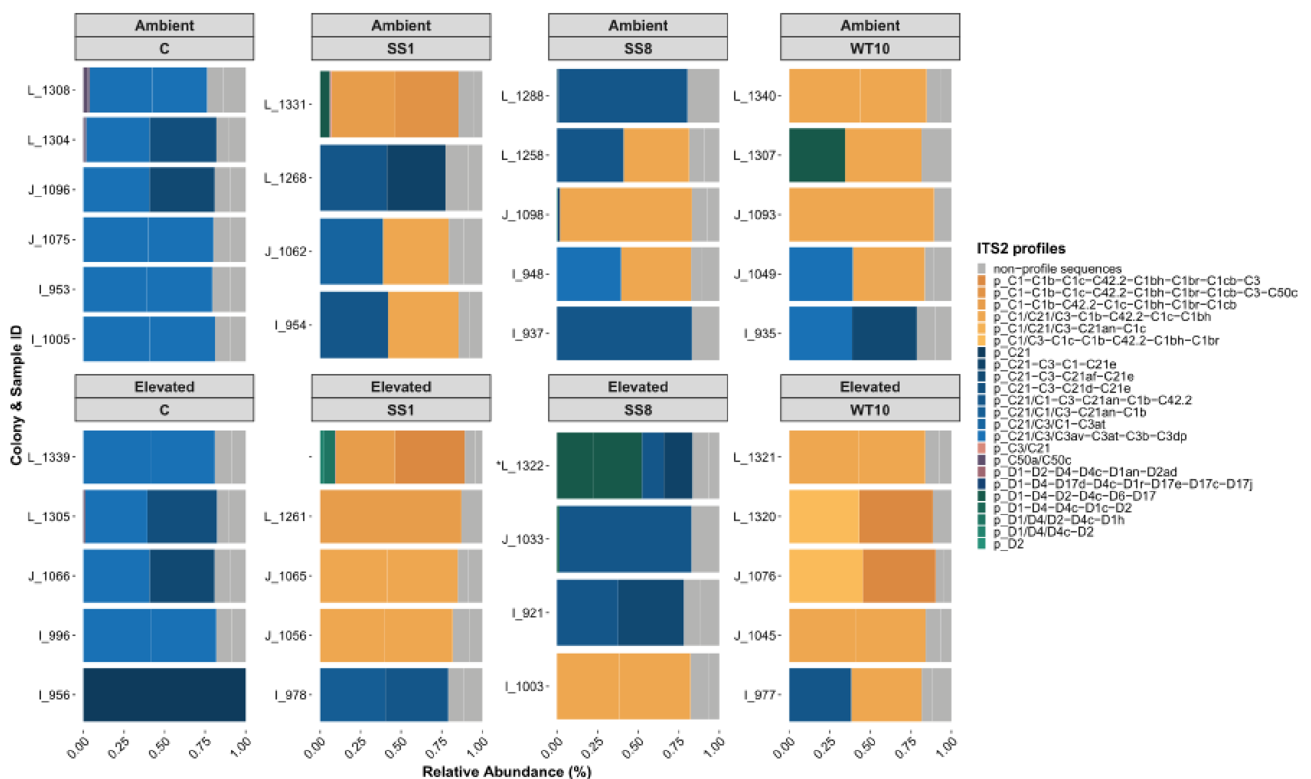

Figure 3. Example of symbiont community composition of corals subjected to mass spectrometry imaging. Individual samples are represented by horizontal bars with the axis label prefix corresponding to coral genotypes (I-L). Inoculum (WT10, SS1, SS8) is represented by yellow bars whereas native symbionts are represented by blue (C21) or green (Durusdinium). Figure modified from (Scharfenstein 2023). Samples are faceted by inoculum treatment (columns: C = control, SS1 and SS8 = heat evolved inocula, and WT10 = wildtype inoculum) and temperature treatment (rows: Ambient = 27 °C and Elevated = 32.26 °C)

2.2 Symbiont data using inoculum as a factor

There was no clear clustering between inoculation and temperature treatment in a heatmap (Figure 4) and PCA (Figure 5). This was unsurprising, because the inoculum did not always end up representing the dominant symbiont of the corals. Therefore, the data were also analysed based on

the abundances of symbiont species present within the sample. Note SS1 and SS8 were grouped into “SS” here, because SS8 at Ambient temperatures (27 °C) had n < 3.

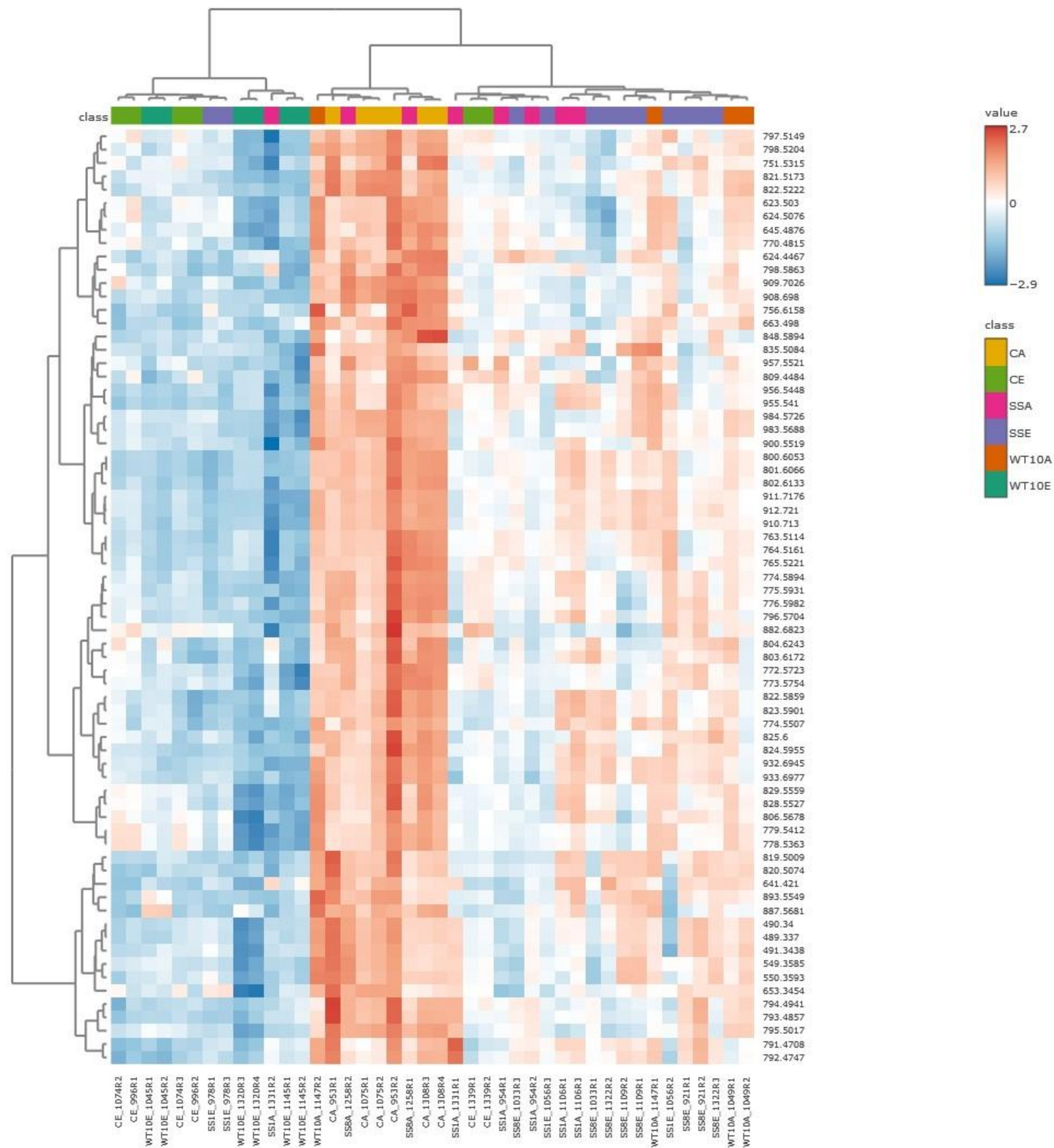

Figure 4 Heatmap of the top 70 symbiont peaks that were significantly different in intensity between coral groups of different inoculum and temperature treatment (total significant peaks: 157 out of 163, ANOVA). Sample and peak similarities were calculated based on Euclidean distance and Ward clustering algorithm. A = ambient, E = elevated. C = controls, SS = heat-evolved, WT10 = wild-type. For example, CA stands for controls at ambient temperature.

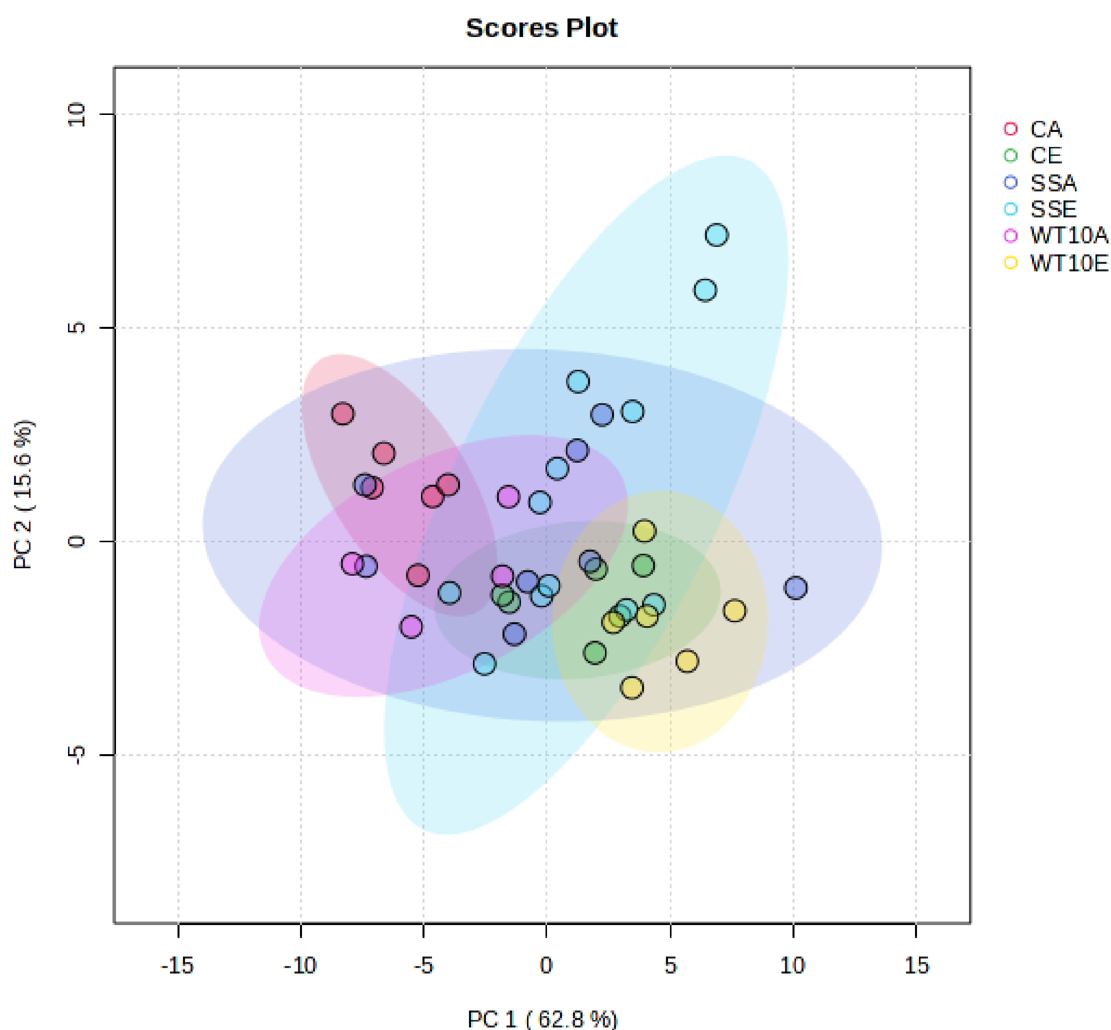

Figure 5 PCA using all 163 symbiont peaks with points coloured according to inoculum treatment x temperature treatment combinations. A = ambient, E = elevated. C = controls, SS = heat-evolved, WT10 = wild-type. For example, CA stands for controls at ambient temperature.

2.3 Symbiont data using dominant symbiont species as a factor

Applying the identity of the dominant symbiont (by relative abundance) as a factor, there was a clearer separation between symbiont C1 (combining SS1, SS8 and WT10 together) vs C21 (the native symbiont), and ambient vs elevated (Figures 6, 7). Of note is that the C1Ambient (SS8A_1258R1 and SS8A_1258R2, indicated in red boxes in Figure 6) samples that clustered with C21Ambient had C21 as near 'co-dominant'. In other words, the proportion of C1 and C21 will likely determine the clustering, and this subsequently requires a gradient analysis for these data and this is currently underway.

Figure 6 Heatmap of the top 70 symbiont peaks that were significantly different in intensity between coral groups of different dominant symbiont species and temperature treatment (total significant peaks: 137 out of 163, ANOVA). Sample and peak similarities were calculated based on Euclidean distance and Ward clustering algorithm. The red boxes highlight smaples SS8A_1258R1 and SS8A_1258R2.

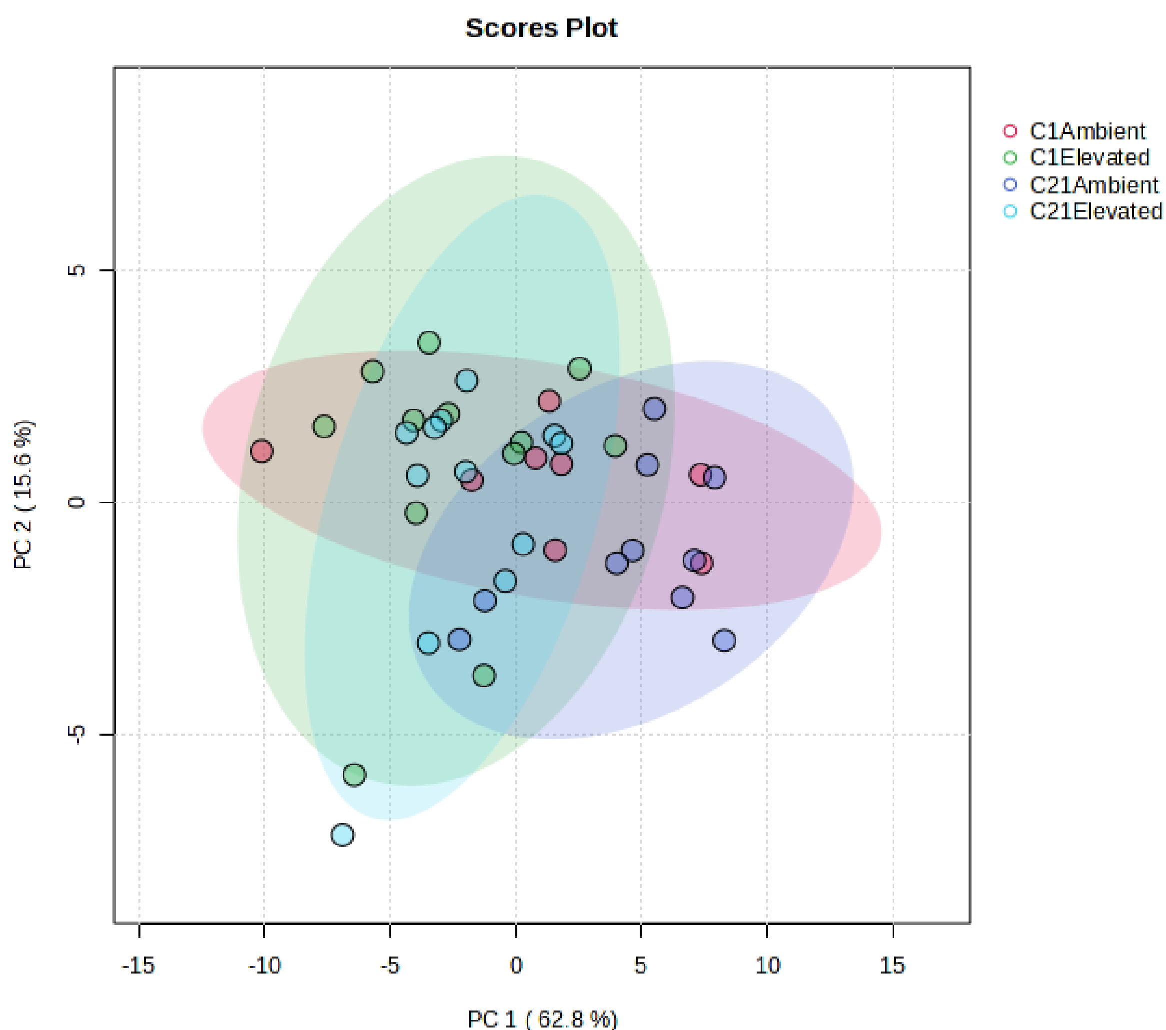

Figure 7 PCA using all 163 symbiont peaks, coloured by symbiont x temperature treatment combinations

2.4 Symbiont data using inoculum as a factor (under elevated temperature only)

Interestingly, heat-evolved SS8 was distinct from WT10 under elevated temperature, based on a heatmap and PCA (Figures 8, 9). SS1 replicates clustered with SS8 or WT10 or control (C). SS1 was not as strongly different from WT10, compared to SS8. The analysis was not repeated for ambient temperature due to the lower duplicate number (i.e., lack of statistical power).

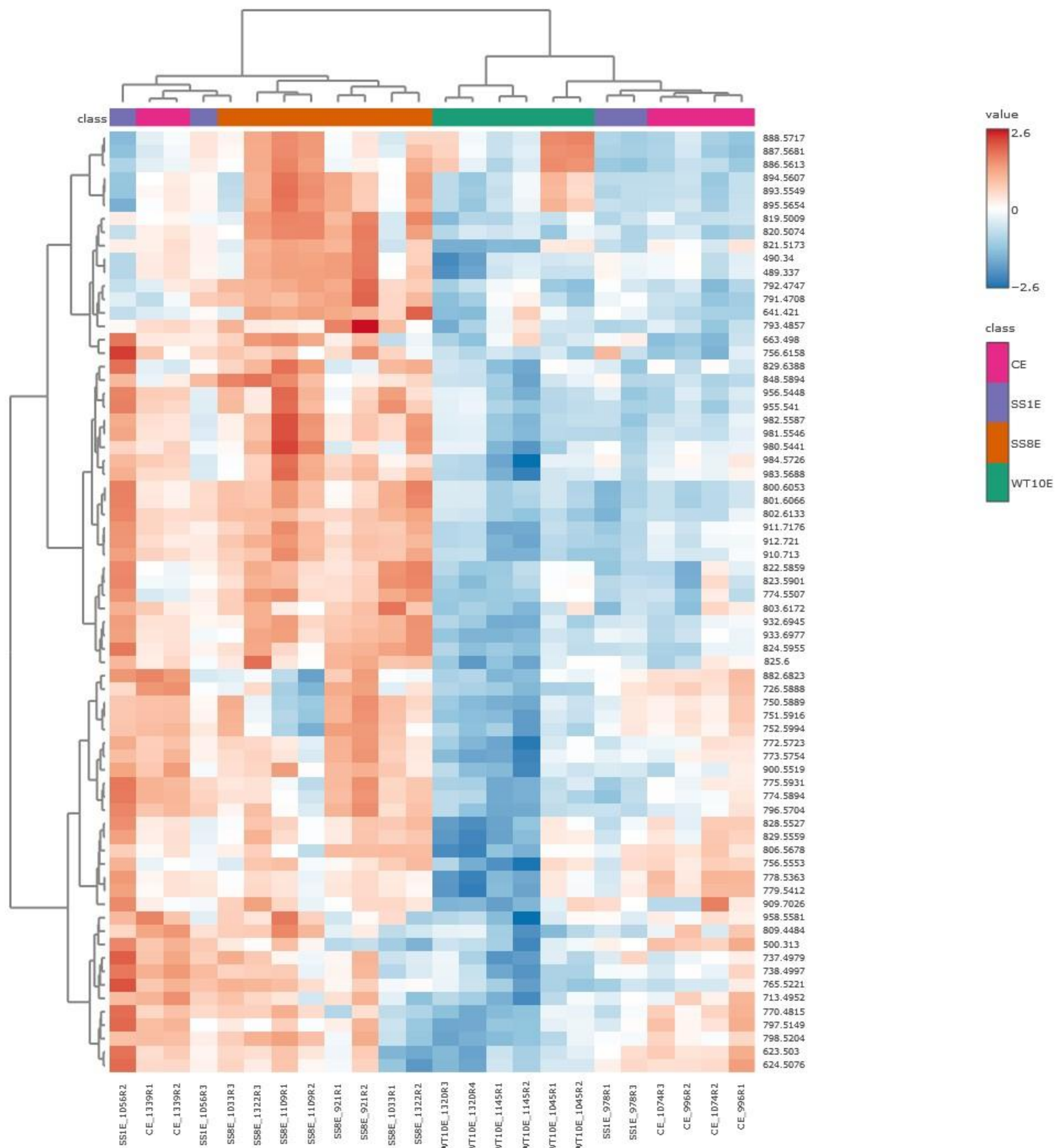

Figure 8 Heatmap of the top 70 symbiont peaks that were significantly different in intensity between coral groups of different dominant symbiont under elevated temperature (total significant peaks: 85 out of 163, ANOVA). C = controls, SS = heat-evolved, WT10 = wild-type.

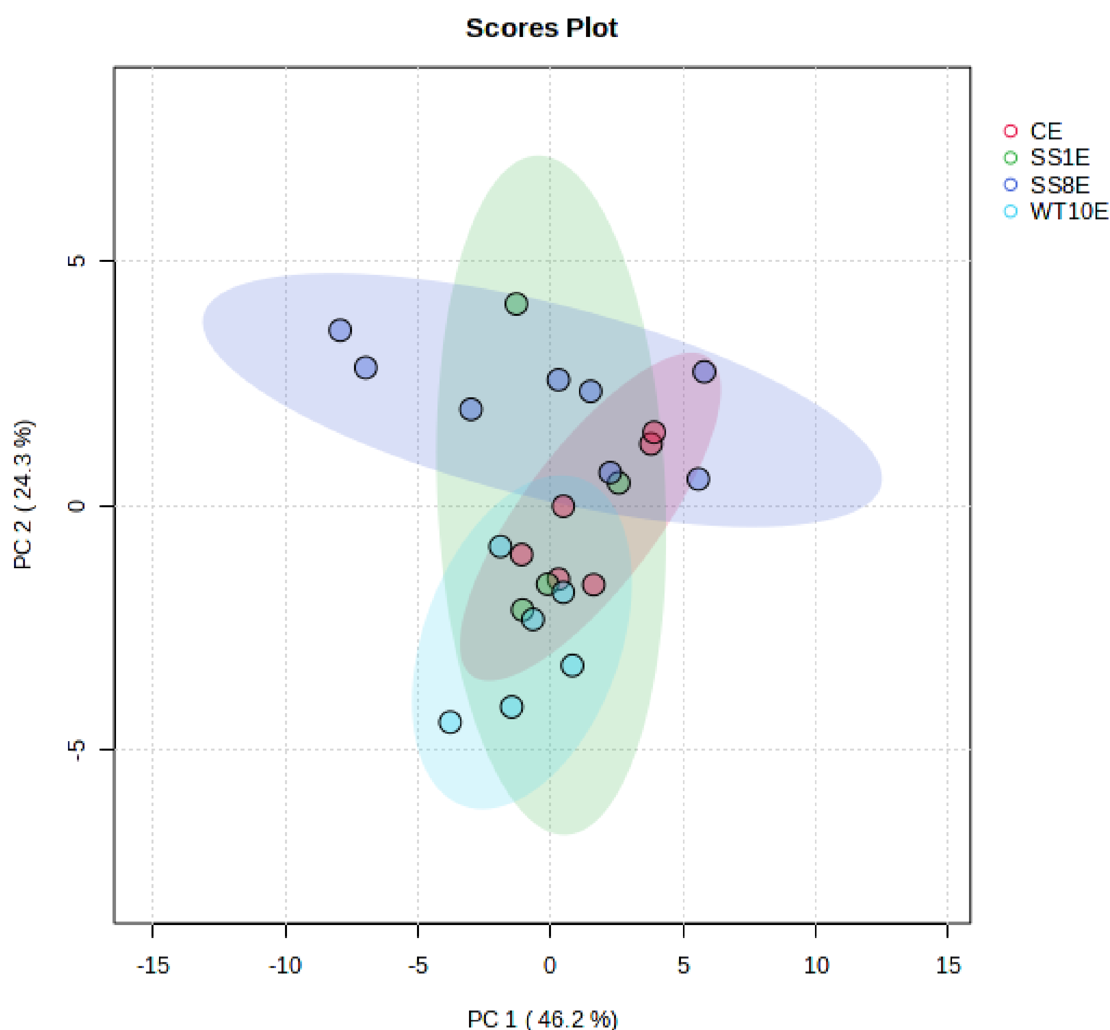

Figure 9 PCA using all 163 symbiont peaks under elevated temperature, coloured by symbiont treatment. Note the separation between SS8 and WT10 under elevated temperature.

2.5 Host data using inoculum as a factor

Overall, there were far fewer metabolites that were significant in the host data than symbiont data. This is not entirely unexpected, as host metabolome changes are often seen in the tricarboxylic acid cycle (TCA) cycle and gluconeogenesis(Rädecker et al. 2021; Williams et al. 2021). MSI is suitable for detecting lipids, but not so much in pathways with polar metabolites in the low m/z range (LC/GCMS are better methods), such as the TCA cycle and glucogenesis. Clustering was observed between ambient and elevated temperature, but not between inoculum treatments (Figures 10, 11).

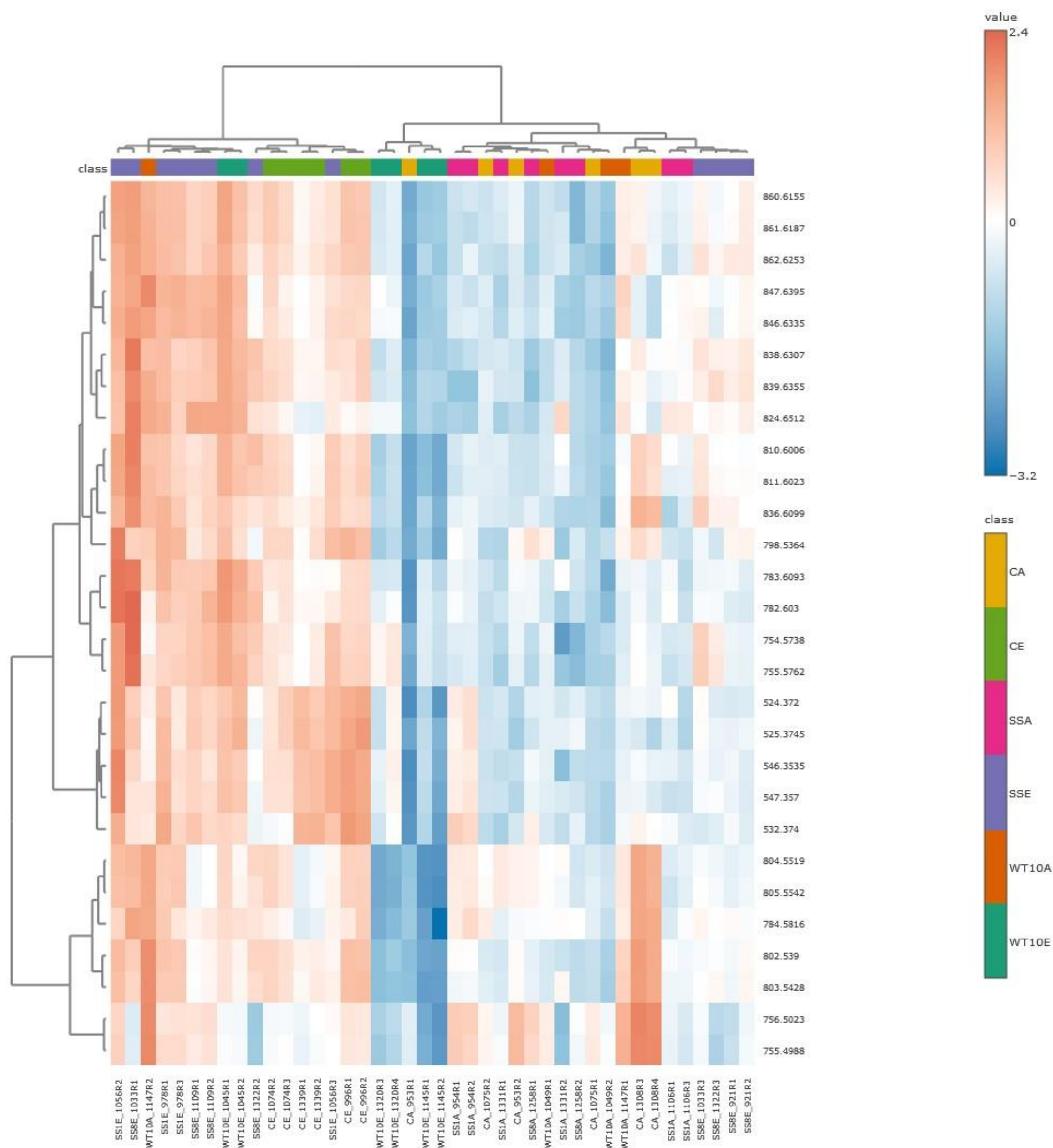

Figure 10 Heatmap of the 28 host peaks (out of 156) that were significantly different in intensity between coral groups of different inocula (ANOVA). Note the separation between ambient and elevated temperature. A = ambient, E = elevated. C = controls, SS = heat-evolved, WT10 = wild-type.

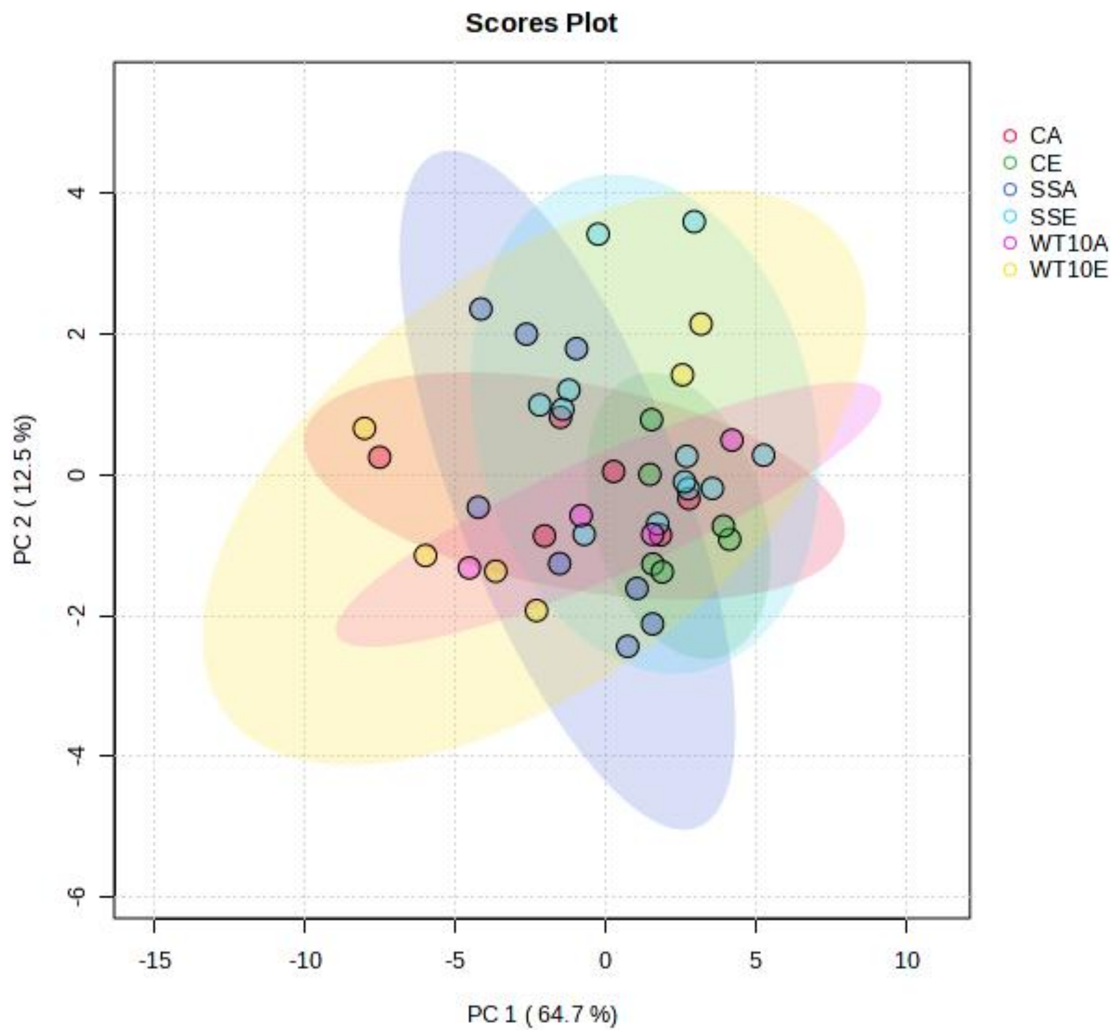

Figure 11 PCA using all 156 host peaks, with points coloured according to inoculum treatment x temperature treatment combinations.

2.6 Host data using dominant symbiont species as a factor

Similarly, there was no clear separation between coral groups inoculated with different algal symbionts in the host data (Figures 12, 13).

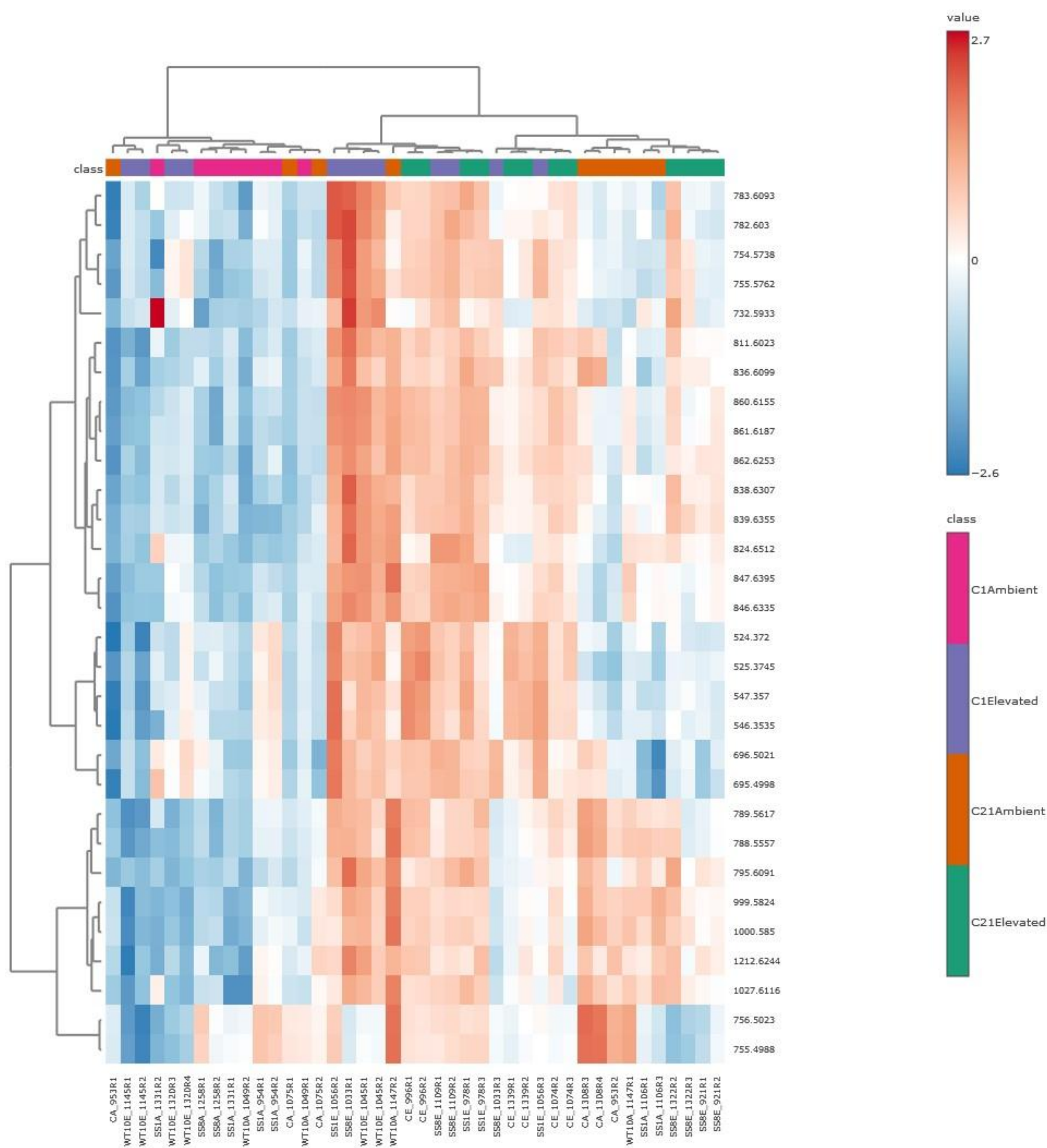

Figure 12 Heatmap of the 30 host peaks (out of 156) that were significantly different in intensity between coral groups of different dominant symbiont (ANOVA).

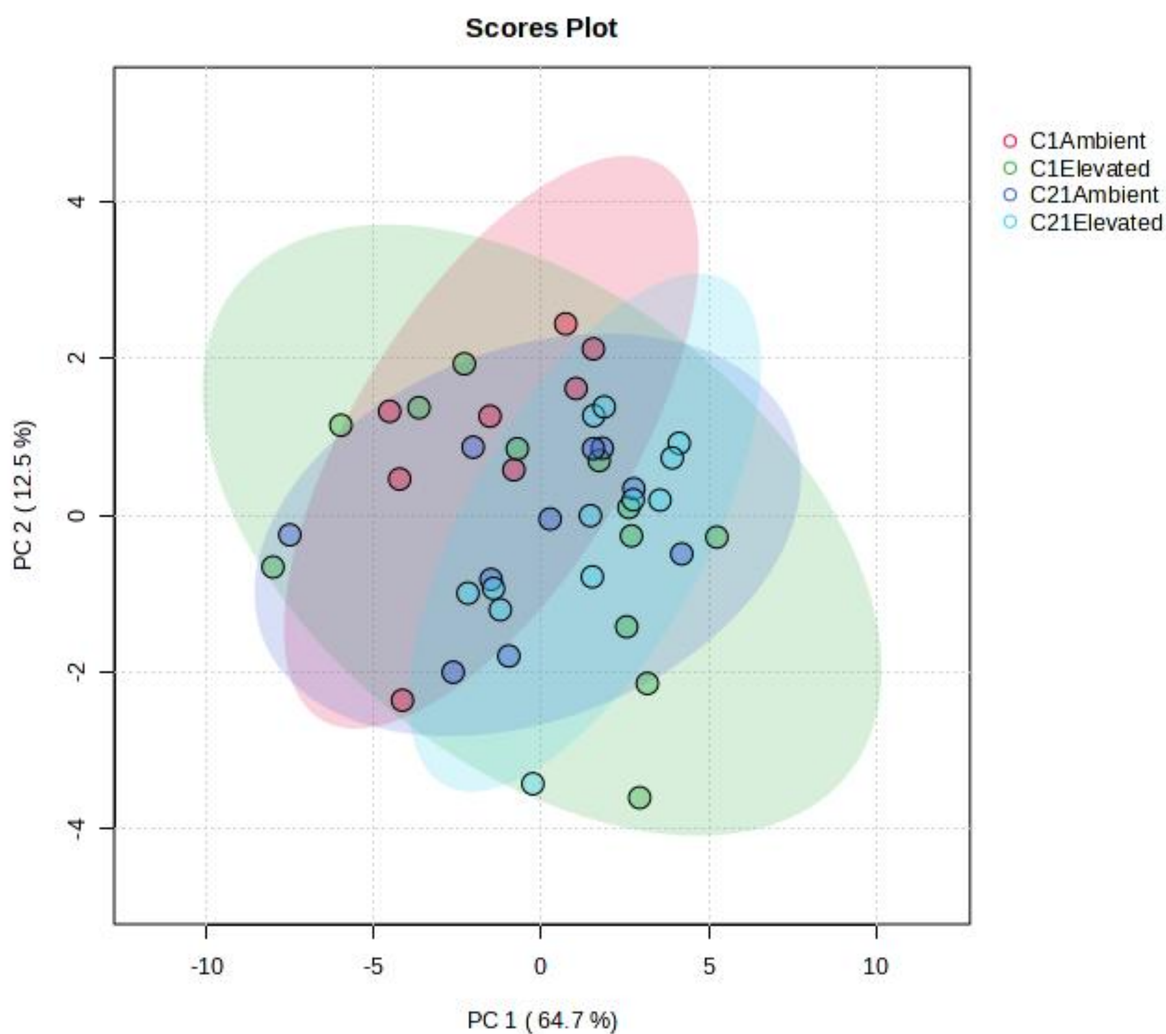

Figure 13 PCA using all 156 host peaks, with points coloured according to dominant photosymbiont x temperature treatment combinations

2.7 Host data using inoculum as a factor (under elevated temperature only)

Consistent with the above, there was no clear separation in the host data, despite ANOVA suggesting that 84 out of 156 peaks were significantly different (Figures 14, 15).

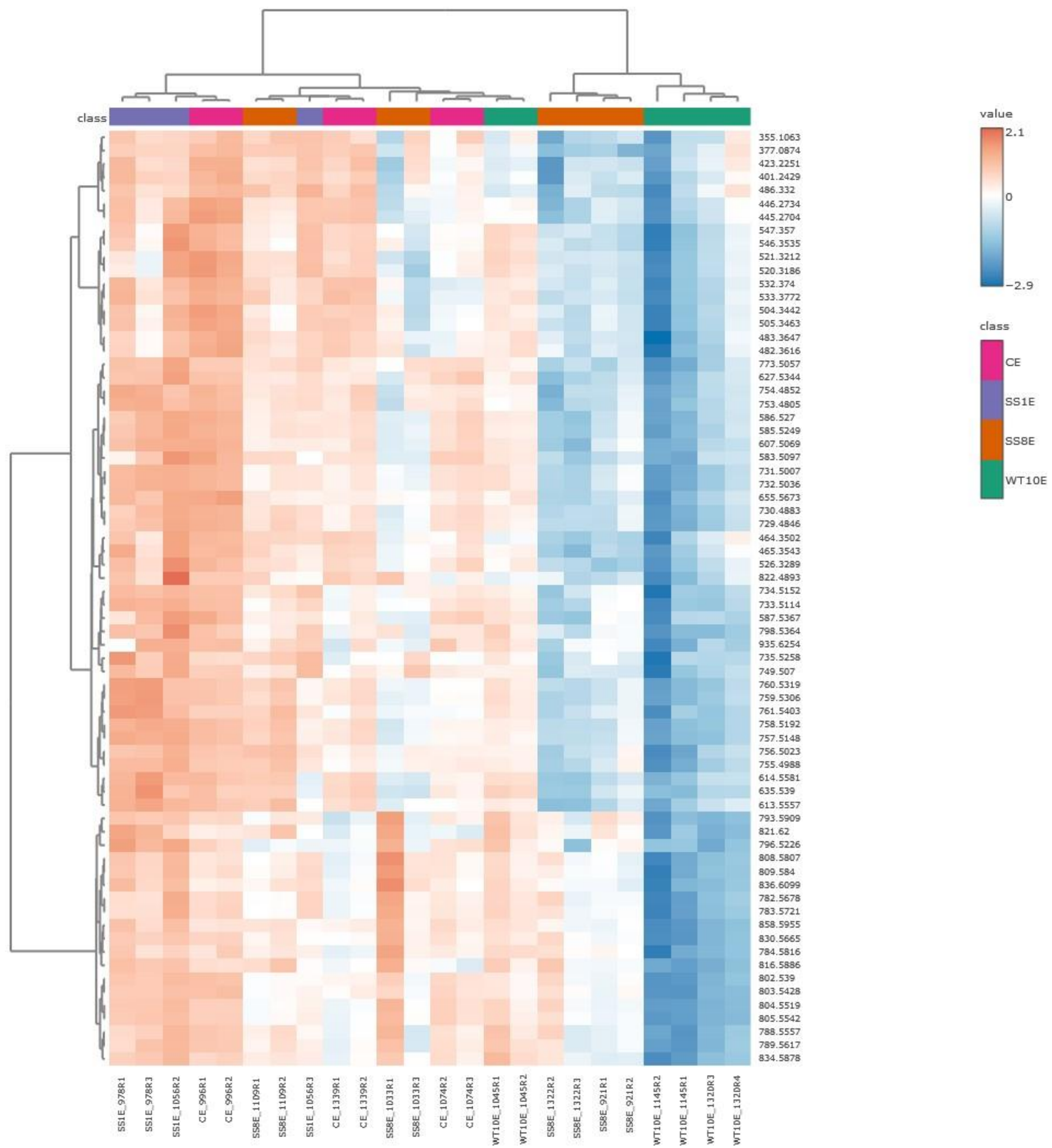

Figure 14 Heatmap of the top 70 host peaks that were significantly different in intensity between coral groups of different dominant symbiont species (total significant peaks: 84 out of 163, ANOVA). C = controls, SS = heat-evolved, WT10 = wild-type.

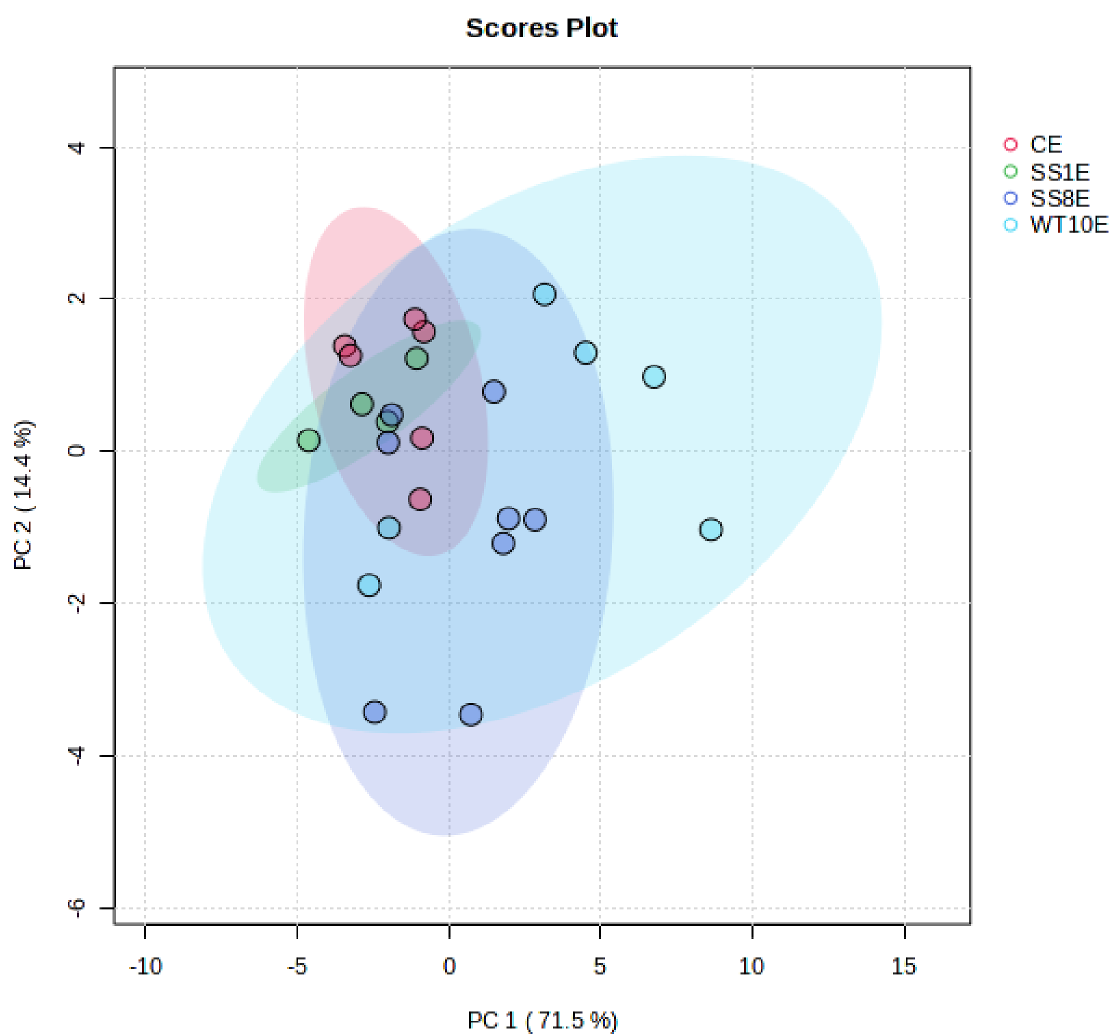

Figure 15 PCA using all 156 host peaks under elevated temperature, with points coloured according to inoculum treatment.

3 Conclusions, Recommendations and Future Work

In summary, the metabolite profiles of the *in hospite* algal symbionts were influenced by their taxonomic identity and the temperature conditions that they were under. They differed between symbiont species under ambient temperature and remained different under elevated temperature. This suggests that there are intrinsic differences in the metabolism of these algal symbiont species (despite the fact that they belong to the same genus), and that future studies should not overlook the potential impacts of diversity within genera. While it is common to use the dominant algal symbiont type as the reference or predictor to host performance, our findings suggest that co-dominance is potentially another important factor to consider. To address this, future studies can use gradient analysis to incorporate the relative abundance of the dominant algal symbiont type into the analysis. Compared to the algal symbionts, there was little difference in the metabolite profile of the host associated with different symbiont types, and this could be linked to the particular mass spectrometry method employed. We recommend future studies to conduct targeted analysis on biochemical products (e.g., carbohydrates) and pathways (e.g., TCA cycle, gluconeogenesis) that are most likely influenced by the algal symbionts based on existing literature.

The data presented in this report will form part of a manuscript on the thermal tolerance differences between *P. daedalea* inoculated with heat-evolved, wildtype and native *Cladocopium* symbionts (Nitschke et al, in prep.). This will provide mechanistic insights into the thermal tolerance differences of corals that harbour different symbiont communities. Such knowledge will guide symbiont manipulation interventions by identifying potential biomarkers associated with coral resilience and vulnerability. Once biomarkers are identified, they can be used to screen for beneficial properties in candidate algal cultures for reef restoration.

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