Ecophysiology of Red Sea Corals in Response to Carbon and Nitrogen Availabilities

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Ecophysiology of Red Sea Corals in Response to Carbon and Nitrogen Availabilities

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This thesis is dedicated to my parents who have made many sacrifices to get me to where I am today.

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Summary

Carbon (C) and nitrogen (N) are essential for coral health, growth and energy production. Yet, maintaining a balanced availability of these elements is critical, as both deficiencies and excesses can have negative consequences for coral survival. This thesis explores how key Red Sea coral taxa, including reef-forming hard corals and a dominant soft coral species, respond to C and N availability, providing insights into their ecophysiology and resilience to environmental challenges such as nutrient pollution. Research Question 1 explored the ecophysiological responses of Red Sea corals to natural C fluxes, as detailed in Chapters 2 and 4. Chapter 2 showed that *Xenia umbellata*'s physiology was negatively impacted by the absence of heterotrophic food, reducing pulsation rates, symbiont density, and mitotic index, though the coral compensated by increasing symbiont chlorophyll-*a* content. Water flow had no significant effect, likely due to its pulsation-driven flow regulation. Chapter 4 revealed that azooxanthellate corals like *Tubastraea coccinea* exhibited significantly higher denitrification rates than zooxanthellate species, as denitrifiers utilised environmental C (e.g., DOC), instead of relying solely on photosynthates. High DOC availability (in addition to other environmental and physiological factors) was identified as one of the key drivers of denitrification in *Acropora* spp., *Millepora dichotoma* and *Tubastrea coccinea*, highlighting the role of C in N cycling processes in corals. These findings emphasise the critical role of both autotrophic and heterotrophic strategies in corals' responses to natural variations in C availability and its influence on biogeochemical processes like denitrification. Research Question 2 investigated the ecophysiological responses of *Xenia umbellata* to excess C availability under eutrophic conditions, as explored in Chapter 3. The study found that excess organic matter (OM) at 20 mg C L^{-1}, provided as dissolved organic matter (DOM) had no negative effect on coral ecophysiology. However, particulate organic matter (POM) in the form of phytoplankton and zooplankton, caused significant damage, including impaired feeding tentacles, reduced pulsation rates, and increased mortality. The severity of these effects was primarily linked to POM dosage, rather than particle size, highlighting *X. umbellata's* vulnerability to coastal eutrophication, where excess POM can harm its ecophysiology. Research Question 3 explored the ecophysiological responses of Red Sea corals to natural fluctuations in N availability, addressed in Chapter 4. High ammonium levels drive denitrification in *Acropora spp.*, as ammonium supports nitrification and nitrate production, a key substrate for denitrifying bacteria. Unexpectedly, *T. coccinea* showed elevated denitrification under low nitrate availability, likely due to co-occurring N_2 fixation and denitrification, characteristic of

oligotrophic Red Sea conditions. These results emphasize that coral responses to N fluxes are highly species-specific and influenced by local nutrient dynamics, underscoring the need to consider both biological and environmental variability when assessing coral reef resilience. This thesis highlights *Xenia umbellata*'s adaptability to low C availability and variable flow but reveals its vulnerability to excess C inputs, exposing soft corals to anthropogenic threats. Species with higher heterotrophic capacities may better withstand inorganic N pollution, potentially driving shifts toward heterotrophic-dominated reefs with significant biodiversity and ecosystem implications. This thesis offers critical insights into the physiological responses of Red Sea corals to ambient and excess nutrient levels, helping to predict reef resilience and shifts in community composition. The findings provide a basis for targeted management strategies to mitigate nutrient-related stress, especially in light of expanding coastal development projects in the Central Red Sea region.

Zusammenfassung

Kohlenstoff (C) und Stickstoff (N) sind für die Gesundheit, das Wachstum und die Energieproduktion von Korallen unerlässlich. Die Aufrechterhaltung eines ausgewogenen Gleichgewichts dieser Elemente ist jedoch entscheidend, da sowohl ein Mangel als auch ein Überschuss negative Folgen für das Überleben der Korallen haben können. In dieser Arbeit wird untersucht, wie die wichtigsten Korallenarten des Roten Meeres, darunter riffbildende Steinkorallen und eine dominante Weichkorallenart, auf die Verfügbarkeit von C und N reagieren, was Einblicke in ihre Ökophysiologie und ihre Widerstandsfähigkeit gegenüber Umweltproblemen wie der Nährstoffverschmutzung ermöglicht. Forschungsfrage 1 untersuchte die ökophysiologischen Reaktionen von Korallen im Roten Meer auf natürliche C-Flüsse, wie in den Kapiteln 2 und 4 beschrieben. Kapitel 2 zeigte, dass die Physiologie von *Xenia umbellata* durch das Fehlen von heterotropher Nahrung negativ beeinflusst wurde, was zu einer Verringerung der Pulsationsrate, der Symbiontendichte und des Mitoseindexes führte, obwohl die Koralle dies durch einen erhöhten Chlorophyll-a Gehalt der Symbionten kompensierte. Wasserströmung hatte keine signifikante Auswirkung, wahrscheinlich aufgrund der pulsationsgesteuerten Strömungsregulierung. Kapitel 4 zeigte, dass azooxanthellate Korallen wie *Tubastraea coccinea* signifikant höhere Denitrifikationsraten aufwiesen als zooxanthellate Arten, da die Denitrifikanten C aus der Umgebung z. B. gelöster organischer Kohlenstoff (DOC) nutzten, anstatt sich ausschließlich auf Photosyntheseprodukte zu verlassen. Eine hohe DOC-Verfügbarkeit (zusätzlich zu anderen Umwelt- und physiologischen Faktoren) wurde als einer der Hauptfaktoren für die Denitrifikation in *Acropora* spp., *Millepora dichotoma* und *Tubastrea coccinea* identifiziert, was die Rolle von C in den N-Zyklus-Prozessen in Korallen unterstreicht. Diese Ergebnisse unterstreichen die entscheidende Rolle sowohl autotropher als auch heterotropher Strategien bei den Reaktionen der Korallen auf natürliche Schwankungen der C-Verfügbarkeit und deren Einfluss auf biogeochemische Prozesse wie die Denitrifikation. Forschungsfrage 2 untersuchte die ökophysiologischen Reaktionen von *Xenia umbellata* auf ein Überangebot an C unter eutrophen Bedingungen, wie in Kapitel 3 beschrieben. Die Studie ergab, dass ein Überschuss an organischer Substanz (OM) von 20 mg C L-1, die als gelöste organische Substanz (DOM) bereitgestellt wurde, keine negativen Auswirkungen auf die Ökophysiologie der Korallen hatte. Die partikuläre organische Substanz (POM) in Form von Phyto- und Zooplankton verursachte jedoch erhebliche Schäden, wie z. B. eine Beeinträchtigung der Tentakel, verringerte Pulsationsraten und erhöhte Sterblichkeit. Die Schwere dieser Auswirkungen hing in erster Linie mit der POM-

Dosierung und weniger mit der Partikelgröße zusammen, was die Anfälligkeit von *X. umbellata* für die Eutrophierung der Küstengebiete verdeutlicht, wo ein Übermaß an POM ihre Ökophysiologie beeinträchtigen kann. Forschungsfrage 3 untersuchte die ökophysiologischen Reaktionen von Korallen im Roten Meer auf natürliche Schwankungen der Stickstoffverfügbarkeit, die in Kapitel 4 behandelt werden. Hohe Ammoniumwerte treiben die Denitrifikation in *Acropora* spp. an, da Ammonium die Nitrifikation und die Nitratproduktion fördert, ein Schlüsselsubstrat für denitrifizierende Bakterien. Unerwarteterweise zeigte *T. coccinea* eine erhöhte Denitrifikation bei geringer Nitratverfügbarkeit, wahrscheinlich aufgrund der gleichzeitigen N2-Fixierung und Denitrifikation, die für oligotrophe Bedingungen im Roten Meer charakteristisch sind. Diese Ergebnisse unterstreichen, dass die Reaktionen der Korallen auf N-Flüsse sehr artspezifisch sind und von der lokalen Nährstoffdynamik beeinflusst werden, was die Notwendigkeit unterstreicht, bei der Bewertung der Widerstandsfähigkeit von Korallenriffen sowohl biologische als auch ökologische Schwankungen zu berücksichtigen. Diese Arbeit unterstreicht die Anpassungsfähigkeit von *Xenia umbellata* bei geringer C-Verfügbarkeit und variabler Strömung, zeigt aber auch ihre Anfälligkeit gegenüber übermäßigem C-Eintrag, wodurch Weichkorallen anthropogenen Bedrohungen ausgesetzt sind. Arten mit höherer heterotropher Kapazität können anorganischer N-Verschmutzung besser widerstehen, was zu einer Verschiebung hin zu heterotrophen Riffen führen könnte, was erhebliche Auswirkungen auf die biologische Vielfalt und das Ökosystem hätte. Diese Arbeit bietet wichtige Einblicke in die physiologischen Reaktionen von Korallen im Roten Meer auf die umgebenden und überhöhten Nährstoffgehalte und hilft bei der Vorhersage der Widerstandsfähigkeit von Riffen und der Veränderung der Zusammensetzung von Gemeinschaften. Die Ergebnisse bilden die Grundlage für gezielte Managementstrategien zur Abschwächung von nährstoffbedingtem Stress, insbesondere im Hinblick auf die zunehmenden Küstenentwicklungsprojekte in der zentralen Rotmeerregion.

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List of publications and manuscripts within the thesis

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Hill, C.E.L., Rücker, J., Eichhorn, N., Mezger, S.D., Caporale, G., El-Khaled, Y. C., Blanco C, S.V., Wild, C., & Tilstra, A. (2024). Excess particulate organic matter negatively affects the ecophysiology of the widespread soft coral *Xenia umbellata. Marine Biology, 171*(12), 238. https://doi.org/10.1007/s00227-024-04563-7

First author manuscripts in preparation for publication

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Contribution of the candidate to multi-author articles and manuscripts included in the thesis

Chapter 2 | **Hill, C. E. L.**, Abbass, S. G., Caporale, G., El-Khaled, Y. C., Kuhn, L., Schlenzig, T., Wild, C., & Tilstra, A. (2023). Physiology of the widespread pulsating soft coral *Xenia umbellata* is affected by food sources, but not by water flow. *Ecology and Evolution*, *13*(9), e10483. https://doi.org/10.1002/ece3.10483

Experimental concept and design	70%
Experimental work and/or acquisition of (experimental) data	100%
Data analysis and interpretation	100%
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Writing of the first and last version of the manuscript	100%

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Chapter 1| General Introduction

1.1 Key players and traits of coral reefs

Coral reefs are unique marine ecosystems that are primarily built by hard corals of the order Scleractinia. These corals secrete calcium carbonate skeletons that gradually accumulate and layer to create solid frameworks overtime (Connell, 1973; Goreau et al., 1979; Stanley, 1981). Whilst occupying less than 0.1% of the ocean floor (Spalding & Grenfell, 1997), coral reefs support approximately 30% of ocean biodiversity, hosting fish, marine mammals and numerous invertebrates of both adult and juvenile life stages (Fisher et al., 2015). Coral reefs underpin the health of the oceans by cycling nutrients like nitrogen (N), phosphorus (P) and carbon (C) (Pellowe et al., 2023) and maintaining water quality (Chen, 2021). They also provide a suite of critical goods and services to humankind including coastal protection, commercial fisheries and tourism (Moberg & Folke, 1999).

Much of the functioning of coral reef ecosystems is directly dependent on the biological communities that inhabit the benthos (Tsikopoulou et al., 2024). Whilst calcifying and slow-growing hard corals are essential for building reef structures, soft corals of the class Octocorallia are also integral to coral reef ecosystems. Soft corals differ from hard corals as they lack a rigid calcium carbonate skeleton, but instead have a fleshy and flexible structure supported by internal sclerites (Rahman & Oomori, 2008). Soft corals are fast-growing and often among the first to recolonise disturbed or degraded reef area (Dinesen, 1985). Soft corals also play a vital role in nutrient cycling of C and N, reinforcing the analogy of coral reefs as "oases in an ocean desert". Whilst soft corals do not calcify like hard corals, soft corals contribute to C sequestration by storing C in their tissues (Widdig & Schlichter, 2001). They also efficiently cycle N, helping to balance N availability and thereby prevent both N limitation and eutrophication (Bednarz et al., 2015; El-Khaled et al., 2021). However, despite their importance, soft corals are less well understood than hard corals.

1.2 Coral reefs of the Red Sea

Coral reefs inhabit tropical and subtropical regions (Levinton, 2022). One such area is the Red Sea, known for its unique environmental conditions compared to other oceanic regions that harbour coral reefs. The Red Sea is a narrow, elongated body of water classified as a marginal sea of the Indian Ocean, extending approximately 2,000 km in length and 250 km in width (Berumen et al., 2019). The Red Sea is recognised as one of the warmest, saltiest and oligotrophic seas in the world (Berumen et al., 2019; Carvalho et al., 2019). Yet, it exhibits significant temporal and spatial variations, with environmental conditions shifting markedly both over seasons and across a north-south gradient (Berumen et al., 2019). Generally, temperatures in the Red Sea are considerably higher than other regions that host coral reefs (Chaidez et al., 2017), reaching summer averages of ~ 33 °C in the central Red Sea (Rich et al., 2022). The oligotrophic state of the Red Sea is characterised by the limited availability of key nutrients such as nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_3^{2-}), all of which are essential for supporting productivity (Acker et al., 2008). These low availabilities are primarily driven by limited freshwater input due to low rainfall and the absence of major river discharges, factors which also contribute to its high salinity (Berumen et al., 2019). These nutrient-poor conditions are further intensified by its geographic isolation, with only a narrow connection to the Indian Ocean, minimising nutrient exchange (Churchill et al., 2014). Additionally, the Red Sea experiences strong stratification, where warm surface waters and cooler deep waters remain separate, restricting nutrient upwelling to the upper layers where most coral reefs reside (Acker et al., 2008). As a result, Red Sea corals have remarkably high tolerance to thermal stress (Evensen et al., 2021; Fine et al., 2013), high salinity (Kleinhaus et al., 2020) and oligotrophic conditions (Rädecker et al., 2015). The Red Sea, therefore, serves as an invaluable natural laboratory for studying coral ecophysiology, focusing on how environmental conditions influence coral physiology -processes such as photosynthesis, respiration and nutrient uptake- and drive their adaptive responses to diverse and challenging stressors.

1.3 Mechanisms of carbon acquisition

1.3.1 Autotrophic feeding

Corals function as holobionts, which are complex meta-organisms consisting of the coral host in close association with a diverse community of fungi, archaea, endolithic algae, viruses, bacteria, Symbiodiniaceae and other protists (Voolstra et al., 2021). Symbiodiniaceae, colloquially known as zooxanthellae, are algal cells that reside within the tissues of some corals (Couce et al., 2012; Freudenthal, 1962). Corals which host these algal partners are known as zooxanthellate corals (Schuhmacher & Zibrowius, 1985). The Symbiodinicaeae engage in a symbiotic relationship with their coral host, providing mutual benefits to both organisms (Muscatine, 1990). The Symbiodiniaceae photosynthetically fix C by converting sunlight and carbon dioxide (CO_2) into organic C. The bulk of C-rich photosynthates (~ 95%) are translocated to the coral host to fuel coral growth and calcification, while the remainder is utilised for their own metabolic needs (Goreau, 1959; Muscatine, 1990; Trench, 1993). This mode of energy acquisition -named autotrophy- is fundamental to the high productivity of coral reef ecosystems and has even been termed the "engine" of the reef ecosystem (Muller-Parker et al., 2015; Roth, 2014). In exchange, the Symbiodiniaceae obtain a protective habitat and access to the host's metabolic waste products, such as carbon dioxide and ammonia, as well as organic compounds like glucose and amino acids which they utilise for photosynthesis and growth (Trench, 1993; Yellowlees et al., 2008). However, the coral-algal symbiosis is highly sensitive to environmental conditions. Whilst autotrophy may benefit from factors such as moderately increased sunlight (Wellington, 1982) high water flow (Finelli et al., 2006; Mass et al., 2010) and low turbidity (Anthony & Fabricius, 2000), the coral-algal symbiosis may break down if exposed to significant or prolonged environmental perturbation. This breakdown manifests as coral bleaching, where the coral host expels its symbionts, exposing the white calcium carbonate skeleton beneath (Douglas, 2003). A myriad of stressors can cause bleaching, such as elevated sea surface temperatures in combination with high solar radiation (Hoegh-Guldberg et al., 2007; Hughes et al., 2003), changes in salinity (Goreau, 1964), excess nutrients (Wiedenmann et al., 2013) and increased sedimentation and pollutants (Coles & Brown, 2003).

1.3.2 Heterotrophic feeding

Numerous studies have found that corals may also acquire C via heterotrophy, obtaining energy from external sources (Houlbrèque & Ferrier-Pagès, 2009). This process includes the uptake of dissolved organic matter (DOM) like sugars and dissolved free amino acids (Ferrier, 1991;

Stephens, 1962). Additionally, it involves the ingestion of particulate organic matter (POM) like plankton of varying types and sizes (Heidelberg et al., 2004), detritus (Anthony & Fabricius, 2000) and microbes such as bacteria (Houlbrèque et al., 2004). Heterotrophic feeding provides additional nutrients like N and P which are crucial for growth but cannot be obtained through autotrophy (Ayukai, 1995). In fact, the photosynthates translocated by Symbiodiniaceae have been nicknamed "junk food" since they are deficient in these nutrients (Falkowski et al., 1984). Corals exhibit varying dependencies on heterotrophy, with most being mixotrophic where they derive nutrition from both autotrophic and heterotrophic feeding strategies (Sturaro et al., 2021). Yet, some corals do not host Symbiodiniaceae, and therefore rely entirely on heterotrophy, acquiring all their C from external organic sources. These corals are known as azooxanthellate (Schuhmacher & Zibrowius, 1985). Soft corals are generally recognised as more heterotrophic than hard corals, as they exhibit lower photosynthetic rates (Fabricius & Klumpp, 1995). This is further corroborated by findings showing that 8 out of 10 zooxanthellate soft coral species from mid-shelf reefs of the Great Barrier Reef could not depend solely on autotrophy to meet their respiratory C requirements (Fabricius & Klumpp, 1995). Environmental factors can increase heterotrophy in corals including nutrient-rich conditions that support plankton growth (Ferrier-Pagès et al., 2003), stronger water flow that delivers more food particles to the coral (Fabricius & Klumpp, 1995), and various forms of environmental stress (Grottoli et al., 2006a). In particular, heterotrophic C can also become a crucial energy source when photosynthetic C fixation is impaired, such as during bleaching events or under limited light conditions found in turbid environments (Grottoli et al., 2006b). In fact, research has shown that heterotrophic feeding promotes rapid recovery following acute stress, as these corals possess a significantly enhanced ability to sustain and replenish their energy reserves in the form of lipids, carbohydrates and proteins (Grottoli et al., 2006b; Rodrigues & Grottoli, 2007). There is also evidence that heterotrophic nutrient supply aids in the restoration of photosynthate translocation, helping to re-establish normal nutrient exchange processes (Tremblay et al., 2016).

1.4 Nitrogen regulation in coral reefs

1.4.1 Nitrogen uptake pathways

Whilst the high productivity of coral reef ecosystems can be attributed to their efficient carbon acquisition mechanisms, this can also be due to their effective uptake of N for growth and biomass production (Muscatine & Porter, 1977). However, corals typically inhabit oligotrophic environments, where the availability of nutrients like N is low (Muscatine & Porter, 1977). This incongruity was first recognised by Charles Darwin during his work on coral reefs in the South Pacific, and has since been termed "Darwin's paradox" (Darwin, 1889). Corals overcome this via efficient mechanisms of N acquisition. To do so, corals satisfy a large amount of their N demand via heterotrophic feeding when sufficient food sources are available (Houlbrèque & Ferrier-Pagès, 2009). Another source of N, specifically for zooxanthellate corals, comes from photosynthates transferred from the Symbiodiniaceae to the coral host (Falkowski et al., 1984). These compounds contain N since Symbiodiniaceae take up both NH_4^+ and NO_3^- (Pernice et al., 2012). Furthermore, N-cycling microbes living in association with the coral host can acquire N via the energetically intensive process of N_2 fixation (Cardini et al., 2015; Lesser et al., 2007; Shashar et al., 1994). This N-cycling pathway is governed by diazotrophic bacteria that utilise nitrogenase, an enzyme complex responsible for converting atmospheric N_2 into bioavailable forms like NH_4^+ (Halbleib & Ludden, 2000) (Figure 1.1). To conserve N within the reef ecosystem, efficient N recycling is vital, ensuring a continuous supply of nitrogenous compounds for the coral host. Symbiodiniaceae recycle the host's metabolic waste products such as NH_4^+, converting them into various nitrogenous compounds that the host can reuse (Kopp et al., 2013; Reynaud et al., 2009; Wang & Douglas, 1999).

1.4.2 Nitrogen removal pathways

A microbially driven process called denitrification has recently been hypothesised to play an important role in alleviating the coral from excess N and in doing so maintain the coral-algal symbiosis and the overall health of the coral (El-Khaled et al., 2020; Tilstra et al., 2019). Coral associated denitrification refers to the process by which denitrifying bacteria living in association with the coral host sequentially convert NO_3^- through various intermediate N compounds into gaseous N_2 (Goering, 1985) (Figure 1.1). Whilst denitrification has been comprehensively studied in other fields (Bremner & Shaw, 1958; Garcias-Bonet et al., 2018; Philippot et al., 2007; Seitzinger et al., 2006), research on denitrification in coral reef ecosystems is still in its infancy. Yet, in recent years more work has emerged, offering foundational insights into the role of denitrification (El-Khaled et al., 2020; Glaze et al., 2022;

Rädecker et al., 2015; Tilstra et al., 2019; Yang et al., 2013). For example, scientists recently identified that denitrification actively occurs in three Red Sea hard coral holobionts (Tilstra et al., 2019) as well as within various benthic components of coral reef ecosystems including turf algae, coral rubble, soft coral, biogenic rock and reef sands when exposed to N enrichment (El-Khaled et al., 2020). Yet, these studies also found substrate- and coral species-specific differences in denitrification activity (El-Khaled et al., 2021; Tilstra et al., 2019). Additionally, findings have shown that denitrification positively correlates with N_2 fixation rates and with algal symbiont density of corals (Tilstra et al., 2019). From these findings, it has been postulated that denitrification may have similarities with N_2 fixing bacteria and be linked to the heterotrophic capacity of the coral (Tilstra et al., 2019).

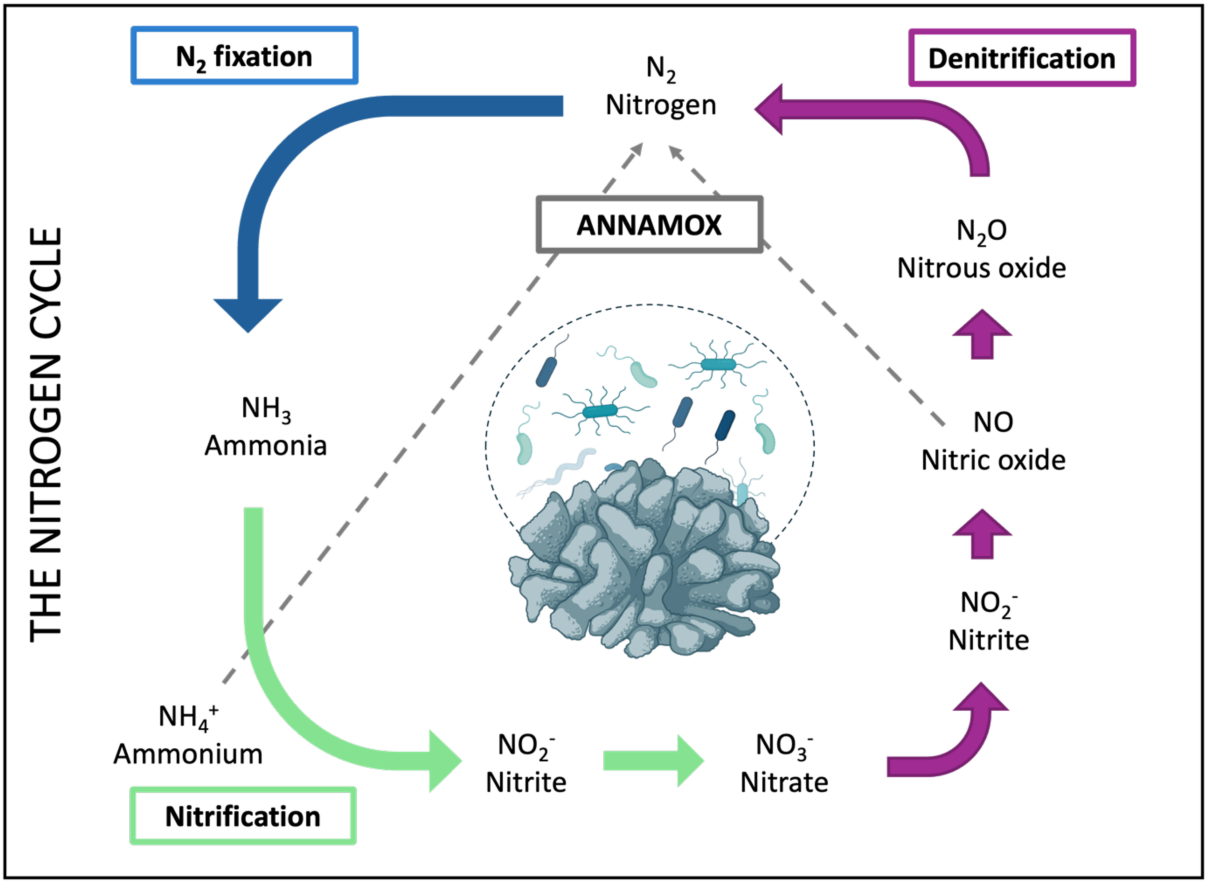

Figure 1.1 Nitrogen cycling pathways associated with coral holobionts. Icons sourced from BioRender.

1.5 Nutrient pollution and its impact on coral health

Coral reefs are increasingly threatened by global-scale climate change and localised stressors (Hoegh-Guldberg, 1999; Wilkinson, 1999). Hard corals, reliant on calcification, are especially

vulnerable to global threats like ocean warming and acidification which trigger coral bleaching and weaken reef frameworks (Anthony et al., 2008; Cornwall et al., 2021; Erez et al., 2011). Importantly, the resilience of corals to bleaching is closely linked to nutrient availability, making nutrient dynamics critical to their ability to cope with these global pressures (Morris et al., 2019). Alarmingly, nutrient pollution in the typically oligotrophic Red Sea has surged (El Nemr & El-Said, 2014; Ghandourah et al., 2023). Rapid population growth and urban development along the coast has resulted in greater discharges of sewage and industrial rich effluents into the sea, leading to nutrient imbalances that exacerbate existing challenges and generate new ones (Orif, 2020). While nutrient pollution encompasses various elements, excess C and N are particularly impactful leading to several ecological consequences in coral reef ecosystems (Bednarz et al., 2020). Excess C can be in the form of dissolved organic matter (DOM) or particulate organic matter (POM). One type of DOM, dissolved organic carbon (DOC), can stimulate the growth of microbes in the mucopolysaccharide layer of hard corals, disrupting their microbiome and causing mortality (Kline et al., 2006; Kuntz et al., 2005). Some of these microbes can be pathogenic, increasing the prevalence of coral diseases and making corals more vulnerable to the effects of stressors like ocean warming and acidification (Haas et al., 2016). However, interestingly, POM does not induce the same negative physiological response as DOM in hard corals. For example, studies have shown that moderate levels of POM offers energy and growth benefits (Dubinsky & Jokiel, 1994; Fabricius, 2005).

Excess N availability can directly reduce calcification and growth of corals (Silbiger et al., 2018) and disrupt the coral-algal symbiosis through several mechanisms (Baker et al., 2018; Wiedenmann et al., 2013). Firstly, excess N causes reduced C translocation from the algal symbionts to the coral host as they allocate more C to their own growth instead (Baker et al., 2018). This causes a proliferation of the symbionts, which shifts the symbiosis from mutualistic to parasitic, thereby increasing the susceptibility of the coral to bleaching (Baker et al., 2018; Cunning & Baker, 2013). Secondly, high N levels without equivalently high P levels may cause P starvation of the algal symbionts. P is essential to maintain the structure and function of the thylakoid membranes in chloroplasts, which are crucial for photosynthesis and C fixation (Wiedenmann et al., 2013). When P is limited, the integrity of these thylakoid membranes is compromised, reducing the symbionts' ability to efficiently perform photosynthesis and increasing the coral's susceptibility to thermal and light stress, leading to coral bleaching (Wiedenmann et al., 2013). Excess N also promotes the growth of fast-growing algae, forming algal blooms that smother corals and create eutrophic environments that result in an overall

loss of biodiversity (Lapointe, 1997). In turn, algae release a DOC into the water, contributing to the pool of excess C (Norrman et al., 1995). Interestingly, soft corals have generally demonstrated greater resilience to nutrient pollution compared to hard corals, as numerous studies have shown that their key physiological traits remain largely unaffected by both organic and inorganic enrichment (Heimburger, 2021; Klinke et al., 2022; Mezger et al., 2022; Simancas-Giraldo et al., 2021; Thobor et al., 2022; Vollstedt et al., 2020).

1.6 Knowledge gaps

C and N are essential for the health and productivity of corals. However, imbalances in their levels can result in substantial coral mortality. Thus, it is crucial to fully understand how C and N availability impact coral ecophysiology. Yet, there are still significant knowledge gaps that remain to be addressed in this context. The key knowledge gaps addressed in this thesis are as follows:

1. *Xenia umbellata* is a well-studied soft coral in the Indo-Pacific and Red Sea, yet its ecophysiological responses to varying levels of natural C availability remain poorly understood. While some studies suggest that *X. umbellata* can fully meet its metabolic demands through autotrophy (Mezger et al., 2022), others argue it has a greater reliance on heterotrophy (Al-Sofyani. & Niaz., 2007). These contrasting findings highlight the need for a deeper understanding of how *X. umbellata* responds to natural fluctuations in C availability, which is critical for elucidating its trophic ecology and adaptability in dynamic marine environments. Additionally, *X. umbellata* inhabits diverse environments across a depth gradient from < 1m to 25 m (Janes, 2013), where water motion varies significantly. Given that water motion influences C acquisition strategies in scleractinian corals and other octocorals (Chang-Feng & Ming-Chao, 1993; Fabricius, 2005; Sebens et al., 1997, 1998), investigating its interaction with the C acquisition of *X. umbellata* could provide new insights into the ecophysiological mechanisms driving its resilience and trophic flexibility as a soft coral.

2. With increasing nutrient pollution in coastal regions, coastal coral reefs are increasingly exposed to an excess of organic matter (OM) in the water column of both dissolved (DOM) and particulate (POM) forms. Whilst DOM, rather than POM, may negatively

impact the ecophysiology of hard corals (Kline et al., 2006; Kuntz et al., 2005), the impact on soft corals remains unclear. Previous studies have investigated the effects of DOM on *X. umbellata* and found no significant ecophysiological impact (Simancas-Giraldo et al., 2021; Vollstedt et al., 2020), yet it remains unknown how *X. umbellata* is affected by excess POM, when C content is standardised between treatments. Understanding how *X. umbellata* responds to excess POM is critical for determining its capacity to tolerate changing C dynamics in nutrient-enriched coastal environments.

3. Denitrification is increasingly recognised as an ecophysiological trait that may alleviate the stress of excess N availability in corals (El-Khaled et al., 2020; Tilstra et al., 2019). However, foundational understanding of how denitrification responds to fluctuations in C and N availability remains limited. Whilst some studies suggest that denitrifying microbes in corals may utilise photosynthates as a C source to fuel their metabolism (Tilstra et al., 2019), the interaction between heterotrophic capacity and denitrification rates has not yet been explored. Furthermore, the effects of natural environmental variability -such as seasonal fluxes in N and C dynamics- on denitrification rates are poorly understood. This foundational knowledge is essential to decipher how these processes operate under normal conditions, which is a prerequisite for predicting and addressing the impacts of global change scenarios.

1.7 Thesis overview | overarching questions and structure

To fill these knowledge gaps, broader overarching questions were asked which were addressed with more targeted research questions within each chapter (Table 1).

1. What are the ecophysiological responses of Red Sea corals to natural fluxes of C availability? **[addressed in chapter 2 and chapter 4]**.

2. What are the ecophysiological responses of Red Sea corals to excess C availability under eutrophic conditions? **[addressed in chapter 3]**.

3. What are the ecophysiological responses of Red Sea corals to natural fluxes of N availability? **[addressed in chapter 4].**

This thesis is divided into five chapters, comprising a general introduction (chapter 1), three main data chapters (chapters 2, 3, & 4) and a general discussion (chapter 5) (Table 1.1).

Table 1.1 A summary of the specific research questions, hypotheses, methodological approach and the species focus of each main data chapter of the thesis. Abbreviations include DOM = dissolved organic matter, POM = particulate organic matter, DOC = dissolved organic carbon.

	Chapter 2	***Chapter 3***	***Chapter 4***
Research question	What are the feeding preferences of the soft coral *X. umbellata*? What are the effects of water flow on the feeding regime of *X. umbellata*?	What are the physiological effects of 20 mg C L^{-1} of dissolved and particulate forms (of varying particle sizes) of OM on the morphology and ecophysiology of *X. umbellata?*	How does denitrification differ among four hard skeleton-forming corals over a temporal scale of one year? How does the heterotrophic capacity of corals affect denitrification? What is the relative influence of external environmental and internal physiological traits on coral-associated denitrification?
Species focus	*Xenia umbellata*	*Xenia umbellata*	*Stylophora pistillata, Acropora* spp., *Millepora dichotoma,* and *Tubastrea coccinea*
Hypothesis	*X. umbellata* will exhibit a negative physiological response when heterotrophic feeding is inhibited, indicating a preference for a mixotrophy. The highest water flow with food addition will yield the best physiological response in *X. umbellata*, promoting both autotrophy and heterotrophy to best meet metabolic needs.	The physiology of *X. umbellata* will be negatively impacted by POM but not by DOM, as excess POM at 20 mg C L^{-1} may harm delicate feeding structures and no longer offer heterotrophic benefits.	Denitrification rates will fluctuate between months, with higher rates during warmer temperatures and higher C and N availability. Denitrification rates will be higher in more autotrophic corals. Both internal physiological traits and external environmental conditions will influence denitrification rates.
Approach	Feeding and water flow was manipulated over 15 days, assessing various physiological parameters. Four water volume exchange rates were crossed with three feeding treatments in a fully factorial design.	C enrichment was manipulated over 28 days, assessing various physiological parameters. A pulse of 20 mg C L^{-1} was provided as one DOM treatment (glucose-DOC), two POM treatments (phytoplankton and zooplankton) and a control.	Four corals encompassing a range of heterotrophic capacities were sampled over a complete year. We assessed denitrification rates, quantified key physiological traits and monitored environmental conditions.

1.8 References

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Chapter 2 | Physiology of the widespread pulsating soft coral *Xenia umbellata* is affected by food sources, but not by water flow

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2.1 Abstract

Coral energy and nutrient acquisition strategies are complex and sensitive to environmental conditions such as water flow. While high water flow can enhance feeding in hard corals, knowledge about the effects of water flow on the feeding of soft corals, particularly those pulsating, is still limited. In this study, we thus investigated the effects of feeding and water flow on the physiology of the pulsating soft coral *Xenia umbellata*. We crossed three feeding treatments *i)* no feeding, *ii)* particulate organic matter [POM] as phytoplankton, and *iii)* dissolved organic carbon [DOC] as glucose, with four water volume exchange rates (200, 350, 500 and 650 Lh^{-1}) over 15 days. Various ecophysiological parameters were assessed including pulsation rate, growth rate, isotopic and elemental ratios of carbon (C) and nitrogen (N) as well as photo-physiological parameters of the Symbiodiniaceae (cell density, chlorophyll-*a* and mitotic index). Water flow had no significant effect but feeding had a substantial impact on the physiology of the *X. umbellata* holobiont. In the absence of food, corals exhibited significantly lower pulsation rates, lower Symbiodiniaceae cell density, and lower mitotic indices compared to the fed treatments, yet significantly higher chlorophyll-*a* per cell and total N content. Differences were also observed between the two feeding treatments, with significantly higher pulsation rates and lower chlorophyll-*a* per cell in the DOC treatment, but higher C and N content in the POM treatment. Our findings suggest that the *X. umbellata* holobiont can be viable under different trophic strategies, though favouring mixotrophy. Additionally, the physiology of the *X. umbellata* may be regulated through its own pulsating behaviour without any positive nor negative effects from different water flow. Thus, this study contributes to our understanding of soft coral ecology, particularly regarding the competitive success and widespread distribution of *X. umbellata*.

Keywords

Trophic ecology, carbon, nitrogen, phytoplankton, current regime

2.2 Introduction

Tropical coral reefs are highly productive and host a huge diversity of organisms. However, they inhabit oligotrophic waters which are deficient in essential nutrients. Therefore, corals rely on several key mechanisms of nutrient and energy acquisition such as autotrophy (Muscatine and Porter, 1977), and heterotrophy (Houlbrèque and Ferrier-Pagès, 2009) in order to meet their metabolic demands. Corals exist on a spectrum where their individual dependence on autotrophy and heterotrophy differs between species. Some corals are purely autotrophic or heterotrophic while others are mixotrophic and derive nutrition from both feeding modes (Fabricius and Klumpp, 1995; Fox *et al.*, 2018; Conti-Jerpe *et al.*, 2020; Sturaro *et al.*, 2021).

Autotrophy in corals is facilitated by their symbiotic relationship with Symbiodiniaceae that photosynthesise and translocate carbon to the coral host (Muller-Parker, D'Elia and Cook, 2015; LaJeunesse *et al.*, 2018). The rate of carbon fixation by Symbiodiniaceae is high, and fundamental amino acids and sugars can be assimilated by the coral within a matter of seconds (Streamer, McNeil and Yellowlees, 1993). Research has even shown that healthy corals that harbour Symbiodiniaceae are able to meet 100% of their daily metabolic demand via autotrophy alone (Grottoli, Rodrigues and Palardy, 2006). Corals can also feed heterotrophically, by actively preying on dissolved [DOM] and particulate organic matter [POM] of varying size classes (Houlbrèque and Ferrier-Pagès, 2009), to obtain nutrients such as nitrogen and phosphorus that support both the coral host and if present, the Symbiodiniaceae (Muscatine and Porter, 1977; Fitt and Cook, 2001). The heterotrophic uptake of POM is facilitated by morphological adaptations such as feeding tentacles, mesenterial filaments, cnidae and even mucus, to effectively capture prey from the water column (Al-Sofyani. and Niaz., 2007; Yosef *et al.*, 2020). The extent to which corals rely on autotrophy and heterotrophy not only varies considerably between species, but is also modulated by the environment (Palardy, Rodrigues and Grottoli, 2008).

Environmental conditions such as water flow have been shown to affect coral feeding (Sebens and Johnson, 1991; Chang-Feng and Ming-Chao, 1993; Fabricius, Genin and Benayahu, 1995; Sebens, Witting and Helmuth, 1997; Sebens *et al.*, 1998; Wijgerde *et al.*, 2012). Corals, as sessile organisms, depend on water motion to supply food items for heterotrophic feeding.

Consequently, heterotrophy in corals can be enhanced with high water flow, as there is an increased flux of food particles across the polyps (Fabricius, Genin and Benayahu, 1995). Flow speeds have also been found to influence food capture efficiency, with zooplankton primarily captured at low flow and phytoplankton captured at higher flows, allowing corals to exploit different food sources under different flow regimes (Orejas *et al.*, 2016). However, higher water flow has variable and not always beneficial effects on nutrient acquisition, with evidence of increased uptake yet also increased efflux of nutrients (Borchardt, Hoffmann and Cook, 1994). In addition, with very high water motion, the mechanical forces on the coral may become too intensive and feeding structures may be swept back (Purser *et al.*, 2010) and possibly damaged (Wainwright and Koehl, 1976; Sebens, 1997), or particles may simply pass over polyps too quickly to be successfully captured (McFadden, 1986; Purser *et al.*, 2010). Autotrophy can accelerate in higher flow environments, with increased oxygen efflux and thereby increased photosynthetic efficiency of the coral (Finelli *et al.*, 2006; Mass *et al.*, 2010). Corals have even been found in low flow conditions, to perform cilia-induced mixing of the coral diffusive boundary layer to remove excess oxygen and prevent oxidative stress (Pacherres *et al.*, 2022).

Whilst there is a considerable amount of literature that covers the effects of water flow on feeding regimes in corals, these studies largely focus on scleractinian corals (Sebens and Johnson, 1991; Sebens, Witting and Helmuth, 1997; Sebens *et al.*, 1998; Wijgerde *et al.*, 2012; Orejas *et al.*, 2016), with substantially less attention paid to soft corals (Chang-Feng and Ming-Chao, 1993; Fabricius, Genin and Benayahu, 1995). In recent years, soft corals have increased in cover in many regions, while scleractinians have concomitantly decreased and/or not recovered from bleaching events at a significant pace (Lenz *et al.*, 2015; Contreras-Silva *et al.*, 2020). This is a consequence of increased environmental stressors that negatively affect scleractinian corals, in combination with soft corals' opportunistic lifestyle involving fast growth rates, extensive asexual reproduction and high fecundity (Fabricius, 1995; Tilot *et al.*, 2008; Haverkort-Yeh *et al.*, 2013). One markedly successful soft coral family is Xeniidae (Ehrenberg, 1828). Research on these corals has shown them to be resilient against numerous global and local change parameters. For example, a study on *Xenia* cf *crassa* (Schenk, 1896) revealed it was not vulnerable to thermal stress, with no evidence of bleaching during the marine heatwave in Australia in 2019 (Steinberg *et al.*, 2022). In another study, *Ovabunda macrospiculata* (Gohar., 1940) demonstrated a resistance to high pCO_2 conditions (Gabay *et al.*, 2014). One species in particular, namely *Xenia umbellata* (Lamarck, 1816), has displayed

resistance to warming (Mezger *et al.*, 2022; Thobor *et al.*, 2022), organic eutrophication (Vollstedt *et al.*, 2020; Simancas-Giraldo *et al.*, 2021) and phosphate enrichment (Klinke *et al.*, 2022; Mezger *et al.*, 2022), with its success across all these studies attributed to its trophic plasticity.

Xenia umbellata is inherently mixotrophic in its feeding strategy, possessing morphological features to support both auto- and heterotrophy. However, whether *X. umbellata* has a preferred or more dominant feeding mode is unclear. Whilst soft corals are generally considered as more heterotrophic (Pupier *et al.*, 2021), *X. umbellata* has demonstrated a higher photosynthetic productivity compared to other soft corals, with the ability to sustain its energetic needs by net autotrophy alone (Mezger *et al.*, 2022). Yet, other studies suggest that *X. umbellata* relies more on heterotrophic suspension feeding because of its morphology and biochemical composition (Al-Sofyani. and Niaz., 2007). *Xenia umbellata* is naturally distributed throughout the Red Sea and the Indo-Pacific (Verseveldt, 1965), where it occupies a range of environments including hard and soft substrates such as reef walls, and sand slopes (Janes, 2014) (Figure 2.1A & B). *Xenia umbellata* also inhabits a depth profile extending from 3 m to 25 m (Janes, 2014), but has been observed at very shallow depths of less than 1 m (Figure 2.1C), where environmental conditions such as water motion vary considerably. Whilst water flow and its interaction with feeding is well-researched for scleractinian corals, knowledge gaps remain for soft corals, as mentioned above. The soft coral *X. umbellata* has repeatedly demonstrated resilience in the face of global change, however, detailed knowledge about its trophic ecology is still lacking. Therefore, *X. umbellata* is the ideal soft coral to use in our investigation into the effects of water flow and feeding.

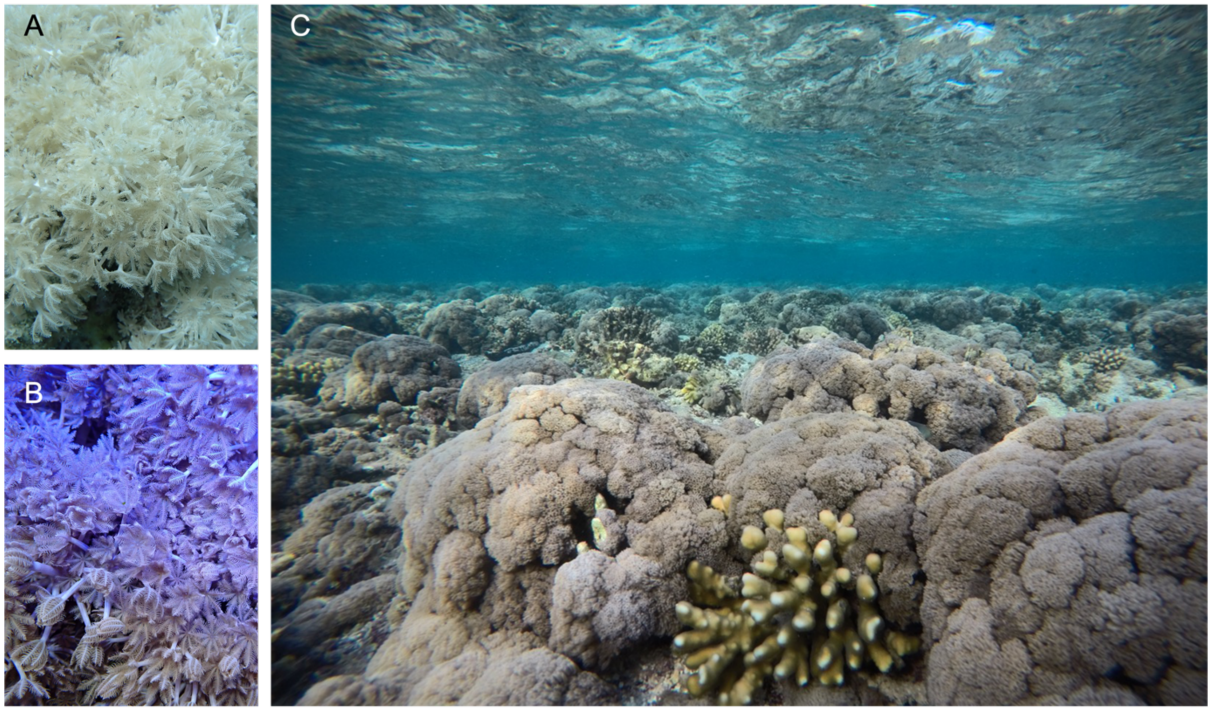

Figure 2.1 A] *Xenia umbellata* photographed *in situ.* B] *X. umbellata* photographed in laboratory conditions in the long-term maintenance tank within the Marine Ecology department at the University of Bremen, Germany. C] An image of a shallow reef (<1 m depth) covered in *X. umbellata* in the central Red Sea, along the coast of Saudi Arabia. Photo credit: A & C: Walter A. Rich, B: Arjen Tilstra.

Our study aimed to *i)* investigate the effects of water flow on the feeding regime of *X. umbellata*, and also aimed to *ii)* determine the feeding preferences of *X. umbellata*. We manipulated feeding and water flow over 15 days and assessed multiple physiological parameters including pulsation, growth, isotopic and elemental ratios as well as photo-physiological parameters of the Symbiodiniaceae, i.e., cell density, mitotic index, and chlorophyll-*a* content. Firstly, we hypothesised that the highest water flow treatment combined with food addition would result in the best physiological responses from *X. umbellata,* because they would be able to optimally perform both autotrophy and heterotrophy, and thereby fully meet their metabolic requirements. Secondly, we hypothesised that *X. umbellata* would have a negative physiological response in the control treatment where they are unable to feed heterotrophically, and through this demonstrate their preference for a mixotrophic feeding strategy for optimal health.

2.3 Methodology

The experiment was conducted within the laboratory facilities of the Marine Ecology Department at the University of Bremen, Germany, from November 2021 to February 2022. The experiment was broken down into three consecutive 15-day phases. Four water volume exchange rates were crossed in a fully factorial design with feeding treatments supplied in phases: *i)* no feeding (Phase 1), *ii)* POM, supplied as phytoplankton (Phase 2) and *iii)* DOC, supplied as glucose (Phase 3) (Figure 2). Within each phase, the four water volume exchange rates were replicated three times, with 6 fragments within each tank (Figure 2).

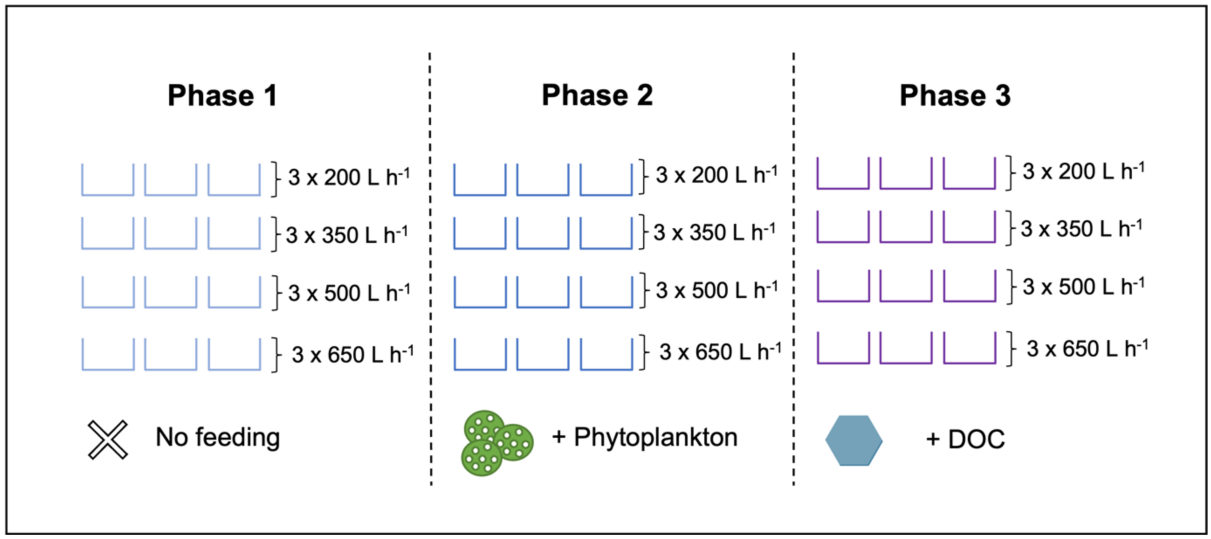

Figure 2.2 A summary of the experimental design. Feeding treatments were provided in 'phases', with no food supplied in phase 1 (control), phytoplankton supplied in phase 2 and DOC supplied in phase 3. Within each experimental phase, four water volume exchange rates (200 Lh^{-1}, 350 Lh^{-1}, 500 Lh^{-1} and 650 Lh^{-1}) were constantly maintained with a replication of three, with a random distribution across 12 experimental tanks. Phases were run consecutively for a duration of 15 days each, using different coral fragments.

2.3.1 *Xenia umbellata* preparation

Xenia umbellata colonies used in our experiment were collected in 2017 from the northern Red Sea. They were kept in a maintenance aquarium under stable temperature, salinity, light, oxygen, pH and nutrient conditions, which were later replicated in the experiment (Figure 2.1B & Table 2.1). These large clonal mother colonies of *X. umbellata*, all of the same genotype,

were fragmented following the 'plug mesh method' outlined by Kim, Wild, and Tilstra (2022) into small fragments. The new fragments were left to heal for 14 days and acclimatise to their surroundings for a further 7 days in stable conditions, within the same maintenance aquarium as described above. Fragments were then examined for quality, and a total of 72 healthy colonies that displayed consistent and regular pulsations were selected and distributed across 12 experimental tanks, with six colonies assigned to each tank on day 0. The fragmentation process was repeated ~21 days in advance of each experimental phase as new fragments were used within each phase. We chose to do this to ensure that corals in all phases had not been exposed to any prior stressors. In addition, this way, corals in all phases were of a similar size, removing this as a potentially confounding factor.

2.3.2 Experimental setup

Each experimental tank (n = 12) was connected to a technical tank (n = 12) positioned at a lower level, behind. Each technical tank contained a skimmer (EHEIM Skim Marine 100; EHEIM GmbH and Co. KG, Germany) connected to an external air pump (EHEIM Air Compressor 100L/H, EHEIM GmbH and Co. KG, Germany), a thermostat (3613 aquarium heater. 75 W 220–240 V; EHEIM GmbH and Co. KG, Germany) connected to a temperature controller (Schego Temperature Controller TRD, max. 1000 W) and a water pump (EHEIM CompactOn 300/1000 pump; EHEIM GmbH and Co. KG, Germany). An exchange of water was constantly maintained between the two tanks via an overflow pipe and a return water pump (set to the desired water flow for the respective treatment, see Table A1). LED lights (Royal Blue—matrix module and Ultra Blue White 1:1—matrix module, WALTRON daytime® LED light, Germany) were secured above all experimental tanks and provided light on a 12:12h light:dark cycle at an intensity of 120 µmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). Black plastic sheets were secured externally on the left and right walls and beneath every experimental tank. This ensured that all tanks were receiving the same quantity of light, regardless of their placement.

2.3.3 Water flow treatments

Within the 12 experimental tanks, four water volume exchange rates of 200 Lh^{-1}, 350 Lh^{-1}, 500 Lh^{-1} and 650 Lh^{-1} were established (here on referred to as water flow treatments), in three

replicates (Table S2.1). Pumps were tested prior to the experiment for an accurate measurement of the volume exchange rate compared to the setting of the pump (Table S2.1). Clod cards were used to assess and confirm the ecological significance of each speed.

2.3.4 Feeding treatments

Feeding treatments were supplied in three phases. In the first phase, no food was supplied to the corals. In the second phase, 1.5 x 10^4 cells mL^{-1} of phytoplankton (Plankton24.de, *Synechococcus* sp.) were administered to each tank daily. This concentration was chosen because it falls within the range of conditions in the central Red Sea, where *X. umbellata* naturally occurs (Kürten *et al.*, 2015). In the third experimental phase, DOC, in the form of glucose (D-Glucose anhydrous, purity: 99%, Fisher Scientific U.K. Limited, Loughborough, UK), was administered to each tank to achieve a constant concentration of 20 mg/L of DOC. This concentration of DOC was chosen because it was higher than ambient levels, thereby providing *X. umbellata* with ample opportunity to feed heterotrophically (Vollstedt et al. 2020). To achieve this concentration, water samples were taken on day 0 of phase 3 and run on a Total Organic Carbon Analyser (TOC-L CPH/CPNPC-Controlled Model, Shimadzu, Japan) to determine the baseline of DOC present in each tank, and glucose was administered accordingly. Water samples were analysed as described above, for the first three days of the experimental phase to determine the average uptake of glucose. Consequently, 2 mg/L of glucose was administered every two days to all experimental tanks throughout phase 3 of the experiment. In all three experimental phases, the skimmers were turned off for two hours every day between 10 am and 12 pm to provide *X. umbellata* with the opportunity to uptake the food.

2.3.5 Maintenance

In all experimental phases, salinity, temperature, and oxygen were measured daily every morning in all tanks, while nutrient concentrations and pH were measured twice per week in the morning before food was supplied (Table 1). Manual adjustments were made when required. In addition, 10% water exchanges were performed daily in all experimental tanks, two hours post-feeding in the afternoon. In the phytoplankton feeding phase however, 50% water exchanges were required daily due to elevated nutrient levels and increased light attenuation because of the treatment.

Table 2.1. Water parameters monitored throughout all three experimental phases, grouped by water flow treatments (represented by their speed in L h^{-1}) and feeding treatments with DOC = dissolved organic carbon. Values are reported as averages ± standard deviation. * Values are displayed as ranges because data are categorical, and therefore, no average/standard deviation could be obtained.

	Phase 1 No feeding				**Phase 2 Phytoplankton**				**Phase 3 DOC**			
	200	**350**	**500**	**650**	**200**	**350**	**500**	**650**	**200**	**350**	**500**	**650**
Temperature [°C]	25.3 ± 0.3	25.3 ± 0.4	25.5 ± 0.2	25.3 ± 1.0	25.3 ± 0.3	25.2 ± 0.9	25.4 ± 0.2	25.2 ± 0.4	25.3 ± 0.3	25.4 ± 0.5	25.4 ± 1.0	25.4 ± 0.2
Salinity [PSU]	35.3 ± 0.3	35.2 ± 0.4	35.2 ± 0.4	34.9 ± 0.6	35.6 ± 0.5	35.4 ± 1.5	35.6 ± 0.4	35.4 ± 0.5	35.3 ± 0.5	35.2 ± 0.5	35.3 ± 0.5	35.1 ± 0.6
Oxygen [mg/L]	6.5 ±0.087	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.6 ± 0.1	6.7 ± 0.2	6.6 ± 0.1	6.6 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1
pH	8.3 ± 0.1	8.3 ± 0.1	8.3 ± 0.1	8.3 ± 0.1	8.3 ± 0.0	8.3 ± 0.0	8.4 ± 0.0	8.3 ± 0.0	8.3 ± 0.0	8.3 ± 0.0	8.3 ± 0.0	8.3 ± 0.0
Phosphate [mg/L] *	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Nitrate [mg/L] *	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5	0.5	0.5	0.5
Nitrite [mg/L]	0.03 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.05 ± 0.1	0.07 ± 0.1	0.07 ± 0.1	0.09 ± 0.1	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
Ammonium [mg/L] *	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Calcium [mg/L]	426.7 ± 17.8	421.7 ± 21.7	423.3 ± 20.6	416.7 ± 23.9	420 ± 14.77	416.7 ± 11.6	418.3 ± 18.0	418.3 ± 10.3	416.7 ± 20.6	428.3 ± 27.6	428.3 ± 23.3	421.7 ± 32.4
Magnesium [mg/L]	1433.3 ± 126.6	1458.3 ± 117.7	1436.7 ± 102.3	1443.3 ± 118.7	1330 ± 63.5	1323.3 ± 63.7	1331.7 ± 50.8	1311.7 ± 57.5	1353.3 ± 156.6	1341.7 ± 94.0	1341.7 ± 175.7	1358.3 ± 32.4
Alkalinity [°dKH]	7.2 ± 1.1	7.4 ± 1.4	7.3 ± 1.2	7.4 ± 1.4	8.4 ± 0.5	8.6 ± 0.7	8.6 ± 0.7	8.5 ± 0.5	7.7 ± 1.0	7.8 ± 1.2	7.8 ± 1.1	7.6 ± 1.0

2.3.6 Ecological assessments

Pulsation

One pulsation cycle was defined as one whole contraction of the polyp (open- fully closed-open) (Vollstedt *et al.*, 2020). To determine the pulsation rate, the number of pulsations displayed within one minute were counted, with use of a stopwatch and a hand-tally counter. On day 15, the pulsation rates were measured on the same three corals within each experimental tank (12 biological replicates per treatment). The measurement was focused on one random polyp per coral and was repeated three times on the same polyp (3 technical replicates). To minimise variability among repeated measurements, one observer coherently performed all pulsation measurements.

Growth rate

One colony within each experimental tank (n = 12) was followed throughout each 15-day phase and counted twice each, resulting in 12 biological replicates, and 2 technical replicates. The number of polyps on the selected colonies were counted at the beginning (day 0) and end (day 15) of each phase. The colony was transferred to a temporary smaller glass jar, and tweezers were used to aid and improve the accuracy of counting. To calculate the growth rate (number of new polyps per day), the equation below was used (Equation 1), where the number of polyps on day 0 *(P_{start})* were subtracted from day 15 *(P_{end})* and divided by the total number of days of the experimental phase *(d)*.

Equation 1

$$Growth\ rate\ (polyps\ day^{-1}) = \left(\frac{P_{end} - P_{start}}{d}\right)$$

2.3.7 Symbiodiniaceae parameters

Xenia umbellata colonies were removed from the experiment on day 15, and stored at -20°C. On the day of analysis, colonies were thawed for approximately 30 minutes in the dark. To obtain a tissue slurry, each colony was homogenised in 10 ml of demineralised [DM] water using a hand-homogeniser (Pupier, Bednarz and Ferrier-Pagès, 2018). An accurate sample volume was determined using a pipette. Two subsamples were created per colony, by

transferring 2 ml of slurry into two Eppendorf tubes. Samples were centrifuged for 10 minutes to separate coral tissue and algal cells. The supernatant was discarded, and the pellet was resuspended in 2 ml of DM water. The centrifugation step was repeated once more. One subsample was used for algal cell counts and mitotic index, while the second subsample was used for chlorophyll-*a*.

Algal cell density and mitotic index

To obtain algal cell counts, an established counting method using a haemocytometer was followed (LeGresley. and McDermott., 2010). In brief, the pellets were resuspended in 2 ml of DM water and vortexed. The haemocytometer was sterilised with ethanol and the coverslip was affixed using DM water. 10 µL of sample was pipetted beneath the coverslip onto the upper and lower counting chambers. Under a light microscope, the algal cells were counted in the four outer squares within both the upper and lower chambers, providing two replicate counts. The counts were normalised to the initial sample volume and per surface area of each colony. Cells in mitosis were also counted simultaneously and divided by the total number of algal cells per sample to obtain the mitotic index.

Chlorophyll-*a* per cell

For the determination of chlorophyll-*a*, the methodology of Jeffrey and Humphrey (1975) was followed. Pellets were resuspended in 2 ml of 90% acetone and vortexed. The samples were then stored in the dark at 4°C for 24 hours. After this, samples were centrifuged for 5 minutes, and 2 ml were transferred into two glass cuvettes (1 ml in each), for two replicate readings. Samples were individually measured at two fixed wavelengths of 663 nm and 630 nm using a Trilogy Fluorometer (Turner Designs) fitted with a chlorophyll-*a* module. Each sample was measured three times. All analyses were performed in a dark room. Measurements were normalised to the initial volume of the sample and to the number of algal cells per colony.

2.3.8 Stable isotope and carbon and nitrogen elemental analyses

One colony of *X. umbellata* per treatment was removed from the experiment on day 0, and on day 15, rinsed with DM water to remove salt, and stored at -20°C until further analysis.

Colonies were weighed, and dried in the oven at 40°C for ~48 hours or until a consistent weight was achieved. Dried tissue was then ground with a pestle and mortar, and ~1 mg was

transferred into a tin cup. Samples were analysed for carbon and nitrogen content, as well as for isotopic ratios of $\delta^{15}N$ and $\delta^{13}C$ (‰) at the Natural History Museum, Berlin with a Flash 1112 EA coupled to a Delta V IRMS via a ConflolV-interface (Thermo Sceintific, Waltham, MA, USA), as described in greater detail in Karcher *et al.*, (2020).

2.3.9 Statistical analyses

Firstly, we tested for normality using visual normality distribution plots and the Shapiro-Wilk normality test. Data that was not normally distributed was transformed either via a log transformation or a tukey ladder of powers transformation. All data was first assessed for significance using a two-way analysis of variance test (2-ANOVA). When water flow was excluded, data was re-assessed using a one-way analysis of variance test (ANOVA). For significant variables i.e., when $p < 0.05$, a post-hoc Tukey HSD test was performed to identify specifically where the significant differences lay.

Due to different starting values of certain response parameters between experimental phases (i.e., pulsation rates, all Symbiodiniaceae parameters and all isotope and elemental parameters), statistical analyses were performed on relative differences within each experimental phase. However, we opted to show absolute values within each figure for transparency and easier understanding. Consequently, statistically significant differences between day 15 data marked on the figures may not visually appear as very different. Figures with visualised relative differences can be found in the supplementary material (S2.2, S2.4 & S2.5).

All data analyses and creation of figures were carried out in R (version 4.2.3) (R Core Team, 2023) using packages ‘dplyr’ (Wickham *et al.*, 2018), ‘ggplot2’ (Wickham, 2016), ‘ggpubr’ (Kassambara, 2020a), ‘RColorBrewer’ (Neuwirth, 2014), ‘wesanderson’ (Ram and Wickham, 2018), ‘gridExtra’ (Auguie and Antonov, 2017), ‘rstatix’ (Kassambara, 2020b), ‘ARTools’ (Kay *et al.*, 2021), and ‘rcompanion’ (Mangiafico, 2017).

2.4 Results

2.4.1 Water flow

Water flow had no observable nor significant effect on any of the measured physiological parameters of *X. umbellata* (see supplementary figures S2.2 – S2.5 and Table S2.6). Consequently, data from the water flow treatments have been pooled and only feeding treatments are presented within the figures below, with a replication of n = 12 per feeding treatment on day 15. On day 0 however, no data could be pooled as on day 0 no water flow treatments were established yet. Therefore, the replication number presented within the results section for day 0 remained as three.

2.4.2 Pulsation

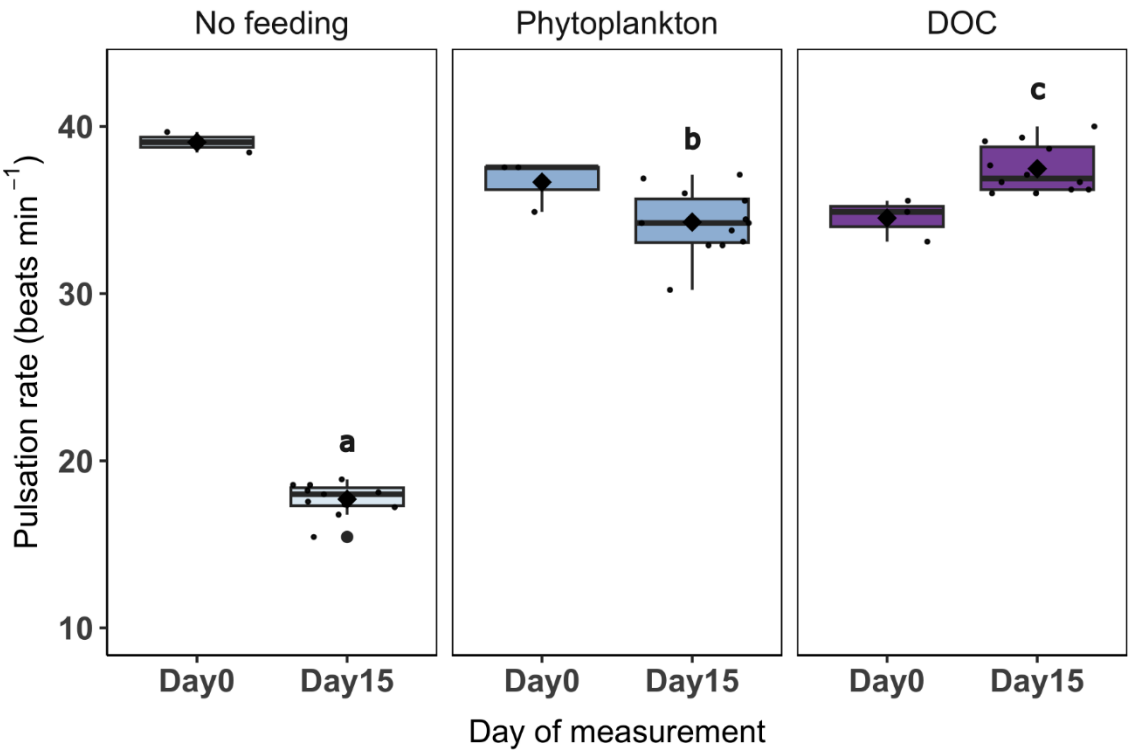

Figure 2.3 The average pulsation rate (beats min^{-1}) of *Xenia umbellata* at the start (day 0) and end (day 15) of the experiment within 'No feeding', 'Phytoplankton' and 'Dissolved organic carbon [DOC]' feeding treatments. On day 0, there are three biological replicates for each feeding treatment, and on day 15 there are 12 biological replicates. The median is represented by the black horizontal line and the mean is indicated by a black diamond. Variables that have different letters are significantly different (based on relative differences between day 15 data, see "Statistical analyses"**)**, whereas variables that have the same letter are not significantly different.

Feeding treatments had a significant effect on pulsation rates (ANOVA, 2, F = 905.2, p < 0.001). Post-hoc testing revealed that all three feeding treatments were significantly different (Tukey, p < 0.001) (Figure 2.3). On day 15, pulsation rates were significantly lowest in the no feeding treatment, where pulsations had decreased by a magnitude of 1.9, from 39 down to 21 beats min^{-1}. Where food was provided, in the phytoplankton and DOC feeding treatments, the pulsation rate remained more stable, with a marginal decrease from 36 beats min^{-1} to 34 beats min^{-1} in the phytoplankton treatment, and with a marginal increase from 34 beats min^{-1} to 37 beats min^{-1} in the DOC treatment (Figure 2.3). On day 15, corals in the DOC treatment had a significantly higher pulsation rate than all other treatments (Tukey, p < 0.001) (Figure 2.3).

2.4.3 Growth Rate

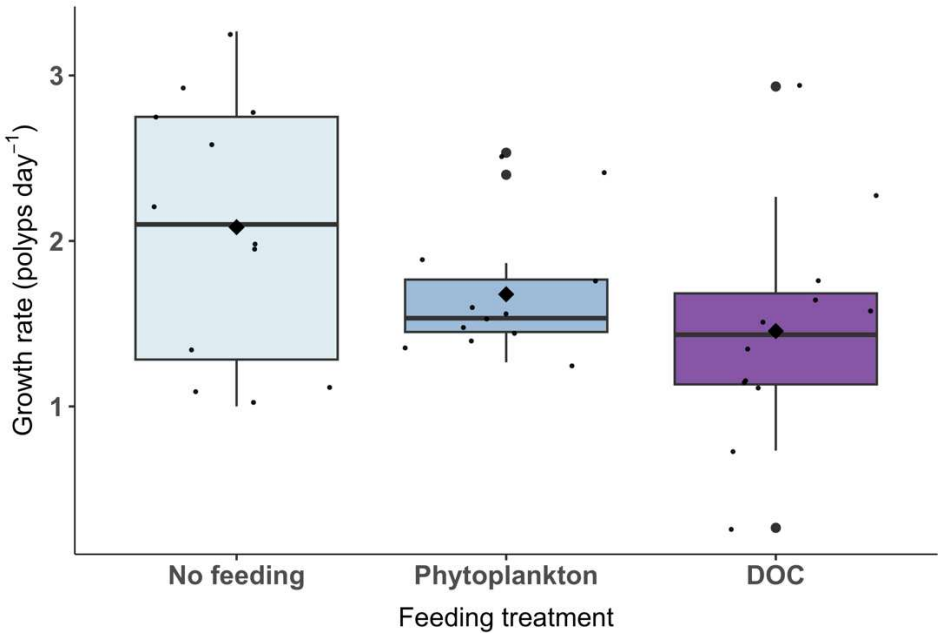

Figure 2.4 The average growth rate of *Xenia umbellata* following 15 days of exposure to 'No feeding' 'Phytoplankton' and 'Dissolved organic carbon [DOC]' feeding treatments. There are 12 biological replicates per feeding treatment. The median is represented by the black horizontal line and the mean is indicated by a large black diamond.

Growth rates were highly variable among treatments, with an average growth of 2.1 polyps per day in the no feeding treatment, 1.7 polyps per day in the phytoplankton treatment and 1.5 polyps per day in the DOC treatment (Figure 2.4). These were not significantly different (ANOVA, 2, F = 2.848, p = 0.07).

2.4.4 Symbiodiniaceae parameters

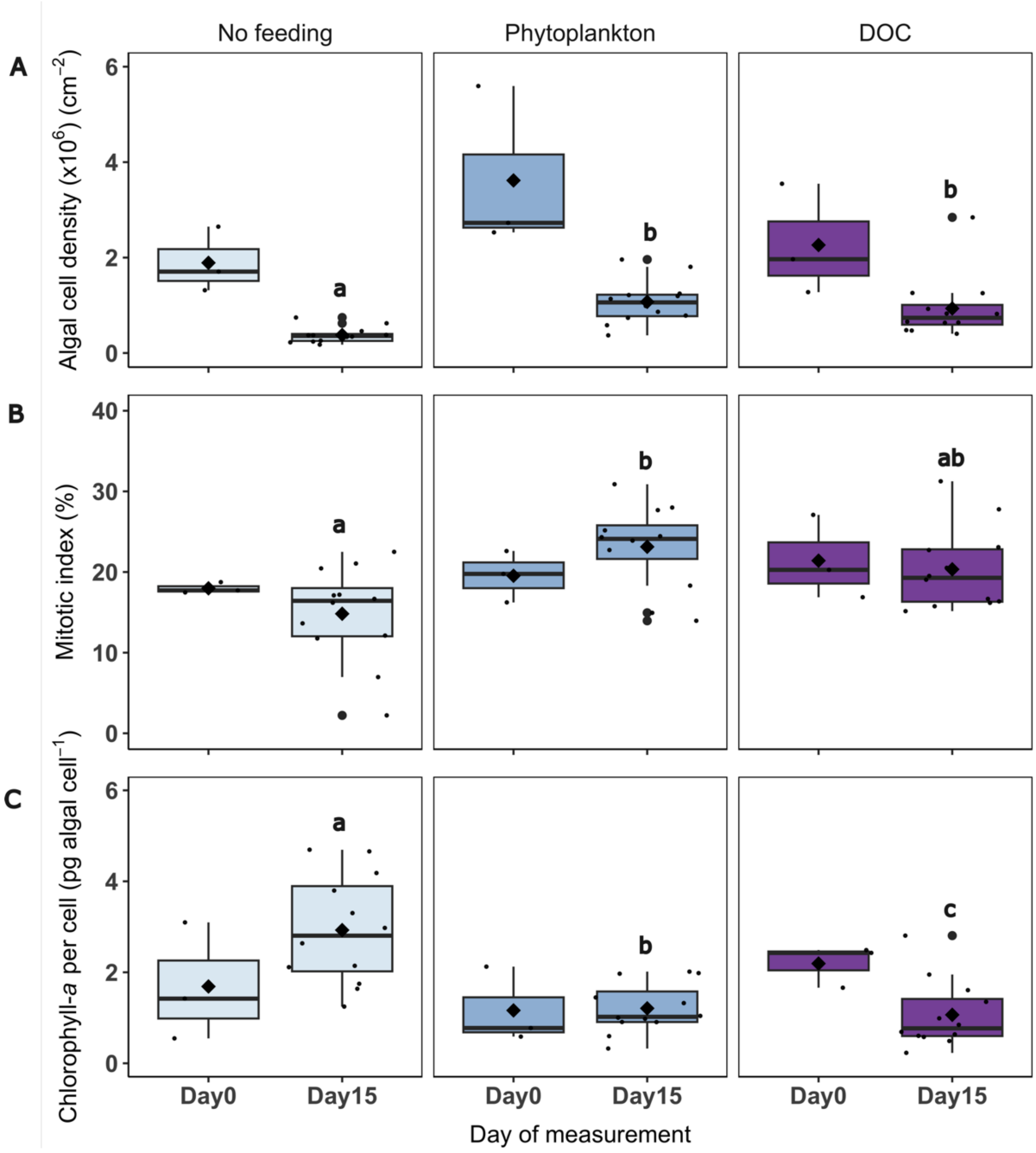

Figure 2.5 Symbiodiniaceae parameters of *Xenia umbellata*, including A] algal cell density (cm^{-2}), B] mitotic index (%) and C] chlorophyll-*a* per algal cell (pg algal $cell^{-1}$) at the start (day 0) and end (day 15) of the experiment within 'No feeding', 'Phytoplankton' and 'Dissolved organic carbon [DOC] feeding treatments. Day 0 for all Symbiodiniaceae parameters have a biological replication of three, and for day 15 have a biological replication of 12 per feeding treatment. The median is represented by the black horizontal line and the mean is indicated by a large black diamond. Variables that have

different letters are significantly different, whereas variables that have the same letter are not significantly different (based on relative differences between day 15 data, see "Statistical analyses").

Algal cell density

Significant differences in algal cell density were observed across feeding treatments (ANOVA, 2, $F = 15.87$, $p < 0.001$) (Figure 2.5A). Whilst the algal cell density decreased throughout the experiment in all treatments, there was a greater drop in the no feeding treatment, where the algal cell density was significantly lower (3.8×10^5 cells cm^{-2}) than in both the phytoplankton (1.1×10^6 cells cm^{-2}) and DOC (9.4×10^5 cells cm^{-2}) feeding treatments on day 15 (Tukey, $p < 0.01$) (Figure 2.5A). Significantly higher algal cell densities were observed when food was supplied as phytoplankton (Tukey, $p < 0.01$) and as DOC (Tukey, $p < 0.001$) compared to the no feeding treatment (Figure 2.5A).

Mitotic index

Overall, there was a significant effect of feeding treatments on the mitotic index of the corals (ANOVA, 2, $F = 5.144$, $p < 0.05$), with a significantly higher number of cells in mitosis in the phytoplankton treatment (23%) compared to the no feeding treatment (15%) on day 15 (Tukey, $p < 0.05$) (Figure 2.5B). There was no significant difference between no feeding and DOC (Tukey, $p > 0.05$) and between phytoplankton and DOC (Tukey, $p > 0.05$) (Figure 2.5B).

Chlorophyll-*a*

Feeding treatments had a strong significant effect on the chlorophyll-*a* content per cell of *X. umbellata* (ANOVA, 2, $F = 16.65$, $p < 0.001$), with all treatments differing significantly (Tukey, $p < 0.05$) (Figure 2.5C). A significantly higher chlorophyll-*a* per cell was found in the no feeding treatment (2.9 pg $cell^{-1}$) compared to both the phytoplankton (1.2 pg $cell^{-1}$) and DOC treatment (1.1 pg $cell^{-1}$) (Figure 2.5C). In addition, the chlorophyll-*a* per cell was also significantly higher in the phytoplankton treatment compared to the DOC (Tukey, $p < 0.05$) (Figure 2.5C).

2.4.5 Isotope and elemental analyses

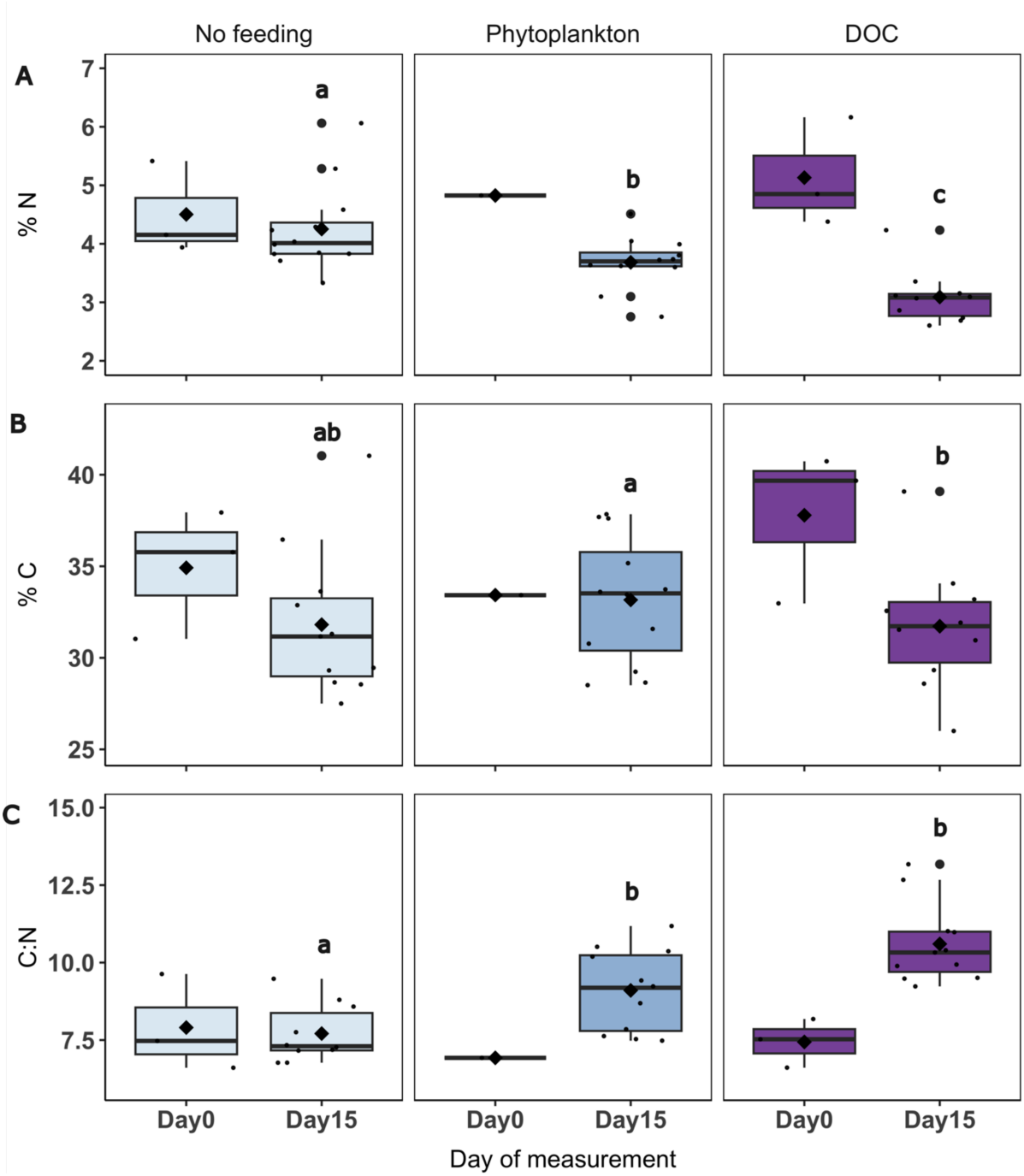

Figure 2.6 Elemental data of *Xenia umbellata* including A] nitrogen content (% N), B] carbon content (% C) and C] carbon to nitrogen ratio (C:N), *following* 15 days of exposure to 'No feeding' 'Phytoplankton' and 'Dissolved organic carbon [DOC]' feeding treatments. For day 0 measurements, there are 3 biological replicates, excluding the phytoplankton treatment where it is reduced to 1, due to technical faults during analysis. For day 15 measurements there are 12 biological replicates. The median is represented by the black horizontal line and the mean is indicated by a large black diamond. Statistical significance is indicated by letters. Variables that have different letters are significantly different,

whereas variables that have the same letter are not significantly different (based on relative differences between day 15 data, see "Statistical analyses").

Significant differences in nitrogen content (%N) were observed across feeding treatments (ANOVA, 2, $F = 12.72$, $p < 0.001$) (Figure 2.6A). A significantly higher nitrogen content was found in corals within the no feeding treatment (4.3%), compared to phytoplankton feeding (3.7%) and compared to DOC (3%) (Tukey, $p < 0.001$) (Figure 2.6A). In addition, the nitrogen content of corals within the phytoplankton treatment was significantly higher than corals in the DOC feeding (3%) (Tukey, $p < 0.05$) (Figure 2.6A).

Significant differences in carbon content (%C) were also observed across feeding treatments (ANOVA, 2, $F = 7.049$, $p < 0.05$) (Figure 2.6B). A significantly higher carbon content was found in corals within the phytoplankton treatment (33%) compared to the DOC treatment (31%) (Tukey, $p < 0.05$) (Figure 2.6B). However, no significant differences were found between corals in no feeding treatment (32%) compared to both food provision treatments (Tukey, $p > 0.05$) (Figure 2.6B).

The carbon to nitrogen ratio (C:N) significantly differed across feeding treatments (ANOVA, 2, $F = 21.7$, $p < 0.001$) (Figure 2.6C). The C:N of corals within the phytoplankton (9.1) and DOC (10.5) feeding treatments was significantly higher than that of corals in the no feeding treatment (7.5) (Tukey, $p > 0.01$) (Fig 6C). However, there was no significant difference in the C:N of corals between phytoplankton and DOC treatments (Tukey, $p > 0.05$) (Figure 2.6C).

Lastly, no observable, nor statistically significant differences were found between feeding treatments in the nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) stable isotope signatures of *X. umbellata* (S2.8A & B).

2.5 Discussion

Overall, we argue that the physiology of *X. umbellata* is unaffected by water flow as no significant effect on any of the observed parameters was found within our study. *Xenia umbellata* was, however, substantially impacted by a lack of heterotrophic food sources, with a significantly reduced pulsation rate, lower Symbiodiniaceae cell density, and lower mitotic

index compared to the fed treatments, yet significantly higher chlorophyll-*a* per cell and N content. Significant differences were also observed between the DOC and phytoplankton treatments, with significantly higher pulsation rates and lower chlorophyll-*a* per cell in the DOC treatment, but higher C and N content in the phytoplankton treatment.

2.5.1 Does water flow affect the physiology or trophic ecology of *Xenia umbellata*?

One key outcome of our study was finding no significant effect of water flow on any of the measured physiological parameters of *X. umbellata* (S2.2-S2.5). Based on previous research about the effects of water flow on feeding regimes in corals, we hypothesised that there would be a positive effect on the physiological responses of *X. umbellata* due to water flow enhancing autotrophy and heterotrophy. These studies, however, have mainly focused on scleractinian corals, or soft corals with distinctly different morphologies to *X. umbellata.* The morphology of *X. umbellata* (and some other species within the Xeniidae family) is unique in that it exhibits continuous non-synchronous pulsation of its polyps, first noted by Lamarck in 1816. Pulsation motions continually thrust water in an upwards direction around the polyp, prompting mixing across the coral-water boundary layer (Kremien *et al.*, 2013), and thereby modulating flow at a local scale around the polyps. Consequently, we suggest that *X. umbellata* does not gain any additional benefit from a high flow environment, nor experience negative effects from a low flow environment because it is able to control flow already at a local scale, via its pulsation behaviour. It should be noted however, that higher water flows than measured in our experiment may occasionally occur, especially in more turbulent shallow water environments that *X. umbellata* occupy. In such environments it is possible that polyps may be blasted and therefore unable to function, thereby making heterotrophic feeding difficult (Purser *et al.*, 2010). We would therefore suggest further experimentation to look into the effects of a more extreme environment, to see whether or not *X. umbellata* still remain unaffected.

2.5.2 Does *Xenia umbellata* exhibit a dominant feeding mode?

Whilst water flow treatments had no significant effect on the assessed physiological parameters, we did observe significant effects of feeding treatments on *X. umbellata*.

Firstly, we found that pulsation rates were significantly different between all feeding treatments (Figure 2.3). Corals in the unfed treatment experienced a large reduction in pulsation rate following 15 days of no food, whereas comparatively, the pulsation rate of the corals in both the fed treatments did not differ substantially from the start of their respective day 0 measurement (Figure 2.3). Given that pulsation can enhance autotrophy (Kremien *et al.*, 2013), we expected to see increased pulsation rates in the no feeding treatment. However, the significantly lower pulsation rate among unfed corals suggests that *X. umbellata* may not have had the energy to sustain its normal range of pulsations nor increase its pulsation rate to enhance autotrophy when lacking a heterotrophic energy source. For example, 12 of the most common symbiotic soft coral genera on the Great Barrier Reef are unable to satisfy their carbon requirements exclusively via autotrophy (Fabricius, Genin and Benayahu, 1995). In addition, it could be that the reduced pulsation rate was intentional by the coral to conserve energy, as a result of low capture success. However, if this was the case, we would also expect to see a reduction in growth rate, yet growth was not negatively impacted and instead increased across all treatments. We would therefore encourage a follow-up study where we correlate pulsation rates with the capture rate of prey to address this hypothesis better.

Although our results suggest that autotrophy was not sufficient to satisfy the energetic needs of *X. umbellata* alone, it is likely that with greater light availability, a higher or sole reliance on autotrophy may have been possible. In our experiment, we supplied a light intensity of 120 µmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). However, much higher light intensities have been recorded in the Red Sea at depths of 1 – 20 m than supplied in our experiment (Haas *et al.*, 2010). For example, in the winter months light intensity can range from 78 µmol quanta m^{-2} s^{-1} (20 m) to 527 µmol quanta m^{-2} s^{-1} (1 m) and even reach 144 µmol quanta m^{-2} s^{-1} (20 m) to 946 µmol quanta m^{-2} s^{-1} (1 m) in the peak of summer (Haas *et al.*, 2010). Although introducing food sources can increase nutrient loading and consequently decrease light availability, this did not occur here as nutrients were consistent across treatments (Table 1), and light was measured steadily at 120 µmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) across all treatments. With *X. umbellata* inhabiting depths as shallow as 1 m (Figure 2.1C), the light intensity in our experiment may not have been sufficiently high to support autotrophy as a sole feeding mode.

Secondly, unfed corals also had a significantly lower algal cell density compared to both food provision treatments (Figure 2.5A), and a significantly lower mitotic index compared to the

POM feeding treatment (Figure 2.5B). Generally, higher algal cell densities and mitotic indices are indications of a healthy coral that has a proliferating and stable supply of symbionts (Belda, Lucas and Yellowlees, 1993). However, there is also evidence to suggest that higher values occur under heat stress in the hard coral *Stylophora pistillata*, and could be a sign of altered resource partitioning (Rädecker *et al.*, 2021). In this instance however, heat stress was not a factor, and therefore we argue that the significantly reduced algal cell density demonstrates a reduction in health of *X. umbellata* in the absence of a heterotrophic food source.

We did, however, observe a significantly higher chlorophyll-*a* content (Figure 2.5C), higher nitrogen content (Fig 6A) and lower carbon to nitrogen ratio (Figure 2.6C) among unfed corals, compared to those in phytoplankton and DOC treatments. This increase in cellular chlorophyll-*a* suggests that *X. umbellata* may have been the holobionts' attempt to optimise its photosynthetic capacity given that autotrophy was the only mode of energy acquisition available in the absence of heterotrophic food. These cellular morphological modifications have been observed previously in corals transplanted from deep to shallow water (Martinez *et al.*, 2020), where autotrophy also became the primary feeding mode, and additionally in other instances where corals have experienced unfavourable conditions for autotrophy and thus needed to optimise light capture (Wall *et al.*, 2020). The higher nitrogen content in unfed corals could therefore be justified by this concomitant increase in chlorophyll-*a*, as chlorophyll-*a* compounds contain nitrogen (Imsande, 1998).

Overall, our data suggests that *X. umbellata* may prefer the presence of a heterotrophic food source in order to maintain optimum health, thereby supporting the notion that it is an inherent mixotroph. Whilst the health of *X. umbellata* appeared to decline when in absence of food, we cannot distinctly say that they are unable to sustain themselves with autotrophy alone because the light intensity supplied within our experiment was at the lower end of their natural range. Therefore, we recommend for future work to repeat our experiment using a range of higher light intensities to thoroughly assess the role of autotrophy for *X. umbellata*, with inclusion of a photosynthesis-irradiance (PI) curve to provide further insight.

2.5.3 Does *Xenia umbellata* have a preferred heterotrophic food source?

The pulsation rate between phytoplankton and DOC feeding treatments differed with a significantly higher pulsation rate among corals exposed to DOC (Figure 2.3). Pulsation is particularly beneficial for the uptake of dissolved matter from the surrounding water (Kremien *et al.*, 2013), therefore justifying the increased pulsation rates observed in the DOC feeding treatment. Furthermore, corals exposed to DOC had a significantly lower concentration of chlorophyll-*a* per algal cell compared to corals in the phytoplankton treatment (Figure 2.5C). This could be because experimentally provided DOC as an available carbon source leads to an excess supply of inorganic carbon, and therefore *X. umbellata* may no longer invest energy into enhancing its photosynthetic apparatus, resulting in lower concentrations of chlorophyll-*a*. A similar ecophysiological response was recently observed among the upside down jellyfish *Cassiopea* sp. where gross photosynthesis was reduced in response to medium (20 mg L^{-1}) and high (40 mg L^{-1}) concentrations of DOC (Tilstra *et al.*, 2022). Lastly, a significantly higher nitrogen (%N) and carbon content (%C) was observed within the phytoplankton feeding treatment compared to the DOC feeding treatment (Figure 2.6A & B). It therefore appears that phytoplankton could serve as a more nutritious food source for *X. umbellata* as digested plankton offers a source of carbon but also organic nitrogen (Ferrier-Pagès *et al.*, 2003) that supports coral growth.

Our study suggests that POM provision in the form of phytoplankton, best supported the health of *X. umbellata* compared to DOM. It is important to highlight however, that we only provided one form of DOM, in the form of DOC, and one form of POM in the form of one species of phytoplankton, excluding other important groups such as dissolved inorganic nitrogen [DIN], dissolved organic nitrogen [DON] and other particulate matter such as zooplankton and other phytoplankton species. Our experiment aimed to determine the trophic preferences of *X. umbellata,* and now upon forming a baseline understanding, future research could build upon this further by assessing a wider range of heterotrophic food sources.

2.5.4 Conclusions

Our study shows that *X. umbellata* is unaffected by water flow and does not gain any additional benefit from high flow nor suffers under low flow regimes. We attribute this to its ability to control water flow at a local scale around its polyps using its pulsation behaviour to continually achieve optimum flow conditions. In addition, we found that *X. umbellata* does not respond

particularly well to an absence of heterotrophic food sources with significantly reduced pulsation, algal cell density and mitotic index. However, our findings suggest that photosynthetic energy generation of the *X. umbellata* holobiont is enhanced via increased chlorophyll-*a* contents per cell when food is scarce. Lastly, we found that the health of *X. umbellata* may be better supported by carbon and nitrogen containing-POM over carbon exclusive-DOM ingestion, as a greater variety of nutrients are offered. Overall, our study demonstrates the flexibility of *X. umbellata* to a variable environment, especially those with variable water flow and food availability.

2.5.5 Ecological implications

Coastal environments are experiencing more eutrophication as a consequence of nutrient loading, with increased levels of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphate (DIP) (Zhao *et al.*, 2021). These higher nutrient levels stimulate excessive phytoplankton growth and an increase in algal biomass (Yunev *et al.*, 2007), which in turn, increases the release of DOC into the water (Mueller *et al.*, 2016). Overall, these increases may favour the success of *X. umbellata* by supporting their heterotrophic feeding, whereas scleractinian corals could be negatively impacted. For example, increased DIN and DIP could cause a reduction in calcification up to 50% (Fabricius, 2005), and increased DOC could cause bleaching (Pogoreutz *et al.*, 2017). Overall, our findings contribute towards a better understanding of how *X. umbellata* is able to occupy such a broad range of habitats with varying environmental conditions, as well as to succeed in the face of global and local change.

2.6 Acknowledgements

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2.7 Author contributions

C.E.L. Hill: Data curation (equal); formal analysis (lead); investigation (lead); methodology (equal); supervision (equal); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **S.G. Abbass:** Data curation (equal); investigation (equal); methodology (equal); supervision (equal); writing – review and editing (equal). **G. Caporale:** Data curation (equal); investigation (equal); methodology (equal); writing – review and editing (equal). **Y.C. El-Khaled:** Methodology (equal); validation (equal); writing – review and editing (equal). **L. Kuhn:** Data curation (equal); investigation (equal); methodology (equal); writing – review and editing (equal). **T. Schlenzig:** Data curation (equal); investigation (equal); methodology (equal); writing – review and editing (equal). **C. Wild:** Conceptualization (supporting); funding acquisition (lead); project administration (lead); resources (lead); writing – review and editing (equal). **A. Tilstra:** Conceptualization (lead); project administration (equal); supervision (lead); writing – review and editing (equal).

2.8 References

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Chapter 3 | Excess particulate organic matter negatively affects the ecophysiology of the widespread soft coral *Xenia umbellata*

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3.1 Abstract

Coastal coral reefs are experiencing rising concentrations of organic matter. While dissolved organic matter (DOM), rather than particulate organic matter (POM), may negatively impact hard corals, the impact on soft corals remains unclear. We examined the physiological effect of 20 mg L^{-1} of organic carbon (C) addition on the widespread Indo-Pacific soft coral *Xenia umbellata* in a series of tank experiments over 28 days. We supplied DOM as glucose, and two POM sources as phytoplankton (2 – 5 µm length) and zooplankton (150 – 200 µm length). We comparatively assessed coral morphology, pulsation, colouration, algal symbiont densities, chlorophyll *a*, oxygen fluxes, and mortality. Corals in the control and DOM enrichment treatments exhibited no morphological or physiological changes. Excess phyto- and zooplankton caused disfigurement of the polyp tentacles and shortening of its pinnules. This coincided with a mortality of 11 and 14%, respectively, a 35% reduction in pulsation rates, and darkening of the polyps (with excess zooplankton), while other assessed response variables remained stable. This suggests that in contrast to many hard corals, the soft coral *X. umbellata* is vulnerable to excess POM rather than DOM, with amplified effects upon exposure to larger particles. Our results suggest that excess POM may damage the delicate feeding apparatus of *X. umbellata*, thereby reducing pulsation and lowering gas exchange. In turn, this can cause nutritional, and ultimately, energy deficiencies by directly affecting heterotrophic and autotrophic feeding. Our findings indicate that the global-change-resilient soft coral *X. umbellata* is vulnerable to local eutrophication, particularly high concentrations of POM.

Keywords

Heterotrophy, autotrophy, octocoral, eutrophication, nutrients

3.2 Introduction

Coral reefs are often referred to as "oases in a desert ocean" (Odum & Barrett, 1971) since they occupy and thrive in oligotrophic environments that lack nutrients, an enigma termed "Darwin's Paradox"(Darwin, 1842). In such challenging conditions, shallow-water corals exhibit adaptations that enable them to effectively navigate these nutrient limitations. For example, the duality of corals, as both auto- and heterotrophs, enables them to effectively acquire nutrients which they efficiently retain and recycle to maintain their productivity (Hutchings et al., 2019). Autotrophic feeding is the main mode of organic carbon (C) acquisition, where coral hosts receive C-rich photosynthates from their algal symbionts (Symbiodiniaceae) (Falkowski et al., 1984; Muscatine et al., 1984; Muscatine & Porter, 1977). The remainder of required nutrients, such as nitrogen (N) and phosphorus (P) can be sourced from heterotrophic feeding on dissolved or particulate organic matter (OM) from the water column (Houlbrèque & Ferrier-Pagès, 2009), or taken up in the form of inorganic nutrients (nitrate, phosphate and ammonium) from the environment (Muscatine & Porter, 1977). Lastly, corals are also able to supplement N via dinitrogen (N_2) fixation, where prokaryotes living in association with the coral host, convert atmospheric N into biologically accessible forms (Cardini et al., 2015). By leveraging both autotrophic and heterotrophic feeding strategies and efficiently acquiring, retaining and recycling essential nutrients, corals can sustain their productivity in oligotrophic environments.

Organic matter is fundamental in coral's survival in oligotrophic environments as it nourishes coral-associated microbes (Haas et al., 2011; Tanaka & Nakajima, 2018; Wild et al., 2010) and provides direct sustenance to the coral host itself when taken up as a heterotrophic food source (Houlbrèque & Ferrier-Pagès, 2009). Organic matter broadly refers to material containing C-based compounds, which can be in dissolved (DOM) or particulate (POM) forms (Romankevich, 1984). The pool of POM is composed of a small proportion of living biomass, such as bacteria and plankton, and a large proportion of detritus including faecal pellets and dead cells (Carlson & Hansell, 2015). Particles can vary in size, but generally, POM will be retained on a filter with a pore size in the range of 0.2 to 0.7 μm (Repeta, 2015). On the other hand, the pool of DOM, is able to pass through a filter of the same pore size (Carlson & Hansell, 2015; Repeta, 2015). Corals can directly uptake DOM as a food source (Houlbrèque & Ferrier-Pagès, 2009; Lange et al., 2023), and it is also considered as a nutrient source for POM such

as phytoplankton and zooplankton (Berman & Bronk, 2003; Grover et al., 2006, 2008). Some corals are avid predators of POM, and are able to ingest ~ 0.5 - 2 prey items per polyp per hour (Sebens et al., 1996). In coastal waters, DOM concentrations are higher on average than POM by 1-2 orders of magnitude (Barrón & Duarte, 2015). Organic matter is therefore essential for coral survival in nutrient-poor environments, serving as a critical food source for both the coral host and its associated microbes.

The accumulations of OM in the ocean are highly dynamic, naturally fluctuating over seasons and changing with weather events that drive upwelling (Bayraktarov & Wild, 2014; Stuhldreier et al., 2015). These are also influenced by run-off from land (allochthonous OM), which can be increased by coastal development (Fabricius et al., 2013). Whilst OM is essential to sustain life in the ocean, an excess of allochthonous OM can trigger eutrophication. Eutrophication is common in coastal areas where excess nutrients enter aquatic ecosystems via sewage, industrial wastewater, and agricultural fertilisers (Laws, 1981; Tuholske et al., 2021). Inorganic nutrients can alleviate primary producers from nutrient limitations, causing excessive growth of POM, which in turn releases DOM exudates into the surrounding water (Smith et al., 2006; Thornton, 2014; Zhang et al., 2023). Overall, naturally occurring seasonal increases in OM combined with excess nutrient input from land can lead to coastal eutrophication.

The effects from high levels of OM have been well documented on reef-building (hard) corals. Studies show that elevated concentrations of DOM disrupt their microbiome. For example, dissolved organic carbon (DOC) enrichment accelerates the growth of microbes in the coral's mucopolysaccharide layer, resulting in mortality (Kline et al., 2006; Kuntz et al., 2005; Mitchell & Chet, 1975). Furthermore, DOC enrichment causes a proliferation of N_2 fixing bacteria (diazotrophs), exponentially increasing N_2 fixation and causing a breakdown of the coral-algal symbiosis (Pogoreutz et al., 2017). On the other hand, hard corals respond differently to excess POM. Instead, limited data suggest that moderate levels of POM offer substantial energy and growth benefits for some hard coral species, with observations including increased tissue thickness, linear extension of the host and increased growth of its algal symbionts (Dubinsky & Jokiel, 1994; Fabricius, 2005). These effects can be attributed to the ability of some corals to become mixotrophic at high turbidity, and actively uptake POM (Anthony & Fabricius, 2000). However, when exposed to extreme levels of POM, photosynthetic pigments may increase, but these benefits may then be negated by smothering and reduced light availability, resulting in decreased gross photosynthesis and respiration,

bleaching, mortality and reduced coral cover in affected regions (Marubini, 1996; Shimoda et al., 1998; Tomascik & Sander, 1985; Walker & Ormond, 1982). Therefore, the effects of OM on hard corals vary depending on the type and quantity of OM in excess.

With many studies focused on hard corals, there is a paucity of research into the effects of various forms of OM on soft corals. Soft corals are generally considered less vulnerable to anthropogenic stressors than hard corals (Inoue et al., 2013), since they have not experienced the same decadal-scale decline in population density (Lenz et al., 2015), and have been reported to increase in areas known to have higher anthropogenic disturbance, such as in Jakarta Bay (Indonesia) and Okinawa (Japan) (Baum et al., 2016; Lalas et al., 2024). However, the vulnerability of soft corals to global and local changes varies by taxon, with emerging evidence indicating that some soft coral species are at risk from sedimental mobilisation and heat stress (Larkin et al., 2021; Maucieri & Baum, 2021). The soft coral *Xenia umbellata*, a widespread and pulsating soft coral native to the Indo-Pacific, including the Red Sea (Lamarck, 1816; Verseveldt, 1965), has demonstrated remarkable resilience to various global and local pressures (Klinke et al., 2022; Mezger et al., 2022; Simancas-Giraldo et al., 2021; Thobor et al., 2022; Vollstedt et al., 2020), while also becoming a common spreader and invader in non-native regions (Toledo-Rodriguez et al., 2024). Therefore, it is crucial to examine how *X. umbellata* responds to local stressors such as enrichment of OM, which frequently accumulates on reefs during eutrophication events. Some research has been carried out into the effects of DOM on the physiology of *X. umbellata* (Simancas-Giraldo et al., 2021; Vollstedt et al., 2020; Xiang et al., 2022). Studies by Vollstedt et al. (2020) and Simancas-Giraldo et al. (2021) both investigated the effect of three concentrations of DOC enrichment (in combination with warming) on the physiology of *X. umbellata*, concluding that DOC had no negative effect on *X. umbellata*, but may potentially serve as an antagonist that increases the resistance of *X. umbellata* up to certain thresholds of ocean warming (Simancas-Giraldo et al., 2021; Vollstedt et al., 2020). One study by Hill et al., (2023) investigated the role of phytoplankton as a heterotrophic food source for *X. umbellata*, finding that it enhanced the C and N content of the coral and promoted optimal health of *X. umbellata* when supplied in combination with light for mixotrophic feeding. It remains unclear, however, how the physiology of *X. umbellata* is affected by excess POM (when equated with DOC for total C content).

In this study, we investigated the effects of 20 mg organic C L^{-1} of dissolved and particulate forms (of varying particle sizes) of OM on the morphology and physiology of *X. umbellata*

under laboratory conditions. A concentration of 20 mg organic C L^{-1} was chosen as it represented *in situ* measurements of polluted reefs (Kline et al., 2006). In total, we supplied three treatments, including one DOM treatment as *i)* glucose-DOC, and two POM treatments as *ii)* phytoplankton, and *iii)* zooplankton. We also included a control treatment where no organic C enrichment was supplied. A range of parameters were assessed including tentacle morphology and ecophysiological parameters such as pulsation rate, colouration, algal cell density, chlorophyll-*a* content per algal cell, oxygen fluxes, and mortality. We hypothesised that the physiology of *X. umbellata* would be affected by POM but not by DOM. We based this hypothesis on previous research that has only found a neutral effect of DOM on the physiology of *X. umbellata* (Simancas-Giraldo et al., 2021; Vollstedt et al., 2020). In addition, we anticipated that 20 mg organic C L^{-1} of POM may not support heterotrophic feeding as previously found with lower provisions of phytoplankton (Hill et al., 2023), but instead, impede heterotrophic feeding by clogging the delicate feeding apparatus of *X. umbellata* (Szmant-Froelich et al., 1982), ultimately affecting the coral's overall physiology. Lastly, we hypothesised that larger particle sizes would initiate greater effects on the physiology of *X. umbellata*, as they are more abrasive than smaller particles on the soft coral tissue (Liefmann et al., 2018). By investigating the effects of excess DOM and POM on the morphology and physiology of *X. umbellata*, we aim to deepen our understanding of how *X. umbellata* responds to coastal eutrophication, and gain insight into how soft corals respond to excess DOM and POM.

3.3 Methodology

3.3.1 *Xenia umbellata* preparation

Colonies of *Xenia umbellata*, sourced from the Red Sea, were maintained within a tank at the University of Bremen for the past four years at a temperature of approximately 26 °C and at a salinity of 35 PSU. Three weeks prior to the start of the experiment, fragments (~ 5 cm in size) of the source colonies were obtained following the 'plug mesh method' (Kim et al., 2022). For the first two weeks, fragments were left to heal in the maintenance tank where the source colonies were kept. One week before the start of the experiment, healed fragments were transferred to experimental aquaria where they were able to acclimatise to their new

surroundings. A total of 264 fragments were used in the experiment, with 22 assigned to each experimental tank. Hereinafter fragments are referred to as colonies.

3.3.2 Experimental setup and maintenance

The experiment was carried out at University of Bremen, Germany, in May 2022 for a duration of 28 days, within the laboratory facilities of the Marine Ecology department. The experimental setup consisted of twelve experimental tanks (n = 12), each connected to its own technical tank, positioned directly behind. The experimental tank and the technical tank combined, held 16 L of water (Figure S3.1). At the beginning of the experiment, tanks were filled with 50% of freshly prepared artificial seawater created by mixing specialised salt (Zoomix Sea Salt, Tropic Marin, Germany) with demineralised water to obtain a salinity of ~ 35 PSU. To ensure that corals were not exposed to an unnaturally sterile environment, the remaining 50% of water was sourced from a long-running maintenance tank, where the colonies of *X. umbellata* were sourced from. Every technical tank contained a thermostat (3613 aquarium heater 75 W 220–240 V; EHEIM GmbH and Co. KG, Germany) connected to a temperature controller (Temperaturregler TRD, max. 1000 W, Schego Schemel & Goetz GmbH & Co, Germany), a skimmer (EHEIM Skim Marine 100; EHEIM GmbH and Co. KG, Germany) powered by an external air pump (EHEIM Air Compressor 100L/H, EHEIM GmbH and Co. KG, Germany), and a water pump (EHEIM CompactOn 1000 pump, EHEIM GmbH and Co. KG, Germany) to maintain the water flow between both tanks via an overflow pipe. A 1 L bottle of demineralised water, fitted with a refill fix nano top up cap (Refill Fix Nano refilling unit, Aqua Medic GmbH, Germany), was inverted and fixed to the side of each technical tank, where it automatically dispensed water into the tank when a reduced water level was detected due to evaporation. Overhead lamps (Royal Blue – matrix module and Ultra Blue White 1:1 – matrix module, 150 W, WALTRON daytime® LED light, Germany) were secured above the tanks, providing light on a 12:12 h light:dark cycle (Table 1). Black sheets were placed on both sides and beneath all experimental tanks to ensure that light remained equal in each tank, regardless of placement (Figure S3.1). Every day, a 50% water exchange was carried out in each tank at the end of the pulse enrichment, and temperature, salinity, oxygen, pH and light intensity (DataLogger Li1400, LI-COR) were measured (Table 1). Nutrient levels, including nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}), magnesium (Mg) and calcium (Ca), were measured twice per week using JBL test kits (TESTLAB MARIN; JBL GmbH & Co. KG, Germany) (Table 1). Manual adjustments were made when a deviation was observed

in temperature and salinity. Furthermore, twice per week, biofouling on the grids and plugs securing the *X. umbellata* colonies was removed using a soft bristle brush, and subsequently, coral colonies were randomly repositioned within the tanks to reduce effects of potential non-uniformities in tank conditions.

3.3.3 Organic carbon treatments

The corals were supplied with a pulse of 20 mg C L^{-1} in three different forms; DOC (D-Glucose anhydrous, purity: 99%, Fisher Scientific UK. Limited, Loughborough, UK), phytoplankton (*Nannochloropsis gaditana*, powder; algova UG, Germany, 2 – 5 µm particle size), and a natural mix of zooplankton (ReefRoids, poly Lab, USA, 150 – 200 µm particle size; Figure S3.2). The concentration of 20 mg C L^{-1} was chosen as it reflected measured C values of polluted reefs, and aligned with former organic C enrichment experiments (Kline et al., 2006; Simancas-Giraldo et al., 2021; Vollstedt et al., 2020; Zelli et al., 2023). The three organic C enrichment treatments and the control treatment were randomly distributed across the 12 experimental tanks, with a replication of three (n = 3). A control treatment was also established, where no additional organic C source was supplied. To establish the DOC treatment, the baseline of DOC already present in each respective tank was measured every morning using a Total Organic Carbon Analyser (TOC-L CPH/CPNPC-Controlled Model, Shimadzu, Japan), and glucose was administered accordingly to reach the target concentration (20 mg C L^{-1}). For the phytoplankton and zooplankton treatments, the exact carbon content (C%) of each was established with elemental analysis prior to the start of the experiment. With the C% of each plankton source, the specific dosage to achieve a concentration of 20 mg C L^{-1} per tank was calculated as 0.7 g for both phytoplankton and zooplankton. Treatments were supplied as a pulse enrichment, where corals were exposed to the treatments for a 2-hour period every morning, approximately from 9 to 11 am, during which skimmers were switched off.

3.3.4 Physiological parameter measurements

Tentacle morphology, pulsation and colouration

On the final experimental day (day 28), microscope images were taken of 3 randomly selected polyps of one colony per tank (n = 3). Images of the feeding tentacles were taken to see if there were any qualitative differences in tentacle appearance across treatments.

Pulsations were recorded on 3 randomly selected polyps of the same colony from each tank, every 7 days. One pulsation was defined as a complete contraction of the polyp, from open to fully closed to open again (Vollstedt et al., 2020). To ensure consistent tracking of the same colony over time without confounding factors, only one colony per tank was monitored. This approach was necessary because other colonies within the tank were subjected to different physiological measurements that either required removal from the tank before the experiment concluded, or induced short term stress, which could have affected the pulsation counts. Pulsations were counted for 30 seconds using a hand tally counter and a stopwatch at the same time every morning to avoid differences due to circadian rhythms (Klinke et al., 2022). The shorter observation period was chosen to save time, as water flow pumps were turned off during counting to avoid any interference. All pulsation counts were recorded by the same individual to minimise observer bias. The overall pulsation rate was calculated as the number of pulsations per minute; therefore, counts were multiplied by two and three technical replicates from the same colony were averaged to obtain one value per colony per tank.

One colony per tank was designated for colouration assessment, and this same colony was photographed every 7 days for re-assessment, equating to n = 3 per treatment. The colonies were individually transferred into a smaller heated tank (25°C) where they were photographed using an Olympus TG6 underwater camera (settings: ISO 100, f/1.4, x4 magnification), at a fixed distance and orientation. Images were later analysed in Photoshop (Adobe Photoshop CC 2015), using the "lasso" tool to select the opening of the gastrovascular cavity, and the tentacles of three opened polyps. Following this, the red, green and blue pixel values (RGB) were analysed for each coral. Given that *X. umbellata* is a soft coral with no calcium carbonate skeleton, it is visually more challenging to determine when they have bleached, and lost algal symbionts (Thobor et al., 2022). Therefore, the RGB score, also referred to as luminosity, was the most suitable method to assess potential changes in colouration. The luminosity ranges from 0 – 255, with 0 being black pigmentation and 255 being white pigmentation (i.e., bleached). RGB values were converted to a percentage out of 100 and inverted, so that a lower value, or a reduction indicates paling/bleaching of the fragment, while higher values correlate with darkening of the fragment (Tilstra et al., 2017). We renamed this parameter 'inverted luminosity'.

Symbiodiniaceae parameters

To assess Symbiodiniaceae parameters, one colony was selected at random from each tank every 7 days throughout the experiment and stored at -20 °C. On the day of analysis, in a darkened room, colonies were first thawed and then blended with a hand-homogeniser (MONIPA™ High Speed Homogenizer FSH-2A) in 10 ml of demineralised water. The exact volume of the tissue slurry was determined using a pipette. The slurry was then aliquoted into two separate 2 ml Eppendorf tubes and centrifuged for 10 minutes to separate the algal cells and coral tissue. The resulting supernatant was discarded, and the pellet was resuspended in 2 ml of demineralised water. The centrifugation step was repeated once more. One subsample was used for algal cell counts, and the other subsample was used for chlorophyll-*a* analysis.

For algal cell density determination, the protocol of LeGresley. & McDermott., (2010) was followed closely. In brief, pellets were resuspended in 2 ml of demineralised water and vortexed to obtain a homogenous solution. A subsample of 10 µL was pipetted onto both the upper and lower sections of a haemocytometer (Improved Neubauer counting chamber, depth 0.1mm). Using a light microscope, the cells in the four outer grids of both the upper and lower chambers were counted in a consistent way, to achieve a replication of two counts per sample. Counts were subsequently normalised to the initial slurry volume and the surface area of the original coral colony.

For chlorophyll-a per algal cell analysis, the protocol of Jeffrey & Humphrey, (1975) was followed. In a darkened room, the pellets obtained in the earlier step were resuspended in 2 ml of 90% acetone and vortexed to obtain a homogenous solution. They were then stored in the dark at 4°C for 24 hours. The following day, samples were centrifuged for five minutes, and each sample was aliquoted into two separate 1 ml glass cuvettes. Both subsamples were individually measured on a UV-Spectrophotometer (GENESYS 150, Fisher Scientific, Germany) at two fixed wavelengths of 663 nm and 630 nm, against a pre-made calibration curve. Both subsamples were measured three times each, and readings were normalised to the initial slurry volume and the total number of Symbiodiniaceae cells in the sample.

Oxygen fluxes

One colony per tank was designated for oxygen flux measurements, and this same colony was incubated every 7 days to assess oxygen fluxes, equating to n = 3 per treatment. On the

measurement day, the plugs of the selected colonies were cleaned with a brush to scrape off any potentially interfering organisms. Oxygen fluxes were measured approximately 1 hour after the daily organic C treatment was administered. A beaker incubation technique was followed. Corals were removed from their tanks and affixed to a stand inside a 160 ml glass beaker. All beakers were filled with water directly from the coral's respective tank and closed underwater to avoid air bubbles interfering with oxygen readings. Beakers were then placed into a water bath equipped with a thermostat (3613 aquarium heater. 75 W 220–240 V; EHEIM GmbH and Co. KG, Germany) to maintain temperature at ~25°C. The water bath was placed on top of a magnetic stirring plate which powered magnetic stir bars (at 190 rpm) within each beaker, to sustain ample water circulation and homogenous oxygen concentrations. Corals were incubated in this setup for 1.5 hours in the light (net photosynthesis [Pnet]) at ~ 100 umol photons m^{-2} s^{-1} PAR (Royal Blue – matrix module and Ultra Blue White 1:1 – matrix module, 150 W, WALTRON daytime® LED light, Germany), and 1.5 hours in the dark (respiration [R]). Oxygen concentration readings were taken with an optode sensor (HACH LDO, HACH HQ 40d, Hach Lange GmbH, Germany) before and after each incubation. In addition to incubating one coral colony per tank, one coral plug with no coral attached (blank plug) was also selected from each experimental tank and incubated in the same setup. The beakers containing blank coral plugs served as controls to account for oxygen fluxes related to biofouling on the plug or micro-organisms within the experimental water. After each incubation, the initial oxygen concentration was subtracted from the final oxygen concentration to determine the oxygen flux, with oxy-light for the light incubation and oxy-dark for the dark incubation. Following this, various normalisation steps were carried out (Equation 1) (adapted from Thobor et al., (2022)). Briefly, to account for size differences between the selected colonies, data was normalised to the surface area of the respective colony (*p*). The surface area (SA) of each colony was determined by counting the number of polyps and multiplying this value by the approximate SA of one polyp (Bednarz et al., 2012; Thobor et al., 2022). Data was also normalised to the jar size (in litres) *(v)*, the incubation length (in hours) *(h)*, and the planktonic background was subtracted (control - light or control - dark). Subsequently, net photosynthesis (Pnet) from the light incubation, and respiration (Resp) from the dark incubation were used to calculate the gross photosynthesis (Pgross) (Equation 2).

Equation 1

$$P_{net}\ or\ Resp = \frac{\frac{(oxy_{(light\ or\ dark)})}{h} - \frac{(control_{(light\ or\ dark)})}{h}}{p * s * v}$$

Equation 2

$$Pgross = Pnet + |Resp|$$

Specific growth rate

The number of polyps of one selected colony from each tank was counted every 3-4 days. To do so, the colony was transferred to a small glass jar filled with water from the respective experimental tank. Tweezers were used to gently separate neighbouring polyps and accurately count them. Specific growth rate (SGR) was calculated following the equation below (Equation 3) (Tilstra et al., 2017; Wijgerde et al., 2012), where P_t is the initial number of polyps, P_{t-1} is the final number of polyps and Δt is the time interval in days. The overall SGR unit is polyp polyp^{-1} day^{-1} which may be abbreviated to d^{-1}.

Equation 3

$$SGR\ (d^{-1}) = \frac{(\ln P_t - \ln P_{t-1})}{\Delta t}$$

Mortality

Colonies were monitored closely daily throughout the experiment. A colony was classified as dead if the entire colony fully dissolved, leaving only the plug behind. The percentage of mortality was calculated from the total number of colonies remaining in each respective tank per week.

3.3.4 Statistical analyses

Data was visualised and analysed using R (version 4.2.3) with the packages 'rstatix' (Kassambara, 2020), 'afex' (Singmann et al., 2024) and 'emmeans'. As a first step, data was evaluated to determine if it satisfied the assumptions required for parametric testing. Normality was assessed via the Shapiro-Wilk test, and homogeneity of variances was examined with Levene's test. Data that failed to meet these assumptions were log transformed and re-assessed. Data that met the assumptions were analysed using a 2-way analysis of variance (ANOVA), while if not normally distributed even following data transformation were analysed with a non-parametric linear mixed effect model (LMM). A random effect term was included for parameters that had repeated measures. Where significant differences were detected, pairwise comparisons of the estimated marginal means were conducted with adjustments for multiple comparisons using the Tukey method. Mortality was the only data parameter with one factor, and due to a non-normal distribution, was analysed using a Kruskal-Wallis (KW) test, followed by a Dunn's test for post-hoc analysis.

3.4 Results

3.4.1 Background environmental parameters

The majority of measured background parameters (Table 1) remained consistent across treatments, except for concentrations of nitrite, nitrate, phosphate, and light intensity (Figure 3.1). Nitrite concentrations significantly differed across treatments (LMM, 3, $F = 16.79$, $p < 0.001$; Figure 3.1a). The average nitrite concentration of the zooplankton treatment (0.14 mg L^{-1}) was significantly higher than all other treatments, including the control (0.02 mg L^{-1}; $p < 0.001$), DOC (0.02 mg L^{-1}; $p < 0.001$) and phytoplankton (0.04 mg L^{-1}; $p < 0.001$; Figure 3.1a). Nitrite also significantly increased over time (LMM, 24, $F = 2.34$, $p < 0.05$) within the zooplankton treatment ($p < 0.001$), with concentrations increasing from 0.02 to 0.37 mg L^{-1} between day 0 to day 28 (Figure 3.1a). Nitrate concentrations significantly differed across treatments (LMM, 3, $F = 16.99$, $p < 0.001$; Figure 3.1b). Similar to nitrite, the average nitrate concentration of the zooplankton treatment (1.84 mg L^{-1}) was significantly higher than all other treatments, including the control (0.5 mg L^{-1}; $p < 0.001$), DOC (0.5 mg L^{-1}; $p < 0.001$) and phytoplankton (0.68 mg L^{-1}; $p < 0.001$; Figure 3.1b). Nitrate also significantly increased over

time (LMM, 24, F = 2.53, $p < 0.001$) within the zooplankton treatment ($p < 0.001$), with concentrations increasing from 0.5 to 3.67 mg L^{-1} between day 0 to day 28 (Figure 3.1b). Phosphate concentrations significantly differed across treatments (LMM, 3, F = 372.33, $p < 0.001$; Figure 3.1c). Average concentrations of phosphate were significantly higher in both phytoplankton (0.55 mg L^{-1}) and zooplankton (0.81 mg L^{-1}) than the control (0.02 mg L^{-1}) and DOC (0.02 mg L^{-1}) treatments ($p < 0.001$; Figure 3.1c). In addition, phosphate was significantly higher in the zooplankton (0.81 mg L^{-1}) compared to the phytoplankton (0.55 mg L^{-1}) treatment ($p < 0.001$; Figure 3.1c). Phosphate also significantly increased over time (LMM, 24, F = 2.53, $p < 0.001$) within the zooplankton ($p < 0.001$) treatment, increasing from 0.02 to 0.94 mg L^{-1} between day 0 to day 28, and likewise in the phytoplankton treatment ($p < 0.001$) from 0.02 to 0.54 mg L^{-1} (Figure 3.1c). Significant differences in light intensity were measured across treatments (LMM, 3, F = 97.67, $p < 0.001$; Figure 3.1d). The average light intensity within the phytoplankton (63 µmol m^{-2} s^{-1}) and zooplankton (86 µmol m^{-2} s^{-1}) treatments was significantly lower than within control (102 µmol m^{-2} s^{-1}) and DOC (104 µmol m^{-2} s^{-1}) treatments ($p < 0.001$; Figure 3.1d). Phytoplankton also had a significantly lower light intensity than the zooplankton treatment ($p < 0.001$; Figure 3.1d). Additionally, light intensity significantly decreased over time (LMM, 81, F = 1.3885, $p < 0.05$), within the phytoplankton treatment ($p < 0.05$) decreasing by 60% from 103 to 41 µmol m^{-2} s^{-1} between day 0 to day 28 Although not significant, light intensity also reduced by 30% in the zooplankton treatment from 101 to 71 µmol m^{-2} s^{-1} between day 0 and 28.

Table 3.1 Background parameters monitored throughout the experiment. Values are reported as mean ± standard deviation. *Parameters that were inconsistent across treatments are displayed graphically in figure 3.1.

	Control	DOC	Phytoplankton	Zooplankton
Temperature [°C]	24.53 ± 0.26	24.50 ± 0.32	24.53 ± 0.23	24.62 ± 0.40
Salinity [PSU]	35.28 ± 0.36	35.24 ± 0.40	35.13 ± 0.43	35.02 ± 0.31
pH	8.36± 0.03	8.34 ± 0.00	8.28 ± 0.04	8.27 ± 0.09
Oxygen [mg L^{-1}]	6.95 ± 0.13	6.89 ± 0.10	6.79 ± 0.16	6.85 ± 0.16
***NO_2 [mg L^{-1}]**	0.02 ± 0.00	0.02 ± 0.01	0.04 ± 0.05	0.14 ± 0.18
***NO_3 [mg L^{-1}]**	0.50 ± 0.00	0.50 ± 0.00	0.68 ± 0.22	1.84 ± 1.97
NH_4 [mg L^{-1}]	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
***PO_4 [mg L^{-1}]**	0.02 ± 0.00	0.02 ± 0.00	0.55 ± 0.33	0.81 ± 0.38
Mg [mg L^{-1}]	1437.78 ± 123.06	1362.22 ±143.00	1382.22 ±186.40	1437.50 ± 196.96
Ca [mg L^{-1}]	455.56 ± 26.03	451.11 ± 20.30	457.78 ± 27.28	452.50 ± 14.88
***Light [µmol m^{-2} s^{-1}]**	101.63 ± 21.53	104.0 ± 13.10	63.45 ± 22.65	86.28 ± 17.51

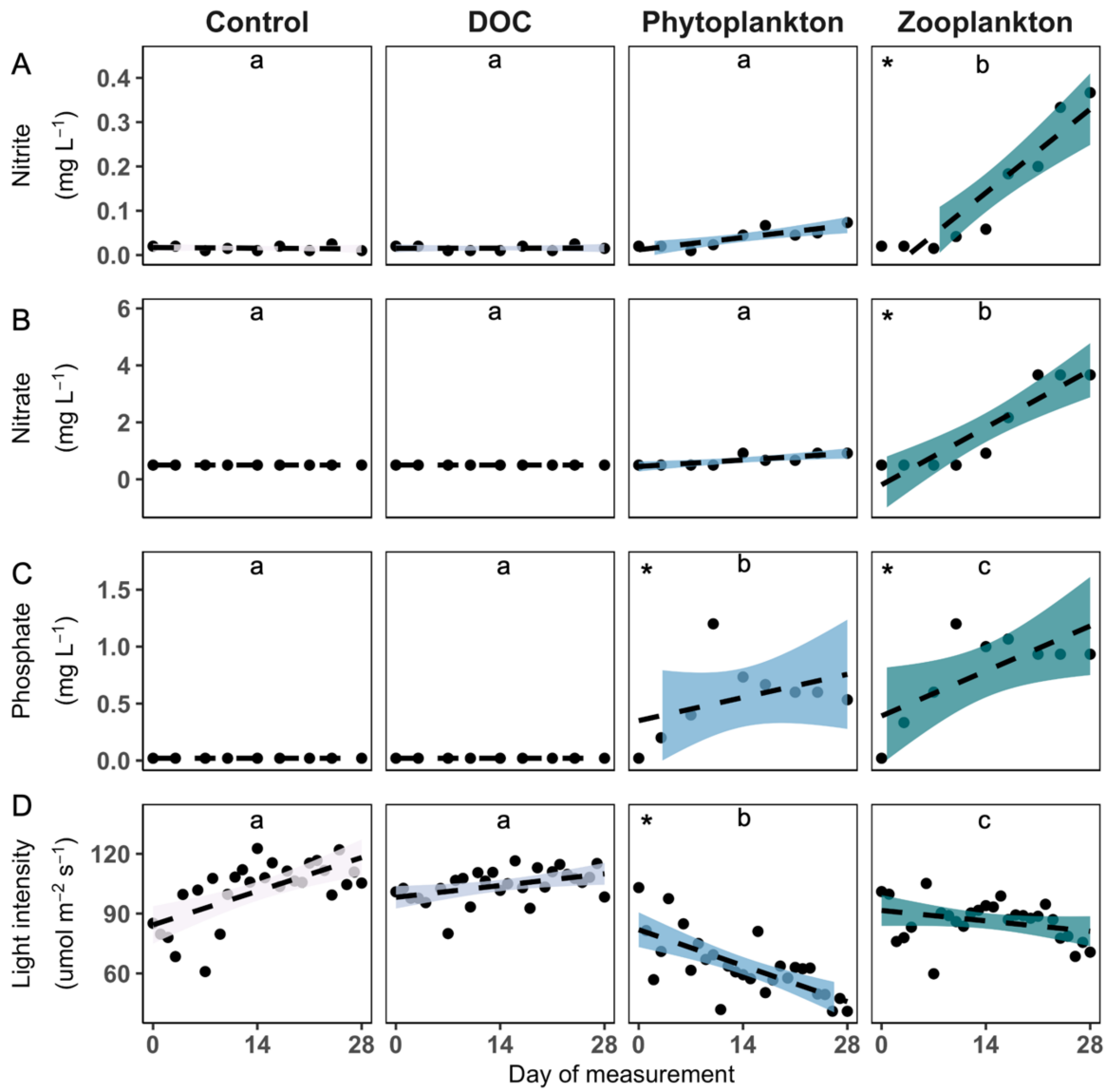

Figure 3.1 Variable background parameters when *Xenia umbellata* was exposed to 20 mg C L^{-1} in three forms (dissolved organic carbon [DOC], phytoplankton, and zooplankton) and a control treatment over time (28 days). Significant differences across treatments are indicated by different letters, whereas the same letter indicates no significant difference. Significant differences within organic carbon treatments over time are indicated by an asterisk (*) in the respective treatment panel.

3.4.2 Tentacle morphology

The morphology of the tentacles appeared visibly different across treatments (Figure 3.2). In the microscope images, the arm and pinnules of each tentacle in the control and DOC treatments are elongated in shape (Figure 3.2a & b). Conversely, in the phytoplankton and

zooplankton treatments, the tentacles are distorted in shape with sharp bends in the arms and have compacted pinnules (Figure 3.2c & d). Overall, damage to the tentacles in the phytoplankton and zooplankton treatments are visible, with more prominent changes in the zooplankton treatment (Figure 3.2d).

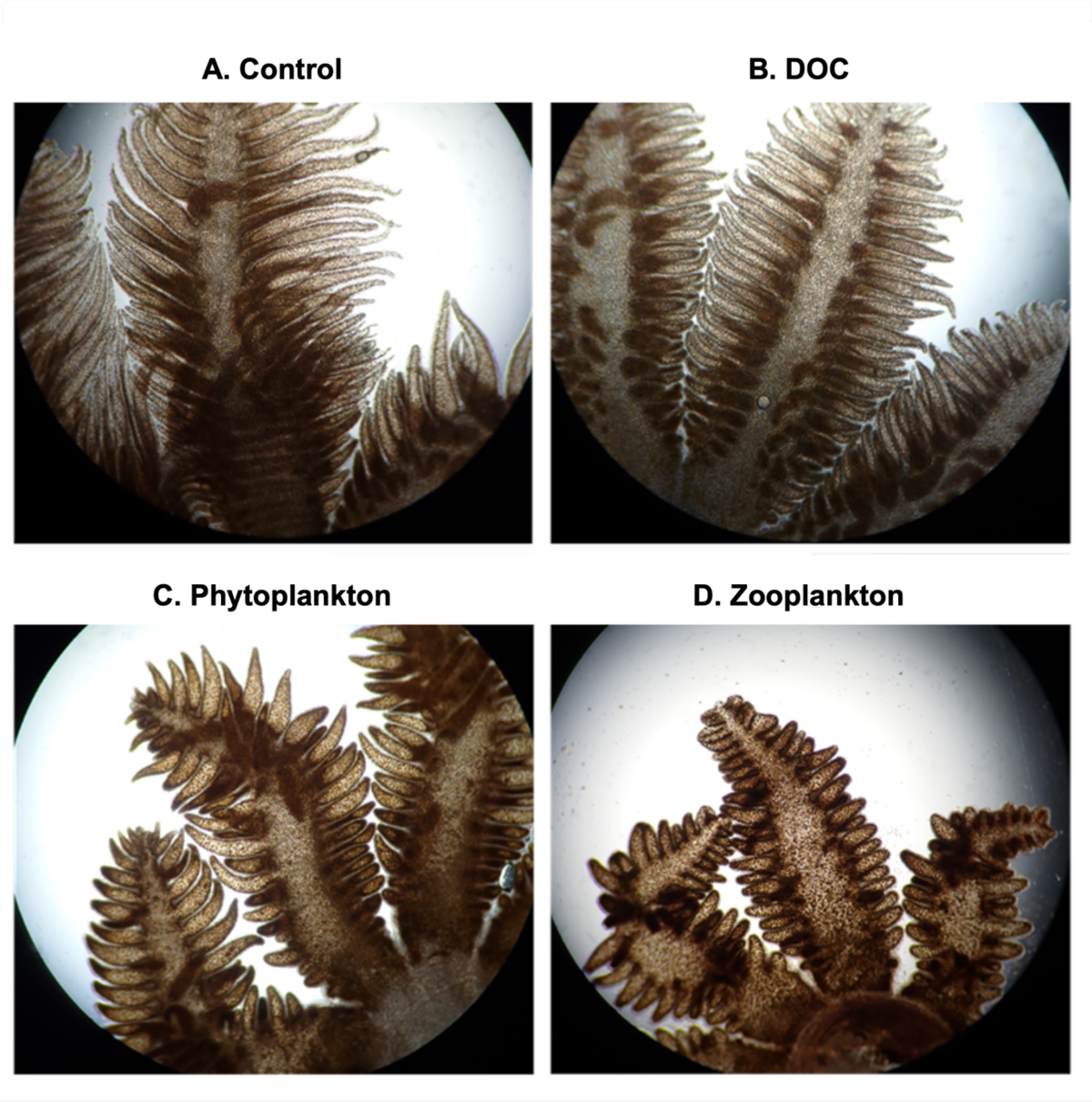

Figure 3.2 Tentacle morphology of a subsample of *Xenia umbellata* , following 28 days of exposure to three different organic carbon enrichment treatments including A] a control treatment where no organic carbon was supplied, B] dissolved organic carbon [DOC], C] phytoplankton, and D] zooplankton. (Magnification x 40).The images shown are representative of the entire population.

3.4.3 Pulsation rate

Pulsation rates significantly differed across treatments (LMM, 3, $F = 47.95$, $p < 0.001$;Figure 3.3). Average pulsation rates were significantly higher in the control (38 beats min^{-1}; $p < 0.001$) and DOC treatments (36 beats min^{-1}; $p < 0.001$), than in the phytoplankton (29 beats min^{-1}; $p < 0.001$) and zooplankton treatments (31 beats min^{-1}; $p < 0.001$; Figure 3.3). Average pulsation rates also significantly differed between the control (38 beats min^{-1}) and DOC treatments (36 beats min^{-1}; $p < 0.05$; Figure 3.3). Additionally, pulsation rates significantly decreased over time (LMM, 12, $F = 9.73$, $p < 0.001$) within the phytoplankton ($p < 0.05$) and zooplankton ($p < 0.05$) treatments, where pulsation rates significantly decreased between day 0 and every subsequent measurement day (day 7, 14, 21 and 28; Figure 3.3). However, significant changes over time were not observed within the control ($p > 0.05$) and DOC ($p > 0.05$) treatments (Figure 3.3).

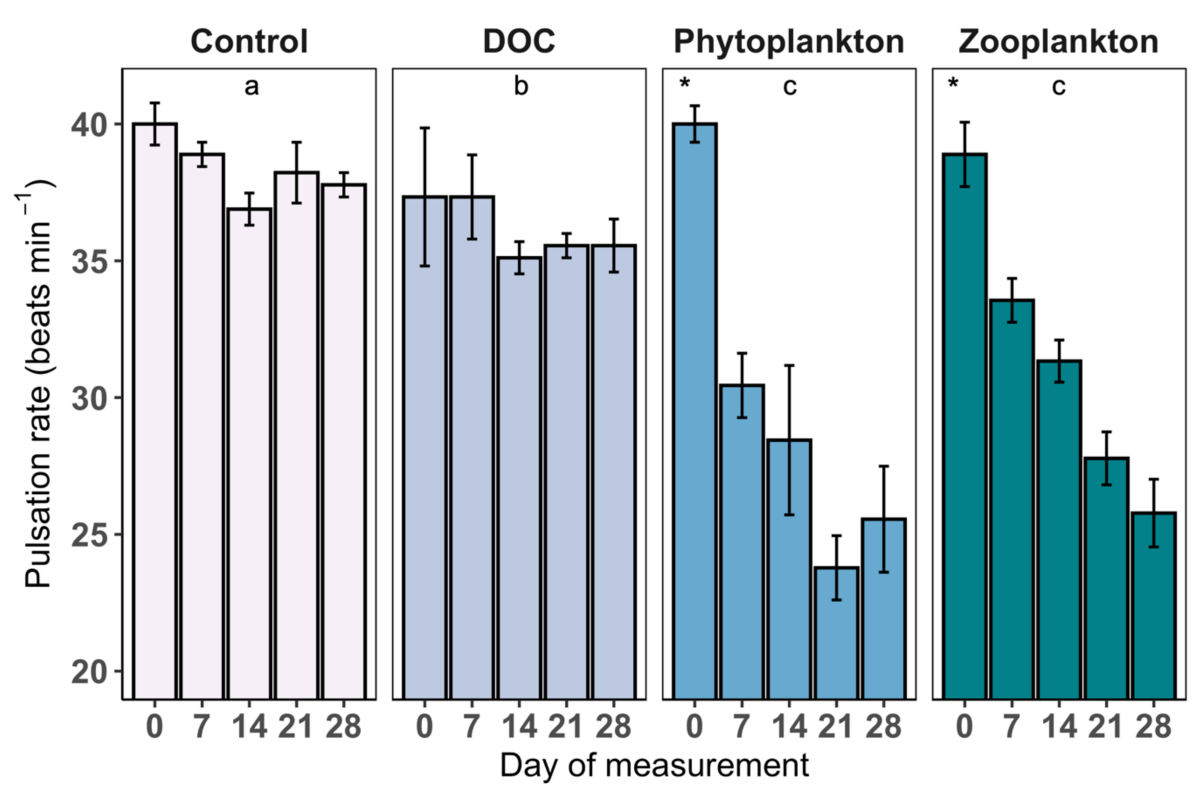

Figure 3.3 The pulsation rate (beats min^{-1}) of *Xenia umbellata* when exposed to 20 mg C L^{-1} in three forms (dissolved organic carbon [DOC], phytoplankton, zooplankton) and a control treatment over time (28 days). Each bar = mean ± standard error. Across treatments, variables that have different letters are statistically different, whereas variables that have the same letter are not significantly different. Significant differences within organic carbon treatments over time are indicated by an asterisk (*) in the respective treatment panel.

3.4.4.Colouration

Colouration, measured as “inverted luminosity”, significantly differed across the organic C enrichment treatments (ANOVA, 3, $F = 27.93$, $p < 0.001$; Figure 3.4). In particular, the average inverted luminosity of corals within the control (36%) and DOC treatment (35%) was significantly lower than those in the phytoplankton treatment (45%; $p < 0.001$) and zooplankton (47%; $p < 0.001$) treatments (Figure 3.4). Furthermore, inverted luminosity significantly increased over time (ANOVA, 12, $F = 1.16$, $p < 0.001$) within the zooplankton treatment ($p < 0.001$), between day 1 (37%) and day 21 (53%) ($p < 0.05$), and between day 1 (37%) and day 28 (53%) ($p < 0.05$; Figure 3.4).

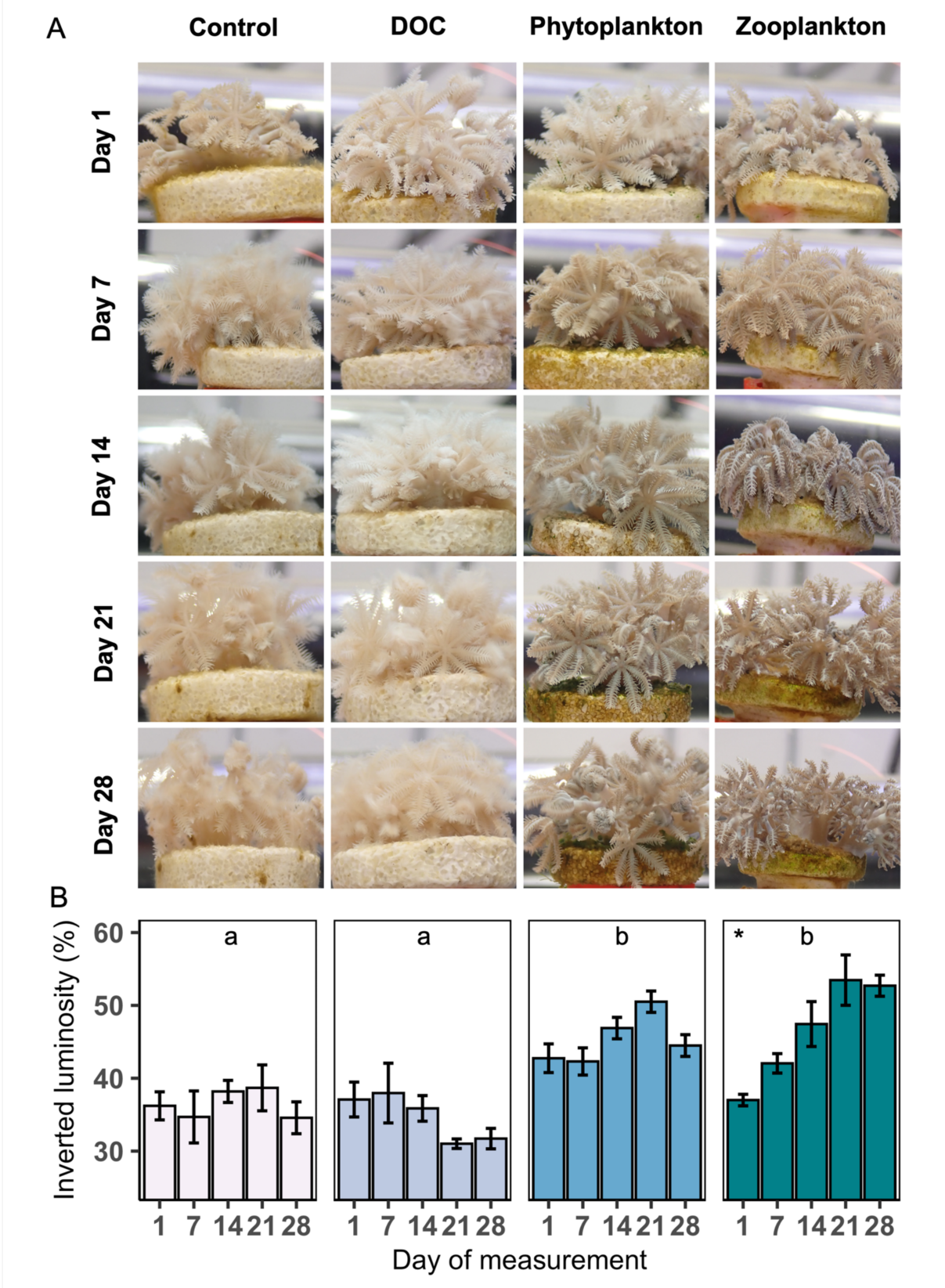

Figure 3.4 The colouration of *Xenia umbellata* when exposed to 20 mg C L^{-1} in three forms (dissolved organic carbon [DOC], phytoplankton, zooplankton) and a control treatment over time (28 days). A) A

subsample of the analysed images, taken by N. Eichhorn. B) Each bar = mean ± standard error. Black pigmentation is signified by higher values, and white pigmentation (i.e., bleaching) by lower values. Across treatments, variables that have different letters are statistically different, whereas variables that have the same letter are not significantly different. Significant differences within organic carbon treatments over time are indicated by an asterisk (*) in the respective treatment panel.

3.4.5 Symbiodiniaceae parameters

The algal cell density significantly differed across organic C enrichment treatments (ANOVA, 3, $F = 5.291$, $p < 0.05$), specifically between the control (1.09×10^5 cm^2) and phytoplankton (1.74×10^5 cm^2) treatment ($p < 0.05$) and between the control (1.09×10^5 cm^2) and zooplankton (2.01×10^5 cm^2) treatment ($p < 0.05$; Figure 3.5a). However, the algal cell density did not significantly change over time within each treatment (ANOVA, 16, $F = 1.447$, $p > 0.05$; Figure 3.5a).

The chlorophyll-*a* per algal cell was highly variable, and consequently had no significant differences across organic C treatments (ANOVA, 3, $F = 2.727$, $p > 0.05$), nor within treatments over time (ANOVA, 16, $F = 1.492$, $p > 0.05$; Figure 3.5b). The minimum average chlorophyll-*a* per cell was observed in the control treatment at 0.76 pg algal cell^{-1}, whilst the maximum was observed in the zooplankton treatment at 1.60 pg algal cell^{-1} (Figure 3.5b).

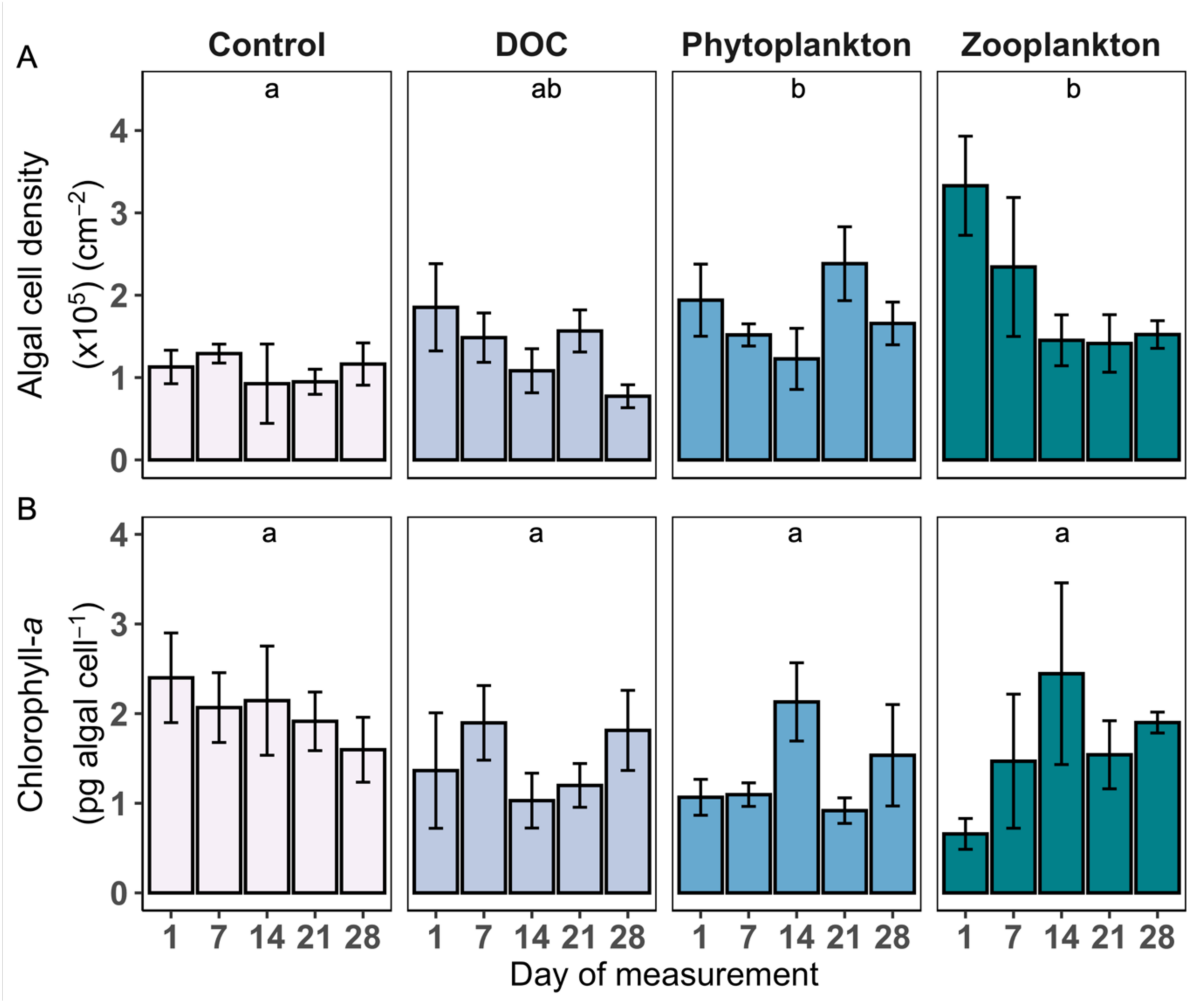

Figure 3.5 Symbiodiniaceae parameters of *Xenia umbellata*, including A) algal cell density (cm^{-2}) and B) chlorophyll-*a* (pg algal $cell^{-1}$) when exposed to 20 mg C L^{-1} in three forms (dissolved organic carbon [DOC], phytoplankton, zooplankton) and a control treatment over time (28 days). Each bar = mean ± standard error. Across treatments, variables that have different letters are statistically different, whereas variables that have the same letter are not significantly different.

3.4.6 Oxygen fluxes

Gross photosynthesis rates did not significantly differ across treatments (LMM, 3, $F = 0.46$, $p > 0.05$); however, rates did significantly differ over time (LMM, 12, $F = 2.64$, $p < 0.05$; Figure 3.6) within the phytoplankton treatment ($p < 0.05$), with a significant decrease in Pgross between day 0 (14.89) and 28 (10.49), between day 14 (25.01) and day 28 (10.49), and between day 21(17.65) and 28 (10.49; Figure 3.6). Additionally, a significant decrease was observed overtime between day 0 (15.81) and 28 (11.88) in the zooplankton treatment ($p < 0.05$; Figure 3.6). However, at day 28, gross photosynthesis rates did not significantly differ across treatments ($p > 0.05$).

Similarly, respiration rates did not differ significantly across treatments (LMM, 3, F = 2.11, p > 0.05); yet rates significantly fluctuated over time (LMM, 12, F = 2.42, p < 0.05; Figure 3.6) within the phytoplankton treatment (p < 0.001), with a significant increase between day 0 (-3.83) and day 14 (-17.10), and day 7 (-4.61) and 14 (-17.10). However, respiration then significantly decreased between day 14 (-17.10) and 28 (-2.25; Figure 3.6).

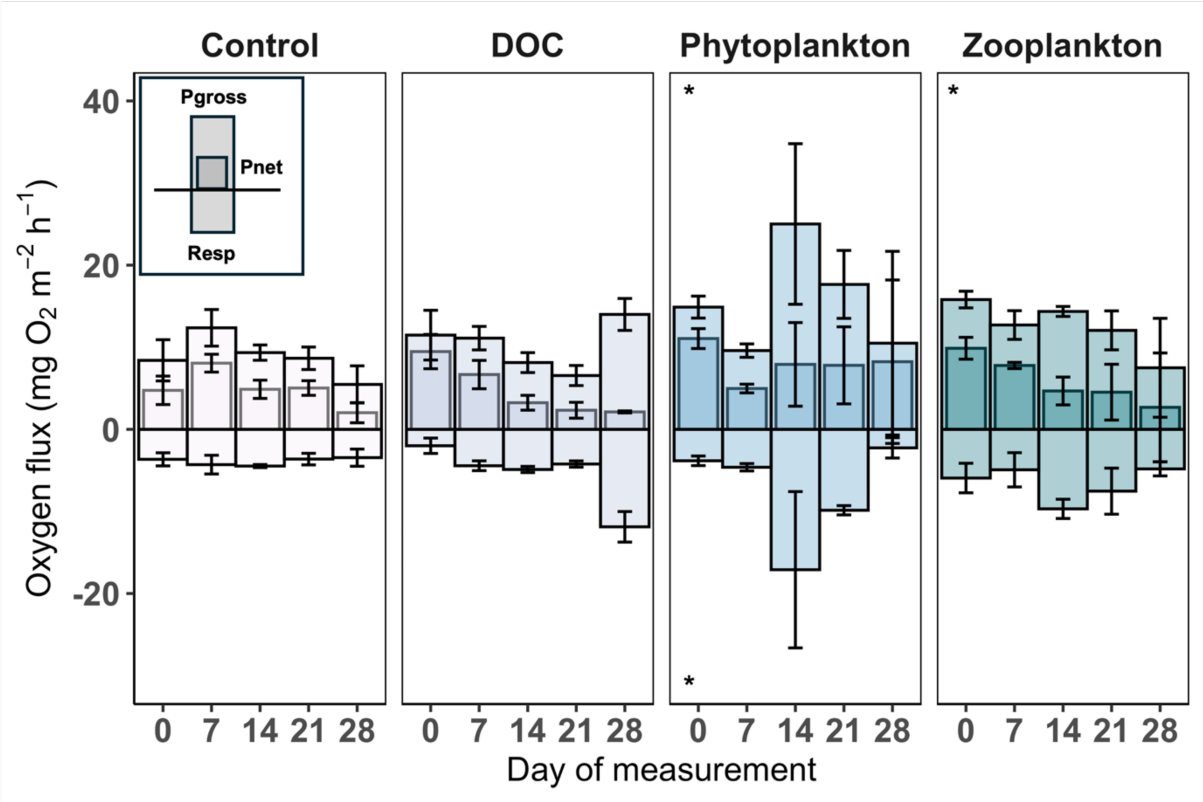

Figure 3.6 Oxygen fluxes (mg O_2 per m^2 h^{-1}) of *Xenia umbellata*, including gross photosynthesis (Pgross), Respiration (Resp) and net photosynthesis (Pnet), when exposed to 20 mg C L^{-1} in three forms (dissolved organic carbon [DOC], phytoplankton, zooplankton) and a control treatment over time (28 days). Each bar = mean ± standard error. Significant differences within organic carbon treatments over time are indicated by an asterisk (*) in the respective treatment panel.

3.4.6 Specific growth rate

The SGRs were highly variable, with no visible nor significant patterns across the organic C enrichment treatments (ANOVA, 3, 0.465, p > 0.05) nor over time within each treatment (ANOVA, 9, 0.419, p > 0.05; S3.3). The minimum average SGR was observed in the

zooplankton treatment at -0.002 d^{-1}, whereas the maximum average was observed in the phytoplankton treatment at 0.02 d^{-1} (S3.3).

3.4.7 Mortality

No mortality was observed in the control (0%) and DOC (0%) treatments, yet mortality did occur in the phytoplankton (11%) and zooplankton (14%) treatments throughout the experiment (Figure 3.7). Mortality was significantly higher in the zooplankton treatment (KW, $H = 7.7478$, $df = 3$, $p < 0.05$) when compared to the control ($p < 0.05$) and DOC ($p < 0.05$) treatments, yet mortality in the phytoplankton treatment was not significantly higher than the control ($p > 0.05$) and DOC ($p > 0.05$) treatments, likely due to high variability (Figure 3.7).

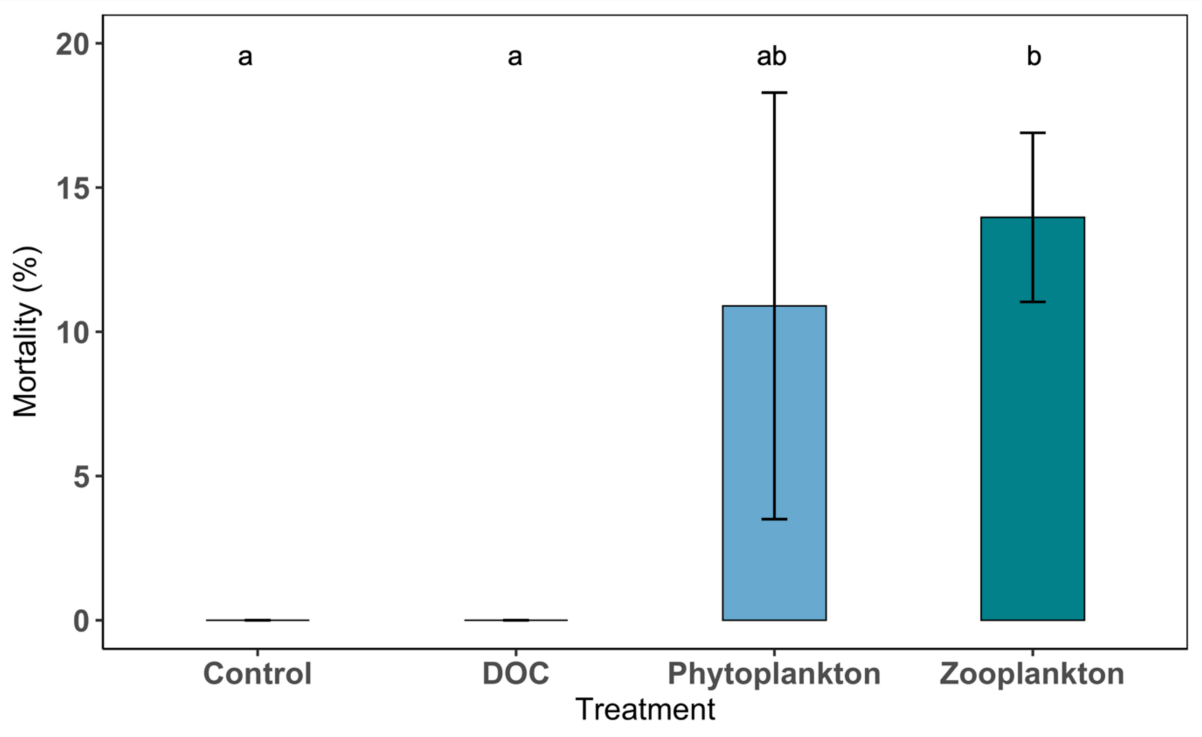

Figure 3.7 The mortality (%) of *Xenia umbellata* when exposed to 20 mg C L^{-1} in three forms (dissolved organic carbon [DOC], phytoplankton, zooplankton) and a control treatment for 28 days. Each bar = mean ± standard error. Across treatments, variables with the same letter are not significantly different.

3.5 Discussion

Our study assessed the morphological and physiological effects of supplying 20 mg L^{-1} of organic C enrichment in both dissolved and particulate forms (of varying particle sizes) on *X. umbellata*. The dissolved form of organic C had negligible effects following 28 days of exposure, whilst particulate forms of organic C, i.e., phytoplankton and zooplankton, had considerable effects on the morphology and physiology of *X. umbellata*. We argue that the observed effects from 20 mg L^{-1} of POM enrichment were primarily caused by damage of delicate feeding apparatus used for heterotrophic feeding, thereby reducing uptake of essential food sources and lowering gas exchange by reduced pulsation. Furthermore, these effects were exacerbated by larger particle sizes.

3.5.1 How does *Xenia umbellata* respond to dissolved forms of organic carbon?

Our study provides further evidence that DOC, in the form of glucose, has no significant effect on the physiology of *X. umbellata*. We observed no significant changes in tentacle morphology, pulsation, colouration, algal cell density, chlorophyll-*a* content per algal cell, oxygen fluxes, mortality nor growth rate (Figures 3.2, 3.3, 3.4, 3.5, 3.6, 3.7 & S3.3). These findings reinforce and build upon previous research, which similarly found no significant impact of 20 mg C L^{-1} of DOC (glucose) on *X. umbellata* (Simancas-Giraldo et al., 2021; Vollstedt et al., 2020). Similar neutral effects of DOC have been observed in other octocorals such as the gorgonian *Pinnogorgia flava,* which also showed no significant ecophysiological response from the same, and higher concentrations of DOC (glucose) enrichment (Zelli et al., 2023). The neutral effects of DOC appear to be unique to octocorals, as other cnidarian-algal symbioses, experience vastly different effects. For instance, hard corals can experience bleaching and mortality due to accelerated growth of microbes living in the mucopolysaccharide layer, causing a disruption between the coral and its microbiome (Kline et al., 2006; Kuntz et al., 2005; Pogoreutz et al., 2017), while *Cassiopea* sp. may shift their trophic strategy more towards heterotrophy upon high DOC exposure (Tilstra et al., 2022). These differences may be explained by the morphology of *X. umbellata* which allow for higher ingestion of DOC, as its polyps provide a higher surface area: volume ratio compared to most hard corals (Siebers, 1982). In turn, this may reduce the pool of DOC available for use by microbes, thus constraining microbial overgrowth and maintaining a harmonious balance between the coral and its associated microbiota (Fabricius & Klumpp, 1995; Zelli et al., 2023). It is important to stipulate, however, that DOC *in situ* consists of multiple sugars such as xylose, mannose, and galactose (to name a few) that we did not include within our experiment. Therefore, whilst our treatment is named

DOC, it consists only of glucose, so we can only comment on the effects of glucose-DOC on *X. umbellata.* Thus, to better understand the effects of DOC on the physiology of *X. umbellata*, multiple sugar types should be tested in combination as this is more reflective of natural DOC enrichment *in situ*.

3.5.2 How does *Xenia umbellata* respond to particulate forms of organic carbon?

The influence on feeding

In POM treatments, *X. umbellata* exhibited significant morphological and physiological changes. For example, injury to the tentacles was visible under a light microscope, where colonies exposed to POM treatments had tentacles with considerably disfigured arms and pinnules compared to colonies in the control and DOC treatments (Figure 3.2). Additionally, there was a significant reduction in pulsation (Figure 3.3) and gross photosynthesis rates over time within both POM treatments (Figure 3.6). As a mixotroph, *Xenia umbellata* relies on both auto- and heterotrophic food sources for complete nutrition (Hill et al., 2023). Unlike hard corals, octocorals uniquely capture POM in a raptorial manner using tentacles and pinnules, and complete ingestion using directional ciliary currents in the mouth and pharynx (Lewis, 1982). Recently, phytoplankton was found to have supported the heterotrophic feeding and health of *X. umbellata* (Hill et al., 2023). However, if present in high quantities, suspended POM sediments could interfere with heterotrophic feeding, by clogging the delicate feeding apparatus (Szmant-Froelich et al., 1982). Consequently, *X. umbellata* colonies exposed to excess POM may have been unable to efficiently capture food sources and could have suffered from nutritional and energetic deficiencies, thereby explaining the significant reduction in the rate of energy-intensive pulsations observed overtime (Figure 3.3; Kremien et al., 2013). The same reduction in pulsation rate was also seen in an earlier experiment when *X. umbellata* had no available heterotrophic food sources to consume (Hill et al., 2023), demonstrating a vast depletion of energy when heterotrophic feeding was impeded. Furthermore, given that pulsations enhance autotrophy by continually breaking the coral – water boundary layer, colonies may also have experienced a parallel reduction in energy acquired from their symbionts due to decreased gaseous exchange (Kremien et al., 2013). This was demonstrated by the significant reduction in gross photosynthesis rates over time within both POM treatments (Figure 3.6). Lastly, the reduced feeding capacity and colony mortality in the POM

treatments created a negative feedback loop, where decreased POM uptake led to its accumulation, further intensifying its harmful effects on the colonies. Overall, the impaired feeding capacity of *X. umbellata* due to damaged feeding apparatus was likely the main factor driving the observed morphological and physiological changes in the POM treatments. Although we acknowledge that the morphological assessments are qualitative, and may lack the same scientific rigor, we evaluated them in conjunction with quantitative physiological measurements, such as gross photosynthesis, pulsation and mortality to provide a more comprehensive and robust analysis of the overall impact of POM on *X. umbellata*.

Negligible effects from fluctuating nutrients and light

In the zooplankton treatment, nitrite, nitrate and phosphate concentrations were significantly higher than in control and DOC treatments, and in phytoplankton treatment phosphate concentrations were also significantly higher than in control and DOC treatments (Figure 3.1). Whilst elevated nitrite levels can negatively affect some marine organisms such as fish (Jensen, 2003), nitrite is not bioavailable to corals in general (Bythell, 1990) and therefore is unlikely to have influenced the physiology of *X. umbellata*. The effects of elevated nitrate and phosphate on corals, on the other hand, have been well-documented, and even investigated specifically on *X. umbellata* (Klinke et al., 2022; Mezger et al., 2022; Thobor et al., 2022). When comparing the concentrations of phosphate and nitrate within our treatments to these former studies, we must be cautious given the analytical methods used in our experiment are less reliable. However, despite these limitations, the highest phosphate concentration (0.81 mg L^{-1}) measured in the current experiment was only marginally higher than the phosphate treatment supplied in former experiments (~ 0.76 mg L^{-1}), where no physiological changes to *X. umbellata* were observed (Klinke et al., 2022; Mezger et al., 2022). Similarly, our peak measured nitrate concentration (1.84 mg L^{-1}) was lower than the maximum nitrate treatment (~2.29 mg L^{-1}) supplied in previous experimental work, again where no physiological changes were observed (Thobor et al., 2022). Given that both previous enrichment experiments found no significant effect of these high concentrations on the physiology of *X. umbellata,* we argue that the inconsistent nutrient concentrations within our experiment had minimal influence on the differential physiological response of *X. umbellata* across treatments.

Additionally, *X. umbellata* colonies within the POM treatments experienced lower light availability than the control and DOC treatment (Figure 3.1d), due to suspended particles

increasing the turbidity of the water and reducing the penetration of light (K. E. Fabricius, 2011). Given the nature of lab experiments, there is considerably less movement and exchange of water than in natural environments. Therefore, we consider the increased turbidity and reduction in light availability to be an artefact of a lab experiment rather than a meaningful effect of POM enrichment on a reef. While gross photosynthesis rates did significantly decline from the start to end of the experiment within the POM treatments, these data were variable and even preceded by increases in photosynthesis (Figure 3.6). Additionally, when we compare the gross photosynthesis rates on the final experimental day (day 28) across treatments, rates in both POM treatments did not significantly differ from those of the control where light remained constant ($p > 0.05$; Figure 3.6). Therefore, we do not consider the reduction of light availability in the POM treatments to have largely affected the rates of gross photosynthesis, and instead attribute this to the reduction in pulsation.

3.5.3 Does particle size matter?

The size of the zooplankton particles (150 – 200 μm in length) was considerably larger than that of the phytoplankton (2 – 5 μm in length; S2). Under zooplankton enrichment, the tentacles of *X. umbellata* appeared more extensively damaged and colonies significantly darkened in their colouration overtime (Figures 3.2, 3.3). Darkening of hard coral tissue is typically attributed to parallel increases in algal cell density or chlorophyll-*a* content per cell (Siebeck et al., 2006). However, these parameters were not significantly higher among colonies in the zooplankton compared to phytoplankton treatments (Figure 3.5). For other octocorals, darkening in colouration has been associated with acute stress, such as when being overgrown by a neighbouring sponge, with darkening of tissue often observed prior to tissue necrosis and death (Mclean et al., 2015). In former studies on the effects of sediments on soft corals, higher tissue abrasion was found on soft corals exposed to sediment plumes with larger particle sizes (Liefmann et al., 2018). This is because larger particles have more abrasive effects due to their larger surface and high encounter rates (Liefmann et al., 2018). It is important to acknowledge, however, that there was a large overlap in the morphological and physiological responses of *X. umbellata* to both small and large size classes of POM, with damaged tentacles, reduced pulsation and photosynthesis rates and mortality observed among colonies in both treatments (Figures 3.2, 3.3, 3.6 & 3.7). It is therefore evident that even small particle sizes of POM can negatively affect *X. umbellata*, with larger particles further exacerbating these effects. Therefore, the primary driver of the effects of POM on *X. umbellata* is more likely the dose of

POM eutrophication rather than the particle size. Our experiment used size classes that represent the extreme ends of the spectrum; therefore, we recommend future studies to include more size classes within the intermediate range (i.e., between 10 and 100 µm) to establish what particle size range is well-tolerated by *X. umbellata*.

3.5.4 Conclusions and ecological significance

No changes to the morphology or physiology of the soft coral *X. umbellata* were observed following 20 mg C L^{-1} of DOM enrichment. This opposes findings of former works of DOM-enrichment on hard corals, where DOM accelerated the growth of microbes within the coral's mucopolysaccharide layer, disrupting the coral's microbiome, causing bleaching and mortality (Kline et al., 2006; Kuntz et al., 2005). We argue that *X. umbellata* was not affected in this way, as it perhaps took up the glucose more efficiently than hard corals via heterotrophic feeding, thereby reducing the pool of DOM available for microbial overgrowth and maintaining a balance between the coral and its associated microbiota. Conversely, significant changes in the morphology and physiology of *X. umbellata* were observed following POM enrichment. In both POM treatments, disfigured tentacles, mortality, and reduced pulsation rates were observed. We attribute these effects to mechanical damage of the delicate feeding apparatus unique to octocorals (Szmant-Froelich et al., 1982), thereby reducing capture of heterotrophic food sources and lowering gas exchange by reduced pulsation and photosynthesis, resulting in nutrient and energy depletion. Furthermore, we observed overlapping effects of both small (phytoplankton) and large (zooplankton) particle size classes on *X. umbellata*, with damaged tentacles, mortality, and reduced pulsation and photosynthesis observed in both treatments. However, larger particles exacerbated these effects with more extensive tentacle damage and darkening in colouration observed. We therefore demonstrate that large particles have more abrasive effects on the tissue of *X. umbellata* due to their larger surface and higher encounter rates. Yet, we argue that the dose of POM plays a larger role than particle size in the morphological and physiological effect of POM on *X. umbellata*. It must be acknowledged however, that our experiment was conducted under laboratory conditions, using laboratory grown coral colonies. Whilst every effort was made to simulate natural conditions, *in situ* research will invariably offer a more accurate representation of natural responses.

Numerous former studies have justified the widespread presence of *X. umbellata* by demonstrating its ability to persist in the face of various common stressors, including

fluctuating water flow conditions (Hill et al., 2023) ocean acidification (Tilstra et al., 2023) inorganic eutrophication (Klinke et al., 2022; Mezger et al., 2022; Thobor et al., 2022) and organic eutrophication in dissolved forms (Simancas-Giraldo et al., 2021; Vollstedt et al., 2020). However, here we demonstrate that *X. umbellata* is not invincible, and vulnerable to local eutrophication, where POM blooms of phyto- and zooplankton accumulate in excess. With increasing coastal development, eutrophication events are occurring more frequently. Management strategies should therefore focus on reducing the run-off of inorganic nutrients from land, which will prevent excessive growth of POM, thereby conserving *X. umbellata*.

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3.7 Authors contributions

C. E.L. Hill: data analysis, visualisation, writing – original draft, writing – review and editing. **J. Rücker:** data collection, writing – review and editing. **N. Eichhorn:** data collection, writing – review and editing. **S. D. Mezger:** data collection, writing- review and editing, supervision. **G. Caporale:** writing – review and editing. **Y. C. El-Khaled:** writing – review and editing. **S. V Blanco C.:** data collection, writing – review and editing. **C. Wild:** writing: review and editing, conceptualisation, supervision, funding. **A. Tilstra:** writing – review and editing, conceptualisation, supervision.

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Chapter 4 | Environmental factors and host physiology drive annual denitrification rates in zooxanthellate and azooxanthellate Red Sea corals

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4.1 Abstract

Nitrogen (N) is fundamental for biomass production in coral reefs, although an N-limited state is crucial for corals to maintain symbiosis with their algal symbionts. Coral-associated denitrifying microbes may help manage excess N, though knowledge about denitrification in corals remains scarce. We thus assessed the temporal dynamics of denitrification over one year in four Red Sea corals including both zooxanthellate and azooxanthellate corals, representing varied trophic strategies. Using acetylene assays, we determined denitrification rates and quantified physiological coral traits and key environmental parameters. Average denitrification rates of the azooxanthellate coral *Tubastrea coccinea* were 19-fold higher than *Stylophora pistillata*, 12-fold higher than *Acropora* spp., and 7-fold higher than *Millepora dichotoma*. Temporal variation was observed among *Acropora* spp., *M. dichotoma* and *T. coccinea*, with generally higher rates in April – August, compared to October – February. Random forest analysis elucidated the six top drivers of denitrification per species. High ammonium availability was the primary driver for *Acropora* spp. (explaining 11% of denitrifying variability), high temperature up to ~ 32°C for *M. dichotoma* (explaining 11%), and low nitrate availability for *T. coccinea* (explaining 17%). However, no denitrifying variation for *S. pistillata* was explained by the measured parameters. Despite species-specific primary drivers, several factors like DOC levels, water chlorophyll-*a*, respiration rates and temperature consistently emerged among the top six drivers across species, indicating considerable overlap in secondary influences. Our findings demonstrate that denitrifiers can utilise environmental carbon rather than solely relying on carbon from symbionts. High denitrification rates in more heterotrophic corals suggest they may cycle nutrients more efficiently and better handle nutrient pollution than more autotrophic species. Species-specific dependencies on environmental conditions may shift the competitive landscape of corals under global change scenarios, benefitting some while disadvantaging others. Denitrification may therefore be an important - yet overlooked - physiological process that plays a role in species' vulnerabilities in the future ocean.

Keywords

Heterotrophy, Nutrient cycling, Anthropogenic impacts, Symbiosis, Biogeochemistry

4.2 Introduction

Nitrogen (N) is essential for corals, supporting protein synthesis, reproduction and photosynthetic efficiency (Babbin et al., 2021). Yet, corals occupy and thrive in oligotrophic environments that have a low availability of N. To sustain their productivity in these environments, corals employ a multifaceted approach to efficiently acquire, process and retain N. They can satisfy much of their N demand through heterotrophic feeding on N-rich prey and particulate organic matter, if available (Houlbrèque & Ferrier-Pagès, 2009). Corals exist as holobionts, living in association with microorganisms such as bacteria, viruses and many other organismal entities (Robbins et al., 2019; Voolstra et al., 2021). Diazotrophic bacteria form part of this intricate microbial community, playing a crucial role in N-fixation and contributing to the coral's N budget. Specifically, diazotrophic bacteria convert atmospheric N_2 into bioavailable ammonium (NH_4^+) that can be used by the coral (Cardini et al., 2015; Lesser et al., 2007; Rädecker et al., 2014; Shashar et al., 1994). In addition, some coral species, termed as zooxanthellate, host symbiotic dinoflagellates from the family Symbiodiniaceae that live in symbiosis with the coral host (Falkowski et al., 1984). The symbionts are capable of taking up nitrate, a process that the coral host itself cannot perform directly as it lacks the appropriate enzymes (Grover et al., 2003; Miller & Yellowlees, 1989). The symbionts supply the coral host with photosynthates containing both carbon (C) and N, albeit with a low N content (Falkowski et al., 1984). The symbionts also recycle metabolic waste products from the host, such as ammonium (Rahav et al., 1989), converting these into amino acids and other nitrogenous compounds that are partially translocated to the coral host (Reynaud et al., 2009; Wang & Douglas, 1999). In contrast, azooxanthellate corals do not host Symbiodiniaceae (Dawson, 2002) and therefore rely solely on heterotrophic feeding and N-fixation for their N supply, without the added benefit of symbiotic N assimilation.

While N is essential for coral health, excess N can have harmful effects (Zhao et al., 2021). Directly, it can reduce calcification and growth of hard corals (Silbiger et al., 2018) and disrupt the coral-algal symbiosis of zooxanthellate species (Rädecker et al., 2015). When more in hospite N is available, the algal symbionts allocate more C to their own growth rather than to the coral host, causing the symbionts to proliferate and become parasitic, which can lead to the onset of coral bleaching (Baker et al., 2018; Cunning & Baker, 2013; Ezzat et al., 2015; Krueger et al., 2020; Marubini & Davies, 1996; Muscatine, 1990; Wooldridge, 2017).

Additionally, excess N, without equivocally high phosphate concentrations, can lead to phosphate starvation of the symbionts (Wiedenmann et al., 2013). The symbionts need phosphate to bolster the stability of their chloroplasts, which serve as their photosynthetic machinery. Under high N:P, the chloroplasts must replace phospholipids within sulpholipids in the thylakoid membranes of the chloroplasts, making them more susceptible to light and heat induced bleaching (Wiedenmann et al., 2013). Indirectly, excess N stimulates pathogenic viruses and bacteria (Lesser et al., 2007; Vega Thurber et al., 2014), promotes macroalgae and phytoplankton growth that competes with corals (D'Angelo & Wiedenmann, 2014; De'ath & Fabricius, 2010), and favours fast-growing species over slow-growing hard corals (Birkeland, 1977). These shifts can reduce overall biodiversity in the long term (Duprey et al., 2016).The effects vary by form of DIN, for example urea-exposed coral recover faster than those exposed to excess nitrate (Burkepile et al., 2020). Additionally, excess ammonium may have mixed effects, offering potential benefits to photosynthesis and calcification of corals at moderate concentrations, yet becoming toxic in higher concentrations (Hoegh-Guldberg, 1994). Conversely, NO_3 may negatively affect both photosynthesis and calcification processes (Fernandes De Barros Marangoni et al., 2020; Shantz & Burkepile, 2014; Zhao et al., 2021).

Environmental N availability often fluctuates naturally, via seasonal cycles (Cardini et al., 2016; Roth et al., 2021; Tilstra et al., 2017), yet has increased exponentially since the industrial revolution via global anthropogenic inputs of many forms (Penuelas et al., 2020). One proposed mechanism by which corals mitigate excess N is the process of denitrification (El-Khaled et al., 2020; Rädecker et al., 2015; Tilstra et al., 2019). Denitrification is a microbial process where nitrate (NO_3^-) is sequentially reduced to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and eventually to dinitrogen gas (N_2) that is released into the atmosphere (Knowles, 1982). Whilst denitrification has been extensively studied in fields such as agricultural and freshwater science (Barton et al., 1999; Malique et al., 2019; Philippot et al., 2007; Pina-Ochoa & Álvarez-Cobelas, 2006; Seitzinger et al., 2006) and even in some marine ecosystems such as seagrasses (Eyre et al., 2016; Garcias-Bonet et al., 2018), its role within coral reef ecosystems remains relatively underexplored. However, this process has received increased attention in recent years, and preliminary insights into denitrification in coral reefs are now emerging. For example, studies have recently revealed that denitrification is an active pathway in multiple Red Sea coral holobionts including *Acropora hemprichii*, *Millepora dichotoma*, *Pleuractis granulosa* (Tilstra et al., 2019), *Stylophora pistillata*, *Pocillopora verrucosa* and among soft corals (El-Khaled, Roth, et al., 2021). Furthermore, denitrification is also an active

pathway among several benthic reef substrates such as coral rubble, biogenic rock, turf algae and reef sediment (El-Khaled et al., 2020; El-Khaled et al., 2021). Yet, these studies both revealed that there are apparent susbstrate and coral species-specific differences in denitrification activity. In addition, Tilstra et al., (2019) demonstrated that denitrification rates and N_2 fixation rates both correlated with algal symbiont density and with each other. Authors therefore speculated that as denitrifiers and diazotrophs are both heterotrophic bacteria (Li et al., 2018), the correlation between these two pathways could be attributed to a shared supply of organic C from the algal symbionts. However, in general, the significance of denitrification in N removal in hard corals is debated, with estimates ranging from limited importance (Glaze et al., 2022) to accounting for ~ 90 % of N_2 production in hard corals (Yang et al., 2024).

Significant knowledge gaps still need to be addressed in the study of coral-associated denitrification. Although previous studies established that denitrification occurs in several Red Sea coral species, these studies measured denitrification 'potential' (El-Khaled, Roth, et al., 2021; Tilstra et al., 2019). This is because activity was inferred using gene copy numbers as a proxy, or denitrification rates were measured after the addition of nitrate which stimulates activity of the denitrification pathway. Therefore, the actual denitrification activity of these corals under natural conditions has not yet been quantified. In addition, the factors driving the observed differences in denitrification between coral species and benthic reef substrates remain unclear, as do the broader mechanisms that drive denitrification overall, including the relative influence of external environmental conditions (i.e., temperature and C and N availability) and host physiology. Furthermore, whilst previous studies have speculated that denitrification might be connected to the heterotrophic capacity of the coral host (El-Khaled et al., 2020; Tilstra et al., 2019), hypothesising that denitrifiers may derive their C from algal symbionts, there has yet to be a direct assessment of denitrification across different corals with varying heterotrophic capacities.

Considering these knowledge gaps, we asked three key questions *i)* How do denitrification rates differ among four zooxanthellate and azooxanthellate skeleton-forming corals over a year? *ii)* What is the relative influence of external environmental and internal physiological traits on coral-associated denitrification rates? And lastly, iii) How does the host heterotrophic capacity influence coral denitrification rates? To assess this, we selected a suite of Red Sea corals that, according to literature, differ in their trophic capacity. We included zooxanthellate corals that have a low heterotrophic capacity, such as *Stylophora pistillata*, *Acropora* spp. and

Millepora dichotoma, and an azooxanthellate coral *Tubastrea coccinea* that is fully heterotrophic (Conti-Jerpe et al., 2020; Creed et al., 2017; Einbinder et al., 2009; Imbs et al., 2020). We sampled these corals bimonthly over a complete year and measured denitrification rates, assessed various physiological parameters and monitored environmental conditions. We hypothesised that denitrification rates among the four coral species assessed in our study would differ, as interspecific variation has been observed between corals in the past (Tilstra et al., 2019). We also expected to see variable denitrification throughout the year, with potentially higher rates exhibited in warmer months as found with other N-cycling pathways (Cardini et al., 2016), and in months with greater nitrate availability (Tilstra et al., 2021). We anticipated that higher denitrification would be found in zooxanthellate corals with a lower heterotrophic capacity that primarily derive C from their algal symbionts, as former research has hypothesised that denitrification of the holobiont may be limited by the photosynthates released from the coral's algal symbionts (Tilstra et al., 2019). Lastly, we hypothesised that both internal physiological traits and external environmental conditions would both influence denitrification rates. In asking these questions, we aim to elucidate the key drivers of denitrification within corals, clarifying the interplay between environmental conditions and the intrinsic physiological characteristics of corals that underpin N-cycling and contribute to the overall health and stability of coral reef ecosystems. This knowledge is crucial for comprehending both the natural dynamics of denitrification and the potential impacts of environmental stressors, such as ocean warming and eutrophication, on microbial community structure and function. Furthermore, the findings will shed light on species-specific differences in denitrification and enhance our understanding of how particular species may withstand global changes.

4.3 Methodology

4.3.1 Collection of corals and water samples

Coral collections were carried out in the central Red Sea at the "Al Fahal Reef", or also known as "The Coral Probiotics Village" (22.30518N, 38.96468E), a mid-shore reef located 15 km offshore from the King Abdullah University of Science and Technology (KAUST), Saudi Arabia (Garcias-Bonet et al., 2024). The area of collections is shallow, with a maximum water depth of 10 m. Four species of Red Sea corals that theoretically differ in their trophic capacity were identified, including three zooxanthellate species *S. pistillata, Acropora* spp., and *M.*

dichotoma that exhibit mixotrophic feeding and an azooxanthellate species *T. coccinea* that has a fully heterotrophic lifestyle (Figure 4.1) (Conti-Jerpe et al., 2020; Creed et al., 2017; Einbinder et al., 2009; Imbs et al., 2020). Five separate colonies (n = 5) of each species were sampled using SCUBA between 5 - 10 m depth, every second month over a one-year timespan, generating six timepoints i.e., April 2022, June 2022, August 2022, October 2022, December 2022, and February 2023. Sampling was consistently carried out in the first two weeks of every sampling month, with *M. dichotoma* and *Acropora* spp. sampled in the first week, and *S. pistillata* and *T. coccinea* sampled in the second week. From each colony, three fragments were cut using pliers and placed into labelled sampling bags, filled with seawater. Out of the three fragments, two were used for incubations (~ 5 cm length) and one was used for physiological assessments (~ 5 cm length). In the case of *T. coccinea*, colonies were too small to sample multiple fragments from, so instead, 15 polyps were sampled bimonthly. On the boat, the fragments to be used in incubations were stored in recirculation aquaria filled with seawater from the sampling site, each being equipped with an air pump to maintain water circulation and oxygen availability. All aquaria were placed in the shade to prevent heat/light stress during transport. The fragments for physiological assessments were kept on ice and later stored at -20 °C in the lab.

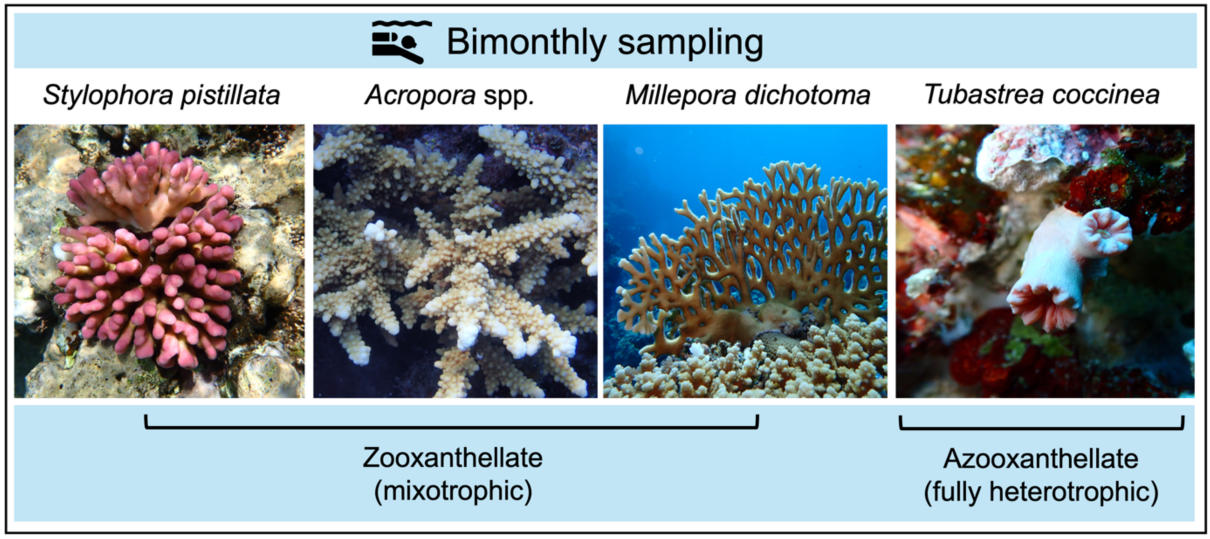

Figure 4.1. Photographs of the zooxanthellate and azooxanthellate coral species included within this study of varying heterotrophic capacities. Images were taken at the sampling site, courtesy of Vivian Bonacker.

4.3.2 Assessment of coral-associated denitrification and other physiological traits

Oxygen fluxes

Approximately 3 hours after corals were sampled from the field, oxygen fluxes were measured via a beaker incubation technique (Bednarz et al., 2012; Herndl & Velimirov, 1986). Corals were secured to stands using rubber bands and placed inside a gas-tight glass beaker. The beaker was filled with seawater that was collected from the sampling site the same morning and closed underwater to exclude air bubbles. The seawater was pre-heated (in a thermal cabinet) to match the *in situ* seawater temperature of the respective sampling month. The same temperature was maintained throughout the incubation by placing the beaker in a water bath heated accordingly. The water bath was placed on top of a magnetic stirring plate that powered stir bars within each beaker at ~ 220 rpm, ensuring adequate water circulation and homogenous oxygen concentrations throughout the beaker. Corals were incubated for ~ 75 minutes in the dark (respiration) and in the light (net photosynthesis). Before and after each incubation, the oxygen concentration of the water in each beaker was recorded using an optode sensor. Two beakers containing the same seawater, but without corals were included within the incubations to account for the planktonic background, i.e., respiration or photosynthesis of microorganisms in the water, serving as controls. The initial oxygen reading was subtracted from the final oxygen reading to assess respiration rates from the dark incubation and net photosynthesis from the light incubation. Data was normalised to the incubation time (h) and the oxygen fluxes of the planktonic background were subtracted. Data was further normalised to the beaker volume in litres (v) and to the surface area of each colony in cm^{-2} (s) determined via a wax-dipping technique (Veal et al., 2010). Thereafter, net photosynthesis (P_{net}) from the light incubation and respiration (R) from the dark incubation were used to calculate gross photosynthesis (P_{gross}) (Equation 2).

Equation 1

$$P_{net}\ or\ R = \frac{\frac{(oxy_{(light\ or\ dark)})}{h} - \frac{(control_{(light\ or\ dark)})}{h}}{v * s}$$

Equation 2

$$P_{gross} = P_{net} + |R|$$

Denitrification rates

On the same day as sampling, following oxygen flux incubations, denitrification rates were determined via acetylene blockage/inhibition assays. This method has been successfully applied to investigate coral reef associated denitrification activities (El-Khaled et al., 2020a). Acetylene blocks the activity of the enzyme nitrous oxide (N_2O) reductase within the denitrification pathway, leading to the accumulation of N_2O which can be used as a proxy for the activity of denitrification of the coral holobiont (Balderston et al., 1976; El-Khaled et al., 2020a; Fedorova et al., 1973; Yoshinari & Knowles, 1976).

Acetylene gas (1 L) and acetylene-enriched seawater (1 L) were freshly generated for use in the assays the day prior. In brief, 12.5 g of calcium carbide (CaC_2) was reacted with 100 ml of MilliQ water in an Erlenmeyer flask sealed with a rubber stopper. The acetylene gas generated from the reaction, passed via silicone tubing into the first Duran gas washing bottle filled with 1 L of MilliQ water, and then subsequently passed into the second 1 L Duran gas washing bottle filled with 1 L of seawater. Remaining gas then passed via silicone tubing into a gas collection bag (Tedlar 1 L sampling bags with polypropylene valve, RESTEK). The 1 L of acetylene-enriched seawater and 1 L of acetylene gas were used to set up the acetylene assays.

Corals were secured to stands using rubber bands and placed inside a gas-tight glass beaker (Figure S4.1a). Beakers were specially adapted for acetylene assays by having an 8 mm hole drilled into the lid. The hole was sealed with a gas-tight rubber stopper, and a hypodermic needle (hypodermic needle with polypropylene hub 30G x 3/4", Tyco Healthcare group, MonojectTM) was permanently inserted through the stopper into the jar. On the outside of the jar, the needle hub was attached to a 2-way stopcock with a luer lock connection (two-way stopcock, BraunTM DiscofixTM) where a gas syringe (50 ml gastight syringe model 1050 TLL PTFE Luer Lock, Hamilton) could be later fitted when required, for gas samples to be taken (Figure S4.1a). Each beaker was filled with seawater (taken from the sampling site the same morning) to 80 % of its capacity, leaving a 20 % headspace. Ten percent of the seawater volume was replaced with acetylene enriched seawater, and likewise, 10% of the beaker headspace was

replaced with acetylene gas (Yoshinari & Knowles, 1976) (Figure S4.1a). The beakers were placed into water baths that were equipped with thermostats (3613 aquarium heater. 75W 220-240 V; EHEIM GmbH and Co.KG) and temperature controllers (Schego Temperature Controller TRD, max. 1000W) and heated to the same temperature as the *in situ* conditions of the respective sampling month (Figure S4.1b). The water bath was placed on top of a magnetic stirring plate that operated magnetic stir bars (at ~ 220 rpm) within each beaker. This ensured that there was sufficient water circulation for the corals throughout the incubation. Corals were incubated in this setup for 12 hours in the light, with light supplied at the same intensity as the in situ conditions of the respective sampling month. Following the light incubation, the acetylene assay was set up from scratch with new coral fragments for a 12 hour dark incubation. New fragments were used to minimise the potential impact of stress on the coral's denitrification rates. Four control beakers containing no corals were included in each incubation run to account for potential background denitrification activity in the seawater. Gas samples (3 ml) were taken from the beaker headspace at the beginning (T0) and end (T12) of each incubation, using a gas syringe (50 ml gastight syringe model 1050 TLL PTFE Luer Lock, Hamilton) and stored in gas tight vials until further measurement.

Gas samples generated from the acetylene assays were measured using a N_2O microsensor (custom-made, Unisense), connected to a multi-channel (fx-6 UniAmp multi-channel 110394, Unisense) (Figure S4.1c). The microsensor (electrochemical) sensor was capable of detecting N_2O in a nanomolar range (detection limit 25 nM). It consisted of a Clark-type microelectrode with a tip size of 50 µm (N2O-NP-804195, Unisense A/S, Aarhus, Denmark) connected to a high-sensitivity picoampermeter (PA 2000, Unisense A/S, Aarhus, Denmark). A voltage of -0.8 V was applied between the cathode and the internal reference anode, whilst N_2O was driven by the external partial pressure to pass through the sensor membrane at the tip (silicone membrane), then it was reduced at the metal cathode surface, while the picoampermeter converted the resulting current to a signal representing the N_2O concentration. Prior to any measurement, the sensor was pre-activated by applying a voltage of -1.3 V for 30 minutes and pre-polarized at -0.8 V for 12 hours until the signal became stable. The sensor was equipped with a front guard made of an ascorbate solution to scavenge incoming O_2 that may interfere with the measuring cathode. The microsensor was calibrated every day prior to usage by a two-point calibration curve consisting of a low point (ambient air: 0.009 µmol/L) and a high point (a known standard:0.575 µmol/L) at a consistent room temperature (21 °C) and pressure (1 bar). To record and visualise measurements, the N_2O microsensor was synced with the Sensor

Trace Suite software (v.1.13) on a computer desktop. To minimise the signal to noise ratio, the N_2O microsensor was fixed in a stable and stationary position using a clamp. The septa of the gas vials were individually pierced by the microsensor, and once no drift in the sesnsor signal was observed, 5 replicate readings were recorded in mV and were automatically converted into µmol/L using the calibration curve. Between each sample, the microsensor tip was rinsed with MiliQ water and dried off (Kimwipes, Kimtech Science). Following this, there was a waiting period of variable length (~ 5 – 15 minutes) while the signal re-stabilised and showed no drift, before the next sample was measured. Gas measurements were subsequently normalised to account for the solubility of N_2O in the milliQ water of the storage vial and seawater of the beaker, and additionally the volume of the beaker (See supplementary). Following this, T0 values were subtracted from T12 values to obtain values of N_2O per 12h. Then the average of five controls were subtracted, to account for background N_2O production potentially from microorganisms in the water. As a next step, values were normalised to surface area (cm^{-2}), which was determined in advance by wax dipping the skeleton of the fragment, post-incubation. Values were then converted to N, by multiplying by two. Next, values from the separate light and dark incubations were added together to generate a rate of N_2O production per day (24h). Lastly, values were converted from µmol to nmol. We therefore ended up with denitrification rates expressed as nmol cm^{-2} d^{-1}.

Symbiont measurements

Firstly, coral tissue was removed from the coral skeleton. To do so, the fragment was held within a sterile clear sampling bag (Whirl-pack sample bag), and an airbrush (model S68 with dual action siphon feed, Master Airbrush) removed the tissue with high pressure air and milliQ water. The airbrush was sterilised with 70 % ethanol and rinsed with milliQ water between samples. Tissue slurries were stored in falcon tubes at -20 °C and defrosted for further analyses when needed. The coral skeletons were kept for surface area determination with the wax-dipping technique (Veal et al., 2010).

For symbiont density determination, a 100 µl aliquot of tissue slurry was centrifuged at 8000 rpm for 5 minutes at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of milliQ water, and vortexed until a homogenous solution was obtained. The sample was then passed through a cell strainer (5 ml polystyrene round-bottom tube with cell strainer cap

25 x 75 mm, Falcon, 30 µm mesh size) in order to remove larger cells/debris. Lastly, 200 µl of sample was pipetted into a 96-well plate, in triplicates. This was repeated for all samples, and each well plate included a positive and negative control. Samples were analysed on a flow cytometer (BD LSRFortessa, Franklin Lakes, New Jersey), with 50 µl of sample read at a 1 µl/s^{-1}flow rate. Values were subsequently normalised to the surface area (cm^{-2}).

For symbiont chlorophyll-*a* determination, all steps were carried out in a dark room to prevent potential light-induced degradation of the chlorophyll. A 500 µl aliquot of tissue slurry was pipetted into a 2 ml Eppendorf tube, and centrifuged at 5000 rpm for 5 minutes at 4 °C. The supernatant was discarded and 2 ml of 90 % acetone was added. Samples were stored for 24 hours at 4 °C. Following this, 200 µl of sample was pipetted into a 96-well plate in duplicates. This was repeated for all samples, and on each plate 2 wells contained 200 µl of pure 90 % acetone, serving as 'blanks'. The well plates were read on a spectrophotometer plate reader (insert brand) at wavelengths of 630 nm and 663 nm. Absorbance readings of the samples were normalised by subtracting the average of the 'blanks', and equations by Jeffrey & Humphrey (1975) were used to calculate the chlorophyll-a and chlorophyll-c concentrations (Equations 3 & 4). The path length of the well plate was also considered, so values were divided by 0.555 (Krueger, 2020). Lastly, data was further normalised according to dilutions and the surface area of the respective fragment.

Equation 3

$$chl\ a\ (\mu g\ ml^{-1}) = 11.47 \times E_{664} - 0.40 \times E_{630}$$

Equation 4

$$chl\ c\ (\mu g\ ml^{-1}) = 24.36 \times E_{630} - 3.73 \times E_{664}$$

Elemental analysis of carbon and nitrogen

Tissue slurry was obtained, via the same method as described in section 3.2.3. The slurry was aliquoted into 3 ml Eppendorf tubes. Firstly, the host fraction was isolated by centrifuging the slurry for 2.5 minutes at 500 x g. The supernatant was then transferred into a new Eppendorf tube (the host fraction), and the pellet in the original Eppendorf tube was kept (symbiont

fraction). The symbiont pellet was resuspended in 3 ml of milliQ and vortexed for 5 – 10 seconds. The new solution was then centrifuged at 400 x g for 2 minutes. The supernatant was removed and discarded. To wash the pellet and remove contaminants, the pellet was resuspended again, vortexed and centrifuged as above. The supernatant was removed, and the pellet was resuspended for the final time, serving as the algal fraction. Both fractions were vortexed to ensure a homogenous solution and 3 ml of each fraction was pipetted onto separate GF/F filters that were fitted to a vacuum filtration assembly. Next, 2 ml of 1 N HCl was pipetted on top of the filter to dissolve any skeleton contaminants. Following this, 2 ml of MilliQ water was pipetted on top of the filter to rinse away the acid. Filters were then placed into sterile and pre-labelled falcon tubes and placed inside a 60 °C drying oven, with the lids off. Samples remained in the drying oven for 48 hours. Tubes were then closed within the oven to avoid entry of moisture into the tubes. On a sterile work surface, the dried mass was scraped off the filter surface into a tin cup. The tin cups were closed using sterile tweezers and weighed before they were placed into a 96-well plate for transportation to the processing facility. Samples were analysed for carbon and nitrogen content, at the Natural History Museum, Berlin with a Flash 1112 EA coupled to a Delta V IRMS via a Conflow lV-interface (Thermo Scientific). Further detail can be found in (Karcher et al., 2020).

4.3.3 Monitoring and measurement of environmental parameters

Nutrients

All glass and plastic sampling equipment, including syringes, beakers, bottles and tubes were acid-washed 24 hours prior to usage, by soaking them in a 4 % hydrochloric acid (HCl) bath. This was a precautionary step to minimise and prevent chances of contaminants or residues from reducing the accuracy of the nutrient measurements. This was especially critical for DOC sampling, where chances of C contamination are high. Twice per sampling month, 2 x 5 L Niskin bottles were used to sample water directly from the average study site depth (8 m). Water was emptied from the Niskin bottles on the boat and subsamples were prepared accordingly for inorganic nutrients (silica, nitrite, nitrate, phosphate), ammonium, dissolved organic carbon (DOC) and chlorophyll-*a*. On the boat, water for inorganic nutrient analysis was passed through 0.22 µM Millex®-GV filters (PVDF Membrane, Merck Millipore Ltd.,

Ireland) into falcon tubes. For the remaining chemical parameters, unfiltered water was directly transferred into containers or tubes, with opaque bottles used for chlorophyll-*a* samples to prevent light-induced degradation of chlorophyll. All water samples were kept on ice during transport on the boat, and in the lab, samples for inorganic nutrient analysis and ammonium were stored at -20 °C, while samples for a chlorophyll-*a* and DOC were stored at + 4 °C until processing.

For inorganics analysis, samples were analysed with a segmented flow analyser (Model AA3 HR, SEAL Analytical IC.) with the following detection limits: phosphate 0.01052, silicates 0.083222, nitrite 0.0217 and nitrate 0.0322 μmol L^{-1}. For ammonium analysis, sampled were analysed using a fluorometer (Turner Designs, Trilogy), with a detection limit of 0.058 μmol L^{-1}.

For DOC analysis, samples were processed within one day of collection. A volume of 500 ml was passed through 0.2 μm GF/F filters (pre-combusted at 450 °C for 4.5 h) affixed to a polycarbonate filter holder. Filtered water was then dispensed into 5 x 40 ml sterile amber glass vials. To inhibit bacterial activity and its associated DOC consumption, each subsample was spiked with 0.1 ml of 85% phosphoric acid (H_3PO_4). Samples were then analysed on a TOC Analyser (TOC-L, Total Organic Carbon Analyser, Shimadzu, Kyoto, Japan).

On the same day as collection, water samples for chlorophyll-*a* (2 L in duplicates) were each passed through GF/F filters, and the filters were stored at -80 °C until further processing. In brief, each filter was placed into a 15 ml falcon tube and immersed in 10 ml of 90 % acetone. Each tube was vortexed for 1 min, sonicated for 10 min in an ice bath and vortexed again for another 30 s. Tubes were then covered in foil to prevent light penetration and stored at 4 °C overnight. The following morning, sonication and vortexing steps were repeated twice more, after which samples were centrifuged at 2500 rpm at 4 °C for 10 min. Once samples had been brought to room temperature, a 2 ml subsample was transferred to a glass cuvette and measured on a spectrophotometer (Turner Design Trilogy fluorometer, San Jose, CA, USA) both before and after the addition of 5 % HCl. The difference between these readings was then calculated as the concentration of chlorophyll-*a* per sample. Solid standards were run before any measurements were taken, and “blanks” of 90 % acetone were run at the beginning, and after every few samples.

Temperature

Seawater temperature was measured continuously throughout the year using Onset Hobo pendant temperature loggers. Four loggers were deployed on the reef, at 5 – 10 m depth and exchanged at every bimonthly sampling.

4.3.4 Data analyses

The software R (version 4.3.2) (R Core Team, 2023) was used to generate figures using packages 'ggplot2' (Wickham, 2016), 'ggpubr' (Kassambara, 2020), 'dplyr' (Wickham et al., 2018), 'RColorBrewer' (Neuwirth, 2014), 'gridExtra' (Auguie & Antonov, 2017), 'cowplot' (Wilke, 2015). Likewise, statistics were also computed in R, using packages 'rstatix' (Kassambara, 2023), 'dunn.test' (Dinno, 2024), 'randomForest' (Liaw & Wiener, 2002) and 'pdp' (Greenwell, 2017). All data was tested for normality via Shapiro-wilk tests, and for homogeneity of variances via the Levene's test. No data was normally distributed, even following transformation, and therefore non-parametric alternatives were used. For example, the temporal dynamics of denitrification when comparing *i)* rates of same species between different months, and *ii)* rates of different species within the same month were assessed using a Kruskal Wallis rank-sum test, followed by a Dunn's test with Bonferroni adjustment for post-hoc analysis. Additionally, we employed a random forest model to identify key environmental and physiological variables influencing denitrification rates across different coral species. The random forest regression model comprised of 500 trees, with 3 variables tried at each split. The variable importance for predicting denitrification was determined by the mean squared error (%MSE), where a high %MSE indicates that the variable is important, as if it were removed, the model's error would significantly increase. Random forest models were run twice on each species. For the first round, we incorporated all the measured environmental and physiological parameters into the model, to identify the top 6 features that are most influential over denitrification. For the second round, we incorporated only these top 6 features, to reduce noise and thereby improve the efficacy of the model. The second round affirmed the order of contribution of these top 6 features in explaining denitrification variance. As a follow-up to the random forest analysis, we plotted partial dependence plots (PDPs) for the top 3 most influential parameters per species. This provided further insight into how these parameters interacted with denitrification.

4.4 Results

Denitrification was detected in all four coral species. *S. pistillata* ranged from 0 – 11.5 nmol N cm^{-2} d^{-1}, *Acropora* spp from 0 - 7.8 nmol N cm^{-2} d^{-1}, *M. dichotoma* from 0 – 6.1 nmol N cm^{-2} d^{-1}, and *T. coccinea* from 0 – 29.1 nmol N cm^{-2} d^{-1}.

4.4.1 Temporal variation within each species

Denitrification rates across coral species showed varying temporal patterns, with some exhibiting significant fluctuations throughout the year. Denitrification rates of *S. pistillata* did not significantly vary over the year (Kruskal-Wallis test, $\chi^2 = 10.725$, df = 5, $p > 0.05$; Figure 4.2a). Denitrification rates of *Acropora* spp. varied significantly over the year (Kruskal-Wallis test, $\chi^2 = 20.905$, df = 5, $p < 0.001$; Figure 4.2b), with significantly higher rates in June (4.1± 0.4 nmol N cm^{-2} d^{-1}) and August (4.8 ± 1.3 nmol N cm^{-2} d^{-1}) compared to October (0.2 ± 0.2 nmol N cm^{-2} d^{-1}) and December (0 ± 0 nmol N cm^{-2} d^{-1}) (Dunn's test, $p < 0.05$). Denitrification rates of *M. dichotoma* also varied significantly over the year (Kruskal-Wallis test, $\chi^2 = 14.513$, df = 5, $p < 0.05$; Figure 4.2c), with significantly higher rates in June (4.4 ± 0.6 nmol N cm^{-2} d^{-1}) compared to August (0.2 ± 0.2 nmol N cm^{-2} d^{-1}) (Dunn's test, $p < 0.05$). Denitrification rates of *T. coccinea* varied significantly over the year (Kruskal-Wallis test, $\chi^2 = 19.651$, df = 5, $p < 0.05$; Figure 4.2d), with significantly higher rates in April (20.5 ± 2.1 nmol N cm^{-2} d^{-1}) and June (16.7 ± 4.5 nmol N cm^{-2} d^{-1}) compared to December (0 ± 0 nmol N cm^{-2} d^{-1}).

4.4.2 Intra-species comparisons

In April, denitrification rates significantly varied among species (Kruskal-Wallis test, $\chi^2 = 11.36$, df = 3, $p < 0.05$; Figure 4.2), as *T. coccinea* (20.5 ± 2.06 nmol N cm^{-2} d^{-1}) had significantly higher denitrification rates than all other species (Dunn's test, $p < 0.05$) including *S. pistillata* (2.2 ± 1.3 nmol N cm^{-2} d^{-1}), *Acropora* spp. (1.2 ± 0.7 nmol N cm^{-2} d^{-1}) and *M. dichotoma* (1.0 ± 0.6 nmol N cm^{-2} d^{-1}).In June, denitrification rates significantly varied among species (Kruskal-Wallis test, $\chi^2 = 8.55$, df = 3, $p < 0.05$; Figure 4.2), as *T. coccinea* (16.7 ± 4.5 nmol N cm^{-2} d^{-1}) also had significantly higher denitrification rates than all others species (Dunn's test, $p < 0.05$) including *S. pistillata* (5.0 ± 2.3 nmol N cm^{-2} d^{-1}), *Acropora* spp. (4.1

± 0.4 nmol N cm^{-2} d^{-1}) and *M. dichotoma* (4.4 ± 0.6 nmol N cm^{-2} d^{-1}). In August, denitrification rates significantly varied among species (Kruskal-Wallis test, χ^2 = 11.77, df = 3, $p < 0.05$; Figure 4.2), as *Acropora* spp. (4.8 ± 1.3 nmol N cm^{-2} d^{-1}) had significantly higher denitrification rates than all other species (Dunn's test, $p < 0.05$) including *S. pistillata* (0.2 ± 0.2 nmol N cm^{-2} d^{-1}), *M. dichotoma* (0.2 ± 0.2 nmol N cm^{-2} d^{-1}) and *T. coccinea* (1.4 ± 0.9 nmol N cm^{-2} d^{-1}). In October, there was no significant variation among species (Kruskal-Wallis test, χ^2 = 9.22, df = 3, $p > 0.05$; Figure 4.2).In December, denitrification rates significantly varied among species (Kruskal-Wallis test, χ^2 = 9.98, df = 3, $p < 0.05$; Figure 4.2), as *M. dichotoma* (1.7 ± 0.8 nmol N cm^{-2} d^{-1}) had significantly higher denitrification than all other species (Dunn's test, $p < 0.05$) including *S. pistillata* (0 ± 0 nmol N cm^{-2} d^{-1}), *Acropora* spp. (0 ± 0 nmol N cm^{-2} d^{-1}), and *T. coccinea* (0 ± 0 nmol N cm^{-2} d^{-1}). Lastly, in February, there was no significant variation among species (Kruskal-Wallis test, χ^2 = 11.77, df = 3, $p > 0.05$; Figure 4.2).

4.4.3 The influence of host heterotrophic capacity on coral denitrification rates

Averaged over the year, denitrification rates of *T. coccinea* were 19-fold higher than *S. pistillata*, 12-fold higher than *Acropora* spp., and 7-fold higher than *M. dichotoma*. Zooxanthellate species *S. pistillata*, *Acropora* spp. and *M. dichotoma* exhibited rates within a similar range to one another, as mentioned above.

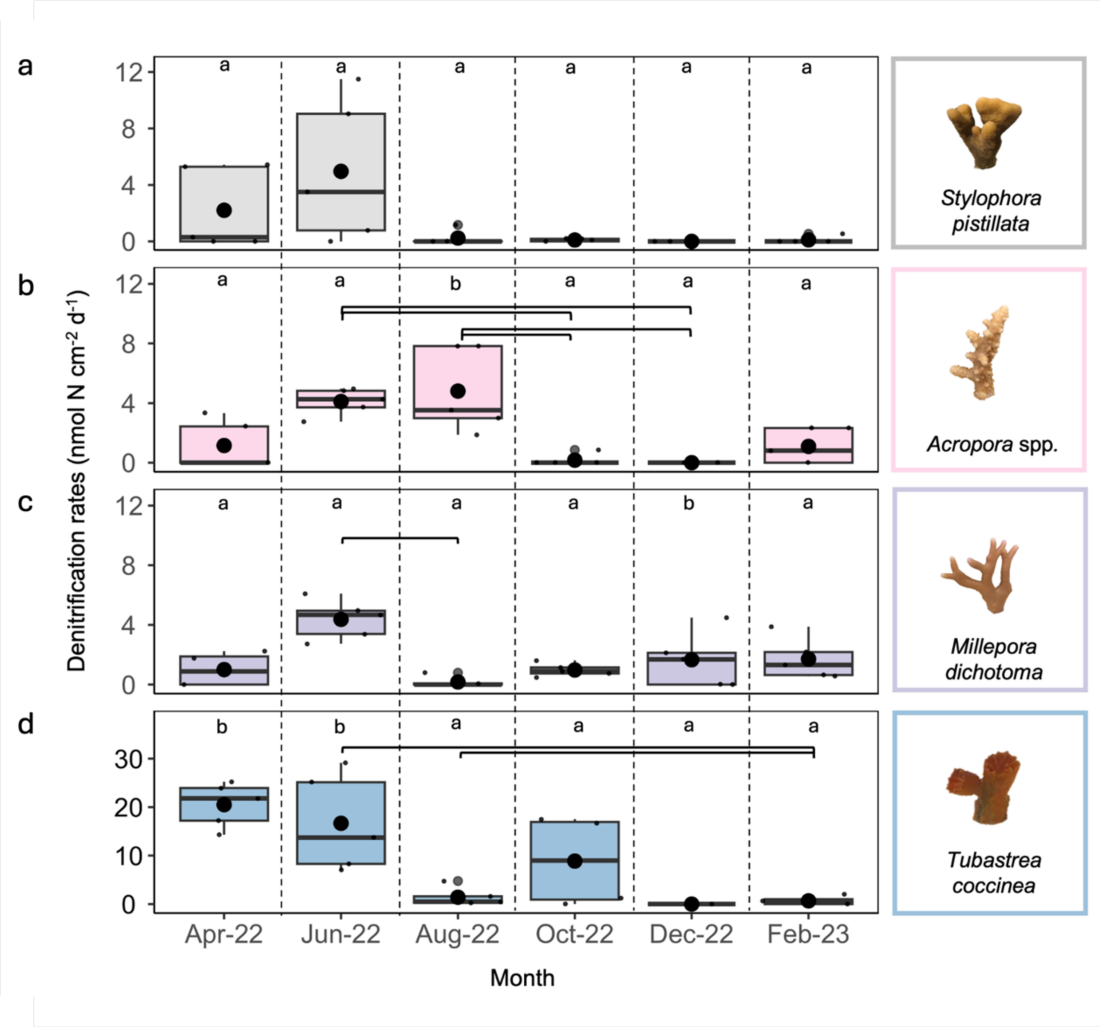

Figure 4.2 A – D] Denitrification rates of zooxanthellate and azooxanthellate over the course of a year. Brackets indicate significant differences between timepoints throughout the year of the same species. Different letters denote significant differences between different species of the same sampling month (visible within the dotted line columns), while the same letter signifies no significant differences. Panels A – C all have the same y axis scales from 0 – 12 nmol N cm^{-2} d^{-1}, yet panel D has a larger scale of 0 – 30 nmol N cm^{-2} d^{-1}.

4.4.4 Natural environmental change throughout the year

Dissolved inorganic nitrogen (DIN) significantly varied throughout the year (ANOVA, $F = 26.29$, $df = 5$, $p < 0.001$; Figure 4.3). Specifically, DIN levels in April (0.4 ± 0.03 µmol L^{-1}) were significantly lower than all other months of the year (Tukey test; $p < 0.05$), with the highest levels observed in December (2.91 ± 0.26 µmol L^{-1}) (Tukey test; $p < 0.05$). Nitrite significantly varied throughout the year (ANOVA, $F = 24.91$, $df = 5$, $p < 0.001$; Figure 4.3), with the lowest levels observed in April (0.02 ± 0.0021 µmol L^{-1}) (Tukey test; $p < 0.05$), and

highest levels observed in August (0.069 ± 0.005 μmol L^{-1}) (Tukey test; $p < 0.05$). Nitrate also significantly varied throughout the year (ANOVA, $F = 43.34$, $df = 5$, $p < 0.001$; Figure 4.3), with lowest levels in April (0.23 ± 0.02 μmol L^{-1}) (Tukey test; $p < 0.05$) and highest levels in December (1.28 ± 0.05 μmol L^{-1}) (Tukey test; $p < 0.05$). Significant variability in annual ammonium levels were observed throughout the year (ANOVA, $F = 10.9$, $df = 5$, $p < 0.001$; Figure 4.3), with lowest levels in April (0.15 ± 0.02 μmol L^{-1}) and June (0.62 ± 0.12 μmol L^{-1}) (Tukey test; $p < 0.05$), and highest levels in December (1.57 ± 0.25 μmol L^{-1}) (Tukey test; $p < 0.05$). Significant changes in water chlorophyll-*a* levels were observed throughout the year (ANOVA, $F = 8.114$, $df = 5$, $p < 0.001$; Figure 4.3), with levels peaking in August (0.37 ± 0.00 μmol L^{-1}) and October (0.43 ± 0.10 μmol L^{-1}) (Tukey test; $p < 0.05$), and lowest levels observed in April (0.12 ± 0.00 μmol L^{-1}), June (0.22 ± 0.01 μmol L^{-1}), December (0.19 ± 0.02 μmol L^{-1}) and February (0.07 ± 0.00 μmol L^{-1}) (Tukey test; $p < 0.05$). Dissolved organic carbon (DOC) levels also showed marked variation throughout the year (ANOVA, $F = 17.63$, $df = 5$, $p < 0.001$; Figure 4.3), with highest levels found in April (139.15 ± 11.43 μmol L^{-1}), followed by June (107.81 ± 1.79 μmol L^{-1}) (Tukey test; $p < 0.05$) and lowest levels in August (74.95 ± 5.38 μmol L^{-1}) and February (75.99 ± 1.34 μmol L^{-1}) (Tukey test; $p < 0.05$). As expected, temperature also exhibited significant variation throughout the year (ANOVA, $F = 433.6$, $df = 5$, $p < 0.001$; Figure 4.3), with each month exhibiting a significantly different temperature

(Tukey test; $p < 0.05$).

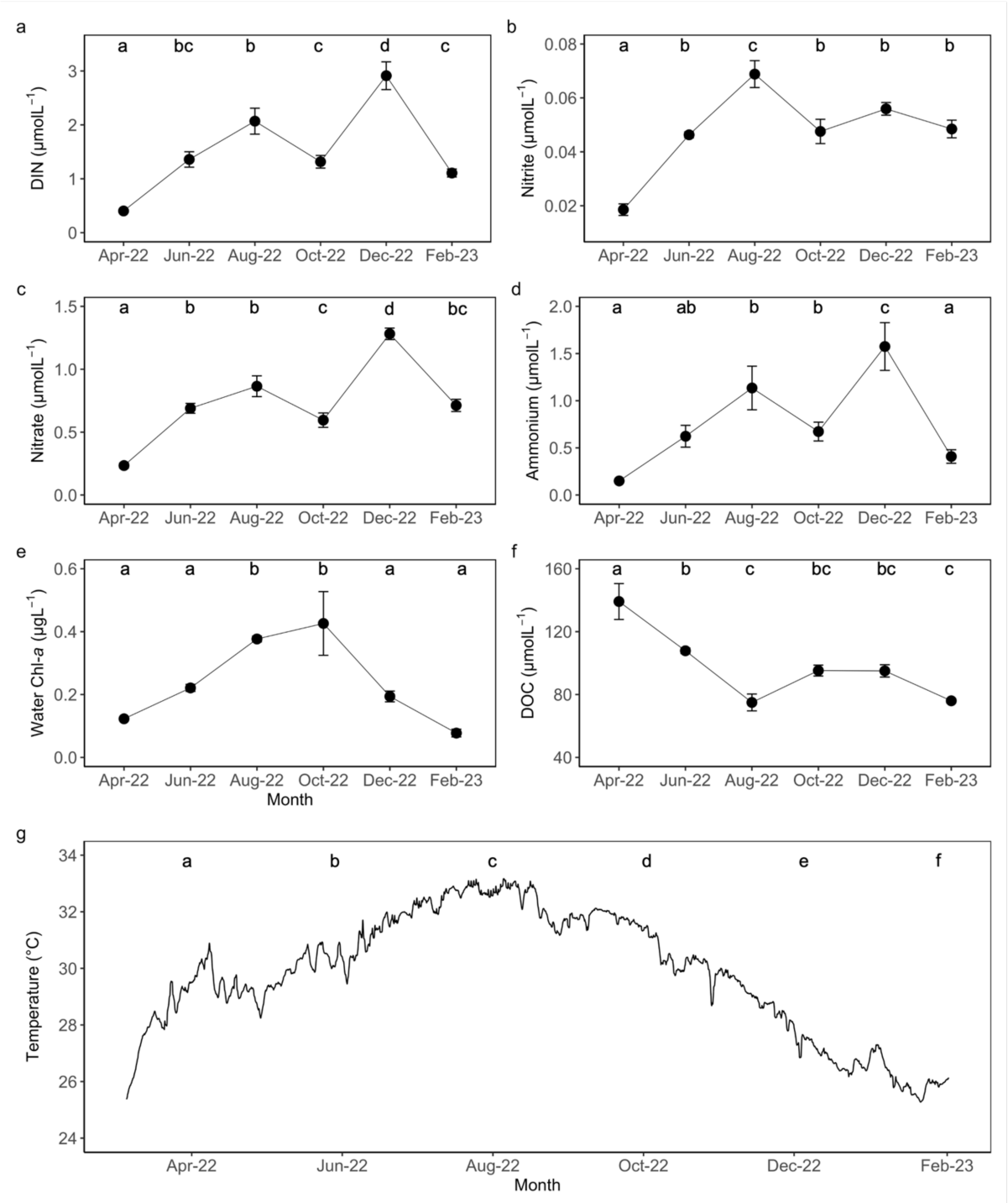

Figure 4.3 *In situ* environmental parameters monitored throughout the sampling year from April 2022 to February 2023, including average values ± standard error of a) Dissolved inorganic nitrogen (DIN), b) nitrite, c) nitrate, d) ammonium e) Water chlorophyll-*a*, f) DOC and a smooth averaged of g) temperature. Letters are used to denote statistical significance, with the same letter indicating no significant difference and different letters indicating a significant difference.

4.4.5 Influence of environmental and physiological factors on denitrification rates

Environmental parameters (nitrate, ammonium, DOC, water chlorophyll-a, and temperature) as well as physiological parameters (symbiont density, symbiont chlorophyll-a, C:N, photosynthesis and respiration) were fed into a random forest model for each species, to determine the most influential features affecting denitrification. The parameters explained varying degrees of denitrification across coral species. In *Acropora* spp, *M. dichotoma* and *T. coccinea*, they explained 51%, 49% and 56% respectively, while for *S. pistillata* they only explained 3% of denitrifying variation. Therefore for *S. pistillata*, we cannot provide any influential features.

For *Acropora* spp., ammonium, respiration, temperature, DOC, water chlorophyll-*a* and gross photosynthesis were identified as the six most influential factors affecting denitrification rates (random forest analysis, Figure 4.4a). Out of the 51% of explained variation, ammonium availability was the strongest predictor, explaining 11% of denitrifying variation, with higher levels promoting increased denitrification (Figure 4.4b). Secondly, increased respiration rates also enhanced denitrification, with a notable increase when respiration rates exceeded - 0.5 μmol O_2 cm^{-2} h^{-1}, explaining 11% of relative denitrifying variation (Figure 4.4c). Elevated temperatures were also associated with higher denitrification rates, explaining 8% of denitrifying variation (Figure 4.4d).

For *M. dichotoma*, temperature, water chlorophyll-*a* concentration, respiration rates, DOC, symbiont total chlorophyll and photosynthesis rates were the six most influential factors affecting denitrification rates (random forest analysis, Figure 4.4e). Out of the 49% of explained denitrifying variation, Temperature was the most influential feature, explaining 11% of the variation. Temperature showed a unimodal relationship, with denitrification increasing as temperature rose to ~ 30 °C, after which it began to decline (Figure 4.4f). Secondly, water chlorophyll-*a* concentration was the second most influential factor, accounting for 9% of variation in denitrification. It demonstrated a unimodal relationship, with denitrification increasing up to a chlorophyll-*a* concentration of 0.2 mg L^{-1} , beyond which it decreased (Figure 4.4g). Respiration rate was the third most influential factor over denitrification rates. Like the pattern observed in *Acropor*a spp., denitrification sharply increased once respiration rates exceeded approximately - 0.5 μmol O_2 cm^{-2} h^{-1} (Figure 4.4h).

Nitrate, DOC, ammonium, respiration rates, water chlorophyll-*a* and temperature had the greatest influence over denitrification rates in *T. coccinea* (random forest analysis, Figure 4.4i). Out of the 56% of variation explained, low nitrate availability had the most influence over denitrification rates, accounting for 17% of denitrifying variation (Figure 4.4j). Secondly, high DOC availability was the second most influential factor over denitrification rates, explaining 12% of variation (Figure 4.4k). Lastly, high ammonium availability ranked third, accounting for 8% of observed variation (Figure 4.4l).

All data parameters that have been fed into the random forest model (e.g., symbiont density and chlorophyll-*a*, elemental C and N ratios, and oxygen fluxes) but not graphically displayed in the result section, are graphically displayed in the supplementary material (See supplementary).

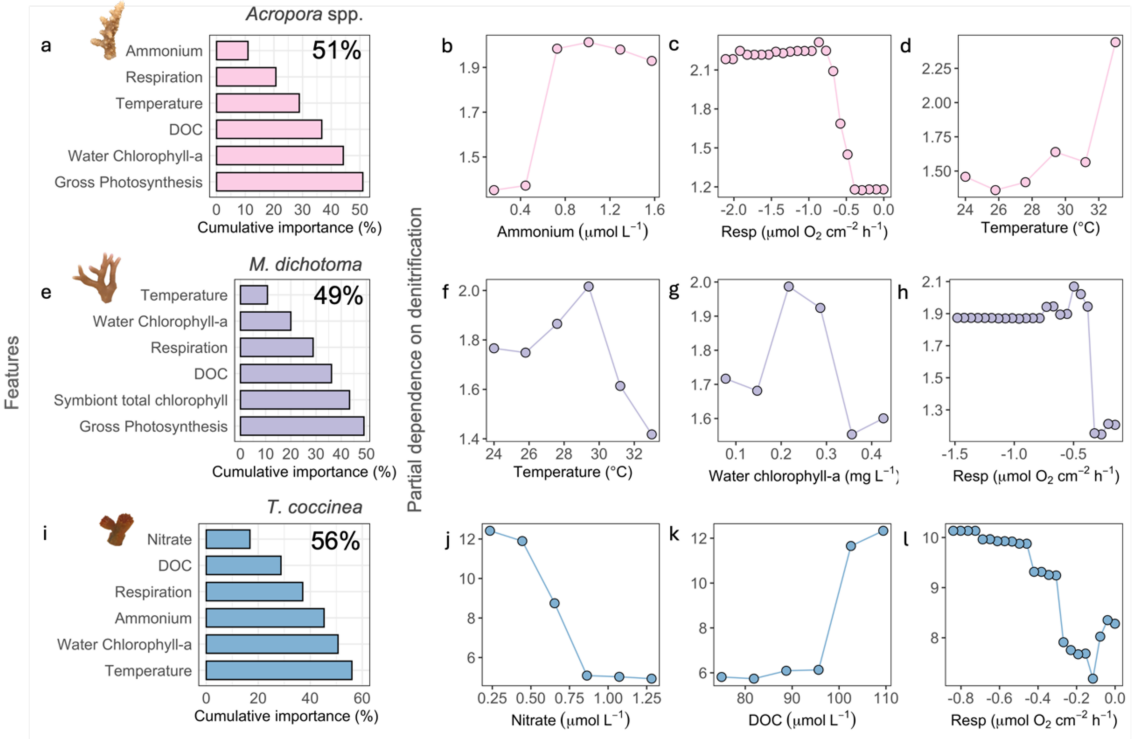

Figure 4.4, Each row represents a separate species, i.e., a – d is *Acropora* spp., e – h is *Millepora dichotoma* and i – l is *Tubastrea coccinea*. In each row, the left bar plot (a, e, i) depicts the top 6 most influential features on denitrification rates, identified by random forest analysis. The percentage in the top right corner of each bar plot indicates the proportion of denitrifying variation that these top 6 features explain. Of this percentage, the cumulative importance of each feature is plotted. In each row there are also 3 partial dependence plots (PDPs) that illustrate the nature of the relationship between the

denitrification and its top three most influential features. *Stylophora pistillata* is missing from this figure as random forest analysis was inffective for this species. Abbreviations include 'DOC' = dissolved organic carbon and 'Resp' = respiration rates.

4.5 Discussion

Our study uncovered several important findings regarding coral associated denitrification. Firstly, we observed significant temporal variation in denitrification rates in all corals except for *S. pistillata,* with generally higher rates in the spring/summer than in the autumn/winter. *Tubastrea coccinea* stood out from the other species, exhibiting denitrification rates 19, 12 and 7-fold higher than *S. pistillata*, *Acropora* spp., and *M. dichotoma,* respectively. Environmental and physiological parameters explained approximately 50% of the variation in denitrification rates for *Acropora* spp,, *M. dichotoma* and *T. coccinea*, but had no explanatory power for *S. pistillata*. While each coral had a unique primary driver, there was considerable overlap among the top six drivers, demonstrating common secondary influencing factors among species.

4.5.1 How do denitrification rates compare to those of other studies?

Former studies that have quantified denitrification in corals have either used acetylene assays and measured the accumulation of N_2O as a proxy for denitrification or used isotope tracer techniques where either NO_2^- or the sum of NO, N_2O and N_2 is used to determine denitrification rates (Table 1). To facilitate more effective comparisons, we have focused solely on rates derived from acetylene assays and involving the same species. Tilstra et al., (2019) previously assessed rates of denitrification via acetylene assays in November 2017, where they quantified rates of two of the same corals as included within our study, namely *Acropora* spp. and *M. dichotoma*. For *Acropora* spp., Tilstra et al., (2019) measured average denitrification rates of 0.38 ± 0.13 nmol N cm^{-2} d^{-1}. Whilst we did not measure denitrification rates in November, we measured rates in the neighbouring months of October and December. For *Acropora* spp., we measured rates of 0.17 ± 0.17 nmol N cm^{-2} d^{-1} in October and 0 nmol N cm^{-2} d^{-1} in December. Therefore, the denitrification rates of our study were overall lower than those measured by Tilstra et al., (2019) A key difference between our studies that may explain this discrepancy is our use of ambient seawater in the acetylene assays, compared to Tilstra et al's., (2019) addition of ~ 3 µmol L^{-1} of DIN to stimulate denitrification. Therefore, the lower availability of DIN in our study may have resulted in lower rates of denitrification in *Acropora* spp. than in past studies. Tilstra et al., (2019) also quantified denitrification of *M. dichotoma*. In November

2017, they measured rates of 0.17 ± 0.10 nmol N cm^{-2} d^{-1}. In our study, we measured higher rates of denitrification at 0.97 ± 0.19 nmol N cm^{-2} d^{-1} in October and 1.66 ± 0.83 nmol N cm^{-2} d^{-1} in December. The higher rates in our study may be due to methodological differences in the normalisation of denitrification rates. For example, our study utilised the wax dipping technique to determine the surface area of fragments, while Tilstra et al., (2019) utilised a modelling software. The variation in surface area methodologies may result in differing surface area estimates, complicating cross-method comparisons. In March 2018, El-Khaled et al., (2021) quantified denitrification of *S. pistillata* with rates of 0.01 ± 0.006 nmol N cm^{-2} d^{-1}. Whilst we did not quantify denitrification in March, we quantified the neighbouring months of February (0.1 ± 0.1 nmol N cm^{-2} d^{-1}) and April (2.2 ± 1.3 nmol N cm^{-2} d^{-1}). The denitrification rates measured within our study are higher than those measured by El-Khaled et al., (2021). This could also be due to differences in the surface area methodology which may lead to differences in the calculated rates of denitrification. It is also worth noting that our study did not measure denitrification in the same month as any of the former studies, making it difficult to draw true comparisons. Indeed, a limitation of our study is that denitrification rates were quantified only within the first two weeks of each bimonthly sampling timepoint. Therefore, our data has limited temporal resolution that may not capture the full effect of denitrification variability throughout the year. We therefore would recommend future work to include a finer temporal scale for this reason.

Table 2 A summary of the denitrification rates of coral-associated denitrification.

Study	**Coral species**	**Denitrification rate converted to nmol N d^{-1} cm^{-2}**	**Method**	**Incubation time**	**Location**	**Date**
Tilstra et al. 2019	*Acropora hemprichii* *Millepora dichotoma* *Pleuractis granulosa*	0.38 ± 0.1 0.17 ± 0.10 0.05 ± 0.02	Combined blockage/reduction acetylene assay + nitrate addition [N_2O accumulation]	24 h 12 h light + 12 h dark	Central Red Sea	Nov 2017
El-Khaled et al. 2020	*Pocillopora verrucosa*	0.04 ± 0.01 (ambient) 0.20 ± 0.06 (eutrophic)	Combined blockage/reduction acetylene assay [N_2O accumulation]	24 h 12 h light + 12 h dark	Central Red Sea	March 2018

El-Khaled et al. 2021	*Pocillopora verrucosa* *Acropora hemprichii* *Stylophora pistillata*	0.09 ± 0.02 0.38 ± 0.1 0.01 ± 0.006	Combined blockage/reduction acetylene assay + nitrate addition [N_2O accumulation]	24 h 12 h light + 12 h dark	Central Red Sea	March 2018
Babbin et al. 2021	*Porites atreoides* *Porites porites* *Orbicella faveolata* *Diploria labrynthiformis* *Acropora palmata*	0.43 1.99 2.35 1.33 0.05	^{15}N incubation experiments [NO_3 -> NO_2^-]	24 h 12 h light + 12 h dark	Cuba, Caribbean	Nov 2017
	Porites astreoides *Porites porites* *Orbicella faveolata* *Diploria labrynthiformis* *Acropora palmata*	2.49 4.10 0.78 2.37 0.05	^{15}N incubation experiments [NO_3 -> NO_2^-]	24h dark	Cuba, Caribbean	Nov 2017
Glaze et al. 2021	*Acropora grandis* *Acropora pulchra* *Montipora digitata* *Porites cylindrica* *Isopora elizabethensis* *Isopora cuneata*	0.08 0 0.06 0.0942 0 0.0148	15N incubation experiments [NO_3^- $\rightarrow NO+N_2O+N_2$]	9 h dark	Great Barrier Reef, Australia	Nov 2017
	Acropora grandis	0 0.0923	15N incubation experiments	5 h light	Great Barrier Reef, Australia	Nov 2017

	Acropora pulchra *Montipora digitata* *Porites cylindrica* *Isopora elizabethensis* *Isopora cuneata*	0 0.0499 0 0.1477	[NO_3^- $\rightarrow NO+N_2O+N_2$]			

4.5.2 How do denitrification rates differ among four skeleton-forming corals over a year?

S. pistillata did not vary significantly throughout the year (Figure 4.2a). However, for *Acropora*, spp., *M. dichotoma* and *T. coccinea*, denitrification rates were generally higher in April – August (spring/summer) than in October – February (autumn/winter) (Figure 4.2b – d). The absence of temporal variation in the denitrification rates of *S. pistillata* is not entirely unexpected, as previous studies indicated that *S. pistillata* physiology remained relatively stable throughout the year, with no significant changes in protein content or skeletal density over time (Rich, 2022). However, for *Acropora*, spp., *M. dichotoma* and *T. coccinea*, it is likely that the observed temporal variation in denitrification was driven by differing sensitivities to environmental and physiological factors (Figure 4.4).

4.5.3 What is the relative influence of environmental and physiological factors on denitrification?

Nitrate is essential to the denitrification process, acting as an electron acceptor for denitrifying bacteria, which sequentially reduces it to dinitrogen gas. Consequently, it is logical that the more available nitrate, the higher the denitrification rates. However, the opposite relationship was apparent in our study, with denitrification rates enhanced by low nitrate availability (Figure 4.4j). A study by El-Khaled et al., (2020) demonstrated that nitrogen cycling processes are more nuanced, showing that multiple N-cycling processes (including opposing pathways like N_2 fixation and denitrification) can be simultaneously active. Therefore, given the oligotrophic

nature of the Red Sea with nitrate concentrations remaining low year-round (0.2 – 1.3 µmol L^{-1}), it could be that N_2 fixation increased in response to low environmental N availability, thereby increasing denitrification as well. It is possible therefore, that denitrification only becomes the dominant pathway at higher nitrate concentrations, while below this threshold, denitrification may co-occur with N_2 fixation, as previously found for other reef substrates (El-Khaled et al., 2021). Future work should therefore focus on determining the nitrate threshold at which these two key N-cycling pathways transition.

Temperature was the top driver of denitrification in *M. dichotoma* and the third most influential factor over denitrification in *Acropora* spp. This is in line with expectations as higher temperatures are well-known to accelerate microbial metabolism and activity (Jørgensen, 2000). More specifically, in seagrass sediments of the Red Sea, denitrification rates have shown a linear increase with temperature (Garcias-Bonet et al., 2018). Additionally, elevated temperatures have been found to increase the activity of diazotrophs that govern N_2 fixation (Bednarz et al., 2015). However, while the denitrification rates of *Acropora* spp. continued to increase into the peak of summer (~ 32 °C), *M. dichotoma* had an upper limit where temperatures beyond 30 °C caused a decline in denitrification, indicating that denitrifiers of *M. dichotoma* may have a lower thermal threshold than that of *Acropora* spp. In previous research, *M. dichotoma* has responded negatively to heat stress, with reduced photo physiological performance when exposed to high temperatures of 34 °C (Banc-Prandi et al., 2022). A recent study by Rädecker et al.(2021) demonstrated that under heat stress, the coral catabolises amino acids, causing an increase in available N, which causes symbionts to proliferate and eventually results in bleaching. However, an increased availability of N may increase denitrification rates. Yet, *M. dichotoma's* sensitivity to higher temperatures may limit the capacity for denitrification to potentially aid in reducing N and maintaining N-limitation for the health of the coral holobiont.

High ammonium availability (Figure 4.4a) was the main driver of denitrification in *Acropora* spp., but not for the other corals (Figure 4.4e & i). Ammonium serves as a substrate for nitrification, where it is sequentially oxidised to nitrite and then nitrate (Wafar et al., 1990). The nitrate produced from this process may then fuel denitrification, explaining the link between high ammonium and increased denitrification. It is possible that *Acropora* spp. hosts more nitrifying microbes than the other corals, allowing it to utilise ammonium more efficiently

for nitrogen cycling. Nitrification has been documented in *Acropora grandis* and *Acropora pulchra* (Glaze et al., 2022), however further research is needed to compare the nitrifying communities of *Acropora* spp. with those of the other corals in our study.

Although high respiration was not identified as the top driver of denitrification for any corals in our study, it was the only parameter that consistently ranked among the top three drivers for all corals analysed using random forest. Although respiration rates are a physiological process carried out by the coral holobiont, they can serve as a proxy for local scale oxygen availability. For instance, higher respiration rates lead to higher oxygen consumption, leading to decreased oxygen availability. Denitrifiers are mostly facultative aerobic heterotrophs (Fernández et al., 2009), capable of switching from oxygen to nitrate as an electron acceptor under anoxic conditions. Therefore, in oxygen-poor environments, denitrifiers are more efficient at denitrification because they rely on nitrate as their electron accepter rather than oxygen, thus driving denitrification and removing N from the system. Other studies corroborate these findings, having also found increased denitrification of coral rubble, turf algae and stony corals when in the absence of light, when respiration rates exceed photosynthesis rates and oxygen availability decreases (Babbin et al., 2021;El-Khaledet al., 2021).

Finally, the water chlorophyll-*a* concentration was the second driver of denitrification in *M. dichotoma,* but did not rank as a top predictor for the other corals. It exhibited a unimodal relationship, where denitrification initially increased in tandem with chlorophyll-*a* content, but eventually became inhibited once the chlorophyll-*a* reached a certain threshold. This is consistent with the findings of previous work that found the same relationship (Zhang et al., 2024; Zhu et al., 2020). Chlorophyll-*a* can enhance denitrification by acting as a redox mediator, facilitating the electron transfer necessary for denitrifying bacteria to reduce nitrate (Lu et al., 2020; Van Der Zee & Cervantes, 2009).

It must be acknowledged, however, that the parameters assessed in this study could not explain all denitrifying variation observed. We must therefore carry out future research to elucidate the remaining drivers of denitrification in corals. Focus could be placed on environmental factors such as pH and salinity, and additional physiological factors such as calcification rates, tissue thickness and density and mucous production rates, as these could influence denitrification rates.

4.5.4 How does the heterotrophic capacity influence coral denitrification rates?

The denitrification rates of the three zooxanthellate corals were similar, with ranges of 0 – 11.5 nmol N cm^{-2} d^{-1} for *S. pistillata*, 0 - 7.8 nmol N cm^{-2} d^{-1} for *Acropora* spp., and 0 – 6.1 nmol N cm^{-2} d^{-1} for *M. dichotoma*. However, these rates were lower than those of the azooxanthellate coral *T. coccinea* (0 – 29.1 nmol N cm^{-2} d^{-1}), and unexpectedly, we observed that the azooxanthellate (and fully heterotrophic) coral *T. coccinea* exhibited an average of 19, 12 and 7-fold higher denitrification rates than *S. pistillata*, *Acropora* spp., and *M. dichotoma* respectively (Figure 4.2). This finding contradicts our hypothesis, in which we expected higher denitrification rates in zooxanthellate corals that are more autotrophic. We anticipated that denitrifiers would utilise the organic carbon (photosynthates) produced by the coral's algal symbionts as their energy source. This is because a previous study found a positive correlation between symbiont density and denitrification (Tilstra et al., 2019), and another found that the relative abundance of denitrifiers was significantly higher in photosymbiotic *Aiptasia* compared to their aposymbiotic counterparts (Xiang et al., 2022). This raises the question of where else denitrifiers are obtaining their carbon from. Given that denitrifiers in *T. coccinea* cannot acquire autotrophically derived C, they may be utilising environmentally derived C. Interestingly, we found that increased DOC availability was the second most influential factor over denitrification rates of *T. coccinea* (Figure 4.4i & 4.4k), and among the top 6 drivers of denitrification in *Acropora* spp. (Figure 4.4a) and *M.dichotoma* (Figure 4.4e), suggesting that DOC may be an important source of C for denitrifiers. While this positive relationship between DOC and denitrification is well-documented in other environments i.e., sediment and freshwater (Bernard-Jannin et al., 2017; Hill et al., 2000; Steinberg, 2013; Zhou et al., 2017), the role of DOC in coral associated denitrification has received less attention. However, a study on excess DOC (glucose) found that denitrifier abundance decreased by an order of magnitude in *Xenia umbellata* but remained unchanged in *Pinnigorgia flava*. (Xiang et al., 2022). If lower relative abundances mean lower denitrifying activity (Tilstra et al 2019), these findings stand in contrast to our study. This discrepancy may be due to differences in the DOC sources: Xiang et al. (2022)used glucose as the sole DOC source, whereas our study utilised seawater sourced from the reef that would have contained a suite of naturally occurring organic compounds. It has been shown that corals host unique denitrifying communities with varying preferences for C sources (Xiang et al., 2022). For example, some taxa like Rhodobacteraceae readily take up glucose (Alonso & Pernthaler, 2006), while others are inhibited by it (Allers et al., 2007). Thus,

it is possible that the corals in the prior experiment may not have favoured glucose, whereas the denitrifiers in the current study may have had access to a preferred carbon source to drive denitrification.

Another explanation for the high denitrification rates of *T. coccinea*, is that heterotrophic corals are known to harbour a greater overall bacterial diversity (Meistertzheim et al., 2016). Therefore, it is plausible that *T. coccinea* may host a broader range of denitrifying bacteria and thus be more capable of achieving higher overall denitrification rates than their zooxanthellate counterparts. However, an assessment of the bacterial community composition would be needed to validate this as an explanation. Furthermore, heterotrophic feeding can increase ammonium availability through metabolic waste products (Goldberg, 2018). This ammonium may then serve as a substrate for nitrification, increasing nitrate availability for denitrifiers (Wafar et al., 1990). The remarkably high denitrification rates of *T. coccinea*, raise the question of how N-limitation benefits its physiology. In zooxanthellate corals, N-limitation is critical for sustaining the coral-algal symbiosis (Rädecker et al., 2015). However, since *T. coccinea* lacks such a symbiotic relationship, its reliance on N-limitation remains unclear. It is possible that denitrification in this species is not a targeted mechanism to promote N-limitation for the coral's benefit, but rather a passive process activated by cues such as increased substrate availability from heterotrophic feeding, as mentioned above.

Overall, these findings provide evidence that dentirifiers can utilise a variety of forms of organic C for energy, enabling azooxanthellate corals to not only denitrify but even outperform those utilising autotrophically derived C. In addition, heterotrophic corals may achieve higher denitrification rates by hosting more diverse denitrifying or increasing ammonium availability via heterotrophic feeding, which enhances nitrification and nitrate availability for dentirifiers. However, our study included only one azooxanthellate species. Therefore, we recommend future studies to assess the denitrification rates of additional azooxanthellate species, as expanding the range of studied species will provide a more comprehensive understanding of how the heterotrophic capacity of the coral host influences denitrification rates.

4.5.4 Conclusions

In this study we sought to address fundamental gaps in the literature surrounding coral-associated denitrification. Using four Red Sea corals with varying heterotrophic capacities, we *i)* assessed the temporal dynamics of denitrification over a year, *iii)* identified key drivers of coral denitrification, and *iii)* investigated the influence of heterotrophic capacity over denitrification. We found that denitrification rates of *Acropora* spp., *M. dichotoma* and *T. coccinea* significantly varied throughout the year displaying a general trend of higher denitrification rates in spring/summer (April – August) and lower rates in autumn/winter (October – February). These trends can be explained by a suite of environmental and physiological factors that influence denitrification rates. Using random forest analysis, we identified the top six parameters that drive denitrification per coral species. Differing amounts of denitrifying variability could be explained per species, with parameters explaining 51% for *Acropora* spp., 49% for *M. dichotoma*, and 56% for *T. coccinea*. Unfortunately, no denitrifying variation for *S. pistillata* could be explained with the measured parameters in our study, as the random forest analysis was ineffective. We found that each coral had a unique primary factor that drove its denitrification rates. For example, *Acropora* spp. was primarily affected by high ammonium availability, *M. dichotoma* by elevated temperature (up to a threshold of ~ 32 °C), and *T. coccinea* by low nitrate availability. Although the primary driver was different per species, there was considerable overlap among the top six features identified for each species, with DOC levels, water chlorophyll-*a* concentrations, respiration rates and temperature consistently emerging as a influential factors for *Acropora* spp., *M. dichotoma* and *T. coccinea*. Overall findings demonstrated that both environmental conditions and physiological traits both influenced denitrification rates of corals. However, it is important to acknowledge that ~ 50% of denitrifying variation in these three corals, and 100% in *S. pistillata*, could not be explained by the measured parameters in our study, highlighting the need for further research to elucidate additional drivers of coral denitrification. Lastly, we discovered that denitrification rates increase with the heterotrophic capacity of the host. This was evidenced by 19, 12 and 7-fold higher denitrification rates measured in the azooxanthellate and fully heterotrophic coral *T. coccinea*, compared to *S. pistillata*, *Acropora* spp. and *M. dichotoma,* respectively. This finding demonstrates that denitrifiers utilise environmental C for denitrification (in the form of DOC) in addition to autotrophically-derived C as formerly hypothesised.

4.5.5 Ecological significance

Our findings enhance understanding of the basic functioning of coral-associated denitrification. The observed temporal variability of denitrification coupled with the influence of environmental factors over denitrification rates underscores the sensitivity of this process to environmental change. As global change accelerates, coral-associated denitrification faces significant vulnerability. For instance, the response of *M. dichotoma* to elevated temperatures suggests that rising sea temperatures could impair denitrification in this species, potentially reducing nutrient cycling and disrupting reef health by altering nutrient dynamics. Furthermore, the finding that denitrification rates increase with the host's heterotrophic capacity, emphasises that corals with higher heterotrophic capacities may be more efficient at nutrient recycling. This may have implications for the resilience of coral ecosystems under varying nutrient conditions, where corals with higher heterotrophic capacities may potentially better cope with nutrient pollution than those that are more autotrophic. However, further research is needed to ascertain whether additional azooxanthellate corals with a high heterotrophic capacity also exhibit high denitrification rates, as our study includes only one. Finally, despite species-specific primary drivers, several factors like DOC levels, water chlorophyll-*a*, respiration rates and temperature consistently emerged among the top six drivers across species, indicating considerable overlap in secondary influences. These findings suggest that management strategies should prioritise mitigating changes in these shared environmental factors to safeguard coral-associated denitrification processes. For instance, efforts to limit excessive DOC inputs from land-based runoff could help maintain balanced nutrient dynamics in reef ecosystems. Additionally, monitoring and controlling water chlorophyll-*a* levels- linked to phytoplankton abundance and eutrophication- can prevent nutrient imbalances that adversely affect dentification.

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4.7 Author contributions

C. E.L. Hill: data collection, data analysis, visualisation, writing – original draft, writing – review and editing. **A. Tilstra:** writing – review and editing, conceptualisation. **Y. C. El-Khaled:** data collection, writing – review and editing. **N. Garcias-Bonet:** data collection, writing- review and editing**. V. A. Bonacker:** data collection, data analysis, writing – review and editing. **A. Novoa Lamprea:** data collection, data analysis, writing – review and editing. **W. A. Rich:** data collection, data analysis, writing – review and editing. **M. Ostendarp:** data analysis, writing: review and editing. **M. D. Fox:** writing – review and editing, supervision. **S. Carvalho:** writing – review and editing, supervision, funding. **R. S. Peixoto:** writing – review and editing, supervision, funding. **C. Wild:** writing – review and editing, conceptualisation, supervision, funding.

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Chapter 5 | General discussion

5.1 Overview

C and N are essential for coral health, growth and energy production (Muscatine & Porter, 1977; Odum & Odum, 1955). Maintaining a balanced availability of these elements is critical, as both deficiencies and excesses can have negative consequences for coral survival (Kline et al., 2006; Rädecker et al., 2015). Understanding how corals regulate C and N availability is crucial for protecting reefs and predicting responses of corals to environmental threats such as nutrient pollution, which can disrupt this balance. This thesis aimed to deepen the understanding of how C and N availability affects the ecophysiology of key Red Sea coral taxa – both reef-forming hard corals and an abundant soft coral species - by addressing fundamental knowledge gaps. To achieve this, three projects were conducted as presented in the thesis data chapters 2 - 4. Chapter 2 was entitled "Physiology of the widespread soft coral *Xenia umbellata* is affected by food sources, but not by water flow", Chapter 3 was entitled "Excess particulate organic matter negatively affects the ecophysiology of the widespread soft coral *Xenia umbellata*" and Chapter 4 was entitled "Environmental factors and host physiology drive annual denitrification rates in zooxanthellate and azooxanthellate Red Sea corals". Each chapter targeted specific questions related to this overarching objective.

5.2 Summary of key findings per chapter. Abbreviations include DOM = dissolved organic matter, POM = particulate organic matter, DOC = dissolved organic carbon.

	Chapter 2	***Chapter 3***	***Chapter 4***
Research question	What are the feeding preferences of the soft coral *X. umbellata*? What are the effects of water flow on the feeding regime of *X. umbellata*?	What are the physiological effects of 20 mg C L^{-1} of dissolved and particulate forms (of varying particle sizes) of OM on the morphology and ecophysiology of *X. umbellata?*	How does denitrification differ among four hard skeleton-forming corals over a temporal scale of one year? How does the heterotrophic capacity of corals affect denitrification? What is the relative influence of external environmental and internal physiological traits on coral-associated denitrification?
Hypothesis	*X. umbellata* will exhibit a negative physiological response when heterotrophic feeding is inhibited, indicating a preference for mixotrophy. The highest water flow with food addition will yield the best physiological response in *X. umbellata*, promoting both autotrophy and heterotrophy to best meet metabolic needs.	The physiology of *X. umbellata* will be negatively impacted by POM but not by DOM, as excess POM at 20 mg C L^{-1} may harm delicate feeding structures and no longer offer heterotrophic benefits.	Denitrification rates will fluctuate between months, with higher rates during warmer temperatures and higher C and N availability. Denitrification rates will be higher in more autotrophic corals. Both internal physiological traits and external environmental conditions will influence denitrification rates.
Key findings	A lack of heterotrophic food sources negatively affected the physiology of *X. umbellata*, with reduced pulsation rates, symbiont density and mitotic index. The physiology of *X. umbellata* was unaffected by all water flow treatments. Phytoplankton was a more nutritious heterotrophic food source as it offers both C and N. *X.umbellata* displayed increased symbiont chlorophyll-*a* contents when no heterotrophic food sources were available.	Excess DOM had no physiological effects on *X. umbellata*, whereas excess POM caused damage to delicate feeding tentacles, a reduction in pulsation rate and mortality as heterotrophic feeding was impeded. Larger particles were more abrasive and had damaging effects on *X. umbellata.* Yet, the dose of POM eutrophication was more influential than particle size.	Temporal variation in denitrification rates were observed for *Acropora* spp., *M. dichotoma* and *T. coccinea.* Generally, rates were higher in April-August than in October-February. Higher denitrification rates were observed in azooxanthellate corals with a higher heterotrophic capacity. Both environmental and physiological factors influenced denitrification rates, with unique top drivers identified for each species.

5.3 Research question 1 | What are the ecophysiological responses of Red Sea corals to natural fluxes of C availability?

[addressed in chapter 2 and chapter 4]

To address this overarching question, chapter 2 examined the effect of three feeding treatments (no heterotrophic food, DOC and phytoplankton) crossed with four water flow regimes on the physiology of *X. umbellata*. Findings revealed that the physiology of *X. umbellata* was negatively impacted by the absence of heterotrophic food sources, with reduced pulsation rates, symbiont density and mitotic index, highlighting the importance of heterotrophic feeding in combination with autotrophy to meet its C demands. However, despite the overall decline in *X. umbellata's* physiological condition in the absence of heterotrophic food, *X. umbellata* also increased its symbiont chlorophyll-*a* content, likely as a compensatory mechanism to enhance autotrophic C acquisition. Furthermore, given that water flow can influence the uptake of C (Chang-Feng & Ming-Chao, 1993; Sebens, 1997; Sebens & Johnson, 1991), enhanced feeding was expected under high flow regimes. However, unexpectedly, there were no effects of water flow on any of the measured physiological parameters. This is likely due to *X. umbellata's* ability to regulate local water flow around its polyps through its continual pulsation behaviour, enabling it to consistently maintain optimal flow conditions. These findings highlight the ecophysiological strategies employed by *X. umbellata* to cope with natural variations in C availability, demonstrating the critical roles of heterotrophic feeding and local water flow regulation in meetings its metabolic demands.

Chapter 4 addressed this overarching question from a different perspective, investigating how a specific ecophysiological parameter -denitrification- is influenced by C availability across four Red Sea coral species. Specifically, the influence of host heterotrophic capacity (i.e., the ability to acquire C through the consumption of external OM, rather than relying on photosynthetically acquired C) on denitrification rates was investigated. This was carried out by examining the denitrification rates of both zooxanthellate (lower heterotrophic capacity) and azooxanthellate species (fully heterotrophic). Previous studies hypothesised that denitrifiers may utilise C from photosynthates as their main energy source, since finding positive correlations between denitrification with Symbiodiniaceae densities among three Red Sea corals (Tilstra et al., 2019). Therefore, we anticipated that zooxanthellate corals would

exhibit higher denitrification rates. However, surprisingly, findings revealed that average denitrification rates of the azooxanthellate (fully heterotrophic coral) *T. coccinea* were higher than its zooxanthellate counterparts, being 19-fold higher than *S. pistillata*, 12-fold higher than *Acropora* spp., and 7-fold higher than *M. dichotoma*. This demonstrated that denitrifiers do not solely utilise C from photosynthates, but also utilise environmental C, enabling azooxanthellate corals to not only denitrify but even outperform those utilising autotrophically derived C. In addition, the denitrification rates of all four corals were examined over a whole year to assess the influence of fluctuating environmental conditions on denitrification rates. Findings revealed that high DOC availability enhanced denitrification rates, being among the top six drivers of denitrification in *Acropora* spp., *M. dichotoma* and *T. coccinea*. Whilst this positive relationship between DOC and denitrification has been identified in other fields (Bernard-Jannin et al., 2017; Hill et al., 2000; Steinberg, 2013; Zhou et al., 2017), this is the first study to uncover the same relationship in corals. These findings provide critical insights into the ecophysiological responses of Red Sea corals to natural variations in C availability, highlighting how both autotrophic and heterotrophic pathways contribute to denitrification and how environmental C availability can play a pivotal role in shaping these processes.

5.4 Research question 2 | What are the ecophysiological responses of Red Sea corals to excess C availability under eutrophic conditions?

[addressed in chapter 3]

To address this question, the study presented in chapter 3 explored how *X. umbellata* responds to excess OM under eutrophic conditions. Corals were exposed to three treatments of excess OM at a concentration of 20 mg C L^{-1}, supplied in the form of DOM (glucose), and in the form of POM (phytoplankton and zooplankton). *X. umbellata* in the control and DOM treatments experienced no significant ecophysiological effects. However, both forms of POM caused significant harm, including damage to feeding tentacles, reduced pulsation rates and increased mortality due to impaired heterotrophic feeding. While larger particles were more abrasive, the severity of the effects was primarily influenced by the dose of POM rather than the particle size, as no negative physiological effects were observed when zooplankton was supplied as a feeding treatment at a lower/natural dosage in chapter 2. These findings highlight the sensitivity

of *X. umbellata* to excess particulate C, demonstrating that whilst *X. umbellata* may be tolerant to numerous environmental change scenarios (Klinke et al., 2022; Mezger et al., 2022; Thobor et al., 2022), it is vulnerable to increasing coastal eutrophication, where particulate OM accumulates.

5.5 Research question 3 | What are the ecophysiological responses of Red Sea corals to natural fluxes of N availability?

[addressed in chapter 4]

High N availability (in the form of ammonium) was identified as the top driver of denitrification in *Acropora* spp. Ammonium serves as a substrate for nitrification, where it is sequentially oxidised to nitrite and then nitrate (Wafar et al., 1990). Nitrate plays a critical role in the denitrification process, serving as the terminal electron acceptor for denitrifying bacteria, which sequentially reduce it to dinitrogen gas. Thus, it is generally accepted that higher nitrate availability leads to increased denitrification rates as shown in previous literature, explaining the link between high ammonium availability driving denitrification rates.

However, for *T. coccinea*, low nitrate availability was identified as the top driver of denitrification rates. Although unexpected, this finding aligns with insights from El-Khaled et al. (2020) who demonstrated that opposing N-cycling pathways such as N_2 fixation and denitrification can co-occur. Therefore, given the relatively oligotrophic nature of the Red Sea, with nitrate ranging from 0.2 – 1.3 µmol L^{-1} throughout the year, N_2 fixation may have increased in response to the low nitrate availability and denitrification may have co-occurred with it, thus explaining the elevated denitrification rates in response to low N availability. However, this only holds true when nitrate availability is low, as when nitrate is in excess, denitrification becomes the dominant pathway and increases as expected (El-Khaled et al., 2021).

These findings highlight the complexity of denitrification processes in Red Sea corals, showing that N availability influences denitrification in highly species-specific ways. Furthermore, these findings demonstrate that while ammonium and nitrate availability can drive

denitrification in some species, other factors such as host-specific physiological traits and other environmental conditions govern denitrification in others, as seen in *M. dichotoma*.

5.6 Limitations and future research directions

Whilst this thesis has answered novel research questions contributing towards the understanding of Red Sea coral physiology in response to C and N availability, there are several limitations of the work that deserve attention. Collectively, the findings of chapters 2 and 3 generate a baseline understanding of the physiological response of *X. umbellata* to limited, moderate and excess C availability. However, a restricted number of feeding treatments (chapter 2) and carbon enrichment treatments (chapter 3) were used due to logistical limitations. Therefore, to establish a more thorough understanding of the physiological response of *X. umbellata* to C availability, a broader range of POM particle sizes and a variety of DOM sugars (e.g., xylose, mannose, galactose) at multiple concentrations should be tested. Furthermore, although *X. umbellata's* health declined in the absence of heterotrophic food (chapter 2), it is unclear whether they are truly unable to sustain themselves via autotrophy alone, as the light intensity supplied throughout the experiment was at the lower end of their natural range. If replicated, the role of autotrophy on *X. umbellata's* physiology should be directly assessed, using a range of light intensities with the addition of a photosynthesis-irradiance (PI) curve to assess performance under different light regimes. Thirdly, the colonies of *X. umbellata* used throughout these experiments were all of one genotype, as they were acquired via fragmentation of one larger colony (chapter 2 & 3). This method has the advantage of removing potential genetic differences between replicates that may introduce variability, but it fails to resolve population-level responses. Future studies employing multiple genotypes should be used for capturing the natural variation within a species, providing more accurate and generalisable conclusions. Additionally, whilst much can be gleaned from lab studies, field experiments may elucidate the complex interactions and environmental influences that occur in natural settings, providing a more comprehensive understanding of how *X. umbellata* responds to real-world environmental variability.

The work presented in chapter 4 only offers a snapshot of denitrification rates and environmental conditions during the first two weeks of each sampling month throughout the year. Repeating this research with finer temporal resolution would help capture diurnal

variability, providing a more detailed understanding of how denitrification rates may or may not vary under shorter timescales. Chapter 4 demonstrated that denitrification is influenced by natural fluxes of C and N availability in an oligotrophic setting. Previous studies have focused on the denitrification potential of Red Sea corals to excess N availability (El-Khaled et al., 2020; Tilstra et al., 2019), yet no studies have quantified the rates of denitrification in response to excess C availability, which is typical of more eutrophic environments. Such assessments would bolster our understanding of how these important species will respond to further stressors and offer insight into future coral reef functioning under various C and N availabilities. Finally, in chapter 4 only one N-cycling pathway (denitrification) was examined. To gain a more thorough understanding of the response of N-cycling to C and N fluctuations, additional pathways such as anaerobic ammonium oxidation (ANAMMOX) and dissimilatory nitrate reduction to ammonium (DNRA) remain understudied in this context.

5.7 Wider ecological implications

Xenia umbellata's capacity to efficiently acquire C when availability is low and under variable flow regimes (chapter 2) likely contributes to its widespread distribution. However, despite its efficient nutrient regulation and ability to withstand numerous stressors (Mezger et al., 2022; Simancas-Giraldo et al., 2021; Thobor et al., 2022), *X. umbellata* is susceptible to disturbance from excess C inputs (in the form of POM), with negative effects on its ecophysiology (chapter 3). This highlights how soft corals -which are often considered to be more tolerant to environmental change (Inoue et al., 2013)- are also under threat by anthropogenic disturbances. In fact, a recent study in the central Red Sea corroborates this, documenting a drastic decline in soft corals following a widespread bleaching event (Gonzalez et al., 2024). The vulnerability of soft corals to certain stressors may thus facilitate phase shifts towards taxa that are able to tolerate such conditions (e.g., turf algae) (Gorgula & Connell, 2004), leading to habitats with lower structural complexity. Considering the findings of chapter 4, corals with a higher heterotrophic capacity may better cope with inorganic N pollution through efficient denitrification, allowing them to survive such disturbances more effectively than corals with a lower heterotrophic capacity. With other environmental threats (e.g., temperature stress and low pH) also favouring the survival of heterotrophic over autotrophic corals (Conti-Jerpe et al., 2020; Grottoli et al., 2006; Hulver et al., 2024; Martinez et al., 2024), it is possible that when also exposed to inorganic N enrichment, reef community composition may shift towards

hosting more heterotrophic species. This may have significant implications for biodiversity by not only winnowing the coral species assemblage, but also causing declines in associated fauna that are highly specialised to affected host species. Overall ecosystem function may also be affected by shifting coral reefs from generally net-autotrophic ecosystems to net-heterotrophic (Courtney et al., 2018), with concomitant changes in biogeochemical cycling of C, N, and other macronutrients.

Increased C and N loading in nearshore environments is often associated with human activity such as sewage outflow, agricultural runoff, or sedimentation from coastal development (Fabricius et al., 2013; Tuholske et al., 2021). The recent launch of numerous large-scale coastal giga-projects in the Kingdom of Saudi Arabia (PIF, 2017) underscores the urgent need to fully understand the effects of excess C and N on Red Sea corals. This thesis provides valuable insights into the effects of ambient and excess nutrient on key aspects of coral physiology, offering knowledge to guide the development of targeted management strategies for coral species with varying sensitivities to these stressors.

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Appendix

Supplementary material | Chapter 2

Table S2.1 Water flow treatment details. Pumps remained consistently on these settings throughout all three experimental phases. The measured pumping speed average ± error.

Water flow treatment	Pump model	Pump setting	Measured pumping speed (Lh^{-1})
1	EHEIM Compact On 300 pump GmbH and Co. KG, Germany	200	205.68 ± 1.62
2	EHEIM Compact On 1000 pump; GmbH and Co. KG, Germany	350	335.26 ± 2.13
3	EHEIM Compact On 1000 pump; GmbH and Co. KG, Germany	500	493.81 ± 4.44
4	EHEIM Compact On 1000 pump; GmbH and Co. KG, Germany	650	649.93 ± 16.21

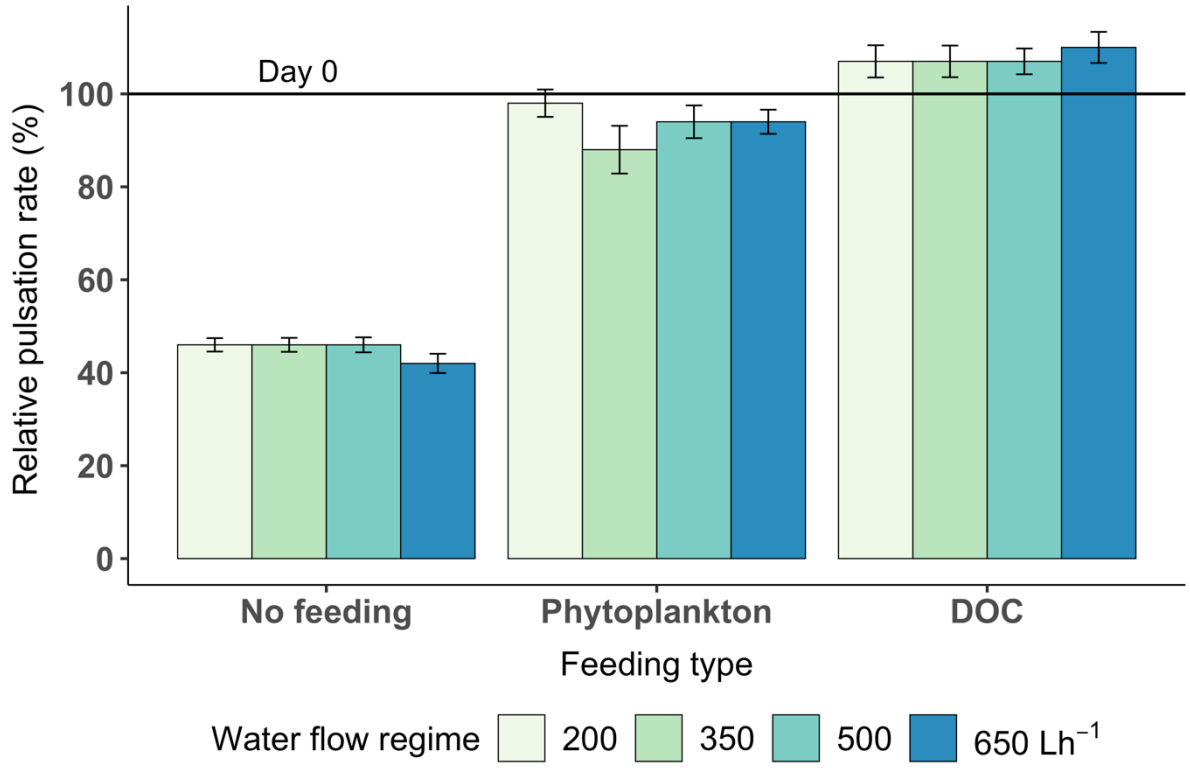

Figure S2.2 The average relative pulsation rates of *Xenia umbellata* on day 15, following exposure to 'no feeding' 'phytoplankton' and 'dissolved organic carbon [DOC]' feeding treatments crossed with four water flow speeds of 200 L h^{-1}, 350 L h^{-1}, 500 L h^{-1} and 650 L h^{-1}. Values are reported as a relative comparison to day 0 of the respective feeding treatment. The average number of pulsations on day 0 of each feeding treatment is equivalent to 100% and is indicated by the horizontal line labelled 'Day 0'. There was no significant effect of water flow. For significant effects of feeding treatments, please see "Results".

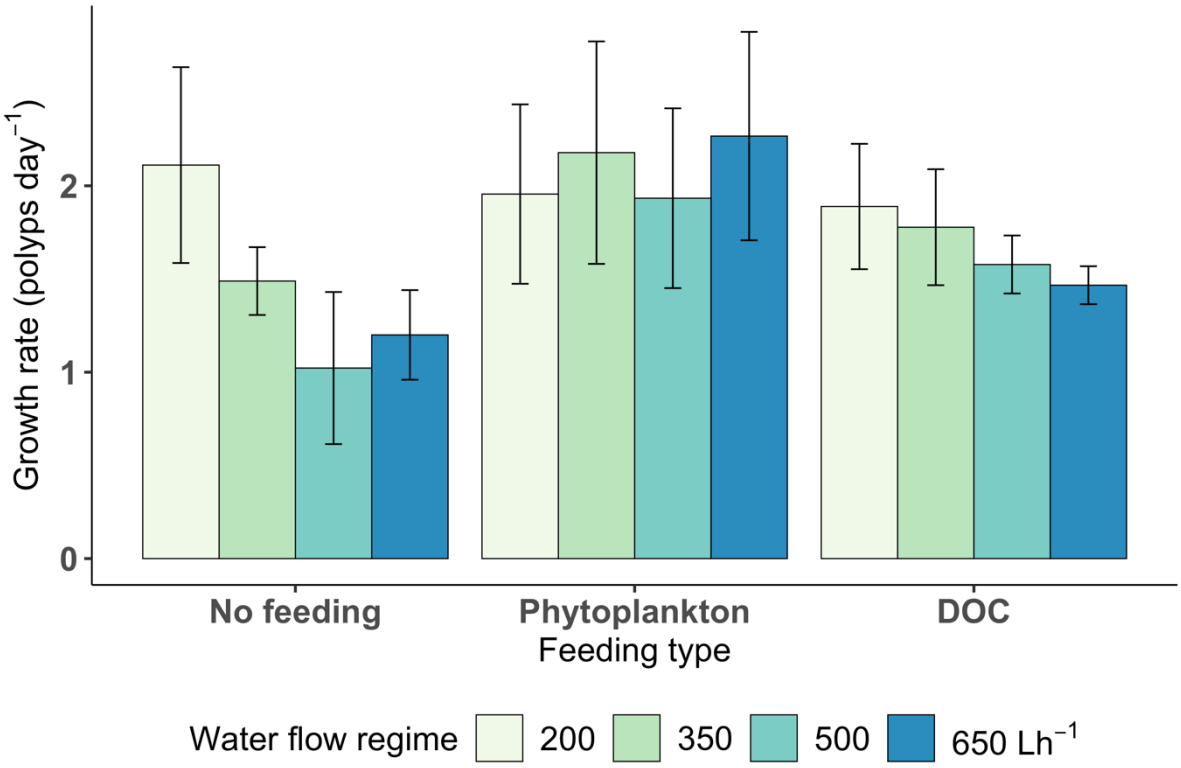

Figure S2.3 The average growth rate of *Xenia umbellata* following 15 days of exposure to 'no feeding' 'phytoplankton' and 'dissolved organic carbon [DOC]' feeding treatments crossed with four water flow speeds of 200 L h^{-1}, 350 L h^{-1}, 500 L h^{-1} and 650 L h^{-1}. There was no significant effect of water flow nor feeding treatments.

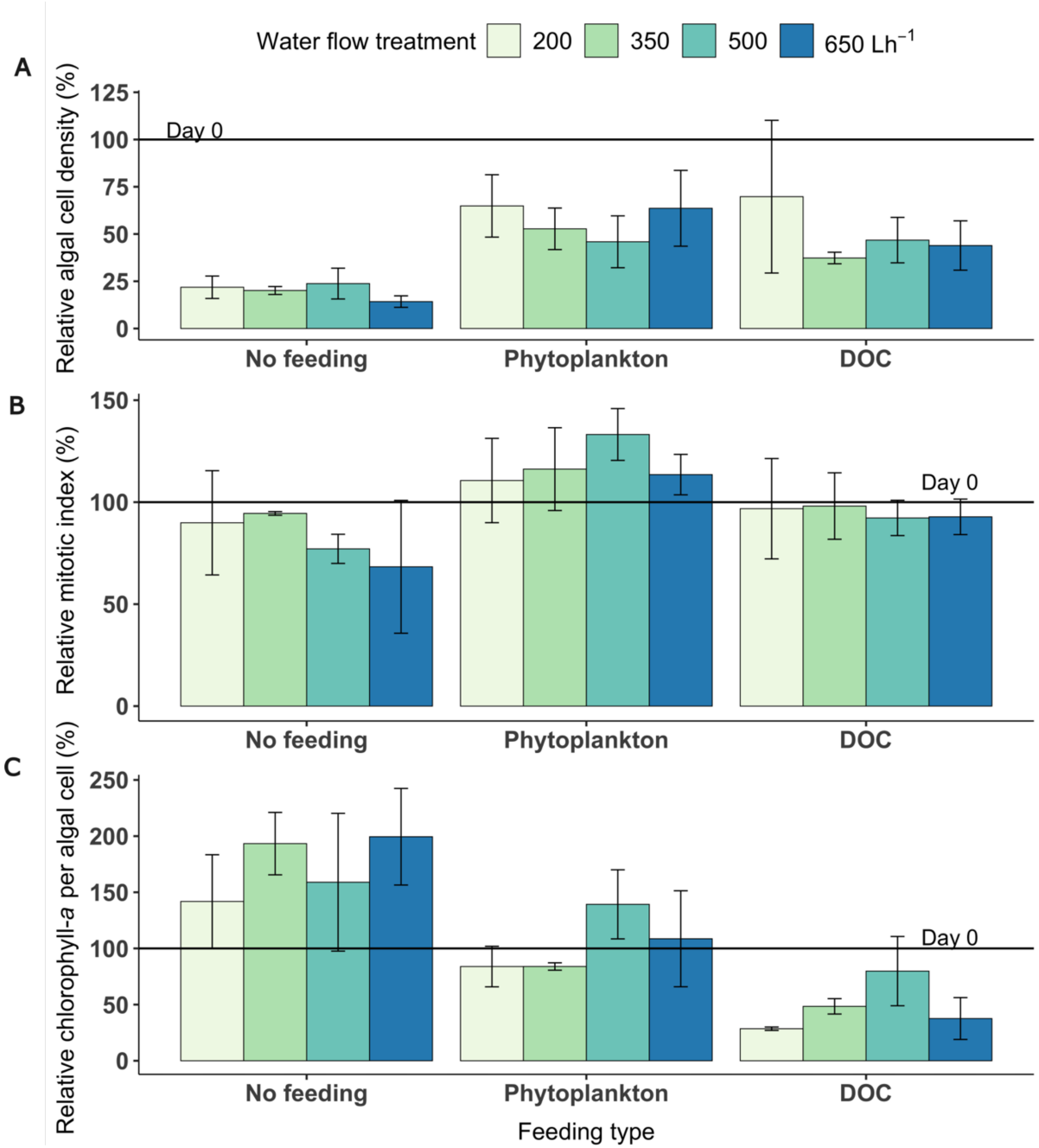

Figure S2.4 Relative Symbiodiniaceae parameters of *Xenia umbellata* including A] algal cell density (%), B] mitotic index (%) and C] chlorophyll-*a* per algal cell (%), following 15 days of exposure to 'no feeding' 'phytoplankton' and 'dissolved organic carbon [DOC]' crossed with four water flow speeds of 200 L h^{-1}, 350 L h^{-1}, 500 L h^{-1} and 650 L h^{-1}. Values are reported as a relative comparison to day 0 of the respective feeding treatment. The average value on day 0 of each experimental phase is equivalent to 100% and is indicated by the horizontal line labelled 'Day 0'. There was no significant effect of water flow. For significant effects of feeding treatments, please see "Results".

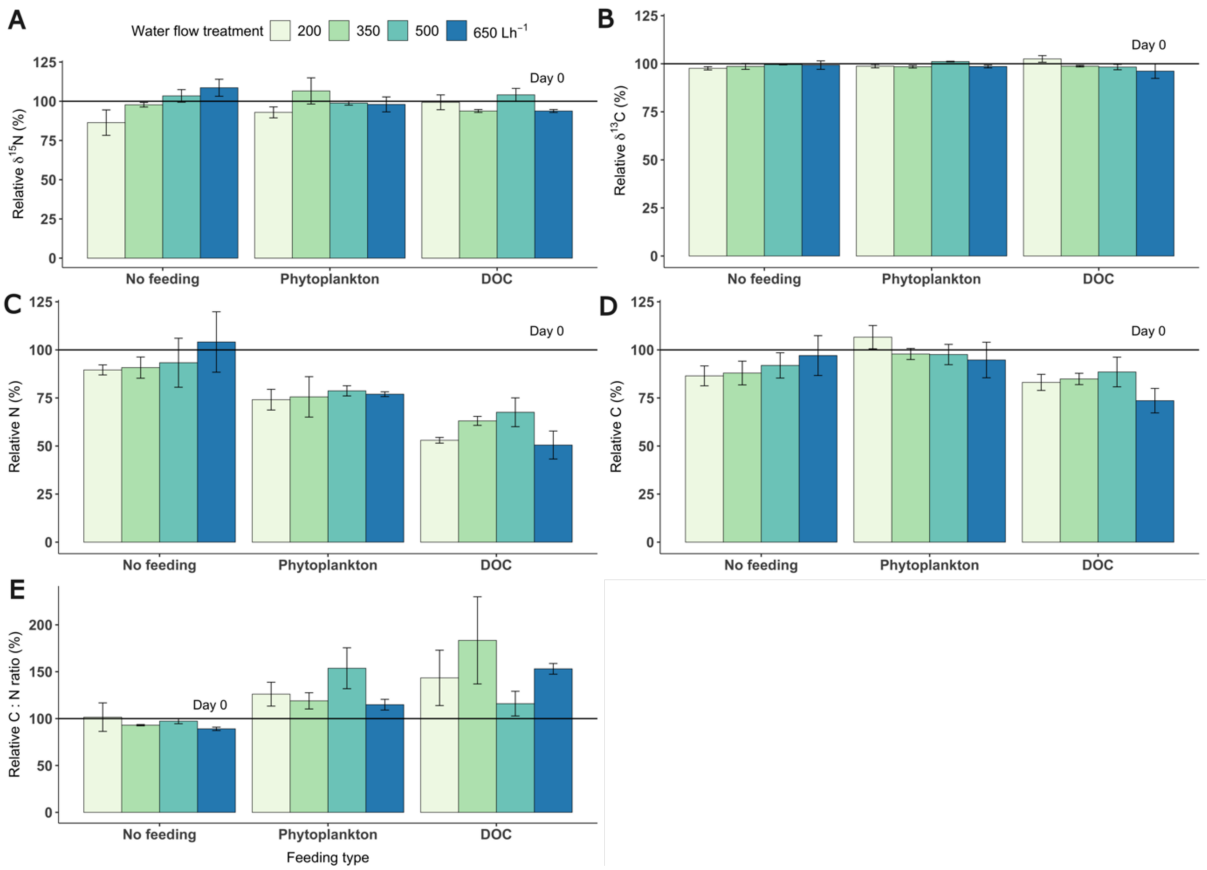

Figure S2.5 Relative isotope and elemental data of *Xenia umbellata* including A] relative nitrogen stable isotope ratio (δ^{15}N (‰)) (%), B] relative carbon stable isotope ratio (δ^{13}C (‰)) (%) C] relative nitrogen content (%N) (%), D] relative carbon content (%C) (%) and E] relative carbon to nitrogen ratio (C:N) (%), following 15 days of exposure to 'no feeding' 'phytoplankton' and 'dissolved organic carbon [DOC]' crossed with four water flow speeds of 200 L h^{-1}, 350 L h^{-1}, 500 L h^{-1} and 650 L h^{-1}. Values are reported as a relative comparison to day 0 of the respective feeding treatment. The average value on day 0 of each experimental phase is equivalent to 100% and is indicated by the horizontal line labelled 'Day 0'. There was no significant effect of water flow. For significant effects of feeding treatments, please see "Results".

Table S2.6 A summary of the statistical output when data was assessed according to water flow and feeding with a 2-way-ANOVA. Significance is indicated by a p value < 0.05 and asterisks indicate the strength of the significance e.g., *** meaning p < 0.0001, ** meaning p < 0.01.

†Whilst water flow appeared to have a significant effect on pulsation, post hoc testing revealed no significant differences between water flow speeds within feeding treatments.

Approach 1: Water flow and feeding					
Statistical test	Parameters	Term	DF	F value	P value
2-way-ANOVA	Pulsation	Water flow Feeding Water flow * feeding	3 2 6	14.947 1011.932 5.422	4.84×10^{-9} ***† $< 2 \times 10^{-16}$ *** 2.57×10^{-5} ***†
2-way-ANOVA	Growth Rate	Water flow Feeding Water flow * feeding	3 2 6	0.799 2.546 0.516	0.5065 0.0994 0.7905
2-way-ANOVA	Symbiodiniaceae density	Water flow Feeding Water flow * feeding	3 2 6	0.263 8.414 0.353	0.8515 0.0017 ** 0.9008
2-way-ANOVA	Mitotic index	Water flow Feeding Water flow * feeding	3 2 6	0.202 4.206 0.279	0.8939 0.0272 0.9411
2-way-ANOVA	Chlorophyll-*a* per cell	Water flow Feeding Water flow * feeding	3 2 6	1.26 15.56 0.73	0.31 4.7×10^{-5} *** 0.63
2-way-ANOVA	Isotope δ N15	Water flow Feeding Water flow * feeding	3 2 6	2.170 0.730 2.102	0.1191 0.4929 0.0924

2-way-ANOVA	Isotope δ C13	Water flow Feeding Water flow * feeding	3 2 6	0.763 0.782 1.479	0.527 0.470 0.231
2-way-ANOVA	Element N %	Water flow Feeding Water flow * feeding	3 2 6	0.563 11.457 0.712	0.644957 0.000353 *** 0.643383
2-way-ANOVA	Element C %	Water flow Feeding Water flow * feeding	3 2 6	0.275 0.807 0.924	0.843 0.459 0.497
2-way-ANOVA	C : N	Water flow Feeding Water flow * feeding	3 2 6	0.723 24.101 1.926	0.549 3.65×10^{-6} *** 0.124

Figure S2.7 A summary of the statistical output when data was assessed according to feeding with a 1-way-ANOVA. Significance is indicated by a p value < 0.05 and asterisks indicate the strength of the significance e.g., *** meaning p < 0.0001, * meaning p < 0.05.

Approach 2: Feeding					
Statistical test	Parameters	DF	F value	P value	Post-hoc Tukey HSD
1-way-ANOVA	Pulsation	2	905.2	$< 2 \times 10^{-16}$ ***	No feeding – DOC (p = 0) Phytoplankton – DOC (p = 0) Phytoplankton – No feeding (p = 0)
1-way-ANOVA	Growth Rate	2	2.848	0.0723	n.a
1-way-ANOVA	Symbio-diniaceae density	2	15.87	1.48×10^{-5} ***	No feeding – DOC (p = 0.0004) Phytoplankton - DOC (p = 0.5) Phytoplankton - No feeding (p = 0.00002)
1-way-ANOVA	Mitotic index	2	5.144	0.0114 *	No feeding – DOC (p = 0.5) Phytoplankton – DOC (p = 0.1) Phytoplankton – No feeding (p = 0.009)
1-way-ANOVA	Chloro-phyll-*a* per cell	2	16.65	$1x\ 10^{-5}$ ***	No feeding – DOC (p = 0.0000058) Phytoplankton – DOC (p = 0.04) Phytoplankton – No feeding (p = 0.008)
1-way-ANOVA	Isotope δ N15	2	0.571	0.571	n.a
1-way-ANOVA	Isotope δ C13	2	0.698	0.505	n.a
1-way-ANOVA	Element N %	2	12.72	8.6×10^{-5} ***	No feeding – DOC (p = 0.00005)

					Phytoplankton – DOC (p = 0.02) Phytoplankton – No feeding (p = 0.07)
1-way-ANOVA	Element C %	2	0.868	0.43	n.a
1-way-ANOVA	C : N	2	21.7	1.48×10^{-6} ***	No feeding – DOC (p = 0.0000009) Phytoplankton – DOC (p = 0.002) Phytoplankton – No feeding (p = 0.01)

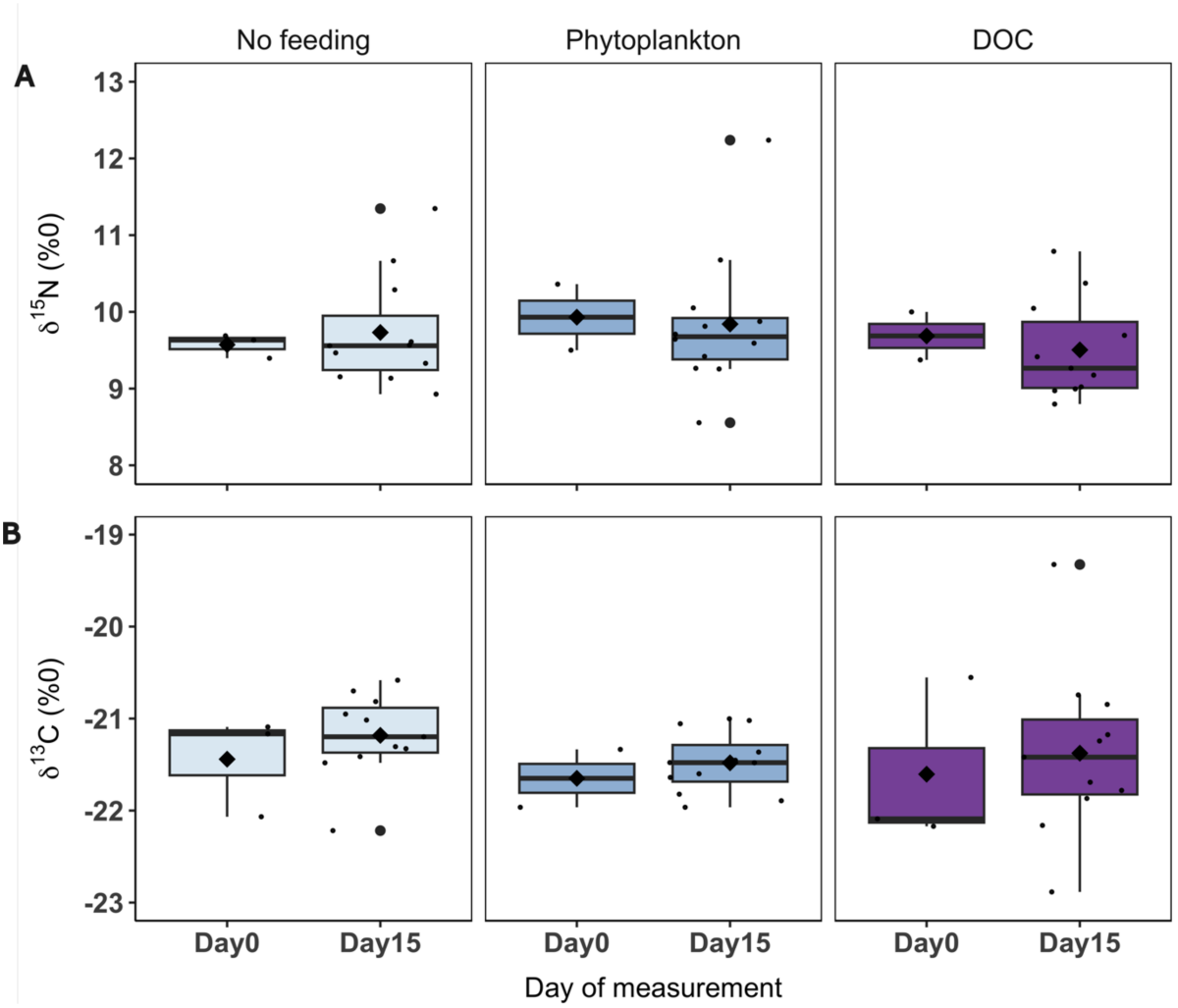

Figure S2.8 Non-significant stable isotope signatures of *Xenia umbellata* including A] nitrogen stable isotope ratio ($\delta^{15}N$ (‰)) and B] carbon stable isotope ratio ($\delta^{13}C$ (‰)), following 15 days of exposure

to ‘No feeding’ ‘Phytoplankton’ and ‘Dissolved organic carbon [DOC]’ feeding treatments. For day 0 measurements, there are 3 biological replicates. For day 15 measurements there are 12 biological replicates. The median is represented by the black horizontal line and the mean is indicated by a large black diamond.

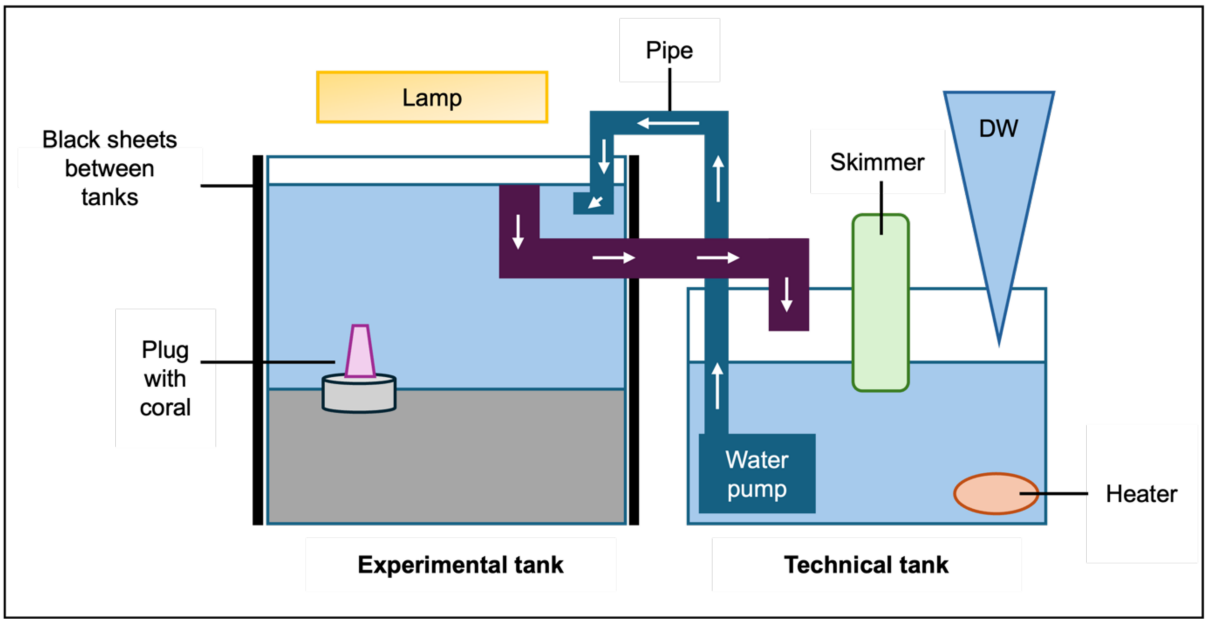

Figure S3.1 A diagram depicting the experimental setup. DW = distilled water dispenser.

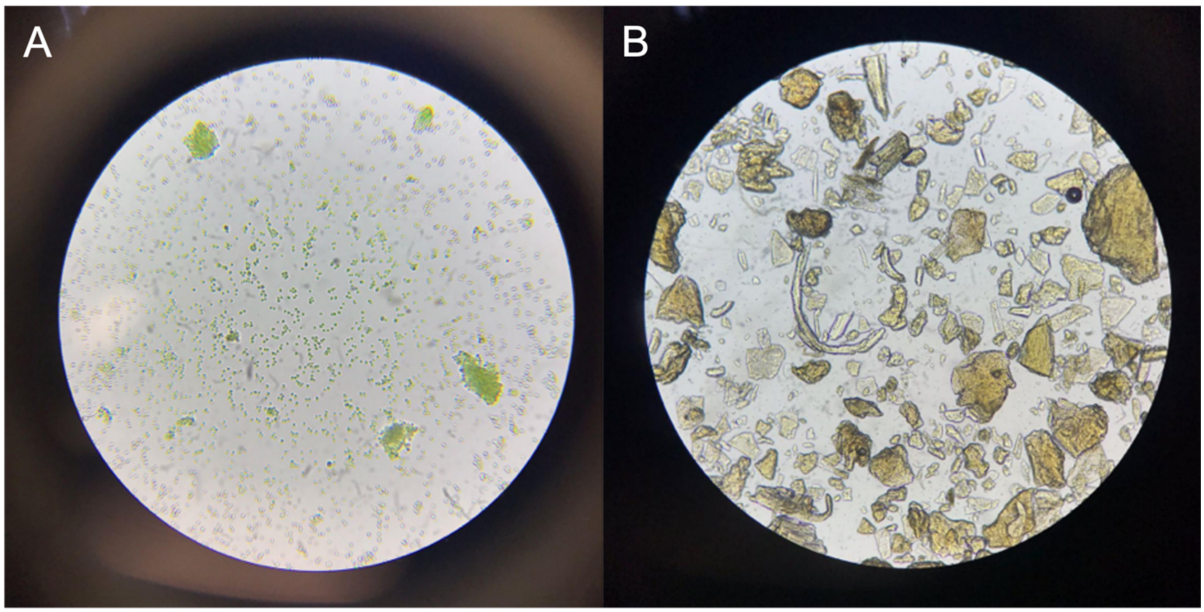

Figure S3.2 Microscope images of the supplied POM enrichment, with A] phytoplankton species *Nannochloropsis gaditana,* particle size: 2 – 5 µm (x 40 magnification), B] natural mix of zooplankton species, particle size 150 – 200 µm (x 10 magnification).

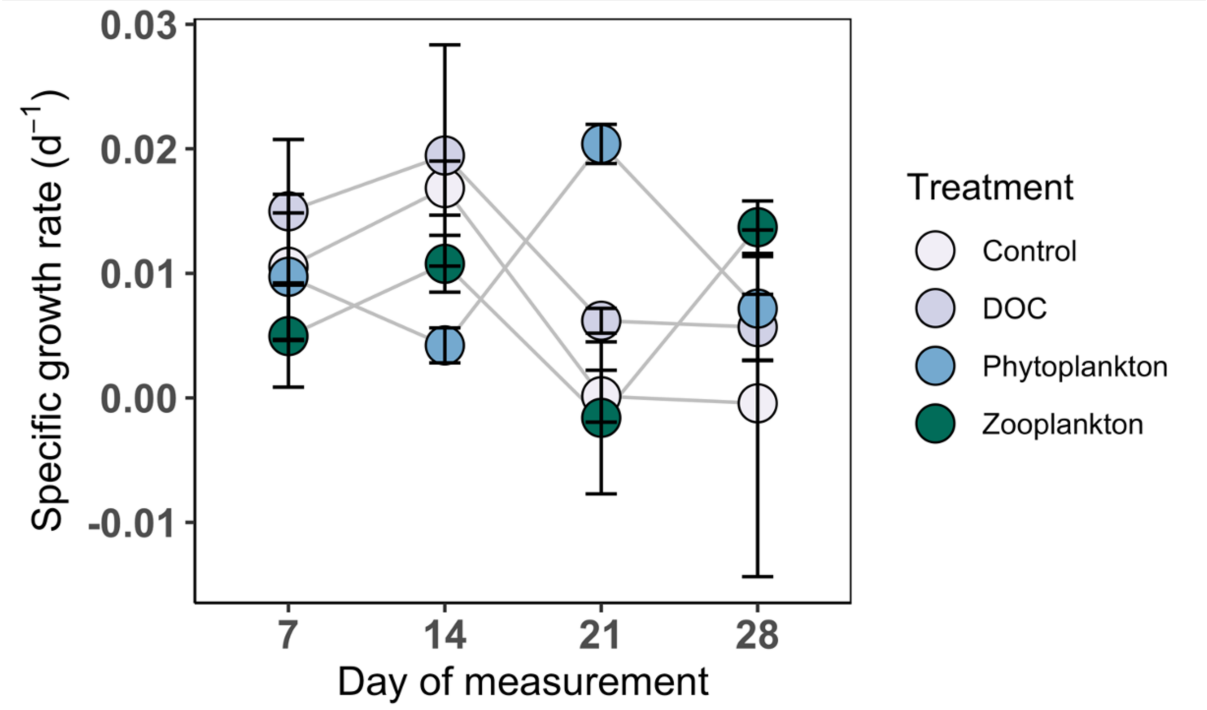

Figure S3.3 Specific growth rate of the *Xenia umbellata* when exposed to 20 mg C L^{-1} of three forms of organic carbon (dissolved organic carbon [DOC], phytoplankton, zooplankton) and a control treatment over time (28 days). Each point = mean ± standard error.

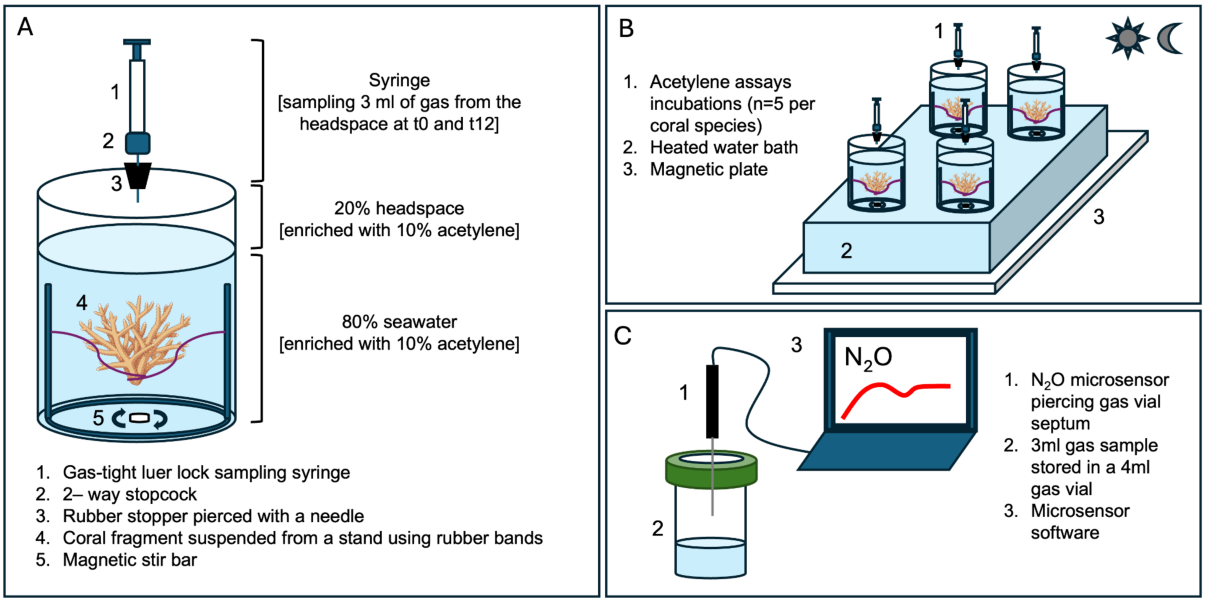

Figure S4.1 Quantification of denitrification. A] The acetylene assay setup per coral fragment. Assays were set up in the same manner for controls, just excluding the coral fragment. B] The acetylene assay incubations, performed for 12 hours in the light and 12 hours in the dark. C] Use of a microsensor to measure nitrous oxide (N_2O) of the gas samples generated from the acetylene assay.

N_2O normalisation equations

[$S^{HS(M)}$] Moles of N_2O in the storage vial headspace (µmol):

$$MV * S^{HS(V)} = S^{HS(M)}$$

[$S^{W(M)}$] Moles of N_2O in the storage vial water: (µmol):

$$MV * BC * S^{W(V)} = S^{W(M)}$$

[$B^{HS(C)}$] Concentration of N_2O in the beaker headspace: (µmol L^{-1}):

$$\frac{S^{HS(M)} + S^{W(M)}}{S^{HS(V)}} = B^{HS(C)}$$

[$B^{HS(M)}$] Moles of N_2O in the beaker headspace (µmol):

$$B^{HS(C)} * B^{HS\,(V)} = B^{HS(M)}$$

[$B^{W(M)}$] Moles of N_2O in the beaker water (µmol):

$$B^{HS(C)} * BC * B^{W(V)} = B^{W(M)}$$

[$B^{T(M)}$] Total moles of N_2O in the whole beaker (µmol):

$$B^{HS(M)} + B^{W(M)}$$

Abbreviations used in above equations:

[*MV*] Microsensor value (µmol L^{-1})
[$S^{HS(V)}$] Storage vial headspace volume (L)
[$S^{HS(M)}$] Storage vial headspace moles (µmol)
[*BC*] Bunsen solubility coefficient*

**The Bunsen solubility coefficient used varied according to the temperature and salinity. These conditions differed between the storage vial (room temperature of 21 ℃ / salinity of 0) and the beaker (variable temperature dependent on the sampling month and salinity of 39). The coefficient can be determined using a table in (Weiss & Price, 1980).*

[$S^{W(V)}$] Storage vial water volume (L)
[$S^{W(M)}$] Storage vial water moles (µmol)
[$B^{HS(C)}$] Beaker headspace concentration (µmol L^{-1})
[$B^{HS(V)}$] Beaker headspace volume (L)
[$B^{HS(M)}$] Beaker headspace moles (µmol)
[$B^{W(V)}$] Beaker water volume (L)
[$B^{W(M)}$] Beaker water moles (µmol)
[$B^{T(M)}$] Beaker total moles (µmol)

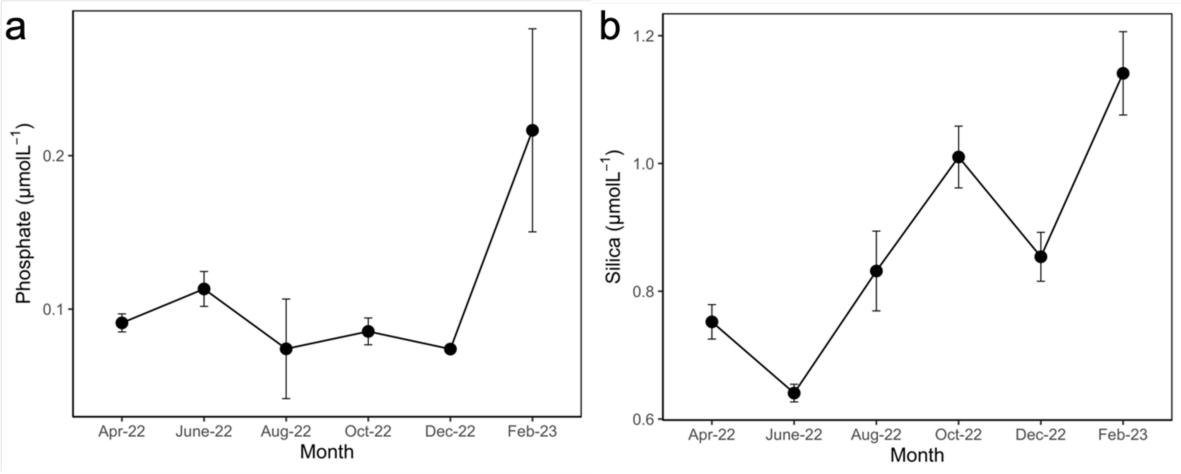

Figure S4.2 a) Phosphate and b) Silica mean concentrations throughout the sampling period April 2022 – February 2023. Bars indicate standard error.

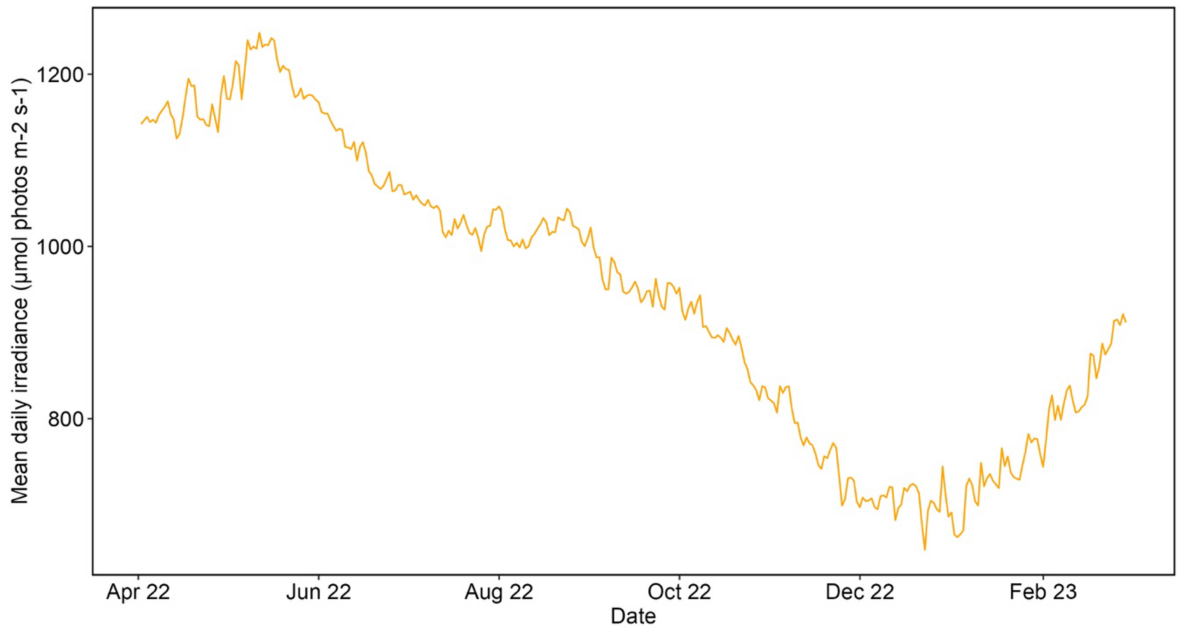

Figure S4.3 Irradiance through the sampling period. Data sourced from the Copernicus ERA5 hourly levels (direct clear sky radiation).

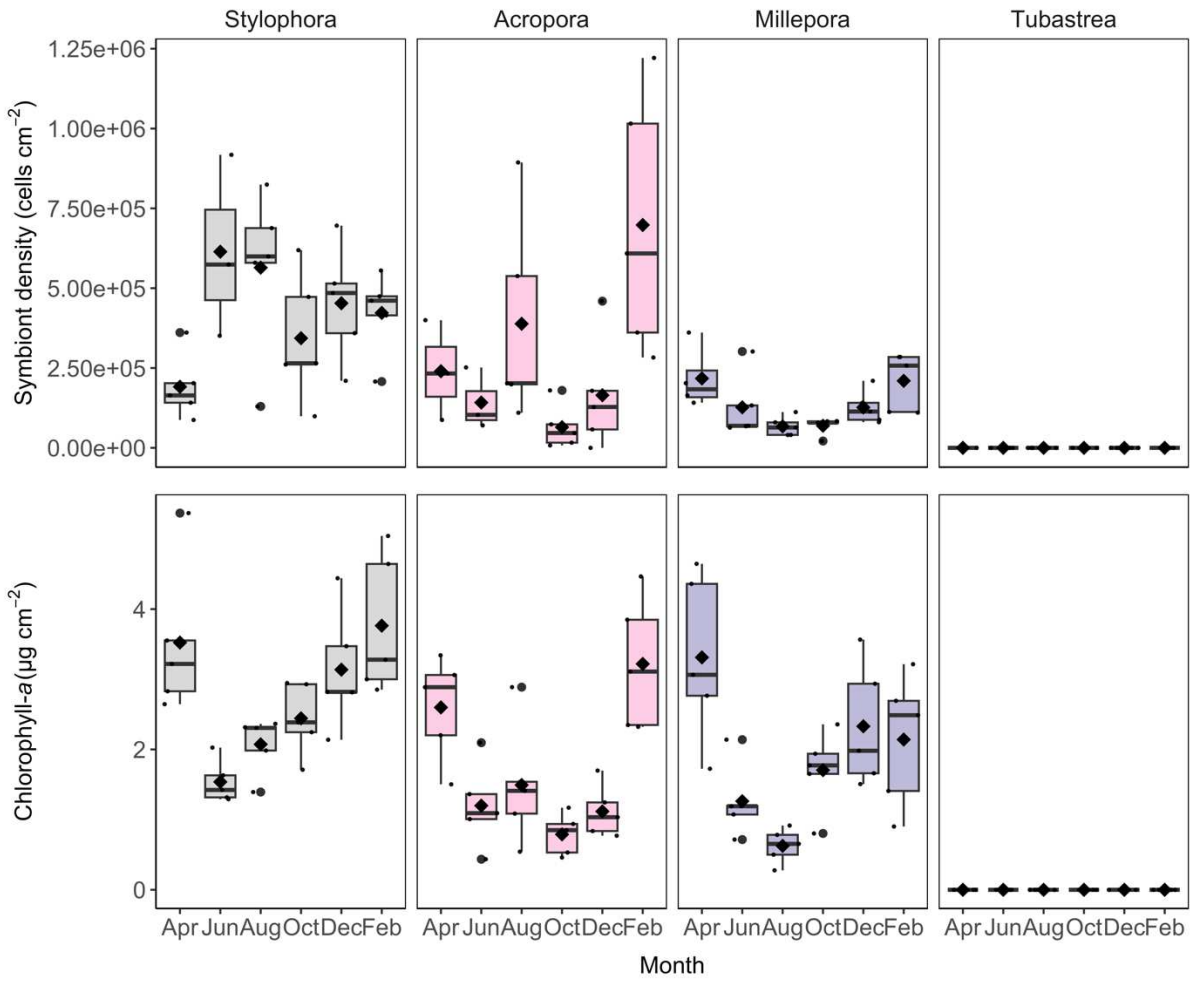

Figure S4.4 a) The symbiont density (cells cm^{-2}), b) the chlorophyll-*a* of four corals (*Stylophora pistillata, Acropora* spp, *Millepora dichotoma* and *Tubastrea coccinea*) at bimonthly time points between April 2022 and February 2023. The large black diamond depicts the mean value of 5 biological replicates per month.

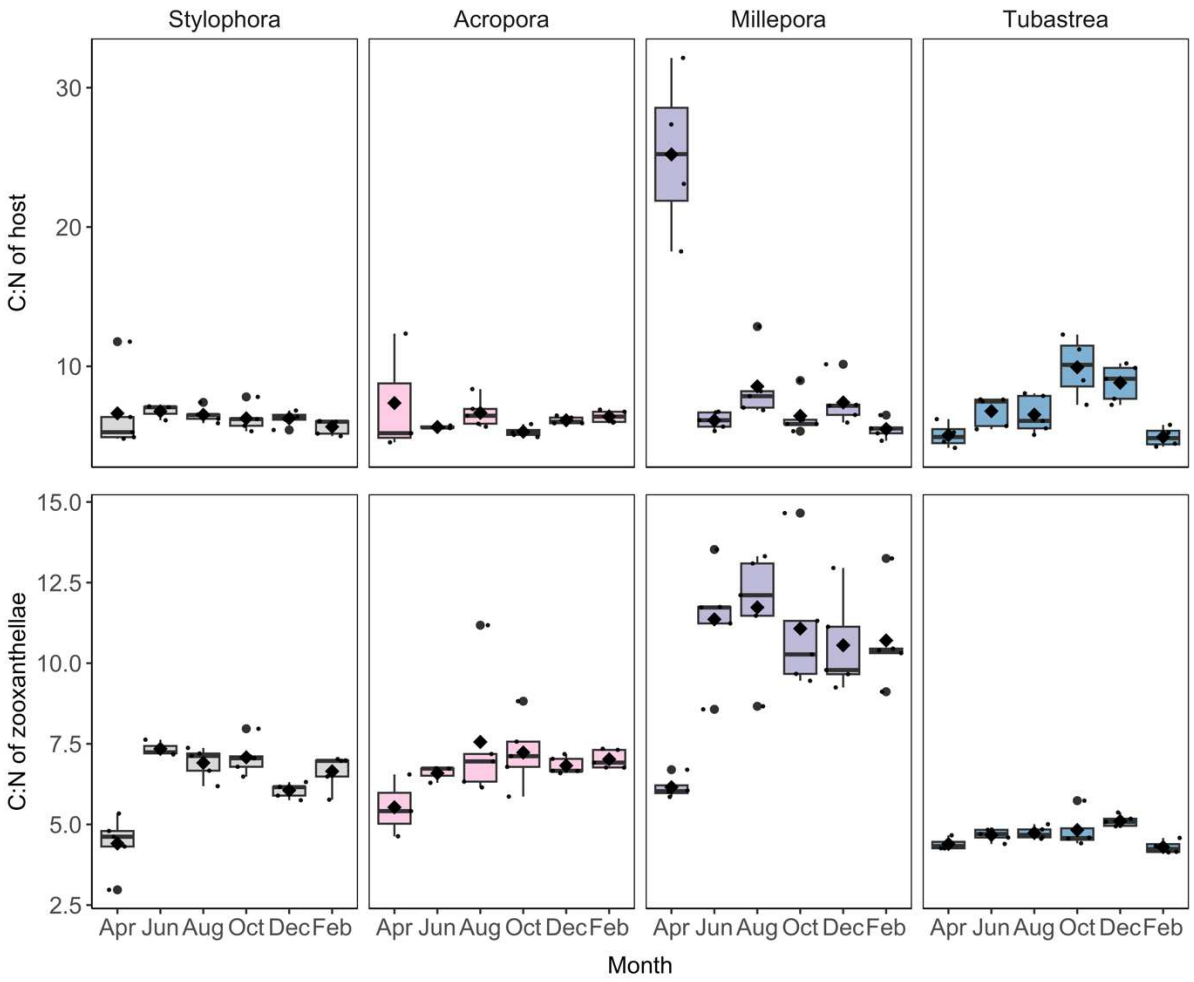

Figure S4.5 a) The C:N of the coral host b) the C:N of the zooxanthellae of four corals (*Stylophora pistillata, Acropora* spp, *Millepora dichotoma* and *Tubastrea coccinea*) at bimonthly time points between April 2022 and February 2023. The large black diamond depicts the mean value of 5 biological replicates per month.

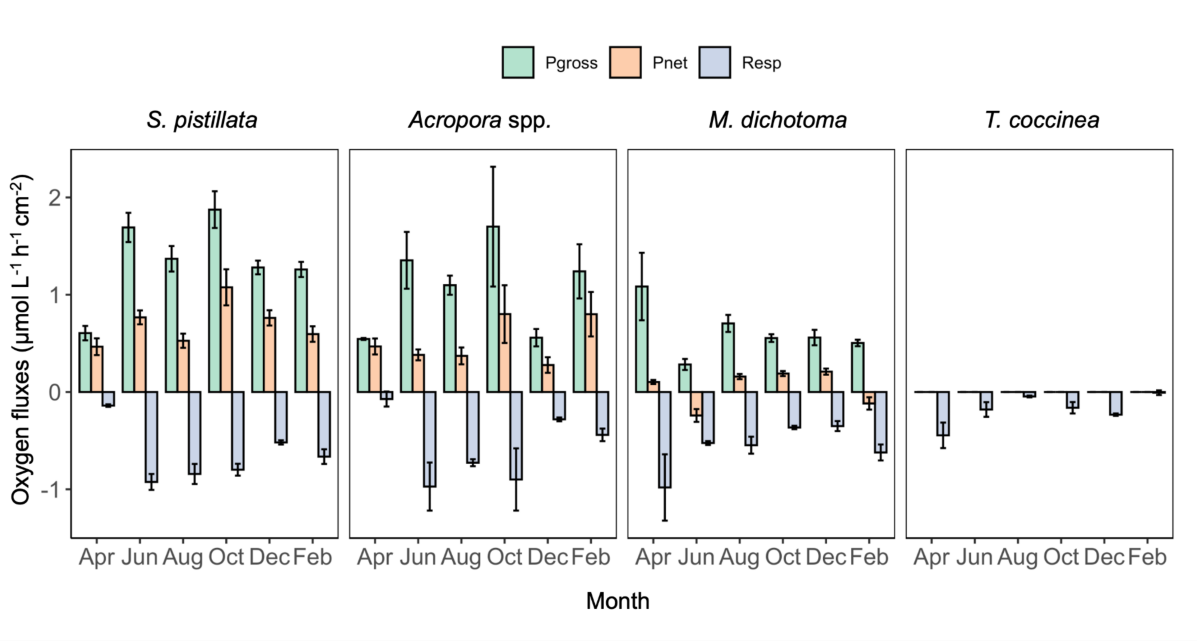

Figure S4.6 The oxygen fluxes of four corals (*Stylophora pistillata, Acropora* spp*, Millepora dichotoma* and *Tubastrea coccinea*) at bimonthly time points between April 2022 and February 2023. Oxygen fluxes include average gross photosynthesis rates (Pgross), average net photosynthesis rates (Pnet) and average respiration rates (Resp). Standard deviation is indicated by error bars.

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