

Exploring manganese supplementation as a way of increasing coral thermal tolerance

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Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Under the Supervision of A. Prof. Emma Camp, Dr Andrei Herdean and Dr Jennifer

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Certificate of Original Authorship

I, Hadley England declare that this thesis is submitted in fulfillment of the requirements for the award of Doctor of Philosophy, in in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work, unless otherwise referenced or acknowledged. In addition, I certify that all information, sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Thesis Structure

This thesis is comprised of an introductory chapter (**Chapter 1**), three data chapters (**Chapters 2 to 4**) in the form of journal manuscripts, and a final synthesis chapter (**Chapter 5**). At the time of submission of this thesis, one data chapter (**Chapter 3**) was published, one data chapter (**Chapter 2**) was under review and one data chapter was prepared for journal submission (**Chapter 4**).

Chapter 1:

General introduction and background of literature.

Chapter 2:

This chapter is presented as the following published research article: **England, H.**, Oakley, C. A., Herdean, A., Hughes, D. J., Songsomboon, K., Matthews, J., & Camp, E. F. (2026). Manganese supplementation maintains cnidarian-dinoflagellate symbiosis under thermal stress. *Communications Biology*.

Chapter 3:

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Chapter 4:

This chapter is presented as the following published research article: **England, H.**, Herdean, A., Matthews, J., & Camp, E. F. (2025) Timing and method of manganese supplementation effects thermal resilience of corals. *Biometals*, <https://doi.org/10.1007/s10534-025-00774-7>

Chapter 5:

General discussion, synthesis of results from all data chapters and recommendations for future research.

Thesis Abstract

Manganese (Mn) is a critical trace element for all life on Earth, particularly for photosynthetic holobionts such as corals where it plays an essential role in the maintenance of symbiosis with Symbiodiniaceae under thermal stress. Corals rely on their relationship with Symbiodiniaceae, exchanging nutrients with them to survive and thrive in oligotrophic waters, where they form large coral reef ecosystems. However, the world's oceans continue to warm due to climate change, and consequently, coral reefs are set to become one of the first functionally extinct ecosystems, placing over 1 billion people at risk of displacement, unemployment and starvation. While traditional conservation strategies such as marine protected areas and coral out planting are playing a vital role in their protection and restoration, they are unlikely to safeguard reefs under the projected climate regimes. As a result, there is growing investment in active intervention strategies that enhance the resilience of corals to thermal stress. One strategy that is being explored involves the use of targeted elemental and nutrient supplementation during periods of thermal stress as a way to enhance coral resilience. However, our understanding of the fundamental role of key trace metals like Mn on coral physiology remains poorly resolved, hindering our understanding of the suitability of such interventions. Consequently, this thesis focuses on advancing knowledge of the essential role of dissolved Mn in Cnidarian-algal symbiosis, exploring the physiological impact non-toxic Mn supplementation has on Cnidarians during periods of thermal stress and developing new phenotyping methods. The knowledge obtained from this thesis looks to advance our understanding on the suitability of Mn supplementation as an active intervention during periods of increased thermal stress.

To achieve the thesis objectives, I first test the ability of non-toxic levels of Mn additions to mitigate the effects of thermal stress using the model Cnidarian, *Exaiptasia diaphana* (**Chapter 2**). I apply proteomic and photophysiological methods to determine what the optimal concentration (up to 15.6 $\mu\text{g L}^{-1}$) of Mn was for *E. diaphana* physiology under both ambient and thermal stress conditions. In this study I identified that Mn supplementation had the ability to significantly increase the thermal resilience of *E. diaphana*, as well as enabling Cnidarians to maintain symbiosis with Symbiodiniaceae. Proteomics data from this chapter challenged the traditional narrative that cnidarian bleaching is primarily driven by the production of reactive oxygen species. This led to the development of a mechanistic model of the potential role of Mn in coral bleaching. Results from this chapter also highlighted the need for a device that is tailored to the assessment of small Cnidarian samples across different elemental gradients.

Consequently, I next developed the Multi Taxa Phenotyping (MTP) system by utilising common laboratory equipment as well as custom protocols and 3D printed holding plates (**Chapter 3**).

This system can retrieve a suite of photophysiological and respirometry data from small Cnidarian samples across a range of different elemental, light and temperature conditions. This device significantly increased my capabilities for further Cnidarian trace metal experiments and was later utilised in Chapter 4. Additionally, this device was benchmarked against another well-known coral phenotyping system where it demonstrated its ability to identify corals that display higher thermal tolerances. The MTP system provides a new tool that reef scientists and restoration practitioners can use in their choice selection for restoration efforts, and within their experimental designs.

Utilising the physiological and technical advancements developed in the two earlier chapters, I then explored how the timing and method of Mn supplementation impacted the thermal performance of a common reef building coral *Acropora millepora* (**Chapter 4**). Using a large-scale flow-through system I tested how a constant supply of Mn ($5 \mu\text{g L}^{-1}$ administered before, during and after the onset of thermal stress), and intermittent pulse Mn dosing ($20 \mu\text{g L}^{-1}$ every second day) affected the thermal resilience of *A. millepora* when supplied at different times relative to the onset of thermal stress. Utilising the MTP system from Chapter 3 coupled with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis I assessed how coral photophysiology, respirometry, and elemental content was impacted as a result of the different treatments. Results indicated that both pre-stress Mn dosing and pulse-dosed Mn supplementation improved the thermal performance of *A. millepora*, and increased Mn accumulation in both the host and Symbiodiniaceae. Results from this chapter provided the first assessment of how Mn dosing frequency and timing can impact the physiological state of corals during thermal stress.

Throughout this thesis, I have demonstrated that dissolved Mn supplementation ($<20\mu\text{g/L}$) has the potential to alleviate the impacts of thermal stress on Cnidarians, although there is phenotypic variation in the response of Cnidarians to Mn supplementation. Findings from this thesis highlight the value of high-throughput phenotyping systems like the MTP system. Further, in this thesis I demonstrated that Mn supplementation primarily impacts the photosystem of Symbiodiniaceae. Overall, through this thesis I provide new knowledge on the role Mn plays in maintaining algal-Cnidarian symbiosis under thermal stress and in turn provide preliminary

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Chapter 1: General introduction and thesis roadmap

1.1 Coral reefs in the Anthropocene

Coral reefs are some of the world's most productive and valuable ecosystems but are under significant and increasing threat (Hughes et al. 2017). Reefs are simultaneously threatened by global stressors, such as ocean warming and acidification (Agostini et al. 2021), and local pressures, such as habitat degradation, eutrophication of waterways, overfishing, invasive pests, diseases, and sedimentation (He & Silliman, 2019; Knowlton & Jackson, 2008). Current trajectories suggest that functional coral reefs could be extinct by 2100 without immediate action on climate change (Ove Guldberg et al. 2022). Service provisions from coral reefs are anticipated to decline under climate change, with concern that they could be lost entirely (Woodhead et al. 2019). Reefs provide food, coastal protection, and resources to over 1 billion people globally (Wong et al. 2022; Deloitte 2018). They support large amounts of biodiversity (Espinoza et al. 2014; Munday et al. 2008), supported indigenous peoples for thousands of years (Kench & Mann, 2017), and play a fundamental role in upholding human rights (Camp et al. 2024).

The health and function of reef ecosystems is largely dependent on healthy corals which are a holobiont, consisting of the coral animal host and its associated symbionts, including dinoflagellates (Symbiodiniaceae), (LaJeunesse et al. 2018), bacteria, and other microorganisms, such as viruses (Rosenberg et al. 2007). Tropical coral reefs are typically found in oligotrophic waters where they have evolved to extract nutrients from the water column (Ferrier-Pagès et al. 2018; Ros et al. 2020), and partner with Symbiodiniaceae who share resources with the coral host such as carbohydrates, fats, amino acids, elements and key metabolites (Grima et al. 2022;

Hillyer et al. 2017; Matthews et al. 2018; Rodriguez et al. 2016; Ros et al. 2020). The species of Symbiodiniaceae associated with the coral can influence the holobionts ability to persist across environmental gradients (e.g. Ros et al. 2020; Camp et al. 2019) and to tolerate stress, including high light (Qin et al. 2019), varied nutrient content (Morris et al. 2019) and warming seawater (Swain et al. 2020).

1.2 Coral bleaching

Ocean warming is causing significant coral loss through mass coral bleaching events (Hughes et al. 2017; Oliver et al. 2018). On the Great Barrier Reef for example, five bleaching events have occurred in the past eight years, resulting in significantly reduced coral cover (Oliver et al. 2018; Reimer et al. 2024) and coral reproductive capacity that collectively impacts the resilience of the ecosystem (Hughes et al. 2017). Coral bleaching occurs when the symbiotic relationship between the coral host and Symbiodiniaceae breaks down (Suggett & Smith 2011). Central to photosynthesis is the protein complex photosystem II (PSII), an essential component of the multicomplex light-dependent reactions of oxygenic photosynthesis (Dasgupta et al. 2008). Light reactions of photosynthesis use energy from the incident light to extract electrons from water molecules by splitting them into molecular oxygen and protons (Schmidt et al. 2016).

Downstream in the process, energy in the form of Adenosine Triphosphate (ATP) and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) is used in light independent reactions (Calvin Benson Buchanan Cycle) to fix carbon dioxide and turn it into sugars. Under ambient conditions, photosynthetic products are shared with the Cnidarian host (Biscéré et al. 2018). It is theorised that during thermal stress, PSII becomes overactive and/or impaired in the Symbiodiniaceae, leading to the production of reactive oxygen species (ROS) (Nielsen et al.

2018). These ROS molecules are vital for cell signalling but also highly reactive and can become damaging if they exceed the organism's antioxidant capacity (Nielsen et al. 2018). For corals under thermal stress, it is thought that they can “leak” from the symbionts into the host, causing oxidative damage to the animal's tissue. This triggers the coral host to expel the Symbiodiniaceae as a protection mechanism, which ultimately results in coral bleaching (Biscéré et al. 2018; Ferrier-Pagès et al. 2018). However, it is important to note as there are many other causes of coral bleaching such as excess/limited light, nutrients, cold shock and changes in salinity where ROS plays a negligible role. Because of this, the role of ROS in coral bleaching is still highly debated (Schlotheuber et al. 2024), with some studies observing little to no increase in ROS during thermal stress. Other theories involving nutrient imbalances are becoming more popular (Rädecker et al. 2015, 2023), as these align with other known bleaching causes and are supported by increasing amounts of data.

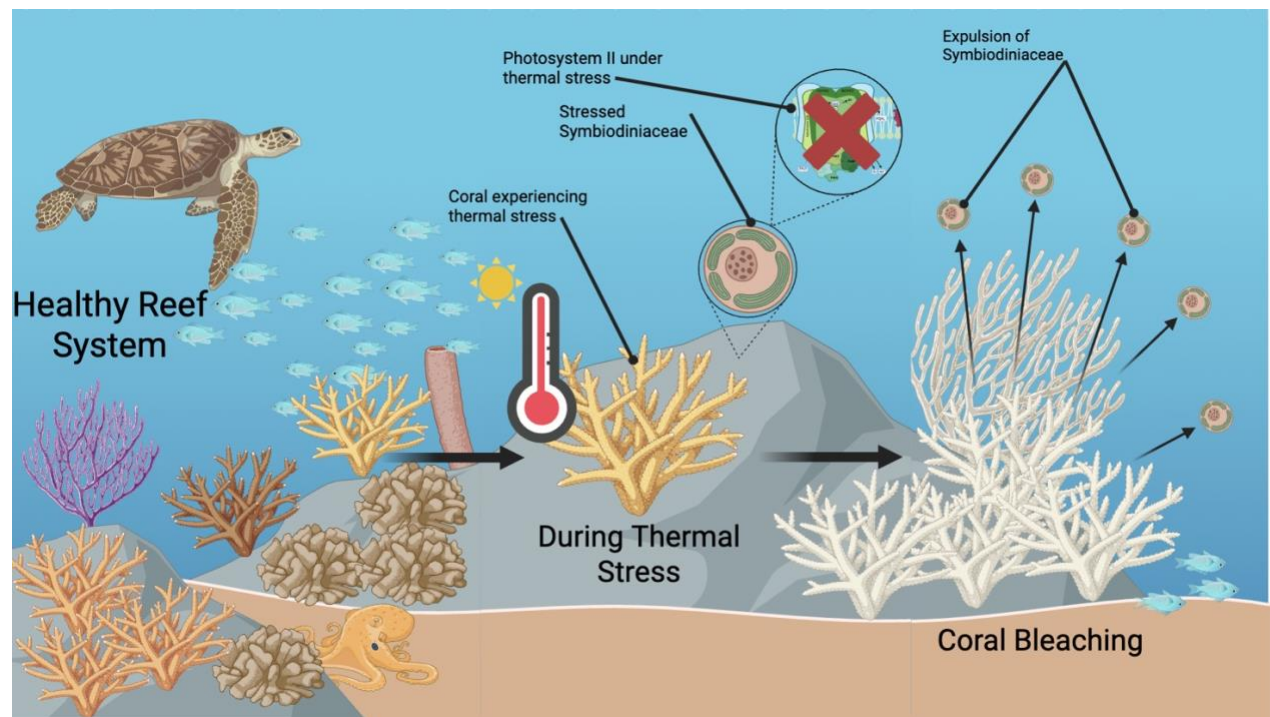


Figure 1.1. Schematic showing how healthy coral reefs are damaged by thermal stress. On the left is a healthy biodiverse reef. The middle shows corals under stress as a result of damage

inside the photosystems of the Symbiodiniaceae. And subsequently, the right shows the result of sustained thermal stress on corals, resulting in mass bleaching and the loss of biodiversity.

1.3 Importance of trace elements

Photosynthetic organisms such as corals rely on a range of nutrients and trace elements, such as dissolved manganese (Mn) to maintain optimum metabolic and photosynthetic processes (Ferrier-Pagès et al. 2018; Montalbetti et al. 2021; Reich et al. 2023a). Recent work has revealed that both the abundance and stoichiometry of trace elements are likely important factors contributing to the thermal response of both the coral (Ferrier-Pagès et al. 2018; Montalbetti et al. 2021) and its Symbiodiniaceae (Biscéré et al. 2018; Camp et al. 2022; Reich et al. 2021). Yet, despite their importance, research on optimal trace element concentrations within reef systems is limited (Reich et al. 2023a), with most studies focusing on their toxicological thresholds (e.g. Golding et al. 2023; Binet et al. 2023) and other elemental requirements (e.g. carbon, nitrogen, phosphorous; Camp et al. 2022; Dunn et al. 2012; Rädercker et al. 2015). Trace elements mostly occur in ultra-low concentrations in reef systems and vary spatially and temporally (Camp et al. 2025). Both physical processes and biological activity are key drivers of trace element availability in seawater, and both are subject to drastic changes under climate change (Henley et al. 2020; Zitoun et al. 2024). Exactly how climate driven shifts in these processes will affect the trace element landscape of our oceans is unknown, leaving a large gap in our knowledge and abilities to combat and account for these changes in the future (Reich et al. 2023). During the late 70's and early 80's there was a flurry of research on trace elements in corals, however this work typically looked at trace metals in coral skeletons as opposed to the host and symbiont requirements (Denton & Burdon-Jones, 1986; Goreau & Yonge, 1997; Swart, 1981). More

recently, studies have revealed that symbionts can accumulate higher amounts of key trace elements to maintain optimal holobiont functioning (Iyagbaye et al. 2022; Reichelt-Brushett & McOrist, 2003). Also, that trace metal requirements and stoichiometries can change significantly in both the coral host and Symbiodiniaceae under stress (Reich et al. 2021 and 2023; Rodriguez et al. 2016). However, our understanding of the optimal trace metals requirements of corals under stress remains poorly understood (Reich et al. 2023). This is surprising given the essential nature of trace elements and the fact that their cycling and bioavailability will be impacted by climate change (Henley et al. 2020; Zitoun et al. 2024), creating a potential risk of mismatch between need and availability (Camp et al. 2025), ultimately leading to environmental conditions which are not suitable for healthy corals and functioning reefs.

1.4 Manganese

Manganese (Mn) is a biologically essential trace element in all organisms, with no exception in oceanic environments where it is typically found in low concentrations. In oligotrophic waters, for example, its concentration is typically around $0.06 \mu\text{g L}^{-1}$ (Morley et al. 1997), whereas in more nutrient rich areas, it can reach up to $7.34 \mu\text{g L}^{-1}$ (Srichandan et al. 2016). At high-levels (e.g. $700 \mu\text{g L}^{-1}$) Mn is lethal to coral (Golding et al. 2023; Summer et al. 2019). Sources of Mn contamination can come from a range of activities that include mining, metal fabrication, herbicides, fossil fuels and terrestrial runoff (Summer et al. 2019). For coastal marine waters, a proposed upper limit of $190 \mu\text{g L}^{-1}$, which offers protection to 99% of marine life has been suggested ANZG (2018). However, despite research conducted on elemental toxic thresholds for Cnidarians (Golding et al. 2023; Iyagbaye et al. 2022; Summer et al. 2019), natural quotas (Camp et al. 2025; Grima et al. 2022) and changing quantities and stoichiometries of Mn for

Cnidarians subjected to stress (Ferrier-Pagès et al. 2018; Montalbetti et al. 2021), remains less resolved. In seawater, Mn occurs primarily in three oxidation states, (1) dissolved Mn^{2+} , (2) Mn^{2+} complexes and (3) particulate Mn^{4+} oxides (Oldham et al. 2019). Mn^{2+} is the most soluble and generally considered the most bioavailable form for marine organisms such as cnidarians. However, oxidized Mn^{3+} and Mn^{4+} forms are commonly associated with particulate phases and mineral oxides that are less readily assimilated (Oldham et al. 2019 and Jones et al. 2020). The speciation and distribution of Mn in seawater is controlled by a range of biotic and abiotic processes including redox reactions, photochemical reduction (Sunda and Huntsman 1994), microbial oxidation (Tebo et al. 2005) and sediment resuspension (Landing and Bruland 1987). These Processes create strong spatial and temporal variability in Mn availability, particularly in coastal systems where terrestrial inputs, sediment interactions and biological activity can elevate dissolved Mn concentrations (Oldham et al. 2019). Climate change driven processes such as ocean warming, deoxygenation, and altered terrestrial runoff regimes may further change Mn cycling and speciation, potentially influencing the amounts of biologically available Mn to marine organisms.

For corals, Mn is crucial for the optimal physiological functioning of the holobiont, playing key roles in maintaining cellular chlorophyll concentrations, supporting photosystem efficiency, and facilitating enzymatic activities (Montalbetti et al. 2021; Reich et al. 2022; Biscéré et al. 2018; Ferrier-Pagès et al. 2018). Mn is an essential trace element for the oxygen evolving complex in PSII of Symbiodiniaceae (Biscéré et al. 2018) and is a cofactor of many important enzymes, including the antioxidant superoxide dismutase (MnSOD) which plays a vital role in the suppression of ROS (Holley et al. 2011). It also supports important cellular processes such as nutrient uptake and assimilation (Goreau & Yonge, 1997), phagocytosis and heterotrophy (Banci,

1997; Wilcox et al. 1998), and cell signaling/responses (Dyken & Shick, 1982; Grasso et al. 2008). In 2018, Bisc  re et al. (2018) showed that concentrations of Mn of 4.1 $\mu\text{g L}^{-1}$, a concentration typically above ambient seawater conditions (Moreton et al. 2009), but below known toxic levels (Binet et al. 2023; Golding et al. 2023), can have a significant positive effect on a coral's thermal tolerance. Further analysis by Montalbetti et al. 2021 on the same coral samples revealed that low-level Mn supplementation significantly impacted the cellular functioning of corals under thermal stress, by altering the expression of stress response proteins such as heat shock proteins, and reducing lipid peroxidation and glutathione reductase levels within the host. However, the optimal levels of Mn for Cnidarian physiological performance, and the molecular implications that accompany Mn enrichment remain unknown. Furthermore, whether the timing of Mn supplementation for Cnidarians under stress impacts their physiological performance remains untested. Collectively this information can advance our understanding on the suitability of Mn supplementation as an active intervention strategy for corals and other symbiotic Cnidarians under heat stress.

1.5 Active intervention strategies

The best way to protect coral reef ecosystems and the valuable economic services they provide from the effects of climate change is by rapid global reduction of greenhouse gas emissions. However, climate change continues to intensify driving the loss of coral reefs globally. Marine protected areas offer protection against over exploitation (Beyer et al. 2018; McClanahan et al. 2024). However, such traditional management is no longer likely to be enough to support coral reefs in the Anthropocene (Suggett et al. 2024). Consequently, active interventions are now being considered and applied worldwide (Peixoto et al. 2024). The feasibility and suitability of any

active interventions require careful consideration of costs, risks, and potential beneficial returns (Camp et al. 2022, 2025), while also addressing issues of scalability and sustainable financing (Suggett et al. 2024).

The aim of active interventions are to directly enhance the resilience of coral reefs through activities such as coral propagation and planting (Suggett et al. 2024), genetic modification (Humanes et al. 2024; van Oppen et al. 2015), nutritional or microbial supplementation (Li et al. 2023; Osman et al. 2025) and directed evolution. The latter process involves targeting priority stock selection for restoration, using phenotyping tools to select species or locations for priority management based on emergent traits (England et al. 2024; **Chapter 3**). Land based phenotyping is significantly more advanced than marine based phenotyping (e.g. high-throughput, hyperspectral imaging, genetic selection) (Yang et al. 2020; Zhao et al. 2019), but progressive efforts are being made in the marine space, for example via the Coral Bleaching Automated Stress System (CBASS) and other technologies that aim to rapidly assess species traits and guide choice selection of management and restoration species and locations (Evensen et al. 2023; Voolstra et al. 2020). To date, selection of locations for priority management have typically been based on observed or predicted refugia (Beyer et al. 2018; McClanahan et al. 2024) and/or high value economic, ecological or cultural sites (Scott et al. 2024). Interestingly, the nutritional landscape of reef sites has been less considered during restoration site selection (Reich et al. 2023b), despite this being a dominant driver of site selection in terrestrial systems (Baer, 2016; Zhang et al. 2023).

Understanding the nutritional requirements of coral, along with how this is likely to change under future climate scenarios, can help scientists evaluate whether current and future seawater compositions meet corals nutritional requirements. Limitations in some key elements have already been documented in the world's oceans (Zitoun et al. 2024), making our understanding of the elemental needs of corals essential, so any mismatches or imbalances can be identified and potentially corrected for (Reich et al. 2023b). For example, in terrestrial agriculture, composition and concentration of crop fertilisers are chosen based on the elementome (the concentration and stoichiometry of elements) of the soil, and the specific requirements of the crop (Meena et al. 2024; Zulfiqar et al. 2021). This tailored approach ensures that plants/crops have access to all critical elements in the exact ratio required under different environments. Such a tailored approach has yet to be applied for coral reefs but is commonplace in coral aquaculture to enhance key coral traits, such as colour (Leal et al. 2014). Before such an approach can be considered for coral reefs, fundamental knowledge on the requirement of elements, such as Mn under normal and stress conditions is required. Consequently, this thesis focuses on exploring how Mn supplementation impacts Cnidarian coral physiology under ambient and heat stress conditions to provide essential knowledge needed to evaluate whether Mn supplementation can contribute to the active intervention toolbox being explored for coral reefs in the Anthropocene.

1.6 Thesis Roadmap

This thesis aimed to advance knowledge on the effects of non-toxic trace metal supplementation of Mn on Cnidarian holobiont physiology during ambient and heat stress conditions (**Chapters 2 and 4**). To achieve this objective, part of the thesis involved developing a phenotyping (**Chapters 3**) technology that allowed assessment of coral thermal performance across a range of

chemical gradients in a controlled trace-metal environment (e.g. free from contamination of trace metals). To achieve the thesis goals I utilised a vast array of analytical techniques ranging from Inductively Coupled Mass Spectrometry (ICP-MS) analysis (**Chapters 2, 3 and 4**), photophysiology (**Chapters 2, 3 and 4**), proteomics (**Chapter 2**) and coral based experimental system design (**Chapters 2, 3 and 4**), as well as a variety of different taxa/species such as *Exaiptasia* (**Chapter 2**), *Acropora kenti*, *Pocillopora damicornis* and *Montipora digitata* (**Chapters 3 and 4**), mangroves, seagrass and algae (**Chapter 3**).

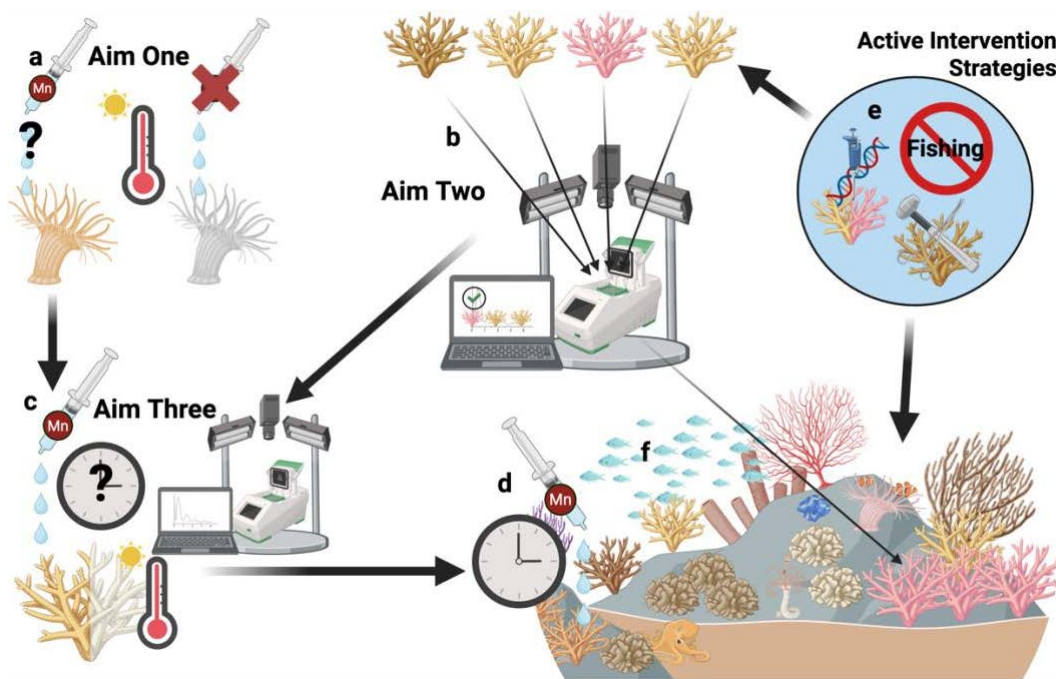


Figure 1.2. Schematic demonstrating how the three thesis aims work together to contribute to the active intervention toolbox. (a) Aim One explored the proteomic and photophysiological effects of different manganese (Mn) concentrations using the model holobiont *Exaiptasia diaphana*. (b) Aim Two involved the development of the Multi-taxa Phenoplate (MTP) system, facilitating further trace element studies on corals (Aim Three) and directly supporting other intervention strategies. (c) Aim Three explored the effects of different methods and timings of Mn delivery on the thermal resilience of the coral *Acropora millepora*. The three chapters aim to advance knowledge on the required concentrations and method of Mn supplementation that may be suitable for coral (d), thus contributing to the broader active restoration toolbox (e) in order to restore and protect thermally tolerant, functional reef systems (f).

Aim 1 (Chapter 2): Utilise a proteomics-based approach to further our understanding of how low-level manganese (Mn) supplementation affects host-algae symbiosis of the Cnidarian model taxa, *Exaiptasia diaphana* under ambient and thermal stress.

Previous studies (Biscéré et al. 2018; Montalbetti et al. 2021) have highlighted the importance of Mn for corals, especially during periods of thermal stress. However, there is little information on what the optimum concentration of Mn is for Cnidarians, with other studies exploring as little as $4.1 \mu\text{g L}^{-1}$ (Biscéré et al. 2018) and as high as $250\text{-}20,000 \mu\text{g L}^{-1}$ (Binet et al. 2023; Golding et al. 2023), with no known studies exploring ranges in between. This leaves a large gap in the literature, especially considering that Mn concentrations in some parts of the ocean have been recorded as high as $11.12 \mu\text{g L}^{-1}$ (Robin et al. 2012). To address this gap, I explored the supplementation of four different concentrations of Mn, 0.5, 4.8, 11.4 and $15.6 \mu\text{g L}^{-1}$ to *Exaiptasia diaphana*, under both elevated (32°C) and ambient (26°C) temperatures. These concentrations were chosen as they are still well below known toxic thresholds (Binet et al. 2023; Golding et al. 2023) but are within the realms of naturally occurring levels (Moreton et al. 2009). Using proteome analysis this study explored how Mn is utilised by *E. diaphana* under ambient and heat stress conditions.

In this chapter, the following hypotheses were tested:

- The addition of Mn up to $15.6 \mu\text{g L}^{-1}$ would benefit *E. diaphana* under heat stress as evidenced by sustained photosynthetic performance relative to no Mn supplementation.
- Mn additions would impact both the Cnidarian host and their Symbiodiniaceae as evidenced by changes in their proteomes.

This chapter is in review: England, H., Oakley, C., Herdean, A, Hughes, D., Matthews, J., Camp, E., (In Review). Manganese supplementation maintains cnidarian-algal symbiosis under thermal stress. *Nature, Communications Biology*.

Aim 2 (Chapter 3): Develop a phenotyping tool for the rapid assessment of coral health under light, temperature and chemical gradients.

Chapter 2 results highlighted a need for a device that enabled high-throughput analysis of small Cnidarian samples across different light, temperature and chemical gradients. Building on established methods designed for small macroalgae samples (Herdean et al. 2022), a custom coral sample adapter plate was created, as well as custom protocols that allowed for the analysis of a suite of photophysiological parameters. The device, a multi-taxa phenotyping (MTP) system greatly increased our capabilities to assess the impacts of Mn supplementation on the thermal tolerance of corals, and as a result was utilised in all subsequent chapters. The hypotheses tested by this chapter were:

- An algal phenotyping system could be customised for multiple taxa, including corals.
- A developed phenotyping system could be expanded to include assessment of chemical gradients.
- A developed phenotyping system could identify corals with higher thermal tolerance.

This chapter has been published in *Nature Scientific Reports*:

England, H., Herdean, A., Matthews, J., Hughes, D. J., Roper, C. D., Suggett, D. J., Voolstra, C. R., & Camp, E. F. (2024). A portable multi-taxa phenotyping device to retrieve physiological performance traits. *Scientific reports*, 14(1), 21826.

Aim 3 (Chapter 4): To assess if the timing and method of manganese (Mn) supplementation has a measurable impact on the thermal response of *Acropora millepora*.

Results from Chapter 2, along with findings from prior studies (e.g. Biscéré et al. 2018) have suggested that maintaining constant Mn levels between 4.8 and 15.6 $\mu\text{g L}^{-1}$ can provide some level of protection for Cnidarians against thermal stress. However, whether Mn must be constantly supplemented throughout the thermal stress period, or if it can be intermittently dosed remains to be tested and formed the main question for this chapter. Here I explored how Mn availability affected the health of corals under ambient and heat stress conditions. Moving on from the use of a model holobiont (*Aiptasia*), I utilised a common reef building coral (*Acropora millepora*) and exposed it to Mn at different times whilst under thermal stress (prior to heat stress – continuous, at the onset of heat stress – continuous, during heat stress – continuous, and intermittent supplementation during heat stress). I used ICP analysis to verify Mn concentrations in the water column, as well as Mn uptake in the host and their Symbiodiniaceae. I also incorporated the MTP device from **Chapter 3** to test the photophysiology of the corals before, during and at the end of the experiment. The hypotheses tested in this experiment were:

- Supplementation of Mn prior to heat stress would result in greater physiological benefits to *A. millepora*.

- The host and Symbiodiniaceae would accumulate Mn differently under thermal stress compared to ambient temperatures.

This chapter is presented as a full article prepared for journal submission.

The knowledge gained from the three aims are synthesised in Chapter 5, where I highlight the trends, patterns and concepts explored in these studies. I also discuss future application of elemental supplementation as well as the MTP system as a useful tool in identification of coral plasticity under environmental change and stress. Such knowledge could be utilised in informing and improving coral reef restoration and management practices in the Anthropocene.

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Chapter 2: Manganese supplementation maintains Cnidarian– dinoflagellate symbiosis under thermal stress.

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2.1 Abstract

Manganese (Mn) is an essential trace element for all photosynthetic life, playing an integral role in their photosystems, metabolism, and antioxidant activity. For cnidarians and their symbionts, most studies focus on the potential toxicity of Mn at high concentrations (e.g. $>700 \mu\text{g L}^{-1}$).

However, there has been less exploration on beneficial, nutritionally relevant levels of Mn. We apply mass spectrometry-based proteomics to explore how dissolved Mn supplementation at increasing concentrations (0.5, 4.8, 11.4, $15.6 \mu\text{g L}^{-1}$) alters the proteome of the sea anemone *Exaiptasia diaphana*, when subjected to ambient (26 °C) and elevated (32 °C) temperatures.

Pulse-amplitude modulated fluorometry demonstrated that Mn supplementation up to $15.6 \mu\text{g L}^{-1}$ mitigates thermal stress to *E. diaphana*, resulting in reduced photochemical damage and symbiont expulsion. Derived photobiology and proteomics data contributes to a mechanistic model for how Mn reduces thermal susceptibility, supporting the viability of Mn additions to enhance the protective capacities of photosynthetic Cnidarians during marine heatwaves.

2.2 Introduction

Reef-building corals typically thrive in oligotrophic waters via evolution of efficient mechanisms and partnerships to retrieve nutrients from the water column (1). For example, coral growth and health are supported through intricate relationships with associated symbionts, including receiving vitamins from bacteria (2) and photosynthetic products (glycerol, glucose, amino acids and lipids) from endosymbiotic dinoflagellates of the family Symbiodiniaceae (3–7). Under natural temperature regimes, photosynthetically fixed carbon is shared with the Cnidarian host (8); in return, the coral host provides the Symbiodiniaceae with a source of inorganic nutrients for photosynthesis and growth such as inorganic carbon, ammonia (NH_3), and phosphate (PO_4^{3-}) (9). However, when subjected to stress, this symbiotic partnership can breakdown, resulting in a loss of Symbiodiniaceae from the holobiont (a process known as bleaching; (10)), resulting in detrimental nutritional impacts for the Cnidarian host (11). While several abiotic stressors can cause bleaching, increasing seawater temperatures and more severe marine heat wave events are the primary cause of coral reef bleaching events (12).

Recent work has revealed that the abundance and stoichiometry of trace elements and nutrients are likely important factors contributing to the resilience of both the coral (1,13) and Symbiodiniaceae to stressors (14–16). Manganese (Mn) is a particularly crucial element with diverse biological functions within both the ocean (where it is a limiting trace element in marine primary production (17,18)) and individual organisms due to its role in many essential physiological processes. As with other microalgae, Mn is required by Symbiodiniaceae within the oxygen evolving complex (OEC), the first protein complex in the light-dependent reactions

of oxygenic photosynthesis (14), which during thermal and light stress undergoes degradation and/or upregulation of reactive oxygen species (ROS) (8). Mn is also important as a cofactor of many enzymes, including the antioxidant enzyme manganese superoxide dismutase (Mn-SOD) which is an important ROS detoxifying enzyme for both the animal host and Symbiodiniaceae (13,19). Mn can also play a key role in photoprotection and regulation (20) as well as aid in the maintenance of chlorophyll concentrations (13,14). Additionally, Mn availability has also been shown to impact macronutrient uptake and assimilation, phagocytosis and heterotrophy (21,22) as well as cell signalling (23,24). However, despite the universal importance of certain trace elements, such as Mn, little research has been conducted to determine potentially beneficial concentrations of trace metals within reef systems (16), particularly when compared to relatively well-studied toxicological thresholds (25).

Mn typically exists in low concentrations (approx. $0.06 \mu\text{g L}^{-1}$; (26) in the oligotrophic waters around coral reefs, but concentrations can exceed $11.2 \mu\text{g L}^{-1}$ in some areas (27). At dramatically higher levels (e.g. $700 \mu\text{g L}^{-1}$) Mn can be lethal to coral (28), and the Australian and New Zealand freshwater and marine water (ANZG, 2018) guidelines, provide a current guideline of $80 \mu\text{g L}^{-1}$ for Mn in marine waters. Sources of Mn contamination include mining, metal fabrication, herbicides, fossil fuels, and terrestrial runoff (28). In 2018, Bisc  re et al. demonstrated that Mn concentrations of $4.1 \mu\text{g L}^{-1}$ had a significant positive effect on coral thermal tolerance by maintaining photosynthetic rates and calcification under thermal stress. Building on this study, it was shown that Mn additions of $4.1 \mu\text{g L}^{-1}$ positively impacted the cellular responses of corals to thermal stress through the decreased expression of heat shock proteins (HSPs) (13) as well as increased rates of calcification and photosynthesis (14).

However, it remains unknown whether higher levels of Mn (yet still below toxicological limits) can further benefit Cnidarian-algal physiological performance, and what protein expression patterns support the maintenance of photosynthetic efficiency, optimum physiological functioning, and thus, symbiosis between both host and symbionts under stressful conditions. We therefore addressed these knowledge gaps using *Exaiptasia diaphana*, ‘Aiptasia’, as a model organism to study the effects of four different concentrations of Mn under both ambient and thermal stress conditions.

As for corals, the sea anemone Aiptasia forms a symbiosis with Symbiodiniaceae. However, in contrast to corals, Aiptasia are easy to propagate in large numbers in laboratory environments (29), render symbiont-free (aposymbiotic, e.g.,(30)), maintain genetic homogeneity, and manipulate the symbiont community (31). Furthermore, a wealth of publicly available data exists for Aiptasia, including its genome, proteome, metabolome, and transcriptome (5,32–34), leading to its wide acceptance as a model organism to study unique interactions of the Cnidarian-algal symbiosis (35). In this study we apply proteomic and photophysiological techniques to Aiptasia to assess how differing concentrations of Mn affects the stability of both Cnidarian symbiosis and photophysiology during thermal stress.

2.3 Methods

2.3.1 Experimental setup

Symbiotic *Exaiptasia diaphana* (AIMS 3 genotype) herein called Aiptasia were sourced from the Australian Institute of Marine Science (AIMS, Townsville, Qld, Australia) and its

Symbiodiniaceae was identified as *Breviolum minutum* (32). Aiptasia were grown in 500 mL glass containers held in a water bath maintained at 26 ± 0.3 °C by Inkbird thermostats (INKBIRD Tech. C.L, China). Lighting consisted of Hydra 52s (Aqua illumination, USA) providing $50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of light on a 12:12h light/dark cycle (typical of their shaded, natural environment on the GBR). Aiptasia were grown in custom artificial seawater (35 ppt), consisting of a salt solution with a modified L1 trace component (Supplementary Tables S2.1 and S2.2) that was replenished in each tank at a rate of 1000 mL day^{-1} via Red Sea peristaltic pumps (Red Sea Aquatics, Israel). The modified L1 trace component mix (Supplementary Tables S2.1 and S2.2) was designed to represent oligotrophic reef conditions and allow for element specific manipulations. Media was freshly made every other day and tested via inductively coupled plasma mass spectrometry (ICP-MS) (Supplementary Table S2.3; see methods below). Aiptasia were fed *Artemia* nauplii (instar 1) twice a week (also grown in the modified media) and following feeding the tanks were cleaned to minimise algal growth. Aiptasia were propagated and genetic homogeneity was maintained by cutting them in half when their oral disks reached a diameter of ca. 6-7 mm, and subsequently were left to regrow (29).

The experimental setup consisted of four water baths, two maintained at a temperature of 26 ± 0.3 °C and two that were incrementally temperature ramped to 32 ± 0.3 °C. Each water bath contained four 500 mL glass containers, one for each manganese concentration (0.5 ± 0.2 , 4.8 ± 0.5 , 11.4 ± 0.8 and $15.6 \pm 0.6 \mu\text{g L}^{-1}$), resulting in a total of eight experimental treatments: 26 °C $0.5 \mu\text{g L}^{-1}$ (control), 26 °C $4.8 \mu\text{g L}^{-1}$, 26 °C $11.4 \mu\text{g L}^{-1}$, 26 °C $15.6 \mu\text{g L}^{-1}$, 32 °C $0.5 \mu\text{g L}^{-1}$, 32 °C $4.8 \mu\text{g L}^{-1}$, 32 °C $11.4 \mu\text{g L}^{-1}$, 32 °C and $15.6 \mu\text{g L}^{-1}$. Manganese chloride was chosen because of its natural bioavailability and ability to dissolve and stay relatively stable in solution.

Modified seawater was delivered to each corresponding container at a rate of 500 mL every 12 hours via a peristaltic pump (Red Sea Aquatics, Israel). Sixteen Aiptasia measuring 5-6 mm were placed into each 500 mL container. Two water baths were then ramped up in temperature from 26 °C to 32 °C at a rate of 0.5 °C per day using controllable thermostats (Inkbird, Australia) and titanium heaters (Schego, Germany). The water baths were then held at this temperature for eight days, until a drop of ca. 30% in values of maximum PSII photochemical efficiency (F_v/F_m) was observed in Aiptasia kept in 0.5 $\mu\text{g L}^{-1}$ of Mn.

2.3.2 PAM fluorometry

Maximum photosystem II (PSII) efficiency (F_v/F_m) was determined after a 20-minute period of dark acclimation using a Waltz Diving PAM (Heinz Walz GmbH, Germany). Measurements were performed every second day until the end of the ramping period, and then daily until the end of experiment between the hours of 12-1 pm. To ensure stray light from the Diving-PAM fluorometer did not affect the dark acclimation of other Aiptasia, individuals were placed into smaller 10 mL wells within the same water baths prior to being measured. A 10 mm spacer was utilised on the end of the fibre optic sensor to maintain a standardised distance between the fibre optic and Aiptasia for each measurement performed. The fibre optic was placed above the centre of each Aiptasia ensuring that the whole organism was captured in each measurement with a total of eight Aiptasia measured for each treatment.

2.3.3 ICP-MS analysis

Water samples from each new batch of media were tested for manganese using an Agilent 7700, ICP-MS (Agilent, USA) (Supplementary Table 2.3). Samples were placed into the SPS 4 autosampler MS (Agilent, USA), the sampling tip was rinsed, and the system purged using 2% nitric acid between samples for 30 seconds. To minimise machine drift and build-up of salt within the machine, an argon humidifier (PerkinElmer, USA) was used, and samples were diluted to 1:5 in a 2% ultra-pure nitric acid solution (Seastar, Canada). Rhodium and Yttrium were used as internal standards and calibration curves (0, 1, 2, 4, 8, 16 and 32 $\mu\text{g L}^{-1}$) were created using a 20 component, ICP-MS standard at 10 $\mu\text{g L}^{-1}$ in 2% HNO_3 + Tr HF (ZeptoMetrix, USA). Spike recoveries were performed by adding 10 $\mu\text{g L}^{-1}$ of Mn to four randomised samples at the end of each run to verify that limited machine drift occurred. Blanks and calibration curves were matrix matched using high purity analytical grade chemicals (Sigma Aldrich, Germany) (Supplementary Table 2.4).

2.3.4 Proteomic analysis

Aiptasia were processed similar to Oakley et al. (36). Frozen Aiptasia were washed in 1 mL HPLC-grade water to remove salts before being mechanically homogenised with a Dounce homogeniser in 500 μL HPLC-grade water. The microalgal fraction was separated by low-speed centrifugation (200 $g \times 30$ seconds) and the supernatant, containing host protein, was transferred to a new tube. The algal pellet was frozen at -80°C until later processing with the same method. The host protein was denatured by addition of sodium deoxycholate to a final concentration of 5% w/w, with 100 mM triethylammonium bicarbonate buffer (TEAB, pH 8.5), and 10 mM tris (2-carboxyethyl)phosphine (TCEP) as a reductant. Samples were incubated at 90–95 $^\circ\text{C}$ for 20

minutes and then disrupted using an ultrasonicator probe (20×2 second pulses) to lyse cells and to denature and dissolve proteins. Lipids and pigments were reduced by an ethyl acetate phase transfer, where the sample was agitated with 1 mL ethyl acetate for 1 minute, followed by centrifugation ($10,000\text{ g} \times 1$ minute). The upper, ethyl acetate layer was removed and the process repeated before the residual ethyl acetate was removed by centrifugation under vacuum for 15 minutes. The remaining aqueous protein sample was transferred to a 0.5 mL 30 kDa molecular weight cutoff filter (Amicon Ultra, Merck Millepore) for filter-aided sample preparation (37).

Each protein sample was concentrated in the filter by centrifugation ($14,000\text{ g} \times 20$ minutes), resuspended in 380 μL 100 mM TEAB in the filter, centrifuged again, and the process repeated before resuspending the sample in 400 μL 100 mM TEAB. A 10 μL subsample was acidified and pelleted to remove deoxycholate, then the protein quantified (Qubit 2.0, ThermoFisher Scientific, USA). 100 μg of the protein sample was reduced with 6 mM TCEP (final) at 37°C for 10 minutes, then alkylated with 20 mM acrylamide (final) at room temperature for 20 minutes. The protein was then digested with 2 μg trypsin (Trypsin Gold, Promega) overnight at 37°C in the filter. Peptides were separated from undigested protein by centrifugation ($14,000\text{ g} \times 20$ minutes) and acidified with 1% formic acid (final) and centrifuged ($10,000\text{ g} \times 2$ minutes) to remove deoxycholate. Peptides were then desalted with C18 tips (Omix Bond Elut, Agilent Technologies), dried by vacuum centrifugation, and stored at 4°C until analysis.

2.3.5 Mass spectrometry

Peptides were resuspended in 0.1% formic acid, dissolved at 37°C for 30 minutes, and analysed by liquid chromatography-tandem mass spectrometry. Samples of 250 ng total peptide were

loaded onto an Acclaim PepMap C18, 50 cm, 75 μm inner diameter, 3 μm particle size, 100 $\text{\AA}$ column (#164570, ThermoFisher Scientific) and Ultimate 3000 liquid chromatograph (ThermoFisher Scientific). Peptides were eluted by a 120-minute gradient from 3%–35% buffer B (buffer A: 0.1% formic acid; buffer B: 80% acetonitrile, 0.1% formic acid) at 300 nL min^{-1} and 55 $^{\circ}\text{C}$. Peptides were analysed with a Lumos Tribrid Hybrid mass spectrometer (ThermoFisher Scientific) by electrospray ionisation at a 1.8 kV spray voltage and a resolution of 120,000. The top 20 MS peaks over a scan range of 375–1400 m/z were analysed by the orbitrap, rejecting +1 charge states and with dynamic exclusion enabled (60 s). Peptides were fragmented by collision-induced dissociation and fragments analysed by the ion trap. The instruments were operated with Chromeleon (v7.3.1), Xcalibur (v4.7), and Tune (v4.1.4244) (ThermoFisher Scientific).

Protein identification was conducted using the Andromeda search engine in MaxQuant (v2.2) against protein models derived from the *Exaiptasia diaphana* genome (32) or *Breviolum minutum* transcriptome (38). False discovery rate (FDR) thresholds were set at 1% for peptide and protein search matches, and a minimum of two peptides per protein were required for identification. Searches assumed trypsin digestion with a maximum of two missed cleavages. Oxidation of methionine and acetylation of the protein N-terminus were specified as variable modifications, and carbamidomethylation of cysteine was specified as a fixed modification. The ‘match between runs’ feature was enabled, and peptide-sequence matches were grouped by parsimony and quantified by label-free quantification intensity.

2.3.6 Protein abundance analysis

Protein abundance analysis was as per (36) where all known false matches were removed and all protein label-free quantification intensity values were log₂- transformed. Differentially abundant proteins (DAPs) were then identified using Limma (39) based of a FDR <0.1 and Log₂-FC of >0.25 or <-0.25. SODs (superoxide dismutase) identified from the proteomics were also analysed due to their known link with manganese and antioxidant activities.

2.3.7 Cell counts

Cell counts of Symbiodiniaceae within individual Aiptasia were performed (36). Subsamples of homogenised Aiptasia were diluted to 1:10 with ASW and measured via fluorescence microscopy (IN Cell 6500 HS; GE Health-care, Sydney Australia, ex. 642 nm), and cells were quantified using IN Carta software (GE Life Science) ($n = 4$).

2.3.8 Statistical analysis

All statistical tests (One Way Analysis of Variance (ANOVA), Tukey post-hoc, Individual t tests, Pearsons, Shapiro-Wilk, Levene's and Non-parametric Kruskal-Wallis test) were conducted in R-Studio (Boston, MA). Principal Component Analysis (PCA) plots (Fig. 2.2a, b) and PERMANOVAs were conducted using Metaboanalyst 5.0 (Canada) incorporating all detected proteins using cube root data transformation and auto scaling. Finally, PANNZER2 (40) was used to annotate identified proteins with GO terms by sequence homology. Gene Ontology (GO) term enrichment was then performed using the package topGO (v. 2.56.0, Alexa et al. 2024) in R (v. 12.0 (R Core Team 2021)). GO terms of DAP's (FDR < 0.1) between specific treatments were tested for enrichment ($p < 0.1$) using Fisher's exact test with a node size of 2.

2.4 Results

2.4.1 Symbiont Cell Densities

Symbiodiniaceae cell densities within *Aiptasia* held at 32 °C were generally lower than those held at 26 °C (ANOVA $F = 2.4$ and $p < 0.05$) (Fig. 2.1a), with the 0.5 $\mu\text{g L}^{-1}$ Mn treatment at 32 °C being significantly (Tukey Post-hoc $p < 0.05$) lower than the control (0.5 $\mu\text{g L}^{-1}$ at 26 °C). Manganese had a significant effect on cell densities under thermal stress (ANOVA $F = 4$ and $p < 0.05$) with Tukey Post-hoc revealing that the 15.6 $\mu\text{g L}^{-1}$ treatment was significantly higher ($p < 0.05$) than 0.5 $\mu\text{g L}^{-1}$ (Fig. 2.1a-c) (Supplementary Table S2.5). A moderate but significant correlation ($p < 0.05$, $R = 0.597$, Pearson's rank test) between increasing Mn concentration and symbiont densities was evident for treatments at 32 °C. Symbiont densities in control (0.5 $\mu\text{g L}^{-1}$ at 26 °C) remained unchanged from the start of experiment (Supplementary Fig 2.1) ($p > 0.05$, independent t test).

2.4.2 Photophysiology

All treatments (0.5, 4.8, 11.4 and 15.6 $\mu\text{g L}^{-1}$) at 32 °C exhibited lower maximum quantum yield of PSII (F_v/F_m) values relative to those at 26 °C (ANOVA $p < 0.05$) (Fig. 2.1d,e) (Supplementary Table S2.6). However, the addition of Mn increased F_v/F_m under the thermal stress treatment (Non-parametric Kruskal-Wallis test 9.611 $p < 0.05$ (ANOVA $F = 4.615$ $p < 0.05$) 0.296), but not at 26 °C (ANOVA $p > 0.05$). After 8 days at 32 °C, F_v/F_m values had dropped 24.2% for the 0.5 $\mu\text{g L}^{-1}$ Mn treatment compared to an 8% decrease for the 15.6 $\mu\text{g L}^{-1}$ Mn treatment (Fig. 2.1e).

Mann-Whitney U tests indicated a significant difference ($p < 0.05$) between the lowest ($0.5 \mu\text{g L}^{-1}$) and three highest Mn treatments (4.8 , 11.4 and $15.6 \mu\text{g L}^{-1}$) when subjected to thermal stress. Overall, Mn addition therefore helped to maintain higher levels of photochemical efficiency under thermal stress whilst having no impact at 26°C .

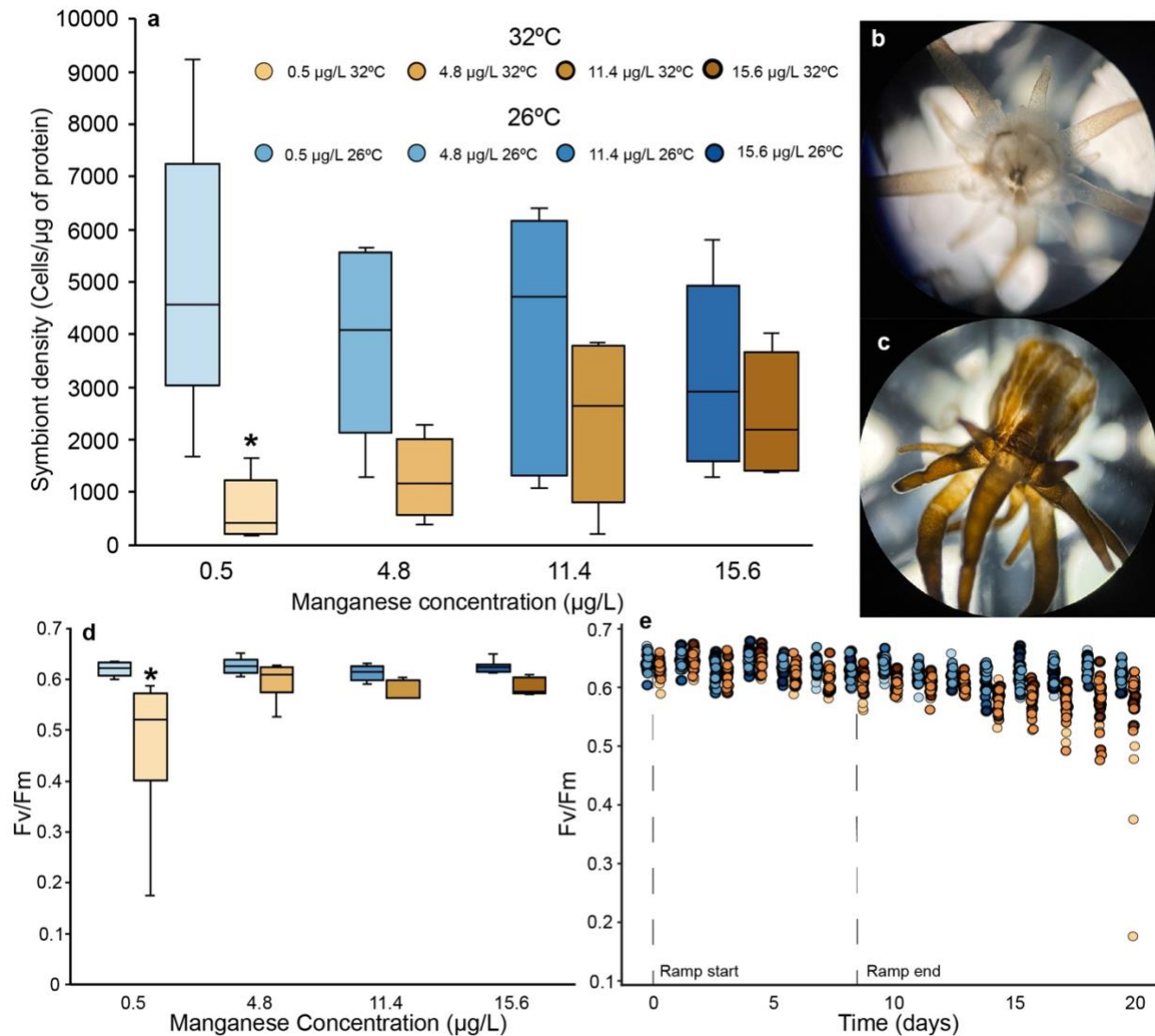


Figure 2.1. Changes in Aiptasia physiology under different levels of manganese (Mn) enrichment and temperature stress. (a) Box plot showing symbiont densities for each treatment (0.5 , 4.8 , 11.4 and $15.6 \mu\text{g L}^{-1}$ at 26 and 32°C) ($n = 6$) at the end of the experiment. Whiskers

indicating the range of data, the box indicating the interquartile range and solid lines indicating median values. (b) Representative photograph of *Aiptasia* maintained at 32°C in 0.5 $\mu\text{g L}^{-1}$ of Mn and (c) Representative photograph of *Aiptasia* maintained at 32°C in 15.6 $\mu\text{g L}^{-1}$ of Mn at the end of experiment. (d) Box plots of average maximum quantum yield of PSII (F_v/F_m) values for each treatment at the end of the experiment ($n = 8$). Whiskers indicating the range of data, the box indicating the interquartile range and solid lines indicating median values. (e) Average F_v/F_m values ($\pm$ SE) for each treatment over the experimental period ($n = 8$). * Indicates significance ($p < 0.05$) between treatments (0.5 $\mu\text{g L}^{-1}$ at 26°C vs 32°C (a) ANOVA) and (0.5 $\mu\text{g L}^{-1}$ vs 4.8, 11.4 and 15.6 $\mu\text{g L}^{-1}$ at 32°C (d) Mann-Whitney U)

2.4.3 Proteomics

A total of 3970 host and 1515 symbiont proteins were detected. Differentially abundant proteins (DAPs) (limma FDR < 0.1 , Log₂- Fold change > 0.25 , < -0.25) were found between temperature treatments (26 °C vs 32 °C) and Mn concentrations at 32 °C (Fig. 2.2) but not at 26 °C. At 32 °C, lower Mn concentrations tended to have more DAPs than higher Mn concentrations compared to the control (0.5 $\mu\text{g L}^{-1}$ 26 °C) for both host and symbiont (Fig. 2.2a-c). This is reflected in PCA plots (Fig. 2a,b) where higher Mn treatments at 32 °C form clusters closer to those at 26 °C. In particular, the 11.4 $\mu\text{g L}^{-1}$ and 15.6 $\mu\text{g L}^{-1}$ treatments at 32 °C for the symbiont proteome appeared to cluster together closer to those at 26 °C than the lower manganese concentrations. PERMANOVAs were conducted where it was shown that both the symbiont ($F = 4.02$, $R = 0.54$ and $p < 0.05$) and host ($F = 3.07$, $R = 0.47$ and $p < 0.05$) proteome were significantly affected by the addition of manganese under thermal stress.

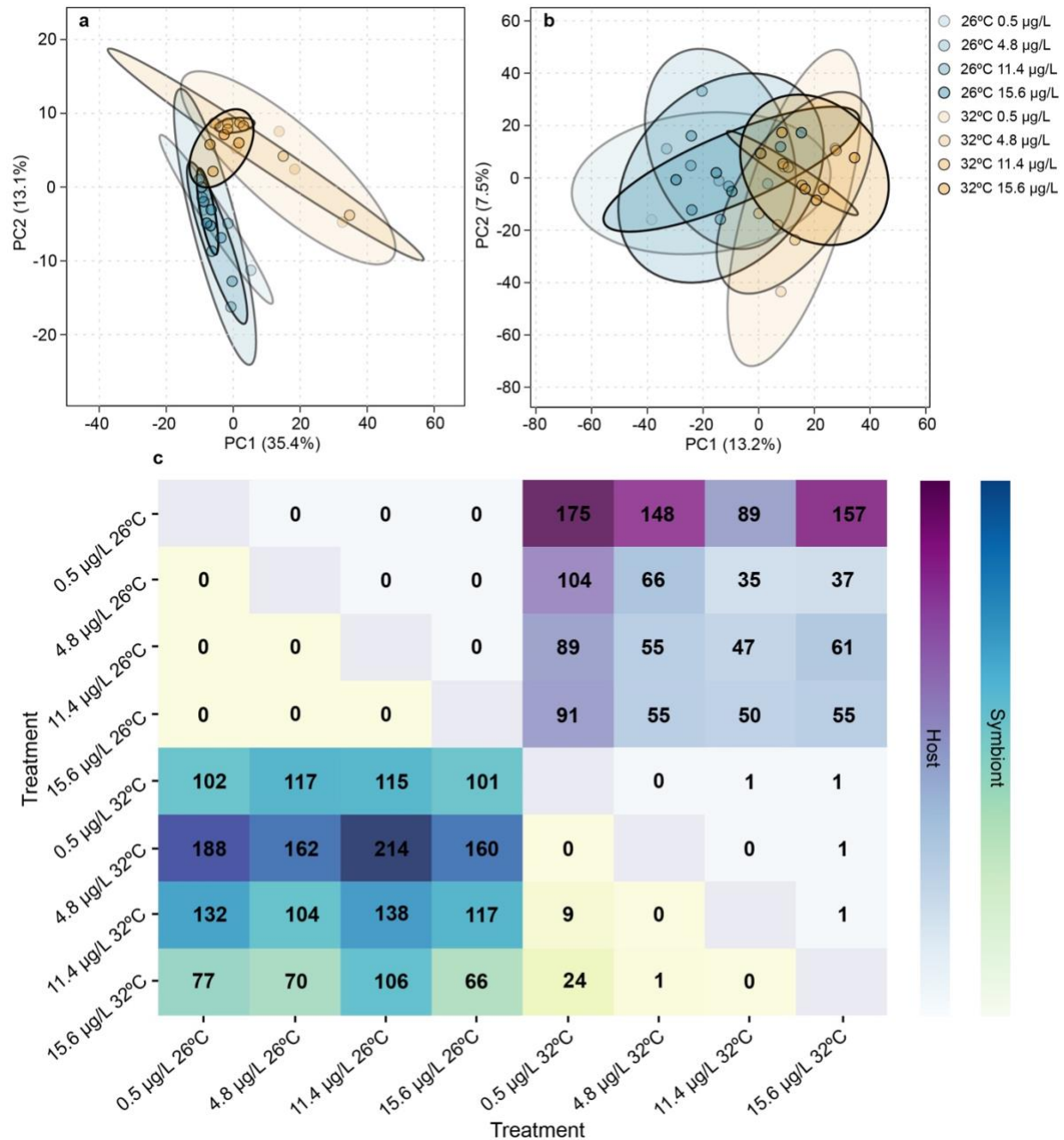


Figure 2.2. Changes in Aiptasia proteomes under different temperatures and manganese concentrations. Principal component analysis (PCA) plots for both (a) symbiont and (b) host protein abundances across all treatments. Gradient indicates manganese (Mn) concentration and colour indicates treatment. (c) Heatmap of differentially abundant proteins (DAPs) (Limma FDR < 0.1, Log₂-Fold change > 0.25, < -0.25) across all treatments for both host and algal symbionts.

2.4.4 Host Proteome

One DAP was found between the Mn treatments at 32 °C, however temperature (26 °C vs 32 °C) had a significant effect with 175 DAPs (Limma FDR < 0.1 Log₂- Fold change > 0.25, < -0.25) observed between 0.5 µg L⁻¹ treatments. HSP 90 alpha-1 and HSP71 were significantly (Limma FDR < 0.1 Log Fold change > 0.25, < -0.25) up-regulated in 0.5 µg L⁻¹ Mn at 32 °C treatments compared to those at 0.5µg L⁻¹ Mn at 26 °C (control), with higher Mn treatments being closer to control levels (Supplementary Table S2.7).

2.4.5 Symbiont Proteome

At 32 °C a total of 24 DAPs were observed between the highest (15.6 µg L⁻¹) and lowest (0.5 µg L⁻¹) Mn treatments in the symbiont proteome (Fig 2.2a,b). DAPs between 32 °C treatments (0.5 – 15.6 µg L⁻¹) in the symbiont proteome consisted of photosynthesis, energy metabolism, protein folding and stress response related proteins (Fig. 2.3a). Heat shock protein (HSP) levels in the symbiont (Fig. 2.3c) were elevated under thermal stress. Interestingly, treatments with the highest Mn concentration (15.6 µg L⁻¹) displayed elevated levels of HSPs compared to the lowest Mn treatment (0.5 µg L⁻¹) (Supplementary Table S2.8).

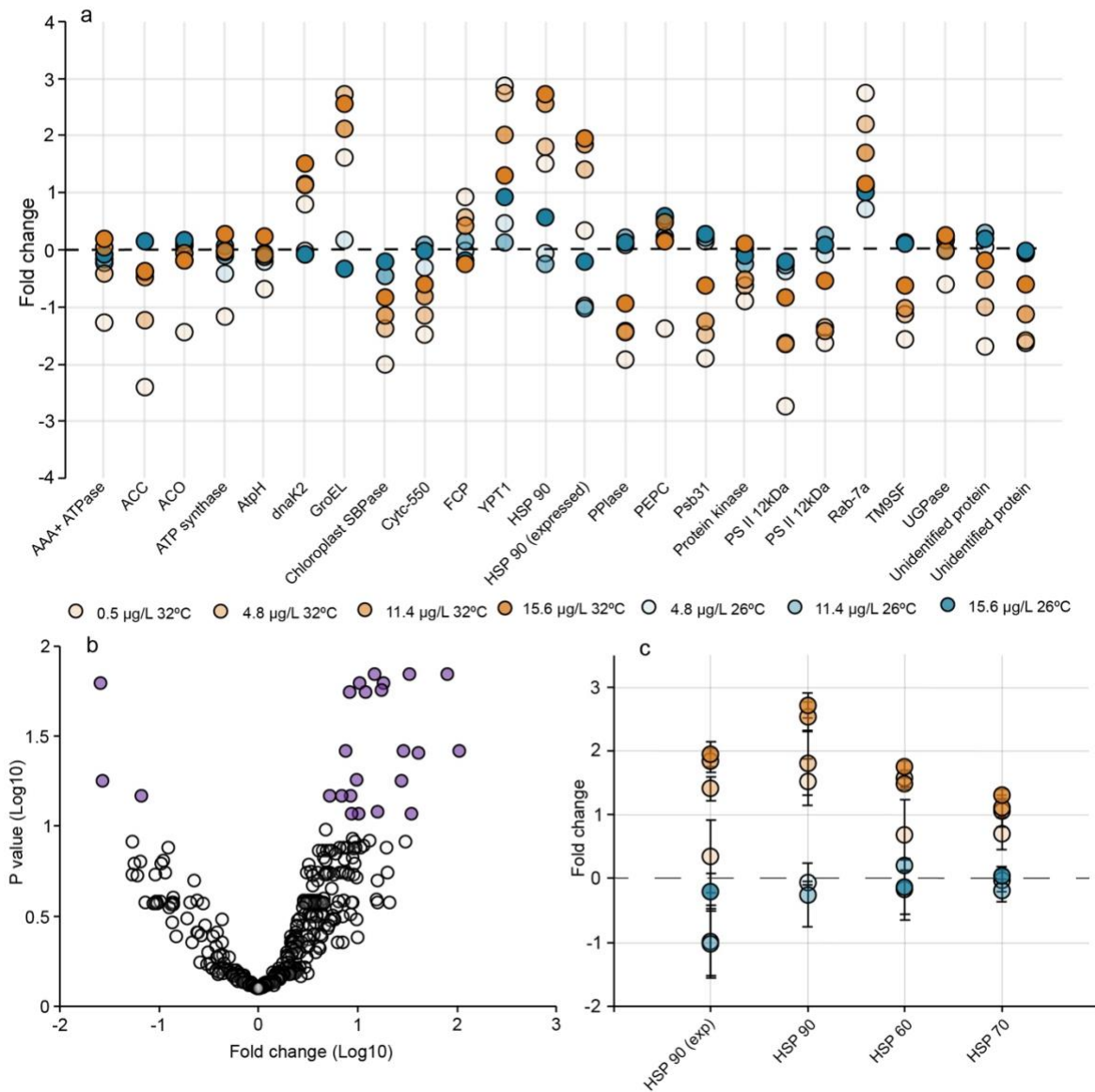


Figure 2.3. Showing differentially abundant proteins (DAP's) and heat shock protein (HSP) abundances between manganese and temperature treatments for *Aiptasia* symbionts. (a) Changes in the proteome of the algal symbiont *Breviolum minutum* that were significantly different between 0.5 and 15.6 $\mu\text{g L}^{-1}$ at 32 °C (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25). Baseline (dotted line) denotes mean protein abundances for control (0.5 $\mu\text{g L}^{-1}$ at 26 °C). (b) Volcano plot of all detected proteins between 0.5 and 15.6 $\mu\text{g L}^{-1}$ 32 °C treatments. Significant proteins are coloured purple (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25). (c) Fold changes for detected heat shock proteins relevant to control (0.5 $\mu\text{g L}^{-1}$ 26 °C denoted by dashed line) with bars indicating Standard Error ($n = 4$).

2.4.6 Gene Enriched Ontology

Gene ontology (GO) analysis was subsequently performed to further understand how treatments influenced the physiological functioning of the *Aiptasia* metaorganism. Enriched GO terms (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25) were compared for both the host and symbiont between control (0.5 µg L⁻¹ at 26 °C) and treatments (0.5 µg L⁻¹ and 15.6 µg L⁻¹ at 32 °C) (Tables 2.1, 2.2 and 2.3). GO terms were sorted into the three main ontologies, “biological processes” (BP), “cellular compartment” (CC) and “molecular function” (MF) with clear differences in GO term classifications being identified between treatments under thermal stress for both the host and symbiont. Addition of Mn during thermal stress had a significant (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25) impact on the abundance and type of enriched GO terms from both the host and symbiont. For symbionts under thermal stress 16 (0.5 µg L⁻¹) and 17 (15.6 µg L⁻¹) GO terms were identified when compared to control (0.5 µg L⁻¹ 26 °C), additionally between 0.5 µg L⁻¹ and 15.6 µg L⁻¹ treatments at 32 °C, 6 GO terms were identified. In the host, zero enriched GO terms were observed between Mn treatments, but were present between control and elevated temperatures. GO Terms between 0.5 µg L⁻¹ treatments consisted of protein folding and structural modification as opposed to the 15.6 µg L⁻¹ treatment at 32 °C which included detoxification terms (Supplementary Table S2.9).

Table 2.1. Gene ontology (GO) terms enriched (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25) between 0.5 µg L⁻¹ and 15 µg L⁻¹ treatments at 32 °C for algal symbionts.

GO ID	GO TERM	DAP'S	P VALUE
Biological Function			
GO:0006457	protein folding	4	0.027
Cellular Compartment			
GO:0005886	plasma membrane	2	0.091
Molecular Function			
GO:0035639	purine ribonucleoside triphosphate binding	10	0.0013
GO:0017111	ribonucleoside triphosphate phosphatase activity	5	0.0242
GO:0140662	ATP-dependent protein folding chaperone	4	0.0024
GO:0051082	unfolded protein binding	3	0.0138

Table 2.2. Selected gene ontology (GO) terms that were enriched (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25) for 0.5 µg L⁻¹ and 15 µg L⁻¹ treatments at 32 °C relative to control (0.5 µg L⁻¹ at 26 °C) for algal symbionts.

GO ID	GO TERM	DAPS	P VALUE
0.5 µg L⁻¹			
Biological Process			
GO:0006457	protein folding	11	0.005
GO:0070887	cellular response to chemical stimulus	5	0.095
GO:0006633	fatty acid biosynthetic process	4	0.013
GO:0046034	ATP metabolic process	4	0.052
GO:0042549	photosystem II stabilization	3	0.001
GO:0015977	carbon fixation	3	0.003
Cellular Compartment			
GO:0009654	photosystem II oxygen evolving complex	3	0.001
GO:0030076	light-harvesting complex	3	0.007
GO:0019898	extrinsic component of membrane	2	0.009
Molecular Function			
GO:0035639	purine ribonucleoside triphosphate binding	26	0.005
GO:0051082	unfolded protein binding	6	0.019
GO:0000287	magnesium ion binding	5	0.055
GO:0140414	phosphopantetheine-dependent carrier activity	2	0.008
GO:0044620	ACP phosphopantetheine attachment site binding	2	0.008
GO:0005200	structural constituent of cytoskeleton	2	0.066

GO:0031072	heat shock protein binding	2	0.066
15.6 µg L ⁻¹			
Biological Process			
GO:0070887	cellular response to chemical stimulus	7	0.025
GO:0006633	fatty acid biosynthetic process	3	0.023
GO:0034514	mitochondrial unfolded protein response	2	0.004
GO:0006556	S-adenosylmethionine biosynthetic process	2	0.004
GO:0045041	protein import into mitochondrial intermembrane space	2	0.004
GO:0009408	response to heat	2	0.019
GO:0006730	one-carbon metabolic process	2	0.031
GO:0006914	autophagy	2	0.031
Cellular Compartment			
GO:0019866	organelle inner membrane	3	0.088
GO:0030076	light-harvesting complex	2	0.035
Molecular Function			
GO:0016887	ATP hydrolysis activity	8	0.011
GO:0009055	electron transfer activity	3	0.085
GO:0004478	methionine adenosyltransferase activity	2	0.004
GO:0044620	ACP phosphopantetheine attachment site binding	2	0.004
GO:0140414	phosphopantetheine-dependent carrier activity	2	0.004
GO:0051087	protein-folding chaperone binding	2	0.034
GO:0031072	heat shock protein binding	2	0.034

2.4.7 Superoxide Dismutase Abundance

SOD abundances were similar across all treatments (temperature and manganese) for the host when compared to control levels ($0.5 \mu\text{g L}^{-1}$ at 26°C). However, in the symbiont, SOD abundance was significantly (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25) lower for 0.5, 4.8 and $11.4 \mu\text{g L}^{-1}$ Mn treatments at 32°C compared to control ($0.5 \mu\text{g L}^{-1}$ at 26°C), whilst the $15.6 \mu\text{g L}^{-1}$ Mn treatment at 32°C maintained abundances of SOD similar to controls.

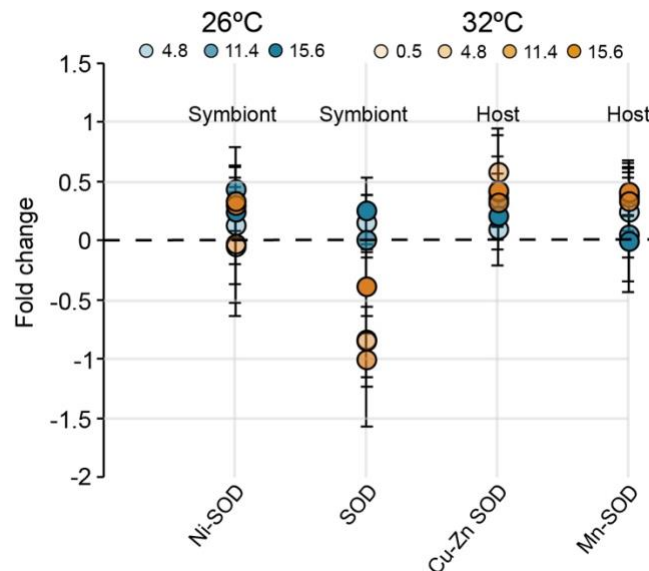


Figure 2.4. Superoxide Dismutase abundances across both host and symbionts under different manganese (0.5, 4.8, 11.4 and 15.6 µg/ L-1) and thermal treatments (26 and 32 °C) when compared to control (0.5 µg/ L-1 at 26 °C) ($\pm$ SD with $n = 4$)

2.5 Discussion

Trace elements are vital for the optimal functioning of many ecosystems (16,41), particularly coral reefs, where essential elements such as Mn are often found in very low concentrations (27). Research into trace elements to date has primarily focused on toxicity (25) with few studies examining beneficial concentrations of these elements and what effects elevated and/or depleted concentrations may have on coral reef taxa (16). We therefore investigated the effects of varying non-toxic Mn concentrations on the model Cnidarian *Exaiptasia diaphana* and its symbiotic microalgae, *Breviolum minutum*, revealing how even small additions in Mn concentration has the capacity to increase their thermal tolerance.

2.5.1 Maintenance of photochemical efficiency and symbiosis

In alignment with previous studies (13,14), *Aiptasia* maintained higher values of F_v/F_m with the addition of Mn under thermal stress, however contrary to these studies, our results indicated that Mn addition maintained algal symbiont densities (Fig. 2.1a,d) (Supplementary Tables S2.5 and S2.6), suggesting that Mn can protect and/or enhance fundamental photophysiological processes required for the transfer of nutrients and ultimate maintenance of symbiosis between algae and host. Therefore, the addition of Mn has the potential to mitigate the impacts of thermal stress in Cnidarians, together with enhancing the efficiency and protection of the photosystems within their algal symbionts (13,14). Similar effects have also been reported in other photosynthetic organisms, where Mn boosts the efficiency and protection of the oxygen evolving complex (OEC) in PSII (20,42–44); this is important as the OEC is often the first target of thermal stress in Symbiodiniaceae (45,46). We found that concentrations of Mn between 4.8-15.6 $\mu\text{g L}^{-1}$ had a beneficial impact, helping to maintain photochemical efficiencies under thermal stress (Fig. 2.1) (Supplementary Table S2.6). F_v/F_m values in the two lowest Mn concentrations at 32 °C generally declined over time with increased exposure to thermal stress, however *Aiptasia* maintained in 4.8 $\mu\text{g L}^{-1}$ of Mn experienced a sudden increase in F_v/F_m (Fig. 2.1e) on the penultimate day of the experiment. Interestingly, this sudden ‘recovery’ of photochemical efficiency coincided with an increase in the rate of symbiont expulsion, suggesting photosynthetic efficiency was potentially increased due to reduced competition for Mn among remaining algal symbionts within the host, aligning with findings of previous studies (13,14,41). The effects of different Mn concentrations were further demonstrated by Iyagbaye et al. (2022) where host and symbiotic algae appear able to partition Mn when exposed to higher concentrations, with the symbiont maintaining a higher concentration than the host tissue (47).

2.5.2 Manganese has a significant effect on the proteome of the Symbiodiniaceae of *Aiptasia* under thermal stress

Consistent with prior studies (36) temperature exerted a pronounced effect on the proteomes of both the host and symbionts (Fig. 2.2a,b). Mn treatments impacted symbionts but not host, suggesting that Mn addition plays a crucial role in preserving the proteostasis of the algal symbiont under thermal stress. Both unique and shared GO terms were identified between 0.5 $\mu\text{g L}^{-1}$ and 15.6 $\mu\text{g L}^{-1}$ Mn treatments at 32 °C, indicating that whilst some processes were common across treatments, Mn addition induced treatment specific changes in the proteome. Enriched GO terms from the symbionts under thermal stress were consistent with those from other studies where terms relating to PSII, protein folding and energy metabolism were all impacted by elevated temperature. Terms such as ‘Photosystem II stabilisation’ and ‘Photosystem II oxygen evolving complex’ were enriched with Differentially abundant proteins (DAPs) under thermal stress at 0.5 $\mu\text{g L}^{-1}$ but not at 15.6 $\mu\text{g L}^{-1}$ Mn (Tables 2.1 and 2.2) compared to control (26 °C), corresponding with F_v/F_m results where treatments at 32 °C with higher Mn concentrations exhibited greater photochemical efficiencies, suggesting that PSII capacity was enhanced by Mn availability. Additionally, GO terms related to protein folding, energy metabolism and other enzymatic activities were enriched between 0.5 $\mu\text{g L}^{-1}$ and 15.6 $\mu\text{g L}^{-1}$ Mn treatments under thermal stress (Table 2.1), indicating that downstream metabolic processes were able to be maintained.

Under thermal stress, only one DAP was found between Mn treatments for the host, however 24 DAPs were observed in the symbiont proteome between the lowest (0.5 $\mu\text{g L}^{-1}$) and highest (15.6

$\mu\text{g L}^{-1}$) Mn concentrations. Interestingly, changes to PSII appeared to be limited to extrinsic proteins with no differences found between intrinsic and core PSII proteins such as D1 and D2 – perhaps partly due to the ability of D1/D2 to be continuously repaired (20). Photosynthetic proteins such as ‘PS II 12kDa’, ‘Psb31’, ‘SBPase’ and ‘Cytochrome C₅₅₀’ were significantly downregulated under thermal stress at $0.5 \mu\text{g L}^{-1}$ when compared to the $15.6 \mu\text{g L}^{-1}$ treatment (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25). Generally, protein abundances at $15.6 \mu\text{g L}^{-1}$ Mn (32 °C) were much closer to those seen under ambient conditions ($0.5 \mu\text{g L}^{-1}$ Mn, 26 °C) (Fig. 2.3a), again suggesting higher levels of Mn helped to maintain functional photosynthetic machinery, particularly the oxygen evolving complex (OEC), as with previous work (20,43) that showed Mn limitation can significantly disrupt the functionality of PSII. Several proteins either incorporate Mn in their functions or work directly in the OEC (48). For example, extrinsic proteins such as cytochrome C₅₅₀ and PS II 12 kDa (PsbU) stabilise the OEC by protecting the Mn cluster from external reductants (49,50) and the presence of PS II 12kDa (PsbU) in *Synechococcus* sp. increased the thermal stability of the OEC (51,52); in the latter case where PsbU may afford protective ability against ROS (53). Both PsbO and Psb31 share common ancestry, however, little is known about the role of Psb31 in higher plants and algae. Limited research suggests Psb31 can serve as a substitute for the Mn stabilising protein PsbO in marine diatoms (54), lending credit to the theory that it arose through functional divergence as a paralog to PsbO.

We observed significant downregulation of these extrinsic proteins (C₅₅₀, PsbU and Psb31), accompanied by no measurable changes in D1 and D2 protein expression and reduced photochemical efficiencies (F_v/F_m) at 32° C and limiting Mn conditions ($0.5 \mu\text{g L}^{-1}$) when

compared to higher manganese treatments ($15.6 \mu\text{g L}^{-1}$, 32°C), suggesting declining photosynthetic capacity may be caused by accumulation of damaged or non-functional OECs.

Other DAPs such as aconitate hydratase B, AtpH protein, acetyl-CoA carboxylase and phosphoenolpyruvate carboxylase represent enzymes involved in metabolic processes such as the Krebs cycle, ATP synthesis, fatty acid metabolism and carbon fixation. All four of these proteins were downregulated in low Mn concentrations ($0.5 \mu\text{g L}^{-1}$) under thermal stress (32°C) when compared to higher Mn levels ($11.4\text{-}15.6 \mu\text{g L}^{-1}$) which showed similar expression rates as Aiptasia kept at 26°C (all Mn treatments) (Limma FDR < 0.1 , Log₂- Fold change > 0.25 , < -0.25) (Fig. 2.3a). Such responses further indicate that these downstream metabolic processes were maintained or enhanced from more efficient photosystem functioning under Mn addition. We also observed upregulation of Rab-7a in our thermally exposed Aiptasia, particularly in lower Mn treatments ($0.5 \mu\text{g L}^{-1}$) where expression rates were significantly higher than those exposed to increased levels ($15.6 \mu\text{g L}^{-1}$) of Mn. Proteins such as Rab-7a (and YPT1) are likely key regulators of vesicular trafficking and are thought to play a direct role in the maintenance of Cnidarian-algal symbiosis through phagocytosis (55,56). Healthy symbionts appear to be able to reject the host derived Rab-7a proteins whilst thermally damaged and photosynthetically impaired algal cells carried this protein (55,56). Interestingly Rab-7a was not detected in over half of the Aiptasia at 26°C thereby supporting previous work that healthy symbionts were less likely to carry this protein.

Overall, heat shock protein (HSP) expression levels were elevated across both host and symbionts at 32°C (all Mn treatments) when compared to 26°C treatments (Fig 2.2c)

(Supplementary Fig. 2.1). Contrary to the host, HSP levels in the symbiont were elevated by addition of Mn under thermal stress, with HSP 90 being significantly higher (Limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25) in the 15.6 µg L⁻¹ treatment compared to 0.5 µg L⁻¹ (Fig. 2.3c). As such, whilst photosynthetic efficiency was higher under elevated Mn (as shown in Fig. 2.3) algal cells may be experiencing a higher demand for protein repair and maintenance processes (Fig. 2.3c) thereby resulting in higher HSP expression amongst Mn-treated *Aiptasia* under thermal stress. Both Biscéré et al. (2018) and Montalbetti et al. (2021) previously showed that corals subjected to increased Mn concentrations exhibit higher thermal tolerance, but no change in SOD activity (13,14). This is interesting as current leading theories suggest that cofactors and enzymes such as SODs play a vital role in the suppression of ROS-induced bleaching. However, similar to previous studies, we observed no significant up-regulation in SOD expression (Fig. 2.5) as a result of either heat stress (57) or Mn treatments (13). Consequently, SOD activity is likely saturated resulting in greater cellular damage from ROS (57) increasing the rate of required repair and folding from HSPs. It has also been shown that HSPs such as Dnak2 can both directly and indirectly support PSII function through the facilitation of protein import, stabilisation and folding in the thylakoid membrane (58,59). The fact that HSP expression levels were reversed for high and low Mn treatments (0.5 µg L⁻¹ vs 15.6 µg L⁻¹ at 32 °C) in the host versus symbiont indicates that although under Mn enrichment the symbiont is exhibiting greater stress responses through elevated HSP expression, it can maintain a better partnership with the host, indicated by lower host HSP expression.

2.5.3 Photosystem II inhibition and repair

Under normal conditions, PSII is in a constant state of damage and repair, with reassembly of the OEC occurring as often as every 15–30 minutes (42). However, increased light and thermal energy can impact the rate of damage and thus, the rate of required PSII reassembly (60). Interestingly, the rate of OEC reassembly, especially the Mn_4Ca cluster, is heavily dependent on cellular concentrations of available Mn^{2+} (42). As PSII breaks down, oxidised Mn ions are lost from the OEC and, without access to free Mn^{2+} , OEC reassembly is inhibited, thus impairing photochemistry and downstream metabolic pathway activity within the symbiont. A drop in photosynthetic metabolic activity inherently reduces the ability of the cell to function and repair, including HSP production (61) (Fig. 2.4a). This was shown in the $0.5 \mu g L^{-1}$ Mn treatment at elevated temperature ($32^\circ C$), where photochemical efficiency, extrinsic protein and HSP abundances were all reduced compared to higher Mn concentrations.

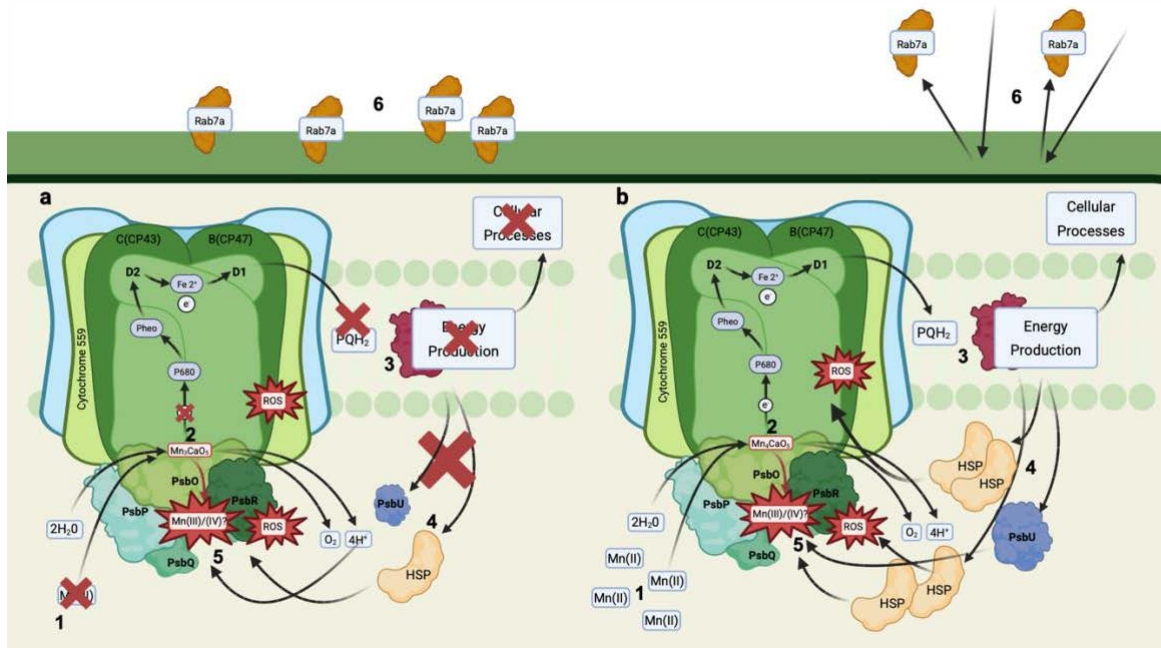


Figure 2.5. Model of Photosystem II ((PSII) depicting hypothesised effects of manganese (Mn) limitation (a) and availability (b) on the oxygen evolving complex (OEC) and downstream processes. Mn-limited environment (a1) and Mn-abundant environment (b1). Loss of Mn ions from the Mn cluster due to thermal stress (a2) and replacement of Mn ions (b2). Resulting loss of

photosynthetic electron transfer and ultimate inhibition of energy production and downstream cellular processes (a3) as opposed to maintenance of electron transport chain, energy production and cellular functioning (b3). Decreased production of heat shock proteins (HSPs) and increased reactive oxygen species (ROS) generation (a4) and increased production of HSPs and PSII extrinsic proteins (b4). Impaired and damaged extrinsic proteins of the OEC are unable to be repaired/folded (a5). Extrinsic proteins can be repaired as a result of increased HSP production and decreased cellular ROS (b5). Increased cellular abundances of host derived Rab-7a proteins leading to phagocytosis/exocytosis (a6). Symbiont can fend off host derived Rab-7a proteins as a result of maintained cellular functioning and thus leading to the maintenance of Cnidarian symbiosis

Our proposed metabolic cascade from Mn limitation would reduce the amount of resource

sharing with the host and the ability of the algal cell to fend off host derived RAB-7a proteins

which are a marker for phagocytosis (55,56), ultimately ending in bleaching (Fig. 2.1b)

(Supplementary Fig S2.2). The results from our study as well as those of (13) support this

hypothesis, as we observed no significant increases in SOD expression above control levels (0.5

$\mu\text{g L}^{-1}$ at 26 °C) (Fig. 2.5) across Mn treatments at 32 °C but significant upregulation of HSPs,

extrinsic proteins and metabolic/energy production proteins, as well as a down regulation in

RAB-7a and YPT1 in high Mn treatments. Further to this, higher expression of Psb31, a known

substitute for PsbO (Mn-stabilising protein) was observed, indicating that the OEC repair

processes were active whilst still maintaining reasonable functionality. Should this theory be

incorrect and SOD did in fact scavenge excess ROS in higher Mn-treated *Aiptasia*, a reduction of

HSP levels alongside an increase in SOD compared to control (0.5 $\mu\text{g L}^{-1}$ at 26 °C) levels should

have been observed, however this was not the case. It was observed in the symbionts under

thermal stress that concentrations of 15.6 $\mu\text{g L}^{-1}$ prevented any significant (limma FDR < 0.1,

Log₂- Fold change > 0.25, < -0.25) decline in SOD activity compared to the control (0.5 $\mu\text{g L}^{-1}$ at

26 °C), however SOD levels were still well below those kept at 26 °C, indicating that levels of

SOD were declining due to the breakdown of other cellular activities and did not increase in line

with theorised increases of ROS.

Despite this, the generation of ROS under thermal stress is known to induce intra and extracellular damage, causing metabolic cascades that play an important role in the breakdown of Cnidarian-algal symbiosis. However, results from both this and previous studies show that it may not be the sole primary cause of holobiont dysregulation (8,11,62). Data here suggests an additional plausible explanation being that the breakdown of the host-symbiont relationship is linked closely to the ability of the algal symbionts to maintain metabolic functioning through continued photosynthetic efficiency, thus maintaining their ability to share metabolites with the Cnidarian host.

It is also important to note that there are many other SOD independent mechanisms for scavenging ROS (catalase, peroxidases, peroxiredoxins and other non-enzymatic antioxidants (63)) that may have played a role in the mitigation of thermal stress. However, our results did not identify any significant differences in their abundances under the conditions tested. Additionally, recent research (8,11,62,64,65) has raised questions about whether ROS is the primary driver of bleaching, suggesting that other processes such as metabolic dysfunction or impaired nutrient cycling may play a more central role. While the ROS model remains widely accepted, these alternative mechanisms warrant further investigation to better understand the complex factors contributing to holobiont breakdown during thermal stress.

2.5.4 Other influences of Manganese

In eukaryotes Mn is vital for mitochondrial ATP synthesis and regulation (66,67) where Mn-dependent enzymes such as pyruvate carboxylase, arginase and Mn-SOD rely on free Mn^{2+} ions to carry out and maintain key metabolic functions (68). Limited information exists on its role within marine taxa but in other organisms Mn is known to substitute/compete with ions such as

Mg²⁺ and Ca²⁺ for binding sites where it can influence mitochondrial function and energy metabolism (69). To date, little to no research has been conducted on corals that combines Mn enrichment and omics analysis, and while we observed no significant proteomics-based changes in these enzymes and proteins this does not imply that low-level Mn enrichment affects PSII only.

2.5.5 Conclusion

Photosynthetic symbiont relationships underpin the existence of modern coral reefs yet are extremely sensitive to environmental changes such as ocean warming. It is therefore important to identify ways in which photosynthetic organisms can both be protected and enhanced as effects of climate change increase. Results from both this and previous studies (13,14,44) have shown that Mn in particular is an essential trace metal directly involved in the photosynthetic apparatus of these autotrophs and has the potential to significantly protect and enhance their performance under thermal stress. Here, we show that maintenance of photosynthetic efficiency and photosynthetic metabolism factors under thermal stress is strongly linked to the concentration of dissolved Mn, with increases in the trace metal directly correlating with enhanced photochemical efficiency and overall holobiont function. This study not only provides empirical evidence of the benefits of increased exogenous Mn availability during thermal stress in Cnidarians but is also the first to characterise its effects at both the physiological and proteomic level in the holobiont. Despite the novelty of this study, future experiments could benefit from additional data such as OJIP analysis and host/symbiont elementomics to help paint a clearer picture on the effect of manganese supplementation. This study provides foundational knowledge that paves the way for future work and for the potential use of manganese supplementation in situ during periods of

thermal stress, with the aim of supporting and increasing the resilience of coral reefs amidst the backdrop of climate change.

2.6 References

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2.6 Supplementary materials

Supplementary Table S2.1. The modified trace metal stock solution recipe.

<i>Chemical</i>	<i>Compound</i>	<i>Stock solution concentration</i>	<i>Amount of stock solution added to trace media solution</i>
EDTA	EDTA		0.008685 g
Zn	ZnSO ₄ ·7H ₂ O	23.00 g/L	0.1 mL
Cu	CuSO ₄ ·5H ₂ O	2.5 g/L	0.17 mL
Fe	FeCl ₂		0.006275 g
Mn	MnCl ₂ ·4H ₂ O	178.1 g/L	0.00125 mL
Ni	NiSO ₄ ·6H ₂ O	2.63 g/L	0.026 mL
V	Na ₃ VO ₄	1.84 g/L	3 mL
Cr	K ₂ CrO ₄	1.94 g/L	0.1 mL
Co	CoCl ₂ ·6H ₂ O	11.9 g/L	0.1 mL
Se	H ₂ SeO ₃	1.29 g/L	0.1 mL
Mo	NaMo	19.9 g/L	0.126 mL

Supplementary Table S2.2. The modified media recipe.

<i>Chemical</i>	<i>Compound</i>	<i>Stock solution concentration</i>	<i>Amount added for 10L of final media</i>
Nitrate	NaNO ₃	75.00 g/L	0.1 mL
Phosphate	NaHP0 ₄ ·H ₂ O	5.00 g/L	0.1 mL
Iodine	Na ₂ SiO ₃ ·9H ₂ O	30.00 g/L	0.1 mL
Vitamins	B ₁₂	0.5mL	0.1 mL
Salt	NaCl		252 g
Hydrous Salts			1000 mL

Magnesium	MgCl ₂ .2H ₂ O	83.76 g/L	
Calcium	CaCl ₂ .2H ₂ O	13.44 g/L	
Strontium	SrCl ₂ .6H ₂ O	0.219 g/L	
Anhydrous Salts			1000 mL
Sulphate	Na ₂ SO ₄	35.51 g/L	
Potassium	KCl	6 g/L	
Bicarb	NaHCO ₃	1.74 g/L	
Bromine	KBr	0.863 g/L	
Boric acid	H ₃ BO ₃	0.230 g/L	
Fluoride	NaF	0.0275 g/L	
Trace Media Solution			10 mL
Manganese Additions			
Mn (5)	MnCl ₂ .4H ₂ O	0.178 g/L	0.853 mL
Mn (10)	MnCl ₂ .4H ₂ O	0.178 g/L	2.247 mL
Mn(15)	MnCl ₂ .4H ₂ O	0.178 g/L	3.12 mL

Supplementary Table S2.3. Target manganese (Mn) concentrations ($\mu\text{g L}^{-1}$). Average ($\pm$ range) achieved concentrations in each new batch of media ($n = 5$), ranges for each treatment over the experimental period ($n = 5$) and mean rates of Mn draw down over 12 hours for each treatment.

<i>Target</i>	<i>Mn concentration ($\mu\text{g L}^{-1}$)</i>			
	<i>0.5</i>	<i>4.1</i>	<i>10.8</i>	<i>15</i>
<i>Concentration in fresh media</i>	0.5 ± 0.2	4.8 ± 0.5	11.44 ± 0.8	15.6 ± 0.6
<i>Concentration after 12 hours</i>	0.5 ± 0.2	3.8 ± 0.5	8.4 ± 2.7	9.0 ± 1.2
<i>Mean draw down</i>	0.1	1.1	3.0	6.6

Supplementary Table S2.4 The recipe for the ICP matrix matching media.

<i>Chemical</i>	<i>Weight (grams)</i>
NaCl	25.2
KCl	0.6
MgCl ₂ .6H ₂ O	8.37
CaCl ₂ .2H ₂ O	1.34
Na ₂ SO ₄	3.55
DH ₂ O	1000

Supplementary Table S2.5. The mean Symbiodiniaceae cells per microgram of host protein $\pm$ standard deviation (SD) ($n = 6$).

		$0.5 \mu\text{g L}^{-1}$	$4.8 \mu\text{g L}^{-1}$	$11.4 \mu\text{g L}^{-1}$	$15.6 \mu\text{g L}^{-1}$
26 °C	<i>Average</i>	5015.2	3837.36	4063.09	3204.35
	<i>SD</i> ($\pm$)	2620.39	1751.46	2375.60	1736.44
32 °C	<i>Average</i>	659.31	1253.17	2346.92	2448.02
	<i>SD</i> ($\pm$)	600.84	732.41	1553.59	1098.10

Supplementary Table S2.6. Mean F_v/F_m values measured at the end of the experiment ($n = 8$) with Standard Deviation (SD).

		$0.5 \mu\text{g L}^{-1}$	$4.8 \mu\text{g L}^{-1}$	$11.4 \mu\text{g L}^{-1}$	$15.6 \mu\text{g L}^{-1}$
26 °C	<i>Average</i>	0.62	0.63	0.61	0.62
	<i>SD</i> ($\pm$)	0.013	0.015	0.014	0.016
32 °C	<i>Average</i>	0.48	0.60	0.59	0.58
	<i>SD</i> ($\pm$)	0.143	0.034	0.016	0.03

Supplementary Table S2.7. Fold changes and SD for heat shock proteins detected in the Mn supplemented hosts relative to control ($0.5 \mu\text{g L}^{-1}$ 26 °C) ($n = 4$).

		26 °C			32 °C			
<i>Manganese concentration ($\mu\text{g L}^{-1}$)</i>		<i>4.8</i>	<i>11.4</i>	<i>15.6</i>	<i>0.5</i>	<i>4.8</i>	<i>11.4</i>	<i>15.6</i>
HSP 90 alpha	Mean	0.02	0.13	0.05	1.02	0.95	0.90	0.62
	SD	0.08	0.19	0.15	0.15	0.10	0.15	0.08
97kDa HSP	Mean	0.04	0.11	0.03	0.45	0.44	0.42	0.22
	SD	0.14	0.12	0.14	0.07	0.08	0.15	0.12
71kDa HSP	Mean	-0.16	-0.01	-0.05	0.55	0.46	0.39	0.45
	SD	0.11	0.09	0.05	0.04	0.07	0.07	0.07
HSP 70	Mean	0.05	0.06	-0.16	0.71	0.60	0.66	0.47
	SD	0.25	0.24	0.21	0.10	0.11	0.09	0.17
HSP 60	Mean	-0.04	-0.04	-0.08	0.23	0.17	0.13	0.12
	SD	0.25	0.12	0.05	0.11	0.10	0.06	0.09

Supplementary Table S2.8. Fold changes and Standard Deviation (SD) for heat shock proteins detected in the Mn supplemented symbionts relative to control ($0.5 \mu\text{g L}^{-1}$ at 26 °C) ($n = 4$).

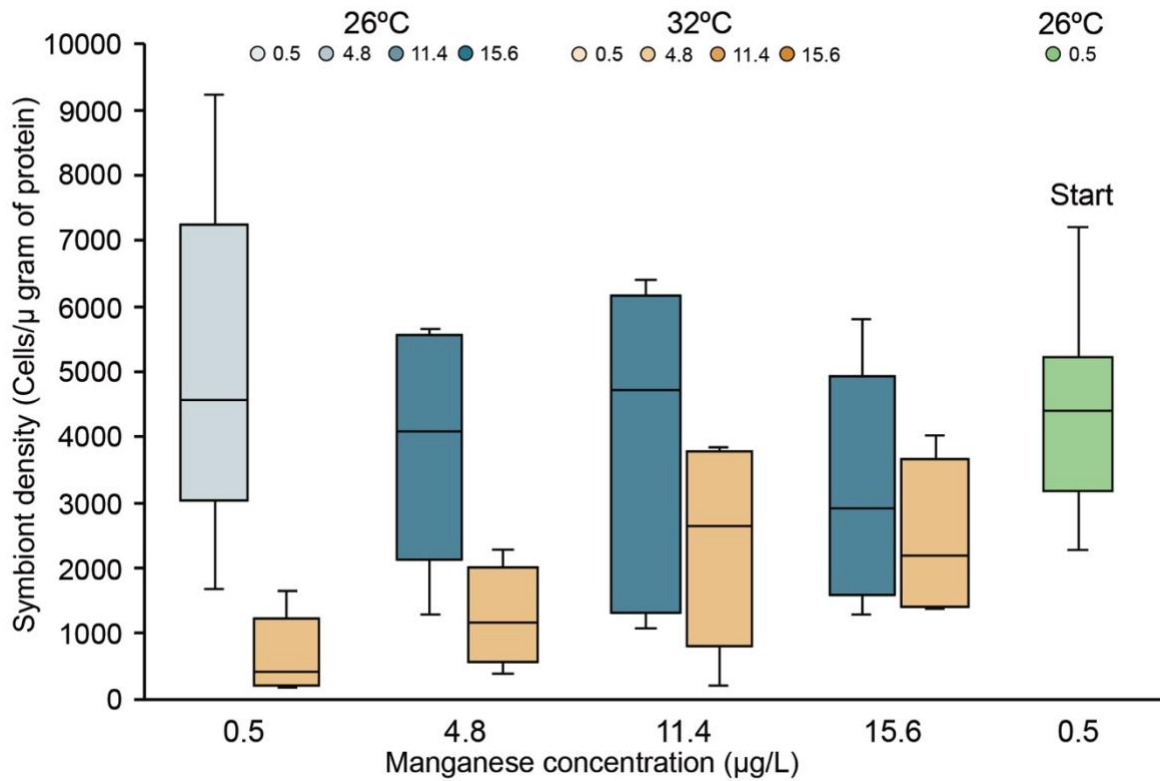
		26 °C			32 °C			
<i>Manganese concentration ($\mu\text{g L}^{-1}$)</i>		<i>4.8</i>	<i>11.4</i>	<i>15.6</i>	<i>0.5</i>	<i>4.8</i>	<i>11.4</i>	<i>15.6</i>

Hsp90 protein, expressed	Mean	-0.99	-1.02	-0.20	0.34	1.41	1.85	1.95
	SD	0.56	0.50	0.28	0.57	0.19	0.19	0.19
Heat shock protein 90	Mean	-0.07	-0.26	N/A	1.52	1.81	2.55	2.72
	SD	0.03	0.49	N/A	0.37	0.49	0.22	0.20
Heat shock protein 60	Mean	-0.16	0.21	-0.13	0.68	1.57	1.49	1.75
	SD	0.48	0.10	0.42	0.56	0.13	0.09	0.09
Heat shock protein 70	Mean	-0.03	-0.19	0.05	0.71	1.06	1.11	1.30
	SD	0.17	0.18	0.14	0.25	0.25	0.14	0.10

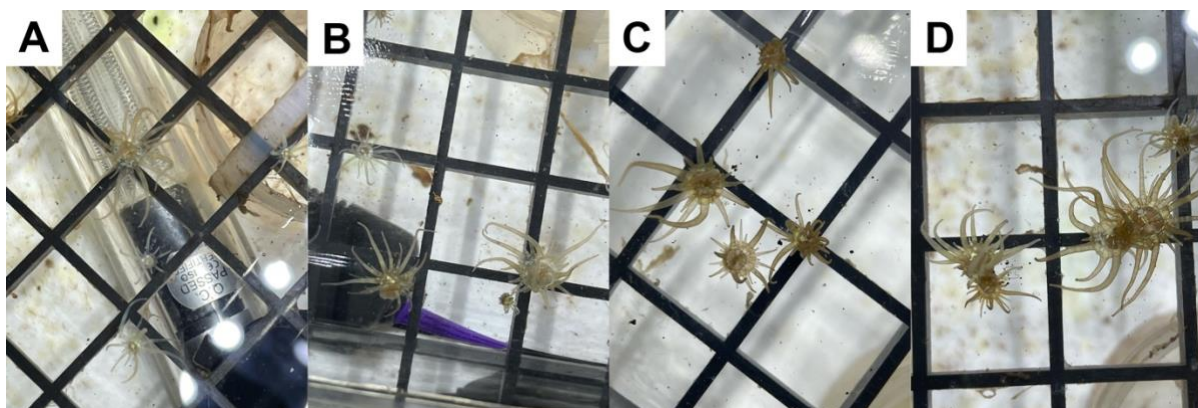
Supplementary Table S2.9. Selected GO terms with two or more significant proteins that were enriched (limma FDR < 0.1, Log₂- Fold change > 0.25, < -0.25) for 0.5 µg L⁻¹ and 15 µg L⁻¹ treatments at 32 °C relative to control (0.5 µg L⁻¹ at 26 °C) for the host.

GO ID	GO TERM	DAPs	P VALUE
0.5 µg L⁻¹			
Biological Process			
GO:0006457	protein folding	6	0.004
GO:0006099	tricarboxylic acid cycle	3	0.017
GO:0006083	acetate metabolic process	2	0.007
GO:0006606	protein import into nucleus	2	0.020
GO:0022900	electron transport chain	2	0.093
GO:0006486	protein glycosylation	2	0.093
Cellular Compartment			
GO:0005759	mitochondrial matrix	3	0.004
GO:0005788	endoplasmic reticulum lumen	2	0.029
GO:0005635	nuclear envelope	2	0.057
Molecular Function			
GO:0051082	unfolded protein binding	3	0.058
GO:0016864	intramolecular oxidoreductase activity, transposing S-S bonds	2	0.016
15.6 µg L⁻¹			
Biological Process			
GO:0006457	protein folding	6	0.003
GO:1901606	alpha-amino acid catabolic process	4	0.018
GO:0006099	tricarboxylic acid cycle	3	0.015
GO:0009063	cellular amino acid catabolic process	3	0.039
GO:0006083	acetate metabolic process	2	0.006
GO:0019805	quinolinate biosynthetic process	2	0.006
GO:0009074	aromatic amino acid family catabolic process	2	0.048
GO:0098869	cellular oxidant detoxification	2	0.073
GO:0022900	electron transport chain	2	0.087
GO:0032271	regulation of protein polymerization	2	0.087
GO:0019748	secondary metabolic process	2	0.088
Cellular Compartment			
GO:0005739	mitochondrion	9	0.003
GO:0005788	endoplasmic reticulum lumen	3	0.003
Molecular Function			
GO:0016491	oxidoreductase activity	21	0.075
GO:0140662	ATP-dependent protein folding chaperone	5	0.000
GO:0031267	small GTPase binding	3	0.027
GO:0050660	flavin adenine dinucleotide binding	3	0.097

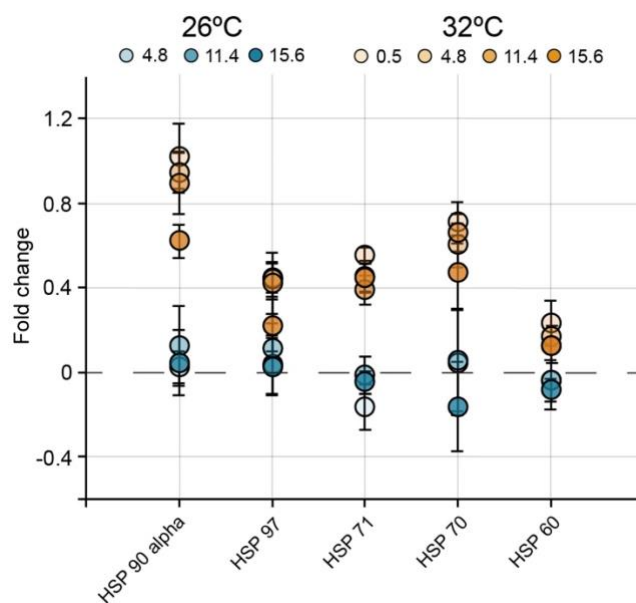
GO:0016864	intramolecular oxidoreductase activity, transposing S-S bonds	2	0.013
GO:0016702	oxidoreductase activity, acting on single donors with incorporation of molecular O ₂	2	0.031
GO:0016620	oxidoreductase activity, acting on the aldehyde or oxo group of donors	2	0.067



Supplementary Figure S2.1. Box and whisker plots showing symbiont densities for each treatment at the end time point ($n = 6$) and at the start of the experiment ($n = 16$)



Supplementary Figure S2.2. Photos showing Aiptasia in different treatments ($0.5 \mu\text{g L}^{-1}$ (a) $4.8 \mu\text{g L}^{-1}$ (b) $11.4 \mu\text{g L}^{-1}$ (c) and $15.6 \mu\text{g L}^{-1}$ (d)) at 32°C at the end of the experiment.



Supplementary Figure S2.3. Showing Fold changes and Standard Deviation (SD) for heat shock proteins found in the host relative to control ($0.5 \mu\text{g L}^{-1}$ 26°C) ($n = 4$).

Chapter 3: A portable multi-taxa phenotyping device to retrieve physiological performance traits.

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3.1 Abstract

Organismal phenotyping to identify fitness traits is transforming our understanding of adaptive responses and ecological interactions of species within changing environments. Here we present a portable Multi-Taxa Phenotyping (MTP) system that can retrieve a suite of metabolic and photophysiological parameters across light, temperature, and/or chemical gradients, using real time bio-optical (oxygen and chlorophyll *a* fluorescence) measurements. The MTP system integrates three well-established technologies for the first time: an imaging Pulse Amplitude Modulated (PAM) chlorophyll *a* fluorometer, custom-designed well plates equipped with optical oxygen sensors, and a thermocycler. We demonstrate the ability of the MTP system to distinguish phenotypic performance characteristics of diverse aquatic taxa spanning corals, mangroves and algae based on metabolic parameters and Photosystem II dynamics, in a high-throughput capacity and accounting for interactions of different environmental gradients on performance. Extracted metrics from the MTP system can not only provide information on the performance of aquatic taxa exposed to differing environmental gradients, but also provide predicted phenotypic responses of key aquatic organisms to environmental change. Further work validating how rapid phenotyping tools such as the MTP system predict phenotypic responses to long term environmental changes *in situ* are urgently required to best inform how these tools can support management efforts.

3.2 Introduction

Photosynthetic organisms underpin the health and functioning of aquatic ecosystems and are increasingly vulnerable to the impacts of climate change^{1,2}. Understanding the intricate interactions between multiple stressors and their effects on the physiological performance of photosynthetic organisms is crucial for predicting their fitness trajectory, survival, and distribution^{3,4}, and in turn the effective management of marine systems. While molecular⁵ and chemical⁶ biomarkers can be used, phenotyping by identifying the expression of photosynthetic traits⁷ possesses a unique advantage that allows use of direct and non-intrusive fluorescence-based metrics^{8–10}. Measurement of chlorophyll *a* fluorescence via active induction protocols has become the standard approach to assess the physiological state and fitness of photosynthetic taxa^{8,9,11} in aquatic systems¹². Most biological pathways of these organisms directly or indirectly depend on photosynthesis as the vast majority of their metabolic requirements are fulfilled through primary production; as such, derived chlorophyll *a* fluorescence parameters can broadly inform organism physiology beyond operation of the photosynthetic machinery^{13,14}. In cases where photosynthetic organisms exist in symbiotic relationships with heterotrophs (e.g., spanning bacteria to invertebrates), employing parallel techniques such as respirometry and chlorophyll *a* fluorescence measurements can be critical to inform complementary fitness traits across multi-taxa partnerships where host metabolism can act discretely from symbiont photophysiology.

Modern aquatic physiological phenotyping tools offer a diverse array of metrics to assess metabolic performance in different environments^{8,10,14}. Common tools include single-turnover and multi-turnover fluorometry protocols^{9,10}, but also hyperspectral imaging¹⁵ and oxygen (O₂)

analysis¹⁶. Phenotyping techniques have been applied in aquatic systems at scales from single cells^{14,17,18} through to entire metazoan individuals⁸. However, high-throughput, multi-analytical phenotyping technology for assessment of organisms within terrestrial systems is far more advanced¹⁹, where rapid on-site phenotyping systems are transforming agriculture practices²⁰; for example, through the identification and selection of water stress tolerant individuals²¹. Recently, a “Coral Bleaching Automated Stress System” (CBASS)^{22,23} was developed to determine thermal thresholds and prospective recovery capacity – both considered key traits (and hence phenotypes) – within an 18-hour period across four distinct thermal cycling profiles, demonstrating high efficacy in resolving differences in coral thermal tolerance²⁴. Beyond the CBASS, however, portable, high-throughput multi-parameter technologies for field assessment of aquatic organisms is lacking. In this work, we address this need by introducing a high-throughput portable phenotyping device that can assess performance of diverse aquatic photosynthetic organisms (algae^{13,17} to holobiont individual²⁵).

Our Multi-Taxa Phenotyping (MTP) system advances a previously described system designed for analysing phenotypes in microalgal cultures using a 96-well plate¹³. The MTP system integrates three well-established technologies for the first time: an imaging Pulse Amplitude Modulated (PAM) chlorophyll *a* fluorometer, custom-designed well plates equipped with optical O₂ sensors, and a thermocycler. This innovative combination is facilitated by an adaptor plate and incorporates customised data acquisition and processing protocols. Collectively the MTP system integrates respirometry metrics with fluorometry-based Photosystem II (PSII) dynamics allowing for a physiological assessment of both partial and entire organisms, thereby broadening application to holobiont (or metaorganism) phenotyping. Having tools that can provide rapid,

real-time, informed decision-making based on observable phenotypic responses to multiple environmental stressors, such as temperature, nutrients, and light⁸ will aid management and conservation of aquatic ecosystems by enabling the identification and selection of resilient individuals that can be utilised in restoration and protection activities²⁶.

3.3 Methods

A Pulse Amplitude Modulation (PAM) imaging fluorometer (Open FluorCam FC 800-O/1010, Photon Systems Instruments, Brno, Czech Republic) fitted with an 8 mm, f/1.4 Manual Iris C-mount lens (VST America, Arlington Heights, IL, U.S.A.) capable of detecting wavelengths 610–780 nm was positioned above a gradient-capable thermocycler (ABI Veriti, Applied Biosystems, Waltham, MA, USA; Fig. 3.1a). The thermocycler was set so that discrete wells were subjected to different temperatures across a user defined gradient (Fig. 3.1b) or held at a single temperature (Fig. 3.1c) depending on the user defined Protocol (see below). Broad spectrum white light was provided by an array of LEDs positioned at a 25° angle and 30 cm distance relative to the MTP sample plate (Fig. 1; Supplementary Fig. S3.1). To integrate the imaging PAM, sample plate and thermocycler for the MTP system, a custom-made adaptor plate was required.

3.3.1 MTP adapter plate

To integrate the thermocycler into the MTP system, a custom-designed adapter plate was manufactured at the University of Technology Sydney using Rigid 10K resin and printed with a Form 2 Desktop SLA printer (Formlabs, Somerville, MA, U.S.A.; Fig. 3.1a). The modified

adapter plate was used to transform a standard 96-well plate into a 24-well plate, combining four adjacent wells with a size of 14 x 14 x 9 mm. Samples of up to 12 x 12 x 8 mm can be adequately contained (Fig. 3.1), enabling assessment of diverse taxa. A Styrofoam insulation ring was applied around the adapter plate to achieve replicable temperatures (see Supplementary Fig. S3.2). The adapter plate was securely attached to the well plate using super glue (Gorilla Glue, Cincinnati, OH, U.S.A.) to ensure watertight conditions. A 2 mm tempered glass sheet, measuring 113–78 mm (Huizhou Konshen Glass Co., Huizhou, Guangdong, China) was recessed within the plate and secured by a custom 3D printed flexible plastic seal (Flexible 80A resin), creating a watertight seal for the plate as well as a mounting surface for the optical O₂ sensors (Supplementary Fig. S3.1). The use of this watertight adapter plate also allowed different chemical gradients to be assessed¹³ (Supplementary Fig. S3.3). Optical O₂ sensors (OXSP5, Pyroscience, Aachen, Germany), with a diameter of 5 mm, were positioned on the underside of the glass lid using clear silicon (All Clear, Selleys, Padstow Australia). These sensors use fluorescence quenching to measure O₂ concentrations and can be directly read by a PAM imaging fluorometer as they utilise an excitation wavelength range of 610–640 nm and an emission wavelength range of 760–790 nm.

Taxa were placed into the wells of the adapter plate, ensuring each sample was not placed directly underneath the O₂ sensor positioned in the corner of each well. The wells were then topped up with seawater to overflowing. The glass lid was then placed on one side and lowered down at an angle and the plastic seal placed around the outside, thus securing the lid to create a watertight seal with no air bubbles trapped inside the well plate (Supplementary Fig. S3.1). When samples were placed in the adaptor plate for O₂ assessment, the plate was filled with 0.2

μm filtered seawater to limit any microbial respiration. Once the adaptor plate was inserted into the thermocycler, one of two protocols were initiated.

3.3.2 Protocol 1

A Multistage Dynamic Photophysiological Response Protocol was developed as a custom PAM Protocol with 3 stages of performance metric retrieval that collectively considered the pre- and post-stress state, as well as performance across a combination of light and/or temperature stress conditions (see Fig. 3.1b).

Stage 1 - (Fig. 3.1b; T0 to T1) consisted of 10 minutes of low light acclimation under $10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ whilst the thermocycler was set to maintain the sample(s) at growth temperature followed by a measurement acquisition of F_q'/F_m' . This provided an initial sample assessment to determine organism photophysiological state prior to initiation of the temperature and light exposure (Fig. 3.1b [T0 to T1]; Supplementary Fig. S3.4).

Stage 2 - (Fig. 3.1b; T1 to T2) consisted of a temperature treatment of the samples under pre-illumination followed by a Rapid Light Curve (RLC). Desired temperatures were then programmed and applied to the thermocycler and samples were maintained at the new temperatures for 10 minutes. A RLC was then initiated, starting with a measurement acquisition of F_q'/F_m' to characterise the effect of temperature exposure independently of light. A sequence of 15 increasing light intensity ($59\text{--}1749 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) steps were applied, with each step applied for 30 seconds (Supplementary Fig. S3.5) yielding the total time for stage 2 of 17.5 minutes. This RLC was used to determine dynamic chlorophyll *a* fluorescence parameters

indicative of photosynthetic performance, specifically: the photochemical efficiency of PSII (F_q'/F_m'), non-photochemical quenching (NPQ), maximum relative electron transport rate ($rETR_{max}$), minimum saturation light intensity (E_K), and the rate of photochemistry in light limiting conditions (α). It was identified that an optimum light step length of 30 seconds was required to ensure $rETR_{max}$ remained high whilst inducing non-photochemical quenching within the samples (Supplementary Fig. S3.5).

Stage 3 - after the RLC completion, the sample was returned to ambient temperature using the thermocycler. Samples were maintained in this state for 10 minutes under low light ($10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and subjected to a further acquisition of F_q'/F_m' (Fig. 3.2a and Fig. 3.3a–b).

From this final F_q'/F_m' measurement any residual NPQ that did not relax during the 10 minutes of stage 3 was determined, thereby providing a measure of the slow-relaxing NPQ (including photoinhibition) post photo- and thermal- exposure (Fig. 3.1b [T2 to T3]). Detailed instrument protocols which include all used settings and time points where the saturation pulses were applied can be accessed online (<https://github.com/HadleyEngland/MTP-Protocol-.git>).

The thermocycler settings for *Protocol 1* consisted of a 10 minute holding period at ambient growth temperature (T0–T1), a 10 minute ramping period where the target temperature gradient was achieved, a 7.5 minute hold at target temperature (T1–T2) and a further 10 minute hold once returned to growth temperature (T2–T3). Well temperatures were measured prior to each experiment to ensure the target temperatures were being achieved using a previously described¹⁷ series of eight micro thermistors (NTC type, EPCOS) connected in a line and controlled by a

microprocessor controller (Uno, Arduino Italy). To measure temperature at T2, the glass lid was briefly lifted, and all sensors were quickly placed into the wells. Each well was measured using two sensors with the mean of the two sensors recorded. This process was performed twice in quick succession, each time starting at a different end of the adapter plate to ensure thermal heterogeneity (Supplementary Fig. S3.2).

3.3.3 Protocol 2

Oxygen Dynamic Response Protocol - was designed to assess O₂ dynamics of taxa under both light and dark conditions, and with the capacity to repeat across varying temperatures (Fig. 3.1c). The initial part of *Protocol 2* involved a 30 minute dark respiration period, followed by a subsequent 30 minute illumination stage at a predetermined light intensity. The light intensity was ascertained from *Protocol 1* and average E_K values for each species were determined using Hennige et al. (2008)²⁷ (Fig. 3.2b). After the adapter plate was placed on the thermocycler the temperature was set to ramp and hold the target temperature for 10 minutes to allow respiration to stabilise and for the thermocycler to reach target temperature. Fluorescence of both the O₂ spot and sample were measured at 3 minute intervals utilising an actinic saturating pulse of light, resulting in a total of 20 measurements during both dark and light phases. Changes in fluorescence of the spots during the light and dark periods (with dark values added to the light values to get gross photosynthesis, see Supplementary Fig. S3.6) were then divided (P_G by R), yielding a photosynthesis (gross, P_G)-to-respiration (P_G:R) ratio (Fig. 3.4a–c). The rETR of each sample was also derived by averaging the rETR measurements from each 3 minute interval (Fig. 3.4d). This facilitated direct comparison between P_G:R and rETR values, enabling a more detailed evaluation of photochemical capacity. Absolute rates of respiration (R)²⁸ (Fig. 3.4e) and

gross photosynthesis (P_G ; Fig. 3.4f) were also achieved by calculating the surface area of each coral fragment using ImageJ²⁹ and carrying out a two-step calibration curve on the optical sensors (Supplementary Fig. S3.7).

O₂ calibration - Prior to the experiment the glass lid with O₂ sensors was placed into a zip lock bag containing ca. 200 mL of 0.2 μm filtered seawater. An aeration stone was then placed inside the bag and nitrogen was bubbled through the water for one hour to completely purge dissolved O₂ and create a 0% O₂ seawater solution. After one hour the air stone was removed, along with any excess nitrogen and the bag sealed tight. The bag containing the glass lid was then placed underneath the imaging PAM where the fluorescence of each individual dot was recorded. The glass lid was then removed, fully dried, and placed under the imaging PAM where another measurement was taken, yielding a 100% O₂ saturated fluorescence reading for each sensor. Using these fluorescence readings as calibration points, the exact O₂ concentrations of each well could then be calculated based on the changes in fluorescence of each sensor over a given time. Returned values were normalised to the corresponding temperature and salinity dependant solubility of O₂²⁸, as well as sample surface area and incubation time to provide a rate measurement ($\mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$) to obtain absolute rates of photosynthesis and respiration across different temperatures.

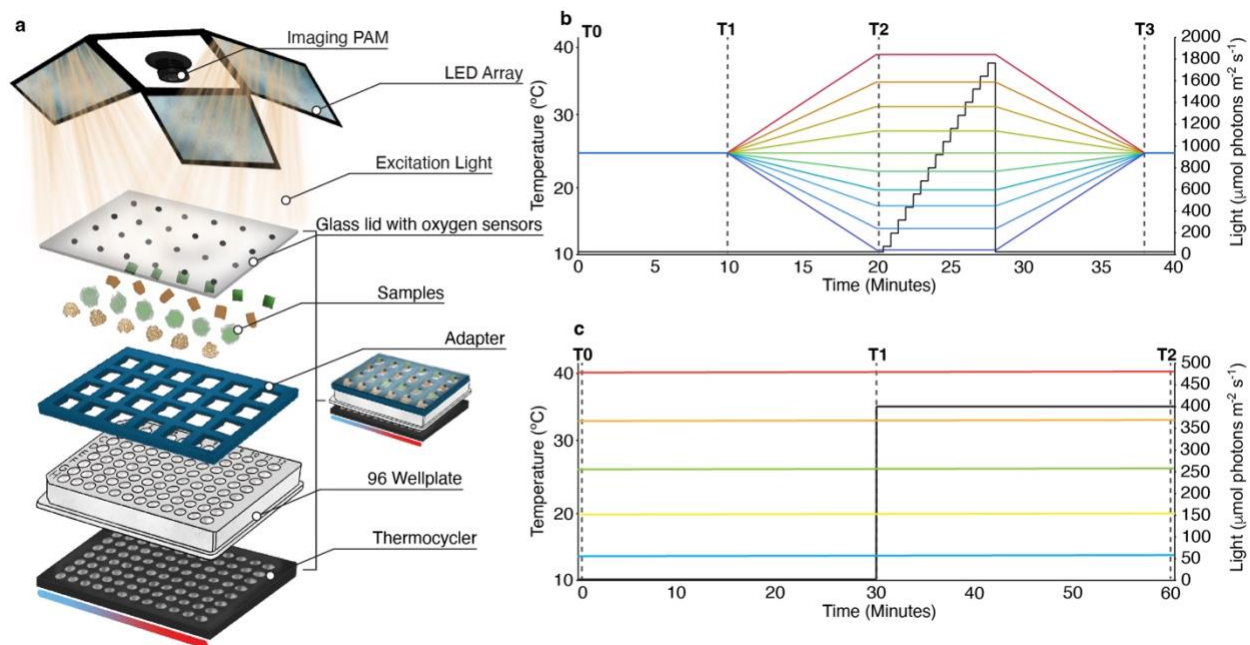


Figure 3.1. The Multi-Taxa Phenotyping (MTP) system and protocols (a) Schematic representation showing the MTP system consisting of the fluorometer imaging device (top), plate assembly (centre) and thermocycler (bottom). (b) *Protocol 1* - a Multistage Dynamic Photophysiological Response Protocol - showing the temperature treatment profile and sample illumination steps for each stage of the procedure. Coloured lines (blue to red gradient) depict temperature profiles, the black line shows illumination treatment (ranging from 10-1749 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and dotted lines show the three critical time points (stages) of the protocol. The different segments of the protocol are: T0 to T1 where sample is maintained at its environmental temperature in low light; T1 to T2 represents the start of temperature gradient and acclimation in low light; T2 to T3 is the rapid light curve (RLC) and the subsequent return of the sample temperature back to initial values from T0 at the end of the RLC; T3 is the final measurement point. (c) *Protocol 2* - *Oxygen Dynamic Response Protocol* - used to assess O₂ dynamics of taxa. Black line indicates light intensity and coloured lines represent a range of potential temperatures applied to the adapter plate depending on user requirements. T0 to T1 is the dark phase where respiration is measured and T1 to T2 is the light phase where photosynthesis is measured.

3.3.4 Multi-taxa application of the MTP system

An initial validation step of the MTP system was to assess whether hypoxic conditions occurred due to the chamber size relative to taxa biomass used. We undertook a series of O₂ drawdown assessments²⁸ on Scleractinian coral. Coral fragments of varying species (*Pocillopora damicornis* and *Seriatopora hystrix*; sourced from the Great Barrier Reef, Queensland (Great

Barrier Reef Aquatics, Cairns)) and biomass (ca. 0.05–0.2 g; Supplementary Fig. S3.7a–b) were placed in the sample plate and incubated in the dark for 45 minutes. O₂ drawdown was measured using the optical O₂ sensors (OXSP5, Pyroscience, Aachen, Germany; Supplementary Fig. S3.7c). The 45 minute timeframe was selected as it was double that of the dark period in any of the MTP Protocols, and thus presented a conservative estimate of O₂ depletion within wells of the MTP system. From this validation it was confirmed that no hypoxic or anoxic conditions (e.g., O₂ sustained below 2 mg L⁻¹ of O₂⁴³) occurred during the Protocols (Supplementary Fig. S3.7) providing confidence in proceeding with further application of the MTP system.

The MTP system was trialed on multiple taxa. Coral (*Acropora kenti*) was sourced from the Great Barrier Reef, Queensland (Great Barrier Reef Aquatics, Cairns) and maintained at the University of Technology Sydney (UTS) aquarium facility for six months under conditions similar to their natural environment³⁰. Mangrove leaves (*Avicennia marina*) were sourced from Towra Aquatic reserve Sydney (under permit #P22/001AR-TP and #P20/0027-1.0) and macroalgae (*Ulva lactuca* and *Sargassum verruculosum*) were sourced from Coogee Beach, Sydney (-33.9215, 151.2585) and stored at the UTS seaweed facility for two days before sampling. As the MTP system was trialed on both temperate and tropical marine taxa, a range of 14–38.6 °C was determined as optimal to retrieve Thermal Performance Curves (Fig. 3.2 and Fig. 3.4). Consequently, the 24-well plate adaptor allowed for six discrete temperatures to be tested simultaneously (Fig. 3.1). As such, we opted to run the MTP *Protocol 1* twice at two complimentary thermal gradients (14–26 °C and 26–38.6 °C), to populate a higher resolution TPC when testing multiple taxa from broad thermal ranges (Fig. 3.2). Prior to use, the temperature was mapped in each well to confirm the exact temperature exposure taxa were

subjected to. Sampling of all taxa was performed between 11 am – 2 pm to minimise the potential effects of circadian rhythm upon photobiology and metabolism³¹. When sampling *A. kenti*, *A. marina*, *S. verruculosum* or *U. lactuca*, small 8–11 mm samples were taken and placed directly into the sample plate together with 0.2 µm filtered seawater from their respective holding aquaria. Mangrove leaves (*A. marina*) were cut into 10 x 10 mm squares and placed into the wells which were filled with distilled water to alleviate any osmotic stress that could occur from using salt water. Once all samples were loaded into the well plate, the glass lid was placed on top, sealing each well and ensuring no air bubbles were trapped under the glass. The plate was then placed immediately onto the thermocycler and *Protocol 1* was initiated (Fig. 3.1b).

A further experiment was undertaken on coral to evaluate how phenotypes derived from the MTP system compared to those obtained utilising the commonly applied CBASS^{22,23} system, noting the two systems have fundamentally different aspects, such as thermal range, ramping rate and extracted photophysiological parameters. Three genotypes of two coral species (*A. kenti* and *P. damicornis*) sourced from the Great Barrier Reef, Queensland (Great Barrier Reef Aquatics, Cairns) were subjected to both the MTP and CBASS protocols. The CBASS assay entailed an 18-hour acute heat stress assay, consisting of a control temperature at 27 °C and three treatments at 30, 33 and 36 °C which were ramped to target temperatures from control over three hours, held at target temperatures for three hours, then ramped down over one hour at which point F_v/F_m values of samples were measured^{22,23}. Fragments of the same six genotypes for both coral species were concurrently analysed using the MTP system and *Protocol 1* with the defined temperature range 23.1 °C to 36.4 °C (Fig. 3a–d). F_q'/F_m' results from T3 of *Protocol 1* of the MTP system were chosen to compare with F_v/F_m values from the CBASS assay as they both

indicated the efficiency and/or damage to PSII after being exposed to a thermal treatment. To assess how low light pre-illumination affected photochemical efficiency under ambient conditions, an additional test of three coral species, *Montipora capricornis*, *Montipora digitata* and *A. kenti*, ($n = 8$ per species), was undertaken (Supplementary Table S3.1).

A final experiment assessed how O₂ dynamics were impacted across a temperature gradient for three coral species (*M. capricornis*, *M. digitata* and *P. damicornis*; $n = 8$ replicates per species). To do this, the above species were exposed to four distinct temperatures (15, 26, 32 and 35 °C). Relative electron transport rates (rETR; Fig. 3.4d), respiration (R; Fig. 3.4e) and gross photosynthesis (P_G; Fig. 3.4f), as well as P_G to R ratios (P_G:R; Fig. 3.4a–c; Supplementary Table S2.2) were calculated using *Protocol 2* of the MTP system.

3.3.5 Data analysis

O₂ dynamics were assessed by one-way Analysis of Variance (ANOVA) and Pearson's Correlation tests conducted in RStudio (RStudio Team (2024) Boston, MA) Assumptions of normality and equal variance were met and checked using Shapiro-Wilk and Levene's tests respectively. Photophysiological parameters F_q/F_m' (T3), rETR_{max} (T2 RLC), residual NPQ (T1 – T3), NPQ_{max} (T2 RLC) and α (T2 RLC) were extracted from the fluorescence images using the FluorCam software. Individual wells were selected in post-processing with all excited pixels within them being averaged and analysed. E_K values were calculated using F_q/F_m' results obtained across the RLC and modelled using an exponential function (Hennige et al. 2008)²⁷. Residual NPQ was calculated from F_m (T1) and F_m' (T3; Fig. 3.1b). Principal Component Analysis plots (PCAs; Fig. 3.3e–f) were created in Metaboanalyst 5.0 (Canada) using cube root

data transformation and auto scaling, incorporating the five above mentioned photophysiological parameters. rETR heatmaps (Fig. 3.2c) were generated using Origin Pro (Northampton, MA, USA) and data smoothed between temperature points.

Thermal performance curves (TPCs) for rETR_{max} values (Fig. 3.2d) were derived using the Gaussian model for all four taxa and created using RStudio. For each taxon, four TPCs were created giving an $n = 4$. From each TPC, T-min and T-max were calculated and indicate the points where rETR_{max} drops to 30% of the organisms maximum rETR_{max} value, thus giving both a low and high thermal limit. Using these values, thermal ranges could then be established by subtracting T-min from T-max. T-opt (the optimal temperature at which the maximum relative rate of photosynthetic electron transport is observed) was also calculated from this model for each taxon. ANOVA and Tukey's post-hoc tests were also conducted on outputs from the Gaussian model (Table 3.1, Supplementary Table S3.3). Mean α values were plotted and TPCs for each taxon were generated using the Briere model^{32,33} (Fig. 3.2e; $n = 4$ per data point) to retrieve T-opt (the temperature at which the maximum rate of photosynthetic efficiency is seen under light limiting conditions). To establish thermal thresholds for each taxon (Fig. 3.2f and Fig. 3.3g–h), F_q'/F_m' values from T3 of *Protocol 1* were modelled using a log-logistic regression model in RStudio^{22,23} to determine the ED50, defined as the temperature at which observed F_q'/F_m' values from T3 decreased by 50% from the modelled median; in doing so, F_q'/F_m' self-normalises for any variance in magnitude inherent across samples. A Tukey's HSD test was used to compare the photosynthetic efficiency measurements retrieved for corals using the MTP and CBASS methods at 36.5 °C and 35.6 °C respectively (Fig. 3.3a–d).

3.4 Results

3.4.1 Multi-taxa application

We analysed four different taxa, *Acropora kenti* (coral tissue fragment), *Avicennia marina* (mangrove leaf), *Sargassum verruculosum* (brown macroalgae), and *Ulva lactuca* (macroalgal frond) to assess the MTP system's ability to extract temperature dependent photophysiological performance metrics across a range of photosynthetic organisms. Prior to any taxa analysis, repeat MTP runs without any biological test material in the wells demonstrated stability in the temperature of wells, with maximum variation of 0.11 °C across three runs at two different temperatures (15 °C and 32 °C). For the multi-taxa comparison, ED50 values ranged from 37.1 °C for *A. kenti* to 44.3 °C for *A. marina* (Fig. 3.2f). Modelled TPCs (Fig. 3.2d; Table 3.1) based on $rETR_{max}$ yielded T-opt that was highest for *U. lactuca* and lowest for *A. kenti* (Supplementary Table S3.4). Taxon ranking based on the F_q'/F_m' derived ED50 (Fig. 3.2f) and $rETR_{max}$ derived T-max values for T2 (Fig. 3.2d, Table 3.1) were the same. T-min and T-max were generally distinct between each taxon ($P < 0.01$; Table 3.1), with thermal range (T-max – T-min) the widest for *A. marina* and narrowest for *S. verruculosum* (Fig. 3.2d, Table 3.1, Supplementary Table S3.4). Thermal range for *A. marina* (23.7 °C) and *U. lactuca* (22.0 °C) was significantly wider than for *A. kenti* (14.9 °C) and *S. verruculosum* (14.5 °C; Tukey HSD < 0.01 ; Supplementary Table S3.3). TPCs based on α indicated a range in T-opt of 22.5 °C–30.2 °C, where T-opt was lowest for *A. marina* and highest for *S. verruculosum*. Finally, E_K values for all taxa were highly variable with temperature (Fig. 3.2b).

Table 3.1. Comparison of mean extracted parameters (rETR_{max}, T-opt, T-min, T-max and thermal range) from a Gaussian thermal performance curve for rETR_{max} of four different taxa (*A. kenti*, *A. marina*, *U. lactuca* and *S. verruculosum*) across a thermal gradient (14–38.6 °C). Mean and standard error (SE) are reported for each parameter ($n = 4$). One-way Analysis of Variance (ANOVA) results are reported for Gaussian model outputs for each parameter to identify significant differences between taxa ($P < 0.05$). Full list of Tukey’s post-hoc results can be found in Supplementary Table S3.3.

Taxon		rETR _{max}	T-opt	T-min	T-max	Thermal range
<i>A. kenti</i>		91.0 ± 3.21	24.7 ± 0.20	17.3 ± 0.37	32.2 ± 0.20	14.9 ± 0.43
<i>A. marina</i>		145.0 ± 5.76	26.8 ± 0.93	14.9 ± 1.31	38.7 ± 0.63	23.7 ± 0.87
<i>U. lactuca</i>		95.8 ± 2.40	27.6 ± 0.44	16.6 ± 0.79	38.6 ± 0.61	22.0 ± 1.09
<i>S. verruculosum</i>		50.5 ± 1.78	27.2 ± 0.23	20.0 ± 0.44	34.5 ± 0.55	14.5 ± 0.89
ANOVA	p-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
	f-value	42.20	5.86	6.50	37.53	31.08

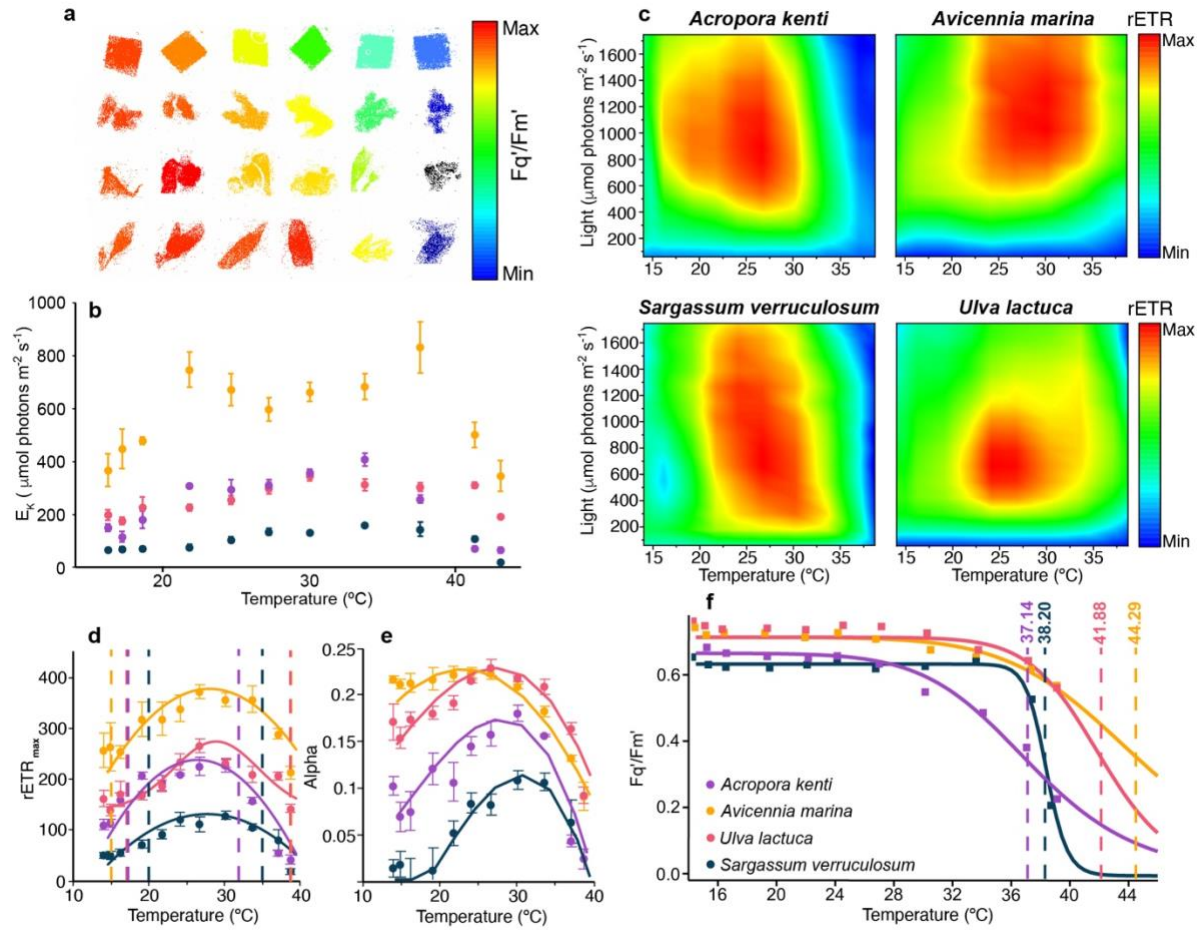


Figure 3.2. Multi-taxa data collected using the MTP system *Protocol 1*. (a) Composite image of four taxa (in rows from top to bottom; *A. marina*, *A. kenti*, *U. lactuca* and *S. verruculosum*) showing effective quantum yield of PSII (F_q'/F_m') values across a temperature gradient (left to right) taken at T3 (see *Protocol 1* in Fig. 3.1b). (b) Scatter plot showing E_K values for *A. kenti*, *A. marina*, *U. lactuca* and *S. verruculosum* across a temperature gradient (14 °C to 38.6 °C). $n = 4$ per data point with standard error of mean shown. (c) Heatmaps showing relative electron transfer rates (rETR) across a gradient of light intensities and temperatures for the four taxa. (d) Maximum rETR values recorded during the RLC ($n = 4$, plots show mean $\pm$ standard error). Dashed lines indicate T-min and T-max values. (e) Alpha (α) values determined from the RLC ($n = 4$, plots show mean $\pm$ standard error). (f) F_q'/F_m' values recorded at T3 on *Protocol 1* with dashed lines indicating ED50 values for each taxon ($n = 4$ biological replicates).

MTP and CBASS derived phenotypes – Phenotypic response patterns were similar across both methods, except for *P. damicornis* genotypes 1 and 2 (P1 and P2, Fig. 3.3g–h) where the MTP but not CBASS extracted a small difference in thermal thresholds. Both methods identified *A. kenti* genotype 2 (A2) and *P. damicornis* genotype 3 (P3) with higher F_v/F_m (CBASS) and F_q'/F_m' (MTP) values than for other genotypes within the same species under thermal stress of 36.5 °C (MTP) and 35.6 °C (CBASS) ($P < 0.05$, Fig. 3.3a–d, Supplementary Table S3.5). The MTP further identified A2 and P3 as a distinct grouping together in the 36.5 °C PCA analysis (Fig. 3.3f) that incorporated a variety of parameters extracted from MTP *Protocol 1* (Fig. 3.3i–l)

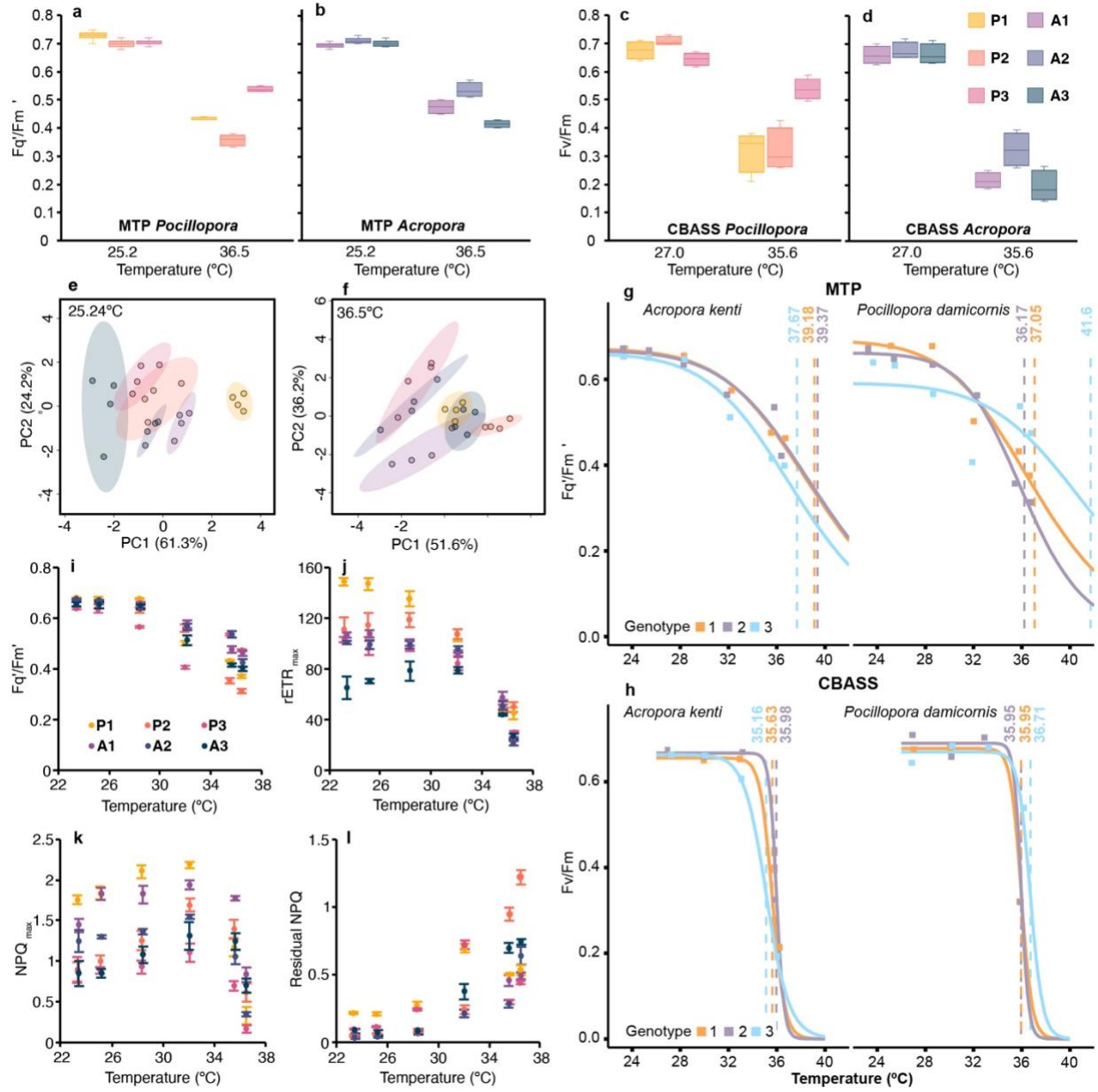


Figure 3.3. Phenotypic differences in photophysiological parameters extracted from corals using the MTP system. Photophysiological parameters are presented for two species ($n = 3$ genotypes) *P. damicornis* (P1, P2, P3) and *A. kenti* (A1, A2, A3) across a temperature gradient. (a and b) Photosynthetic efficiency of PSII for *P. damicornis* and *A. kenti* when exposed to 25.2 °C and 36.5 °C for MTP (taken from T3, Protocol 1) and (c and d) for CBASS at 27.0 °C and 35.6 °C. (e and f) Principal Component Analysis (PCA) plots incorporating F_q'/F_m' , $rETR_{max}$, α , NPQ_{max} and residual NPQ values across two different temperatures; groupings of the same genotype are depicted by coloured ovals. (i – l) show genotype data presented as mean $\pm$ standard error ($n = 4$ replicates per temperature). (i) F_q'/F_m' values taken from T3. (j) $rETR_{max}$ values taken during the rapid light curve. (k) NPQ_{max} values taken during the rapid light curve. (l) Residual NPQ values

from T3. (g and h) Log logistic regression models fitted for each species (*P. damicornis* and *A. kenti*) for the MTP system and CBASS ($n = 4$ replicates per temperature point).

3.4.2 Coral oxygen dynamics across a thermal gradient

$P_G:R$ for all three species were highest at 26 °C before all falling below one at 35 °C (Fig. 3.4a-c; Supplementary Table S3.2). Both *M. capricornis* and *M. digitata* exhibited higher mean $P_G:R$ (4.72 and 4.81) than *P. damicornis* (3.39) at 26 °C ($n = 8$). Average rETR values reached a maximum for *M. capricornis* and *P. damicornis* at 26 °C and 32 °C for *M. digitata* (Fig. 3.4d). Respiration rates (R) generally increased with temperature for all species (Fig. 3.4e) and were highest at 35 °C for *M. capricornis* and *M. digitata* compared to 26 °C for *P. damicornis*. There was a strong correlation between R and P_G at 15 °C ($r = 0.95$, $P = 0.001$), 26 °C ($r = 0.95$, $P = 0.001$), 32 °C ($r = 0.96$, $P = 0.001$) and 35 °C ($r = 0.98$, $P = 0.001$). Values of P_G were highest for all three species at 26 °C before rapidly declining at 35 °C. P_G for all species was directly compared to average rETR values across a temperature gradient of 15–35 °C (Fig. 3.4g). Both *M. capricornis* and *P. damicornis* showed correlation between P_G and rETR ($r = 0.68$, $P < 0.05$ and $r = 0.69$, $P < 0.05$), but less correlation occurred for *M. digitata* ($r = 0.36$). Higher $P_G:R$ correlated with higher rETR values across the four temperatures ($r = 0.63$, $n = 96$, $P < 0.05$; Fig. 3.4h) with the highest rates for both parameters occurring at 26 °C and the lowest at 35 °C. The rETRs were highest at 26 °C for *M. capricornis* and *P. damicornis*, compared to at 32 °C for *M. digitata*.

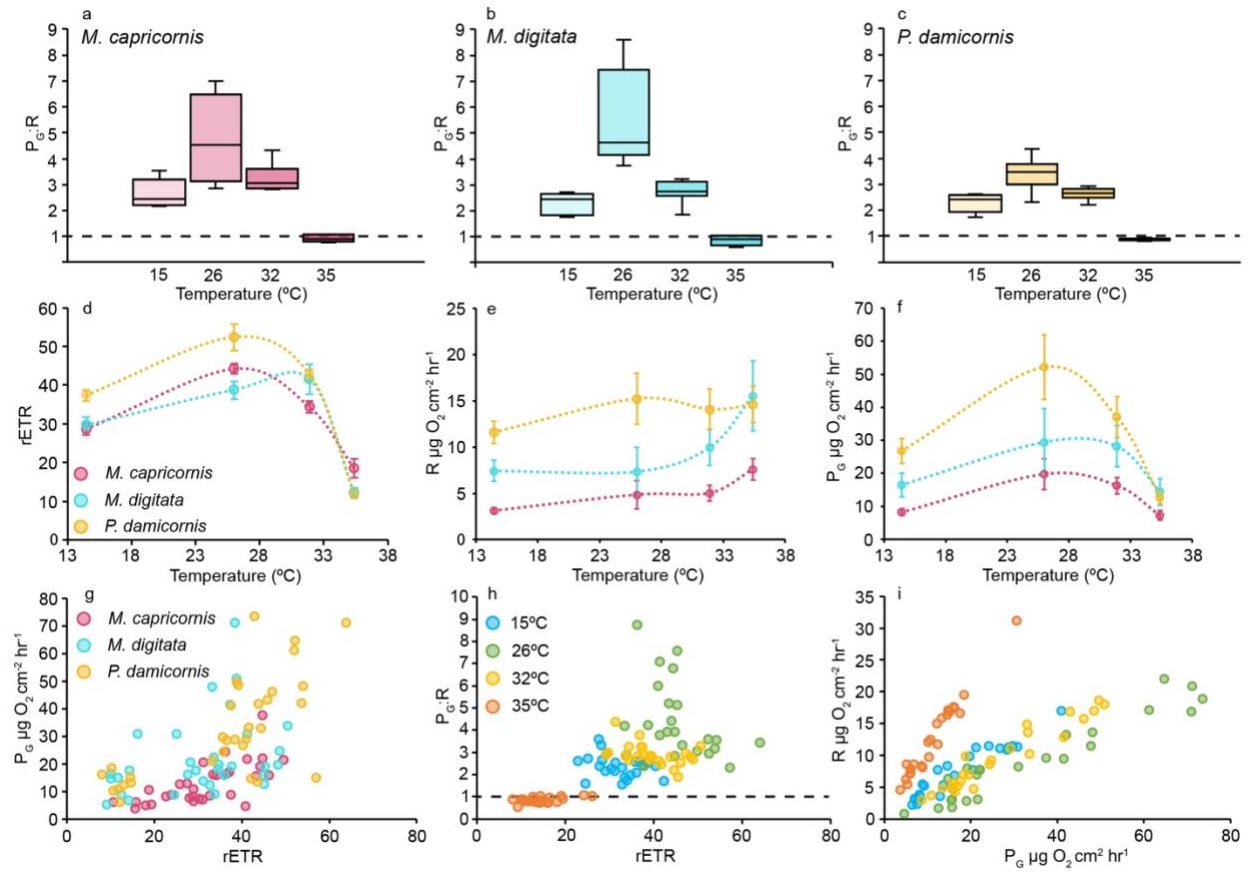


Figure 3.4. Retrieved oxygen dynamics. (a–c) Gross photosynthesis to respiration ($P_G:R$) ratios across four temperatures for each species (*M. capricornis*, *M. digitata* and *P. damicornis*). The dashed lines indicate a value of 1, below which metabolic needs are no longer met from primary production. Box plots show mean as the horizontal line, and upper and lower quartiles, with whiskers indicating maximum and minimum values. $n = 8$ replicates were used per species at each temperature. (d) Average rETR values from three different species (*M. capricornis*, *M. digitata* and *P. damicornis*) were assessed using Protocol 2 at 15, 26, 32 and 35 °C and $280 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Dotted lines indicate 3rd order polynomials. (e) Average respiration rates (R ; $\mu\text{g O}_2 \text{ cm}^{-2} \text{ hr}^{-1}$) measured using Protocol 2 under 30 minutes of darkness across different temperatures. (f) Average rates of gross photosynthesis (P_G ; $\mu\text{g O}_2 \text{ cm}^{-2} \text{ hr}^{-1}$) obtained using Protocol 2 under 30 minutes of $280 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of light across different temperatures. (g) The relationship between gross photosynthesis (P_G) and mean rETR for each species ($n = 32$). (h) The relationship between gross photosynthesis (P_G) to respirations rates ($P_G:R$) and mean rETR values at different temperatures for all three corals combined ($r = 0.63$, $n = 96$, $P < 0.05$). (i) The relationship between respiration (R) and gross photosynthesis (P_G) for all species across four different temperatures ($n = 24$).

3.5 Discussion

International commitments to restore nature, such as the Kunming-Montreal Global Biodiversity Framework, are resulting in intensifying efforts to repair degraded ecosystems³⁴, but these restoration efforts are occurring within the context of intensifying climate change³⁵. Thus, to maximise the success of restoration efforts, decisions need to be made on which taxa are chosen³⁶ as well as which areas are prioritised for restoration³⁷. In terrestrial systems, high throughput phenotyping has been one tool assisting the decision making process^{20,21}, and more recent examples such as CBASS are being applied in marine ecosystems^{22,23}. Here we describe a new system, comprised of three pieces of laboratory equipment customised to work together to retrieve a wealth of physiological parameters across user defined gradients.

In this manuscript we described the design, optimisation and implementation of the MTP system to identify differences in thermal performances both within and across different taxa. When designing and optimising the MTP system we undertook numerous validation steps centred around the integration of existing technologies to a single portable system. These included establishing the thermocycler protocols and undertaking thermal mapping of the MTP plate (Supplementary Fig. S3.2). Adaptability of the thermocycler temperature settings enables users to define target temperatures within the bounds of the thermocycler range. In order to increase the range and resolution of retrieved thermal performance curves (TPCs), multiple MTP runs can be performed. We further validated the integration of optical O₂ sensing and PAM fluorometry (Supplementary Fig. S3.7) and assessed the effect of well size and sample biomass on O₂ dynamics (Supplementary Fig. S3.7). As recommended by previous incubation studies²⁸, measuring O₂ drawdown rates prior to application of the MTP system is recommended to

optimise material biomass to the well plate volume. O₂ production and drawdown rates may vary from a variety of factors (e.g., by taxa, handling procedures and environmental history³⁸) making it inappropriate to prescribe a set material size. One challenge with the current system is the absence of flow within the wells, meaning that there is no external disruption of sample boundary layers that could affect rate measurements. Recent research, however, has uncovered that coral has the capability to augment the mixing of O₂ in their lower boundary layer by utilising the movement of their cilia. This occurs independently of external flow speeds, demonstrating that flow alone does not determine how the boundary layer is disrupted³⁹. Additionally, a recent study utilising similar well volumes and designs also did not incorporate flow or mixing within the wells when assessing O₂ dynamics of macroalgae⁴⁰. Our data indicated immediate responses in O₂ levels to shifts between darkness and light (see Supplementary Fig. S3.7–8) within the current protocol durations. Since all runs of the MTP protocol lacked flow, it ensured standardised conditions. However, it's important to exercise caution when extrapolating rates derived from the MTP system to other studies that incorporated flow.

The MTP system has customisable (e.g., temperature, time, light) protocols based on user needs, but two specific protocols were established for our current study: *Protocol 1* – A Multistage Dynamic Photophysiological Response Protocol and *Protocol 2* – An Oxygen Dynamic Response Protocol. The various stages of *Protocol 1* enabled application of numerous interacting abiotic variables to better predict how taxa will respond to future conditions¹³.

Protocol 1 incorporates a dark-light-dark sequence to minimise O₂ depletion in the wells (Fig. 3.1b). *Protocol 1* was designed around incorporation of a Rapid Light Curve (RLC) approach that is commonly used in photophysiological research. RLCs provide valuable insights into the

light-dependent processes and efficiencies of photoautotrophic activity by rapidly modulating light intensities and measuring the corresponding changes in photochemical activity⁴¹.

Preliminary analysis on corals identified that an optimum light step length of 30 seconds was required to ensure $rETR_{max}$ remained high whilst inducing non-photochemical quenching within the samples (Supplementary Fig. S3.5). Low-light pre-illumination at $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was included in the stages between measurements of the protocol to ensure all samples were at the same light state as well as to counteract the effects of dark acclimation (restriction of the Calvin cycle and consumption of ATP and NADPH), prime the photosystems and mimic natural conditions. The use of pre-illumination was also decided upon out of a consideration of practicality and reproducibility. Dark acclimated F_v/F_m measurements taken by hand with instruments such as the Walz diving PAM require the user to be able to see the samples, implying that there is generally always some amount of light surrounding the samples. This stray light is often overlooked and has the potential to influence the measurements that are taken. The ability to completely dark acclimate a sample for a F_v/F_m measurement is not always possible, especially when undertaking field work with varying light conditions. By adding a constant, yet small amount of pre-illumination both before and during the PAM measurement, accuracy and reproducibility of specific lighting conditions can be ensured. Notably, this aspect of the protocol is user defined, and can be changed as required.

Protocol 2, an Oxygen Dynamic Response Protocol can provide unique insight into holobiont physiology beyond standard photochemistry metrics^{30,42}. While O_2 readings can be extracted during *Protocol 1*, longer incubation times were necessary at given light intensities (e.g., dark versus light) to extract O_2 dynamics that could be used to quantify useful descriptions of

metabolic rates (e.g., net photosynthesis and respiration rates). Optical O₂ sensors (see methods) are widely utilised^{43,44}, including in conjunction with other chlorophyll *a* fluorescence-based methods such as the Walz diving PAM³⁰. In integrating O₂ sensing, the MTP system therefore can retrieve absolute rates of net photosynthesis and respiration, although this requires time-consuming and separate semi-continuous calibration (see methods) and sample-specific surface area normalisation²⁸. We therefore propose using P_G:R (gross photosynthesis to respiration ratio) since it is a self-normalising value to retain the MTP high throughput capacity.

3.5.1 Coral oxygen dynamics across a thermal gradient

O₂ consumption rates are a critical measure of energy metabolism and a vital indicator of mitochondrial health and therefore overall cellular regulation or dysfunction⁴². The ability of the MTP system to measure high-throughput O₂ dynamics across environmental gradients provides valuable insights into the overall health and functionality of complex organisms such as coral holobionts, where one photobiological metric may be limited in explaining the intricate relationship between the heterotrophic coral host and its autotrophic algal symbionts and how it affects phenotypic performance⁴⁵. Inclusion of O₂ sensing alongside fluorometry in the MTP system broadens our capacity for a physiological assessment of entire organisms, and thus holobiont (or metaorganism) phenotyping. Rates of P_G were highest for all three species at 26 °C before rapidly declining at 35 °C, indicating downregulation and/or photoinhibition of PSII apparatus⁴⁶. P_G:R for all three species were highest at 26 °C before all falling below one at 35 °C (Supplementary Table S3.2), indicating a shift in their metabolic activities away from being primarily autotrophic to heterotrophic⁴⁷. Both *M. capricornis* and *M. digitata* exhibited higher mean P_G:R (4.72 and 4.81) than *P. damicornis* (3.39) at 26 °C (*n* = 8), indicating that *M.*

capricornis and *M. digitata* met more of their metabolic requirements through photosynthetic activity⁴⁶ than *P. damicornis*. Further, we observed that higher O₂ production correlated with higher rETR across all species, an outcome in agreement with prior studies that similarly showed good linear correlation between photosynthetic rates measured via chlorophyll *a* fluorescence and O₂^{48,49}. However, under higher temperatures this correlation broke down, indicating an offset between P_G and rETR which can be caused by a shift in electron distribution, giving an indication of how much P_G O₂ is being recycled via alternative electron pathways such as the Mehler reaction^{50,51}, and thus providing important information on tolerance thresholds⁴⁵. Rates of P_G and R for all corals (Fig. 3.4i) showed a strong correlation across all temperatures, indicating that metabolic rates are similarly governed by temperature for all coral⁵².

3.5.2 Multi-taxa comparison

Our results showed that *A. kenti* exhibited lower thermal tolerance than other taxa sampled, supporting previous work on coral exhibiting lower thermal tolerance than marine algae and plants^{53,54}. TPCs have been extensively applied to establish the breadth and optimum temperature performance for some marine taxa more (e.g., marine algae⁵⁵) than others (e.g., coral⁵⁶). Multiple modelling approaches have been applied across TPC studies^{32,33}, and model suitability varies depending on the input parameter^{32,57}. Heatmaps for rETR_{max} from all four taxa (Fig. 3.2c) were created to visualise how photochemical capacity changed with temperature and light interactions, revealing species-specific responses, and hence insight into, specific environmental niche spaces. Notably, the ranking of taxa performance based on T-opt for α was different to that of T-opt for rETR_{max}, which was unsurprising given they describe different aspects of how photochemical efficiency is governed by light availability. Together these metrics

can also provide unique insights into the thermal niche of an organism as well as an indication that such reductions in photosystem capacity and efficiency due to thermal stress could be a good indicator of photosystem downregulation or damage. For example, *A. kenti* T-opt for $rETR_{max}$ was 24.7 °C, while T-opt for α was 27.5 °C suggesting efficient photochemistry is sustained under higher temperatures when light is limiting. Previous work has also demonstrated differences in TPCs based on the parameter used; it is thus important for users of the MTP system to select appropriate parameters to model TPCs dependent on their research question⁵⁷. Other parameters retrieved from *Protocol 1* – (Fig. 3.3i–l) not used for TPCs can still provide important information on taxa environmental history; for example, E_K (the minimum saturating light intensity) reflects the light history of an organism⁵⁸. E_K values for all taxa were highly variable with temperature (Fig. 3.2b), highlighting the need to account for temperature history in parallel when describing E_K . Collectively the multi-taxa comparison using the MTP system (*Protocol 1*) identified differences in photophysiological performance across temperatures, but in alignment with previous studies, and where phenotypic ranking varies depending on the parameter used⁵⁷. Taxa with smaller thermal niches have the potential to be more impacted by climate change⁵⁹; thus, understanding predicted thermal niches can support conservation planning and has been highlighted as a research priority, particularly for marine taxa²⁶. However, future consideration is needed to validate how the MTP predicted thermal responses reflect observed thermal niches in the environment.

3.5.3 MTP and CBASS derived phenotypes

Conceptually, MTP and CBASS are similar in that both systems can be utilised to test thermal performance across a defined temperature range (although MTP is more centred around thermal

optima, whereas CBASS is more centred around thermal limits). The MTP approach differs from CBASS in that it is a much faster assay, assesses a broader range of temperatures, and retrieves a suite of photophysiological parameters to create a thermal performance curve (Fig. 3.3i-l). The 18-hour CBASS assay allows for assessment of two distinct parameters: i) heat stress response (after 7 hours, directly following the heat-hold) and ii) recovery response (after 18 hours, following an overnight reprieve). The difference between the 7-hour and 18-hour responses can provide insight into putative resilience potential (e.g., some corals exhibit recovery of F_v/F_m after 18 hours, while others further lose photosynthetic efficiency). Studies using the CBASS approach typically use a PAM (Walz, Germany) to capture F_v/F_m (maximum dark acclimated photochemical efficiency) as the photophysiological response parameter, although any continuous variable can be used for the purpose of thermal threshold modelling, such as coral whitening, protein biomass, or similar²². Modelling of CBASS response variables are used to determine the temperature at which a 50% reduction of the trait under consideration (e.g., F_v/F_m or colour) occurs (reported as the ED50 thermal tolerance threshold^{21,22}). One important consideration when applying the same CBASS models to MTP data is that if observed photochemical efficiency does not drop significantly enough (e.g., temperature exposures were not high enough to elicit at least a 50% decline in the test parameter) to capture the ED50 value, it will be predicted rather than observed.

Whilst F_v/F_m (CBASS) and F_q'/F_m' (MTP) are both measures of photosystem efficiency, F_v/F_m is a measure of maximum dark-acclimated efficiency and F_q'/F_m' is a measure of light acclimated efficiency (Supplementary Table S3.6). These photophysiological nuances do not allow for a direct comparison in photochemical results, rather a broader comparison of observed phenotypes.

Despite fundamental differences in the two methods, the phenotypic response patterns were generally the same across both systems, suggesting that shorter timeframes (e.g., 45 minutes (MTP) versus 18 hours (CBASS)) may be suitable to capture acute thermal stress response phenotypes. Values of ED50 from the CBASS were generally lower, however, than those from the MTP, most likely reflecting more accumulated stress from longer experimental time of the CBASS. Notably, both the CBASS and MTP methods expose taxa to acute heat stress, and such a short experimental assay time may not always be sufficient to accurately depict organismal responses to environmental stressors over longer time periods⁶⁰. However, the use of MTP and other analogues^{22,23} rapidly provide users with valuable information on the overall health and potential resilience of an organism when exposed to environmental change. Whilst recent work²⁴ has shown good correlation between CBASS predicted and real world observed phenotypic performance, further testing is required downstream to verify this link. Additionally, while the equipment used (Imaging PAM and thermocycler) are standalone items available for laboratories, their cost could make the MTP system prohibitive, and therefore custom built, low-cost variations should be considered in the future.

3.5.4 Conclusion

This paper demonstrates the potential value of high-throughput phenotyping systems, such as the MTP system, to advance knowledge on the responses of key aquatic organisms to environmental change, thus providing a powerful and much needed tool to research, restoration and industry stakeholders. We acknowledge that the MTP system could be cost-prohibitive for some end-users, so further efforts to customise cheaper versions that overcome limits of the current system (e.g., no flow) is an area for further development. The MTP system combination of portability

and high throughput capacity across defined conditions allows for the extraction of a wide range of physiological parameters, providing insights into aquatic organism functioning across varying and predicted environmental conditions. Further, we provide a series of protocols which other researchers can tailor to suit their target taxa and research questions. Whilst this device has shown strong potential in predicting taxon and phenotype specific thermal performance, future studies validating how rapid phenotyping tools such as the MTP system predict phenotypic responses to long term environmental changes *in situ* are needed to fully understand the value of rapid-assay phenotyping.

3.6 Acknowledgements

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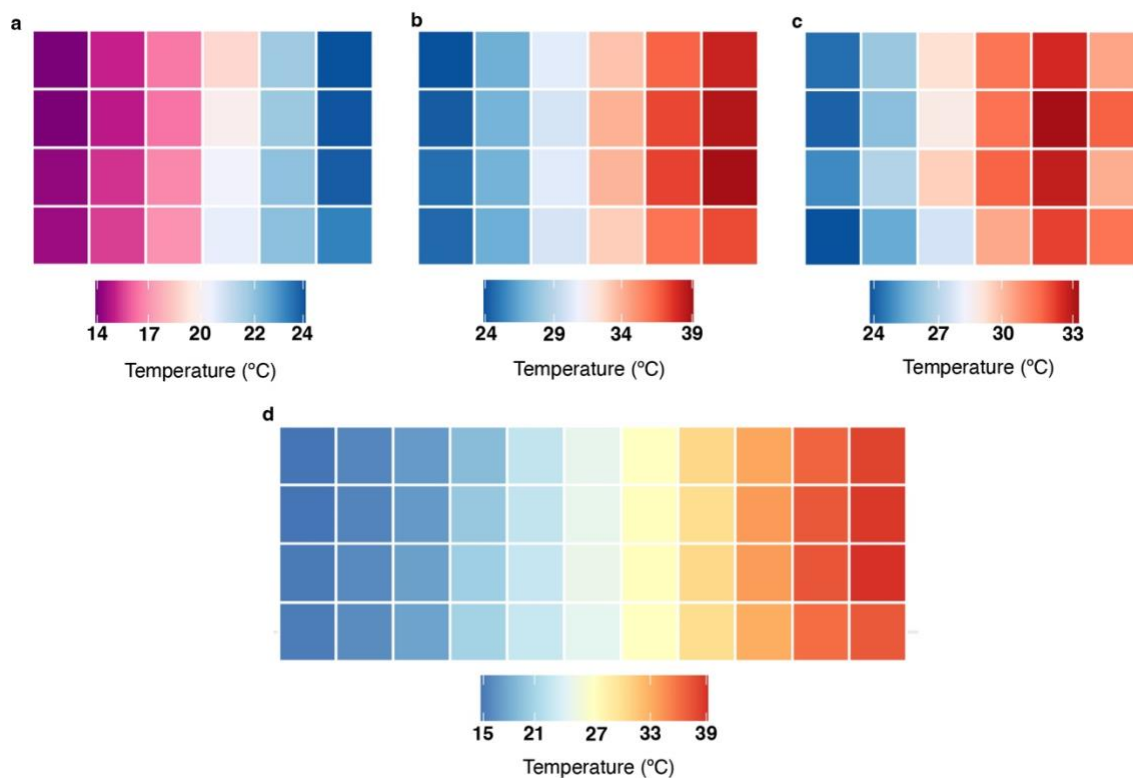
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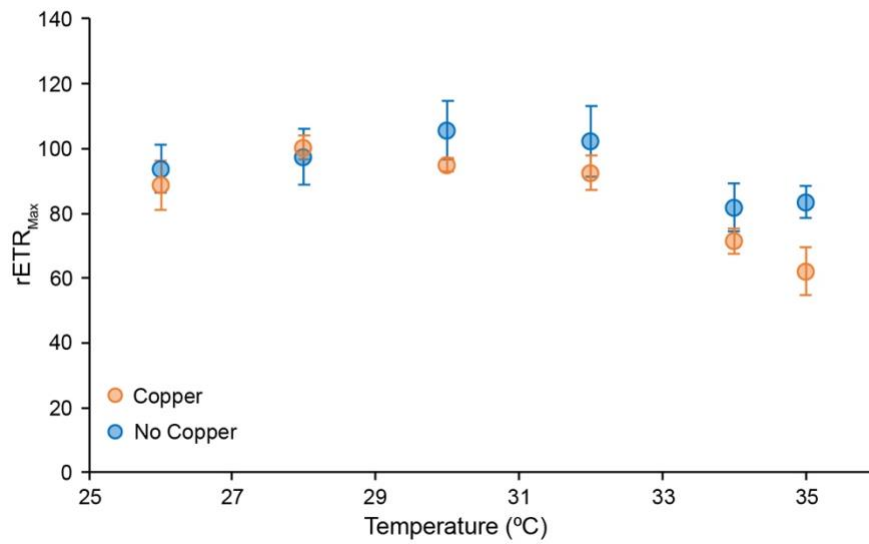
3.8 Supplementary Materials



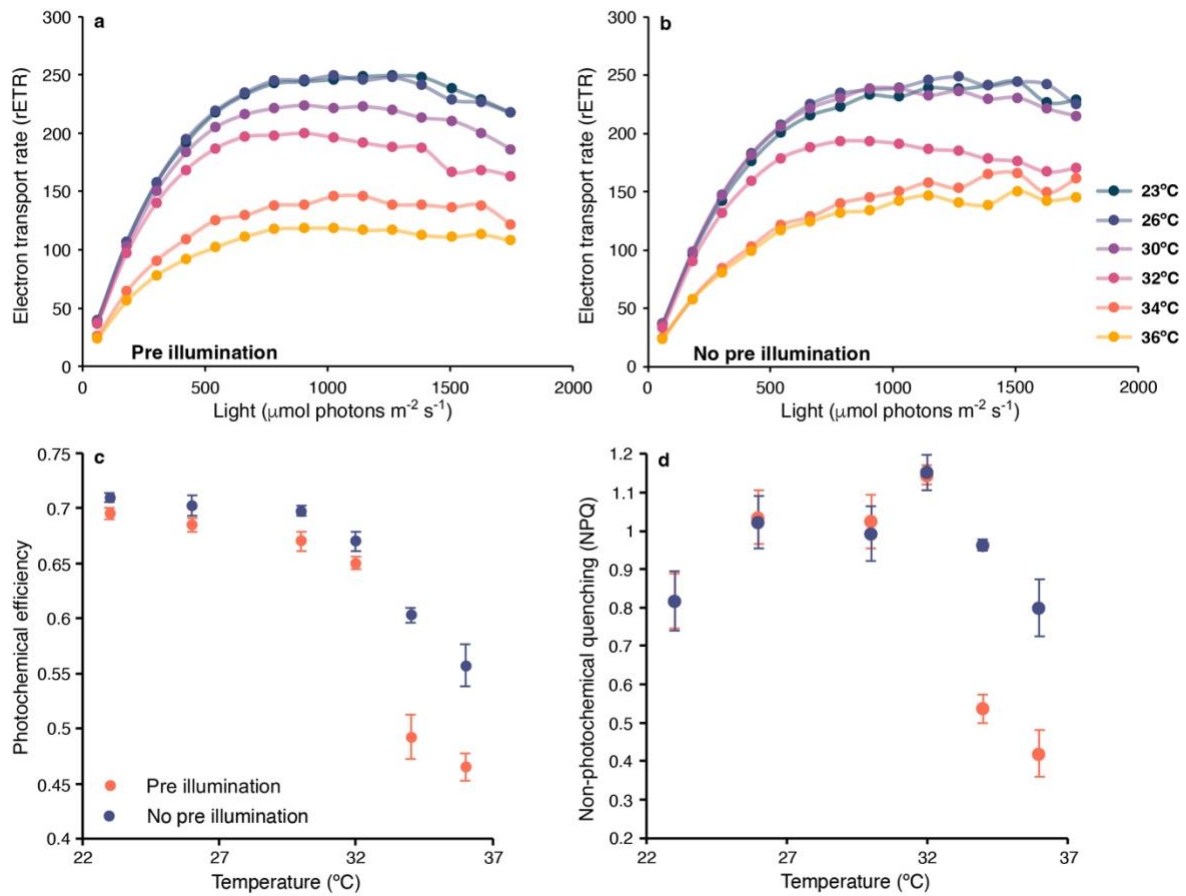
Supplementary Figure S3.1. (a) MTP adapter with coral fragment placed in each well. (b) Flexible seal ensuring the glass lid is held down and watertight conditions are maintained. (c-d) Pictures of the MTP plate filled with water and left for 90 minutes showing absence of air bubbles. (e) MTP adapter plate printed in Rigid 10K resin.



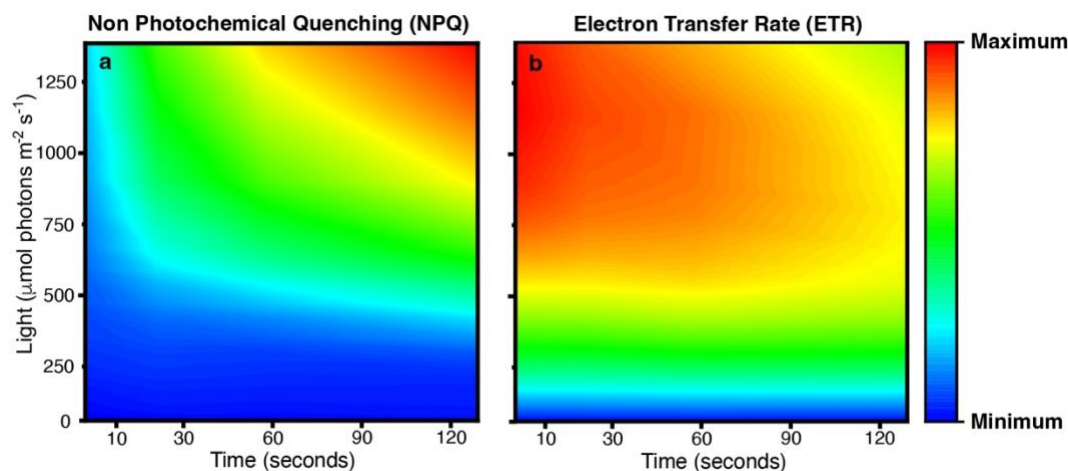
Supplementary Figure S3.2. Heat maps showing thermal gradients of the Multi Taxa Phenoplate (MTP) applied by the thermocycler. (a) Mapping of temperatures 14 – 24 °C (lower temperature gradient), (b) Mapping of temperatures 24 – 39 °C (higher temperature gradient), (c) thermal map of plate without Styrofoam insulation ring added, and (d) Constructed gradient map when lower and temperature plates are combined. All wells were measured a total of four times (twice with two different sensors) with results averaged to ensure accurate temperatures were recorded.



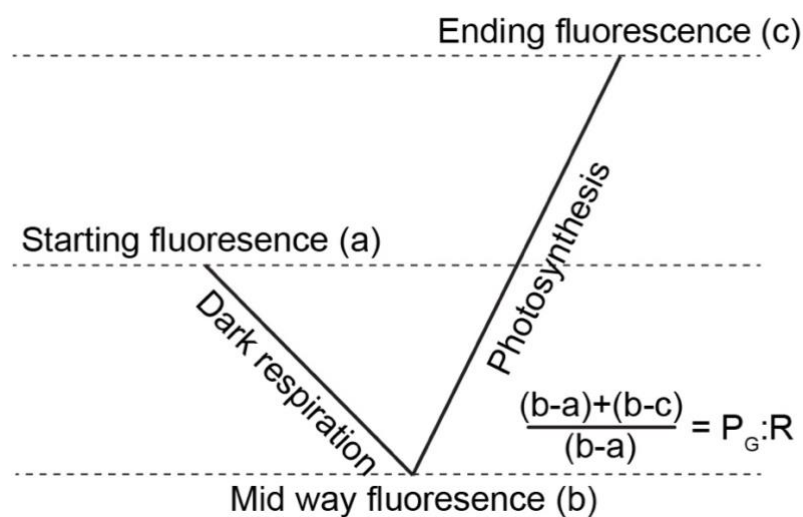
Supplementary Figure S3.3. Comparison of rETR_{max} values between copper and non-copper treated samples for *Montipora digitata* across a thermal gradient. Copper treated samples were exposed to 2,000 ppm of copper (in the form of CuSO₄ · 5H₂O), with both copper and non-copper treated samples being incubated at 26 °C for one hour in darkness before initialising Protocol 1 on the MTP. Each data point represents $n = 4$.



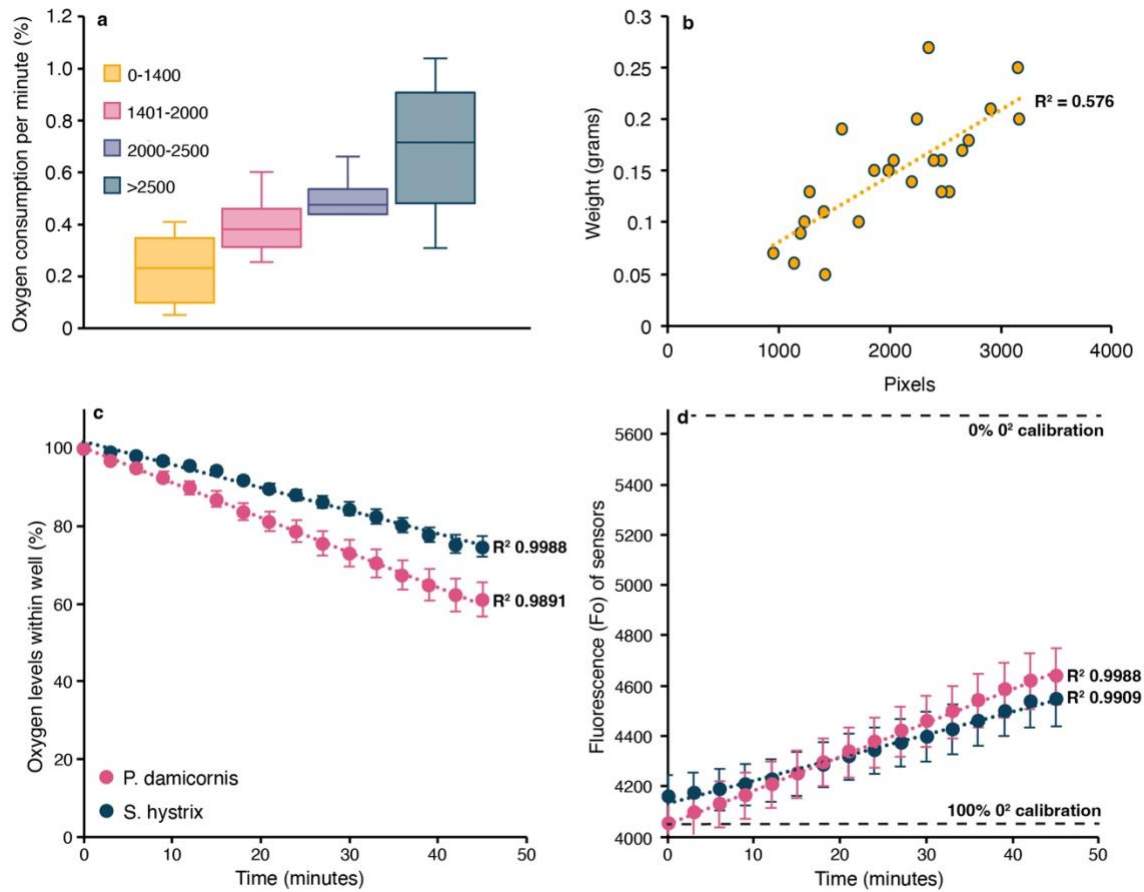
Supplementary Figure S3.4. Differences in photophysiological responses with and without pre-illumination showing rapid light curves (RLCs) across a temperature gradient for samples (*Seriatopora hystrix*) treated with $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of pre-illumination (F_v'/F_m') and no pre-illumination F_v/F_m ($n = 4$ per temperature with bars showing standard error). (a and b) Differences between RLCs for pre and no pre-illumination treated samples. (c) There was a significant difference ($P < 0.05$) between pre (F_v'/F_m') and no pre-illumination (F_v/F_m) for photosynthetic efficiency at T2 for temperatures 30, 34 and 36 $^{\circ}\text{C}$. (d) For NPQ only temperatures 34 $^{\circ}\text{C}$ and 36 $^{\circ}\text{C}$ were significantly different ($P < 0.05$) between treatments.



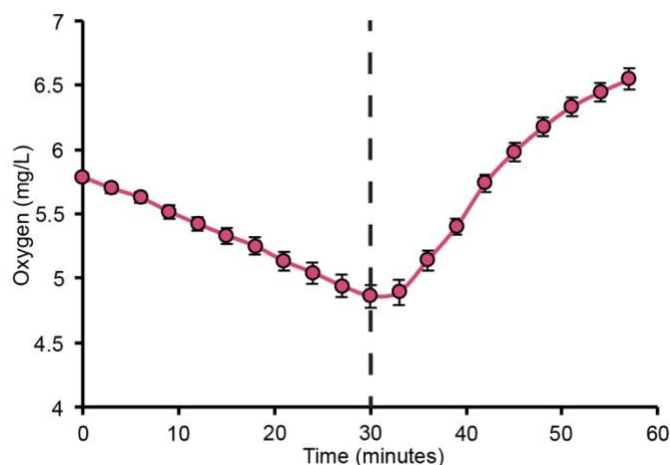
Supplementary Figure S3.5. Heat maps showing differences in rapid light curve (RLC) step times across a light gradient. (a) Changes in non-photochemical quenching (NPQ) across different RLC step times (10, 30, 60 and 120 seconds) and light intensities. (b) showing changes in ETR across different RLC step times (10, 30, 60 and 120 seconds) and light intensities. $n = 24$ technical replicates per step time across six genotypes from two different coral species *Seriatopora hystrix* and *Pocillopora damicornis* with all samples at 26 °C.



Supplementary Figure S3.6. Diagram and equation showing how gross photosynthesis to respiration rates ($P_G:R$) are calculated using Protocol 2 of the Multi Taxa Phenoplate (MTP) system. Fluorescence decreases with increasing oxygen allowing $P_G:R$ to be calculated as shown in the equation.



Supplementary Figure S3.7. Respirometry metrics obtained from the multi taxa phenoplate (a) respiration rates of *Seriatopora hystrix* in MTP wells at 26 °C and when normalised to pixel count (illuminated pixels of sample as measured by the imaging PAM), ($n = 6$) and (b) coral weight (grams) compared to pixel count. Based on these results, sample sizes chosen for analysis were kept to ca. 10x5x5 mm (< 2500 pixels and < 0.2 grams) or less to avoid hypoxic conditions (< 2 mg L⁻¹ O₂) during the dark stage of protocol 1. (c) O₂ depletion in wells over time for two species (*S. hystrix* and *Pocillopora damicornis*) ca. 10x5x5 mm in size (bars indicating standard error) and (d) showing corresponding change in fluorescence of O₂ sensors over time ($n = 24$).



Supplementary Figure S3.8. Average rates of oxygen consumption and production in the Multi Taxa Phenoplate (MTP) wells for three species of coral (*M. capricornis*, *M. digitata* and *P. damicornis*). Dashed line indicates point where light was turned on and photosynthesis began with bars indicating standard error ($n = 24$).

Supplementary Table S3.1. Differences in mean photochemical efficiency values between dark and low light acclimated coral samples from three different species (*Montipora capricornis*, *Montipora digitata* and *Acropora kenti*) at 26 °C ($n = 8$). * indicates significant difference between light and dark samples ($P < 0.05$, ANOVA)

		<i>Montipora digitata</i>		<i>Montipora capricornis</i>		<i>Acropora kenti</i>	
		Low light	Dark	Low light	Dark	Low light	Dark
<i>Treatment</i>		F_q/F_m	F_v/F_m	F_q/F_m	F_v/F_m	F_q/F_m	F_v/F_m
<i>Photochemical Efficiency</i>	<i>Mean</i>	0.64*	0.61	0.43	0.44	0.60	0.62
	<i>SD</i>	0.02	0.02	0.03	0.03	0.05	0.04

Supplementary Table S3.2. Mean and standard error ($n = 8$) gross photosynthesis to respiration rates ($P_G:R$) across four different temperatures (15, 26, 32 and 35 °C) for three coral taxa (*M. capricornis*, *M. digitata* and *P. damicornis*) obtained from protocol 2 on the Multi Taxa Phenoplate (MTP) system.

Species	Temperature (°C)			
	15	26	32	35
Average $P_G:R$	2.7	4.7	3.3	0.9

<i>Montipora capricornis</i>	Standard Error	0.27	0.83	0.27	0.07
<i>Montipora digitata</i>	Average Pg:R	2.2	4.8	2.8	0.9
	Standard Error	0.24	1.09	0.21	0.08
<i>Pocillopora damicornis</i>	Average Pg:R	2.3	3.4	2.6	0.9
	Standard Error	0.17	0.31	0.12	0.02

Supplementary Table S3.3. Results of Tukey's post-hoc test for multiple taxa. Mean values from Supplementary Table S3.2 were used.

Parameter	Comparison	diff	lwr	upr	P. adj
rETRmax	<i>A. marina</i> – <i>A. kenti</i>	54.03	25.75	82.30	0.0005
	<i>S. verruculosum</i> – <i>A. kenti</i>	-52.99	-81.27	-24.72	0.0006
	<i>U. lactuca</i> – <i>A. kenti</i>	4.85	-23.43	33.12	0.955
	<i>S. verruculosum</i> – <i>A. marina</i>	-107.02	-135.30	-78.75	0.0000
	<i>U. lactuca</i> – <i>A. marina</i>	-49.18	-77.46	-20.91	0.0011
	<i>U. lactuca</i> – <i>S. verruculosum</i>	57.84	29.57	86.11	0.0003
T-opt	<i>A. marina</i> – <i>A. kenti</i>	2.11	-0.148	4.37	0.0697
	<i>S. verruculosum</i> – <i>A. kenti</i>	2.53	0.27	4.79	0.026
	<i>U. lactuca</i> – <i>A. kenti</i>	2.92	0.659	5.18	0.0110
	<i>S. verruculosum</i> – <i>A. marina</i>	0.42	-1.84	2.68	0.944
	<i>U. lactuca</i> – <i>A. marina</i>	0.81	-1.45	3.07	0.719
	<i>U. lactuca</i> – <i>S. verruculosum</i>	0.39	-1.873	2.65	0.956
T-min	<i>A. marina</i> – <i>A. kenti</i>	-2.29	-5.74	1.144	0.247
	<i>S. verruculosum</i> – <i>A. kenti</i>	2.73	-0.71	6.17	0.140
	<i>U. lactuca</i> – <i>A. kenti</i>	-0.66	-4.09	2.79	0.940
	<i>S. verruculosum</i> – <i>A. marina</i>	5.02	1.58	8.46	0.004
	<i>U. lactuca</i> – <i>A. marina</i>	1.64	-1.79	5.08	0.513
	<i>U. lactuca</i> – <i>S. verruculosum</i>	-3.38	-6.82	0.06	0.0545
T-max	<i>A. marina</i> – <i>A. kenti</i>	6.52	4.31	8.72	0.000
	<i>S. verruculosum</i> – <i>A. kenti</i>	2.34	0.13	4.54	0.037
	<i>U. lactuca</i> – <i>A. kenti</i>	6.48	4.28	8.69	0.000
	<i>S. verruculosum</i> – <i>A. marina</i>	-4.18	-6.39	-1.97	0.001
	<i>U. lactuca</i> – <i>A. marina</i>	-0.03	-2.24	2.18	0.999
	<i>U. lactuca</i> – <i>S. verruculosum</i>	4.15	1.94	6.36	0.001
Thermal Range	<i>A. marina</i> – <i>A. kenti</i>	8.81	5.22	12.40	0.00005
	<i>S. verruculosum</i> – <i>A. kenti</i>	-0.39	-3.98	3.20	0.988
	<i>U. lactuca</i> – <i>A. kenti</i>	7.14	3.55	10.73	0.0003
	<i>S. verruculosum</i> – <i>A. marina</i>	-9.20	-12.79	-5.611	0.00003
	<i>U. lactuca</i> – <i>A. marina</i>	-1.67	-5.27	1.918	0.532
	<i>U. lactuca</i> – <i>S. verruculosum</i>	7.53	3.94	11.12	0.0002

Supplementary Table S3.4. Table showing retrieved Thermal Performance Curve (TPC) parameters from Gaussian models for multiple taxa ($n = 4$ replicates per taxon), each with 12 discrete temperature points making up the TPC.

Species	Replicate	rETRmax	T-opt (°C)	T-min (°C)	T-max (°C)	Thermal Range (°C)
<i>Acropora kenti</i>	1	98.1	24.4	16.8	32.1	15.3
	2	94.4	25.2	18.4	32.1	13.7
	3	87.3	24.3	16.9	31.7	14.8
	4	84.0	24.9	17.0	32.7	15.7
<i>Avicennia marina</i>	1	157.4	25.2	12.1	38.3	26.1
	2	144.8	28.2	16.9	39.6	22.7
	3	129.7	28.7	17.5	39.8	22.2
	4	148.1	25.2	13.4	37.0	23.7
<i>Ulva lactuca</i>	1	102.6	27.9	18.1	37.6	19.4
	2	93.3	27.7	17.2	38.2	21.0
	3	95.6	26.41	14.4	38.4	24.0
	4	91.7	28.5	16.7	40.4	23.7
<i>Sargassum verruculosum</i>	1	52.0	27.8	19.5	36.1	16.6
	2	50.7	27.1	20.3	33.9	13.7
	3	53.7	27.3	21.1	33.6	12.5
	4	45.5	26.7	19.0	34.3	15.2

Supplementary Table S3.5. Results of Tukey post-hoc analysis for both Multi Taxa Phenoplate (MTP) and Coral Bleaching Automated Stress System (CBASS) treatment at 35.6 °C. Mean values ($n = 4$ per genotype) were used.

Treatment	Comparison	diff	lwr	upr	P. adj
MTP <i>Acropora kenti</i>	A2 – A1	0.06	0.02	0.10	0.009
	A3 – A1	-0.06	-0.10	-0.02	0.009
	A3 – A2	-0.12	-0.16	-0.08	<0.001
MTP <i>Pocillopora damicornis</i>	P2 – P1	-0.08	-0.10	-0.05	<0.001
	P3 – P1	0.11	0.08	0.13	<0.001
	P3 – P2	0.18	0.15	0.21	<0.001
CBASS <i>Acropora kenti</i>	A2 – A1	0.11	0.01	0.21	0.028
	A3 – A1	-0.02	-0.12	0.08	0.828
	A3 – A2	-0.13	-0.23	-0.03	0.011
CBASS <i>Pocillopora damicornis</i>	P2 – P1	-0.00	-0.13	0.13	0.998
	P3 – P1	0.22	0.09	0.35	0.003
	P3 – P2	0.22	0.09	0.35	0.003

Supplementary Table S3.6. Definitions and derivations for parameters presented in manuscript.

Parameter	Definition	Derivation
F_v/F_m	Dark acclimated maximum photochemical efficiency	$(F_m - F_o) / F_m'$
E	Light intensity ($\mu\text{mol photons m}^{-2}\text{s}^{-1}$)	
E_k	Minimum saturating light intensity ($\mu\text{mol photons m}^{-2}\text{s}^{-1}$)	
F_q'/F_m'	Light acclimated photochemical efficiency of photosystem II	$(F_m - F_t) / F_m'$
$rETR_{\text{max}}$	Maximum relative electron transport rate	$(F_q'/F_m') \times E$
α (Alpha)	Initial slope of the Rapid Light Curve; rate of photosynthesis in light limiting conditions	
NPQ_{max}	Maximum non-photochemical quenching observed during the RLC	$(F_m - F_m') / F_m'$
Residual NPQ	NPQ that did not relax after the 10 minutes recovery following the RLC	$(F_m(T1) - F_m'(T3)) / F_m'(T3)$

Chapter 4: Timing and method of manganese (Mn) supplementation affects thermal resilience of corals

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HE, AH, JM and EFC conceived the project. HE conducted the experiment and collected data. HE visualised data and prepared the figures. EFC, JM, and AH supervised the project. HE led the writing, with input and editing from AH, JM and EFC. EFC acquired the funding and administered the project.

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4.1 Abstract

As coral reefs continue to decline globally, new interventions such as nutritional supplementation are being considered. Exogenous supply of nutritional levels of the dissolved essential element manganese (Mn) has increasingly been shown to enhance the resilience of photosynthetic Cnidarians to thermal stress, however questions around the timing of Mn delivery remain unanswered. Here we explore how the timing of Mn additions affects the thermal response of the coral *Acropora millepora*. Coral health was assessed using a variety of different metrics including photochemical efficiency, oxygen dynamics, elementomics and symbiont densities. Results indicated that highest thermal resilience of *A. millepora* occurred when they were exposed to either pulse ($19.34 \mu\text{g L}^{-1}$ every second day) or constant ($4.24 \mu\text{g L}^{-1}$) Mn supply 7 days before heat stress exposure (32°C). ($19.34 \mu\text{g L}^{-1}$). Pulse dosed corals maintained significantly higher photosynthetic efficiency and photosynthesis to respiration rates, as well as increased Symbiodiniaceae densities compared to thermally stressed corals without Mn supplementation. Elemental analysis revealed that both coral tissue and Symbiodiniaceae in Mn-exposed corals accumulated significantly more Mn than non-Mn treated corals. Collectively, our results suggest that intermittent (pulse) Mn exposure prior to the onset of thermal stress may provide the greatest support for thermal resilience in corals. This study advances understanding on how essential elements like Mn could be strategically delivered as an active intervention to enhance coral tolerance during periods of thermal stress.

4.2 Introduction

The world's oceans continue to warm due to anthropogenic climate change, threatening the many unique ecosystems within it (Hughes et al. 2017). Coral reefs cover less than 1% of the ocean

(Arora, 2024; Lyons et al. 2024), yet provide food, jobs and protection to over 1 billion people globally, many of whom live under the poverty line (Camp et al. 2024; Sing Wong et al. 2022). Much akin to “a canary in a coal mine” these ecosystems are set to be the first to go extinct as they are detrimentally impacted by the effects of climate change (Klein et al. 2024). Increasing global temperatures, combined with ocean acidification, deoxygenation, eutrophication and pollution collectively contribute to the ever-growing pressures on these systems. Reef systems are made up of colonies of animals which consist of a host (coral) and symbiotic algae (Symbiodiniaceae). Under optimal environmental conditions the host and symbiont maintain harmonious symbiosis, sharing food and metabolites with each other (Matthews et al. 2017; Rådecker et al. 2021). However, during marine heatwaves this relationship begins to destabilise, leading to the expulsion of the Symbiodiniaceae from the host in what is known as coral bleaching (Ferrier-Pagès et al. 2018; Rådecker et al. 2021). Whilst coral bleaching is a natural phenomenon, the rate and intensity at which mass global bleaching events are now occurring is unprecedented (Sully et al. 2019) and is ultimately compromising the resilience and recovery of reef systems (Hughes, Kerry, et al. 2017). As of 2025, there have been seven mass global bleaching events (Australian Institute of Marine Science, 2024) and without immediate action on climate change there are fears reef systems could be functionally extinct by the year 2100 (Setter et al. 2022).

Unfortunately, even if fossil fuel emissions were to stabilise today, it is predicted that oceans will still need substantial time to equilibrate (Jones et al. 2014; Zhai & de Zhao, 2016); time it is feared that many reef systems do not have. Consequently, beyond traditional marine management like marine parks (Keller et al. 2009) a range of active interventions, aimed at

boosting the resilience of reefs are being explored (Anthony et al. 2020). The toolbox of active interventions being considered are diverse, ranging from bioengineering and geoengineering to coral planting and propagation (Suggett et al. 2024; van Oppen et al. 2015). One strategy more recently being considered is optimising coral nutrition to confer fitness benefits during stress (Becker et al. 2021). Under non-stressful reef conditions, the coral holobiont maintains a symbiotic state between the host and Symbiodiniaceae, with optimal nutritional exchange (Matthews et al. 2017; Rådecker et al. 2021). However, under heat stress, this relationship breaks down. The trigger for this breakdown remains highly debated (Dungan et al. 2022), but recent work has shown that altered nutritional cycling is likely a contributing factor (Ferrier-Pagès et al. 2018). Specifically, that under thermal stress, damage to the photosystems within the symbiotic algae causes a shift from them being mutualistic to parasitic, resulting in their expulsion by the host in order to maintain its own nutrient reserves (Rådecker et al. 2021). It has consequently been proposed that active interventions which can supplement nutrients to minimise competition between Symbiodiniaceae and the Cnidarian host, could help sustain symbiosis longer (Ferrier-Pagès et al. 2018; Matthews et al. 2017; Rådecker et al. 2021).

Manganese (Mn) is a vital trace element, utilised by all oxygenic life on Earth (Robbins et al. 2023). It is not only utilised by many different enzymes that perform biologically essential services, but Mn also forms the basis of all photosynthetic life through its role in photosystem II (PSII). However, despite it being one of the Earth's most abundant elements, it is often scarce and hard to come by in biologically useable forms, particularly in surface waters such as reef ecosystems where it can be a limiting nutrient (Balaguer et al. 2022; Browning et al. 2021). Little is known about trace metal fluxes in reef ecosystems, however nutrient cycling is often

competitive with many different taxa competing for and exchanging nutrients and trace elements as soon as they become available (Reich et al. 2023). As a result, many organisms such as corals have adapted to accumulate nutrients and elements such as Mn from the surrounding environment (Reich et al. 2023). They can do this by acquisition through heterotrophic feeding, or through direct uptake from the surrounding water (Ezzat et al. 2016; Reynaud et al. 2009). It has been shown that exogenous Mn supplementation can enhance coral (Biscéré et al. 2018; Montalbetti et al. 2021) and Aiptaisa (Chapter 2) physiological performance under thermal stress at levels as low as 4.1 – 15.6 $\mu\text{g L}^{-1}$. These previous studies have supplied Mn from the start of heat stress in a continuous exposure. Whether Mn can similarly provide benefits to Cnidarians when provided at different times during thermal stress (e.g. prior to or during heat stress), and in different modes (e.g. pulse versus continuous dosing), remains unresolved, and has practical implications for the viability of Mn dosing as an active intervention during heat stress. Consequently, this study aimed to address whether administering low level Mn enrichment before, during and after the onset of thermal stress, as well as intermittent supplementation versus continuous supplementation impacted the physiological response of the coral *Acropora millepora* to heat stress. Knowledge from this study furthers our understanding on the methods and viability of Mn supplementation as a possible active intervention for corals during thermal stress.

4.3 Methods

4.3.1 Experimental Setup

The experiment was conducted at the Monsoon Aquatics coral farm in Bundaberg, Australia. Twelve *Acropora millepora* colonies were collected from the Great Barrier Reef (16.38.396/146.00.334) at 5-10 m depth. The colonies were kept in a shaded outdoor raceway for one month prior to being fragmented into 4-5 cm fragments that were subsequently attached to ceramic frag plugs before being left for an additional month to recover. Fourteen replicate tanks were setup, each with a volume of 68.5 L and containing a wavemaker (Jebao, China), 600-watt titanium heater (Schego, Germany), thermostat (InkBird, China), LED light (Maxspec) providing 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of light on a 12:12 cycle, and a pump (Aquaone). Additionally, each tank had a continuous supply of fresh seawater (0.2 μm filtered supplied via underground pipe from coast) which was turned over at a rate of twice daily. For treatments with Mn added, a peristaltic dosing pump (Reef Dose, RedSea) was setup, supplying each tank with a designated amount of Mn (CAS: 13446-34-9, MERCK, Germany) as described below. Salinity, pH and temperature were all measured daily using a portable water quality meter (WTW, Weilheim, Germany) (supplementary materials).

4.3.2 Experimental Treatments

A total of seven different treatments were setup, with technical tank replicates for each. Treatment conditions were as follows: 1) tanks kept at 26 °C (control) with no additional Mn added to the native seawater (No Mn 26 °C), 2) tanks kept at 26 °C with Mn dosed once an hour for the duration of the experiment (e.g. from the 1 week holding period through to the end of the experiment), maintaining an average concentration of 4.77 $\mu\text{g L}^{-1}$. For treatments 3-7, tanks were

ramped to 32 °C at a rate of 0.5 °C per day after an initial hold period of 1 week at 26 °C. 3) Treatment 3 tanks did not have any Mn supplied to them for the whole duration of the experiment (No Mn 32 °C), 4) tanks had Mn dosed every hour, maintaining an average concentration of 4.24 $\mu\text{g L}^{-1}$ for the duration of the experiment (e.g. from the 1 week holding period through to the end of the experiment) (Mn 32 °C), 5) tanks had Mn dosed every hour, maintaining an average concentration of 4.74 $\mu\text{g L}^{-1}$ from the end of ramping when temperatures reached 32 °C (Mn ER 32 °C), 6) tanks had Mn dosed every hour, maintaining an average concentration of 4.44 $\mu\text{g L}^{-1}$ from when corals showed a 30% drop in photosynthetic efficiency (F_v/F_m) from control (No Mn 26 °C) values (Mn30% 32 °C), 7) tanks had Mn dosed every second day from the 1 week holding period through to the end of the experiment, bringing the total concentration of the tanks to an average of 19.34 $\mu\text{g L}^{-1}$ immediately after dosing (Mn dosed 32 °C), which was subsequently diluted to ambient seawater concentrations via the turnover with fresh seawater. In each experimental treatment, two fragments from each colony were included to divide genotypic replication across the treatments and tanks, and to allow for sampling across two timepoints (the start of the experiment, and at the end of the experiment). Early in the experiment a couple of fragments died across random treatments due to unrelated reasons, this gave us a final $n = 10-12$ across all treatments.

4.3.3 Manganese Stock Solution

For all Mn supplementation a standard stock solution was used. The stock solution was created by adding 72 grams of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ with Milli-Q water (18.2 $\text{M}\Omega \cdot \text{cm}$, Satorius Lab Instruments GmbH and Co. Goettingen, Germany) to make up 1000 mL. An aliquot (2.85 mL) of this stock solution was then added to 997.15 mL of reverse osmosis deionized (RODI) water to make up

the final dosing solution. For treatments with continuous Mn supplementation this solution was delivered via peristaltic pumps, matching the rate of seawater flow to a final target concentration in each tank of ca. 5 $\mu\text{g L}^{-1}$. For pulse dosed tanks, 24 mL of the final Mn stock solution was added once every second day, bringing the total Mn concentration of each tank to ca. 20 $\mu\text{g L}^{-1}$. These concentrations were chosen as they were still well below toxic thresholds and previous studies had found benefits at or around these concentrations (Biscéré et al. 2018; Montalbetti et al. 2021) and (Chapter 2)).

4.3.4 Photophysiology assessment

Maximum photosynthetic efficiency (F_v/F_m) was measured as a proxy of coral health (Bhagooli et al. 2021; Ralph & Gademann, 2005). Measurements were taken every night at 7 pm, 1 hour after the lights turned off for the duration of the experiment (from start to the end).

Measurements were taken using the AquaPen (Photon Systems Instruments, Czech Republic). The AquaPen was held at a standardised distance (1 cm) and angle (90°) to the coral, avoiding the top 1 cm where symbiont density is less due to new growth. Fast fluorescence induction kinetics (OJIP) analysis was conducted utilising the same device and sampling techniques at both the start and end of the experiment.

4.3.5 Photosynthesis and Respiration Measurements.

Photosynthesis to respiration ratios were measured at the beginning and end of the experiment using the MTP system as described in Chapter 3 (England et al. 2024). The MTP system consists of an imaging pulse amplitude modulated (PAM) fluorometer (Open FluorCAM, Photon Systems

Instruments, Czech Republic) mounted above a thermocycler (ABI Veriti, UK) with customisable protocols that allow rates of net photosynthesis and respiration to be determined by changes in oxygen levels detected with fluorescent oxygen sensors (OXSP5, PyroScience, Aachen, Germany). Small 1 cm sub-samples were cut off of each fragment and placed into the MTP well-plate. These corals were then subjected to a 30-minute period of darkness to assess respiration and a subsequent 30-minute period of 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of light to measure resulting photosynthesis. Rates of oxygen draw down and production were obtained by normalising the changes in fluorescence from the oxygen sensors as per England et al. (2024). These changes in sensor fluorescence were then analysed to determine photosynthesis to respiration (P:R) ratios; ratios were used as they do not require normalisation to coral surface area, thus supporting the high throughput nature of the MTP system (England et al. 2024).

4.3.6 Elemental analysis

At the end of the experiment, fragments were snap frozen in Whirl-Pak bags (Nasco, USA) with liquid nitrogen and stored at -80°C until they were analysed at the University of Technology Sydney (UTS). All bags, falcon tubes, tools and pipettes were trace metal cleaned, rinsed in 10% hydrochloric acid, followed by 2x rinses in Milli-Q water, 1x rinse in 2% HNO_3 (Baseline, 67-69 % w/w, Seastar Chemicals, BC Canada) and two additional rinses in Milli-Q water. Falcon tubes were then labelled and weighed. Buffer (7 mL of 0.1 N sterile TRIS buffer (MERCK, Germany) for elemental analysis) was then added to Whirl-Pak bags containing frozen coral fragments and thawed over ice. Coral tissue was removed from the skeleton via air picking (Grima et al. 2022) and the resulting slurry was transferred into prepared falcon tubes. A 500 μL aliquot of slurry was removed from each tube and stored at 4°C separately for host protein and

cell count quantification. Falcon tubes were centrifuged (Rotanta 460R centrifuge, Hettich, Germany) at 10 °C and 2000 rpm for 10 minutes and the resulting top layer of host tissue (supernatant) transferred to another pre-weighed tube. The remaining algae pellet was resuspended twice with an additional 1.5 mL of TRIS buffer, centrifuged again and the remaining supernatant placed into the tube containing host tissue. All tubes containing both host and symbiont fractions were then frozen at -80 °C before being placed in the freeze drier (Alpha 2-4 LD plus freeze dryer (Christ, Germany)) for three days to dehydrate with the resulting dry mass being weighed. The resultant dry mass was then digested in 1.5 mL of 65% HNO₃ at 100 °C for two hours to completely dissolve the solid material (Ali et al. 2011; Wang et al. 2024). After digestion a further 13.5 mL of Milli-Q water was added for a total volume of 15 mL. A 1 mL aliquot was taken and added to 9 mL of Milli-Q water for a final dilution of 1:100. The resulting dilution was analysed via ICP-MS (7700, Agilent, USA) utilising the same methods as below.

4.3.7 Protein Quantification and Cell Counts

Aliquots of 500 µL of coral slurry were centrifuged using a MIKRO 185 Centrifuge (Hettich, Germany) at 2000 rpm and 4 °C for 10 minutes. The resulting top layer of host tissue was removed via pipette and diluted in an additional 500 µL of Milli-Q water and total protein content was analysed as per Matthews et al. (2024) via a Bradford assay (Bradford, 1976). The remaining algae pellet was resuspended in 1000µL of TRIS buffer and individual cells were counted using a hemocytometer (Camp et al. 2025) (Neubauer Haemocytometer, Fisher Scientific, Loughborough, UK) in triplicate. Resulting cell counts were then normalised to host protein to give cells per µg of protein.

4.3.8 ICP-MS Seawater Analysis

Falcon tubes (Corning, USA) were preprepared by rinsing in 10% hydrochloric acid (Suprapur, Merck, Germany), followed by 2x rinses in Milli-Q water, 1x rinse in 2% HNO₃ and 2 additional rinses in Milli-Q water. They were then prefilled with 8mL of 2% HNO₃ and stored until use. Tank water from all treatments was collected every three days by syringe with a 0.2 µm filter (Sartorius, Germany), filtering 2mL into the preprepared centrifuge tubes which were then sealed and stored at 4 °C. Water samples were placed into the autosampler (SPS-4, Agilent, USA), and then analysed via ICP-MS as per England et al. (2025). Both sampling tip and lines were purged with 2% HNO₃ for 30 seconds between samples. An argon humidifier (Elegra, Perkin Elmer, USA) was used to minimise machine drift and build-up of salt on the ICP-MS cones. Internal standards consisted of Rhodium and Yttrium with the calibration curve (0, 0.19, 0.39, 0.78, 1.57, 3.125, 6.25, 12.5, 25 and 50 µg L⁻¹) created using a 20 component ICP-MS standard at 10 µg L⁻¹ (ZeptoMetrix, USA). All blanks and calibration curves were acid and matrix matched using high purity analytical grade chemicals (Supplementary Table S2.4). Three spike recoveries were performed to verify limited machine drift by adding 10 µg L⁻¹ of Mn into randomised samples at the end of each run.

4.4 Results

4.4.1 Photophysiology

After 25 days, corals kept at ambient temperature (26 °C) had significantly higher F_v/F_m than those at 32 °C irrespective of Mn treatment (0.65 vs 0.24-0.45) (Table 4.1) (Fig.4.1). Corals kept at 32 °C with no Mn had the lowest F_v/F_m out of all treatments, being significantly lower than

both Mn 32 °C and Mn Pulsed 32 °C treatments ($p < 0.05$ Mann-Whitney U). OJIP fluorescence transients were also distinct between corals kept at 26 °C and those at 32 °C, with the Mn pulsed treatment showing the highest rates of fluorescence out of all 32 °C treatments (Fig. 4.2).

Table 4.1. F_v/F_m results taken from the final timepoint. (*indicates significant difference ($P < 0.05$) compared to No Mn 32 °C) ($n = 10-12$)

	NO MN 26 °C	MN FULL TIME 26°C	NO MN 32 °C	MN FULL TIME 32°C	MNER 32 °C	MN30% 32 °C	MN PULSED 32 °C
MEAN	0.65*	0.65*	0.24	0.39*	0.32	0.37	0.45*
SE	0.01	0.01	0.05	0.03	0.06	0.04	0.05

4.4.2 Symbiodiniaceae Cell Densities

All treatments exposed to 32 °C (except Mn Pulsed) displayed significantly ($p < 0.05$; Kruskal-Wallis test followed by Dunn's multiple comparison with Benjamini-Yekutieli correction) lower Symbiodiniaceae cell densities than both 26 °C treatments. No-Mn 26 °C treatment had a slightly higher average cell density count than the Mn 26 °C treatment (2425.75 vs. 2038.71 cells μg^{-1} of host) (Fig. 4.1c and Table 4.2). Corals exposed to thermal stress (32 °C) without Mn exhibited the lowest cell densities at 508.51 cells per μg of host, with the Mn Pulsed and Mn full time treatments having the two highest out of the 32 °C treatments (Table 4.2).

Table 4.2. Cell density of Symbiodiniaceae, per μg of host protein across all treatments ($n = 10 - 12$)

	NO MN 26°C	MN FULL TIME 26°C	NO MN 32°C	MN FULL TIME 32°C	MNER 32°C	MN30% 32°C	MN PULSED 32°C
MEAN	2425.75	2038.71	508.51	815.41	587.97	717.03	1056.99
SE	252.87	162	133.53	180.12	129.88	129.01	265.56

4.4.3 Photosynthesis to Respiration rates

Photosynthesis to respiration rates (P:R) taken at the conclusion of the experiment (Table 4.3), showed that both the 26 °C treatments were significantly higher than all 32 °C treatments ($p < 0.05$ Fishers LSD). No Mn 32 °C had the lowest ratios out of all treatments with Mn Pulsed 32 °C being significantly higher, having the highest P:R ratios out of all 32 °C treatments ($p < 0.05$ Fishers LSD).

Table 4.3. Photosynthesis to respiration ratios (P:R) for coral holobiont across all treatments ($n = 10 - 12$)

	NO MN 26°C	MN FULL TIME 26°C	NO MN 32°C	MN FULL TIME 32°C	MNER 32°C	MN30% 32°C	MN PULSED 32°C
MEAN	3.94	3.5	1.07	1.57	1.41	1.35	1.94
SE	0.47	0.25	0.16	0.27	0.22	0.29	0.27

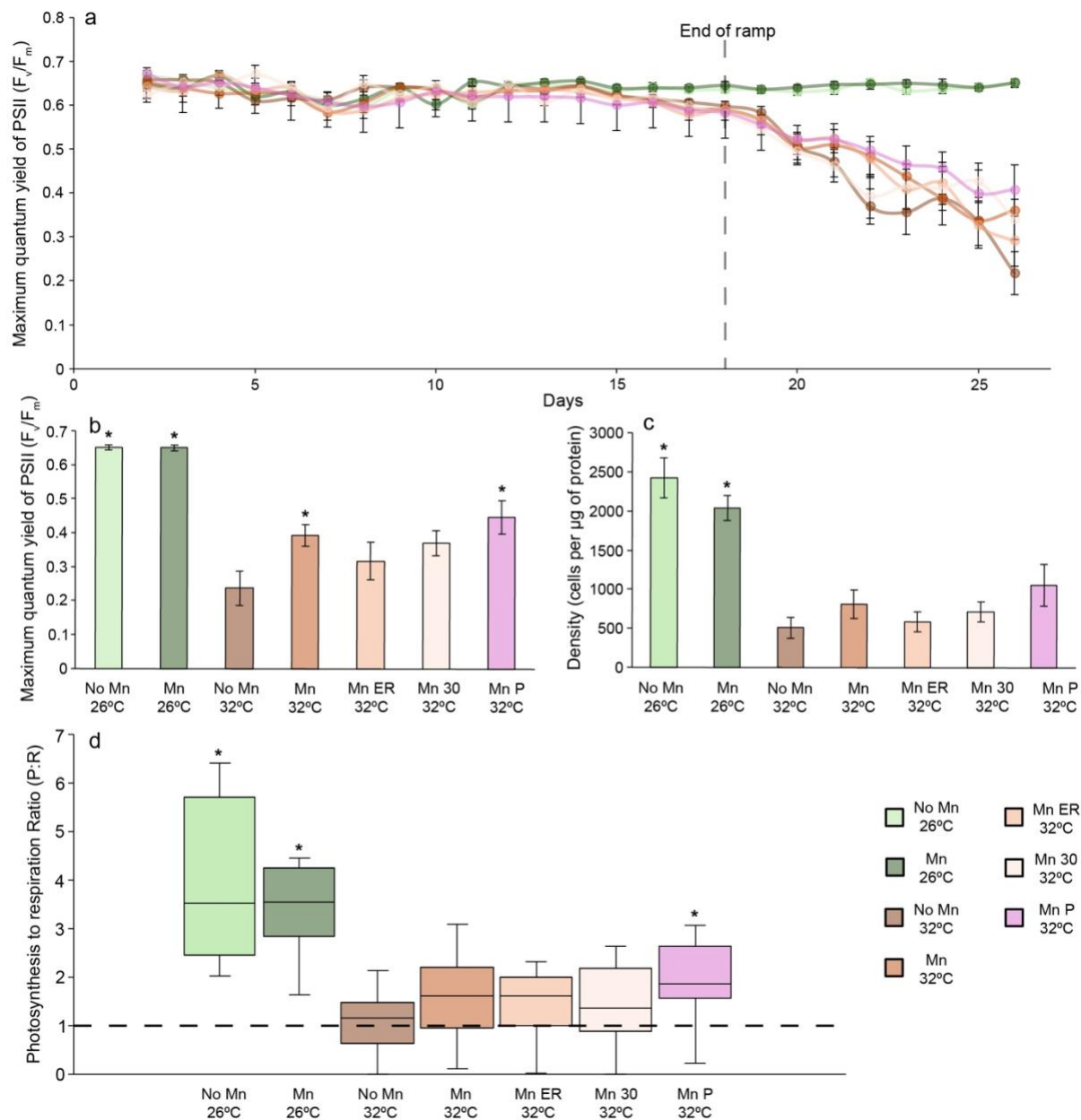


Figure 4.1. Photophysiological and physiological changes of *A. millepora* because of different manganese (Mn) and temperature treatments. (a) Changes in average F_v/F_m over the course of the experiment for all treatments. (b) Average F_v/F_m results from the final timepoint of the experiment (* indicates significant difference between No Mn 32 °C treatment, error bars indicate standard error of mean ($p < 0.05$ Mann Whitney U) $n = 10-12$). (c) Cell densities (Cells μg^{-1} of host protein) for all treatments (* indicates significant difference between No Mn 32 °C treatment, error bars indicate standard error of mean. ($n = 10-12$)) (d) P:R ratios for all treatments at the final timepoint (dashed line indicates point at which rates of photosynthesis match metabolic demands) (* indicates significant difference between No Mn 32 °C treatment (($p < 0.05$ Fishers LSD) $n = 10-12$)).

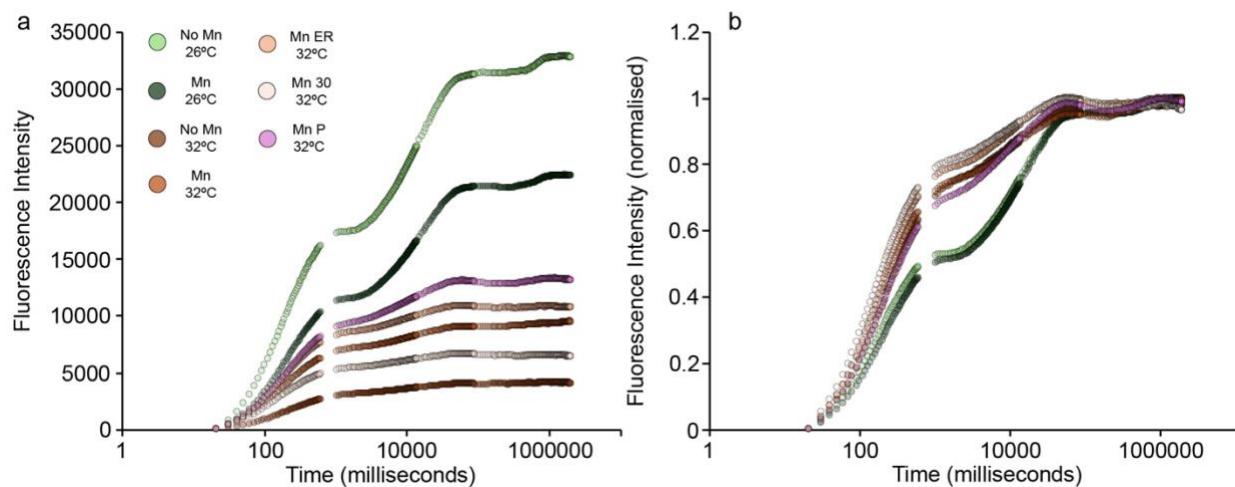


Figure 4.2. OJIP fluorescence transients for all treatments and temperatures. (a) single normalised fluorescence kinetics for all treatments showing differences in mean fluorescence values. (b) double normalised mean fluorescence kinetics for all treatments. ($n = 10-12$)

4.4.4 Host and Symbiont Manganese content

Both heat and Mn treatments had significant impacts on the Mn content of both the host and Symbiodiniaceae symbiont (Kruskal wallis test $p < 0.05$). Symbionts under thermal stress with no added Mn contained significantly less Mn than all other treatments (Mann-Whitney U, $p < 0.05$). Symbionts exposed to constant Mn at 32 °C contained the highest concentrations of Mn out of all treatments and significantly more than No Mn and Mn30% at 32 °C (Table 4.4). All other Mn treatments at 32 °C contained more Mn than No Mn treatment at 32 °C. In symbionts, both Mn and No Mn treatments at 26 °C contained similar concentrations relative to other treatments ($39.88 \mu\text{g L}^{-1}$ and $58.83 \mu\text{g L}^{-1}$). Mn treatments had a significant impact on Mn concentrations within the host at both 26 °C and 32 °C (Mann-Whitney U, $p < 0.05$). Host tissue exposed to constant Mn dosing at 26 °C had significantly higher Mn concentrations than treatments without Mn at both 26 °C and 32 °C. Treatments without Mn had significantly lower Mn concentrations

than others, with the No Mn 32 °C treatment having the lowest concentration of Mn out of all treatments. The amount of time corals were exposed to Mn under thermal stress also had a significant impact (Mann-Whitney U, $p < 0.05$) on tissue Mn content, with the constantly dosed treatment being significantly higher than Mn30% and pulse dosed treatments.

Table 4.4. Manganese (Mn) content of both coral host and symbiont under different temperature and Mn treatments in $\mu\text{g gram}^{-1}$ of dry weight. Values are averages of 10-12 biological replicates.

		<i>No Mn 26°C</i>	<i>Mn 26°C</i>	<i>No Mn 32°C</i>	<i>Mn 32°C</i>	<i>MnER 32°C</i>	<i>Mn30% 32°C</i>	<i>Mn Pulsed 32°C</i>
<i>Symbiont</i>	<i>MEAN</i>	39.88	58.83	16.44	120.69	76.51	38.23	72.62
	<i>SEM</i>	8.82	11.91	2.62	38.02	38.19	5.74	26.38
<i>Host</i>	<i>MEAN</i>	0.99	3.54	0.76	3.35	2.79	2.1	2.05
	<i>SEM</i>	0.15	0.48	0.15	0.61	0.59	0.26	0.25

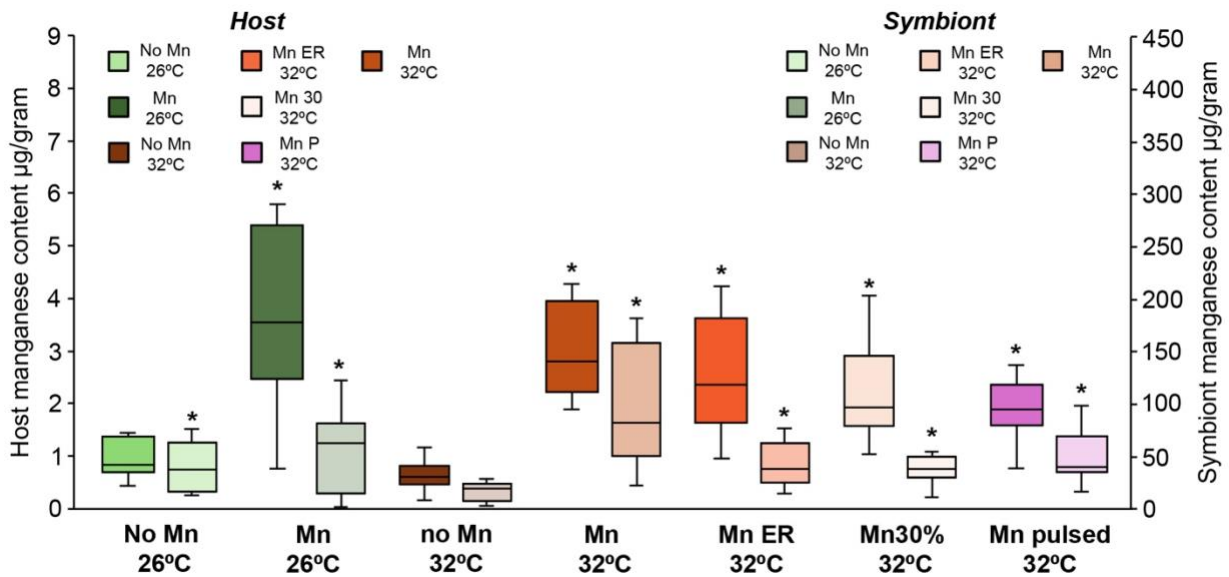


Figure 4.3. Manganese (Mn) concentrations in host and symbiont across different temperatures and treatments. whiskers indicate the range of data, the box indicating the interquartile range and solid lines indicating median values. (*Indicates significant differences between treatment and No Mn 32 °C), ((Mann-Whitney U, $p < 0.05$), $n = 10-12$).

4.4.5 Seawater manganese concentrations

Table 4.5. Average manganese (Mn) concentrations ($\mu\text{g L}^{-1}$) for all treatments measured via ICP-MS ($n = 3-6$) and standard error of mean. ¹indicates treatment after 24h.

	NO Mn 26°C	Mn 26°C	NO Mn 32°C	Mn 32°C	MnER 32°C	Mn30% 32°C	Mn PULSED 32°C	¹ Mn PULSED 32°C
MEAN	0.18	4.77	0.13	4.24	4.74	4.44	19.34	0.30
SEM	0.02	0.73	0.03	0.94	1.17	1.01	0.32	0.12

4.5 Discussion

The timing and mode of trace metal supplementation to corals has a significant impact on potential feasibility of supplementation as a potential active intervention. Studies to date that have looked at low level Mn supplementation for corals have used continuous Mn additions (Biscéré et al. 2018; Montalbetti et al. 2021). Here, the constant supply of Mn at ca. $5 \mu\text{g L}^{-1}$ was compared to pulse doses of Mn at $20 \mu\text{g L}^{-1}$ supplied at different times relative to the onset of thermal stress.

4.5.1 Physiological Effects of Manganese Supplementation

In agreement with Chapter 2 and previous studies (Biscéré et al. 2018; Montalbetti et al. 2021), both constant and pulse dosed Mn supplementation significantly increased the maximum quantum yield of PSII of coral symbionts under thermal stress ($p < 0.05$ Mann-Whitney U)(Fig. 4.1a,b) (Table 4.1). Similar trends, whilst not significant, were also observed for symbiont density measurements where constant and pulse dosed corals displayed higher symbiont counts than thermally treated corals with no Mn (Fig. 4.1c) (Table 4.2). While all corals exposed to Mn

under thermal stress had higher rates of photosynthesis to respiration than those without Mn supplementation, only the pulse dosed treatment was significantly higher ($p < 0.05$ Fishers LSD)(Fig.4.1d) (Table 4.3). These results are echoed by the OJIP transients (Fig.4.2), which provide detailed insights into the efficiency and functional state of photosystem II during the first stages of photosynthesis. The overall reduction in amplitude in the NoMn 32 °C treatment is indicative of lower cell density or a downregulation of photosynthetic components in response to insufficient Mn to build the Oxygen Evolving Complex (OEC). This results in impaired photosynthetic efficiency and disruption to the electron transport chain. However, the Mn Pulsed treatment had the highest photosynthetic efficiency out of all thermal treatments (Fig. 4.2a), suggesting that Mn plays a vital role in the retention of functional PSII centers, enabling a more efficient photosynthetic apparatus under thermal stress. This is not surprising given the essential role of Mn in the oxygen evolving complex (Hakala et al. 2005). When double normalised, the increase in the J step becomes noticeable, (Fig. 4.2b) indicating potential limitations on the acceptor side of PSII (Qa-Qb), which then limits electron transport through the rest of the photosystem leading to less pronounced J, I and P steps. However, it must be noted that use of OJIP analysis on coral presents a unique challenge. Photo damaged Symbiodiniaceae are quickly expelled by the host, making in-situ OJIP analysis of dysfunctional algal symbionts incredibly difficult, as only functional/healthy symbionts are maintained by the host (Fujise et al. 2014). These results are consistent with those of other studies that have explored the impacts of Mn enrichment and/or deficiency on photosynthetic organisms (Biscéré et al. 2018; Schmidt et al. 2016). It is hypothesised that under extreme stress the Mn cluster begins to break down and loses Mn ions, potentially at a higher oxidation rate, thus causing further damage to the photosystems (Schmidt et al. 2016). However, in the presence of excess Mn^{2+} ions it is thought the OEC is able

to actively repair the Mn cluster, thus maintaining down-stream photosynthetic and metabolic processes (Dasgupta et al. 2008) (Chapter 2). This theory is supported by the F_v/F_m and cell density results (Fig. 4.1a,b and c) (Table 4.1) where corals with greater exposure (higher concentrations and longer periods) to Mn maintained higher photosynthetic efficiency compared to those with less, indicating that the presence of free and abundant Mn^{2+} ions both before and during thermal stress potentially enhanced the protection and recovery of PSII.

4.5.2 Accumulation of Mn by Host and Symbiont

For both host and symbiont, Mn treated corals accumulated more Mn within their cells when exposed to Mn enrichment however, under thermal stress alone (No Mn 32 °C) Mn levels in the symbionts were severely depleted compared to the control (No Mn 26 °C) (Fig. 4.3) (Table 4.4). Both previous literature (Smythers et al. 2023) and (Chapter 2) has stated that stressed photosystems require higher amounts of free Mn^{2+} to maintain efficient functioning. This could explain why symbionts that accumulated more Mn under thermal stress maintained higher rates of photochemical efficiency (Fig. 4.2a and 4.3) (Table 4.1 and 4.4). At 26 °C with Mn supplementation, the host accumulated on average over three times more Mn (0.99 vs $3.54 \mu g g^{-1}$) (Table 4.4) than those at 26 °C but without Mn supplementation. Meanwhile, Mn concentrations within the symbiont remained similar (39.88 (No Mn 26 °C) vs $58.83 \mu g g^{-1}$ (Mn 26 °C)), indicating that under ambient conditions the host primarily accumulates Mn, whilst the symbiont selectively accumulates as required (such as under thermal stress conditions). Generally, the later Mn was administered to the corals, the less Mn accumulation occurred for both host and symbiont (Fig. 4.3 and Table 4.4), indicating that early Mn supplementation

(before the onset of thermal stress) provided corals with more opportunity to accumulate Mn reserves, and thus greater thermal protection (Fig. 4.1 and Table 4.1, 4.2 and 4.3).

4.5.3 Mediation of thermal stress by Manganese

Mn plays a key role in mitigating reactive oxygen species (ROS) related damage as a component of the antioxidant enzyme manganese superoxide dismutase (Mn-SOD) (Biscéré et al. 2018; Holley et al. 2011). However, it is currently debated whether the production of excess ROS is the primary driver of coral bleaching, and while it plays an important role in the breakdown of symbiosis between the host and symbiont, other theories suggest that symbiotic dysfunction is primarily driven by the breakdown and/or imbalance of nutrient/elemental cycling between host and symbiont (Chapter 2) (Dungan et al. 2022; Ferrier-Pagès et al. 2018; Nielsen et al. 2018; Rådecker et al. 2021; Schlottheuber et al. 2024). This highlights the importance of trace metals such as Mn, which play a direct role in both the mitigation of ROS induced damage via antioxidants, as well as directly facilitating the production of energy in symbionts through its essential role in the oxygen evolving complex (Schmidt et al. 2016). Given the constant requirement of this trace element for optimal physiological functioning, it is surprising that little work has explored how often, and how much is needed during periods of heightened stress such as bleaching events, especially considering that natural trace element fluxes are expected to change under climate change (Zitoun et al. 2024). As coral reefs continue to decline globally, we must develop a toolbox of active intervention approaches. Management strategies, such as Mn and other nutritional supplementation, could form part of a broader reef protection and rehabilitation framework. However, there are still several important factors to consider before this becomes a viable management strategy. Results here are only from one coral species, and

future work should expand this testing to more coral species with diverse trophic strategies. Furthermore, the fact that Mn is quickly taken up from the surrounding waters through both biological activity (Balaguer et al. 2022) and natural oxidation processes (Robbins et al. 2023) creates significant challenges when trying to target specific concentrations over large areas. Furthermore, whilst this and other studies (Biscéré et al. 2018; Montalbetti et al. 2021) found that continuous supply of Mn significantly increased the thermal tolerance of corals, the practicality of administering and monitoring consistent rates of Mn to target areas remains difficult. Instead, as data from this study shows, Mn could be periodically pulse-dosed at concentrations well below toxic levels, thus alleviating the need for constant supply whilst still providing corals with protection against thermal stress. As with all active interventions, considerations of the associated risks, costs and ethics are ultimately required to ensure any intended benefits outweigh potential negative consequences.

4.5.4 Conclusion

Results presented here indicate that early, constant and pulse dosing of low-level Mn supplementation below toxic concentrations (Binet et al. 2023; Golding et al. 2023) can significantly increase the thermal resilience of *Acropora millepora*. These results also show that the coral host and symbiont differentially accumulate Mn depending on environmental conditions, indicating that whilst Mn supplementation might be beneficial during periods of thermal stress, it may be detrimental under normal temperature conditions. However, further work is needed to determine if these patterns are consistent across multiple species, and if other

environmental variables effect the efficiency or potential potency of exogenous Mn supplementation.

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Chapter 5: General discussion and concluding remarks

5.1 Summary and key findings

Manganese (Mn) is an important element for all life on Earth, and whilst the role of Mn is well documented for plants and other terrestrial organisms, limited research has been undertaken on its importance for Cnidarians such as corals, with most studies having only investigated Mn at toxic levels, leaving a large knowledge gap on the requirements of this trace element, particularly under thermal stress (Binet et al. 2023; Biscéré et al. 2018; Golding et al. 2023; Iyagbaye et al. 2022). This is notable as the availability of trace metals such as Mn is expected to change drastically under the effects of climate change (Zitoun et al. 2024). Therefore, it is important for us to understand how much, and how often corals require essential elements like Mn, to maintain optimum physiological functioning under both ambient and elevated temperatures (Ferrier-Pagès et al. 2018; Reich et al. 2023).

Work in this thesis focused on addressing these gaps in knowledge and in this final chapter (5), I synthesise key findings and outcomes from **Chapters 2, 3 and 4** and discuss the implications of these findings for our understanding of coral physiology. I also explore how this new knowledge could be utilised by stakeholders and potentially integrated into current and new active reef restoration models and strategies.

Firstly **Chapter 2** combined proteomics and photophysiological measurements to explore the roles that different concentrations of Mn (up to 15.6 $\mu\text{g L}^{-1}$) play in mitigating thermal stress. Throughout this chapter, it was evident that the primary target of Mn supplementation was within

the photosystems of Symbiodiniaceae, where I observed sustained photochemical efficiency, increased symbiont densities and significant PSII based proteome changes. Based on these results I developed a mechanistic model which showed how Mn potentially interacts with and supports optimal functioning of PSII within the symbiotic algae of a model Cnidarian (*Exaiptasia diaphana*) under thermal stress. The model provides a new hypotheses that challenges the ROS theory of symbiotic dysfunction under thermal stress that future work can look to further validate. Additionally, methodological challenges from **Chapter 2** highlighted the need for a device, tailored to Cnidarian samples that allow for high-throughput screening across different chemical and temperature gradients. As a result, I developed the Multi-Taxa Phenotyping (MTP) system as part of **Chapter 3**. This increased our capabilities to assess the impacts of various trace metal treatments on small Cnidarian samples. The MTP system can extract thermal performance metrics, allowing users to identify individuals with higher thermal tolerances. This MTP system has proven to be a valuable tool, not only for trace element work but also in the active restoration space where it allows users to identify and select naturally resilient corals that can then be utilised in out planting activities. Once I had developed a high-throughput system capable of analysing small Cnidarian samples across different chemical gradients, we expanded our experimental design to determine when, how long and how often corals need to be supplemented with Mn in order to receive a boost in thermal tolerance (**Chapter 4**). For this experiment I collaborated with Monsoon Aquatics to design and build a large scale, flow through experimental system capable of maintaining common reef building corals (*Acropora millepora*). Using knowledge and skills gained from **Chapter 2** about optimal concentrations of Mn as well as my tailored Cnidarian health assessment tool (MTP) from **Chapter 3**, I exposed *A. millepora* to constant Mn enrichment (ca. 5 $\mu\text{g L}^{-1}$) at different times

(before during and after thermal stress), as well as intermittently pulse dosing every second day (ca. 20 $\mu\text{g L}^{-1}$). Results from **Chapter 4** indicated that corals can benefit from both constant supply and intermittent dosing of Mn from before the onset of thermal stress, significantly contributing to our knowledge on the required frequency and timing of Mn supplementation.

Overall, results from **Chapters 2, 3 and 4** further our knowledge on the physiological impacts of Mn supplementation (at non-toxic levels) for Cnidarians under ambient and thermal stress conditions. Findings from the thesis also address key knowledge gaps around the required concentrations, frequencies and timings of Mn supplementation to sustain algal-Cnidarian symbiosis under thermal stress. This knowledge could directly support the development of Mn supplementation based active intervention strategies.

5.2 Impacts of Manganese supplementation on cnidarian holobiont physiology

Cnidarians are a holobiont, consisting of the animal host, symbiotic algae (Symbiodiniaceae), bacterial and viral communities (Matthews et al. 2020; Thompson et al. 2015). Mn is a critical trace element for Cnidarians where it plays vital roles in metabolism, immune responses, enzymatic activity and photosynthesis (Browning et al. 2021; Chen et al. 2018; Holley et al. 2011). Whilst these holobionts utilise and share resources such as Mn, questions remain around trace metal allocation and use, particularly under stress conditions that cause the breakdown of the Cnidarian-Symbiodiniaceae symbiosis. It must be noted that whilst this thesis explored the effects of Mn supplementation on the Cnidarian host and its Symbiodiniaceae, the bacterial microbiome of the holobiont could also be potentially impacted. Whilst it was beyond the scope

of this thesis to explore these impacts, future research should be undertaken as bacteria are known to play an important role in trace element cycling (Glaze et al. 2022).

In **Chapter 2** I utilised proteomic and photophysiological analysis, where I observed that Mn supplementation up to $15.6 \mu\text{g L}^{-1}$ significantly impacted both photosynthetic efficiency and the proteome of PSII. As a result, I hypothesised that Mn supplementation primarily targets PSII within the Symbiodiniaceae, specifically the oxygen evolving complex (OEC), which consists of a cluster of four manganese and 1 calcium ion (Roose et al. 2016; Schmidt et al. 2016). This Cluster forms the foundation of the entire photosystem, and under normal conditions, facilitates the oxidation of water and supplies electrons to the electron transport chain (Roose et al. 2016). This in turn provides the algae with energy, thus enabling nutrient sharing between host and symbiont, supporting functional symbiosis (Ferrier-Pagès et al. 2018). However, under thermal stress it has been shown that the OEC is primarily damaged and releases its Mn ions, resulting in inactivation of PSII, and its ultimate inhibition of photosynthesis (Dasgupta et al. 2008; de Bang et al. 2015; Smythers et al. 2023). This damage to the OEC was observed in **Chapter 2**, where treatments under thermal stress with no Mn Supplementation exhibited a significant downregulation in PSII extrinsic proteins, whilst no difference was observed in Mn supplemented samples. The resulting reduction in photosynthetic function then creates a metabolic cascade as the Symbiodiniaceae are no longer able to produce energy, reducing and potentially reversing the nutrient cycling between it and its host (Matthews et al. 2017; Rådecker et al. 2021, 2023). Additionally, this switch from mutualism to parasitism can cause an increase in host derived Rab-7a proteins (also observed in **Chapter 2** in non-Mn supplemented samples), a signal for exocytosis and the ultimate expulsion from the host (Baker et al. 2018; Chen et al.

2003). However, when Mn is supplemented under thermal stress, algae can potentially differentially uptake larger concentrations of Mn and supply PSII with free Mn ions (**Chapter 4**) (Vojvodić et al. 2023). This abundance of available Mn around the OEC could enable the extrinsic proteins of PSII to better stabilise itself, replacing Mn ions from the cluster that have been lost due to damage (de Bang et al. 2015; Hakala et al. 2005). This theory is further supported by the similar results of sustained photochemical efficiency and maintenance of symbiosis in Chapters 2 and 4, where Mn treated samples maintained higher F_v/F_m and symbiont densities than those without Mn supplementation.

This mechanistic theory of Cnidarian symbiotic dysfunction provides a complimentary and potentially alternative theory to the traditional reactive oxygen species (ROS) driven narrative, which is increasingly being challenged (Rädecker et al. 2015, 2023; Schlotheuber et al. 2024). Traditionally, coral bleaching was thought to be primarily driven by oxidative damage induced by stray ROS produced by the host and symbiont during thermal stress (Downs et al. 2002; Dungan et al. 2022; Krueger et al. 2015). Organisms have developed numerous ways to counteract ROS, including synthesis of the antioxidant enzyme MnSOD, which binds to and quenches cellular ROS (Biscéré et al. 2018; Holley et al. 2011). Initially it was thought that supplementing the coral hosts with Mn would allow them to produce more MnSOD and thus quench more ROS. However, despite Mn supplementation reducing thermal stress, both the proteomics results from **Chapter 2** and those of others showed no significant increase in SOD activity under Mn enrichment (Montalbetti et al. 2021). Findings from my thesis, in conjunction with other studies (Ferrier-Pagès et al. 2018; Rädecker et al. 2021, 2023; Schlotheuber et al. 2024) suggest that multiple mechanisms may ultimately contribute to coral bleaching.

Additionally, throughout **Chapters 2 and 4**, little impact was observed in the host. Whilst this could be true, further analysis utilising different techniques could be required to identify these impacts.

Despite Mn playing a key role in maintaining Cnidaria-algal symbiosis under thermal stress (Monalbetti et al. 2021; Biscéré et al. 2018; Ferrier-Pagès et al. 2018), results from **Chapter 4** show that corals need to accumulate Mn over an extended period in order to receive any benefits. It was observed that the shorter the duration of Mn supplementation, the less Mn accumulated within the tissues of both the host and symbionts. Additionally, results from **Chapter 4** showed that Mn does not need to be continuously supplied and could be intermittently pulse dosed to achieve the same benefits. Together, Chapters **2 and 4** provide an insight into the fundamental role that Mn plays in maintaining algal-Cnidarian symbiosis, highlighting the potential for targeted elemental and nutritional subsidies as a way of maintaining and strengthening Cnidarian symbiosis under thermal stress.

5.3 Methods, considerations and contributions.

The integration of photophysiology, elementomics and proteomics into Cnidarian research posed numerous challenges in my work, and as a result, methods development formed an integral part of this thesis.

In **Chapter 2** I utilised the model Cnidarian *E. diaphana* alongside proteomics, elementomics and photophysiological assessment to examine the effects of Mn enrichment under thermal stress. However, obtaining physiological metrics was challenging as there are limited high-throughput, trace metal-clean devices tailored to small Cnidarian samples. This highlighted the need for a device that can assess the physiology of small Cnidarian samples in a high throughput way, so I developed the Multi Taxa Phenotyping (MTP) system. The creation of this device allowed me to design larger experiments with significantly more replication, tailored for trace metal studies such as the experiments described in **Chapter 4**. This system also allowed for a broader range of metrics than what is typically available, broadening the analysis I could undertake in future experiments. Additionally, as highlighted in **Chapters 1 and 3**, the MTP system directly contributes to the active intervention landscape by providing practitioners with a rapid phenotyping device that can aid in stock selection for out planting. And whilst we compared it directly to a well-known phenotyping device (CBASS) in **Chapter 3** (Evensen et al. 2023; Voolstra et al. 2020), caution must be taken when comparing results from a rapid/acute thermal stress test (MTP) and trying to infer real world traits and responses to chronic thermal stress, typical of what is experienced during mass bleaching events.

Designing experiments based around manipulation of trace metals posed many challenges, particularly as these experiments required concentrations of Mn below sub-parts per billion. This meant that every aspect of an experiment had to be considered, such as using inert materials for holding tanks, pumps with ceramic shafts instead of stainless steel, washing hands before placing them in water and trace metal cleaning every single item before use. Considerations also included finding seawater with minimal trace metal contamination. When trying to source

suitable seawater for **Chapter 2** it became apparent through ICP-MS testing that most artificial seawater salts contained higher than normal levels of trace metals. Because of this I had to modify a common L1 media (Guillard and Hargraves, 1993) (**Chapter 2**) to replicate trace metal concentrations typical of oligotrophic reef waters. This involved sourcing analytical grade chemicals to make up over 100 L of artificial media each week, as well as testing each batch via ICP-MS.

Throughout this thesis it was important that I could accurately test media samples to ensure that target concentrations of Mn were being achieved. To do this I used an ICP-MS (7700, Agilent, USA) machine and used a series of methods for ICP analysis of trace metals in seawater. Testing large amounts of saltwater is difficult because of the matrix effects that come from the high salt content of the sample. To combat this, I used an argon-humidifier (Elegra, Perkin Elmer, USA), matrix matched blanks (**Chapter 2**), internal standards, certified reference materials, spike recoveries, diluted samples and custom carrier gas mixes. Collectively these methods allowed me to detect Mn concentrations well below sub parts per billion and formed an integral part of Chapters 2 and 4.

Alongside learning and applying multiple different analytical techniques, I also had to grow and maintain several different species of Cnidarians. It is well known that keeping corals in enclosed aquarium systems, particularly stoney type corals, requires both specialised equipment and knowledge. Corals like *Acropora millepora*, require specific water, light and flow parameters. This means that aquarium water either needs to be supplemented to counteract the draw down of macro and trace elements by the corals or be continually replaced. Considering the nature of

experiments conducted in **Chapters 2** and **4**, I chose to continually replace water in the experimental tanks as this would also help to not only limit the build-up of Mn, but also any other waste produced in the experiment, mitigating any potential toxic effects.

However, as outlined above and in **Chapter 2**, sourcing large quantities of oligotrophic seawater was difficult in Sydney. So, for **Chapter 4**, the decision was made to work with a coral farm (Monsoon Aquatics) based in Bundaberg, Queensland. The facility not only had access to unlimited natural, oligotrophic seawater, but also had the space needed to set up much larger (68.5 L) aquarium systems. This enabled me to move beyond the use of a model organism and utilise a common reef building coral.

Throughout the experiments in **Chapters 2** and **4**, it became apparent that maintaining consistent low levels of free Mn^{2+} in biologically active experimental tank systems was difficult. Because Mn is such an important element for all life, it is often taken up from the environment extremely quickly due to biological activity (Browning et al. 2021; Jones et al. 2020; Singh et al. 2023). This is part of the reason why natural levels in of Mn in the ocean are extremely low. I first noticed the rapid uptake of Mn in **Chapter 2** when measuring concentrations in the experimental tanks via ICP-MS after 12 hours. Similar trends were also observed in **Chapter 4** where levels of Mn in the Pulse Dosed treated dropped from $19.34 \mu\text{g L}^{-1}$ to less than $1 \mu\text{g L}^{-1}$ over a 24-hour period. This rapid uptake of Mn from the water column presents several important considerations for its future use in either experimental or active management settings.

5.4 Integration with Active Intervention strategies

As climate change continues to threaten coral reefs around the globe, development of new active intervention strategies is becoming crucial to ensure their survival (Anthony et al. 2020; Camp, 2022; Hughes et al. 2017). Novel active management strategies are increasingly being considered as it is expected that coral reefs will continue to decline, as traditional management strategies struggle to maintain functioning reef systems (Eddy et al. 2021; Mcleod et al. 2019). However, relative to other interventions such as coral out planting, probiotics and genetic modification, limited research has been conducted on the suitability of elemental supplementation as a means of enhancing the thermal tolerance of corals (Montalbetti et al. 2021). This highlights several knowledge gaps which need to be addressed for supplementation-based strategies to be considered. This is contrary to agricultural sciences where the importance of trace metals such as Mn in crop health has been well established (Zulfiqar et al. 2021). Mn deficiency in crops can be a major issue, and as a result, many studies have investigated the effects of Mn supplementation, which is now utilised throughout the world as a common method of crop management (Alejandro et al. 2020; Schmidt et al. 2016; Zulfiqar et al. 2021). And whilst the supplementation of trace metals is common practice in coral aquaculture, its use as a reef intervention strategy is rarely considered, potentially due to the extreme complexity that comes with trying to artificially supplement large areas of marine habitat and while managing any unintended consequences. As Chapters (2 and 4) highlighted, non-toxic, low level Mn supplementation (up to $20 \mu\text{g L}^{-1}$) has the potential to significantly increase the thermal resilience of Cnidarians. And whilst there were no notable detrimental impacts of Mn supplementation under either ambient or thermal stress conditions, there was high phenotypic variability in response to Mn supplementation. This is expected as there is considerable variation in bleaching responses in nature (Burgess et al.

2021). This thesis only assessed Mn supplementation impacts on two Cnidarian species, and consequently, future work is needed to see if the results from this thesis can be replicated across other Cnidarians and importantly to ensure no detrimental impacts for non-target species.

Additionally, Mn supplementation should not be utilised as a standalone solution, but instead as a complimentary strategy within a broader restoration toolbox. For example, if combined with other strategies such as nutritional supplementation (Becker et al. 2021), thermal preconditioning (Majerová & Drury, 2022), probiotics (Thatcher et al. 2022), genetic/selective breeding (Humanes et al. 2024) or phenotype selection (England et al. 2024), Mn enrichment may help to further stabilise Cnidarian symbiosis under thermal stress. Additionally, the supplementation of Mn before and/or during thermal stress may help to increase the effectiveness of other intervention strategies. For example, thermally resilient corals could be selected using devices such as the MTP system, and then out planted at sites identified as natural refuges (McClanahan et al. 2024). Mn could then be combined with other nutritional and or microbial supplementation, providing a multi-level, intervention strategy, mirroring the agricultural industry. Additional benefits of supplementation-based intervention alongside traditional strategies also means that Mn can be supplemented only when it is required, and as shown in **Chapter 4**, can be done so intermittently.

However, some challenges remain, such as timing of Mn delivery, which I have shown significantly affects rates of both accumulation and photochemical efficacy. Exploring field-scale delivery methods possess many challenges and further research is required. Whilst Mn is an important trace element for all life, it can also be toxic in large enough concentrations (Binet et

al. 2023; Golding et al. 2023). Additionally, there are concerns around other potential biological impacts such as enhancing toxic algal blooms which have the capacity to damage sensitive ecosystems (Manic et al. 2024). However, targeted use and developments in microencapsulation, carrier compounds and other biotic/abiotic delivery vectors could potentially help reduce the potential for environmental contamination.

Ultimately, whilst its relative low cost and scalability potentially make it a great option that can be integrated into existing restoration workflows, there are still many limitations which must be considered.

5.5 Limitations and Remaining Knowledge Gaps

In this thesis I provide strong evidence for the physiological benefits of low-level Mn supplementation during thermal stress for Cnidarians however, several limitations must be acknowledged. Firstly, the experimental work was conducted on a limited number of taxa, which included an established model organism (*E. diaphana*) and (*Acropora millepora*), and whilst these species responded well to Mn supplementation, others may not. Additionally, both studies were conducted in controlled laboratory conditions however, physiological responses to Mn supplementation could potentially vary in natural conditions and may be species specific. The natural environment where factors such as seasons, temperature, flow, water chemistry, microbial communities and light are constantly changing will play a significant role in Mn metabolism and uptake (Yu et al. 2024). All these things have the potential to impact overall trace element requirements and uptake, making Mn supplementation potentially unpredictable. In addition to this, the long-term implications and environmental retention of Mn supplementation is poorly

understood (Dey et al. 2023). My thesis focused only on the short-term impacts of Mn supplementation, leaving questions around the persistence of Mn in both the corals and the surrounding environment, as well as the potential trade-offs in recovery, energy allocation, symbiont-microbial communities and or unintended impacts on other reef organisms. However, these risks presented by Mn supplementation are not unique to this intervention. For example, the use of probiotics also carries risks which have been systematically addressed to increase confidence in its application and such approaches could be utilized to reduce risk with Mn supplementation (Peixoto et al. 2017). It must also be noted that supplementation of full-size reef systems is currently impractical due to the scale of the intervention. Instead, Mn based supplementation would be more suitable alongside other, smaller site-based restoration works, creating a series of reef refugia that can then seed and populate the areas around it.

Whilst my thesis focused primarily on the effects of Mn, there is a whole range of other vitally important trace elements, nutrients and proteins which are required for maintaining functional Cnidarian symbiosis, often at specific stoichiometric balances (Ferrier-Pagès et al. 2018; Reich et al. 2023; Rodriguez & Ho, 2018). Despite the importance of trace metals, we are only just beginning to understand how the elemental landscape of the holobiont shifts under climate change. Additionally, the risks of not applying interventions such as Mn supplementation should also be weighed against the risk of doing nothing at all (Peixoto et al. 2024).

5.6 Future Directions

Building upon the findings of this thesis, future research should further explore the cellular mechanisms behind the protective role that Mn plays in the Cnidarian holobiont. Utilising techniques like X-ray Absorption Spectroscopy (XCAS) (Pingitore Jr. et al. 2002) and Cryo-

Electron Microscopy (Cryo-EM) (Zhao et al. 2024) could help explain how and where Mn is utilised and any other cellular implications within the holobiont. This will help researchers to tailor specific supplements that target heat sensitive processes within the holobiont. Additionally, identifying the role that the broader elementome plays in mitigating the effects of thermal stress and producing a multi-element supplement, could help to amplify the impacts of Mn supplementation.

Future work could prioritise translating the findings of this thesis into actionable reef management strategies, ensuring that the physiological benefits of Mn supplementation observed in controlled experiments can be effectively and safely applied in real-world environments. To do this, comprehensive in situ, field trials and mesocosm-based experiments to test delivery methods, uptake dynamics and broader ecosystem impacts must be conducted. Evaluation of long-term physiological consequences of Mn supplementation on both target and non-target species should also be undertaken as to ensure there are no long-term detrimental impacts.

Although this thesis provides strong evidence (**Chapters 2 and 4**) that Mn supplementation can enhance the thermal tolerances of Cnidarians, important challenges remain regarding its scalability and practical application at the reef or site-wide level. One of the biggest concerns is the risk that large-scale or poorly targeted Mn application could result in localised environmental imbalance, potentially leading to the damage of the surrounding ecosystem and sensitive species. Therefore, future work should also address the ecotoxicological thresholds, broader microbiome shifts, site-specific risk assessments and feasibility of targeted dosing approaches to ensure that any field deployment of Mn-based supplementation is both effective and ecologically responsible.

5.7 Final Remarks

This thesis contributes a critical piece to the growing field of active reef restoration by demonstrating that trace metal supplementation (specifically Mn) can be a viable and impactful strategy for increasing the thermal resilience of Cnidarians. Through the integration of elementomics, proteomics, photophysiology and further methods development, my research has not only deepened our understanding of how Mn supports Cnidarian-algal symbiosis during thermal stress, but also provides practical tools and knowledge that can be directly applied within restoration activities. In this thesis I have, i) demonstrated that Mn availability significantly impacts the photophysiology and proteome of Cnidarians, primarily through supporting primary photosynthetic processes, ii) designed and validated a novel Cnidarian health assessment device that significantly contributes to our restoration toolbox, and, iii) demonstrated that both the timing and method of Mn delivery significantly impacts the physiological response of coral to thermal stress.

The work carried out in this thesis underscores the need to build upon lessons learnt from other industries that are facing similar challenges. While obstacles remain before real-world implementation of Mn supplementation could be undertaken, this thesis provides a strong foundation for Mn supplementation as a viable, and much needed additional tool that could support reef ecosystems amidst the ever-changing backdrop of climate change (Peixoto et al. 2024). However, without urgent and substantial reductions in fossil fuel use and greenhouse gas emissions, such restoration efforts will remain temporary solutions, serving as band-aids, delaying the fate of coral reefs until climate conditions surpass those of which they are capable of withstanding.

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