Microbe-host associations as drivers of benthic carbon and nitrogen cycling in a changing Mediterranean Sea

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For my beloved grandparents, who made me love nature.

SUMMARY

Seagrasses, such as the endemic Mediterranean species *Posidonia oceanica*, are critical components of coastal marine ecosystems, providing essential ecosystem services, including carbon sequestration, nutrient cycling, and habitat formation. *P. oceanica* forms extensive meadows that serve as biodiversity hotspots and play a crucial role in mitigating climate change through long-term carbon storage. Despite their ecological significance, the interactions between *P. oceanica* and associated organisms, as well as their combined contributions to biogeochemical cycling, remain poorly understood, particularly under changing environmental conditions. This thesis explores the carbon and nitrogen cycling processes within the *P. oceanica* holobiont, focusing on the epiphytic and microbial communities, microbial driven metabolic processes, and the interaction between *P. oceanica* and larger associated invertebrates, such as the sponge *Chondrilla nucula*. Through field and laboratory experiments, this work demonstrates the significant role of epiphytic algae in the primary production of the seagrass holobiont, contributing a substantial portion of net primary production. Nitrogen cycling processes such as N_2 fixation, nitrification, and denitrification in the seagrass phyllosphere were quantified, revealing their importance in meeting the N demands of the seagrass holobiont, especially under natural ocean acidification conditions. Experiments near marine CO_2 vents indicated that ocean acidification accelerates net primary production and nitrogen cycling, while the structure of the microbial community associated with *P. oceanica* leaves remains largely stable. The facultative mutualism between *P. oceanica* and the sponge *C. nucula* further highlights the complexity of the seagrass holobiont. *P. oceanica* releases dissolved organic carbon, which meets a portion of the sponge's respiratory carbon demand. Conversely, *C. nucula* releases dissolved inorganic nitrogen, including ammonium and nitrate generated by microbial nitrification, which supports seagrass growth. Stable isotope analysis suggests that the association facilitates nutrient exchange, with *P. oceanica* preferentially absorbing sponge-derived ammonium, while epiphytes may benefit from sponge-produced nitrate. This dynamic reduces seasonal fluctuations in productivity, stabilizing the seagrass ecosystem during periods of senescence. Sponge-associated nitrification contributes to the nitrogen budget of the seagrass holobiont, potentially reducing nutrient limitations in oligotrophic Mediterranean waters. The microbiome of *C. nucula* plays a key role in these processes, harboring nitrifiers that mediate the production of nitrate. High-throughput sequencing revealed taxonomic diversity among microbes associated with both the sponge and seagrass, including microorganisms involved in carbon and nitrogen cycling processes. These microbial communities not only mediate nutrient exchange within the seagrass-sponge association but also contribute to the overall resilience and productivity of the ecosystem. This thesis highlights the intricate interactions within the *P. oceanica* holobiont and its nested ecosystem with *C. nucula*. These findings underscore the importance of microbial and epiphytic communities in maintaining the resilience and productivity of seagrass meadows, particularly in nutrient-poor environments like the Mediterranean Sea. This research enhances our understanding of the biogeochemical processes that support seagrass ecosystem stability and provides valuable insights to guide conservation efforts in the face of climate change and anthropogenic pressures.

ZUSAMMENFASSUNG

Seegräser, wie die endemische mediterrane Art *Posidonia oceanica*, sind wichtige Bestandteile mariner Küstenökosysteme und erbringen wichtige Ökosystemleistungen, darunter Kohlenstoffbindung, Nährstoffkreisläufe und Lebensraumbildung. *P. oceanica* bildet ausgedehnte Seegraswiesen, die als Biodiversitäts-Hotspots dienen und durch langfristige Kohlenstoffspeicherung eine entscheidende Rolle im Kampf gegen den Klimawandel spielen. Trotz ihrer herausragenden ökologischen Bedeutung sind Interaktionen zwischen *P. oceanica* und den mit ihr assoziierten Organismen sowie ihr gemeinsamer Beitrag zu biogeochemischen Kreisläufen, insbesondere unter veränderten Umweltbedingungen, nach wie vor nur unzureichend erforscht. In dieser Arbeit werden Prozesse im Kohlenstoff- und Stickstoffkreislauf innerhalb des *P. oceanica*-Holobionten untersucht, wobei der Schwerpunkt auf den epiphytischen und mikrobiellen Gemeinschaften, mikrobiellen Stoffwechselprozessen und der Beziehung zwischen *P. oceanica* und größeren wirbellosen Tieren, wie dem Schwamm *Chondrilla nucula*, liegt. Anhand von Feld- und Laborexperimenten zeigt diese Arbeit die bedeutende Rolle epiphytischer Algen bei der Produktivität des Seegras-Holobionten, die einen erheblichen Anteil an der Nettoprimärproduktion haben. Stickstoffkreislaufprozesse, wie N_2-Fixierung, Nitrifikation und Denitrifikation in der Phyllosphäre des Seegrases, wurden quantifiziert und ihre Bedeutung für die Deckung des Stickstoffbedarfs des Seegras-Holobionten insbesondere unter Ozeanversauerungs-Bedingungen aufgezeigt. Experimente in der Nähe von marinen CO_2-Quellen zeigten, dass Ozeanversauerung die Nettoprimärproduktion und den Stickstoffkreislauf ankurbelt, während die Struktur der mit den Blättern von *P. oceanica* assoziierten mikrobiellen Gemeinschaft weitgehend unverändert bleibt. Der fakultative Mutualismus zwischen *P. oceanica* und dem Schwamm *C. nucula* verdeutlicht die Komplexität des Seegras-Holobionten. *P. oceanica* setzt gelösten organischen Kohlenstoff frei, der einen Teil des Kohlenstoffbedarfs für die Respiration des Schwamms deckt. Umgekehrt setzt *C. nucula* gelösten anorganischen Stickstoff frei, darunter Ammonium und Nitrat, das durch mikrobielle Nitrifikation entsteht und das Seegraswachstum unterstützt. Die Analyse stabiler Isotope deutet darauf hin, dass die Seegras-Schwamm-Verbindung den Nährstoffaustausch erleichtert, wobei *P. oceanica* bevorzugt vom Schwamm stammendes Ammonium aufnimmt, während die Epiphyten möglicherweise durch vom Schwamm stammendes Nitrat profitieren. Diese Dynamik verringert saisonale Produktivitätsschwankungen und stabilisiert das Seegras-Ökosystem in Zeiten der Seneszenz. Die mit dem Schwamm assoziierte Nitrifikation trägt zum Stickstoffhaushalt des Seegras-Holobionten bei, wodurch die Nährstoffbeschränkungen in oligotrophen Gewässern wie dem Mittelmeer verringert werden könnten. Das Mikrobiom von *C. nucula* spielt bei diesen Prozessen eine Schlüsselrolle, denn es beherbergt Nitrifikanten, die die an der Produktion von Nitrat beteiligt sind. Die Hochdurchsatz-Sequenzierung ergab eine große taxonomische Vielfalt von Mikroorganismen, die mit dem Schwamm und dem Seegras assoziiert sind, einschließlich solcher, die an Kohlenstoff- und Stickstoffkreislaufprozessen beteiligt sind. Diese mikrobiellen Gemeinschaften vermitteln nicht nur den Nährstoffaustausch innerhalb der Seegras-Schwamm-Assoziation, sondern tragen auch zur allgemeinen Widerstandsfähigkeit und Produktivität des Ökosystems bei. In dieser Arbeit werden die komplexen Interaktionen innerhalb des *P. oceanica* – Holobionten und seines verschränkten

Ökosystems mit *C. nucula* aufgezeigt. Diese Ergebnisse unterstreichen die Bedeutung der mikrobiellen und epiphytischen Gemeinschaften für die Erhaltung der Widerstandsfähigkeit und Produktivität von Seegraswiesen, insbesondere in nährstoffarmen Umgebungen wie dem Mittelmeer. Durch die Verbesserung unseres Verständnisses dieser Interaktionen liefert diese Arbeit wichtige Erkenntnisse über die biogeochemischen Prozesse, die die Stabilität von Seegrasökosystemen unterstützen, und liefert grundlegende Informationen für Naturschutzmaßnahmen unter dem Druck des Klimawandels und anderer anthropogener Stressoren.

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My love and fascination for nature started with my grandparents showing me various animals, plants, and mushrooms in the Harz – a part of the Central German Uplands. Since then, my curiosity evolved, and my path eventually led me from the mountains to the sea. My parents might have preferred a field of research that wouldn't have made me move to the other end of Germany, but here we are ;-). And Bremen is always worth a visit! I'm forever grateful for my wonderful parents and sisters; your guidance and encouragement have made me the person I am today.

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Berlinghof, J., Peiffer, F., Marzocchi, U., Munari, M., Quero, G. M., Dennis, L., Wild, C., & Cardini, U. (2022). The role of epiphytes in seagrass productivity under ocean acidification. *Scientific Reports*, 12(6249). https://doi.org/10.1038/s41598-022-10154-7

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Manuscripts in preparation for submission included in this thesis:

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DECLARATION ON THE CONTRIBUTION OF THE CANDIDATE TO A MULTI AUTHOR ARTICLE/ MANUSCRIPT WHICH IS INCLUDED AS A CHAPTER IN THE SUBMITTED DOCTORAL THESIS

Contribution of the candidate is given in % of the total workload (up to 100 % for each category).

Chapter 2: The role of epiphytes in seagrass productivity under ocean acidification

Experimental concept and design	0 %
Experimental work and/or acquisition of (experimental) data	0 %
Data analysis and interpretation	90 %
Preparation of figures and tables	100 %
Drafting of the manuscript	80 %

Chapter 3: Accelerated nitrogen cycling on Mediterranean seagrass leaves at volcanic CO_2 vents

Experimental concept and design	0 %
Experimental work and/or acquisition of (experimental) data	10 %
Data analysis and interpretation	50 %
Preparation of figures and tables	40 %
Drafting of the manuscript	50 %

Chapter 4: Reciprocal nutritional benefits in a sponge-seagrass association

Experimental concept and design	5 %
Experimental work and/or acquisition of (experimental) data	10 %
Data analysis and interpretation	10 %
Preparation of figures and tables	0 %
Drafting of the manuscript	5 %

Chapter 5: Nitrification in a seagrass-sponge association

Experimental concept and design	80 %
Experimental work and/or acquisition of (experimental) data	70 %
Data analysis and interpretation	80 %
Preparation of figures and tables	100 %
Drafting of the manuscript	90 %

1. General Introduction

The Ecology of Seagrasses

Seagrasses form a distinct ecological group of aquatic angiosperms, encompassing four families (Cymodoceaceae, Hydrocharitaceae, Posidoniaceae, and Zosteraceae), and comprising approximately 72 species that are specially adapted to thrive in marine environments (Short et al., 2011). They occur in coastal regions of all continents, except Antarctica (Short et al., 2007), forming dense meadows that enhance the structural complexity of coastal ecosystems and provide valuable ecosystem services (Hemminga & Duarte, 2000; Nordlund et al., 2016).

In the Mediterranean Sea, one of the most prominent species is the endemic seagrass *Posidonia oceanica*, commonly known as Neptune grass. It forms extensive seagrass meadows in shallow coastal areas and can be found down to 45 meters below sea level (Marbà et al., 2014). *Posidonia oceanica* has a number of morphological characteristics that are well-suited to its life in the marine environment. Its leaves are elongated, ribbon-shaped, and covered by a protective cuticle. The leaves are connected to the rhizomes by a lignified leaf sheath that also shields the plant's basal meristem. The rhizome, an elongated stem-like structure, allows clonal propagation and connects multiple leaf bundles, contributing to the formation of dense seagrass meadows (Hemminga & Duarte, 2000).

Over time, the leaf canopy traps sediment and to avoid being buried, rhizomes of *P. oceanica* grow vertically. The deposited structure of living and dead rhizomes and roots, together with the sediment filling the gaps, is known as the seagrass 'matte'. Sediment trapping and vertical rhizome growth cause the matte, and thus the seafloor, to rise between 10 and 100 cm per century over time (Boudouresque et al., 2016).

Fig. 1.1. *Posidonia oceanica* with exposed rhizomes and matte. Credit: Dimitris Poursanidis / Ocean Image Bank

Posidonia oceanica plays a crucial role in the Mediterranean ecosystem and provides important ecosystem services. It acts as a key habitat provider for a wide range of marine organisms and contributes to the structural complexity of coastal habitats and provides essential breeding and feeding grounds for various fish species, invertebrates, and other marine flora (Boudouresque et al., 2006; Campagne et al., 2015). *Posidonia oceanica* is also important for water purification through filtration, protection against coastal erosion, water oxygenation, nutrient cycling, carbon sequestration, and has a considerable recreational value (Campagne et al. 2015 and references therein). The estimated economic value of Mediterranean *P. oceanica* meadows based on these ecosystem services ranges from 284 to 514 €/ha/year (Campagne et al., 2015).

BIOGEOCHEMICAL CYCLING OF CARBON AND NITROGEN IN SEAGRASSES

One of the most remarkable features of seagrass meadows is their high binding capacity of organic carbon (C). Over the past decades, there has been growing interest in C sequestration by coastal ecosystems (mangroves, tidal marshes, and seagrasses meadows); the so-called 'blue carbon' (Nellemann et al., 2010). Carbon sequestration by seagrasses is estimated for ca. 10 % of total blue carbon, although seagrass meadows cover less than 0.2% of the area of the world's oceans (Duarte et al., 2005; Fourqurean et al., 2012). Seagrass restoration and protection are therefore now widely recognized as effective strategies for reducing carbon dioxide (CO_2) emissions and mitigating future climate change scenarios (Duarte et al., 2013; Greiner et al., 2013; Macreadie et al., 2021). Because of its high primary production and long-term C storage in its matte, *Posidonia oceanica* is recognized as one of the most effective seagrasses for C fixation and sequestration (Boudouresque et al., 2006; Pergent-Martini et al., 2021). With the potential for inorganic and organic C to exist in these seagrass mattes for millennia (Monnier et al., 2020), C sequestration in *P. oceanica* meadows can even rival peatlands (Strack, 2008) or mangroves (Bouillon et al., 2008),

Seagrasses also play an important role as highly effective nutrient recyclers (Hemminga et al., 1991; Welsh, 2000). Nitrogen (N) cycling within seagrass ecosystems is of considerable importance, not only for the well-being of the seagrass plants themselves but also for the entire ecosystem (Hemminga et al., 1991). The N cycle in seagrass habitats is highly complex (see Fig. 1.2.), consisting of numerous interrelated processes that eventually transform atmospheric nitrogen gas (N_2) into biologically accessible forms, such ammonium (NH_4^+) and nitrate (NO_3^-), and vice versa (Herbert, 1999).

Nitrogen input, primarily through N_2 fixation by diazotrophic microorganisms, turns atmospheric N_2 into NH_3, and thus provides essential bioavailable N sources not only to the seagrass but also to the diverse organisms that graze upon it (Heck & Valentine, 2006). Studies show that *P. oceanica* meadows are usually net sources of dissolved organic nitrogen which is important in the N cycle and budget of the Mediterranean Sea (Barrón & Duarte, 2009).

Nitrification is a two-step process occurring under aerobic conditions and converts NH_4^+ first to NO_2^- and then to NO_3^-. This NO_3^- can serve as substrate for canonical denitrification, a multi-step process in which NO_3^- is first converted to NO_2^-, then to NO and N_2O, and finally to N_2 gas under hypoxic or anoxic conditions (Ward, 2008). Seagrass sediments, especially in warmer (sub-)tropical regions offer an environment where nitrification and

denitrification can be tightly coupled (Eyre et al., 2013; Hoffman et al., 2019). Via the seagrass rhizosphere, O_2 diffuses into the sediment, creating a mosaic of oxic and anoxic microenvironments. Additionally, the relatively high organic content of seagrass sediments, resulting from the input of organic particulate matter and root exudation processes, offers an abundant substrate for NH_4^+ regeneration, promoting nitrification and thus enhancing denitrification (Caffrey & Kemp, 1990). N-loss pathways, such as denitrification or the anaerobic ammonium oxidation (anammox), can regulate excess nutrients in seagrass sediments and thus mitigate excess N loads (Garcias-Bonet et al., 2018).

While N cycling in seagrass sediments (Herbert, 1999; Holmer, 2019; Salk et al., 2017) or the rhizosphere (Lehnen et al., 2016; Mohr et al., 2021) has been the focus of extensive research, precise quantification of N cycling processes associated with other parts of the plant, like the leaves, is still sparse.

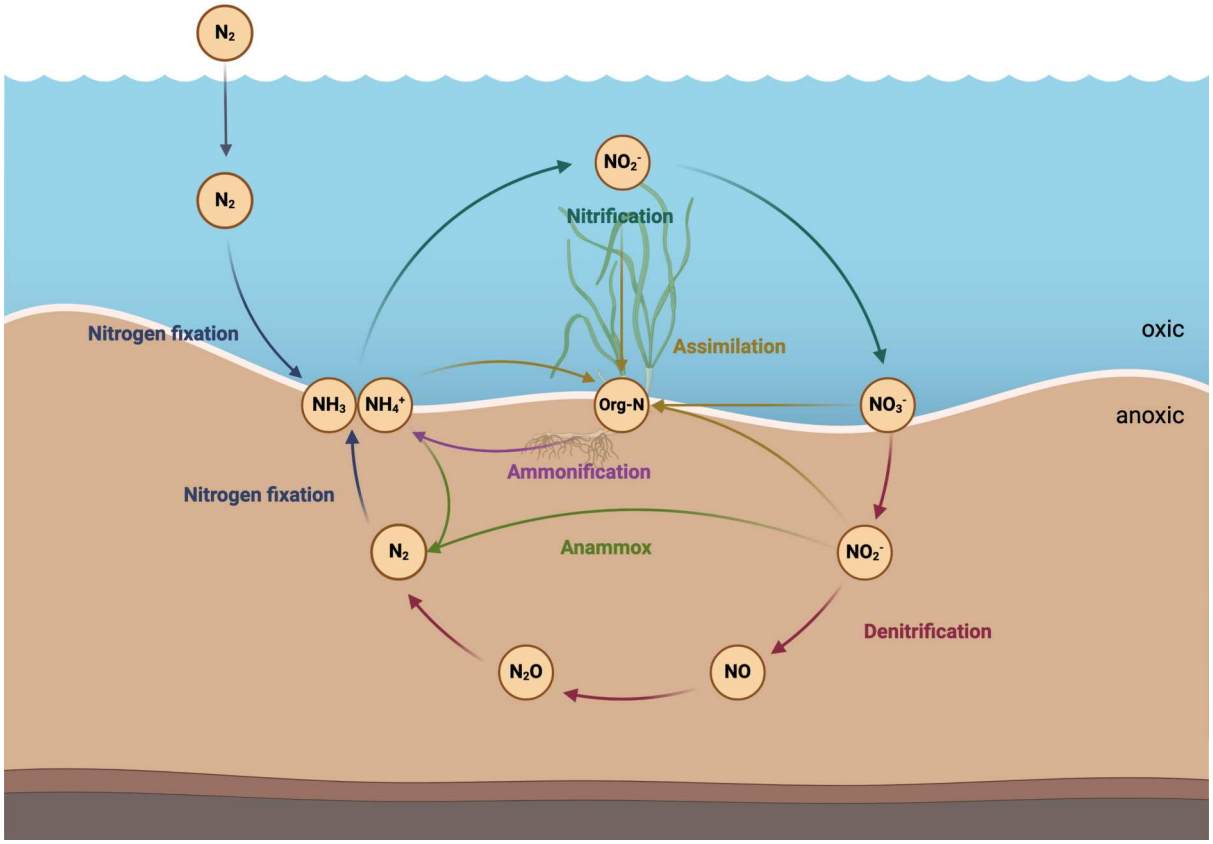

Fig. 1.2. A simplified illustration of the marine nitrogen cycle. Created with BioRender.com

The Effects of Climate Change on Seagrasses

The Mediterranean Sea, being semi-enclosed and relatively shallow, is often referred to as a hotspot for climate change, for example experiencing seawater warming 20% faster than the global average (Vafeidis et al., 2020). Seawater warming has significant effects on seagrasses at the molecular, physiological, morphological, and ecosystem level (Nguyen et al., 2021). Ocean warming can induce heat stress, reducing their photosynthetic capacity and thus affecting growth rates, leaf and shoot quantity, and overall resilience (e.g., Koch et al., 2013; Lee et al., 2007; Nguyen et al., 2021). Long-term warming may also result in local and regional extinctions, especially for species that are adapted to cooler environments (Marbà & Duarte, 2010). Additionally, warmer

waters in combination with eutrophication can promote algal blooms and invasive species, that have a higher temperature tolerance and thus outcompete the seagrass plants (Burkholder et al., 2007; Lee et al., 2007).

At the same time, the Mediterranean Sea is able to absorb relatively more anthropogenic CO_2 per unit area than the global ocean because it is more alkaline and because deep waters are ventilated over shorter timescales (Schneider et al., 2010), allowing rapid penetration of CO_2 in its interior. Thus, the Mediterranean Sea may experience significant ocean acidification (OA, i.e. the decrease in seawater pH resulting from increased dissolution of atmospheric CO_2) over the current century, with seawater pH predicted to decrease between 0.245 and 0.462 units by 2100, depending on the IPCC SRES scenario (Goyet et al., 2016; IPCC, 2023).

Ocean acidification negatively impacts many habitat-forming species, such as calcifying algae, corals, or molluscs, with cascading effects on the entire marine ecosystem (Doney et al., 2009; Kroeker et al., 2013; Riebesell et al., 2000). Marine macrophytes on the other hand may benefit from increased CO_2 concentrations as their photosynthetic rates are often C-limited at current CO_2 levels (M. Koch et al., 2013). However, OA can have complex effects on seagrasses. Several studies showed increased primary production, growth, and shoot density under increased CO_2 concentrations (Cox et al., 2015; Egea et al., 2018; Hernán et al., 2016; Zimmerman et al., 2017), but also increased herbivory on the leaves (Cox et al., 2016; Scartazza et al., 2017). Our knowledge about the effects of OA on the biogeochemical cycling of C and N associated with seagrass is still limited.

Not only the seagrass, but also organisms growing on the leaf surface are affected by changing environmental conditions. Calcareous epiphytes including encrusting red algae, bryozoans, foraminifers, and spirorbids decline under low pH, while non-calcareous invertebrates like hydrozoans or tunicates can benefit (Donnarumma et al., 2014; Gravili et al., 2021; Mecca et al., 2020). This community shift in the epiphytic community can have cascading effects on the seagrass trophic network and ecosystem functioning. While the effects of OA on the macro-epiphytic community have received some attention, there is still a knowledge gap regarding the effects of reduced pH on the seagrass microbiome and microbe-associated metabolic processes.

THE SEAGRASS HOLOBIONT

Similar to other benthic marine organisms, seagrasses such as *P. oceanica* harbour diverse and abundant communities of microorganisms and larger eukaryotic endo- and epiphytes (Borowitzka et al., 2006). These organisms play an important role in shaping seagrass physiology and health (see Fig. 1.3), while also exerting control over biogeochemical processes in seagrass meadows (Seymour et al., 2018; Tarquinio et al., 2019; Ugarelli et al., 2017). The close relationship between seagrasses and associated organisms supports the idea that they together form a 'holobiont', a concept first described by Margulis (1991). In the holobiont, the host organism and its associated partners represent a biological unit in which each member is supported for its success (Margulis, 1991). However, the spectrum of ecological interactions occurring between seagrasses and associated organisms encompasses a wide range, from mutualistic relationships to parasitism (Seymour et al., 2018).

On and within seagrass leaves, roots, rhizomes, and the surrounding sediments, discrete populations of bacteria, fungi, microalgae, archaea, and viruses exist (Borowitzka et al., 2006). Seagrasses and their associated

microbiome interact within various microniches across different plant parts and the nearby sediments. These microenvironments host-specific microbial communities adapted to local conditions, influencing the seagrass both positively and negatively (Seymour et al., 2018).

Seagrass leaves host diverse microbial communities, including bacteria, fungi, and protists. The seagrass phyllosphere is primarily colonized by surrounding heterotrophic bacterioplankton, particularly by groups typically capable of degrading polymers and known for surface attachment and biofilm formation (Ugarelli et al., 2017). Seagrass-associated microorganisms dominate and vary seasonally, with different species peaking in summer and winter. Bacteria can promote leaf growth by providing nutrients and controlling algal growth (Celdrán et al., 2012), while others break down aging leaves (Barnabas, 1992). The most abundant and diverse epiphytes on seagrasses are algae, ranging from unicellular diatoms and dinoflagellates, present on nearly all seagrasses, to large macrophytes (Borowitzka et al., 2006). Epiphytic algae are important primary producers within the seagrass ecosystem and contribute significantly to the food web. They can comprise more than 50% of the primary production in seagrass meadows (Hasegawa et al., 2007; Wear et al., 1999). However, our knowledge of the role of epiphytes in the productivity and biogeochemical cycling within the seagrass holobiont, especially in response to environmental changes, is still limited. Seagrasses also host a variety of other epiphytes, including protozoa, sponges, bryozoans, hydroids, and ascidians. Additionally, sessile invertebrates such as sponges, crustaceans and molluscs can inhabit the seagrass phyllosphere (Borowitzka et al., 2006).

Microorganisms can also live endophytically within seagrass leaves and roots, residing between and inside plant cells. These microbes can be symbionts, benefiting the plant, or pathogens that may become harmful under certain conditions (Seymour et al., 2018). Few studies have explored the diversity of seagrass endophytes, but research on *Posidonia oceanica* indicates a low number of bacterial taxa, suggesting high specialization for an endophytic lifestyle (Garcias-Bonet et al., 2012). Endophytes in roots resemble microbes found in surrounding anoxic sediments and are organized by oxygen gradients (Seymour et al., 2018).

The seagrass rhizosphere harbors about twice the bacterial abundance and biomass compared to nearby non-vegetated sediments. Unlike the phyllosphere, the rhizosphere microbiome shows significant differences from adjacent sediment communities and is more diverse (Ugarelli et al., 2017 and references therein). Seagrass roots release dissolved organic carbon (DOC, Sogin et al., 2022) and oxygen (Brodersen et al., 2015), influencing the microbial activity and sediment chemistry. Oxygen transported from shoots leaks into the sediment, creating a redox gradient that expands oxic zones below the sediment surface, providing a dynamic environment for a diverse group of microorganisms (Seymour et al., 2018 and references therein).

Microorganisms colonizing seagrass surfaces have to withstand plant defense and competition from other microbes (Egan et al., 2013), but benefit from dissolved organic nutrients, such as C, N, and phosphorus (P) released by the plant (Tarquinio et al., 2019 and references therein). Seagrass roots and rhizomes exude up to 11% of organic C produced during photosynthesis, which can be metabolized by microorganisms (Holmer et al., 2001; Moriarty et al., 1986). Seagrasses can also provide iron (Brodersen et al., 2017), crucial for bacterial communication and biofilm formation (Vlamakis et al., 2013). Seagrasses also excrete

dimethylsulfoniopropionate (DMSP, Borges & Champenois, 2015), which some seagrass-associated microorganisms can use as source of sulfur (Egan et al., 2013).

Seagrass growth can be limited by nutrient availability, particularly N and P (Touchette & Burkholder, 2000). Microorganisms associated with seagrasses therefore play a key role in enhancing nutrient access, for example, via N_2 fixation or by mineralizing organic compounds (Evrard et al., 2005; Welsh, 2000). Cyanobacteria on seagrass leaves and sulfate-reducing bacteria on the roots are capable of N_2 fixation and can contribute significantly to the plant's N demand (Agawin et al., 2016; Welsh, 2000). Sulfate-reducing bacteria are able to mineralize organic nutrients by reducing sulfate, supporting nutrients for seagrass growth (Holmer et al., 2001). Their activity leads to toxic hydrogen sulfide accumulation that is highly toxic to seagrasses (Bagarinao, 1992) and potential cause of global seagrass die-offs (Borum et al., 2005; M. S. Koch & Erskine, 2001). Seagrasses can counter this by translocating photosynthetically produced oxygen from the leaves to the roots, where it leaks into the sediment and promotes sulfide oxidation (Borum et al., 2006). Nitrate-reducing sulfur-oxidizing bacteria may help oxidizing sulfides and mitigating their toxic effects (Lee & Dunton, 2000). Additionally, some bacterial taxa associated with seagrass roots are able to solubilize inorganic phosphorus from insoluble compounds, thus increasing nutrient availability for the plant (Jose & Jebakumar, 2014).

Some seagrass-associated microorganisms can also produce phytohormones that regulate various aspects of plant growth, including seed germination, flowering, and fruit production (e.g., Celdrán et al., 2012; Werner & Schmülling, 2009). Furthermore, microorganisms associated with seagrasses can produce a variety of bioactive metabolites that can protect the plant host from pathogens and biofouling (Armstrong et al., 2001). A prominent example of a seagrass pathogen is the protist *Labyrinthula* spp., the cause of wasting disease that decimated up to 90% of *Zostera marina* in the Northern Hemisphere (Sullivan et al., 2013).

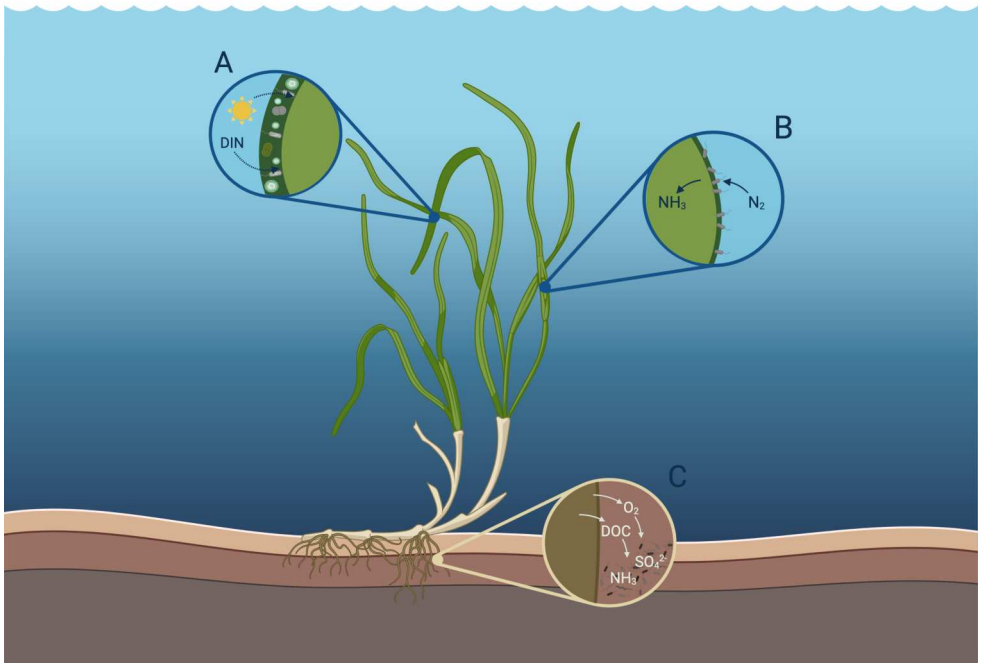

Fig. 1.3. Example of important seagrass-microbe interactions. A: Epiphytic growth of algae, fungi, and heterotrophic microbes on leaves reduces incident irradiance and access to dissolved inorganic nitrogen (DIN) from the water column. B: N_2-fixing microbial biofilm enhances N uptake through the leaves. C: O_2 and DOC released from the plant stimulate microbial processes, including sulfur cycling and N_2 fixation, in the roots and rhizosphere. Adapted from Seymour et al. (2018). Created with BioRender.com

THE SEAGRASS NESTED ECOSYSTEM

Recent studies highlight the importance of nested facilitative interactions in seagrass ecosystems, particularly facultative mutualisms with invertebrates and their microbial communities, especially under stressful conditions (Malkin & Cardini, 2021). For example, sediment-dwelling macrofauna like lucinid bivalves and their microbial symbionts can enhance seagrass resilience by promoting nutrient recycling and detoxifying harmful sulfides (Cardini et al., 2022). These nested interactions, or networks of holobionts, are referred to as nested ecosystems (Pita et al., 2018).

Among the benthic holobionts that commonly associate with seagrasses are marine sponges (Ávila et al., 2015; Soest et al., 2012), which can grow in close association with the plant. Sponges are filter feeders, capable of turning over many thousands of litres of water per day (Godefroy et al., 2019). They often host dense and diverse microbial communities themselves (Thomas et al., 2016), and are classified into two categories (i.e. high or low microbial abundance – HMA or LMA) depending on the number of extracellular bacteria that populate their mesohyl matrix (Hentschel et al., 2006). The microbial community of marine sponges plays a vital role in nutrient cycling (especially C and N), vitamin synthesis, and defense (Hentschel et al., 2012). Photosynthetic symbionts such as cyanobacteria provide organic C to the sponge (Arillo et al., 1993).

Sponges excrete large amounts of dissolved inorganic nitrogen (DIN), as they produce ammonia as a metabolic waste product (Corredor et al., 1988; Diaz & Ward, 1997). Many symbionts in the sponge microbiome play a role in N metabolism, especially aerobic processes such as ammonia oxidation, nitrification, or N_2 fixation, but also anaerobic processes (e.g., denitrification, anammox) (Pita et al., 2018 and references therein).

Sponges take up dissolved organic matter (DOM) produced by primary producers, such as corals, algae, or seagrasses (De Goeij et al., 2013; Rix et al., 2017). This DOM is converted into particulate organic matter (POM) and released by the sponge as detritus or taken up by sponge-associated organisms. Through this ‘sponge loop’, sponges act as intermediaries, efficiently retaining and redistributing nutrients in coastal environments, thus supporting their overall productivity and stability (De Goeij et al., 2013).

Seagrasses are known to release substantial amounts of DOM into the surrounding seawater and sediments (Sogin et al., 2022), that can be taken up by the sponges and their microbiome. The plants can also provide physical substrate for sponge growth (Archer et al., 2015). Seagrasses on the other side may benefit from dissolved inorganic nitrogen (DIN) released by sponges through ammonium excretion, N_2 fixation, or nitrification (Davy et al., 2002; Jiménez & Ribes, 2007; Fiore et al., 2010; Rix et al., 2015), which can help to overcome N-limitation in oligotrophic areas. Understanding the mechanisms and rates of C and N cycling in these seagrass-sponge associations is crucial for unraveling the complexities of nutrient dynamics in coastal ecosystems and holds implications for ecosystem functioning.

SPECIFIC KNOWLEDGE GAPS

1|The seagrass phyllosphere is colonized by a multitude of photosynthetically active epiphytes, and their biomass often represents a significant proportion of the primary producer biomass (Borowitzka et al., 2006). However, our knowledge of the extent of their contribution to the primary production of the seagrass holobiont is still limited. Like the seagrass plants, epiphytes are facing environmental changes, such as OA. Growth and primary production of marine macrophytes generally benefit from increased CO_2 concentrations, as they are often C-limited under current C concentrations (M. Koch et al., 2013). The effects of OA on the epiphyte community and their contribution to the holobiont productivity with potential consequences for the ecosystem, are not well understood.

2|Biogeochemical cycling of N in seagrass sediments (Herbert, 1999; Holmer, 2019; Salk et al., 2017) and in the seagrass rhizosphere (Lehnen et al., 2016; Mohr et al., 2021) plays an important role in the seagrass ecosystem. However, precise quantification of N cycling processes associated with other plant parts such as the seagrass phyllosphere, and their contribution to the N demand of the seagrass holobiont, is still sparse. Nitrogen cycling processes, such as N_2 fixation or nitrification, can be negatively affected by changes in the seawater pH (Wannicke et al., 2018; Wyatt et al., 2010). Our knowledge of the effects of OA on N cycling processes associated with seagrass leaves is sparse. There is also still a knowledge gap regarding the effects of a reduced pH on the seagrass microbiome and its role in the functioning of the seagrass holobiont.

3|Seagrasses interact with a multitude of organisms, forming holobiont networks often described as nested ecosystems (Pita et al., 2018). In the Mediterranean Sea, the high microbial abundance (HMA) sponge *Chondrilla nucula* is frequently associated with *P. oceanica*. However, a clear categorization of the seagrass-sponge relationship within the spectrum of symbiotic interactions—ranging from mutualism to competition—remains absent. Additionally, the mechanisms governing the seagrass-sponge-microbe interaction, particularly in relation to primary production and the cycling of dissolved organic carbon (DOC), dissolved organic nitrogen (DON), and inorganic nutrients, are yet to be explored.

4|Sponge-associated nitrification is producing the bulk of the DIN released by the sponge (Diaz & Ward, 1997; Southwell et al., 2008) and could potentially be important for the seagrass holobiont when growing in association. Understanding the mechanisms and rates of nitrification in these seagrass-sponge associations is crucial for unraveling the complexity of nutrient dynamics in coastal ecosystems and has implications for ecosystem functioning.

AIMS AND APPROACH

The overarching aim of this thesis is to address some of the critical knowledge gaps in C and N cycling in the seagrass holobiont and nested ecosystem, especially under changing environmental conditions. This PhD thesis includes four main studies investigating C and N cycling and microbial transformations in the phyllosphere of the *P. oceanica* holobiont under OA (1, 2) as well as in the nested association between *P. oceanica* and the sponge *C. nucula* (3, 4) to answer the following research questions:

1|How does the epiphytic community contribute to seagrass productivity? What are the effects of OA on the productivity of seagrass leaves and their epiphytes? What is the ecological relevance?

2|What are the rates of key microbial N cycling processes under ambient and OA conditions in the *P. oceanica* phyllosphere and how do these processes contribute to the N demand of the seagrass holobiont? How does OA affect the diversity of the microbial community on *P. oceanica* leaves? What role does the microbial community on *P. oceanica* leaves play in N cycling?

3|How does the association between *P. oceanica* and the sponge *C. nucula* affect primary production, inorganic and organic nutrient fluxes in the seagrass holobiont? What are the potential benefits for the seagrass and the sponge?

4|What are the potential nitrification rates in the association between *P. oceanica* and *C. nucula*? Does the microbiome of *C. nucula* harbor nitrifying microorganisms and can microbial nitrification contribute to the seagrass holobiont N demand?

Fieldwork was carried out in the Gulf of Naples (Tyrrhenian Sea, Italy). To answer parts 1 and 2, experiments with *P. oceanica* were carried out at the eastern coast of Ischia Island. This site is characterized by the presence of submarine CO_2 vents of volcanic origin, which naturally generate a gradient in CO_2 concentration and pH. Laboratory incubation experiments with *P. oceanica* leaves from vent and ambient pH sites were performed to assess net primary production and respiration of seagrass leaves and epiphytes. Field experiments with incubation chambers were conducted to measure net community production and respiration directly in the seagrass meadow. Stable isotope tracers of ^{15}N were used in laboratory incubation experiments to quantify N cycling processes in the *P. oceanica* phyllosphere. 16s rRNA gene amplicon sequencing was used to investigate the diversity of the microbial community associated with the *P. oceanica* phyllosphere, and the microorganisms potentially involved in N transformation processes.

Parts 3 and 4 were carried out in the area of Bacoli in the Gulf of Naples (Tyrrhenian Sea, Italy). Here, patches of *P. oceanica* meadows with a high abundance of *C. nucula* growing in close association with the seagrass exist. Underwater surveys were combined with incubation experiments for assessing fluxes of oxygen, organic and inorganic nutrients. Incubation experiments with stable isotope tracers of ^{15}N were used to assess potential nitrification rates (PNR) of the seagrass-sponge association. These experiments were complemented with 16s rRNA gene amplicon sequencing to explore the diversity of the sponge microbial community, with a specific focus on nitrifying microorganisms.

THESIS STRUCTURE AND OUTLINE

In this dissertation, I investigated various aspects of host-microbe interactions on marine seagrasses, aiming to enhance our understanding of the role of these associations in benthic C and N cycling under changing environmental conditions. **Chapters 1 and 6** are the general introduction and discussion of this thesis, which establish and discuss the main research questions. All other chapters are either published manuscripts, or manuscripts in preparation for publication.

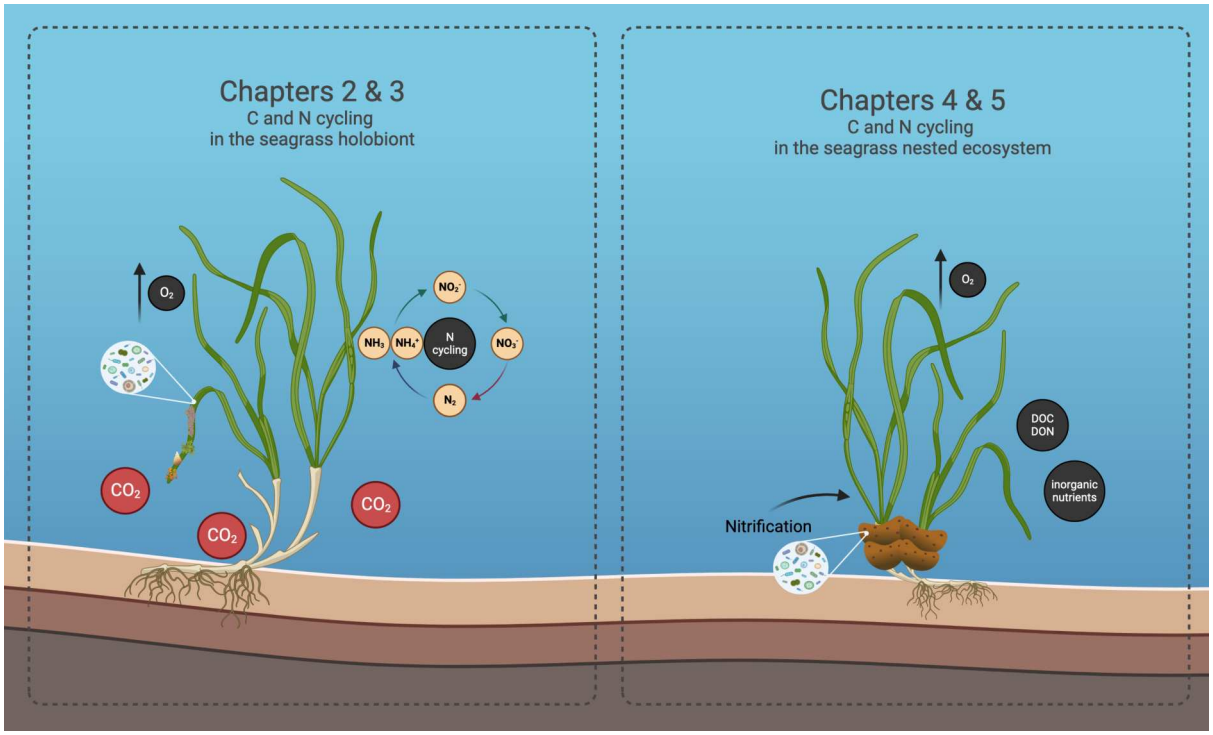

Fig. 1.4. Overview of thesis chapters. Chapters 2 & 3 investigate C and N cycling in the *P. oceanica* holobiont under OA, while chapters 4 & 5 focus on C and N cycling in a seagrass nested ecosystem with the sponge *C. nucula*. Created with BioRender.com

In **Chapter 2** we investigated the contribution of seagrass epiphytes to net primary production and respiration of *Posidonia oceanica* leaves in incubation experiments. Additionally, we assessed how natural exposure to increased CO_2 concentrations at volcanic vents affects oxygen fluxes in the seagrass phyllosphere and the *in situ* seagrass community, as well as on the epiphytic community cover and composition.

Chapter 3 quantifies rates of key nitrogen cycling processes (N_2 fixation, nitrification, and denitrification) in the *P. oceanica* phyllosphere, how these rates are affected by naturally increased CO_2 concentrations, and how these processes contribute to the overall N demand of the seagrass holobiont. We also investigated the microbial community structure associated with *P. oceanica* leaves under CO_2 exposure with a focus on potential players involved in N cycling processes.

Chapter 4 presents the association between *P. oceanica* and the sponge *Chondrilla nucula* and explores potential mutualistic benefits. We investigated whether this association has an effect on seagrass shoot density and sponge cover with underwater surveys, or on fluxes of oxygen, carbon, and nitrogen at the community level, using incubation experiments.

In **Chapter 5** we quantified potential nitrification rates in the association between *P. oceanica* and *C. nucula* in incubation experiments using stable isotopes of ^{15}N and explored the potential of *C. nucula*-associated nitrification to contribute to the N demand of the *P. oceanica* holobiont. Additionally, we investigated the sponge microbiome with a specific focus on potential nitrifiers, using 16s rRNA sequencing.

REFERENCES

Agawin, N. S. R., Ferriol, P., Cryer, C., Alcon, E., Busquets, A., Sintes, E., Vidal, C., & Moyà, G. (2016). Significant nitrogen fixation activity associated with the phyllosphere of Mediterranean seagrass Posidonia oceanica: First report. *Marine Ecology Progress Series*, *551*, 53–62. https://doi.org/10.3354/MEPS11755

Archer, S. K., Stoner, E. W., & Layman, C. A. (2015). A complex interaction between a sponge (Halichondria melanadocia) and a seagrass (Thalassia testudinum) in a subtropical coastal ecosystem. *Journal of Experimental Marine Biology and Ecology*, *465*, 33–40. https://doi.org/10.1016/J.JEMBE.2015.01.003

Arillo, A., Bavestrello, G., Burlando, B., & Sarà, M. (1993). Metabolic integration between symbiotic cyanobacteria and sponges: A possible mechanism. *Marine Biology*, *117*(1), 159–162. https://doi.org/10.1007/BF00346438

Armstrong, E., Yan, L., Boyd, K. G., Wright, P. C., & Burgess, J. G. (2001). The symbiotic role of marine microbes on living surfaces. *Hydrobiologia*, *461*, 37–40.

Ávila, E., Ávila-García, A. K., & Cruz-Barraza, J. A. (2015). Temporal and small-scale spatial variations in abundance and biomass of seagrass-dwelling sponges in a tropical estuarine system. *Marine Ecology*, *36*(3), 623–636. https://doi.org/10.1111/maec.12171

Bagarinao, T. (1992). Sulfide as an environmental factor and toxicant: Tolerance and adaptations in aquatic organisms. *Aquatic Toxicology*, *24*(1), 21–62. https://doi.org/10.1016/0166-445X(92)90015-F

Barnabas, A. D. (1992). Bacteria on and within leaf blade epidermal cells of the seagrass *Thalassodendron ciliatum* (Forssk.) Den Hartog. *Aquatic Botany*, *43*(3), 257–266. https://doi.org/10.1016/0304-3770(92)90071-P

Barrón, C., & Duarte, C. M. (2009). Dissolved organic matter release in a Posidonia oceanica meadow. *Marine Ecology Progress Series*, *374*, 75–84. https://doi.org/10.3354/MEPS07715

Borges, A. V., & Champenois, W. (2015). Seasonal and spatial variability of dimethylsulfoniopropionate (DMSP) in the Mediterranean seagrass *Posidonia oceanica*. *Aquatic Botany*, *125*, 72–79. https://doi.org/10.1016/j.aquabot.2015.05.008

Borowitzka, M. A., Lavery, P., & Van Keulen, M. (2006). Epiphytes of Seagrasses. In *Seagrasses: Biology, Ecology and Conservation* (pp. 441–461).

Borum, J., Pedersen, O., Greve, T. M., Frankovich, T. A., Zieman, J. C., Fourqurean, J. W., & Madden, C. J. (2005). The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, Thalassia testudinum. *Journal of Ecology*, *93*(1), 148–158. https://doi.org/10.1111/j.1365-2745.2004.00943.x

Borum, J., Sand-Jensen, K., Binzer, T., Pedersen, O., Greve, T., Larkum, A., Orth, R., & Duarte, C. (2006). Oxygen Movement in Seagrasses. In *Seagrasses: Biology, Ecology and Conservation* (pp. 255–270). https://doi.org/10.1007/1-4020-2983-7_10

Boudouresque, C. F., Mayot, N., & Pergent, G. (2006). The outstanding traits of the functioning of the Posidonia oceanica seagrass ecosystem. *Biol. Mar. Medit.*, *13*(4), 109–113.

Boudouresque, C. F., Pergent, G., Pergent-Martini, C., Ruitton, S., Thibaut, T., & Verlaque, M. (2016). The necromass of the Posidonia oceanica seagrass meadow: Fate, role, ecosystem services and vulnerability. *Hydrobiologia*, *781*(1), 25–42. https://doi.org/10.1007/s10750-015-2333-y

Bouillon, S., Borges, A. V., Castañeda-Moya, E., Diele, K., Dittmar, T., Duke, N. C., Kristensen, E., Lee, S. Y., Marchand, C., Middelburg, J. J., Rivera-Monroy, V. H., Smith III, T. J., & Twilley, R. R. (2008). Mangrove production and carbon sinks: A revision of global budget estimates. *Global Biogeochemical Cycles*, *22*(2). https://doi.org/10.1029/2007GB003052

Brodersen, K. E., Koren, K., Moßhammer, M., Ralph, P. J., Kühl, M., & Santner, J. (2017). Seagrass-Mediated Phosphorus and Iron Solubilization in Tropical Sediments. *Environmental Science & Technology*, *51*(24), 14155–14163. https://doi.org/10.1021/acs.est.7b03878

Brodersen, K. E., Nielsen, D. A., Ralph, P. J., & Kühl, M. (2015). Oxic microshield and local pH enhancement protects ostera muelleri from sediment derived hydrogen sulphide. *New Phytologist*, *205*(3), 1264–1276. https://doi.org/10.1111/nph.13124

Burkholder, J. M., Tomasko, D. A., & Touchette, B. W. (2007). Seagrasses and eutrophication. *Journal of Experimental Marine Biology and Ecology*, *350*(1), 46–72. https://doi.org/10.1016/j.jembe.2007.06.024

Caffrey, J., & Kemp, W. (1990). Nitrogen cycling in sediments with estuarine populations of Potamogeton perfoliatus and Zostera marina. *Marine Ecology Progress Series*, *66*, 147–160. https://doi.org/10.3354/meps066147

Campagne, C. S., Salles, J.-M., Boissery, P., & Deter, J. (2015). The seagrass Posidonia oceanica: Ecosystem services identification and economic evaluation of goods and benefits. *Marine Pollution Bulletin*, *97*, 391–400. https://doi.org/10.1016/j.marpolbul.2015.05.061

Cardini, U., Marín-Guirao, L., Montilla, L. M., Marzocchi, U., Chiavarini, S., Rimauro, J., Quero, G. M., Petersen, J. M., & Procaccini, G. (2022). Nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning in contaminated sediments. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.918675

Celdrán, D., Espinosa, E., Sánchez-Amat, A., & Marín, A. (2012). Effects of epibiotic bacteria on leaf growth and epiphytes of the seagrass Posidonia oceanica. *Marine Ecology Progress Series*, *456*, 21–27. https://doi.org/10.3354/MEPS09672

Corredor, J. E., Wilkinson, C. R., Vicente, V. P., Morell, J. M., & Otero, E. (1988). Nitrate release by Caribbean reef sponges. *Limnology and Oceanography*, *33*(1), 114–120. https://doi.org/10.4319/lo.1988.33.1.0114

Cox, T. E., Gazeau, F., Alliouane, S., Hendriks, I. E., Mahacek, P., Le Fur, A., & Gattuso, J. P. (2016). Effects of in situ CO2 enrichment on structural characteristics, photosynthesis, and growth of the Mediterranean seagrass Posidonia oceanica. *Biogeosciences*, *13*(7), 2179–2194. https://doi.org/10.5194/bg-13-2179-2016

Cox, T. E., Schenone, S., Delille, J., Diaz-Castaneda, V., Alliouane, S., Gattuso, J.-P., & Gazeau, F. (2015). Effects of ocean acidification on Posidonia oceanica epiphytic community and shoot productivity. *Journal of Ecology*, *103*, 1594–1609. https://doi.org/10.1111/1365-2745.12477

De Goeij, J. M., Van Oevelen, D., Vermeij, M. J. A., Osinga, R., Middelburg, J. J., De Goeij, A. F. P. M., & Admiraal, W. (2013). Surviving in a Marine Desert: The Sponge Loop Retains Resources Within Coral Reefs. *Science*, *342*(6154), 108–110. https://doi.org/10.1126/science.1241981

Diaz, M. C., & Ward, B. B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series*, *156*, 97–107.

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean Acidification: The Other CO 2 Problem. *Annu.Rev.Mar.Sci.*, *1*, 169–192. https://doi.org/10.1146/annurev.marine.010908.163834

Donnarumma, L., Lombardi, C., Cocito, S., & Gambi, M. C. (2014). Settlement pattern of Posidonia oceanica epibionts along a gradient of ocean acidification: An approach with mimics. *Mediterranean Marine Science*, *15*(3), 498–509. https://doi.org/10.12681/MMS.677

Duarte, C. M., Losada, I. J., Hendriks, I. E., Mazarrasa, I., & Marbà, N. (2013). The role of coastal plant communities for climate change mitigation and adaptation. *Nature Climate Change 2013 3:11*, *3*(11), 961–968. https://doi.org/10.1038/nclimate1970

Duarte, C. M., Middelburg, J. J., & Caraco, N. (2005). Major role of marine vegetation on the oceanic carbon cycle. *Biogeosciences*, *2*(1), 1–8. https://doi.org/10.5194/bg-2-1-2005

Egan, S., Harder, T., Burke, C., Steinberg, P., Kjelleberg, S., & Thomas, T. (2013). The seaweed holobiont: Understanding seaweed–bacteria interactions. *FEMS Microbiology Reviews*, *37*(3), 462–476. https://doi.org/10.1111/1574-6976.12011

Egea, L. G., Jimé Nez-Ramos, R., Herná Ndez, I., Bouma, T. J., & Brun, F. G. (2018). Effects of ocean acidification and hydrodynamic conditions on carbon metabolism and dissolved organic carbon (DOC) fluxes in seagrass populations. *PLoS ONE*, 1–20. https://doi.org/10.1371/journal.pone.0192402

Evrard, V., Kiswara, W., Bouma, T. J., & Middelburg, J. J. (2005). Nutrient dynamics of seagrass ecosystems: 15N evidence for the importance of particulate organic matter and root systems. *Marine Ecology Progress Series*, *295*, 49–55. https://doi.org/10.3354/meps295049

Eyre, B. D., Maher, D. T., & Squire, P. (2013). Quantity and quality of organic matter (detritus) drives N2 effluxes (net denitrification) across seasons, benthic habitats, and estuaries. *Global Biogeochemical Cycles*, *27*(4), 1083–1095. https://doi.org/10.1002/2013GB004631

Fourqurean, J. W., Duarte, C. M., Kennedy, H., Marbà, N., Holmer, M., Mateo, M. A., Apostolaki, E. T., Kendrick, G. A., Krause-Jensen, D., Mcglathery, K. J., & Serrano, O. (2012). Seagrass ecosystems as a globally significant carbon stock. *Nature Geoscience*, *5*, 505–509. https://doi.org/10.1038/NGEO1477

Garcias-Bonet, N., Arrieta, J. M., de Santana, C. N., Duarte, C. M., & Marbà, N. (2012). Endophytic bacterial community of a Mediterranean marine angiosperm (Posidonia oceanica). *Frontiers in Microbiology*, *3*. https://doi.org/10.3389/fmicb.2012.00342

Garcias-Bonet, N., Fusi, M., Ali, M., Shaw, D. R., Saikaly, P. E., Daffonchio, D., & Duarte, C. M. (2018). High denitrification and anaerobic ammonium oxidation contributes to net nitrogen loss in a seagrass ecosystem in the central Red Sea. *Biogeosciences*, *15*(23), 7333–7346. https://doi.org/10.5194/BG-15-7333-2018

Godefroy, N., Le Goff, E., Martinand-Mari, C., Belkhir, K., Vacelet, J., & Baghdiguian, S. (2019). Sponge digestive system diversity and evolution: Filter feeding to carnivory. *Cell and Tissue Research*, *377*(3), 341–351. https://doi.org/10.1007/s00441-019-03032-8

Goyet, C., Hassoun, A., Gemayel, E., Touratier, F., Saab, M. A.-A., & Guglielmi, V. (2016). Thermodynamic Forecasts of the Mediterranean Sea Acidification. *Mediterranean Marine Science*, *17*(2), Article 2. https://doi.org/10.12681/mms.1487

Gravili, C., Cozzoli, F., & Gambi, M. C. (2021). Epiphytic hydroids on Posidonia oceanica seagrass meadows are winner organisms under future ocean acidification conditions: Evidence from a CO2 vent system (Ischia Island, Italy). *European Zoological Journal*, *88*(1), 472–486. https://doi.org/10.1080/24750263.2021.1899317

Greiner, J. T., McGlathery, K. J., Gunnell, J., & McKee, B. A. (2013). Seagrass Restoration Enhances "Blue Carbon" Sequestration in Coastal Waters. *PLOS ONE*, *8*(8), e72469. https://doi.org/10.1371/journal.pone.0072469

Hasegawa, N., Hori, M., & Mukai, H. (2007). Seasonal shifts in seagrass bed primary producers in a cold-temperate estuary: Dynamics of eelgrass Zostera marina and associated epiphytic algae. *Aquatic Botany*, *86*(4), 337–345. https://doi.org/10.1016/J.AQUABOT.2006.12.002

Heck, K. L., & Valentine, J. F. (2006). Plant–herbivore interactions in seagrass meadows. *Journal of Experimental Marine Biology and Ecology*, *330*(1), 420–436. https://doi.org/10.1016/j.jembe.2005.12.044

Hemminga, M. A., & Duarte, C. M. (2000). Seagrass Ecology. In *Seagrass Ecology*. Cambridge University Press. https://doi.org/10.1017/cbo9780511525551

Hemminga, M. A., Harrison, P. G., & Van Lent, F. (1991). The balance of nutrient losses and gains in seagrass meadows. *Marine Ecology Progress Series*, *71*(1), 85–96.

Hentschel, U., Piel, J., Degnan, S. M., & Taylor, M. W. (2012). Genomic insights into the marine sponge microbiome. *Nature Reviews Microbiology*, *10*(9), 641–654. https://doi.org/10.1038/nrmicro2839

Hentschel, U., Usher, K. M., & Taylor, M. W. (2006). Marine sponges as microbial fermenters. *FEMS Microbiology Ecology*, *55*(2), 167–177. https://doi.org/10.1111/j.1574-6941.2005.00046.x

Herbert, R. A. (1999). Nitrogen cycling in coastal marine ecosystems. *FEMS Microbiology Reviews*, *23*(5), 563–590. https://doi.org/10.1111/j.1574-6976.1999.tb00414.x

Hernán, G., Ramajo, L., Basso, L., Delgado, A., Terrados, J., Duarte, C. M., & Tomas, F. (2016). Seagrass (Posidonia oceanica) seedlings in a high-CO 2 world: From physiology to herbivory. *Scientific Reports*, *6*(38017). https://doi.org/10.1038/srep38017

Hoffman, D. K., McCarthy, M. J., Newell, S. E., Gardner, W. S., Niewinski, D. N., Gao, J., & Mutchler, T. R. (2019). Relative Contributions of DNRA and Denitrification to Nitrate Reduction in Thalassia testudinum Seagrass Beds in Coastal Florida (USA). *Estuaries and Coasts*, *42*(4), 1001–1014. https://doi.org/10.1007/s12237-019-00540-2

Holmer, M. (2019). Productivity and Biogeochemical Cycling in Seagrass Ecosystems. In G. M. E. Perillo, E. Wolanski, D. R. Cahoon, & C. S. Hopkinson (Eds.), *Coastal Wetlands (Second Edition)* (pp. 443–477). Elsevier. https://doi.org/10.1016/B978-0-444-63893-9.00013-7

Holmer, M., Andersen, F. Ø., Nielsen, S. L., & Boschker, H. T. S. (2001). The importance of mineralization based on sulfate reduction for nutrient regeneration in tropical seagrass sediments. *Aquatic Botany*, *71*(1), 1–17. https://doi.org/10.1016/S0304-3770(01)00170-X

IPCC. (2023). *IPCC, 2023: Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (First). Intergovernmental Panel on Climate Change (IPCC). https://doi.org/10.59327/IPCC/AR6-9789291691647

Jose, P. A., & Jebakumar, S. R. D. (2014). *Molecular phylogeny and plant growth promoting traits of endophytic bacteria isolated from roots of seagrass Cymodocea serrulata*.

Koch, M., Bowes, G., Ross, C., & Zhang, X.-H. (2013). Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Global Change Biology*, *19*, 103–132. https://doi.org/10.1111/j.1365-2486.2012.02791.x

Koch, M. S., & Erskine, J. M. (2001). Sulfide as a phytotoxin to the tropical seagrass *Thalassia testudinum*: Interactions with light, salinity and temperature. *Journal of Experimental Marine Biology and Ecology*, *266*(1), 81–95. https://doi.org/10.1016/S0022-0981(01)00339-2

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M., & Gattuso, J.-P. (2013). Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Global Change Biology*, *19*, 1884–1896. https://doi.org/10.1111/gcb.12179

Lee, K.-S., & Dunton, K. H. (2000). Diurnal changes in pore water sulfide concentrations in the seagrass *Thalassia testudinum* beds: The effects of seagrasses on sulfide dynamics. *Journal of Experimental Marine Biology and Ecology*, *255*(2), 201–214. https://doi.org/10.1016/S0022-0981(00)00300-2

Lee, K.-S., Park, S. R., & Kim, Y. K. (2007). Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: A review. *Journal of Experimental Marine Biology and Ecology*, *350*(1), 144–175. https://doi.org/10.1016/j.jembe.2007.06.016

Lehnen, N., Marchant, H. K., Schwedt, A., Milucka, J., Lott, C., Weber, M., Dekaezemacker, J., Seah, B. K. B., Hach, P. F., Mohr, W., & Kuypers, M. M. M. (2016). High rates of microbial dinitrogen fixation and sulfate reduction associated with the Mediterranean seagrass Posidonia oceanica. *Systematic and Applied Microbiology*, *39*(7), 476–483. https://doi.org/10.1016/J.SYAPM.2016.08.004

Macreadie, P. I., Costa, M. D. P., Atwood, T. B., Friess, D. A., Kelleway, J. J., Kennedy, H., Lovelock, C. E., Serrano, O., & Duarte, C. M. (2021). Blue carbon as a natural climate solution. *Nature Reviews Earth & Environment*, *2*(12), 826–839. https://doi.org/10.1038/s43017-021-00224-1

Malkin, S., & Cardini, U. (2021). Facilitative interactions on the rise: Cable bacteria associate with diverse aquatic plants. *New Phytologist*, *232*(5), 1897–1900. https://doi.org/10.1111/nph.17664

Marbà, N., Díaz-Almela, E., & Duarte, C. M. (2014). Mediterranean seagrass (Posidonia oceanica) loss between 1842 and 2009. *Biological Conservation*, *176*. https://doi.org/10.1016/j.biocon.2014.05.024

Marbà, N., & Duarte, C. M. (2010). Mediterranean warming triggers seagrass (Posidonia oceanica) shoot mortality. *Global Change Biology*, *16*(8), 2366–2375. https://doi.org/10.1111/j.1365-2486.2009.02130.x

Margulis, L. (1991). *Symbiosis as a Source of Evolutionary Innovation—Symbiogenesis and symbionticism*. MIT Press.

Mecca, S., Casoli, E., Ardizzone, G., & Gambi, M. C. (2020). Effects of ocean acidification on phenology and epiphytes of the seagrass Posidonia oceanica at two CO2 vent systems of Ischia (Italy). *Mediterranean Marine Science*, *21*(1), 70–83. https://doi.org/10.12681/MMS.20795

Mohr, W., Lehnen, N., Ahmerkamp, S., Marchant, H. K., Graf, J. S., Tschitschko, B., Yilmaz, P., Littmann, S., Gruber-Vodicka, H., Leisch, N., Weber, M., Lott, C., Schubert, C. J., Milucka, J., & Kuypers, M. M. M. (2021). Terrestrial-type nitrogen-fixing symbiosis between seagrass and a marine bacterium. *Nature 2021*, 1–5. https://doi.org/10.1038/s41586-021-04063-4

Monnier, B., Pergent, G., Valette-Sansevin, A., Boudouresque, C. F., Mateo, M. Á., & Pergent-Martini, C. (2020). The Posidonia oceanica matte: A unique coastal carbon sink for climate change mitigation and implications for management. *Vie Milieu*.

Moriarty, D. J. W., Iverson, R. L., & Pollard, P. C. (1986). Exudation of organic carbon by the seagrass *Halodule wrightii* Aschers. And its effect on bacterial growth in the sediment. *Journal of Experimental Marine Biology and Ecology*, *96*(2), 115–126. https://doi.org/10.1016/0022-0981(86)90237-6

Nellemann, C., Corcoran, E., Duarte, C., Young, C. D., Fonseca, L., & Grimsdith, G. (2010). Blue Carbon: The role of healthy oceans in binding carbon. *Center for Coastal and Ocean Mapping*. https://scholars.unh.edu/ccom/132

Nguyen, H. M., Ralph, P. J., Marín-Guirao, L., Pernice, M., & Procaccini, G. (2021). Seagrasses in an era of ocean warming: A review. *Biological Reviews*, *96*(5), 2009–2030. https://doi.org/10.1111/brv.12736

Nordlund, L. M., Koch, E. W., Barbier, E. B., & Creed, J. C. (2016). Seagrass Ecosystem Services and Their Variability across Genera and Geographical Regions. *PLOS ONE*, *11*(10), e0163091. https://doi.org/10.1371/journal.pone.0163091

Pergent-Martini, C., Pergent, G., Monnier, B., Boudouresque, C.-F., Mori, C., & Valette-Sansevin, A. (2021). Contribution of *Posidonia oceanica* meadows in the context of climate change mitigation in the Mediterranean Sea. *Marine Environmental Research*, *165*, 105236. https://doi.org/10.1016/j.marenvres.2020.105236

Pita, L., Rix, L., Slaby, B. M., Franke, A., & Hentschel, U. (2018). The sponge holobiont in a changing ocean: From microbes to ecosystems. *Microbiome*, *6*(46). https://doi.org/10.1186/s40168-018-0428-1

Riebesell, U., Zondervan, I., Rost, B., Tortell, P. D., Zeebe, R. E., & Morel, F. M. M. (2000). Reduced calcification of marine plankton in response to increased atmospheric CO2. *Nature*, *407*, 364–367. https://doi.org/10.1038/35030078

Rix, L., de Goeij, J. M., van Oevelen, D., Struck, U., Al-Horani, F. A., Wild, C., & Naumann, M. S. (2017). Differential recycling of coral and algal dissolved organic matter via the sponge loop. *Functional Ecology*, *31*(3), 778–789. https://doi.org/10.1111/1365-2435.12758

Salk, K. R., Erler, D. V., Eyre, B. D., Carlson-Perret, N., & Ostrom, N. E. (2017). Unexpectedly high degree of anammox and DNRA in seagrass sediments: Description and application of a revised isotope pairing technique. *Geochimica et Cosmochimica Acta*, *211*, 64–78. https://doi.org/10.1016/J.GCA.2017.05.012

Scartazza, A., Moscatello, S., Gavrichkova, O., Buia, M. C., Lauteri, M., Battistelli, A., Lorenti, M., Garrard, S. L., Calfapietra, C., & Brugnoli, E. (2017). Carbon and nitrogen allocation strategy in Posidonia oceanica is altered by seawater acidification. *Science of the Total Environment*, *607*, 954–964. https://doi.org/10.1016/j.scitotenv.2017.06.084

Schneider, A., Tanhua, T., Körtzinger, A., & Wallace, D. W. R. (2010). High anthropogenic carbon content in the eastern Mediterranean. *Journal of Geophysical Research: Oceans*, *115*(C12). https://doi.org/10.1029/2010JC006171

Seymour, J. R., Laverock, B., Nielsen, D. A., M., T.-T. S., & Macreadie, P. I. (2018). The Microbiology of Seagrasses. In *Seagrasses of Australia* (pp. 343–392). Springer International Publishing. https://doi.org/10.1007/978-3-319-71354-0

Short, F., Carruthers, T., Dennison, W., & Waycott, M. (2007). Global seagrass distribution and diversity: A bioregional model. *Journal of Experimental Marine Biology and Ecology*, *350*, 3–20. https://doi.org/10.1016/j.jembe.2007.06.012

Short, F. T., Polidoro, B., Livingstone, S. R., Carpenter, K. E., Bandeira, S., Bujang, J. S., Calumpong, H. P., Carruthers, T. J. B., Coles, R. G., Dennison, W. C., Erftemeijer, P. L. A., Fortes, M. D., Freeman, A. S., Jagtap, T. G., Kamal, A. H. M., Kendrick, G. A., Judson Kenworthy, W., La Nafie, Y. A., Nasution, I. M., … Zieman, J. C. (2011). Extinction risk assessment of the world's seagrass species. *Biological Conservation*, *144*(7), 1961–1971. https://doi.org/10.1016/j.biocon.2011.04.010

Soest, R. W. M. V., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., Voogd, N. J. D., Santodomingo, N., Vanhoorne, B., Kelly, M., & Hooper, J. N. A. (2012). Global Diversity of Sponges (Porifera). *PLOS ONE*, *7*(4), e35105. https://doi.org/10.1371/journal.pone.0035105

Sogin, E. M., Michellod, D., Gruber-Vodicka, H. R., Bourceau, P., Geier, B., Meier, D. V., Seidel, M., Ahmerkamp, S., Schorn, S., D'Angelo, G., Procaccini, G., Dubilier, N., & Liebeke, M. (2022). Sugars dominate the seagrass rhizosphere. *Nature Ecology and Evolution*, *6*, 866–877. https://doi.org/10.1038/S41559-022-01740-Z

Southwell, M. W., Weisz, J. B., Martens, C. S., & Lindquist, N. (2008). In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. *Limnology and Oceanography*, *53*(3), 986–996. https://doi.org/10.4319/lo.2008.53.3.0986

Strack, M. (Ed.). (2008). *Peatlands and Climate Change*. International Peat Society.

Sullivan, B. K., Sherman, T. D., Damare, V. S., Lilje, O., & Gleason, F. H. (2013). Potential roles of *Labyrinthula* spp. In global seagrass population declines. *Fungal Ecology*, *6*(5), 328–338. https://doi.org/10.1016/j.funeco.2013.06.004

Tarquinio, F., Hyndes, G. A., Laverock, B., Koenders, A., & Säwström, C. (2019). The seagrass holobiont: Understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. *FEMS Microbiology Letters*, *366*(6), 1–15. https://doi.org/10.1093/femsle/fnz057

Thomas, T., Moitinho-Silva, L., Lurgi, M., Björk, J. R., Easson, C., Astudillo-García, C., Olson, J. B., Erwin, P. M., López-Legentil, S., Luter, H., Chaves-Fonnegra, A., Costa, R., Schupp, P. J., Steindler, L., Erpenbeck, D., Gilbert, J., Knight, R., Ackermann, G., Victor Lopez, J., … Webster, N. S. (2016). Diversity, structure and convergent evolution of the global sponge microbiome. *Nature Communications*, *7*(1), Article 1. https://doi.org/10.1038/ncomms11870

Touchette, B. W., & Burkholder, J. A. M. (2000). Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, *250*, 133–167. https://doi.org/10.1016/S0022-0981(00)00195-7

Ugarelli, K., Chakrabarti, S., Laas, P., & Stingl, U. (2017). The Seagrass Holobiont and Its Microbiome. *Microorganisms*, *5*(81). https://doi.org/10.3390/microorganisms5040081

Vafeidis, A. T., Abdulla, A. A., Bondeau, A., Brotons, L., Ludwig, R., Portman, M., Reimann, L., Vousdoukas, M., & Xoplaki, E. (2020). *First Mediterranean Assessment Report – Chapter 6: Managing future risks and building socio-ecological resilience*. Zenodo. https://doi.org/10.5281/ZENODO.7101119

Vlamakis, H., Chai, Y., Beauregard, P., Losick, R., & Kolter, R. (2013). Sticking together: Building a biofilm the Bacillus subtilis way. *Nature Reviews Microbiology*, *11*(3), 157–168. https://doi.org/10.1038/nrmicro2960

Wannicke, N., Frey, C., Law, C. S., & Voss, M. (2018). The response of the marine nitrogen cycle to ocean acidification. *Global Change Biology*, *24*, 5031–5043. https://doi.org/10.1111/gcb.14424

Ward, B. B. (2008). Nitrification in Marine Systems. In *Nitrogen in the Marine Environment* (pp. 199–261). https://doi.org/10.1016/B978-0-12-372522-6.00005-0

Wear, D. J., Sullivan, M. J., Moore, A. D., & Millie, D. F. (1999). Effects of water-column enrichment on the production dynamics of three seagrass species and their epiphytic algae. *Marine Ecology Progress Series*, *179*, 201–213. https://doi.org/10.3354/MEPS179201

Welsh, D. T. (2000). Nitrogen fixation in seagrass meadows: Regulation, plant-bacteria interactions and significance to primary productivity. *Ecology Letters*, *3*, 58–71.

Werner, T., & Schmülling, T. (2009). Cytokinin action in plant development. *Current Opinion in Plant Biology*, *12*(5), 527–538. https://doi.org/10.1016/j.pbi.2009.07.002

Wyatt, N. J., Kitidis, V., Woodward, E. M. S., Rees, A. P., Widdicombe, S., & Lohan, M. (2010). Effects of high CO2 on the fixed nitrogen inventory of the Western English Channel. *Journal of Plankton Research*, *32*(5), 631–641. https://doi.org/10.1093/PLANKT/FBP140

Zimmerman, R. C., Hill, V. J., Jinuntuya, M., Celebi, B., Ruble, D., Smith, M., Cedeno, T., & Swingle, W. M. (2017). Experimental impacts of climate warming and ocean carbonation on eelgrass Zostera marina. *Marine Ecology Progress Series*, *566*, 1–15. https://doi.org/10.3354/MEPS12051

RESOURCES

- ChatGPT version 3.5, OpenAi: https://www.openai.com/chat (assisting with the text structure)
- DeepL Translate, Deepl SE: https://www.deepl.com/translator (translation of text passages)
- DeepL Write, Deepl SE: https://www.deepl.com/write (rephrasing of text passages)

CHAPTER 2

Set up of incubation chambers in a *Posidonia oceanica* meadow at Ischia Island (Italy). Photo by Ulisse Cardini.

CHAPTER 2

THE ROLE OF EPIPHYTES IN SEAGRASS PRODUCTIVITY UNDER OCEAN ACIDIFICATION

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ABSTRACT

Ocean Acidification (OA), due to rising atmospheric CO_2, can affect the seagrass holobiont by changing the plant's ecophysiology and the composition and functioning of its epiphytic community. However, our knowledge of the role of epiphytes in the productivity of the seagrass holobiont in response to environmental changes is still very limited. CO_2 vents off Ischia Island (Italy) naturally reduce seawater pH, allowing to investigate the adaptation of the seagrass *Posidonia oceanica* L. (Delile) to OA. Here, we analyzed the percent cover of different epiphytic groups and the epiphytic biomass of *P. oceanica* leaves, collected inside (pH 6.9–7.9) and outside (pH 8.1–8.2) the CO_2 vents. We estimated the contribution of epiphytes to net primary production (NPP) and respiration (R) of leaf sections collected from the vent and ambient pH sites in laboratory incubations. Additionally, we quantified net community production (NCP) and community respiration (CR) of seagrass communities in situ at vent and ambient pH sites using benthic chambers. Leaves at ambient pH sites had a 25% higher total epiphytic cover with encrusting red algae (32%) dominating the community, while leaves at vent pH sites were dominated by hydrozoans (21%). Leaf sections with and without epiphytes from the vent pH site produced and respired significantly more oxygen than leaf sections from the ambient pH site, showing an average increase of 47 ± 21% (mean ± SE) in NPP and 50 ± 4% in R, respectively. Epiphytes contributed little to the increase in R; however, their contribution to NPP was important (56 ± 6% of the total flux). The increase in productivity of seagrass leaves adapted to OA was only marginally reflected by the results from the in situ benthic chambers, underlining the complexity of the seagrass community response to naturally occurring OA conditions.

KEYWORDS: *P. oceanica*; CO_2 vents; benthic chambers; oxygen fluxes

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INTRODUCTION

Seagrasses are among the most important marine ecosystem engineers, providing various ecosystem services and maintaining human well-being. The habitat-forming seagrass *Posidonia oceanica*, endemic to the Mediterranean Sea, provides protection from coastal erosion, wastewater treatment and supports fisheries by providing habitats and nursery grounds for a broad range of fish and invertebrates (Boudouresque et al., 2006; Campagne et al., 2015). *Posidonia oceanica* meadows have high primary production rates, while decomposition rates in the seagrass sediments are rather low, creating an effective long-term carbon (C) sink (Duarte et al., 2013). Thus, *P. oceanica* meadows can be regarded as autotrophic ecosystems, releasing substantial amounts of oxygen (O_2) while intensively sequestering carbon dioxide (CO_2) (Barrón et al., 2006).

Coastal development and climate change have been causing a decline of 13 - 50% of *P. oceanica* meadows in the Mediterranean since 1960 (Marbà et al., 2014). Under worst-case global warming scenarios, it is predicted that by 2050 *P. oceanica* will lose 75% of suitable habitats, and by 2100 it is at risk of functional extinction (Chefaoui et al., 2018; Marbà & Duarte, 2010). As a consequence of habitat degradation, and therefore, increased seagrass decomposition, the organic C stored in the sea meadows can be emitted as CO_2 to the atmosphere (Chefaoui et al., 2018; Lovelock et al., 2017). Ocean acidification (OA, the decrease in seawater pH due to increased dissolution of atmospheric CO_2) is an additional climate change stressor, expected to impact habitat-forming species with cascading effects on the whole marine ecosystem (Kroeker et al., 2013; Zunino et al., 2021). Marine calcifying organisms such as calcifying algae, corals, or mollusks are negatively affected by OA (Doney et al., 2009; Kroeker et al., 2013; Riebesell et al., 2000). Conversely, marine macrophytes may benefit from the increased CO_2 concentration since their photosynthetic rates are often C limited at current ocean CO_2 levels (Koch et al., 2013). Indeed, mesocosm studies with Zostera spp. and *Thalassia hemprichii* showed increased primary production, growth, and shoot density under increased CO_2 availability (Egea et al., 2018; Jiang et al., 2010; Zimmerman et al., 2017) and *Cymodocea nodosa* showed significantly higher seagrass productivity in naturally acidified seawater (Apostolaki et al., 2014). Furthermore, by removing CO_2 from the water column through photosynthetic activity, seagrass meadows can increase pH in their surroundings, thus locally buffering OA (Bergstrom et al., 2019; Hendriks et al., 2014).

The effects of OA on *P. oceanica* remain unclear. A short-term laboratory study showed that early life stages of the plant benefit from future predicted CO_2 concentrations and displayed bigger seed size, improved photosynthetic performance, and higher C storage in their belowground tissues (Hernán et al., 2016). However, seedlings grown under high CO_2 concentrations were preferred by herbivorous fish, which could potentially offset the positive effects (Hernán et al., 2016). While a reduced seawater pH significantly increased the net productivity of adult plants in laboratory experiments (Cox et al., 2015), it did not affect leaf biometrics, photosynthetic rates, and leaf growth in mesocosm experiments (Cox et al., 2016). *Posidonia oceanica* meadows near CO_2 vents that have long-term adaptation to a reduced seawater pH exhibit higher shoot densities but lower leaf lengths, while their photosynthetic performance is similar at vent and ambient pH sites (Hall-Spencer et al., 2008; Mecca et al., 2020).

Epiphytic algae, invertebrates, and microorganisms living in close association with the seagrass plant form a biological unit called a holobiont (Tarquinio et al., 2019; Ugarelli et al., 2017). Epiphytes are key players in the seagrass phyllosphere (Brodersen & Kühl, 2022), modulating light-harvesting, gas, and nutrient exchange between the plant and the surrounding water and affecting key biogeochemical processes within the holobiont, such as C and nitrogen fixation, or transport of oxygen and dissolved organic carbon (DOC) (Seymour et al., 2018; Ugarelli et al., 2017). Under ambient pH conditions, *P. oceanica* leaves are colonized by a large variety of epiphytes, ranging from bacteria, such as Cyanobacteria (Ruocco et al., 2018) or Planctomycetes (Kohn et al., 2020), to fleshy and encrusting red, brown, and green algae (Casola et al., 1987) and calcifying invertebrates. Ocean acidification shifts the community structure from encrusting algal epiphytes to fleshy algae and non-calcifying invertebrates, such as hydrozoans and tunicates (Cox et al., 2015; Martin et al., 2008; Mecca et al., 2020). This shift in epiphyte community structure can have cascading effects on the associated communities and the functioning of the seagrass ecosystem, such as by affecting the light availability of the plant and key biogeochemical processes (Tarquinio et al., 2019; Ugarelli et al., 2017).

Several studies have investigated the phenology of the epiphytic community found along pH gradients at CO_2 vents in the field, finding reduced abundances of calcareous organisms under reduced pH (Hall-Spencer et al., 2008; Martin et al., 2008; Mecca et al., 2020). However, our knowledge of the role of epiphytes in the productivity of the seagrass holobiont in response to environmental changes is still limited. The present study aims to assess the effects of OA conditions on the productivity of seagrass communities along the natural CO_2 vents off Ischia Island and to disentangle the role of the epiphytic community vs. the plant host on seagrass productivity under OA.

MATERIAL AND METHODS

STUDY AREA

The experiments were conducted in September 2019 and September 2020 at Ischia Island in the Gulf of Naples (Tyrrhenian Sea, Italy). The island is characterized by systems of submarine CO_2 vents of volcanic origin. The gas emitted from the seafloor is composed of CO_2 (90.1 - 95.3%), N_2 (3.2 - 6.6%), O_2 (0.6 - 0.8%), Ar (0.08 - 0.1%), and CH_4 (0.2 - 0.8%), and it does not contain toxic sulfur compounds nor does it affect the surrounding water temperature or salinity (Foo et al., 2018; Hall-Spencer et al., 2008). One study area was located at the shallow vent system at Castello Aragonese (CA), where the vents occur at 0.5 - 3 m depth. Here, we selected two sites characterized by two different pH regimes (‘vent pH’ and ‘ambient pH’) at approximately 3 m water depth with similar light levels (Table 2.1). The vent pH site was in a venting area on the south side (40°43'50.5"N 13°57'47.2"E) and the ambient pH site was located on the north side of the Castello (40°43'54.8"N 13°57'47.1"E). Another study area for *in situ* incubations was located at Chiane del lume (CdL), where vents occur at 10 - 12 m depth (Table 2.1). Here, the vent pH site was located at the level of Grotta Tisichello (40°42' 53.56"N 13°58' 2.37"E) and the ambient pH site about 680 m north (40°43.248'N 13° 57.916'E).

Table 2.1. Environmental parameters (mean ± SE, n) measured at vent and ambient pH sites at Castello Aragonese (CA) and Chiane del Lume (CdL).

	Vent pH				Ambient pH			
	CA		CdL		CA		CdL	
Variable	Mean ± SE	*n*	Mean ± SE	*n*	Mean ±SE	*n*	Mean ± SE	*n*
T (°C)	22.95 ± 0.07	4	25.23 ± 0.02	3	22.95 ± 0.05	4	25.32 ± 0.15	3
Light (lux)	20001 ± 271	3	4898 ± 2996	3	17807 ± 3349	3	6213 ± 1057	3
pH	7.34 ± 0.04	2	7.92 ± 0.01	3	8.17 ± 0.02	2	8.18 ± 0.01	2
DO (mg L^{-1})	8.50 ± 0.11	4	8.87 ± 0.55	3	8.79 ± 0.22	4	8.71 ± 0.06	3
DOC (μM)	NA		143.79 ± 1.38	2	NA		139.74 ± 4.03	6
DON (μM)	NA		7.13 ± 0.06	2	NA		7.67 ± 0.83	6
NH_4^+	NA		0.61 ± 0.04	2	NA		0.44 ± 0.1	6
NO_3^-	NA		0.20 ± 0.14	2	NA		0.20 ± 0.04	6

Temperature, light, pH, and DO were continuously measured with data loggers (between 12 am and 2 pm of the respective incubation day). DOC, DON, NH_4^+, and NO_3^- were analyzed from samples collected on the respective sampling day.

EPIPHYTE NPP AND R

To assess the epiphytic contribution to seagrass productivity, we collected *P. oceanica* shoots in September 2019 at the vent and ambient pH sites of Castello Aragonese and transported them directly into the laboratory. We selected leaves with homogenous coverage of epiphytes and cut off 3 cm long sections of the central part of the leaf, avoiding both young and heavily grazed and senescent parts of the plant. Epiphytes were scraped off with a scalpel from half of the leaves, taking care not to damage the plant tissue. A total of 28 *P. oceanica* leaf sections were incubated (from the vent and ambient pH sites, covered by epiphytes (+Epi) or with epiphytes removed (-Epi), in light or dark incubation) to assess net primary production (NPP), gross primary production (GPP), and respiration (R). Leaf sections were transferred into transparent 24 ml glass vials filled with seawater from the respective pH site. The pH of the water was checked and adjusted if needed to the original site values by CO_2 bubbling. Half of the vials were incubated in the light to assess NPP, and the others were incubated wrapped in aluminum foil to assess R in the dark. We incubated the vials on a shaker (Stuart orbital shaker SSL1; 30 rpm) under artificial light at 360 μmol m^{-2} s^{-1}, upside down with the transparent bottom exposed to the light source and leaf sections standing vertically within the vials. Incubations were conducted in a temperature-controlled room at 25°C. Oxygen concentrations were measured at the beginning and the end of the incubation (5-6 h) using a fiber-optic oxygen sensor (FireStingO2, PyroScience, Germany), making sure oxygen did not drop below 50% saturation. Temperature and pH were measured at the beginning and the end of the incubation using a pH meter (Multi 3430, WTW, Germany). PH values increased during the light incubations from 6.96 to 7.33 in the vent pH treatment and from 8.02 to 8.25 in the ambient pH. In the dark incubations, pH values remained stable during the incubation in the vent pH and decreased from 8.02 to 7.91 in the ambient pH treatment. At the end of the experiment, we scraped off the epiphytes of the incubated leaf sections, and seagrass leaves and epiphytes were dried at 60°C for 48h and weighed separately. NPP and R were normalized to biomass (dry weight) since it reflects the different treatments (with and without epiphytes).

NCP AND CR

Natural seagrass communities were incubated *in situ* in September 2019 at Castello Aragonese (CA) and in September 2020 at Chiane del Lume (CdL) to assess their productivity. We estimated net community production (NCP), community respiration (CR), and nutrient fluxes during incubations with benthic chambers using the design by Olivé et al. (2016), which allows avoiding sediment disturbance, dilution, continuous stirring, or gaseous head-space while ensuring mixing through water motion. The chambers consisted of an internal PVC cylinder (13 cm diameter) inserted into the plastic bag to maintain the cylindrical shape and standardize the chamber volume (10 L), a bottom cylinder inserted approximately 10-15 cm into the sediment, and a gas-tight polyethylene plastic bag with a sampling port to draw water samples. The chambers (n = 4) were deployed randomly within each station (CA and CdL, each with a vent and ambient pH site) by scuba divers with a minimum distance of 3 m to assure independence between the replicates. The incubations were performed during the central hours of the days, between 11.00 am and 3.00 pm. During the incubations, we measured temperature, pH, dissolved oxygen, and light intensity continuously inside the chambers, using data loggers (Onset Computer Corporation, USA). We covered the chambers with opaque polyethylene bags to exclude light and started the dark incubation to assess R. After approx. 1.5 h, we removed the covers and recorded the light incubation for another 1.5 - 2 h to assess NCP (Olivé et al., 2016). We collected water samples to analyze inorganic and organic nutrients with 50 ml acid-washed syringes through the sampling port immediately after the deployment of the chambers, after the dark incubation, and after the light incubation. Additionally, we took water samples from the water column inside the seagrass meadow and ca. 1 m above. The water samples were used for the analysis of dissolved inorganic nitrogen (DIN: ammonium, nitrate, and nitrite), dissolved inorganic phosphate (DIP), DOC, and dissolved organic nitrogen (DON). For DIN and DIP determination, we filtered the water through a cellulose acetate membrane filter (pore size: 0.22 µm) into 20 ml HDPE vials and stored upright at -20°C until analysis with a Continuous Flow Analyzer (Flowsys, SYSTEA SpA., Italy). We filtered the water samples for DOC and DON determination through precombusted GF/F filters into acid-washed HDPE vials, immediately acidifying the samples with 80 µl of 18.5% HCl and storing them at 4°C until analysis on a total organic carbon analyzer (TOC-L with TNM-L Unit, Shimadzu Corporation, Japan). We counted the total number of *P. oceanica* shoots and leaves within each incubation chamber and measured the leaf length and width *in situ*.

EPIPHYTE BIOMASS AND COMMUNITY STRUCTURE

We collected 20 *P. oceanica* leaves at vent pH and 20 at ambient pH sites at Castello Aragonese and directly transported them into the laboratory for community identification. We took high-resolution pictures with a stereoscope (Zeiss AxioCam 208 color) from both sides of a subset of the leaves (approx. 1 cm width and 3 cm length). We analyzed the community structure by identifying major groups and estimated percent cover using the software CPCe 4.1, counting 25 random points per frame (20 leaves per site x 2 sides of the leaf = 80 frames in total). Subsequently, we carefully scraped off the epiphytes with a scalpel, dried the leaves and epiphytes at 60°C for 48 h, and weighed them separately to estimate the *P. oceanica* leaf and epiphyte biomass.

DATA ANALYSIS

NPP and R rates in the laboratory incubations were calculated as:

$$NPP\ or\ R\ (\mu M\ O_2\ g^{-1}\ h^{-1}) = \frac{([O_2]_{final} - [O_2]_{initial}) * V}{DW * t} \quad \text{(I)}$$

where $[O_2]$ is the oxygen concentration (µmol L^{-1}) in the light (NPP) and the dark (R) incubations, *V* is the volume of the vials (24 mL), *DW* is the dry weight of the seagrass leaf biomass (g), and *t* is the incubation time (h). Gross primary production (GPP) was calculated as:

$$GPP\ (mM\ O_2\ m^{-2}\ h^{-1}) = NPP + R \quad \text{(III)}$$

In situ, NCP and CR were calculated as:

$$NCP\ or\ CR\ (mM\ O_2\ m^{-2}\ h^{-1}) = \frac{\Delta DO * V}{A} \quad \text{(II)}$$

where *ΔDO* is the slope obtained from the linear regression of the oxygen concentrations (mmol L^{-1} h^{-1}) during the light (NCP) and dark (CR) incubations, *V* is the volume of the benthic chamber (10 L), and *A* is the chamber area (0.013 m^2).

The daylight NCP and night CR budgets were calculated from the NCP and CR rates during 24 h, considering an 11:13 light/darkness photoperiod. NCP daily budgets were calculated as the sum of daylight NCP and night CR budgets (Olivé et al., 2016).

STATISTICAL ANALYSIS

We tested the effects of pH (vent pH vs. ambient pH), treatment (-Epi vs. +Epi), and their interaction on the productivity in a two-way ANOVA (Type II) and used estimated marginal means (EMMs) for posthoc pairwise comparison of the fitted means. We tested for normality and homogeneity of variances before each analysis using Shapiro-Wilk's and Levene's tests. ANOVA Type II was performed despite the unbalanced design, as the test is considered robust to moderate departures from unequal sample sizes when the homogeneity of variances is met (Langsrud, 2003).

We tested the effects of the pH (vent pH vs. ambient pH) on the community productivity, the total epiphyte cover, and the percent cover of the individual epiphytic groups using one-way ANOVAs (Type II). We tested for normality and homogeneity of variances before each analysis using Shapiro-Wilk's and Levene's tests and removed outliers and used generalized linear models (GLM) with Poisson or Quasi Poisson distribution when normality and homogeneity were not met. All statistical analyses were performed with RStudio (version 3.5.3) using the packages *car*, *ggplot2*, and *emmeans*(RStudio Team, 2021).

RESULTS

EPIPHYTE BIOMASS AND COMMUNITY STRUCTURE

The epiphytic biomass of the leaf sections in the laboratory incubations (Fig. 2.1a) was 2.8-fold higher at ambient pH sites compared to vent pH sites ($F_{1,13}$= 96.52, p<0.001, R^2 = 0.87). The epiphytic biomass of the leaves collected from the benthic chambers after the *in situ* incubations (Fig. 2.1b) was on average 0.7-fold higher at ambient pH sites compared to vent pH sites ($F_{1,11}$= 9.48, p=0.011, R^2 = 0.37). The total epiphyte cover and the community composition differed between vent and ambient pH sites (Fig. 2.1c). Leaves from ambient pH sites showed a 25% higher total epiphyte cover ($F_{1,78}$=4.20, p=0.043, R^2 = 0.04) and were mainly covered with encrusting red algae (32%) followed by hydrozoans (7%). Leaves from vent pH sites were primarily covered with hydrozoans (21%), followed by encrusting red algae (12%). The coverage of calcifying groups such as bryozoans and encrusting red algae was higher at ambient pH sites, namely 41x higher for bryozoans (GLM, Chisq=18.21, df=1, p<0.001) and 2.7x higher for encrusting red algae (GLM, Chisq=32.27, df=1, p<0.001). Non-calcifying groups, such as fleshy green algae and hydrozoans, showed higher coverage at vent pH sites. Fleshy green algae (GLM, Chisq=4.47, df=1, p=0.034) were only present at vent pH sites and the coverage of hydrozoans was 2.9x increased at vent pH sites (GLM, Chisq=21.991, df=1, p<0.001).

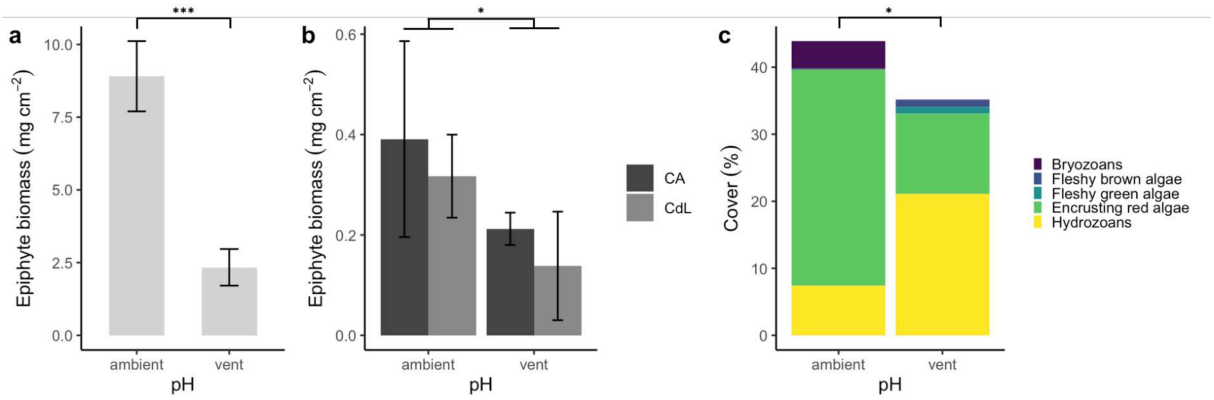

Fig. 2.1. Epiphyte biomass and community structure. (a) Epiphyte biomass per leaf section from vent and ambient pH sites in the laboratory incubations; (b) epiphyte biomass (whole leaf) per leaf area in the benthic chambers at vent and ambient pH sites at Castello Aragonese (CA) and Chiane del Lume (CdL); (c) percent cover of the epiphytic groups at Chiane del Lume (only species groups with >1% cover are shown). Error bars indicate 95% confidence intervals. Stars show significant differences; number of stars show significance level (* p<0.05, ** p<0.01, *** p<0.001).

CONTRIBUTION OF EPIPHYTES TO SEAGRASS PRODUCTIVITY IN LABORATORY INCUBATIONS

We found that NPP (Fig. 2.2a) significantly increased in the presence of epiphytes ($F_{1,10}$= 51.92, p<0.001, R^2 = 0.82), which contributed on average 56 ± 6% (mean ± SE) to NPP regardless of pH. At the same time, NPP increased on average by 47 ± 21% in leaves from the vent pH site ($F_{1,10}$= 8.81, p=0.014, R^2 = 0.82) compared to the ambient pH site, regardless of the presence/absence of epiphytes. GPP (Fig. 2.2b) followed a similar pattern and increased in the presence of epiphytes ($F_{1,10}$= 23.60, p<0.001, R^2 = 0.69) and in leaves from the vent pH site ($F_{1,10}$= 7.07, p=0.024, R^2 = 0.69). Respiration (Fig. 2.2c) was not affected by the presence/absence of epiphytes but increased on average by 50 ± 4% in leaves from the vent pH site ($F_{1,11}$= 5.80, p=0.035, R^2 = 0.25) compared to the ambient pH site.

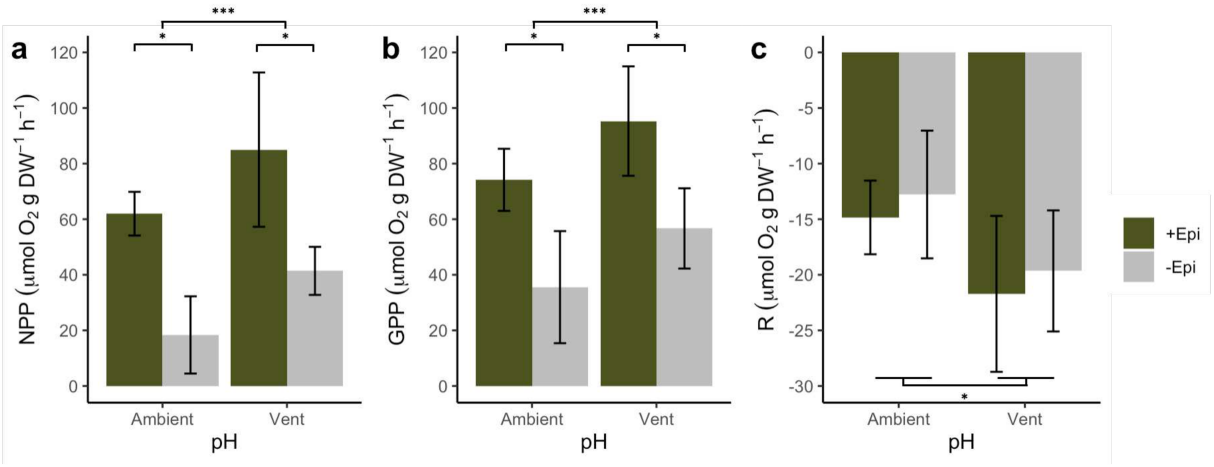

Fig. 2.2. Ex situ net primary production (a), gross primary production (b), and respiration (c) of leaves with epiphytes (+Epi, green) and without epiphytes (-Epi, grey), from vent and ambient pH sites, normalized by seagrass leaf biomass (dry weight). Negative values represent oxygen consumption, while positive values show oxygen production. Error bars indicate 95% confidence intervals. Stars show significant differences; number of stars show significance level (* p<0.05, ** p<0.01, *** p<0.001).

IN SITU NET COMMUNITY PRODUCTION AND RESPIRATION

To assess the productivity of natural seagrass communities under OA, we measured *in situ* oxygen production in light vs. dark incubations at vent vs. ambient pH sites. We observed no significant differences between daylight, night, or daily budgets at the vent and ambient pH sites at Castello Aragonese or Chiane del Lume (Table 2.2).

DOC, DON, NH_4^+, NO_2^-, and NO_3^- fluxes did not change during dark and light incubations and were not affected by seawater pH (Table S2.1). The PO_4^- flux slightly decreased during the light incubations at the vent and ambient pH sites by 0.019 ± 0.0005 (mean ± SE) µM h^{-1}.

The morphology of the seagrass meadows differed significantly between the two sites (Table S2.2). The seagrass at the vent sites displayed a higher shoot density but shorter average leaf length and width, causing the leaf area index not to differ significantly between the vent and ambient pH sites.

Table 2.2. Daily metabolic budgets (mean ± SE) of *P. oceanica* at vent and ambient pH sites at Castello Aragonese (CA) and Chiane del Lume (CdL).

	Location	*n*	Daylight budget (mmol O_2 m^{-2})	Night budget (mmol O_2 m^{-2})	Daily budget (mmol O_2 m^{-2} day^{-1})
Vent pH	CA	4	243.90 ± 60.07	-165.34 ± 18.99	70.60 ± 55.45
	CdL	3	233.89 ± 35.55	-200.07 ± 56.81	33.81 ± 24.10
Ambient pH	CA	4	207.52 ± 34.55	-176.88 ± 22.99	30.63 ± 20.10
	CdL	3	211.68 ± 19.73	-201.02 ± 41.23	10.66 ± 23.25
(Olivé et al., 2016)	Revellata Bay (Calvi, France)	3	143.34 ± 12.53	-81.68 ± 6.51	61.67 ± 14.12

Daylight budget was calculated from net community production (NCP) and night budget from community respiration (CR), assuming an 11:13 light: dark cycle. Negative values mean a net consumption of O_2. The daily budget was calculated as the sum of daylight and night budget. The budgets calculated by Olivé et al. (2016) after 1.5-2h incubation time were added for comparison.

DISCUSSION

EPIPHYTIC COMMUNITIES DIFFER BETWEEN SEAGRASS LEAVES FROM VENT AND AMBIENT PH SITES

The epiphytic communities in the studied vent area showed significant differences among pH conditions (Fig.2.1). The overall epiphyte cover was 25% higher under ambient pH conditions. Encrusting red algae showed a reduced coverage from 32% under ambient pH conditions to 12% under vent pH conditions. The coverage of the non-calcifying hydrozoans increased from 7% to 21% under OA conditions. This shift from coralline to non-calcifying organisms was also found by Mecca et al. (2020) in the vent system of Castello Aragonese. Several studies showed that encrusting red algae (Corallinales) are especially vulnerable to acidification due to the sensitivity of their carbonate skeleton (Donnarumma et al., 2014; Hall-Spencer et al., 2008; Martin et al., 2008). On the other side, hydrozoans show a higher tolerance to reduced seawater pH and can, therefore, outcompete more pH-sensitive species (Gravili et al., 2021). In contrast to Mecca et al. (2020), we found that also bryozoans were negatively affected by low pH conditions. Bryozoans are calcifying organisms as well, but due to organic tissue protecting their skeleton and different mineralogical composition, they are less sensitive to OA than coralline algae. However, Rodolfo-Metalpa et al. (2010) found reduced calcification rates under very low pH conditions (pH 7.43) and high mortality rates when low pH was combined with high seawater temperatures (25 -28°C).

The epiphytic biomass was significantly higher at leaf sections from ambient pH sites (Fig. 2.1). This is attributable to the higher epiphytic coverage at ambient pH sites and the difference in epiphytic calcium carbonate mass (Martin et al., 2008). The differences in epiphytic biomass between the vent and ambient pH sites of seagrass leaves collected from within the benthic chambers were not as pronounced as those of leaf sections in the laboratory incubations (0.7-fold increase instead of 2.8-fold increase at vent pH sites) and displayed lower values. This results from high variability in epiphytic growth on *P. oceanica* leaves *in situ*, including young (non-epiphytized) and senescent portions that were excluded from the laboratory incubations.

EPIPHYTES CONTRIBUTE TO LEAF NPP UNDER VENT AND AMBIENT PH CONDITIONS

In our laboratory incubations, epiphytes accounted for 50% of *P. oceanica* leaf NPP under vent pH and 62% under ambient pH conditions (Fig. 2.2). Several studies have found that epiphytes contribute up to 60% to photosynthesis and primary production for different seagrass species, such as *Halodule wrightii, Syringodium filiforme, Thalassia testudinum* (Wear et al., 1999), and *Zostera marina* (Hasegawa et al., 2007). The epiphytic community of *P. oceanica* can be highly diverse, with 430 epiphyte species recorded on its leaves (Piazzi et al., 2016). These are accompanied by a diverse prokaryotic community within the leaf biofilm (Kohn et al., 2020). Among this community, many members are phototrophs, such as the abundant Corallinales, Ochrophyta, Chlorophyta, diatoms, and cyanobacteria (Piazzi et al., 2016). In leaves from the ambient pH site, the bulk of the measured epiphytic NPP on the leaves was likely attributable to Corallinales, which covered large portions of the leaf surface and are found to be the most abundant epiphytic group (ca. 30% cover) at ambient pH around the Castello Aragonese in Ischia (Mecca et al., 2020). Conversely, epiphytic organisms other than Corallinales

are likely responsible for the contribution to NPP in leaves from the vent pH site. Within the diverse epiphytic consortium, heterotrophic bacteria can also indirectly contribute to primary production, helping to overcome the shortcoming of limiting nitrogen and phosphorous (Celdrán et al., 2012). However, with our experimental approach, it was not possible to determine micro-epiphytes that occur within the biofilm of the leaf surface, such as cyanobacteria, dinoflagellates, foraminifers, or planctomycetes (Kohn et al., 2020; Piazzi et al., 2016). Albeit, these epiphytic communities can also turn into a threat to the plant if coastal eutrophication and global warming result in their overgrowth on the seagrass phyllosphere (Brodersen et al., 2020; Noisette et al., 2020). In these cases, leaf epiphytes can lead to a strong O_2 build-up, increased oxidative stress, reduced light conditions in the leaf micro-environment in the light, or reduced internal plant aeration and production of phytotoxic nitric oxide in the dark (Costa et al., 2015; Noisette et al., 2020). Moreover, thick biofilms can thermally stress the underlying plan leaf tissue when the seagrass is already close to its upper thermal limits (Noisette et al., 2020).

While epiphytes clearly drove NPP in our laboratory incubations, R was not affected by the presence/absence of epiphytes (Fig.2.2). This is in agreement with the results of Costa et al. (2015), who observed no effects of epiphytes on R of *P. oceanica* shoots. By contrast, Brodersen et al. (2020) found lower R rates in leaves of *Zostera marina* with epiphytes as a consequence of the reduced diffusive O_2 uptake of epiphyte-covered seagrass leaves.

NPP AND R OF SEAGRASS LEAF SECTIONS INCREASE UNDER OA CONDITIONS

The relationship between decreasing pH and increasing production of *P. oceanica* has been investigated over a wide range of pH from 7.9 to 5.5 (Cox et al., 2015; Guilini et al., 2017; Hall-Spencer et al., 2008), using a variety of methods. Other seagrass species, such as Zostera spp. (Egea et al., 2018; Palacios & Zimmerman, 2007), *Thalassia hemprichii* (Jiang et al., 2010), and *Cymodocea nodosa* (Apostolaki et al., 2014) also showed stimulation in productivity under lower pH conditions. NPP, GPP, and R were significantly higher in leaves from vent pH sites in our laboratory experiments (Fig. 2.2). On average, NPP increased by 47 $\pm$ 21% (mean $\pm$ SE) and R by 50 $\pm$ 4%, suggesting that the *P. oceanica* holobiont is indeed C-limited at current seawater inorganic C concentrations. However, increased seagrass productivity is not necessarily expected to translate into net growth of the meadow. Accordingly, an increased vulnerability of *P. oceanica* leaves to grazing by herbivores (Mecca et al., 2020) is attributed to the more labile organic composition of the seagrass holobiont (Scartazza et al., 2017) and, as our data indicate, the absence of calcareous epiphytes at vent pH sites. Additionally, so far it is not entirely clear whether it is the plant or its epiphytes that are mainly benefiting from the increased CO_2 concentrations. Since epiphytic fleshy algae respond positively to increased CO_2 availability (Koch et al., 2013), they could compete with their plant host for similar resources under OA conditions. Hansen et al. showed that epiphytes of the seagrass *Zostera marina* can have a competitive advantage under elevated CO_2 at seawater temperatures up to 22°C. Additionally, epiphytic biofilms reduced the photosynthetic efficiency of the seagrass especially under higher temperatures (27°C) (Hansen et al., 2022). Competition between seagrasses and filamentous algal epiphytes has been also shown under high CO_2 and high light (Burnell et al., 2014) as well as in polluted conditions (Mabrouk et al., 2013). In our laboratory incubations, epiphytic contribution to NPP was 62% in leaves

from the ambient pH site and 50% in those from the vent pH site (Fig. 2.2). Furthermore, NPP of leaves from the vent pH site was higher than NPP of leaves from the ambient pH site by 26% with epiphytes present and by 68% with epiphytes removed. While the plant directly benefits from increased CO_2 concentrations and reduced shading by calcareous epiphytes, the lower epiphytic contribution to NPP in the CO_2 vents is likely a combined result of changes in biomass, community composition as well as species-specific rates.

PRODUCTIVITY OF THE SEAGRASS COMMUNITY IS ONLY MARGINALLY AFFECTED BY OA

P. oceanica meadows at the vent pH sites showed higher shoot density but shorter leaf length and width than at ambient pH sites (Table S2.2). Increased shoot density and shorter leaf length under vent pH conditions have been reported for *P. oceanica* and other seagrass species (Hall-Spencer et al., 2008; Martin et al., 2008; Palacios & Zimmerman, 2007). These changes in seagrass morphology under OA have been associated with increased grazing pressure by herbivores, such as the fish *Sarpa salpa*, sea urchins, or other invertebrates (Garrard et al., 2014; Hernán et al., 2016). As a reaction to high grazing activity, *P. oceanica* invests energy-rich compounds produced by photosynthesis into shoot recruitment rather than belowground C storage (Scartazza et al., 2017). Fluxes of organic (DOC, DON) and inorganic (NH_4^+, NO_2^-, NO_3^-) nutrients did not differ between vent and ambient pH sites in the dark and the light incubations (Table S2.1) but showed high variability among the benthic chambers. Phosphate consumption was higher during light incubations than dark incubations at both vent and ambient pH sites. Phosphate is essential for effective photosynthesis and therefore actively taken up by seagrasses (Touchette & Burkholder, 2000). Our estimates for *P. oceanica* metabolic daylight, night, and daily budgets are in the same order of magnitude as those reported by Olivé et al. (2016) using similar chambers and incubation times (Table 2.2). While our daily budgets agree well with their results, we found higher daylight and night budgets. This can be an effect of different light intensities during the incubations and different plant biomasses within the incubation chambers. Our incubations were carried out in September, while Olivé et al. (2016) carried out their incubations in October in Calvi (France), at a higher latitude than our station in Ischia (Italy). Despite differences in morphology, and differently from what we have reported for our laboratory experiments, there was no statistically significant increase in productivity of in situ seagrass communities at the vent pH sites. However, we saw a pattern of higher autotrophy at the vent pH sites of CA and CdL compared to the respective ambient pH sites, which resulted in more than two-fold average daily budgets under OA conditions. When normalizing the in-situ productivity to biomass (Fig. S2.1), we saw a pattern of higher productivity and respiration at the vent site of CA. In contrast, productivity did not differ between the ambient and vent sites of CdL. The different patterns of in-situ productivity between the two locations are probably a result of their differences in depth and hence light intensity as well as the different bubbling intensity of the CO_2 vents and, therefore, pH ranges. The location of CdL is deeper (10 - 12 m) than CA (3 m), resulting in a 3-fold lower light intensity (see Table 2.1). At CdL, the pH range between the ambient and vent site is not as high as for CA (7.92 - 8.18 and 7.34 - 8.17, respectively). Eventually, high variability in benthic metabolism prevented discerning significant differences. When logistically feasible, follow-up studies should thus consider an increased replication when measuring benthic metabolism *in situ*.

Seagrasses are not only colonized by epiphytes living on the leaf surface but also at the roots and rhizomes of the plant (Piazzi et al., 2016; Ugarelli et al., 2017). Additionally, various phototrophic and heterotrophic organisms inhabit the *P. oceanica* belowground habitat (Borg et al., 2006). These organisms were unaccounted for in our laboratory experiments while they were included in the *in situ* benthic incubation chambers. A recent study on rocky benthic communities from the same CO_2 vents in Ischia found functional vulnerability (i.e., decrease in functional diversity following the loss of species) to OA to be more pronounced than the corresponding decrease in taxonomic diversity, identifying heterotrophic feeding strategies among the functional entities that are most vulnerable to OA (Teixidó et al., 2018). If similar scenarios apply to the *P. oceanica* communities, this may explain our results, suggesting increased autotrophy at the vents. This, however, may not translate into more C sequestration, as the more labile organic composition of the seagrass holobiont (Scartazza et al., 2017) and the absence of calcareous epiphytes at vent pH sites leads to increased grazing and C remineralization. Additional experiments with more replication throughout the year would provide helpful insights about seasonal patterns that might occur.

CONCLUSIONS

In summary, the present study demonstrates that natural CO_2 enrichment clearly affects the epiphyte community structure and the productivity of both seagrass leaves and their epiphytic community. Epiphytes contributed significantly to NPP under vent and ambient pH conditions but not to seagrass respiration. However, this was only marginally translated to changes in NCP or CR at the community level *in situ*. Our results show the high complexity of host-epiphyte interactions and their response to environmental changes such as OA. A comparison with other studies shows that this response is highly dependent upon spatial and temporal scales, the species themselves, and the environmental characteristics of the site. However, it is clear that studies that seek to understand seagrass biology and ecology cannot disregard the role of its associated epiphytes.

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AUTHORS CONTRIBUTION

F.P., U.M., C.W., and U.C. planned the research and designed the study. F.P., U.M., M.M., G.M.Q., L.D., and U.C. conducted experimental/lab work. J.B. and F.P. analyzed the data. J.B. prepared the figures. J.B., F.P., and U.C. wrote the article, and all authors reviewed the paper. C.W. and U.C. provided funding.

REFERENCES

Apostolaki, E. T., Vizzini, S., Hendriks, I. E., & Olsen, Y. S. (2014). Seagrass ecosystem response to long-term high CO2 in a Mediterranean volcanic vent. *Marine Environmental Research*, *99*, 9–15. https://doi.org/10.1016/j.marenvres.2014.05.008

Barrón, C., Duarte, C. M., Frankignoulle, M., & Vieira Borges, A. (2006). Organic Carbon Metabolism and Carbonate Dynamics in a Mediterranean Seagrass (Posidonia oceanica) Meadow. *Estuaries and Coasts*, *29*(3), 417–426.

Bergstrom, E., Silva, J., Martins, C., & Horta, P. (2019). Seagrass can mitigate negative ocean acidification effects on calcifying algae. *Scientific Reports*, *9*(1932). https://doi.org/10.1038/s41598-018-35670-3

Borg, J. A., Rowden, A. A., Attrill, M. J., Schembri, P. J., & Jones, M. B. (2006). Wanted dead or alive: High diversity of macroinvertebrates associated with living and "dead" Posidonia oceanica matte. *Marine Biology*, *149*, 667–677. https://doi.org/10.1007/s00227-006-0250-3

Boudouresque, C. F., Mayot, N., & Pergent, G. (2006). The outstanding traits of the functioning of the Posidonia oceanica seagrass ecosystem. *Biol. Mar. Medit.*, *13*(4), 109–113.

Brodersen, K. E., Koren, K., Peter Revsbech, N., & Kühl, M. (2020). Strong leaf surface basification and CO 2 limitation of seagrass induced by epiphytic biofilm microenvironments. *Plant Cell Environment*, *43*, 174–187. https://doi.org/10.1111/pce.13645

Brodersen, K. E., & Kühl, M. (2022). Effects of Epiphytes on the Seagrass Phyllosphere. *Frontiers in Marine Science*, *9*, 1–10. https://doi.org/10.3389/fmars.2022.821614

Burnell, O. W., Russell, B. D., Irving, A. D., & Connell, S. D. (2014). Seagrass response to CO2 contingent on epiphytic algae: Indirect effects can overwhelm direct effects. *Oecologia*, *1*, 871–882. https://doi.org/10.1007/s00442-014-3054-z

Campagne, C. S., Salles, J.-M., Boissery, P., & Deter, J. (2015). The seagrass Posidonia oceanica: Ecosystem services identification and economic evaluation of goods and benefits. *Marine Pollution Bulletin*, *97*, 391–400. https://doi.org/10.1016/j.marpolbul.2015.05.061

Casola, E., Scardi, M., Mazzella, L., & Fresi, E. (1987). Structure of the Epiphytic Community of Posidonia oceanica Leaves in a Shallow Meadow. *Marine Ecology*, *8*(4), 285–296. https://doi.org/10.1111/j.1439-0485.1987.tb00189.x

Celdrán, D., Espinosa, E., Sánchez-Amat, A., & Marín, A. (2012). Effects of epibiotic bacteria on leaf growth and epiphytes of the seagrass Posidonia oceanica. *Marine Ecology Progress Series*, *456*, 21–27. https://doi.org/10.3354/MEPS09672

Chefaoui, R. M., Duarte, C. M., & Serrão, E. A. (2018). Dramatic loss of seagrass habitat under projected climate change in the Mediterranean Sea. *Global Change Biology*, *24*(10), 4919–4928. https://doi.org/10.1111/gcb.14401

Costa, M. M., Barrote, I., Silva, J., Olivé, I., Alexandre, A., Albano, S., & Santos, R. (2015). Epiphytes Modulate Posidonia oceanica Photosynthetic Production, Energetic Balance, Antioxidant Mechanisms, and Oxidative Damage. *Frontiers in Marine Science*, *2*(111). https://doi.org/10.3389/fmars.2015.00111

Cox, T. E., Gazeau, F., Alliouane, S., Hendriks, I. E., Mahacek, P., Le Fur, A., & Gattuso, J. P. (2016). Effects of in situ CO2 enrichment on structural characteristics, photosynthesis, and growth of the Mediterranean seagrass Posidonia oceanica. *Biogeosciences*, *13*(7), 2179–2194. https://doi.org/10.5194/bg-13-2179-2016

Cox, T. E., Schenone, S., Delille, J., Diaz-Castaneda, V., Alliouane, S., Gattuso, J.-P., & Gazeau, F. (2015). Effects of ocean acidification on Posidonia oceanica epiphytic community and shoot productivity. *Journal of Ecology*, *103*, 1594–1609. https://doi.org/10.1111/1365-2745.12477

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean Acidification: The Other CO 2 Problem. *Annu.Rev.Mar.Sci.*, *1*, 169–192. https://doi.org/10.1146/annurev.marine.010908.163834

Donnarumma, L., Lombardi, C., Cocito, S., & Gambi, M. C. (2014). Settlement pattern of Posidonia oceanica epibionts along a gradient of ocean acidification: An approach with mimics. *Mediterranean Marine Science*, *15*(3), 498–509. https://doi.org/10.12681/MMS.677

Duarte, C. M., Kennedy, H., Marbà, N., & Hendriks, I. (2013). Assessing the capacity of seagrass meadows for carbon burial: Current limitations and future strategies. *Ocean and Coastal Management*, *83*, 32–38. https://doi.org/10.1016/j.ocecoaman.2011.09.001

Egea, L. G., Jimé Nez-Ramos, R., Herná Ndez, I., Bouma, T. J., & Brun, F. G. (2018). Effects of ocean acidification and hydrodynamic conditions on carbon metabolism and dissolved organic carbon (DOC) fluxes in seagrass populations. *PLoS ONE*, 1–20. https://doi.org/10.1371/journal.pone.0192402

Foo, S. A., Byrne, M., Ricevuto, E., & Gambi, M. C. (2018). The carbon dioxide vents of Ischia, Italy, a natural system to assess impacts of ocean acidification on marine ecosystems: An overview of research and comparisons with other vent systems. *Oceanography and Marine Biology*, *56*, 237–310. https://doi.org/10.1201/9780429454455-4

Garrard, S. L., Gambi, M. C., Scipione, M. B., Patti, F. P., Lorenti, M., Zupo, V., Paterson, D. M., & Buia, M. C. (2014). Indirect effects may buffer negative responses of seagrass invertebrate communities to ocean acidification. *Journal of Experimental Marine Biology and Ecology*, *461*, 31–38. https://doi.org/10.1016/J.JEMBE.2014.07.011

Gravili, C., Cozzoli, F., & Gambi, M. C. (2021). Epiphytic hydroids on Posidonia oceanica seagrass meadows are winner organisms under future ocean acidification conditions: Evidence from a CO2 vent system (Ischia Island, Italy). *European Zoological Journal*, *88*(1), 472–486. https://doi.org/10.1080/24750263.2021.1899317

Guilini, K., Weber, M., De Beer, D., Schneider, M., Molari, M., Lott, C., Bodnar, W., Mascart, T., De Troch, M., & Vanreusel, A. (2017). Response of Posidonia oceanica seagrass and its epibiont communities to ocean acidification. *PLoS ONE*, *12*(8). https://doi.org/10.1371/journal.pone.0181531

Hall-Spencer, J. M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S. M., Rowley, S. J., Tedesco, D., & Buia, M.-C. (2008). Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature*, *454*. https://doi.org/10.1038/nature07051

Hansen, A. B., Pedersen, A. S., Kühl, M., & Brodersen, K. E. (2022). Temperature Effects on Leaf and Epiphyte Photosynthesis, Bicarbonate Use and Diel O 2 Budgets of the Seagrass Zostera marina L. *Frontiers in Marine Science*, *9*(822485). https://doi.org/10.3389/fmars.2022.822485

Hasegawa, N., Hori, M., & Mukai, H. (2007). Seasonal shifts in seagrass bed primary producers in a cold-temperate estuary: Dynamics of eelgrass Zostera marina and associated epiphytic algae. *Aquatic Botany*, *86*(4), 337–345. https://doi.org/10.1016/J.AQUABOT.2006.12.002

Hendriks, I. E., Olsen, Y. S., Ramajo, L., Basso, L., Steckbauer, A., Moore, T. S., Howard, J., & Duarte, C. M. (2014). Photosynthetic activity buffers ocean acidification in seagrass meadows. *Biogeosciences*, *11*, 333–346. https://doi.org/10.5194/bg-11-333-2014

Hernán, G., Ramajo, L., Basso, L., Delgado, A., Terrados, J., Duarte, C. M., & Tomas, F. (2016). Seagrass (Posidonia oceanica) seedlings in a high-CO 2 world: From physiology to herbivory. *Scientific Reports*, *6*(38017). https://doi.org/10.1038/srep38017

Jiang, Z. J., Huang, X.-P., & Zhang, J.-P. (2010). Effects of CO 2 Enrichment on Photosynthesis, Growth, and Biochemical Composition of Seagrass Thalassia hemprichii (Ehrenb.) Aschers. *Journal of Integrative Plant Biology*, *52*(10), 904–913. https://doi.org/10.1111/j.1744-7909.2010.00991.x

Koch, M., Bowes, G., Ross, C., & Zhang, X.-H. (2013). Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Global Change Biology*, *19*, 103–132. https://doi.org/10.1111/j.1365-2486.2012.02791.x

Kohn, T., Rast, P., Kallscheuer, N., Wiegand, S., Boedeker, C., Jetten, M. S. M., Jeske, O., Vollmers, J., Kaster, A.-K., Rohde, M., Jogler, M., & Jogler, C. (2020). The Microbiome of Posidonia oceanica Seagrass Leaves Can Be Dominated by Planctomycetes. *Front. Microbiol*, *11*, 1458. https://doi.org/10.3389/fmicb.2020.01458

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M., & Gattuso, J.-P. (2013). Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Global Change Biology*, *19*, 1884–1896. https://doi.org/10.1111/gcb.12179

Langsrud, Ø. (2003). ANOVA for unbalanced data: Use Type II instead of Type III sums of squares. *Statistics and Computing*, *13*, 163–167.

Lovelock, C. E., Atwood, T., Baldock, J., Duarte, C. M., Hickey, S., Lavery, P. S., Masque, P., Macreadie, P. I., Ricart, A. M., Serrano, O., & Steven, A. (2017). Assessing the risk of carbon dioxide emissions from blue carbon ecosystems. *Front Ecol Environ*, *15*(5), 257–265. https://doi.org/10.1002/fee.1491

Mabrouk, L., Hamza, A., Brahim, B., & Bradai, M.-N. (2013). Variability in the structure of epiphyte assemblages on leaves and rhizomes of Posidonia oceanica in relation to human disturbances in a seagrass meadow off Tunisia. *Aquatic Botany*, *108*, 33–40. https://doi.org/10.1016/j.aquabot.2013.03.002

Marbà, N., Díaz-Almela, E., & Duarte, C. M. (2014). Mediterranean seagrass (Posidonia oceanica) loss between 1842 and 2009. *Biological Conservation*, *176*. https://doi.org/10.1016/j.biocon.2014.05.024

Marbà, N., & Duarte, C. M. (2010). Mediterranean warming triggers seagrass (Posidonia oceanica) shoot mortality. *Global Change Biology*, *16*(8), 2366–2375. https://doi.org/10.1111/j.1365-2486.2009.02130.x

Martin, S., Rodolfo-Metalpa, R., Ransome, E., Rowley, S., Buia, M.-C., Gattuso, J.-P., & Hall-Spencer, J. (2008). Effects of naturally acidified seawater on seagrass calcareous epibionts. *Biol. Lett*, *4*, 689–692. https://doi.org/10.1098/rsbl.2008.0412

Mecca, S., Casoli, E., Ardizzone, G., & Gambi, M. C. (2020). Effects of ocean acidification on phenology and epiphytes of the seagrass Posidonia oceanica at two CO2 vent systems of Ischia (Italy). *Mediterranean Marine Science*, *21*(1), 70–83. https://doi.org/10.12681/MMS.20795

Noisette, F., Depetris, A., Kühl, M., & Brodersen, K. E. (2020). Flow and epiphyte growth effects on the thermal, optical and chemical microenvironment in the leaf phyllosphere of seagrass (Zostera marina). *Journal of the Royal Society Interface*, *17*. https://doi.org/10.1098/rsif.2020.0485rsif20200485

Olivé, I., Silva, J., Costa, M. M., & Santos, R. (2016). Estimating Seagrass Community Metabolism Using Benthic Chambers: The Effect of Incubation Time. *Estuaries and Coasts*, *39*, 138–144. https://doi.org/10.1007/s12237-015-9973-z

Palacios, S. L., & Zimmerman, R. C. (2007). Response of eelgrass Zostera marina to CO2 enrichment: Possible impacts of climate change and potential for remediation of coastal habitats. *Marine Ecology Progress Series*, *344*, 1–13. https://doi.org/10.3354/MEPS07084

Piazzi, L., Balata, D., & Ceccherelli, G. (2016). Epiphyte assemblages of the Mediterranean seagrass Posidonia oceanica: An overview. *Marine Ecology*, *37*, 3–41. https://doi.org/10.1111/maec.12331

Riebesell, U., Zondervan, I., Rost, B., Tortell, P. D., Zeebe, R. E., & Morel, F. M. M. (2000). Reduced calcification of marine plankton in response to increased atmospheric CO2. *Nature*, *407*, 364–367. https://doi.org/10.1038/35030078

Rodolfo-Metalpa, R., Lombardi, C., Cocito, S., Hall-Spencer, J. M., & Gambi, M. C. (2010). Effects of ocean acidification and high temperatures on the bryozoan Myriapora truncata at natural CO2 vents. *Marine Ecology*, *31*(3), 447–456. https://doi.org/10.1111/J.1439-0485.2009.00354.X

RStudio Team. (2021). RStudio. In *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.* [Computer software].

Ruocco, N., Mutalipassi, M., Pollio, A., Costantini, S., Costantini, M., & Zupo, V. (2018). First evidence of Halomicronema metazoicum (Cyanobacteria) free-living on Posidonia oceanica leaves. *PLoS ONE*, *13*(10). https://doi.org/10.1371/journal.pone.0204954

Scartazza, A., Moscatello, S., Gavrichkova, O., Buia, M. C., Lauteri, M., Battistelli, A., Lorenti, M., Garrard, S. L., Calfapietra, C., & Brugnoli, E. (2017). Carbon and nitrogen allocation strategy in Posidonia oceanica is altered by seawater acidification. *Science of the Total Environment*, *607*, 954–964. https://doi.org/10.1016/j.scitotenv.2017.06.084

Seymour, J. R., Laverock, B., Nielsen, D. A., M., T.-T. S., & Macreadie, P. I. (2018). The Microbiology of Seagrasses. In *Seagrasses of Australia* (pp. 343–392). Springer International Publishing. https://doi.org/10.1007/978-3-319-71354-0

Tarquinio, F., Hyndes, G. A., Laverock, B., Koenders, A., & Säwström, C. (2019). The seagrass holobiont: Understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. *FEMS Microbiology Letters*, *366*(6), 1–15. https://doi.org/10.1093/femsle/fnz057

Teixidó, N., Gambi, M. C., Parravacini, V., Kroeker, K., Micheli, F., Villéger, S., & Ballesteros, E. (2018). Functional biodiversity loss along natural CO 2 gradients. *Nature Communications*, *9*(5149). https://doi.org/10.1038/s41467-018-07592-1

Touchette, B. W., & Burkholder, J. A. M. (2000). Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, *250*, 133–167. https://doi.org/10.1016/S0022-0981(00)00195-7

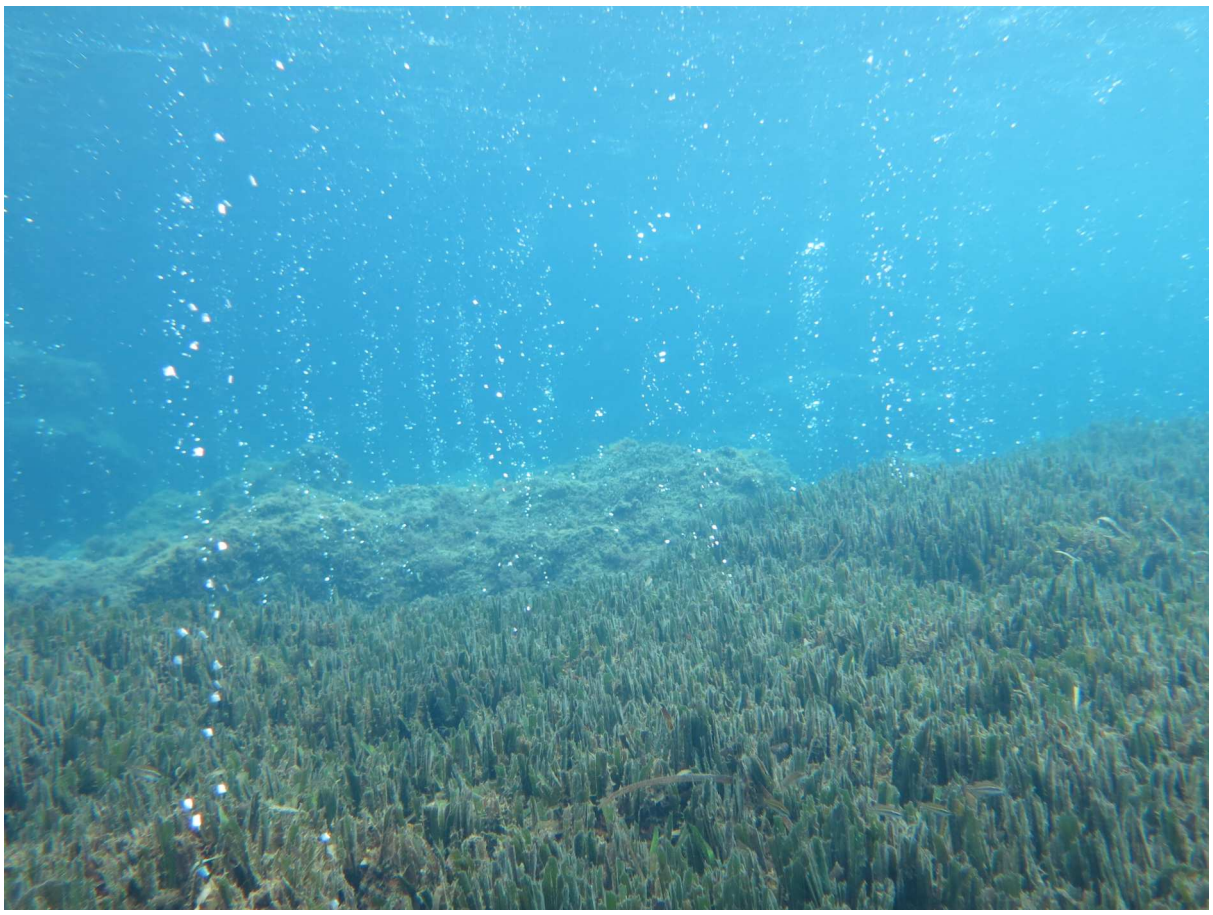
Ugarelli, K., Chakrabarti, S., Laas, P., & Stingl, U. (2017). The Seagrass Holobiont and Its Microbiome. *Microorganisms*, *5*(81). https://doi.org/10.3390/microorganisms5040081

Wear, D. J., Sullivan, M. J., Moore, A. D., & Millie, D. F. (1999). Effects of water-column enrichment on the production dynamics of three seagrass species and their epiphytic algae. *Marine Ecology Progress Series*, *179*, 201–213. https://doi.org/10.3354/MEPS179201

Zimmerman, R. C., Hill, V. J., Jinuntuya, M., Celebi, B., Ruble, D., Smith, M., Cedeno, T., & Swingle, W. M. (2017). Experimental impacts of climate warming and ocean carbonation on eelgrass Zostera marina. *Marine Ecology Progress Series*, *566*, 1–15. https://doi.org/10.3354/MEPS12051

Zunino, S., Libralato, S., Canu, D. M., Prato, G., & Solidoro, C. (2021). Impact of Ocean Acidification on Ecosystem Functioning and Services in Habitat-Forming Species and Marine Ecosystems. *Ecosystems*. https://doi.org/10.1007/s10021-021-0060

CHAPTER 3



Posidonia oceanica meadow growing at the CO_2 vents at Ischia Island (Italy). Photo by Ulisse Cardini.

CHAPTER 3

ACCELERATED NITROGEN CYCLING ON MEDITERRANEAN SEAGRASS LEAVES AT VOLCANIC CO_2 VENTS

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ABSTRACT

Seagrass meadows form highly productive and diverse ecosystems in coastal areas worldwide, where they are increasingly exposed to ocean acidification (OA). Efficient nitrogen (N) cycling and uptake are essential to maintain plant productivity, but the effects of OA on N transformations in these systems are poorly understood. Here we show that complete N cycling occurs on leaves of the Mediterranean seagrass *Posidonia oceanica*, with OA affecting both N gain and loss while the epiphytic microbial community structure remains largely unaffected. Daily leaf-associated N_2 fixation contributes to 35% of the plant's N demand under ambient pH, while it contributes to 45% under OA. Nitrification potential is only detected under OA, and N-loss via N_2 production increases, although the balance remains decisively in favor of enhanced N gain. Our work highlights the role of the N-cycling microbiome in seagrass adaptation to OA, with key N transformations accelerating towards increased N gain.

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INTRODUCTION

Seagrass meadows are highly productive ecosystems worldwide, often occurring in nutrient-limited coastal areas (Hemminga & Duarte, 2000). They are among the most ecologically and economically valuable ecosystems on Earth (Björk et al., 2008). Providing habitat, breeding grounds, and food for a wide range of organisms, they are considered 'hotspots' for biodiversity (Hyman et al., 2019). They also play an important role in sequestering large amounts of carbon, comparable to terrestrial forests (Fourqurean et al., 2012). In particular, the Mediterranean seagrass *Posidonia oceanica* can contribute to climate change mitigation through its effective CO_2 uptake and large sequestration capacity (Duarte et al., 2013) and may even act as a buffer against ocean acidification (OA) by temporarily raising the seawater pH through its daylight photosynthesis (Hendriks et al., 2014). This is relevant since the Mediterranean Sea has a higher capacity to absorb anthropogenic CO_2 than other oceans due to its particular CO_2 chemistry and active overturning circulation (Lacoue-Labarthe et al., 2016). The pH of the Mediterranean Sea in the Western basin is predicted to decrease between 0.245 under the most optimistic scenario of the "Special Report: Emissions Scenarios" (SRES) and 0.462 units under the most pessimistic SRES scenario (IPCC, 2007).

Generally, marine plants are expected to benefit from increased CO_2 concentrations as their photosynthetic rates are undersaturated at current CO_2 levels (Koch et al., 2013). However, OA has multifaceted effects on *P. oceanica*. Photosynthetic performance of *P. oceanica* seedlings and net leaf productivity increase under high pCO_2 (Berlinghof et al., 2022; Cox et al., 2015; Hernán et al., 2016), while OA has little effect on the net community production of *P. oceanica* but results in increased shoot density and shorter leaf length due to increased herbivory (Berlinghof et al., 2022; Cox et al., 2016; Scartazza et al., 2017). Calcareous epiphytes such as encrusting red algae, bryozoans, foraminifers, and spirorbids decline or even disappear under OA, while non-calcareous invertebrates such as hydrozoans and tunicates benefit (Berlinghof et al., 2022; Donnarumma et al., 2014; Gravili et al., 2021; Mecca et al., 2020).

Much less attention has been paid to the effects of OA on the biogeochemical cycling of elements other than carbon, such as nitrogen (N). Nitrogen is an essential nutrient for all living organisms and can be a limiting factor for primary production in marine seagrasses (Hemminga et al., 1991), with its availability depending on diverse N transformation processes that are performed by a complex network of metabolically diverse microorganisms (Kuypers et al., 2018). Seawater pH affects N speciation and concentration, which in turn affects metabolic processes and N transformations (Wannicke et al., 2018; Wyatt et al., 2010). Dinitrogen (N_2) fixation by N_2-fixing bacteria and archaea (i.e., diazotrophs) has often been found to increase under OA (Hutchins et al., 2009; Wannicke et al., 2018). The reason is not always clear, but in phototrophs, it may involve more energy being redirected to the demanding N_2 fixation process owing to the down-regulation of carbon-concentrating mechanisms (Kranz et al., 2010; Levitan et al., 2007; Wannicke et al., 2018). Autotrophic microbial nitrification can be highly sensitive to pH, and nitrification in the open ocean is considerably reduced by OA (Beman et al., 2011). Dissimilatory nitrate reduction processes (e.g., denitrification or anaerobic ammonium oxidation - anammox), which are modular and involve many different bacterial groups often found in low-pH environments,

are thought to be less affected by OA, with rates showing contrasting results at low seawater pH (Wannicke et al., 2018).

Many N-cycling microorganisms can be found in close association with *P. oceanica*, together forming a holobiont (Tarquinio et al., 2019; Ugarelli et al., 2017). Seagrass-associated microbes can enhance the N access via ammonification and genes for microbial ammonification can be found ubiquitously in this system (Pfister et al., 2023). N_2 fixation by associated diazotrophic microorganisms can be crucial in providing the N required for seagrass photosynthesis and growth when its availability is limited (Agawin et al., 2016; Mohr et al., 2021). Diazotrophic bacteria have been detected in the rhizosphere of *P. oceanica* (Garcias-Bonet et al., 2016) with high rates of root-associated N_2 fixation reported (Lehnen et al., 2016). Analogous to many land plants that associate with diazotrophs, a recent study shows that *P. oceanica* lives in symbiosis with an N_2-fixing γ-proteobacterium in its roots, providing N in exchange for sugars, that can fully sustain plant biomass production during its primary growth season (Mohr et al., 2021). Apart from this root-symbiosis, N_2 fixation has been shown to occur associated with all parts of *P. oceanica*, both above and below ground (Agawin et al., 2019).

Overall, although rhizosphere N cycling has been the focus of extensive research, precise quantification of N transformations on seagrass leaves, as well as an evaluation of the effects of OA, are still lacking. Phyllospheric N_2 fixation can considerably contribute to the N demand of *P. oceanica* and the N budget in the Mediterranean Sea (Agawin et al., 2016; Mohr et al., 2021). Besides N_2 fixation, we hypothesize that seagrass leaves could also be suitable sites for nitrification. For example, Ling et al. (2018) found a diverse community of ammonia-oxidizing archaea (AOA) and bacteria (AOB) associated with different parts of the seagrass *Thalassia hemprichii*, including leaf tissues. Moreover, anoxic parts within µm to mm-thick biofilms on the leaf surface could provide potential microhabitats for N loss pathways, such as denitrification (Brodersen & Kühl, 2022; Noisette et al., 2020) or anammox performed by groups such as Planctomycetes, which were found to dominate the microbiome of *P. oceanica* leaves at some locations (Kohn et al., 2020).

Here, we investigate the effects of long-term natural OA occurring at volcanic CO_2 vents on the epiphytic prokaryotic community of *P. oceanica* leaves and quantify rates of the key N cycling processes by the plant phyllosphere. We test the effects of pH and the presence/absence of epiphytes in multifactorial laboratory incubations (see Fig. S3.1), using N stable isotope tracers to quantify N_2 fixation, nitrification potential, and anammox and denitrification potential, and net nutrient fluxes to quantify assimilatory processes by leaves and epiphytes. We complement these analyses with 16s rRNA gene amplicon sequencing to explore the diversity of the phyllosphere microbial community and the potential players involved in N transformation processes on seagrass leaves.

RESULTS AND DISCUSSION

COMPLETE MICROBIAL N CYCLING OCCURS IN THE *P. OCEANICA* PHYLLOSPHERE

Incubation experiments with ^{15}N stable isotope labeling reveal that all key microbial N cycling processes occurred in the phyllosphere of *P. oceanica,* with microbial epiphytes contributing to a net N gain in all conditions by the holobiont. To quantify rates of N_2 fixation by the phyllosphere diazotrophic community, we incubated leaf sections with and without epiphytes in $^{15}N_2$-enriched seawater. We detected clear $^{15}N_2$ incorporation in epiphyte tissue in the light incubations, ranging from 0.12 ± 0.05 nmol cm^{-2} h^{-1} (mean ± SE) at the ambient site to 0.62 ± 0.15 nmol N cm^{-2} h^{-1} at the vent site (Fig. 3.1a). $^{15}N_2$ incorporation was 409% higher at the vent site ($F_{1,13}$ = 5.80, p = 0.03, R^2 = 0.52) and in the same order of magnitude as N_2 fixation rates measured *in situ* in minimally disturbed *P. oceanica* meadows (Agawin et al., 2017). Corresponding to dry weight-based rates of up to 131.08 nmol N g DW^{-1} h^{-1}, these rates are also comparable to N_2 fixation rates measured by root symbionts of *P. oceanica* under ambient pH (Lehnen et al., 2016; Mohr et al., 2021). Conversely, we observed significant $^{15}N_2$ incorporation in only one of four replicates in the dark. We did not observe a significant transfer of fixed N to the *P. oceanica* plant tissues in the limited time frame of the experiment, neither in the light nor in the dark (Figs. S3.2, S3.3).

We explored the potential of the phyllosphere microbiome to nitrify in ^{15}N-NH_4^+ incubation experiments. While there was a strong variability among samples (Fig. S3.4), we found significant (>2.5 x SD) potential nitrification rates (PNR) at the vent site when epiphytes were present (Fig. 3.1b), ranging from 0.031 ± 0.007 pmol N cm^{-2} h^{-1} (mean ± SE) in the dark to 0.058 ± 0.004 pmol N cm^{-2} h^{-1} in the light. However, these rates were only marginal compared to the other N transformation processes. PNR was 86% higher in the light ($F_{1,13}$ = 67.00, p < 0.001, R^2 = 0.83). In contrast, we found no significant PNR in incubations with epiphytes from the ambient site, neither in the light nor in the dark. The plant can compete with nitrifiers for N, as NH_4^+ is typically readily taken up by *P. oceanica* (Lepoint et al., 2002), making the leaf phyllosphere a challenging environment for nitrifying prokaryotes. Our measurements of PNR in *P. oceanica* leaves are of relevance, as they indicate that a community of nitrifiers exists that can compete with the plant for NH_4^+ uptake. However, with PNR of up to 0.058 ± 0.004 pmol N cm^{-2} h^{-1}, their net contribution to NH_4^+ or NO_2^- oxidation contributes only marginally to the N budget of the *P. oceanica* phyllosphere.

Previous studies suggested that anoxic parts within thick biofilms on the surface of seagrasses could be suitable microhabitats for microbial-mediated N-loss pathways, such as denitrification and anammox (Brodersen & Kühl, 2022; Noisette et al., 2020). Using incubation experiments of leaf sections amended with ^{15}N-NO_3^-, we report $^{29}N_2$ production rates ranging from 2.43 ± 0.53 pmol N cm^{-2} h^{-1} at the ambient site in the dark to 7.14 ± 2.07 pmol N cm^{-2} h^{-1} at the vent site in the light (Fig. 3.1c) when epiphytes were present. $^{29}N_2$ production was 134% higher at the vent site ($F_{1,13}$ = 10.82, p = 0.006, R^2 = 0.39), while the light/dark treatment had no effect. A significant production rate of $^{30}N_2$ was only detected at the vent site in the light with epiphytes present (18.84 ± 3.33 pmol N cm^{-2} h^{-1}; Fig. 3.1d). Based on these results, we calculated daily budgets of total N-N_2 loss (sum of $^{29}N_2$ and $^{30}N_2$ production) of up to 4.01 ± 0.74 µmol N m^{-2} d^{-1} (or 0.401 ± 0.074 nmol N cm^{-2} d^{-1}) at the vent site. These rates

are significant, and comparable to N loss rates reported from seagrass sediments by Salk et al. (2017),who measured denitrification rates of 0.10 nmol N cm^{-2} d^{-1} and anammox rates of 0.43 nmol N cm^{-2} d^{-1}. The presence of Planctomycetes and detectable rates of $^{29}N_2$ in $^{15}N\text{-}NO_3^-$ amended incubations suggest that anammox may play an important role as an N loss pathway on seagrass leaves.

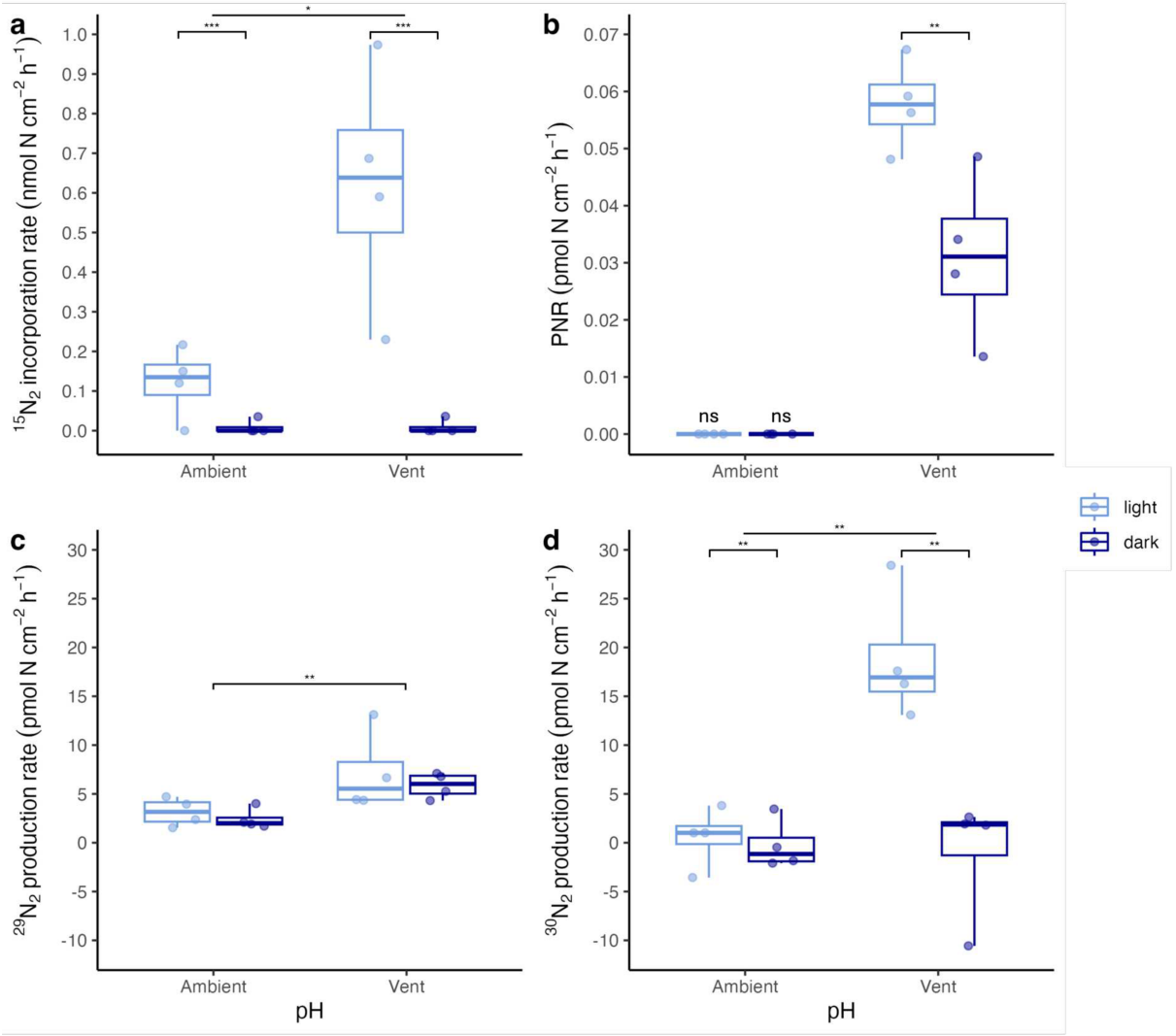

Fig. 3.1. Epiphyte-mediated nitrogen transformations in light and dark incubations from the ambient and vent site. Epiphytic $^{15}N_2$ fixation rates (**a**), potential nitrification rates (PNR) in incubations with epiphytes (**b**), $^{29}N_2$ and $^{30}N_2$ production rate in incubations with epiphytes (**c**, **d**). The center line denotes the median value (50th percentile), the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles. Letters indicate significant differences between treatments, ns indicates enrichment was not significant, n=4.

P. oceanica can assimilate fixed N as NH_4^+ or NO_3^- (Lepoint et al., 2002) but shows a higher affinity for NH_4^+ (Touchette & Burkholder, 2000). While NH_4^+ uptake rates were unaffected by the presence or absence of epiphytes (Fig. S3.5a, b), NO_3^- consumption rates (Fig. S3.5c, d) were increased by 147 - 270 % in the presence of epiphytes. This is probably due to active NO_3^- uptake because NO_3^- loss via denitrification or anammox and nitrification activity was three orders of magnitude lower (Fig. 3.1c, d). Conversely to NH_4^+ uptake, NO_3^- uptake rates were affected by the presence of epiphytes, suggesting that epiphytes may preferentially use this form of N as a strategy to avoid competition for N with the plant, combining active NO_3^- uptake and N_2 fixation.

DISTINCT MICROBIAL COMMUNITIES CONTRIBUTE TO SEAGRASS PHYLLOSPHERE N CYCLING

The 16s rRNA gene amplicon sequencing of the phyllosphere-associated microbiome revealed a diverse microbial community differing between the water column and seagrass leaf community, but not between ambient and vent pH (see Fig. S3.6 and Table S3.1) and including many members potentially involved in N transformation processes on *P. oceanica* leaves.

The leaves were dominated by the phylum *Proteobacteria* with the classes *Alphaproteobacteria* (20-22%) and *Gammaproteobacteria* (9-15%) across both pH sites (Fig. 3.2). Among the predominant orders were *Rhodobacterales* (9%), which are commonly found as first colonizers on marine surfaces and seagrasses, probably due to their ability to be opportunistic and persist in rapidly changing environments (Dang et al., 2008; Mejia et al., 2016; Trevathan-Tackett et al., 2020). About 1.5% of this clade were identified as *Epibacterium*, a genus of common bacteria in coastal areas that have the potential to assimilate ammonium and that also expresses antibacterial activity towards other marine bacteria (Matallana-Surget et al., 2018). Other ammonia oxidizers, such as the strain HIMB11 were identified in the water column (Durham et al., 2014). *Rhodobacterales* also include (putative) N_2 fixers in both terrestrial (Li et al., 2023) and marine (Lesser et al., 2018; Moynihan et al., 2022) environments. We found *Rhizobiales* accounting for 5% of the total leaf community, a taxonomic order that includes a diversity of N_2-fixing microbes that form symbiotic relationships with terrestrial plants (Lindström & Mousavi, 2020) and are known for promoting plant health and growth (Avis et al., 2008). One of the identified genera within this clade was *Pseudovibrio,* a common member of animal and macrophyte holobionts, with the capacity to undergo complete denitrification and, in some species, assimilatory nitrate reduction and probably another regulator of the microbial community through their antibiotic metabolite production (Blanchet et al., 2017).

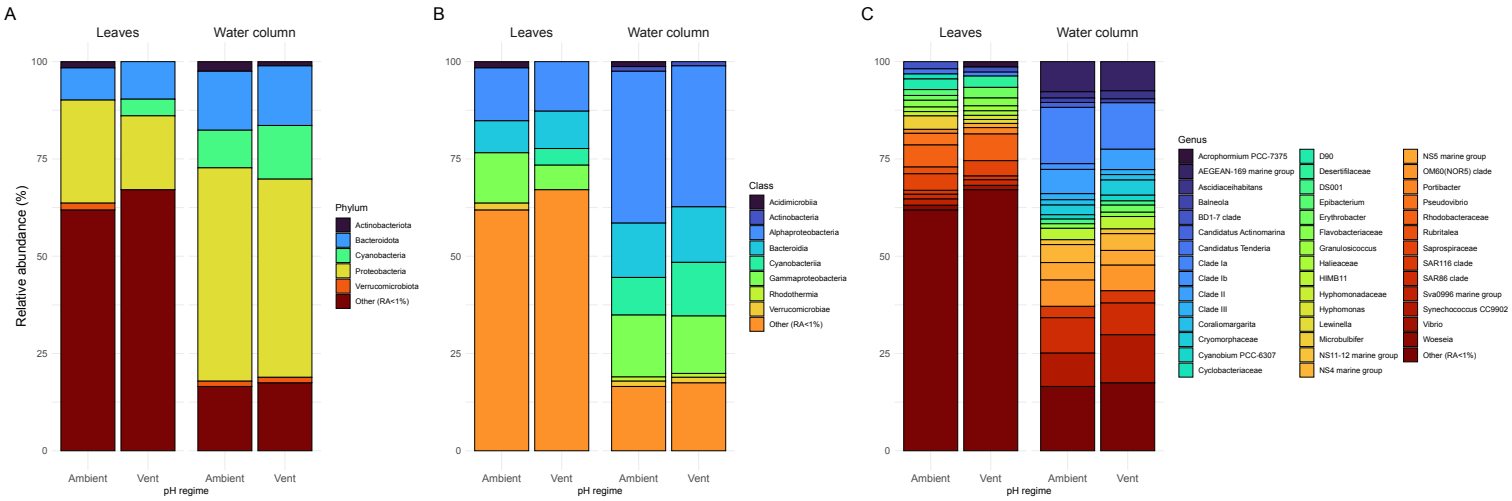

Fig. 3.2. Average relative abundances of prokaryotic taxa. Prokaryotic phyla (a), classes (b), and genera (c) on leaves and water column samples from both pH regimes.

Cyanobacteria accounted for 2-14% of the total leaf community (Fig. 3.2). Especially the orders *Phormidesmiales* and *Cyanobacteriales* had a large effect in the differential abundance analysis (Fig. 3.3). Higher N_2 fixation rates under light conditions suggest a diazotrophic community dominated by species that can cope with O_2 production from daytime photosynthesis, which would otherwise irreversibly inhibit the enzyme nitrogenase. Among the genera that can sustain N_2 fixation in the light (Bergman et al., 2013; Berrendero et al., 2016), the leaves from

both pH regimes comprised sequences for *Schizothrix* (0.22% on leaves vs. 0.01% in the water column) and *Trichodesmium* (up to 0.5% on leaves vs. 0.002% in the water column).

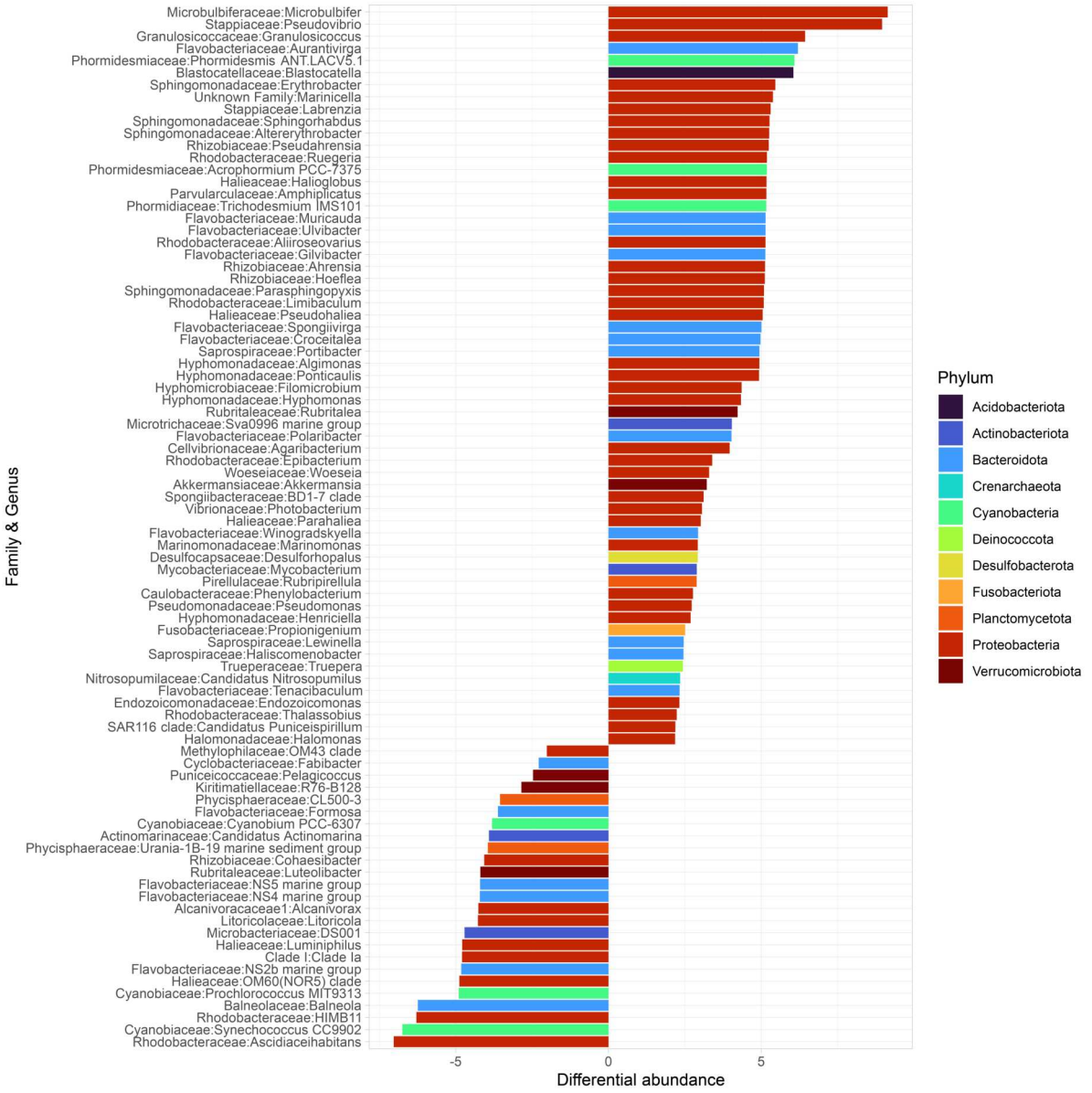

Fig. 3.3. Differential taxonomic order abundance in pooled leaf and water column samples. Positive values mean differential abundance in the leaves and negative values in the water column.

Among the predominant orders in the phylum, *Bacteroidota* (17%) was the order *Flavobacterales* (8%). They are also frequently found as early colonizers on marine surfaces and seagrasses (Mejia et al., 2016; Trevathan-Tackett et al., 2020). In other studies, some photosynthetic and light-dependent members of *Bacteroidota* that harbor the nifH gene, e.g., *Chlorobaculum* and *Chlorobium* (Agawin et al., 2017) , were more abundant on leaves than in the water column. Other heterotrophic bacterial N_2 fixers that may depend on seagrass photosynthetic exudates (Agawin et al., 2017) were found on *P. oceanica* leaves within the *Desulfobacterota* phylum. As part of the *P. oceanica* leaf microbiome, these groups are likely to collectively contribute to N_2 fixation as a consortium of (directly or indirectly) light-dependent N_2 fixers.

Granulosicoccus was the phylotype with the largest effect detected in the differential abundance analysis (Fig. 3.3). It has been often found as part of the phyllosphere microbiome of macroalgae and seagrasses (Crump et al., 2018; Sanders-Smith et al., 2020; Weigel et al., 2022) having the potential for dissimilatory nitrate reduction to ammonium and the synthesis of vitamins that are needed by their macroalgae host (Weigel et al., 2022). Among the potential denitrifiers, the gammaproteobacterium *Marinicella* was predominantly detected on *P. oceanica* leaves; it often contributes to denitrification in *Synechococcus*-dominated biofilms and anammox-concentrating reactors (Van Duc et al., 2018; Yin et al., 2021; Zhang et al., 2021).

Planctomycetes accounted for 2% of the microbial leaf community (Fig. 3.2) and were more abundant on the leaves than in the water column (Fig. 3.3). *Planctomycetes* are commonly found on macroalgae across the globe (Bondoso et al., 2017; Lage & Bondoso, 2014) and can even dominate the *P. oceanica* leaf microbiome (Kohn et al., 2020). Members of this phylum have been linked to N_2 fixation in surface ocean waters (Delmont et al., 2018). Among *Planctomycetes* are also members that can utilize anammox to gain energy by anaerobically oxidizing NH_4^+ with NO_2^- as the electron acceptor (Jetten et al., 2009; Strous et al., 1999). There is also potential for their participation in nitrification, as the family Gemmataceae and several others that we detected in both the leaves and water column harbor the genes to code for the nitronate monooxygenase (Rambo et al., 2020).

Finally, we found significantly higher relative abundances of the families Nitrosomonadaceae, Nitrospiraceae, Nitrospinaceae (AOB), and Nitrosopumilales (AOA) in the phyllosphere of *P. oceanica* (Fig. S3.7, Table S3.2), all of which include nitrifying members (Hutchins & Capone, 2022; Kuypers et al., 2018). In particular, we found a higher relative abundance of *Nitrosopumilales* (family *Nitrosopumilaceae*) on leaves, which often show a higher affinity for ammonia than AOB (Jung et al., 2022; Martens-Habbena et al., 2009), further indicating that competition for NH_4^+ plays a major role on seagrass leaves.

OCEAN ACIDIFICATION ACCELERATES N CYCLING TOWARDS HIGHER N_2 FIXATION AND N UPTAKE

Our results show that OA occurring at natural CO_2 vents accelerated key N transformation processes associated with the phyllosphere of *P. oceanica*, while the prokaryotic community structure remained largely unaffected. To quantify N transformation rates under OA conditions, we incubated leaf sections from CO_2 vents, where the plant and its epiphytic community are acclimated to long-term CO_2 enrichment and lower pH (vent pH = 7.80 ± 0.14; ambient pH = 8.08 ± 0.04). We found that daylight N_2 fixation was significantly higher on leaves acclimated to low pH (Fig. 3.1a). The positive response of N_2 fixation rates to elevated CO_2 concentrations is supported by several studies with planktonic diazotrophs, such as *Trichodesmium*, *Crocosphaera*, and *Nodularia* (see review papers by Kroeker et al. (2013); Liu et al. (2010); Wannicke et al. (2018)). A widely accepted explanation for the positive influence of elevated CO_2 concentrations on some diazotrophs is their ability to reallocate energy from the downregulation of carbon-concentrating mechanisms to N_2 fixation (Kroeker et al., 2013; Wannicke et al., 2018).

Notably, potential nitrification (PNR) was only detected under OA conditions in our incubations (Fig. 3.1b). Reduced pH is generally expected to negatively affect ammonium oxidation in the first step of nitrification

(Beman et al., 2011; Kitidis et al., 2011). However, some studies showed that increasing CO_2 levels could lead to higher autotrophic nitrification rates by reducing CO_2 limitation (Hutchins et al., 2009) and that a diverse nitrifier community, such as that found in estuarine and coastal sediments, could adapt to a wider range of pH values (Fulweiler et al., 2011).

Ocean acidification is generally not expected to have a major, direct effect on denitrification and anammox, as both processes occur in anaerobic environments that already have elevated CO_2 concentrations and low pH values (Hutchins et al., 2009; Wannicke et al., 2018). However, on *P. oceanica* leaves under high CO_2 conditions, an increase in both C (see Berlinghof et al., 2022) and N_2 fixation, as well as nitrification, may have favored the formation of anoxic microniches on the leaf biofilm and generated organic C and oxidized N compounds available for metabolism by denitrifying bacteria.

We observed that NH_4^+ uptake rates were increased by 62 – 97% at the vent site and NO_3^- uptake rates were increased by 330 - 412 % (Fig. S3.5c, d). At the ambient site, we measured higher epiphyte cover and lower net primary production and respiration (Berlinghof et al., 2022), which can affect nutrient uptake rates. Apostolaki et al. (2012) showed that N uptake in leaves decreases with increasing epiphyte load, suggesting that epiphyte overgrowth inhibits leaf N uptake in *P. oceanica*. On the other hand, the seagrass may adapt to an increased N demand due to higher productivity under OA. This agrees with Ravaglioli et al. (2017), who found overexpression of N transporter genes after nutrient addition at low pH, suggesting increased N uptake by the seagrass.

While N cycling on the *P. oceanica* phyllosphere accelerated under high CO_2, the prokaryotic community structure remained largely unaffected. Similarly, Banister et al. (2021) found that the leaf-associated microbiome of the seagrass *Cymodocea nodosa* was stable across pH gradients at a comparable Mediterranean CO_2 vent site. The microbial community of *P. oceanica* was also found to be stable in environments differing in other geomorphological traits, e.g., depth, substrate, and turbidity (Rotini et al., 2023). Conversely, colonization experiments using an inert substrate showed marked differences in coastal microbial biofilms between natural pH and vent-exposed sites (Lidbury et al., 2012). A stable microbial community in our study supports the hypothesis of a microbiome that is regulated by interactions with its plant host (Crump et al., 2018), while our biogeochemical measurements suggest the presence of coupled metabolisms between the seagrass and its microbiome contributing to plant health and adaptation in a high-CO_2 world.

PHYLLOSPHERE N CYCLING CONTRIBUTES TO THE HOLOBIONT N DEMAND

We calculated daily rates in mmol N m^{-2} d^{-1} of plant and epiphyte-mediated N-cycling processes at vent and ambient pH based on a 12:12 light/dark cycle (Fig. 3.4a, b). We further calculated the percentage of daily primary production of the *P. oceanica* holobiont (plant + epiphytes) that can be supported by leaf-associated N_2 fixation (Fig. 3.4c, d).

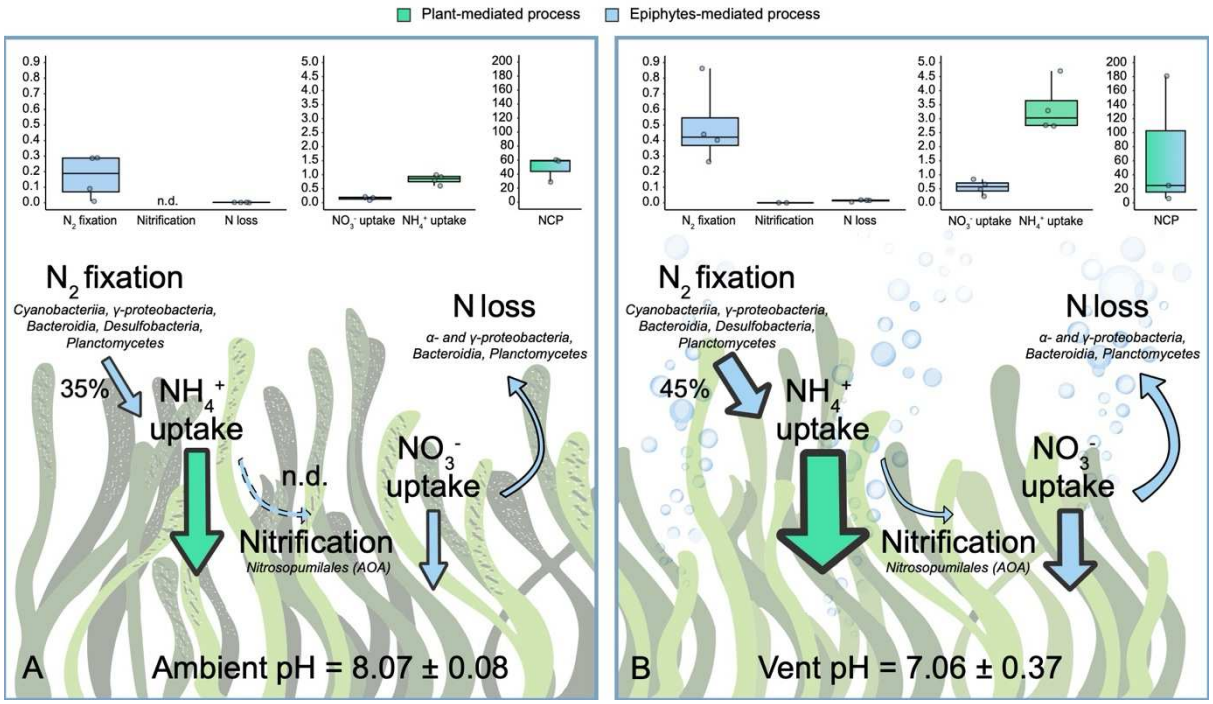

Fig. 3.4. Overview of N cycling processes under ambient and vent pH conditions. The metabolic rates (in mmol m^{-2} $_{meadow\ area}$ d^{-1}) for plant- and epiphyte-mediated processes under ambient (A) and vent (B) pH conditions, based on a 12:12 h light and dark cycle, are depicted in the upper portion of each panel. Data distribution is shown in a box plot format, with the center line denoting the median value (50th percentile), the box encapsulating the interquartile range (25th to 75th percentiles), and whiskers indicating the 5th and 95th percentiles. Nitrification was not detectable (n.d.) at the ambient site. The lower portion of each panel employs arrow size to convey the relative differences in N cycling processes. Additionally, the % contribution of N_2 fixation to the estimated N demand of the plant, as well as relevant taxa in the microbial community for each N cycling process, are provided for further context.

Although NCP, and thus the seagrass N demand, was higher under OA, the contribution of N_2 fixation to meeting this demand was increased at vent pH. N_2 fixation contributed with 169 ± 71 mmol N m^{-2} d^{-1} to 35 % of the seagrass N demand at ambient pH and with 493 ± 129 mmol N m^{-2} d^{-1} to 45 % at vent pH (Fig. 3.4). The contribution of N_2 fixation to the seagrass N demand has been reported to be highly variable over seasonal (e.g., Agawin et al., 2017; Cardini et al., 2018) and spatial (Agawin et al., 2017) gradients. Integrating the seasonal values over a year, Agawin et al. (2017) calculated that ca. 15% of the annual plant N demand can be provided by aboveground N_2 fixation in *P. oceanica* meadows. Further research (e.g., using NanoSIMS or longer-term incubations) should investigate how much of the N fixed by the epiphytic diazotrophs is actually transferred to the plant host.

A large fraction of the *P. oceanica* holobiont N demand was obtained through NH_4^+ uptake with 829 ± 87 mmol N m^{-2} d^{-1} at the ambient and 3376 ± 461 mmol N m^{-2} d^{-1} at the vent site (Fig. 3.4). NH_4^+ uptake was considered being plant-mediated, because the presence of epiphytes had no significant effect (Fig S3.5). NO_3^- uptake, primarily attributed to the epiphytic community, contributed with 159 ± 37 mmol N m^{-2} d^{-1} at the ambient and 555 ± 139 mmol N m^{-2} d^{-1} at the vent site. NO_3^- uptake rates were comparable to the annual average NO_3^- leaf uptake by Lepoint et al. (2002) (1.2 g N m^{-2} yr^{-1} = 235 mmol N m^{-2} d^{-1}). Conversely, our NH_4^+ uptake rates were higher than their maximum values obtained in spring months (1300 mg N m^{-2} h^{-1} = 2227 mmol N m^{-2} d^{-1}; Lepoint et al., 2002). However, they also show that large seasonal differences can occur, with values ranging from 0 to

2227 mmol N m^{-2} d^{-1}. The total N gain in our study (N_2 fixation + NH_4^+ and NO_3^- uptake - N loss) was 1115 ± 194 mmol N m^{-2} d^{-1} at the ambient and 4410 ± 727 mmol N m^{-2} d^{-1} at the vent site. Thus, OA tipped the balance decisively in favor of increased N gain.

Taken together, our results show that major N cycling processes occur on *P. oceanica* leaves, and that epiphytes contribute to net N uptake by the holobiont. Ocean acidification occurring at the investigated volcanic CO_2 vent accelerates N cycling, while the prokaryotic community structure remains largely unaffected. At a vent pH (~ 7), high rates of microbial daylight N_2 fixation on the phyllosphere of *P. oceanica* can partially sustain the increased C-fixation and thus N demand of the holobiont. Further experiments at comparable sites with reduced pH should investigate whether our results can be generalized to a broader spatial scale. Access to diverse N sources may help to avoid competition within the holobiont. Adaptation of marine plants to environmental changes is fundamental for their survival; here we show that functional plasticity of their N-cycling microbiome is a key factor in regulating seagrass holobiont functioning on a changing planet.

METHODS

STUDY AREA AND SAMPLING

The study area is located at the islet of Castello Aragonese on the northeastern coast of the island of Ischia (Tyrrhenian Sea, Italy). This site is characterized by the presence of submarine CO_2 vents of volcanic origin, which naturally generate a gradient in CO_2 concentration and pH, without affecting the surrounding water temperature or salinity (Foo et al., 2018; Hall-Spencer et al., 2008). Around the islet, meadows of *P. oceanica* occur at depths of 0.5 - 3 m, also extending into vent zones with low pH. We selected two sites characterized by different pH regimes (vent pH = 7.80 ± 0.14; ambient pH = 8.08 ± 0.04; Table S3.3) at approximately 3 m water depth. We restricted our study locations to these sites because not many vent sites have comparable levels of CO_2, depth, light, and hydrodynamics. Increasing the number of locations would have increased confounding factors, potentially affecting the reliability and consistency of our data. The vent pH site was located in a vent area on the south side (40°43'50.5"N 13°57'47.2"E) and the ambient pH site was located on the north side of the bridge (40°43'54.8"N 13°57'47.1"E).

For the incubation experiments, shoots of *P. oceanica* were collected at each site at three days in September 2019 and transported directly to the laboratory. Sections of the central part of the leaf (3 cm in length) were cut off, selecting leaves with homogeneous epiphyte coverage, and avoiding heavily grazed and senescent parts of the plant, as described in Berlinghof et al. (2022). Macro-epiphytes and biofilm were carefully removed from half of the seagrass leaves with a scalpel, ensuring the removal of the majority of microbial epiphytes and taking special care not to damage the plant tissue. Leaf sections from the vent pH and ambient pH sites, with epiphytes present (n = 4) or removed (n = 3), were used for dark and incubations. Focusing on the leaves allowed us to control for the community composition within the phyllosphere exposed to oxygen-rich seawaters and avoid contrasting processes occurring between the mainly oxidized aboveground phyllosphere and the mainly reduced belowground rhizosphere.

Samples for microbial community analysis were collected in October 2019 at the vent (n = 3) and ambient site (n = 4) described above. Before disturbing the plants, we collected 5 L of seawater from the water column above the plants at each site. Whole seagrass plants were collected, and the central part of the leaf was cut off with sterile tools, washed with sterile NaCl solution [0.8 % m/v] to remove loosely attached microorganisms, and transferred to 15 mL falcon tubes with sterile tweezers. The falcon tubes were kept in dry ice during transport to the laboratory (SZN Villa Dohrn, Ischia, Italy) and then stored at -20°C. In the laboratory, the seawater was immediately filtered on 0.2 µm cellulose nitrate membrane filters (n = 2 at each site) and the filters were stored at -20°C until further genetic analysis.

PROKARYOTIC DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

DNA from seagrass and seawater samples was extracted using the Qiagen DNeasy Powersoil Kit (Qiagen). For seawater, the entire membrane filters were used, while for seagrass, we cut approximately 1 g of the central part of the leaf. Leaf samples were placed into 2 mL vials containing 600 µL of sterile NaCl solution [0.8 % m/v] and were vortexed three times for 30 s according to the protocol of the Seagrass Microbiome Project (https://seagrassmicrobiome.org). The solution was transferred to the Powerbead columns (Qiagen) and then processed according to the manufacturer's instructions with slight modifications to increase DNA yield and quality, as described in Basili et al. (2020). The extracted DNA samples were quantified using a microvolume spectrophotometer (Thermo Scientific NanoDrop 2000c) and stored at -20 °C until processing.

Illumina MiSeq sequencing (2 x 300 bp paired-end protocol) of the hypervariable V4 region of the 16S rRNA gene was performed using the 515FB and 806RB bacteria- and archaea-specific primers (Walters et al., 2016). The primers were removed from the raw sequence data using cutadapt v2.8 (Martin, 2011) and the fastq files were processed using the R package *DADA2* (Callahan et al., 2016; R Core team, 2023). Quality filtering and denoising of the trimmed fastq files was performed using the following parameters: "truncLen = c(200, 200), maxEE = c(2, 2), truncQ = 2, ndmaxN = 0). Paired-end reads were then merged into amplicon sequence variants (ASVs); chimeric sequences were identified and removed. Prokaryotic taxonomy assignment was performed using the SILVA v138 database (Quast et al., 2012). The complete pipeline is openly available in the research compendium accompanying this paper at https://github.com/luismmontilla/embrace. The sequences are available in the NCBI SRA database as the BioProject ID PRJNA824287.

BIOINFORMATICS AND DATA ANALYSIS OF THE SEQUENCING DATA

The ASV matrix was analyzed as a compositional dataset, as described in detail in other works (Gloor et al., 2017; Quinn et al., 2018). Briefly, we transformed the raw pseudo-counts using the centered-log ratio to handle the data in a Euclidean space. We then tested the null hypothesis of no effect of the factors described above on the prokaryotic community associated with *P. oceanica* using a permutation-based multivariate analysis of variance (PERMANOVA) derived from a Euclidean distance matrix. We performed this test using the *vegan* package for R (Anderson, 2001; Oksanen et al., 2020). In addition, we performed a differential abundance analysis of the ASVs (pooled leaf vs water column samples) using the ANOVA-like differential expression method implemented in the

ALDEX2 package for R (Fernandes et al., 2013). This algorithm produces consistent results, whereas other analyses can be variable depending on the parameters set by the researcher or required by the dataset (Nearing et al., 2022).

DINITROGEN FIXATION

The $^{15}N_2$-enriched seawater addition method was used to determine N_2 fixation rates (Klawonn et al., 2015). The $^{15}N_2$ gas (Cambridge Isotope Laboratories Inc.) was tested negative for contamination with ^{15}N–labeled ammonium. Stock solutions of 0.22 µm filtered and $^{15}N_2$-enriched water from the two study sites (vent and ambient pH) were prepared and gently transferred to 24 mL glass vials to minimize gas exchange with the atmosphere. Subsequently, one section of a seagrass leaf with (n = 4) and without epiphytes (n = 3) was added per vial and the vials were sealed without leaving any headspace. Additionally, vials with 0.22 µm filtered but unenriched site water containing leaves with epiphytes served as controls to account for potential variation in the natural abundance of ^{15}N in epiphytes or leaves (n = 3, see also Fig. S3.1 for the experimental design). The vials were incubated on a shaker (Stuart Orbital Shaker SSL1; 30 rpm); vials for dark incubations were covered with aluminum foil. Incubations were performed in a temperature-controlled room at 22°C. After an incubation period of T0 = 0 h, T1 = 5 h, and T2 = 9 h light/ 8 h dark, three or four vials from each treatment were opened for sampling. At the beginning and end of the incubation, oxygen concentrations in the incubation vials were measured without opening the vials using a fiber-optic oxygen sensor with sensor spots (FireStingO2, PyroScience), and pH was measured using a pH meter (Multi 3430, WTW).

For tissue analysis, epiphytes were removed from seagrass leaves with a scalpel, transferred separately into Eppendorf tubes, and freeze-dried for 72 h. They were then homogenized in a mortar, weighed, and transferred into tin cups to determine carbon (%C) and nitrogen content (%N), and ^{15}N incorporation. Water samples were transferred to 12 mL exetainers (Labco Ltd) and fixed with 200 µL of 7 M $ZnCl_2$ for $^{29}N_2$ and $^{30}N_2$ analyses to calculate atom% excess of the medium. In addition, samples for the analysis of dissolved inorganic nitrogen (DIN: NH_4^+, NO_2^-, NO_x^-) and PO_4^{3-} were transferred to 20 ml HDPE vials and stored at -20°C until further analysis.

Carbon (%C) and nitrogen (%N) content and the isotopic composition ($\delta^{13}C$, $\delta^{15}N$) in seagrass leaves and epiphyte tissue were analyzed by isotope ratio mass spectrometry (IRMS, Delta plus V, Thermo Scientific) coupled to an elemental analyzer (Flash EA1112, Thermo Scientific) at Aarhus University (Denmark). $^{15}N_2$ fixation rates were calculated according to Montoya et al. (1996):

$$^{15}N_{excess} = {}^{15}N_{sample} - {}^{15}N_{NA} \quad \text{(I)}$$

$$N_2 \text{ fixation} = (\text{atom\%}(^{15}N_{excess}) / \text{atom\%}(^{15}N_{medium})) \times (PN_{sample} / t) \quad \text{(II)}$$

$^{15}N_{sample}$ is the ^{15}N content of the samples after exposure to $^{15}N_2$ enriched seawater, and $^{15}N_{NA}$ is the ^{15}N content in natural abundance samples without $^{15}N_2$ exposure. The enrichment of samples ($^{15}N_{excess}$) was considered significant for samples with a value greater than 2.5 times the standard deviation of the mean of the natural abundance samples. $^{15}N_{medium}$ is the enrichment of the incubation medium at the end of the incubations. With

our approach, we achieved an enrichment of ~16.0 atom %^{15}N in the incubation vials. PN_{sample} is the N content of the sample (µg), and t represents the incubation time (h). $^{15}N_2$ fixation rates were normalized per seagrass leaf area (cm^2). The C:N molar ratio was determined as C:N= (% C/12) / (% N/14).

Dissolved nutrient concentrations (NH_4^+, NO_2^-, NO_x^-, PO_4^-) were measured with a continuous flow analyzer (Flowsys, SYSTEA S.p.A.). NO_3^- concentrations were calculated as the difference between NO_x^- and NO_2^-. Subsequently, nutrient fluxes were calculated as the difference between final and initial nutrient concentrations, corrected for controls, and normalized to leaf area.

POTENTIAL NITRIFICATION RATES

Nitrification potential was determined using stock solutions of 0.22 µm filtered water from the study sites (vent and ambient pH site) with an ambient NH_4^+ concentration of 0.65 µM that was enriched with $^{15}NH_4^+$ (≥98 atom %^{15}N) to a final concentration of 20 µM. The incubation was performed as described above (see also Fig. S3.1 for the experimental design) with sampling times at T0 = 0h, T1 = 2h, T2 = 5h, and T3 = 9 h light/ 8 h dark. Water samples were filtered at 0.22 µm, transferred to 15 mL polypropylene tubes, and stored at -20°C for the analysis of NO_3^- production. Vials with 0.22 µm filtered site water with 20 µM $^{15}NH_4^+$ but without leaves served as controls for background microbial activity in the water column (n = 3).

Isotopic samples for $^{15}NO_3^-$ production were analyzed by isotope ratio mass spectrometry (IRMS) using a modified version of the Ti(III) reduction method described by Altabet et al. (2019). Sample aliquots for nitrification analysis (3 mL) were acidified by adding 10 µL of 2.5 nM sulfanilic acid in 10% HCl to each 1 mL of sample, then added to 3 mL of the international standard USGS-32 ($\delta^{15}N$ = +180‰) in a 12 mL exetainer, so that the final concentration of USGS-32 was 0.1 ppm NO_3-N (~7 µM NO_3^-). After combining the sample with the standard, the exetainer headspace was flushed with argon for 2 minutes. NO_3^- was then converted to nitrous oxide (N_2O) for stable N isotope analysis by adding 200 µL zinc-treated 30% $TiCl_3$. The exetainers were immediately sealed with a gas-tight, pierceable, chlorobutyl rubber septum and the final reaction volume was 6.15 mL. The Ti(III)-treated samples were left at room temperature for >12 h to convert NO_3^- to N_2O. The headspace of the exetainer was sampled with a double-holed needle using a CTC PAL autosampler and a modified flush-fill line of a GasBench device (Thermo Scientific). The flush rate was ca. 25 mL min^{-1} and the flushing time was 5.5 min. The headspace sample was passed through a magnesium perchlorate and ascarite trap to remove water and CO_2, respectively, and then collected in a sample loop (50 cm PoraPlot Q; ø = 0.53 mm; Restek) submersed in liquid nitrogen. N_2O in the sample was then separated from CO_2 and other gases by injecting onto a Carboxen 1010 PLOT column (30 m × 0.53 mm, 30 µm film thickness, Supelco; temp = 90 °C, flow rate 2.6 mL min^{-1}) with helium as carrier gas. The sample was then transferred to a MAT253 PLUS IRMS via a Conflo interface (ThermoScientific). $\delta^{15}N$ values were determined relative to the N_2O working gas, and then corrected for linearity according to the peak height relationship and the titanium-to-sample ratio (Altabet et al., 2019); the absolute value of the linear correction term was <1.3‰ for all samples. The corrected values were then normalized to the $\delta^{15}N$-air scale by simultaneous analysis of the international standards USGS32, USGS34, and USGS35. The $\delta^{15}N$ value of NO_3^- in the sample was finally determined via a mass balance of the relative NO_3^-

concentrations of the sample and USGS32, the measured $\delta^{15}N$ value of the mixture, and the accepted $\delta^{15}N$ value of USGS32. The external precision of the $\delta^{15}N$ measurement (± one standard deviation of the mean) determined for an in-house standard was 1.1‰.

Potential nitrification rates (PNR) were calculated using an equation modified from Beman et al. (2011):

$$^{15}N_{excess} = {}^{15}N_t - {}^{15}N_0 \quad \text{(III)}$$

$$PNR = (atom\%(^{15}N_{excess}) / atom\%(^{15}N_{medium})) \times ([NO_3^-] / t) \quad \text{(IV)}$$

$^{15}N_t$ is the ^{15}N content of the samples in the NO_3^- pool measured at time t, and $^{15}N_0$ is the ^{15}N content in the NO_3^- pool measured at the beginning of the incubations. The enrichment of samples ($^{15}N_{excess}$) was considered significant for samples with a value greater than 2.5 times the standard deviation of the mean of the T_0 samples. $^{15}N_{medium}$ is the enrichment of the incubation medium at the end of the incubations. Based on the NH_4^+ concentrations measured before and after the addition of $^{15}NH_4^+$, this resulted in a theoretical enrichment of ~95.9 atom %^{15}N in the incubation medium. $[NO_3^-]$ is the concentration of NO_3^- (µM) and t is the incubation time (h). Potential nitrification rates were normalized per seagrass leaf area (cm^2) and corrected for the rates in control incubations without organisms.

Potential Anammox and Denitrification rates

To determine the rates of N loss via N_2 production (combined denitrification and anammox), stock solutions of 0.22 µm filtered water from the two study sites (vent and ambient pH) with an ambient NO_3^- concentration of 1.94 µM were enriched with $^{15}NO_3^-$ (≥98 atom %^{15}N) to a final concentration of 10 µM. The incubation was performed as described above (see also Fig. S3.1 for the experimental design), with sampling times at T0 = 0 h, T1 = 2 h, T2 = 5 h, and T3 = 9 h light/ 8h dark. Vials with 0.22 µm filtered site water from each of the study sites with 10 µM $^{15}NO_3^-$ but without leaves served as controls for background microbial activity in the water column (n = 3). Water samples were transferred into 12 mL exetainers and fixed with 200 µL of 7 M $ZnCl_2$ for $^{29}N_2$ and $^{30}N_2$ analyses.

Isotopic samples for $^{29}N_2$ and $^{30}N_2$ production were analyzed by gas chromatography-isotope ratio mass spectrometry (GasBench, Thermo Scientific). $^{29}N_2$ and $^{30}N_2$ concentrations were calculated via linear regression of a standard curve with N_2 air standards. Production rates of ^{15}N-enriched N_2 gas were calculated from the difference in $^{29}N_2$ or $^{30}N_2$ concentrations between T1 (2 h) and T2 (5 h), as we observed a lag phase from T0 to T1. Because the changes in $^{29}N_2$ and $^{30}N_2$ concentrations were very small (Table S3.4), we decided to report $^{29}N_2$ and $^{30}N_2$ production rates instead of further transforming the data to calculate denitrification or anammox rates. $^{29}N_2$ and $^{30}N_2$ production rates were normalized to seagrass leaf area (cm^2) and corrected for the rates in control incubations without organisms.

Holobiont N demand calculations

To calculate daily metabolic rates of plant and epiphyte-mediated N cycling processes, we integrated rates of N_2

fixation, nitrification potential, N loss (denitrification and anammox), NO_3^-, and NH_4^+ uptake in the light and dark incubations assuming a daily 12:12 h light/dark cycle. This way we obtained daily rates in mmol N $m^{-2}_{leaf\ area}$ d^{-1} at vent and ambient pH. We further used net community productivity (NCP) from Berlinghof et al.[12] (using a photosynthetic quotient of 1), C:N ratios (Fig. S3.8), average leaf density and dry weight per leaf at the ambient and vent site (Table S3.5) to calculate the potential percentage of daily primary production of the seagrass holobiont (plant + epiphytes) that can be supported by leaf-associated N_2 fixation.

STATISTICS AND REPRODUCIBILITY

For the incubation experiments, we used central sections of *P. oceanica* leaves from the vent and ambient pH site with epiphytes present (n = 4) or removed (n = 3) in the dark and incubations (see Fig S1). Samples were not measured repeatedly; for every sampling timepoint, a new incubation vial was opened and measured.

We tested for normality and homogeneity of variances before each analysis using Shapiro-Wilk's and Levene's tests and transformed data or removed outliers if normality and homogeneity of variances were not met. We tested the effects of pH (vent pH vs. ambient pH), treatment (with and without epiphytes), and their interaction on the $^{15}N_2$ incorporation rates, potential nitrification rates (PNR), $^{29}N_2$ and $^{30}N_2$ production rates, and the nutrient fluxes using two-way ANOVAs (type II). We tested the effects of pH (vent pH vs. ambient pH) on the C:N ratios of leaves and epiphytes using a one-way ANOVA (type II). All statistical analyses were performed with R (R Core team, 2023, version 4.1.2) using the packages *car* and *emmeans*.

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AUTHOR CONTRIBUTIONS

F.P., G.M.Q., U.M., C.W., and U.C. designed the study. F.P., G.M.Q., U.M., and U.C. performed the experiments. J.B., U.M., and T.B.M. performed the mass spectrometry analyses. L.M.M. and G.M.Q. performed the molecular analyses. F.M. and M.A. performed the nutrient analyses. J.B. and L.M.M. analyzed the results. J.B., L.M.M., and U.C. wrote the manuscript with contributions from all co-authors.

REFERENCES

Agawin, N. S. R., Ferriol, P., Cryer, C., Alcon, E., Busquets, A., Sintes, E., Vidal, C., & Moyà, G. (2016). Significant nitrogen fixation activity associated with the phyllosphere of Mediterranean seagrass Posidonia oceanica: First report. *Marine Ecology Progress Series*, *551*, 53–62. https://doi.org/10.3354/MEPS11755

Agawin, N. S. R., Ferriol, P., & Sintes, E. (2019). Simultaneous measurements of nitrogen fixation in different plant tissues of the seagrass Posidonia oceanica. *Marine Ecology Progress Series*, *611*, 111–127. https://doi.org/10.3354/MEPS12854

Agawin, N. S. R., Ferriol, P., Sintes, E., & Moyà, G. (2017). Temporal and spatial variability of in situ nitrogen fixation activities associated with the Mediterranean seagrass Posidonia oceanica meadows. *Limnology and Oceanography*, *62*(6), 2575–2592. https://doi.org/10.1002/LNO.10591

Altabet, M. A., Wassenaar, L. I., Douence, C., & Roy, R. (2019). A Ti(III) reduction method for one-step conversion of seawater and freshwater nitrate into N2O for stable isotopic analysis of 15N/14N, 18O/16O and 17O/16O. *Rapid Communications in Mass Spectrometry*, *33*(15), 1227–1239. https://doi.org/10.1002/rcm.8454

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*(1), 32–46.

Apostolaki, E. T., Vizzini, S., & Karakassis, I. (2012). Leaf vs. Epiphyte nitrogen uptake in a nutrient enriched Mediterranean seagrass (Posidonia oceanica) meadow. *Aquatic Botany*, *96*, 58–62. https://doi.org/10.1016/j.aquabot.2011.09.008

Avis, T. J., Gravel, V., Antoun, H., & Tweddell, R. J. (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. *Soil Biology and Biochemistry*, *40*(7), 1733–1740. https://doi.org/10.1016/j.soilbio.2008.02.013

Banister, R. B., Schwarz, M. T., Fine, M., Ritchie, K. B., & Muller, E. M. (2021). Instability and Stasis Among the Microbiome of Seagrass Leaves, Roots and Rhizomes, and Nearby Sediments Within a Natural pH Gradient. *Microbial Ecology*. https://doi.org/10.1007/s00248-021-01867-9

Basili, M., Quero, G. M., Giovannelli, D., Manini, E., Vignaroli, C., Avio, C. G., & De Marco, R. (2020). Major Role of Surrounding Environment in Shaping Biofilm Community Composition on Marine Plastic Debris. *Frontiers in Marine Science*, *7*(262). https://doi.org/10.3389/fmars.2020.00262

Beman, J. M., Chow, C. E., King, A. L., Feng, Y., Fuhrman, J. A., Andersson, A., Bates, N. R., Popp, B. N., & Hutchins, D. A. (2011). Global declines in oceanic nitrification rates as a consequence of ocean acidification. *PNAS*, *108*(1), 208–213. https://doi.org/10.1073/PNAS.1011053108

Bergman, B., Sandh, G., Lin, S., Larsson, J., & Carpenter, E. J. (2013). Trichodesmium– a widespread marine cyanobacterium with unusual nitrogen fixation properties. *FEMS Microbiology Reviews*, *37*(3), 286–302. https://doi.org/10.1111/j.1574-6976.2012.00352.x

Berlinghof, J., Peiffer, F., Marzocchi, U., Munari, M., Quero, G. M., Dennis, L., Wild, C., & Cardini, U. (2022). The role of epiphytes in seagrass productivity under ocean acidification. *Scientific Reports*, *12*(6249). https://doi.org/10.1038/s41598-022-10154-7

Berrendero, E., Valiente, E. F., Perona, E., Gómez, C. L., Loza, V., Muñoz-Martín, M. Á., & Mateo, P. (2016). Nitrogen fixation in a non-heterocystous cyanobacterial mat from a mountain river. *Scientific Reports*, *6*(1), 30920. https://doi.org/10.1038/srep30920

Björk, M., Short, F. T., Mcleod, E., & Beer, S. (2008). *Managing seagrasses for resilience to climate change*. IUCN, Gland, Switzerland. https://scholars.unh.edu/jel

Blanchet, E., Prado, S., Stien, D., Oliveira da Silva, J., Ferandin, Y., Batailler, N., Intertaglia, L., Escargueil, A., & Lami, R. (2017). Quorum Sensing and Quorum Quenching in the Mediterranean Seagrass Posidonia oceanica Microbiota. *Frontiers in Marine Science*, *4*. https://doi.org/10.3389/fmars.2017.00218

Bondoso, J., Godoy-Vitorino, F., Balagué, V., Gasol, J. M., Harder, J., & Lage, O. M. (2017). Epiphytic Planctomycetes communities associated with three main groups of macroalgae. *FEMS Microbiology Ecology*, *93*(3), fiw255. https://doi.org/10.1093/femsec/fiw255

Brodersen, K. E., & Kühl, M. (2022). Effects of Epiphytes on the Seagrass Phyllosphere. *Frontiers in Marine Science*, *9*, 1–10. https://doi.org/10.3389/fmars.2022.821614

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cardini, U., Van Hoytema, N., Bednarz, V. N., Al-Rshaidat, M. M. D., & Wild, C. (2018). N2 fixation and primary productivity in a red sea Halophila stipulacea meadow exposed to seasonality. *Limnol. Oceanogr*, *63*, 786–798. https://doi.org/10.1002/lno.10669

Cox, T. E., Gazeau, F., Alliouane, S., Hendriks, I. E., Mahacek, P., Le Fur, A., & Gattuso, J. P. (2016). Effects of in situ CO2 enrichment on structural characteristics, photosynthesis, and growth of the Mediterranean seagrass Posidonia oceanica. *Biogeosciences*, *13*(7), 2179–2194. https://doi.org/10.5194/bg-13-2179-2016

Cox, T. E., Schenone, S., Delille, J., Diaz-Castaneda, V., Alliouane, S., Gattuso, J.-P., & Gazeau, F. (2015). Effects of ocean acidification on Posidonia oceanica epiphytic community and shoot productivity. *Journal of Ecology*, *103*, 1594–1609. https://doi.org/10.1111/1365-2745.12477

Crump, B. C., Wojahn, J. M., Tomas, F., & Mueller, R. S. (2018). Metatranscriptomics and amplicon sequencing reveal mutualisms in seagrass microbiomes. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2018.00388

Dang, H., Li, T., Chen, M., & Huang, G. (2008). Cross-ocean distribution of Rhodobacterales bacteria as primary surface colonizers in temperate coastal marine waters. *Applied and Environmental Microbiology*, *74*(1), 52–60. https://doi.org/10.1128/AEM.01400-07

Delmont, T. O., Quince, C., Shaiber, A., Esen, Ö. C., Lee, S. T., Rappé, M. S., McLellan, S. L., Lücker, S., & Eren, A. M. (2018). Nitrogen-fixing populations of Planctomycetes and Proteobacteria are abundant in surface ocean metagenomes. *Nature Microbiology*, *3*(7), 804–813. https://doi.org/10.1038/s41564-018-0176-9

Donnarumma, L., Lombardi, C., Cocito, S., & Gambi, M. C. (2014). Settlement pattern of Posidonia oceanica epibionts along a gradient of ocean acidification: An approach with mimics. *Mediterranean Marine Science*, *15*(3), 498–509. https://doi.org/10.12681/MMS.677

Duarte, C. M., Kennedy, H., Marbà, N., & Hendriks, I. (2013). Assessing the capacity of seagrass meadows for carbon burial: Current limitations and future strategies. *Ocean and Coastal Management*, *83*, 32–38. https://doi.org/10.1016/j.ocecoaman.2011.09.001

Durham, B. P., Grote, J., Whittaker, K. A., Bender, S. J., Luo, H., Grim, S. L., Brown, J. M., Casey, J. R., Dron, A., Florez-Leiva, L., Krupke, A., Luria, C. M., Mine, A. H., Nigro, O. D., Pather, S., Talarmin, A., Wear, E. K., Weber, T. S., Wilson, J. M., … Rappé, M. S. (2014). Draft genome sequence of marine alphaproteobacterial strain HIMB11, the first cultivated representative of a unique lineage within the Roseobacter clade possessing an unusually small genome. *Standards in Genomic Sciences*, *9*(3), 632–645. https://doi.org/10.4056/sigs.4998989

Fernandes, A. D., Macklaim, J. M., Linn, T. G., Reid, G., & Gloor, G. B. (2013). ANOVA-Like Differential Expression (ALDEx) Analysis for Mixed Population RNA-Seq. *PLoS ONE*, *8*(7), e67019. https://doi.org/10.1371/journal.pone.0067019

Foo, S. A., Byrne, M., Ricevuto, E., & Gambi, M. C. (2018). The carbon dioxide vents of Ischia, Italy, a natural system to assess impacts of ocean acidification on marine ecosystems: An overview of research and comparisons with other vent systems. *Oceanography and Marine Biology*, *56*, 237–310. https://doi.org/10.1201/9780429454455-4

Fourqurean, J. W., Duarte, C. M., Kennedy, H., Marbà, N., Holmer, M., Mateo, M. A., Apostolaki, E. T., Kendrick, G. A., Krause-Jensen, D., Mcglathery, K. J., & Serrano, O. (2012). Seagrass ecosystems as a globally significant carbon stock. *Nature Geoscience*, *5*, 505–509. https://doi.org/10.1038/NGEO1477

Fulweiler, R. W., Emery, H. E., Heiss, E. M., & Berounsky, V. M. (2011). Assessing the Role of pH in Determining Water Column Nitrification Rates in a Coastal System. *Estuaries and Coasts*, *34*, 1095–1102. https://doi.org/10.1007/s12237-011-9432-4

Garcias-Bonet, N., Arrieta, J. M., Duarte, C. M., & Marbà, N. (2016). Nitrogen-fixing bacteria in Mediterranean seagrass (Posidonia oceanica) roots. *Aquatic Botany*, *131*, 57–60. https://doi.org/10.1016/j.aquabot.2016.03.002

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., & Egozcue, J. J. (2017). Microbiome Datasets Are Compositional: And This Is Not Optional. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.02224

Gravili, C., Cozzoli, F., & Gambi, M. C. (2021). Epiphytic hydroids on Posidonia oceanica seagrass meadows are winner organisms under future ocean acidification conditions: Evidence from a CO2 vent system (Ischia Island, Italy). *European Zoological Journal*, *88*(1), 472–486. https://doi.org/10.1080/24750263.2021.1899317

Hall-Spencer, J. M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S. M., Rowley, S. J., Tedesco, D., & Buia, M.-C. (2008). Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature*, *454*. https://doi.org/10.1038/nature07051

Hemminga, M. A., & Duarte, C. M. (2000). Seagrass Ecology. In *Seagrass Ecology*. Cambridge University Press. https://doi.org/10.1017/cbo9780511525551

Hemminga, M. A., Harrison, P. G., & Van Lent, F. (1991). The balance of nutrient losses and gains in seagrass meadows. *Marine Ecology Progress Series*, *71*(1), 85–96.

Hendriks, I. E., Olsen, Y. S., Ramajo, L., Basso, L., Steckbauer, A., Moore, T. S., Howard, J., & Duarte, C. M. (2014). Photosynthetic activity buffers ocean acidification in seagrass meadows. *Biogeosciences*, *11*, 333–346. https://doi.org/10.5194/bg-11-333-2014

Hernán, G., Ramajo, L., Basso, L., Delgado, A., Terrados, J., Duarte, C. M., & Tomas, F. (2016). Seagrass (Posidonia oceanica) seedlings in a high-CO 2 world: From physiology to herbivory. *Scientific Reports*, *6*(38017). https://doi.org/10.1038/srep38017

Hutchins, D. A., & Capone, D. G. (2022). The marine nitrogen cycle: New developments and global change. *Nature Reviews Microbiology*, *20*(7), 401–414. https://doi.org/10.1038/S41579-022-00687-Z

Hutchins, D. A., Mulholland, M. R., & Fu, F. (2009). Nutrient Cycles and Marine Microbes in a CO_2-Enriched Ocean. *Oceanography*, *22*(4), 128–145. https://doi.org/10.2307/24861030

Hyman, A. C., Frazer, T. K., Jacoby, C. A., Frost, J. R., & Kowalewski, M. (2019). Long-term persistence of structured habitats: Seagrass meadows as enduring hotspots of biodiversity and faunal stability. *Proc. R. Soc. B*, *286*(20191861). https://doi.org/10.1098/rspb.2019.1861

IPCC (Ed.). (2007). *Emissions scenarios: Summary for policymakers;a special report of IPCC Working Group III$Intergovernmental Panel on Climate Change*. Intergovernmental Panel on Climate Change.

Jetten, M. S. M., Niftrik, L. van, Strous, M., Kartal, B., Keltjens, J. T., & Op den Camp, H. J. M. (2009). Biochemistry and molecular biology of anammox bacteria. *Critical Reviews in Biochemistry and Molecular Biology*, *44*(2–3), 65–84. https://doi.org/10.1080/10409230902722783

Jung, M.-Y., Sedlacek, C. J., Dimitri Kits, K., Mueller, A. J., Rhee, S.-K., Hink, L., Nicol, G. W., Bayer, B., Lehtovirta-Morley, L., Wright, C., de la Torre, J. R., Herbold, C. W., Pjevac, P., Daims, H., & Wagner, M. (2022). Ammonia-oxidizing archaea possess a wide range of cellular ammonia affinities. *ISME*, *16*, 272–283. https://doi.org/10.1038/s41396-021-01064-z

Kitidis, V., Laverock, B., McNeill, L. C., Beesley, A., Cummings, D., Tait, K., Osborn, M. A., & Widdicombe, S. (2011). Impact of ocean acidification on benthic and water column ammonia oxidation. *Geophysical Research Letters*, *38*(L21603). https://doi.org/10.1029/2011GL049095

Klawonn, I., Lavik, G., Böning, P., Marchant, H. K., Dekaezemacker, J., Mohr, W., & Ploug, H. (2015). Simple approach for the preparation of 15−15 N 2-enriched water for nitrogen fixation assessments: Evaluation, application and recommendations. *Frontiers in Microbiology*, *6*(769). https://doi.org/10.3389/fmicb.2015.00769

Koch, M., Bowes, G., Ross, C., & Zhang, X.-H. (2013). Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Global Change Biology*, *19*, 103–132. https://doi.org/10.1111/j.1365-2486.2012.02791.x

Kohn, T., Rast, P., Kallscheuer, N., Wiegand, S., Boedeker, C., Jetten, M. S. M., Jeske, O., Vollmers, J., Kaster, A.-K., Rohde, M., Jogler, M., & Jogler, C. (2020). The Microbiome of Posidonia oceanica Seagrass Leaves Can Be Dominated by Planctomycetes. *Front. Microbiol*, *11*, 1458. https://doi.org/10.3389/fmicb.2020.01458

Kranz, S. A., Levitan, O., Richter, K.-U., Prášil, O., Berman-Frank, I., & Rost, B. (2010). Combined Effects of CO 2 and Light on the N2-Fixing Cyanobacterium Trichodesmium IMS101: Physiological Responses. *Plant Physiology*, *154*, 334–345. https://doi.org/10.1104/pp.110.159145

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M., & Gattuso, J.-P. (2013). Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Global Change Biology*, *19*, 1884–1896. https://doi.org/10.1111/gcb.12179

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Microbial Biogeochemistry*, *16*, 263–276. https://doi.org/10.1038/nrmicro.2018.9

Lacoue-Labarthe, T., Nunes, P. A. L. D., Ziveri, P., Cinar, M., Gazeau, F., Hall-Spencer, J. M., Hilmi, N., Moschella, P., Safa, A., Sauzade, D., & Turley, C. (2016). Impacts of ocean acidification in a warming Mediterranean Sea: An overview. *Regional Studies in Marine Science*, *5*, 1–11. https://doi.org/10.1016/J.RSMA.2015.12.005

Lage, O. M., & Bondoso, J. (2014). Planctomycetes and macroalgae, a striking association. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00267

Lehnen, N., Marchant, H. K., Schwedt, A., Milucka, J., Lott, C., Weber, M., Dekaezemacker, J., Seah, B. K. B., Hach, P. F., Mohr, W., & Kuypers, M. M. M. (2016). High rates of microbial dinitrogen fixation and sulfate reduction associated with the Mediterranean seagrass Posidonia oceanica. *Systematic and Applied Microbiology*, *39*(7), 476–483. https://doi.org/10.1016/J.SYAPM.2016.08.004

Lepoint, G., Millet, S., Dauby, P., Gobert, S., & Bouquegneau, J. M. (2002). Annual nitrogen budget of the seagrass Posidonia oceanica as determined by in situ uptake experiments. *Marine Ecology Progress Series*, *237*, 87–96. https://doi.org/10.3354/meps237087

Lesser, M. P., Morrow, K. M., Pankey, S. M., & Noonan, S. H. C. (2018). Diazotroph diversity and nitrogen fixation in the coral Stylophora pistillata from the Great Barrier Reef. *The ISME Journal*, *12*(3), 813–824. https://doi.org/10.1038/s41396-017-0008-6

Levitan, O., Rosenberg, G., Setlik, I., Setlikova, E., Grigel, J., Klepetar, J., Prasil, O., & Berman-Frank, I. (2007). Elevated CO2 enhances nitrogen fixation and growth in the marine cyanobacterium Trichodesmium. *Global Change Biology*, *13*, 531–538. https://doi.org/10.1111/j.1365-2486.2006.01314.x

Li, Y., Lei, S., Cheng, Z., Jin, L., Zhang, T., Liang, L.-M., Cheng, L., Zhang, Q., Xu, X., Lan, C., Lu, C., Mo, M., Zhang, K.-Q., Xu, J., & Tian, B. (2023). Microbiota and functional analyses of nitrogen-fixing bacteria in root-knot nematode parasitism of plants. *Microbiome*, *11*(1), 48. https://doi.org/10.1186/s40168-023-01484-3

Lidbury, I., Johnson, V., Hall-Spencer, J. M., Munn, C. B., & Cunliffe, M. (2012). Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent ecosystem. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2012.02.011

Lindström, K., & Mousavi, S. A. (2020). Effectiveness of nitrogen fixation in rhizobia. *Microbial Biotechnology*, *13*(5), 1314–1335. https://doi.org/10.1111/1751-7915.13517

Ling, J., Lin, X., Zhang, Y., Zhou, W., Yang, Q., Lin, L., Zeng, S., Zhang, Y., Wang, C., Ahmad, M., Long, L., & Dong, J. (2018). Community Composition and Transcriptional Activity of Ammonia-Oxidizing Prokaryotes of Seagrass Thalassia hemprichii in Coral Reef Ecosystems. *Front. Microbiol*, *9*, 7. https://doi.org/10.3389/fmicb.2018.00007

Liu, J., Weinbauer, M. G., Maier, C., Dai, M., & Gattuso, J. P. (2010). Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. *Aquatic Microbial Ecology*, *61*(3), 291–305. https://doi.org/10.3354/AME01446

Martens-Habbena, W., Berube, P. M., Urakawa, H., de la Torre, J. R., & Stahl, D. A. (2009). Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature*, *461*(7266), 976–979. https://doi.org/10.1038/nature08465

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Matallana-Surget, S., Werner, J., Wattiez, R., Lebaron, K., Intertaglia, L., Regan, C., Morris, J., Teeling, H., Ferrer, M., Golyshin, P. N., Gerogiorgis, D., Reilly, S. I., & Lebaron, P. (2018). Proteogenomic Analysis of Epibacterium Mobile BBCC367, a Relevant Marine Bacterium Isolated From the South Pacific Ocean. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.03125

Mecca, S., Casoli, E., Ardizzone, G., & Gambi, M. C. (2020). Effects of ocean acidification on phenology and epiphytes of the seagrass Posidonia oceanica at two CO2 vent systems of Ischia (Italy). *Mediterranean Marine Science*, *21*(1), 70–83. https://doi.org/10.12681/MMS.20795

Mejia, A., Rotini, A., Lacasellab, F., Bookman, R., Thaller, M., Shem-Tovd, R., Wintersd, G., & Migliore, L. (2016). Assessing the ecological status of seagrasses using morphology, biochemical descriptors and microbial community analyses. A study in Halophila stipulacea (Forsk.) Aschers meadows in the northern Red Sea. *Ecological Indicators*, *60*, 1150–1163. https://doi.org/10.1016/j.ecolind.2015.09.014

Mohr, W., Lehnen, N., Ahmerkamp, S., Marchant, H. K., Graf, J. S., Tschitschko, B., Yilmaz, P., Littmann, S., Gruber-Vodicka, H., Leisch, N., Weber, M., Lott, C., Schubert, C. J., Milucka, J., & Kuypers, M. M. M. (2021). Terrestrial-type nitrogen-fixing symbiosis between seagrass and a marine bacterium. *Nature 2021*, 1–5. https://doi.org/10.1038/s41586-021-04063-4

Montoya, J. P., Voss, M., Kähler, P., & Capone, D. G. (1996). A Simple, High-Precision, High-Sensitivity Tracer Assay for N2 Fixation. *Applied and Environmental Microbiology*, *62*(3), 986–993. https://doi.org/10.1128/AEM.62.3.986-993.1996

Moynihan, M. A., Goodkin, N. F., Morgan, K. M., Kho, P. Y. Y., Lopes dos Santos, A., Lauro, F. M., Baker, D. M., & Martin, P. (2022). Coral-associated nitrogen fixation rates and diazotrophic diversity on a nutrient-replete equatorial reef. *The ISME Journal*, *16*(1), 233–246. https://doi.org/10.1038/s41396-021-01054-1

Nearing, J. T., Douglas, G. M., Hayes, M. G., MacDonald, J., Desai, D. K., Allward, N., Jones, C. M. A., Wright, R. J., Dhanani, A. S., Comeau, A. M., & Langille, M. G. I. (2022). Microbiome differential abundance methods produce different results across 38 datasets. *Nature Communications*, *13*(1), 342. https://doi.org/10.1038/s41467-022-28034-z

Noisette, F., Depetris, A., Kühl, M., & Brodersen, K. E. (2020). Flow and epiphyte growth effects on the thermal, optical and chemical microenvironment in the leaf phyllosphere of seagrass (Zostera marina). *Journal of the Royal Society Interface*, *17*. https://doi.org/10.1098/rsif.2020.0485rsif20200485

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O' Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. J. (2020). *vegan: Community ecology package*.

Pfister, C. A., Cardini, U., Mirasole, A., Montilla, L. M., Veseli, I., Gattuso, J.-P., & Teixido, N. (2023). Microbial associates of an endemic Mediterranean seagrass enhance the access of the host and the surrounding seawater to inorganic nitrogen under ocean acidification. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-47126-4

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

Quinn, T. P., Erb, I., Richardson, M. F., & Crowley, T. M. (2018). Understanding sequencing data as compositions: An outlook and review. *Bioinformatics*. https://doi.org/10.1093/bioinformatics/bty175

R Core team. (2023). *R: A language environment for statistical computing*.

Rambo, I. M., Dombrowski, N., Constant, L., Erdner, D., & Baker, B. J. (2020). Metabolic relationships of uncultured bacteria associated with the microalgae Gambierdiscus. *Environmental Microbiology*, *22*(5), 1764–1783. https://doi.org/10.1111/1462-2920.14878

Ravaglioli, C., Lauritano, C., Buia, M. C., Balestri, E., Capocchi, A., Fontanini, D., Pardi, G., Tamburello, L., Procaccini, G., & Bulleri, F. (2017). Nutrient Loading Fosters Seagrass Productivity Under Ocean Acidification. *Scientific Reports*, *7*(13732). https://doi.org/10.1038/s41598-017-14075-8

Rotini, A., Conte, C., Winters, G., Vasquez, M. I., & Migliore, L. (2023). Undisturbed Posidonia oceanica meadows maintain the epiphytic bacterial community in different environments. *Environmental Science and Pollution Research*, *30*(42), 95464–95474. https://doi.org/10.1007/s11356-023-28968-x

Salk, K. R., Erler, D. V., Eyre, B. D., Carlson-Perret, N., & Ostrom, N. E. (2017). Unexpectedly high degree of anammox and DNRA in seagrass sediments: Description and application of a revised isotope pairing technique. *Geochimica et Cosmochimica Acta*, *211*, 64–78. https://doi.org/10.1016/J.GCA.2017.05.012

Sanders-Smith, R., Segovia, B. T., Forbes, C., Hessing-Lewis, M., Morien, E., Lemay, M. A., O'Connor, M. I., & Parfrey, L. W. (2020). Host-Specificity and Core Taxa of Seagrass Leaf Microbiome Identified Across Tissue Age and Geographical Regions. *Frontiers in Ecology and Evolution*, *8*. https://doi.org/10.3389/fevo.2020.605304

Scartazza, A., Moscatello, S., Gavrichkova, O., Buia, M. C., Lauteri, M., Battistelli, A., Lorenti, M., Garrard, S. L., Calfapietra, C., & Brugnoli, E. (2017). Carbon and nitrogen allocation strategy in Posidonia oceanica is altered by seawater acidification. *Science of the Total Environment*, *607*, 954–964. https://doi.org/10.1016/j.scitotenv.2017.06.084

Strous, M., Fuerst, J. A., Kramer, Evelien, H. M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K. T., Webb, R., Kuenen, J. G., & Jetten, M. S. M. (1999). Missing lithotroph identified as new planctomycete. *Letters to Nature*, *400*, 446–449.

Tarquinio, F., Hyndes, G. A., Laverock, B., Koenders, A., & Säwström, C. (2019). The seagrass holobiont: Understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. *FEMS Microbiology Letters*, *366*(6), 1–15. https://doi.org/10.1093/femsle/fnz057

Touchette, B. W., & Burkholder, J. A. M. (2000). Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, *250*, 133–167. https://doi.org/10.1016/S0022-0981(00)00195-7

Trevathan-Tackett, S. M., Allnutt, T. R., Sherman, C. D. H., Richardson, M. F., Crowley, T. M., & Macreadie, P. I. (2020). Spatial variation of bacterial and fungal communities of estuarine seagrass leaf microbiomes. *Aquatic Microbial Ecology*, *84*, 59–74. https://doi.org/10.3354/ame01926

Ugarelli, K., Chakrabarti, S., Laas, P., & Stingl, U. (2017). The Seagrass Holobiont and Its Microbiome. *Microorganisms*, *5*(81). https://doi.org/10.3390/microorganisms5040081

Van Duc, L., Song, B., Ito, H., Hama, T., Otani, M., & Kawagoshi, Y. (2018). High growth potential and nitrogen removal performance of marine anammox bacteria in shrimp-aquaculture sediment. *Chemosphere*, *196*, 69–77. https://doi.org/10.1016/j.chemosphere.2017.12.159

Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J. A., Jansson, J. K., Caporaso, J. G., Fuhrman, J. A., Apprill, A., & Knight, R. (2016). Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. *mSystems*, *1*(1). https://doi.org/10.1128/mSystems.00009-15

Wannicke, N., Frey, C., Law, C. S., & Voss, M. (2018). The response of the marine nitrogen cycle to ocean acidification. *Global Change Biology*, *24*, 5031–5043. https://doi.org/10.1111/gcb.14424

Weigel, B. L., Miranda, K. K., Fogarty, E. C., Watson, A. R., & Pfister, C. A. (2022). Functional Insights into the Kelp Microbiome from Metagenome-Assembled Genomes. *mSystems*, *7*(3), e01422-21. https://doi.org/10.1128/msystems.01422-21

Wyatt, N. J., Kitidis, V., Woodward, E. M. S., Rees, A. P., Widdicombe, S., & Lohan, M. (2010). Effects of high CO2 on the fixed nitrogen inventory of the Western English Channel. *Journal of Plankton Research*, *32*(5), 631–641. https://doi.org/10.1093/PLANKT/FBP140

Yin, S., Li, J., Dong, H., & Qiang, Z. (2021). Unraveling the nitrogen removal properties and microbial characterization of "*Candidatus* Scalindua"-dominated consortia treating seawater-based wastewater. *Science of The Total Environment*, *786*, 147470. https://doi.org/10.1016/j.scitotenv.2021.147470

Zhang, Z., Nair, S., Tang, L., Zhao, H., Hu, Z., Chen, M., Zhang, Y., Kao, S.-J., Jiao, N., & Zhang, Y. (2021). Long-Term Survival of Synechococcus and Heterotrophic Bacteria without External Nutrient Supply after Changes in Their Relationship from Antagonism to Mutualism. *mBio*, *12*(4), 10.1128/mbio.01614-21. https://doi.org/10.1128/mbio.01614-21

CHAPTER 4

Biodiversity in a *Posidonia oceanica* meadow. Photo by Ulisse Cardini.

CHAPTER 4

RECIPROCAL NUTRITIONAL BENEFITS IN A SPONGE-SEAGRASS ASSOCIATION

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ABSTRACT

Sponges commonly form associations within seagrass meadows, but their potential impact on seagrass productivity and nutrient cycles remains poorly understood. This study investigates the association between the demosponge Chondrilla nucula and the Mediterranean seagrass Posidonia oceanica in two sampling occasions during the plant growth (spring) and senescence (autumn) seasons at a small inlet near Naples, Italy, where the sponge grows conspicuously within the seagrass bed. We found a non-linear relationship between the benthic cover of the sponge and the seagrass, with higher C. nucula cover linked to intermediate P. oceanica cover, suggesting spatial dependence. P. oceanica showed higher net primary production (NPP) in spring, while C. nucula was net heterotrophic in spring but exhibited slightly positive NPP in autumn. NPP remained stable when the two organisms were associated, regardless of the season. C. nucula consistently contributed inorganic nutrients to the association in the form of phosphate, ammonium, and substantial nitrate, recycling nutrients that potentially benefited P. oceanica in its growth season. In return, the seagrass consistently provided dissolved organic carbon, which aided sponge nutrition in spring. These findings suggest reciprocal benefits in the interaction between C. nucula and P. oceanica, with nutrient exchange facilitating a facultative mutualism that potentially supports and stabilizes the productivity of the seagrass ecosystem.

KEYWORDS: *Posidonia oceanica*; *Chondrilla nucula*; holobiont; facilitation; oxygen fluxes; nutrient fluxes; stable isotopes analyses

A modified version of this chapter is in preparation for publication in *Limnology and Oceanography*.

INTRODUCTION

Seagrasses are vital ecosystem engineers that create habitats for diverse marine life. Seagrass meadows support significantly more species than unvegetated areas, particularly among fish and invertebrate communities (Heck et al., 2008), while fostering complex epibenthic assemblages (Whippo et al., 2018). Many invertebrates use these meadows as sources of organic matter (Kharlamenko et al., 2001) and as shelter (Boström & Bonsdorff, 1997), and they also host diverse microbiomes that perform key ecosystem functions, contributing to so-called nested ecosystems (Malkin & Cardini, 2021; Mcfall-Ngai et al., 2013; Pita et al., 2018). Positive species interactions are recognized as crucial drivers of community structure and ecosystem functioning in seagrass ecosystems (Cardini et al., 2019, 2022; Gagnon et al., 2021; Malkin & Cardini, 2021). However, although this topic has been well explored in terrestrial environments, substantial knowledge gaps remain in marine systems (e.g., Bulleri, 2009).

Among seagrass-associated invertebrates, sponges play a crucial role in nutrient cycling. They consume dissolved organic carbon (DOC) and transform it into detritus, making it available for higher trophic levels through what is known as the sponge loop (De Goeij et al., 2013; Rix et al., 2017). High-microbial abundance (HMA) sponges, in particular, absorb dissolved organic matter (DOM) and release nitrate (NO_3^-), ammonium (NH_4^+), and phosphate (PO_4^{3-}), which are essential for nutrient recycling (Maldonado et al., 2012). This function makes sponges beneficial partners for primary producers (PP), such as seagrasses and macroalgae, which release large amounts of DOM into their surroundings (Barrón et al., 2014). At the same time, the growth of PP is often limited by inorganic nitrogen (Touchette & Burkholder, 2000), underscoring the potential significance of sponge-PP interactions.

Sponge-PP associations have been documented in various marine environments. On coral reefs, sponges absorb DOC from corals and macroalgae, returning inorganic nutrients in a reciprocal, mutually beneficial relationship (Campana et al., 2021; Pawlik & McMurray, 2020; Rix et al., 2017). Similar associations occur in mangrove ecosystems, where sponges release nitrogen that supports mangrove growth, while receiving carbon from mangrove roots, establishing facultative mutualisms (Ellison et al., 1996).

In seagrass meadows, sponges have been shown to enhance growth and nutrient content of primary producers, as demonstrated in the association of the sponge *Ircina felix* with the seagrass *Thalassia testudinum* and other non-dominant seagrass species (Archer et al., 2021). Another example involves *T. testudinum* benefiting from nutrients like NH_4^+ and PO_4^{3-} released by the sponge *Halichondria melanadocia* (Archer et al., 2015, 2018). However, despite growing research interest in sponge-PP associations, only a limited number of studies have been dedicated to these interactions to date, leaving key ecological processes insufficiently understood. In particular, a quantification of the effect of sponge-seagrass associations on seagrass productivity and nutrient cycles remains largely unexplored, and the extent to which these interactions enhance or stabilize seagrass primary production under varying environmental conditions is still unclear. Addressing these gaps is essential for understanding the resilience of seagrass ecosystems and their capacity to adapt to global change.

In the Mediterranean Sea, the seagrass *Posidonia oceanica* forms extensive meadows that extend from the surface down to about 40 m depth. Among the variety of associated biodiversity, shallow *P. oceanica* meadows frequently host the demosponge *Chondrilla nucula* (Pansini & Pronzato, 1985). *C. nucula* is widespread across the Mediterranean Sea and is classified as a high-microbial-abundance (HMA) sponge (Erwin et al., 2012), harboring a rich and diverse microbiome dominated by cyanobacteria (Mazzella et al., 2024). Similar microbiome compositions have been found in *C. nucula* populations across other Mediterranean locations (Thiel et al., 2007) and in the congeneric *C. caribensis* from the Caribbean (Hill et al., 2006), suggesting stable core bacterial assemblages in *Chondrilla* spp. across regions. Phylogenetic analyses identified cyanobacterial symbionts in *C. nucula* and proposed them as *Candidatus Synechococcus spongiarum* for *C. nucula* from the Mediterranean and C. australiensis from Australia (Usher et al., 2004). In the Caribbean species *C. caribensis*, these symbionts provide nutritional benefits through photosynthate translocation and algal cell ingestion (Hudspith et al., 2022).

This study investigates the association between *C. nucula* and *P. oceanica* in the central Tyrrhenian Sea (Italy), focusing on a coastal site in the Gulf of Pozzuoli. Here, *C. nucula* grows abundantly at the base of seagrass shoots and expands laterally to adjacent rhizomes, despite the availability of alternative substrates nearby. We explore whether the *C. nucula-P. oceanica* association can be characterized as a facultative mutualism, hypothesizing that DOM released by *P. oceanica* supports sponge nutrition, while the sponge provides a source of inorganic nutrients to the seagrass.

To test this hypothesis, we: (i) conducted a spatial distribution analysis of *C. nucula* within the seagrass meadow, (ii) quantified net fluxes of oxygen, organic, and inorganic nutrients in closed chamber incubations, and (iii) used stable isotope analyses to examine potential signals of nutrient transfer in the sponge-seagrass association. These experiments evaluated the effect of each organism, both individually and in association, during the plant growth (spring) and senescence (autumn) seasons. Together, these approaches helped to gain clarity on the nature of this sponge-seagrass association and determine whether nutrient exchange plays a role, supporting the characterization of this association as a facultative mutualism.

METHODS

STUDY AREA AND BENTHIC COVER

Experiments were conducted in the "Schiacchetiello" inlet (40.7938 N, 14.0870 E, Southern Tyrrhenian Sea, Mediterranean), located in the municipality of Bacoli, Italy. Here, the seagrass *P. oceanica* grows at depths of 0-6 m, forming a patchy meadow. In many patches, the sponge *C. nucula* is found growing at the base of seagrass shoots, enveloping the rhizomes (Fig. 4.1 A-D). We conducted video transects by snorkeling along the longest distance across the shallow (0.5 - 2 m) seagrass patches in November 2021. Video footage was processed using FFMPEG (https://ffmpeg.org/) to extract frames, from which 82 images were randomly selected from high quality images. We used these images to estimate the cover percentage of the primary benthic substrates within replicated 0.25 m^2 quadrats, resulting in a total surveyed area of 20.5 m^2. Inorganic and organic nutrient concentrations, as well as light intensity and temperature data, were collected upon each sampling occasion

with methods as those reported in the following section to characterize environmental conditions at the study site where sampling took place for the following incubations.

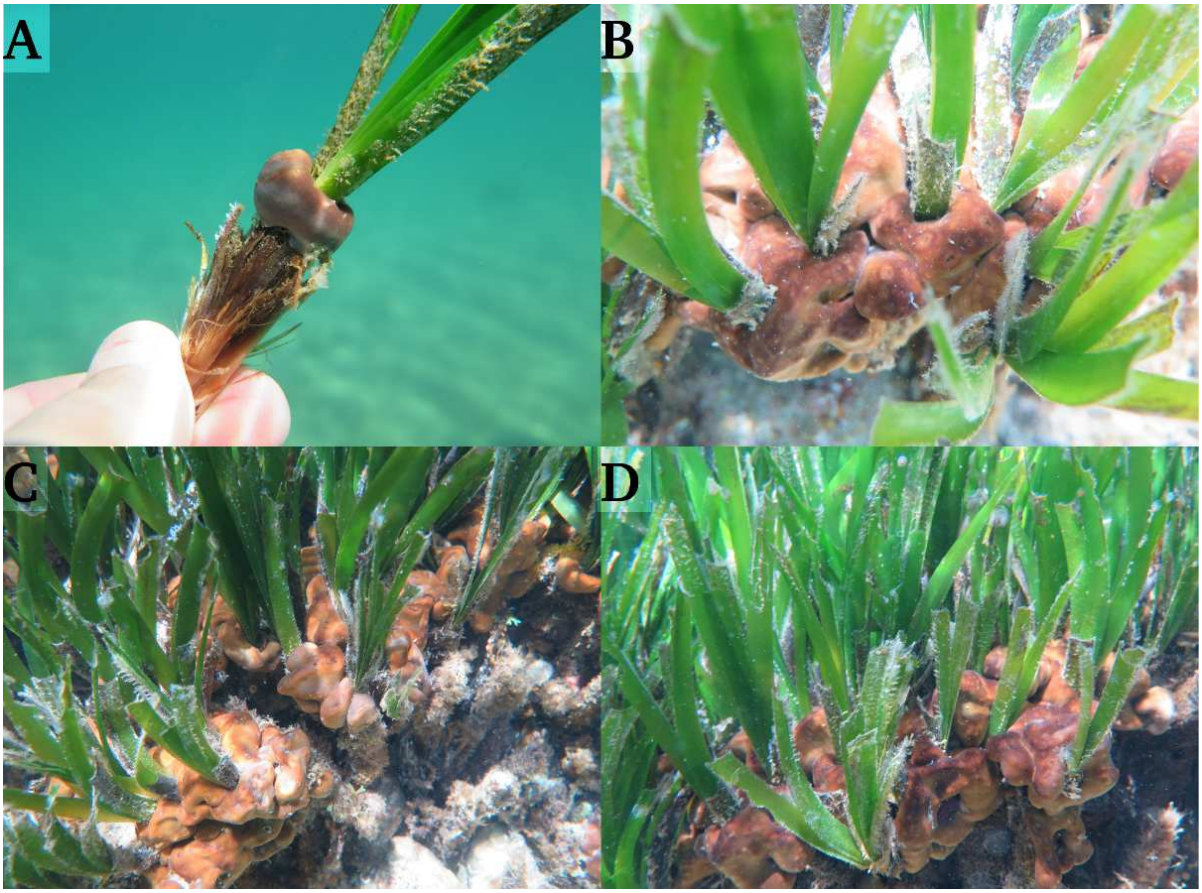

Fig. 4.1. Photographic documentation of the association between *P. oceanica* and *C. nucula* at the study site. (A) Close-up of a *P. oceanica* shoot with a *C. nucula* bundle surrounding the transition zone between the leaves and foliar sheath. (B) Detail of sponge bundles firmly attached to individual shoots. (C, D) Large *C. nucula* colonies covering multiple shoots and fusing into extensive, contiguous growths. Photos: U. Cardini.

Table 4.1. Environmental conditions at the study site in the two seasons at the time of the incubation experiments. Bold *p* values indicate significant differences between the two seasons in the respective variable (t-test).

Season	NH_4^+ (µM)	NO_x^- (µM)	PO_4^{3-} (µM)	DOC (µM)	DON (µM)	Light intensity (LUX)	Temperature (°C)
Autumn	0.98 ± 0.01	0.97 ± 0.08	0.05 ± 0.01	117.3 ± 12.5	6.4 ± 2.9	49371 ± 42228	19.5 ± 0.1
Spring	0.39 ± 0.05	0.66 ± 0.03	0.06 ± 0.01	81.9 ± 4.0	6.2 ± 0.2	66313 ± 34418	20.2 ± 0.1
p value	**0.003**	**0.009**	0.129	**0.004**	0.943	0.314	**<0.001**

OXYGEN AND NUTRIENT FLUXES

P. oceanica shoots (hereafter "seagrass"), *C. nucula* bundles ("sponge"), and seagrass shoots hosting *C. nucula* ("association") were collected from shallow beds (~1.5 m depth) for *in situ* closed-chamber incubations, as in Pfister et al. (2023). These experiments were performed during midday hours on one sampling occasion in each of two seasons: autumn (November 2021) and spring (May 2022). We acknowledge that the lack of within-season replication limits our ability to assess intra-seasonal variability. However, the study was designed to capture distinct differences between two key periods in the plant's life cycle: the growth season (spring) and the senescence season (autumn). These represent critical phases of biological activity, and the experiments were intended to provide a comparative snapshot of these distinct functional states. In autumn, incubations were conducted using 0.55 L cylindrical chambers (n = 3) for ~3.75 hours; in spring, we used 1.1 L chambers (n = 4) for ~6 hours to adjust for longer leaf length in this season and to maintain a similar biomass to volume ratio. Control chambers (n = 3) containing only seawater were included to measure fluxes from the water column community. Another full set of chambers were wrapped in three layers of black polyethylene to block light and incubated as "dark" chambers alongside the "light" chambers. The chambers were held in floating crates to maintain exposure to natural sunlight (for the "light" chambers) and seawater temperature while allowing for gentle wave action to prevent stratification. HOBO data loggers were used to monitor light intensity and temperature both within the chambers and externally, ensuring conditions resembled those found *in situ*. Discrete measurements with a LICOR light sensor allowed verifying that light chambers in autumn (when light intensity was lower, see Table 4.1) received light levels well above saturation irradiance (~400 µmol quanta m^{-2} s^{-1}). At the beginning and end of each incubation, O_2 concentrations inside the chambers were measured using a portable digital meter (WTW Multi 3430 Set K). After the incubations, 30 mL of seawater from each chamber was collected for dissolved organic carbon (DOC) and nitrogen (DON) analysis, and 20 mL was collected for inorganic nutrient measurements (NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}). Samples were collected with acid-washed syringes and filtered immediately: DOC/DON samples were filtered using pre-combusted GF/F glass microfiber filters (pore size: 0.7 µm), acidified with 80 µL HCl (6 M), and refrigerated at 4°C until analysis. Inorganic nutrient samples were filtered through 0.22 µm PES membranes, frozen *in situ*, and stored at -20°C. DOC/DON was analyzed using a TOC-L Analyzer with TN unit (Shimadzu Corporation, Japan), while inorganic nutrients were measured with a continuous flow analyzer (Flowsys, SYSTEA SpA). Sponge and seagrass samples from each chamber were lyophilized for dry weight determination. Hourly flux rates for oxygen and nutrients were calculated as the change in analyte concentrations over incubation time corrected for the signal in the controls and the effective seawater volume in the chamber, standardized to the dry weight of the organisms and expressed in µmol analyte g DW^{-1} h^{-1}. Daily rates were expressed in µmol analyte g DW^{-1} d^{-1} considering a light:dark photoperiod of 12h light and 12h dark.

STABLE ISOTOPE ANALYSES

To examine potential signals of nutrient transfer in the sponge-seagrass association, in spring 2022 we collected samples of *P. oceanica* (leaves and epiphytes) and *C. nucula*, when growing associated vs non-associated. Epiphytes were gently scraped off seagrass leaves and stored in Eppendorf tubes. All samples were placed in

acid-washed vials and lyophilized for 48 hours. Dried tissues were ground to a fine powder using a tissue lyser, then acid-fumed before being weighed into silver capsules for isotope analysis. Samples were analyzed using a Flash Elemental Analyzer (Thermo Scientific) equipped with a single reactor (1020°C), along with a MAT 253 Plus isotope ratio mass spectrometer (IRMS) interfaced with a Conflo IV system (Thermo Scientific, Bremen, Germany). The $\delta^{13}C$ and $\delta^{15}N$ values were normalized to Vienna Pee Dee Belemnite and atmospheric air, respectively, after correcting for blanks, ion source linearity, and standardizing against laboratory working standards and international reference materials (IAEA-600, IAEA-603). Precision was typically <0.1‰ for $\delta^{13}C$ and 0.2‰ for $\delta^{15}N$. The molar C:N ratios (mol:mol) were calculated from C and N weights in the capsules (µg) and based on their respective molecular weights.

DATA ANALYSIS

Net primary production (NPP) and respiration (R) were determined based on hourly O_2 fluxes in light and dark incubations, respectively. To compute integrated rates of gross primary production (GPP = NPP + |R|), daily net community production (NCP = GPP × 12 - |R| × 24), and daily fluxes for each nutrient (Daily Flux = light flux × 12 + dark flux × 12), we generated analytical combinations of the observed values for light and dark fluxes, assuming equal duration of daylight or darkness. Each pair of independent values was combined using the respective formulas to compute the distribution of integrated rates (n = 9 for autumn, n = 16 for spring). This output provided a comprehensive distribution of the potential outcomes based on the input datasets. We examined potential asymmetries in the dependence between seagrass and sponge cover using the "qad" package (Griessenberger et al., 2022) and modeled their relationship using a generalized additive model (GAM) with the "mgcv" package (Wood, 2011). Permutation-based analysis of variance (PERMANOVA) using Euclidean distance were performed on each response variable (Anderson, 2017) to test the effects of *Community* (seagrass, sponge, association) and *Season* (autumn, spring) on hourly and daily oxygen fluxes as well as daily nutrient fluxes, while separate PERMANOVAs assessed hourly nutrient fluxes with *Condition* (light, dark) as an additional factor. $\delta^{13}C$ and $\delta^{15}N$ values and C:N ratios were tested for differences among *Sample* types (*P. oceanica* leaves, *P. oceanica* epiphytes, *C. nucula*) and *Association* types (associated vs non-associated) using PERMANOVA. Statistical differences in environmental conditions among both seasons for light intensity, temperature, organic (DOC, DON) and inorganic (NH_4^+, NO_x^-, PO_4^{3-}) nutrients were assessed using Welch's t-test. All data analyses were performed with R software (R Core team, 2023).

RESULTS

ENVIRONMENTAL CONDITIONS AND SEAGRASS-SPONGE ASSOCIATION PATTERNS

Environmental variables at the sampling site were higher in autumn than in spring for NH_4^+, NO_2^-, NO_3^-, and DOC concentrations, as well as for seawater temperature (Table 4.1). The relationship between seagrass and sponge cover was non-linear (Fig. 4.2), and our model revealed a cubic curve (edf = 3.7, $p = 6.55 \times 10^{-5}$), with a peak in

sponge cover (~10% and up to 30%) at intermediate levels of seagrass cover (~75%; Fig. 4.2). The asymmetry analysis supported this trend with significant q coefficients (Table S4.1).

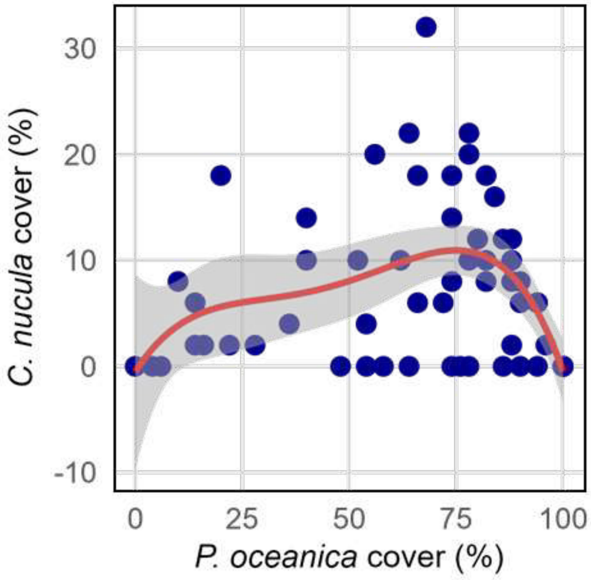

Fig. 4.2. Relationship between *P. oceanica* cover (%) and *C. nucula* cover (%) based on field observations. Each point represents a discrete measurement, with *P. oceanica* cover plotted on the x-axis and *C. nucula* cover on the y-axis. A fourth-degree polynomial regression (red line) models the relationship, with shaded gray ribbons representing the 95% confidence interval.

PRIMARY PRODUCTION AND RESPIRATION RATES

Hourly rates of net primary production (NPP) were highest in the seagrass, followed by the seagrass-sponge association, and lowest in the sponge (Fig. 4.3a, Table S4.2). A seasonal effect was particularly evident in the seagrass, where NPP in autumn was 37% lower than in spring (11.1 ± 3.6 vs 17.5 ± 3.5 µmol O_2 g DW^{-1} h^{-1}, respectively; Fig. 4.3a, Table S4.3). Conversely, NPP rates in the sponge were higher (and slightly positive) in autumn compared to spring (0.2 ± 2.6 vs. -2.8 ± 2.2 µmol O_2 g DW^{-1} h^{-1}, respectively; Fig. 4.3a, Table S4.3), whereas NPP rates in the association showed no seasonal variation. Hourly respiration (R) rates were highest (more negative) in the sponge, followed by the seagrass-sponge association, and lowest in the seagrass (Fig. 4.3b, Table S4.2). Again, a seasonal effect was pronounced in the seagrass, with R rates in autumn 2-fold higher (more negative) than in spring (-3.8 ± 0.4 vs. -1.8 ± 0.3 µmol O_2 g DW^{-1} h^{-1}, respectively; Fig. 4.3b, Table S4.3), as well as in the sponge (-6.1 ± 1.1 vs. -3.6 ± 1.3 µmol O_2 g DW^{-1} h^{-1}, respectively; Fig. 4.3b, Table S4.3). R rates in the association showed little seasonal variation (Fig. 4.3b). Daily rates of net community production (NCP) were highest in the seagrass treatment across both seasons, while the association showed intermediate values, and the sponge exhibited negative values (Fig. 4.3c, Table S4.2). Daily NCP was 54% lower in autumn compared to spring in the seagrass (87.2 ± 38.0 vs. 189.0 ± 37.7 µmol O_2 g DW^{-1} day^{-1}, respectively; Fig. 4.3c, Table S4.3), while it remained stable across seasons in the sponge (-70.8 ± 28.8 vs. -76.0 ± 28.7 µmol O_2 g DW^{-1} day^{-1}, respectively; Fig. 4.3c, Table S4.3) and in the association (73.6 ± 29.5 vs. 78.8 ± 19.6 µmol O_2 g DW^{-1} day^{-1}, respectively; Fig. 4.3c, Table S4.3).

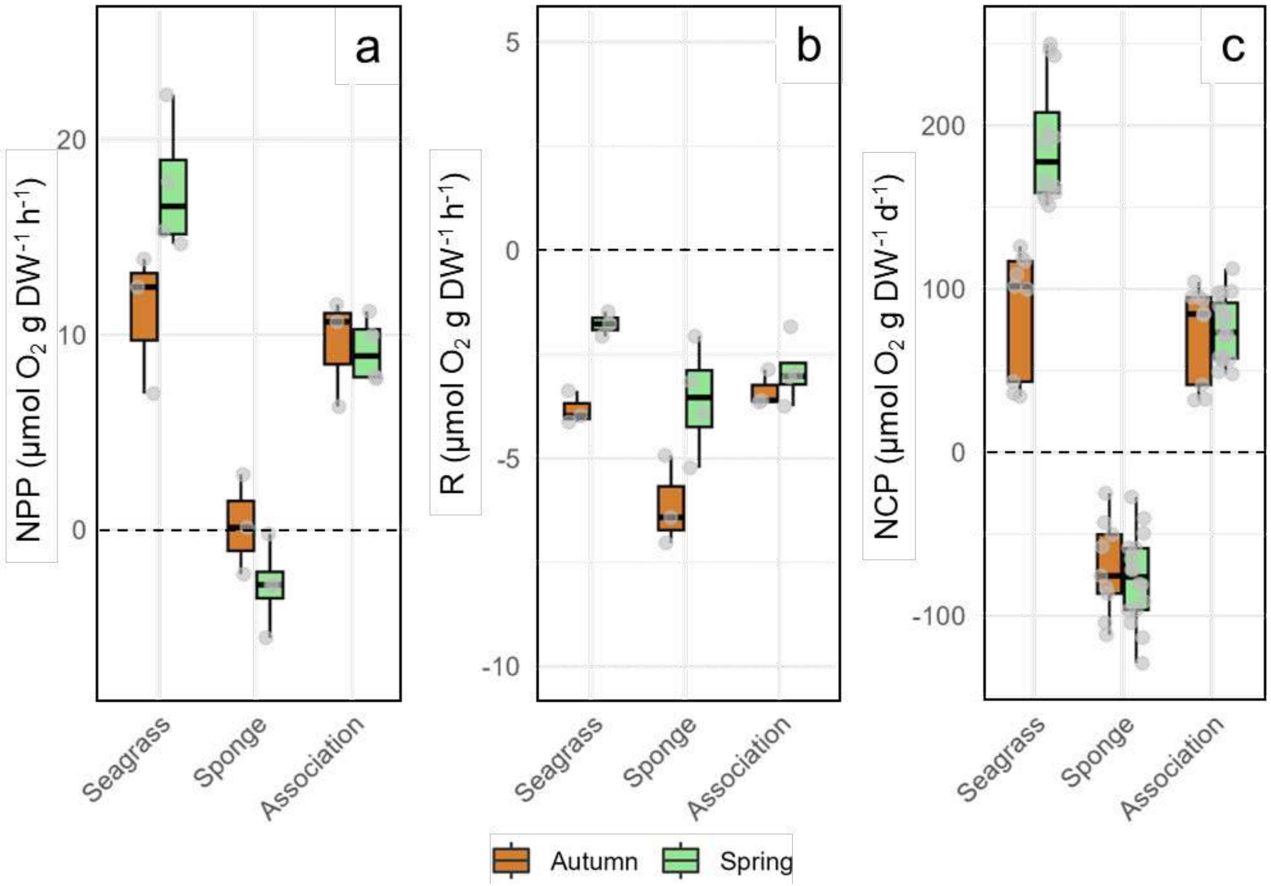

Fig. 4.3. Seasonal variability in net primary production – NPP (a), dark respiration – R (b), and daily net community production – NCP (c) across the different '*Community*' types (*P. oceanica, C. nucula*, and their association). Each panel displays the distribution of metabolic rates for two seasons (Autumn and Spring). Boxplots represent the interquartile range (IQR) with the median marked by a horizontal line. Whiskers extend to 1.5 × IQR, and outliers are shown as individual points (jittered for clarity). Positive values indicate oxygen production, while negative values reflect oxygen consumption. NPP and R are expressed in µmol O_2 g DW^{-1} h^{-1}, while NCP is expressed in µmol O_2 g DW^{-1} d^{-1}.

ORGANIC NUTRIENT FLUXES

Daily DOC fluxes (Fig. 4.4a, Table S4.4) showed the highest release rates in the seagrass treatment for both seasons, particularly in spring (autumn = 40.2 ± 7.9, spring = 63.1 ± 17.3 µmol C g DW^{-1} day^{-1}, Table S4.5). In contrast, the sponge exhibited DOC release in autumn and uptake in spring (27.3 ± 8.6 µmol C g DW^{-1} day^{-1} and -28.4 ± 24.9 µmol C g DW^{-1} day^{-1}, respectively, Table S4.5). The association also showed seasonality in DOC fluxes, with significant release in autumn (40.9 ± 18.2 µmol C g DW^{-1} day^{-1}) and a shift to minimal net uptake in spring (-1.4 ± 20.5 µmol C g DW^{-1} day^{-1}; Fig. 4.4a, Table S4.5). For the most part, daily DON fluxes (Fig. 4.4b, Table S4.4) showed release across treatments and seasons. In autumn, the highest DON release was in the sponge (7.6 ± 2.4 µmol N g DW^{-1} day^{-1}), with intermediate values in the association and the lowest in seagrass (3.5 ± 1.2 µmol N g DW^{-1} day^{-1}, Table S4.5). During spring, the seagrass exhibited the highest DON release (10.8 ± 3.2 µmol N g DW^{-1} day^{-1}), with intermediate values in the sponge and the lowest in the association (0.8 ± 1.7 µmol N g DW^{-1} day^{-1}; Fig. 4.4b, Table S4.5). Hourly DOC fluxes (Fig. S4.1, Table S4.4, S4.5) were higher in the light than in the dark in the seagrass and association *Community* types, with highest values for the seagrass in the light in spring. The sponge showed release in autumn but uptake in spring, with no clear differences between dark and

light. Hourly DON fluxes were higher in the dark than in the light in autumn for all three *Community* types (Fig. S4.1, Table S4.4, S4.5), while they were higher in the light than in the dark in the seagrass and in the association in spring.

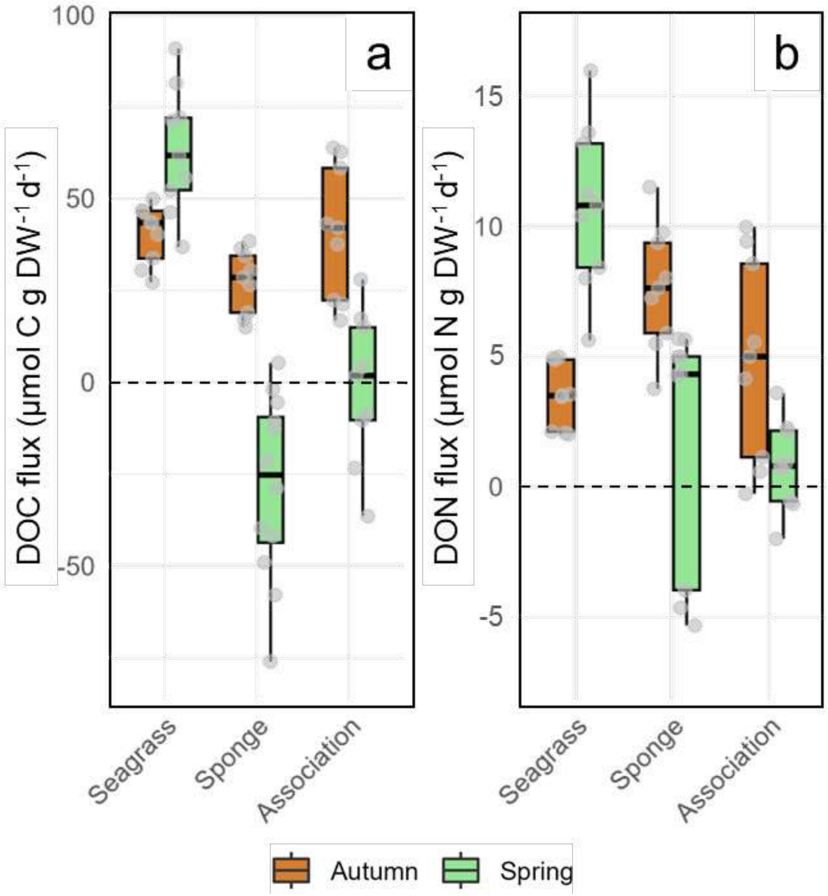

Fig. 4.4. Seasonal variability in dissolved organic carbon – DOC (a) and dissolved organic nitrogen – DON (b) daily fluxes across the different '*Community*' types (*P. oceanica, C. nucula*, and their association). Each panel shows the distribution of nutrient fluxes for two seasons (Autumn and Spring). Boxplots represent the interquartile range (IQR) with the median indicated by a horizontal line. Whiskers extend to 1.5 × IQR, with points outside this range plotted as individual data points (jittered for visibility). Negative values indicate nutrient uptake, and positive values indicate release. Flux rates are expressed in µmol g DW^{-1} d^{-1}.

INORGANIC NUTRIENT FLUXES

Daily NH_4^+ fluxes (Fig. 4.5a) showed constant uptake in the seagrass throughout the *Seasons* (autumn and spring = -0.3 ± 0.4 and -1.6 ± 0.5 µmol NH_4^+ g DW^{-1} day^{-1}, respectively; Table S4.6, S4.7). Conversely, the sponge showed consistent NH_4^+release (autumn and spring = 0.7 ± 0.6 and 2.4 ± 0.7 µmol NH_4^+ g DW^{-1} day^{-1}, respectively), while the association showed release in autumn (1.6 ± 0.6 µmol NH_4^+ g DW^{-1} day^{-1}) and a flux close to net zero in spring (0.4 ± 0.9 µmol NH_4^+ g DW^{-1} day^{-1}; Table S4.6, S4.7). The seagrass showed low but consistent NO_X^- uptake (autumn = -0.2 ± 0.1, spring = -1.2 ± 0.3 µmol NO_X^- g DW^{-1} day^{-1}; Fig. 4.5b), while the sponge displayed strong and consistent NO_X^- release (autumn = 13.6 ± 5.4, spring = 16.7 ± 5.5 µmol NO_X^- g DW^{-1} day^{-1}), with the association showing intermediate but positive fluxes in both seasons (autumn = 4.6 ± 1.9, spring = 6.5 ± 2.3 µmol NO_X^- g DW^{-1} day^{-1}; Table S4.6, S4.7). In autumn, both the seagrass and the sponge showed NH_4^+ release in the light and

NH_4^+ uptake in the dark (Fig. S4.2, Table S4.6, S4.7), while this pattern was not maintained in spring. Conversely, NO_X^- fluxes were highest in the sponge with higher release in the dark than in the daylight, across both autumn and spring (Fig. S4.2, Table S4.6, S4.7). PO_4^{3-} fluxes were low (close to detection limit) and variable, with no clear difference between daylight and dark but mainly uptake in the seagrass and release in the sponge and the association (Fig. S4.2, Table S4.6, S4.7).

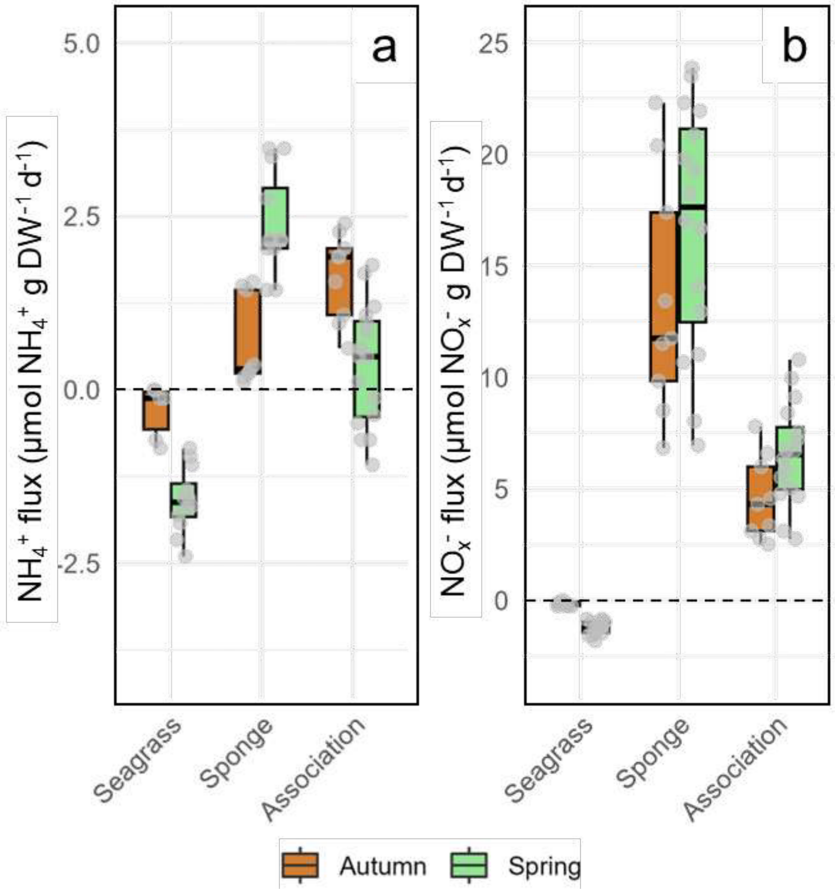

Fig. 4.5. Seasonal variability in ammonium (NH_4^+), and nitrate + nitrite (NO_X^-) daily fluxes across the different 'Community' types (*P. oceanica, C. nucula*, and their association). Each panel shows the distribution of nutrient fluxes for two seasons (Autumn and Spring). Boxplots represent the interquartile range (IQR) with the median indicated by a horizontal line. Whiskers extend to 1.5 × IQR, with points outside this range plotted as individual data points (jittered for visibility). Negative values indicate nutrient uptake, and positive values indicate release. Flux rates are expressed in µmol g DW^{-1} d^{-1}.

STABLE ISOTOPE ANALYSIS

$\delta^{13}C$ of the seagrass ranged from -13.7 ± 1.3‰ when associated with the sponge to -13.9 ± 0.8‰ when non-associated, with no statistical differences between the two groups (n = 20; Fig. S4.3, Table S4.8). Concurrently, we detected an increase in $\delta^{15}N$ of ca. 1‰ in *P. oceanica* living associated with the sponge, from 4.3 ± 1.0‰ to 5.3 ± 0.7‰ (Fig. S4.3, Table S4.8, S4.9). The sponge *C. nucula* showed similar $\delta^{13}C$ and $\delta^{15}N$ values for non-associated sponges (-19.2 ± 0.5‰ and 6.6 ± 0.7‰) vs sponges associated with the seagrass (-19.0 ± 0.5‰ and 6.7 ± 0.6‰, n=20; Fig. S4.3). Plant epiphytes had $\delta^{13}C$ values similar to those of the sponge (-18.8 ± 2.4‰ and -19.6 ± 2.2‰, n=10, for associated vs non-associated plants, respectively) but showed an increase in their $\delta^{15}N$

of ca. 1‰ when the plant was associated with the sponge (from 5.9 ± 1.0‰ to 7.3 ± 1.5‰, Fig. S4.3, Table S4.8, S4.9).

DISCUSSION

This study provides new insights into an association between the seagrass *P. oceanica* and the sponge *C. nucula*, revealing key aspects of their interaction. We found that the association displays non-linear spatial dependence, with higher sponge abundance linked to intermediate seagrass cover. Further, we found evidence that the sponge benefited from DOC released by the seagrass in spring, while contributing substantial inorganic nitrogen, which may support seagrass productivity. These findings support the hypothesis of a facultative mutualism between *P. oceanica* and *C. nucula*, advance our understanding of the ecological dynamics within *P. oceanica* meadows, and highlight the importance of sponges in maintaining meadow stability and nutrient cycling.

THE SPONGE-SEAGRASS ASSOCIATION SHOWS SPATIAL DEPENDENCE

We found evidence that the association between *C. nucula* and *P. oceanica* displays non-linear spatial dependence, with the maximum sponge cover occurring at intermediate seagrass cover (~75%) and areas of both low and high seagrass cover corresponding to minimal sponge presence. This pattern suggests that at intermediate levels of seagrass cover, there is a favorable balance of available substrate and resource availability for both organisms at this site. These results align with Archer et al. (2015), who reported that intermediate seagrass cover offers sufficient substrate for sponge colonization without significantly reducing water flow or light, which could otherwise impair sponge nutrition and/or seagrass photosynthesis. This is relevant because *C. nucula*, similarly to its congeneric species from the Caribbean and Australia, is a photophilic HMA sponge with a rich microbiome dominated by autotrophic cyanobacteria, which contribute to its energy production (Hudspith et al., 2022; Mazzella et al., 2024; Usher et al., 2004). However, a previous report found that photoautotrophy could account for only a small fraction of the total daily carbon uptake in the Caribbean congeneric *C. caribensis* (ca. 7%), while DOC uptake contributed the most to the sponge diet (ca. 92%, Hudspith et al., 2022). At our study site, this may provide a competitive advantage for *C. nucula* to associate with a large primary producer, such as *P. oceanica*, which is known to release large amounts of DOM (Barrón & Duarte, 2009). The asymmetric spatial dependence of the sponge and seagrass indicates neutrality for *P. oceanica* toward the presence of the sponge. However, this neutrality could be the result of a balance between positive and negative effects, rather than the absence of interaction, as Mathis & Bronstein (2020) suggested. In particular, the seagrass may compete with the sponge for space, while at the same time may benefit from its efficient nutrient recycling capacity.

ASSOCIATION WITH THE SPONGE STABILIZES MEADOW PRODUCTIVITY

NPP measurements show that the sponge was near zero metabolic balance in autumn but shifted to net heterotrophy in spring. In contrast, the seagrass and the seagrass-sponge association remained autotrophic throughout both seasons. Our respirometry results are consistent with rates and seasonal dynamics described in previous studies (Berlinghof et al., 2022; Koopmans et al., 2020; Olivé et al., 2016), providing confidence that the data collected on these occasions are representative of broader seasonal processes, and confirming a shift

for the plant from a highly productive growth phase in spring to a senescent phase in autumn. Sponges where symbiont photosynthesis exceeds holobiont respiration are termed “net phototrophic” and are estimated to have >50% of their daily respiratory needs met by their photosynthetic partners (Wilkinson & Trott, 1985). Although microbial diversity in *C. nucula* was not assessed in this study, our rates are to be attributed to cyanobacterial symbiont photosynthesis (Usher et al., 2004), which contributed significantly to sponge nutrition, providing approximately 52% of daily respiratory carbon demand in autumn while only a minor fraction in spring (Fig. 4.6). This underscores the importance of mixotrophy in this sponge, which, similar to what has been reported for a congeneric species in the Caribbean, may rely heavily on DOM uptake (Hudspith et al., 2022). *P. oceanica* showed strong seasonality in productivity (NPP and NCP), but this variation was significantly less pronounced when *C. nucula* was present, indicating a buffering effect by the sponge due to its increased autotrophy in autumn. It is known that biodiversity can enhance productivity, resource use, and stability of seagrass ecosystems (Duffy, 2006). Similarly to land plants, where species interactions that present asynchrony in species fluctuations result in niche partitioning or facilitation and increase both productivity and temporal stability (Isbell et al., 2009), meadows colonized by *C. nucula* may exhibit lower primary production relative to non-colonized meadows during productive seasons, but increased sponge activity during the plant senescence season may buffer the ecosystem against nutrient limitations, favoring nutrient recycling and promoting long-term stability.

NUTRIENT FLUXES BETWEEN SEAGRASS AND SPONGE UNDERPIN THE ASSOCIATION

P. oceanica contributed significant amounts of DOM (as DOC and DON) to its surrounding environment, particularly in spring, concomitant with the highest plant NPP rates. *P. oceanica* is known to enhance DOC fluxes relative to adjacent unvegetated sediments, as these plants produce nonstructural carbohydrates in excess (Barrón & Duarte, 2009; Sogin et al., 2022). In particular, we estimate that the plant released approximately 46% and 33% of its NCP as DOC in autumn and spring, respectively. This is lower than the 71% estimate by Barrón and Duarte (2009), for a *P. oceanica* community in Mallorca Island (Spain), although their estimate also included contributions from allochthonous inputs.

DON was for the most part released by all community types across both seasons, albeit with high variability. The pattern mirrored that of DOC fluxes, with the highest DON release observed in seagrass during spring, coinciding with its growth season, and a tendency for DON uptake by the sponge and the association also in spring. DON fluxes in benthic organisms such as seagrasses and sponges have been rarely documented. The rates measured here are lower but comparable to those reported by Liu et al. (2018) for the tropical seagrasses *Thalassia hemprichii* and *Enhalus acoroides*, attributed to the leaching of nonstructural carbohydrates and other labile organic matter from seagrass leaves. In addition to seagrass leaching, epiphytes and sponges may also contribute DON to the surrounding environment. These sources of DON may have supported sponge heterotrophy in spring, as well as microbial processes such as ammonification (Pfister et al., 2023) and nitrification (Berlinghof et al., 2024), thereby facilitating nitrogen cycling within the studied system.

Concurrently, we detected net DOC uptake by *C. nucula* during spring, under both light and dark conditions, while the sponge released DOC in autumn. DOC uptake/release aligns with the sponge's mixotrophic condition, shifting between autotrophy-dominated in autumn (DOC release) and heterotrophy-dominated (DOC uptake) in spring, as indicated by our measurements of NPP. If we assume linear DOC removal by the sponge in response to rising DOC concentrations in the environment (Ribes et al., 2023) and similar DOC release rates by the plant when associated with the sponge compared to when it is not associated, we can estimate that the DOC released by the seagrass in spring may have covered approximately 33% of the sponge's respiratory carbon demand (Fig. 4.6), a decrease from 92% in the congeneric low-light dwelling *C. caribensis* (Hudspith et al., 2022). Given the difference in depth niches between the two species and the shallow depth at our study site, it is reasonable to expect that *C. nucula* relies more on photoautotrophy compared to its congeneric species from the Caribbean. This reliance on photoautotrophy was estimated to cover approximately 52% of its respiratory carbon needs in autumn and around 10% in spring (Fig. 4.6).

While the seagrass contributed DOC, which was taken up by the sponge in spring, the sponge excreted significant amounts of NH_4^+ and NO_3^- potentially benefiting plant growth. Indeed, we measured substantial uptake of both NH_4^+ and NO_3^- by the seagrass, particularly during spring, which coincides with its peak productivity season. HMA sponges are particularly known for contributing dissolved inorganic nutrients to their surroundings, primarily in the forms NH_4^+ and NO_3^- and PO_3^{4-} (Maldonado et al., 2012). Notably, *C. nucula* exhibited substantial NO_x^- fluxes in both autumn and spring. These fluxes are likely the result of microbial nitrification within the sponge's body. Numerous studies have demonstrated that sponges often associate with ammonium-oxidizing and nitrite-oxidizing microorganisms (Jiménez & Ribes, 2007; Schläppy et al., 2010; Southwell et al., 2008), and *C. nucula* at our study site is probably no exception. Specifically, NO_2^- and NO_3^- production rates in *C. nucula* aligned closely with those reported in previous studies from the Caribbean (Corredor et al., 1988; Diaz & Ward, 1997), and showed that 98% of NO_x^- was released as NO_3^-. This pattern indicates the coexistence of ammonia-oxidation and nitrite-oxidation within the sponge holobiont. Further studies are needed to quantify these processes using stable isotope labeling. However, if we conservatively assume that the plant's uptake remains constant when in association with the sponge compared to when it is not, we can estimate that the sponge contributed approximately 10% to the plant N demand in spring through NO_x^- uptake and about 13% through NH_4^+ uptake (Fig. 4.6).

Our analysis of stable isotope data reveals that both the plant and its epiphytes exhibited higher $\delta^{15}N$ values when associated with the sponge compared to when they were found alone. Significant isotopic fractionation occurs during microbial nitrification, resulting in the product NO_3^- being depleted in $\delta^{15}N$ while the residual NH_4^+ becomes enriched in $\delta^{15}N$ (Casciotti & Buchwald, 2012). In fact, the isotopic composition of NO_3^- expelled from sponges *in situ* has lower $\delta^{15}N$ values than NO_3^- from the ambient water column due to nitrification (Southwell et al., 2008). Therefore, in our study, the increase in $\delta^{15}N$ values in both the plant and its epiphytes when associated with the sponge may have resulted from the preferential incorporation of $\delta^{15}N$-enriched residual NH_4^+ excreted by the sponge, which becomes enriched in ^{15}N during microbial nitrification. Seagrasses often exhibit preferential uptake of NH_4^+, with NO_3^- uptake rates representing only a small fraction of total nitrogen

uptake (Alexandre et al., 2011, 2014), also because NO_3^- incorporation implies active transport with an associated energetic cost (Touchette & Burkholder, 2000). However, this pattern may not prevent the *P. oceanica* holobiont from benefiting also from the released NO_3^-. This seagrass species exhibits a complex nitrogen budget that involves both uptake and recycling processes (Berlinghof et al., 2024; Pfister et al., 2023), which may enable *P. oceanica* to utilize NO_3^- particularly in spring (Lepoint et al., 2002). This capability may allow the plant to meet its nitrogen requirements even in the nutrient-poor conditions of the Mediterranean Sea.

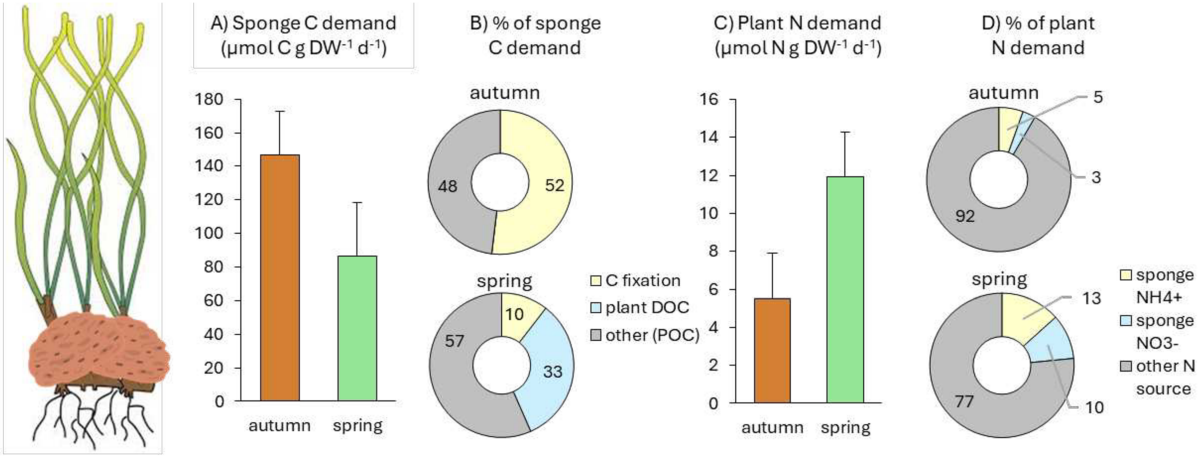

Fig. 4.6. Photoautotrophic and heterotrophic nutrient recycling within the *P. oceanica* - *C. nucula* association. (A) Seasonal variation in the sponge daily respiratory carbon (C) demand (µmol C g DW^{-1} d^{-1}), shown as mean ± SD. (B) Potential contribution (%) of heterotrophic dissolved organic carbon (DOC) and photoautotrophic C fixation to the sponge respiratory C demand, with the remaining fraction hypothesized to originate from particulate organic carbon (POC) via filter-feeding. (C) Seasonal variation in the plant daily nitrogen (N) demand estimated from net community production (NCP) and C:N ratios (µmol N g DW^{-1} d^{-1}), shown as mean ± SD. (D) Potential contribution (%) of sponge ammonium (NH_4^+) and nitrate+nitrite (NO_x^-) release to the plant total daily N demand.

CONCLUSIONS

Our study highlights the ecological significance of the association between the seagrass *P. oceanica* and the sponge *C. nucula*, offering new evidence of spatial dependence and nutrient exchange between these organisms. The results suggest that intermediate seagrass cover promotes sponge colonization while ensuring favorable conditions for both organisms. The association is characterized by a dynamic seasonal balance between autotrophy and heterotrophy, with the sponge benefiting from seagrass-derived DOC and contributing inorganic nitrogen that likely enhances seagrass productivity in spring. This facultative mutualism stabilizes meadow productivity by buffering seasonal fluctuations and underscores the critical role of sponges in nutrient recycling within seagrass ecosystems. Future research should focus on tracing nutrient flows between the two species using stable isotope labeling, quantifying the contribution of microbial nitrification to the sponge's nitrogen output, as well as assessing the stability of the association under environmental stressors. By deepening our understanding of these interactions, we can better predict how such associations may respond to global changes and contribute to the stability of seagrass ecosystems.

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AUTHOR CONTRIBUTION STATEMENT

Conceptualization: UC; Formal analysis: UC, LMM, GZ-H; Investigation: UC, LMM, JB, EG, MF, IO; Resources: UC, FM, TM; Data Curation: UC, LMM, GZ-H, FM, TM; Writing - Original Draft: UC, LMM, GZ-H; Writing - Review & Editing: all co-authors; Visualization: UC, LMM, GZ-H; Supervision: UC, CW, SF, IO; Project administration: UC; Funding acquisition: UC, CW.

REFERENCES

Alexandre, A., Georgiou, D., & Santos, R. (2014). Inorganic nitrogen acquisition by the tropical seagrass alophila stipulacea. *Marine Ecology*, *35*(3), 387–394. https://doi.org/10.1111/maec.12128

Alexandre, A., Silva, J., Bouma, T. J., & Santos, R. (2011). Inorganic nitrogen uptake kinetics and whole-plant nitrogen budget in the seagrass *Zostera noltii*. *Journal of Experimental Marine Biology and Ecology*, *401*(1), 7–12. https://doi.org/10.1016/j.jembe.2011.03.008

Anderson, M. J. (2017). Permutational Multivariate Analysis of Variance (PERMANOVA). In *Wiley StatsRef: Statistics Reference Online* (pp. 1–15). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118445112.stat07841

Archer, S. K., English, P. A., Campanino, F. M., & Layman, C. A. (2021). Sponges facilitate primary producers in a Bahamas seagrass system. *Marine Biology*, *168*(11), 162. https://doi.org/10.1007/s00227-021-03977-x

Archer, S. K., Hensel, E., & Layman, C. A. (2018). Ambient nutrient availability drives the outcome of an interaction between a sponge (Halichondria melanadocia) and seagrass (Thalassia testudinum). *Journal of Experimental Marine Biology and Ecology*, *503*, 86–91. https://doi.org/10.1016/j.jembe.2018.02.005

Archer, S. K., Stoner, E. W., & Layman, C. A. (2015). A complex interaction between a sponge (Halichondria melanadocia) and a seagrass (Thalassia testudinum) in a subtropical coastal ecosystem. *Journal of Experimental Marine Biology and Ecology*, *465*, 33–40. https://doi.org/10.1016/J.JEMBE.2015.01.003

Barrón, C., Apostolaki, E. T., & Duarte, C. M. (2014). Dissolved organic carbon fluxes by seagrass meadows and macroalgal beds. *Frontiers in Marine Science*, *1*. https://doi.org/10.3389/fmars.2014.00042

Barrón, C., & Duarte, C. M. (2009). Dissolved organic matter release in a Posidonia oceanica meadow. *Marine Ecology Progress Series*, *374*, 75–84. https://doi.org/10.3354/MEPS07715

Berlinghof, J., Montilla, L. M., Peiffer, F., Quero, G. M., Marzocchi, U., Meador, T. B., Margiotta, F., Abagnale, M., Wild, C., & Cardini, U. (2024). Accelerated nitrogen cycling on Mediterranean seagrass leaves at volcanic CO2 vents. *Communications Biology*, *7*(341), 1–11. https://doi.org/10.1038/s42003-024-06011-0

Berlinghof, J., Peiffer, F., Marzocchi, U., Munari, M., Quero, G. M., Dennis, L., Wild, C., & Cardini, U. (2022). The role of epiphytes in seagrass productivity under ocean acidification. *Scientific Reports*, *12*(6249). https://doi.org/10.1038/s41598-022-10154-7

Boström, C., & Bonsdorff, E. (1997). Community structure and spatial variation of benthic invertebrates associated with *Zostera marina* (L.) beds in the northern Baltic Sea. *Journal of Sea Research*, *37*(1), 153–166. https://doi.org/10.1016/S1385-1101(96)00007-X

Bulleri, F. (2009). Facilitation research in marine systems: State of the art, emerging patterns and insights for future developments. *Journal of Ecology*, *97*(6), 1121–1130. https://doi.org/10.1111/j.1365-2745.2009.01567.x

Campana, S., Hudspith, M., Lankes, D., de Kluijver, A., Demey, C., Schoorl, J., Absalah, S., van der Meer, M. T. J., Mueller, B., & de Goeij, J. M. (2021). Processing of Naturally Sourced Macroalgal- and Coral-Dissolved Organic Matter (DOM) by High and Low Microbial Abundance Encrusting Sponges. *Frontiers in Marine Science*, *8*. https://doi.org/10.3389/fmars.2021.640583

Cardini, U., Bartoli, M., Lücker, S., Mooshammer, M., Polzin, J., Lee, R. W., Micić, V., Hofmann, T., Weber, M., & Petersen, J. M. (2019). Chemosymbiotic bivalves contribute to the nitrogen budget of seagrass ecosystems. *The ISME Journal*, *13*(12), 3131–3134. https://doi.org/10.1038/s41396-019-0486-9

Cardini, U., Marín-Guirao, L., Montilla, L. M., Marzocchi, U., Chiavarini, S., Rimauro, J., Quero, G. M., Petersen, J. M., & Procaccini, G. (2022). Nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning in contaminated sediments. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.918675

Casciotti, K., & Buchwald, C. (2012). Insights on the marine microbial nitrogen cycle from isotopic approaches to nitrification. *Frontiers in Microbiology*, *3*. https://www.frontiersin.org/articles/10.3389/fmicb.2012.00356

Corredor, J. E., Wilkinson, C. R., Vicente, V. P., Morell, J. M., & Otero, E. (1988). Nitrate release by Caribbean reef sponges. *Limnology and Oceanography*, *33*(1), 114–120. https://doi.org/10.4319/lo.1988.33.1.0114

De Goeij, J. M., Van Oevelen, D., Vermeij, M. J. A., Osinga, R., Middelburg, J. J., De Goeij, A. F. P. M., & Admiraal, W. (2013). Surviving in a Marine Desert: The Sponge Loop Retains Resources Within Coral Reefs. *Science*, *342*(6154), 108–110. https://doi.org/10.1126/science.1241981

Diaz, M. C., & Ward, B. B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series*, *156*, 97–107.

Duffy, J. E. (2006). Biodiversity and the functioning of seagrass ecosystems. *Marine Ecology Progress Series*, *311*, 233–250. https://doi.org/10.3354/meps311233

Ellison, A. M., Farnsworth, E. J., & Twilley, R. R. (1996). Facultative Mutualism Between Red Mangroves and Root-Fouling Sponges in Belizean Mangal. *Ecology*, *77*(8), 2431–2444. https://doi.org/10.2307/2265744

Erwin, P. M., Pita, L., López-Legentil, S., & Turon, X. (2012). Stability of Sponge-Associated Bacteria over Large Seasonal Shifts in Temperature and Irradiance. *Applied and Environmental Microbiology*, *78*(20), 7358–7368. https://doi.org/10.1128/AEM.02035-12

Gagnon, K., Christie, H., Didderen, K., Fagerli, C. W., Govers, L. L., Gräfnings, M. L. E., Heusinkveld, J. H. T., Kaljurand, K., Lengkeek, W., Martin, G., Meysick, L., Pajusalu, L., Rinde, E., van der Heide, T., & Boström, C. (2021). Incorporating facilitative interactions into small-scale eelgrass restoration—Challenges and opportunities. *Restoration Ecology*, *29*(5), e13398. https://doi.org/10.1111/rec.13398

Griessenberger, F., Trutschnig, W., & Junker, R. R. (2022). qad: An R-package to detect asymmetric and directed dependence in bivariate samples. *Methods in Ecology and Evolution*, *13*(10), 2138–2149. https://doi.org/10.1111/2041-210X.13951

Heck, K. L., Carruthers, T. J. B., Duarte, C. M., Hughes, A. R., Kendrick, G., Orth, R. J., & Williams, S. W. (2008). Trophic Transfers from Seagrass Meadows Subsidize Diverse Marine and Terrestrial Consumers. *Ecosystems*, *11*(7), 1198–1210. https://doi.org/10.1007/s10021-008-9155-y

Hill, M., Hill, A., Lopez, N., & Harriott, O. (2006). Sponge-specific bacterial symbionts in the Caribbean sponge, Chondrilla nucula (Demospongiae, Chondrosida). *Marine Biology*, *148*, 1221–1230. https://doi.org/10.1007/s00227-005-0164-5

Hudspith, M., De Goeij, J. M., Streekstra, M., Kornder, N. A., Bougoure, J., Guagliardo, P., Campana, S., Van Der Wel, N. N., Muyzer, G., & Rix, L. (2022). Harnessing solar power: Photoautotrophy supplements the diet of a low-light dwelling sponge. *ISME*. https://doi.org/10.1038/s41396-022-01254-3

Isbell, F. I., Polley, H. W., & Wilsey, B. J. (2009). Biodiversity, productivity and the temporal stability of productivity: Patterns and processes. *Ecology Letters*, *12*(5), 443–451. https://doi.org/10.1111/j.1461-0248.2009.01299.x

Jiménez, E., & Ribes, M. (2007). Sponges as a source of dissolved inorganic nitrogen: Nitrification mediated by temperate sponges. *Limnology and Oceanography*, *52*(3), 948–958. https://doi.org/10.4319/lo.2007.52.3.0948

Kharlamenko, V. I., Kiyashko, S. I., Imbs, A. B., & Vyshkvartzev, D. I. (2001). Identification of food sources of invertebrates from the seagrass Zostera marina community using carbon and sulfur stable isotope ratio and fatty acid analyses. *Marine Ecology Progress Series*, *220*, 103–117. https://doi.org/10.3354/meps220103

Koopmans, D., Holtappels, M., Chennu, A., Weber, M., & De Beer, D. (2020). High Net Primary Production of Mediterranean Seagrass (Posidonia oceanica) Meadows Determined With Aquatic Eddy Covariance. *Frontiers in Marine Science*, *7*, 118. https://doi.org/10.3389/fmars.2020.00118

Lepoint, G., Millet, S., Dauby, P., Gobert, S., & Bouquegneau, J.-M. (2002). Annual nitrogen budget of the seagrass Posidonia oceanica as determined by in situ uptake experiments. *Marine Ecology Progress Series*, *237*, 87–96. https://doi.org/10.3354/meps237087

Liu, S., Jiang, Z., Zhou, C., Wu, Y., Arbi, I., Zhang, J., Huang, X., & Trevathan-Tackett, S. M. (2018). Leaching of dissolved organic matter from seagrass leaf litter and its biogeochemical implications. *Acta Oceanologica Sinica*, *37*(8), 84–90. https://doi.org/10.1007/s13131-018-1233-1

Maldonado, M., Ribes, M., & Van Duyl, F. C. (2012). Nutrient Fluxes Through Sponges. In *Advances in Marine Biology* (Vol. 62, pp. 113–182). Elsevier. https://doi.org/10.1016/B978-0-12-394283-8.00003-5

Malkin, S., & Cardini, U. (2021). Facilitative interactions on the rise: Cable bacteria associate with diverse aquatic plants. *New Phytologist*, *232*(5), 1897–1900. https://doi.org/10.1111/nph.17664

Mathis, K. A., & Bronstein, J. L. (2020). Our Current Understanding of Commensalism. *Annual Review of Ecology, Evolution, and Systematics*, *51*(Volume 51, 2020), 167–189. https://doi.org/10.1146/annurev-ecolsys-011720-040844

Mazzella, V., Dell'Anno, A., Etxebarría, N., González-Gaya, B., Nuzzo, G., Fontana, A., & Núñez-Pons, L. (2024). High microbiome and metabolome diversification in coexisting sponges with different bio-ecological traits. *Communications Biology*, *7*(1), 1–18. https://doi.org/10.1038/s42003-024-06109-5

Mcfall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Loso, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *PNAS*, *110*(9), 3229–3236. https://doi.org/10.1073/pnas.1218525110/-/DCSupplemental

Olivé, I., Silva, J., Costa, M. M., & Santos, R. (2016). Estimating Seagrass Community Metabolism Using Benthic Chambers: The Effect of Incubation Time. *Estuaries and Coasts*, *39*, 138–144. https://doi.org/10.1007/s12237-015-9973-z

Pansini, M., & Pronzato, R. (1985). Distribution and Ecology of Epiphytic Portfera in two Posidonia oceanica (L.) DELILE Meadows of the Ligurian and Tyrrhenian Sea. *Marine Ecology*, *6*(1), 1–11. https://doi.org/10.1111/j.1439-0485.1985.tb00316.x

Pawlik, J. R., & McMurray, S. E. (2020). The Emerging Ecological and Biogeochemical Importance of Sponges on Coral Reefs. *Annual Review of Marine Science*, *12*(Volume 12, 2020), 315–337. https://doi.org/10.1146/annurev-marine-010419-010807

Pfister, C. A., Cardini, U., Mirasole, A., Montilla, L. M., Veseli, I., Gattuso, J.-P., & Teixido, N. (2023). Microbial associates of an endemic Mediterranean seagrass enhance the access of the host and the surrounding

seawater to inorganic nitrogen under ocean acidification. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-47126-4

Pita, L., Rix, L., Slaby, B. M., Franke, A., & Hentschel, U. (2018). The sponge holobiont in a changing ocean: From microbes to ecosystems. *Microbiome*, *6*(46). https://doi.org/10.1186/s40168-018-0428-1

R Core team. (2023). *R: A language environment for statistical computing*.

Ribes, M., Yahel, G., Romera-Castillo, C., Mallenco, R., Morganti, T. M., & Coma, R. (2023). The removal of dissolved organic matter by marine sponges is a function of its composition and concentration: An in situ seasonal study of four Mediterranean species. *Science of The Total Environment*, *871*, 161991. https://doi.org/10.1016/j.scitotenv.2023.161991

Rix, L., de Goeij, J. M., van Oevelen, D., Struck, U., Al-Horani, F. A., Wild, C., & Naumann, M. S. (2017). Differential recycling of coral and algal dissolved organic matter via the sponge loop. *Functional Ecology*, *31*(3), 778–789. https://doi.org/10.1111/1365-2435.12758

Schläppy, M.-L., Schöttner, S. I., Lavik, G., Kuypers, M. M. M., de Beer, D., & Hoffmann, F. (2010). Evidence of nitrification and denitrification in high and low microbial abundance sponges. *Marine Biology*, *157*(3), 593–602. https://doi.org/10.1007/s00227-009-1344-5

Sogin, E. M., Michellod, D., Gruber-Vodicka, H. R., Bourceau, P., Geier, B., Meier, D. V., Seidel, M., Ahmerkamp, S., Schorn, S., D'Angelo, G., Procaccini, G., Dubilier, N., & Liebeke, M. (2022). Sugars dominate the seagrass rhizosphere. *Nature Ecology and Evolution*, *6*, 866–877. https://doi.org/10.1038/S41559-022-01740-Z

Southwell, M. W., Weisz, J. B., Martens, C. S., & Lindquist, N. (2008). In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. *Limnology and Oceanography*, *53*(3), 986–996. https://doi.org/10.4319/lo.2008.53.3.0986

Thiel, V., Leininger, S., Schmaljohann, R., Brümmer, F., & Imhoff, J. F. (2007). Sponge-specific Bacterial Associations of the Mediterranean Sponge Chondrilla nucula (Demospongiae, Tetractinomorpha). *Microb. Ecol.*, *54*, 101–111. https://doi.org/10.1007/s00248-006-9177-y

Touchette, B. W., & Burkholder, J. A. M. (2000). Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, *250*, 133–167. https://doi.org/10.1016/S0022-0981(00)00195-7

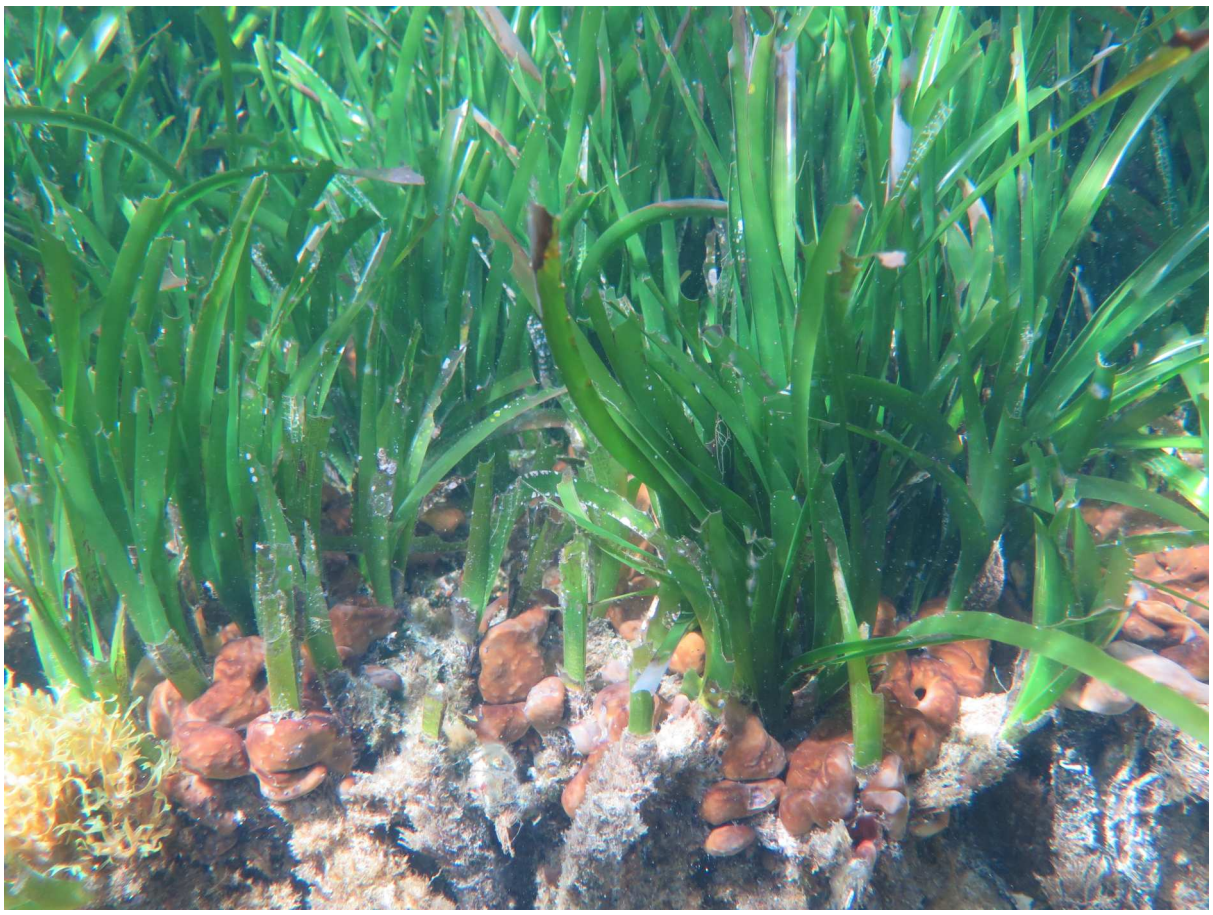
Usher, K. M., Fromont, J., Sutton, D. C., & Toze, S. (2004). The Biogeography and Phylogeny of Unicellular Cyanobacterial Symbionts in Sponges from Australia and the Mediterranean. *Microb. Ecol.*, *48*, 167–177. https://doi.org/10.1007/s00248-003-1062-3

Whippo, R., Knight, N. S., Prentice, C., Cristiani, J., Siegle, M. R., & O'Connor, M. I. (2018). Epifaunal diversity patterns within and among seagrass meadows suggest landscape-scale biodiversity processes. *Ecosphere*, *9*(11), e02490. https://doi.org/10.1002/ecs2.2490

Wilkinson, C., & Trott. (1985). Light as a factor in determining the distribution of sponges across the central Great Barrier Reef. 125–130. Proceedings of the 5th International Coral Reef Congress.

Wood, S. N. (2011). Fast Stable Restricted Maximum Likelihood and Marginal Likelihood Estimation of Semiparametric Generalized Linear Models. *Journal of the Royal Statistical Society Series B: Statistical Methodology*, *73*(1), 3–36. https://doi.org/10.1111/j.1467-9868.2010.00749.x

CHAPTER 5



Posidonia oceanica and *Chondrilla nucula* growing in association in the Gulf of Naples (Italy). Photo by Ulisse Cardini.

CHAPTER 5

NITRIFICATION IN A SEAGRASS-SPONGE ASSOCIATION

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ABSTRACT

In the Mediterranean Sea, the demosponge *Chondrilla nucula* can occur in close association with the native seagrass *Posidonia oceanica*. *C. nucula* harbors a diverse and abundant microbial community, including potential nitrifiers. Thus, the sponge may contribute to the nitrogen (N) demand of the seagrass holobiont. In this study, we investigated potential nitrification rates (PNR) and inorganic N fluxes within this association at a site where *C. nucula* covered 18 ± 3 % of the seagrass meadow area, during plant growth (spring) and senescence (autumn). Using incubation experiments with ^{15}N-labeled ammonium, we measured PNR and inorganic N of the seagrass-sponge association, and of sponge and seagrass independently, under light and dark conditions. We supplemented these experiments with 16s rRNA gene amplicon sequencing to characterize the microbial community of the sponge. PNR was exclusively measured when the sponge was present (alone or in association with the seagrass). PNR was highest in the dark and when *C. nucula* was associated with the seagrass, ranging from 21 ± 7 to 267 ± 33 nmol N g DW^{-1} h^{-1} in spring and autumn, respectively. Sponge-mediated PNR can support 8% of the N demand of the *P. oceanica* holobiont during growth and 47 % during senescence. We identified key nitrifying bacterial and archaeal groups as members of the sponge's microbial community. While *C. nucula* released inorganic N, potentially sustaining the seagrass, it benefitted from dissolved organic carbon released by *P. oceanica*. These results suggest that the interaction between *C. nucula* and *P. oceanica* is mutually beneficial, ultimately supporting and stabilizing the seagrass ecosystem.

A modified version of this chapter is in preparation for publication in *ISME Communications*.

INTRODUCTION

Seagrasses are marine flowering plants that form vital ecosystems in coastal regions around the world, providing a range of ecological services and supporting high biodiversity (Costanza et al., 1997; Nordlund et al., 2016). Seagrasses can be considered as holobionts, forming complex symbiotic relationships with a diverse microbiome that includes bacteria, fungi, and other microorganisms living on the plant surfaces and within their tissues. These microbial communities are crucial for plant physiology and health because of their role in nutrient cycling, access to sunlight, or as protection against pathogens (e.g., Seymour et al., 2018; Tarquinio et al., 2019; Ugarelli et al., 2017). For instance, leaf epiphytes contribute to plant nitrogen (N) requirements by fixing atmospheric N_2 and converting into bioavailable forms (e.g., Agawin et al., 2016; Mohr et al., 2021; Welsh, 2000), while sulfate-reducing bacteria in the rhizosphere contribute to nutrient mineralization (Holmer et al., 2001; Nielsen et al., 2001). The seagrass holobiont is further embedded in a 'nested ecosystem' (see Pita et al., 2018), where larger organisms, such as lucinid clams or sponges and their respective microbiome, interact with seagrasses and their associated microbes (Cardini et al., 2022; Malkin & Cardini, 2021). These nested interactions create a complex web of relationships that fundamentally contribute to the overall functioning of seagrass ecosystems.

Marine sponges (Porifera) represent one of the oldest and most primitive multicellular organisms on Earth. As filter feeders, they feed on microorganisms and can host dense and diverse microbial communities in their mesohyl matrix (Thomas et al., 2016). In recent years, there has been a growing emphasis on researching sponges and their prokaryotic symbionts, investigating their role in the biogeochemical cycling of nutrients (Maldonado et al., 2012 and references therein; Pita et al., 2018) and particularly on quantifying fluxes of dissolved inorganic nitrogen (DIN) released by sponges. Sponge-associated nitrification was hereby found to be the process producing the bulk of the DIN released (Diaz & Ward, 1997; Southwell, Weisz, et al., 2008).

Sponges are commonly found in seagrass meadows (Ávila et al., 2015; Soest et al., 2012) and can grow in very close association with the plants. However, the mechanisms and potential benefits of this association in terms of nutrient cycling still need to be investigated. Seagrasses are known to release large quantities of dissolved organic matter (DOM) into the surrounding seawater and sediments (Barrón & Duarte, 2009; Sogin et al., 2022). Sponges have the ability to take up DOM, which is then recycled to particulate organic matter (POM) that is released by the sponge and can be taken up by higher trophic levels. This process is also known as the "sponge loop", a benthic counterpart of the oceanic microbial loop (De Goeij et al., 2013; Rix et al., 2017). The seagrass on the other hand may benefit from the release of DIN by the sponges via ammonium excretion, nitrogen fixation, or nitrification (Davy et al., 2002; Jiménez & Ribes, 2007; Fiore et al., 2010; Rix et al., 2015), as primary production in oligotrophic areas is often N-limited. Studies of an association between the seagrass *Thalassia testudinum* and the sponge *Halichondria melanadocia* in the Caribbean Sea revealed a context depended commensal relationship, balancing between the negative shading effect of the sponge for the seagrass with positive effects of N and phosphorus supplied by the sponge (Archer et al., 2015). This way, sponges can facilitate the growth of primary producers (Archer et al., 2021). At the same time, the sponge benefits from the substrate for growth provided by the plant (Archer et al., 2015).

A similar association can be found in the Mediterranean Sea between the demosponge *Chondrilla nucula* and the endemic seagrass *Posidonia oceanica*. The sponge can be found growing in very close association with the seagrass, attached to the lower part of the leaves (see Fig. 4.1 in Chapter 4). Belonging to the high microbial abundance (HMA) sponges, *C. nucula* harbors a distinct and diverse procaryotic community, including Cyanobacteria, Acidobacteria, Gamma-, and Deltaproteobacteria (Hill et al., 2006; Thiel et al., 2007) and also potential nitrifiers (Mazzella et al., 2024). Studies showed that *C. nucula* can release high amounts of DIN (17 - 44 nmol DIN g dry wt^{-1} min^{-1}, Diaz & Ward, 1997; 141 $\pm$ 26 µmol NO_3^- + NO_2^- L^{-1} sponge h^{-1}, Hoer et al., 2018; 600 nmol NO_3^- dry wt^{-1} h^{-1}, Corredor et al., 1988). The excretion of nitrate or nitrite is taken as first evidence of the presence of microbial nitrifiers in the sponges (Corredor et al., 1988; Diaz & Ward, 1997; Jiménez & Ribes, 2007). Understanding the mechanisms and rates of nitrification in these associations is important for unraveling the complexity of N dynamics in coastal ecosystems, with consequences for biodiversity and ecosystem functioning.

Nitrification is a pivotal process in the nitrogen (N) cycle and plays a fundamental role in shaping the nutrient dynamics of marine ecosystems. This biological transformation involves the oxidation of ammonia (NH_3) to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-), each process mediated by distinct groups of microorganisms (Ward, 2008). The first step, the oxidation from ammonia to nitrite, is performed by ammonia-oxidizing bacteria (AOB) or archaea (AOA), while the second step, the oxidation of nitrite to nitrate, is carried out by nitrite-oxidizing bacteria (NOB) (Ward, 2008). These nitrifying microorganisms can be found in the open ocean (Beman et al., 2013; Francis et al., 2005; Wuchter et al., 2006), coastal sediments (Freitag & Prosser, 2003; Park et al., 2008), but also marine invertebrates, such as sponges (Bayer et al., 2007; Hoffmann et al., 2009). Measurements of high nitrification rates based on the release of nitrite and nitrate have been reported from several tropical and temperate sponges (Bayer et al., 2008; Diaz & Ward, 1997; Hoffmann et al., 2009; Jiménez & Ribes, 2007; Nemoy et al., 2021).

In this study, we investigated the process of sponge-associated microbial nitrification as an indicator of a potential mutualism in the association between *P. oceanica* and *C. nucula*. We quantified potential nitrification rates (PNR) and net fluxes of inorganic and organic nutrients within the association and the organisms alone in incubation experiments, using ^{15}N labeled ammonium, both in the light and in the dark. We complement these analyses with 16s rRNA gene amplicon sequencing to explore the diversity of the sponge microbial community, and the potential players involved in nitrification.

METHODS

STUDY SITE AND SAMPLING

The incubation experiments were performed in May and October 2022 at the Schiacchetiello inlet (40°47'36.9"N 14°05'13.4"E) in the area of Bacoli (Tyrrhenian Sea, Italy). Here, shallow patches (0-6 m depth) of a *P. oceanica* meadow with a high *C. nucula* coverage exist (Table 5.1). The site is characterized by high human pressure due to tourism (e.g., boat anchoring in the meadows) and eutrophication due to a nearby mussel farm. We collected the aboveground part of *P. oceanica* shoots when growing alone, small specimens of *C. nucula* (max. 5 cm ∅)

growing alone and both when growing in association. We selected *P. oceanica* shoots in the central part of the meadow patches to avoid edge effects. *C. nucula* was carefully removed from the substrate to avoid any damage to the tissue. Shoots of *P. oceanica* with the sponge growing attached to the lower part of the leaves (see Fig. 4.1, Chapter 4) were considered as association. We made sure that the organisms stayed submerged in the water until further use.

Table 5.1. Environmental parameters (mean ± SE) measured in May and October 2022. Temperature and light were continuously measured with data loggers (between ca. 10 am and 5 pm of the respective incubation day). DOC, DON, NH_4^+, and NO_3^- were analyzed from samples collected on the respective sampling day (n = 3). Sponge coverage and shoot density were collected in autumn at 8 meadow patches with 8 random subplots each (25 x 25 cm).

	Spring	Autumn
Water temperature (°C)	21.03 ± 0.01	24.52 ± 0.04
Light (Lux)	58441 ± 2748	39007 ± 1498
Dissolved oxygen (mg/L)	8.10 ± 0.03	7.52 ± 0.12
DOC (μM)	106.46 ± 4.91	134.37 ± 8.03
DON (μM)	7.82 ± 0.68	11.25 ± 0.29
NH_4^+ (μM)	12.74 ± 3.09	2.76 ± 1.75
NO_3^- (μM)	2.06 ± 1.23	2.21 ± 0.03
Sponge coverage (%)	---	18.33 ± 2.72
Seagrass shoot density (m^{-2})	---	349.00 ± 16.71

Samples for the microbial community analysis of the sponge were collected in May 2022 at the site described above. Five specimens of *C. nucula* growing alone and in association were removed from the substrate, cut into pieces with sterile scalpels, and washed with sterile filtered seawater to remove rubble or organisms attached. They were then transferred into sterile 50 mL Falcon tubes filled with stabilizing buffer solution (RNA*later*) and stored on dry ice until transferred to the laboratory, where they were stored at -20°C until further analysis. For the microbial community of the water column, we collected 5 L of seawater from the sampling site and filtered 5 x 1 L on 0.22 μm cellulose nitrate membrane filters. The filters were transferred into sterile 15 mL Falcon tubes filled with RNA*later* and stored on dry ice until transfer to the laboratory, where they were stored at -20°C until further analysis.

Incubation experiment with stable isotopes

PNR was determined by amending site water with 5 μM $^{15}NH_4^+$ (≥98 atom %^{15}N) tracing solution. For the incubations, we filled acid-washed polyethylene chambers (1100 mL) with site water and added seagrass, sponge, or the association (n = 4) for incubating in the light or the dark. We added 500 μL of a 10 mM $^{15}NH_4^+$ stock solution to each chamber, closed them without air bubbles, and gently inverted them. This resulted in an enrichment of 60.19 atom% ^{15}N-NH_4^+ in spring and of 76.51 atom% in autumn in the incubation chambers. ^{15}N-NH_4^+-enriched chambers (also 5 μM) without organisms served as controls for background processes in the water column (n = 2). Chambers with the seagrass-sponge association but without ^{15}N-NH_4^+- enrichment served as controls for our isotope enrichment method (n = 2).

T_0 samples for the analysis of O_2 production were taken from the bottom of the chambers to reduce gas exchange in 12 mL exetainers (Labco Ltd.) using acid-washed syringes and tubes and were subsequently fixed

with 100 µL 7 M $ZnCl_2$. T_0 samples for the analysis of dissolved inorganic nutrients (DIN: NH_4^+, NO_2^-, NO_x^- and PO_4^{3-}), dissolved organic carbon and nitrogen (DOC, DON), and $^{15}NO_3^-$ concentrations were taken in triplicates from an extra incubation chamber with 5 µM ^{15}N-NH_4^+-enrichment without organisms added. The chambers were refilled with site water, closed without air bubbles, and placed upside down in two plastic crates, making sure the seagrass shoots and sponges were placed the right way around and to not create shading from the chamber lids or the crates. One crate was covered with black plastic bags for the dark incubation; then both crates were placed for 5-6 h floating in the water to ensure a stable temperature, light availability, and mixing via wave activity (16 chambers per crate, 32 chambers in total). Temperature and light were continually measured during the incubation with HOBO data loggers (Onset Computer Corporation) inside a control chamber and in the water column.

At the end of the incubation, the chambers were opened and T_{final} samples for O_2 analysis were taken as described above. Samples for the analysis of $^{15}NO_3^-$ production were filtered with 0.2 µm disposable syringe filters into 50 mL acid-washed Falcon tubes and stored on dry ice until transported to the laboratory, where they were frozen at -20°C until further analysis. Samples to analyze DIN were filtered with 0.2 µm disposable syringe filters into 20 mL acid-washed HDPE vials and stored on dry ice until transported to the laboratory, where they were frozen at -20°C until further analysis. Samples to analyze DOC and DON were filtered with precombusted (400°C, 4 to 5 h) 0.7 µm GF/F filters into 30 mL acid-washed HDPE vials, fixed with 80 µL 18% HCl, and stored in cool boxes until transferred to the laboratory, where they were stored at 6°C until further analysis. The biomass (seagrass and sponges) was collected in zip-lock bags and stored in cool boxes until further processing. In the lab, the biomass samples were then processed by separating seagrass leaves, seagrass epiphytes, and sponges for measuring the wet weight. After the samples were freeze-dried for 24 h, the dry weight of each tissue type was measured. To measure background C and N concentrations at the study site, 1000 mL of water column samples for T_0 as well as the remaining water of the incubation chambers at T_{final} was filtered with precombusted 0.7 µm GF/F filters. The filters were stored in 15 mL centrifuge tubes on dry ice until transport to the laboratory, where they were frozen at -20°C until further analysis.

POTENTIAL NITRIFICATION RATES (PNR)

Isotopic samples for $^{15}NO_3^-$ production were analyzed by isotope ratio mass spectrometry (IRMS, ThermoScientific) using the Ti(III) reduction method as described in (Berlinghof et al., 2024). Potential nitrification rates (PNR) were calculated using an equation modified from (Beman et al., 2011):

$$^{15}N_{excess} = {}^{15}N_t - {}^{15}N_0 \quad \text{(I)}$$

$$PNR = (atom\%(^{15}N_{excess}) / atom\%(^{15}N_{medium})) \times ([NO_3^-] / t) \quad \text{(II)}$$

$^{15}N_t$ is the ^{15}N content of the samples in the NO_3^- pool measured at time t, and $^{15}N_0$ is the ^{15}N content in the NO_3^- pool measured at the beginning of the incubations. The enrichment of samples ($^{15}N_{excess}$) was considered significant for samples with a value greater than 2.5 times the standard deviation of the mean of the T_0 samples. $^{15}N_{medium}$ is the enrichment of the incubation medium at the end of the incubations. Based on the NH_4^+

concentrations measured before and after the addition of $^{15}NH_4^+$, this resulted in an enrichment of ~95.9 atom $\%^{15}N$ in the incubation medium. [NO_3^-] is the concentration of NO_3^- (μM) and t is the incubation time (h). PNR was corrected for the rates in control incubations without organisms. Since PNR was only detected in incubations where the sponge was present, they were normalized to sponge dry weight (g) in the SG + SP and SP treatments; and to seagrass dry weight (g) in the SG treatments.

Stable Isotope Analysis

We collected samples of *P. oceanica* (leaves and epiphytes, in autumn additionally seagrass meristem tissue) and *C. nucula*, when growing associated vs non-associated. Epiphytes were carefully scraped off the seagrass leaves and stored in Eppendorf tubes. All samples were stored in acid-washed vials and lyophilized. The samples were ground to a fine powder using a tissue lyser, acid-fumed with HCl and then weighed into silver capsules for isotope analysis. The samples were analyzed using a Flash Elemental Analyzer (Thermo Scientific) equipped with a single reactor (1020°C), along with a MAT 253 Plus isotope ratio mass spectrometer (IRMS) interfaced with a Conflo IV system (Thermo Scientific, Bremen, Germany). The $\delta^{13}C$ and $\delta^{15}N$ values were corrected for blanks, ion source linearity, standardized against laboratory working standards and international reference materials (IAEA-600, IAEA-603) and normalized to Vienna Pee Dee Belemnite and atmospheric air, respectively. Precision was typically <0.1‰ for $\delta^{13}C$ and 0.5‰ for $\delta^{15}N$. The molar C:N ratios (mol:mol) were calculated from C and N weights in the capsules (μg) and based on their respective molecular weights.

Fluxes of oxygen, inorganic and organic nutrients

Oxygen concentrations from T_0 and T_{final} sampling were measured with a membrane-introduction mass spectrometry (MIMS, Bay Instruments, LLC). All samples were measured in technical quadruplicates and 0.2 μM filtered seawater (20°C, salinity = 38 psu) was used as standard to calculate the O_2 concentrations from the atomic mass of 32. The lowest oxygen saturation in the dark incubations dropped to 38% of the initial O_2 concentration and the highest in the light incubation increased to 179%.

Dissolved inorganic nutrient concentrations (NH_4^+, NO_2^-, NO_3^-, PO_4^-) were measured with a continuous flow analyzer (Flowsys, SYSTEA SpA.). NO_3^- concentrations were calculated as the difference between NO_x^- and NO_2^-. DOC and DON concentrations were measured with a TOC-L Analyzer with TN unit (Shimadzu Corporation, Japan). Net nutrient fluxes were calculated as the difference between final and initial nutrient concentrations, corrected for controls, and normalized to biomass dry weight.

Data analysis

To compute daily, integrated rates of nutrient fluxes (daily flux = light flux × 12 + dark flux × 12), we generated analytical combinations of the observed light and dark fluxes, assuming a daily 12:12 h light/dark cycle. Each pair of independent values was combined to calculate the distribution of integrated rates (n=4). The results present a thorough distribution of potential outcomes derived from the input data. Permutation-based analysis of variance (PERMANOVA) using Euclidean distance was performed on each response variable (Anderson, 2017) to

test the effects of *community* (seagrass, sponge, association) and *season* (spring vs autumn) on PNR, O_2, inorganic and organic nutrient fluxes. $\delta^{13}C$, $\delta^{15}N$ values and C:N ratios were tested for differences among *tissue* types (*P. oceanica* leaves, *P. oceanica* meristem, *P. oceanica* epiphytes, *C. nucula*), *association* types (associated vs non-associated) and *season* (spring vs autumn) using PERMANOVA (n=8). Pairwise comparisons were performed using Tukey's honest significant difference (HSD) test. All statistical analyses were performed with R version 4.2.3 (R Core team, 2023) using the packages *car* and *emmeans*.

HOLOBIONT N DEMAND CALCULATIONS

To calculate how much DIN *C. nucula* can provide via nitrification and ammonification for the N demand of the *P. oceanica* holobiont (plant + epiphytes), we integrated PNR in light and dark incubations assuming a daily 12:12 h light/dark cycle. We further used the daily O_2 budget (using a photosynthetic quotient of 1) and C:N ratios of seagrass leaf and epiphyte tissue to calculate the potential percentage of daily primary production of the seagrass holobiont that can be supported by sponge-mediated PNR.

PROKARYOTIC DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

DNA from sponge and seawater samples was extracted using the Qiagen DNeasy Powersoil Kit (Qiagen) following a modified version of the method described by Taylor et al. (2004). Sponge tissue was grounded, resuspended in sterile distilled water and left for 1 hour. The tissue was transferred into a fast-prep tube (or tube containing 0,5 g of silica beads) and 1 ml of extraction buffer, 0.015 g of PVPP, 300 microliters of chloroform-Isoamylic (24:1) was added. The fast-prep tube was centrifuged at 15.000 g for 30 min, the supernatant was collected and precipitated over night at room temperature with 3M sodium acetate (0.1 x sample volume) and isopropanol (0.7 x sample volume). Then the samples were centrifuged at 14.000 rpm for 30 min, and the pellets were washed twice with 70% ethanol, dried at 37°C, and re-suspended in 50 µl Tris HCL (pH 8; 10 mM). The water filters were cut in little pieces and transferred sterile into a 50 ml Falcon tube. Then 2.25 ml of extraction buffer and 100 microliter of Proteinase K (Stock 100 µg/µl) was added, and the samples were incubated at 37°C on a shaker for 30 minutes and then at 55°C for 30 min in a water bath. 0.25 ml of SDS 20% were added to each sample and they were incubated for 5 minutes in dry ice or -80° and 3x5 minutes in a water bath at 65°C. The samples were centrifuged for 10 minutes at 7000 rpm, and the supernatant was transferred into a new sterile 50 ml Falcon tube. 900 µl extraction buffer and 100 µl of SDS 20% were added to the pellet in the old Falcon tube, vortexted, incubated for 5 minutes at 65°C, centrifuge for 10 minutes at 7000 rpm, and the supernatant was transferred to the supernatant previously taken. Chloroform:Isoamyl alcohol (24:1; 1 x sample volume) was added, centrifuged for 10 minutes at 7000 rpm, and the supernatant was transferred into a new sterile 15 ml Falcon tube. Isopropanol (0.6 x sample volume) was added and precipitate overnight. Then each sample was splitted into 3 x 2 mL Eppendorf tubes, centrifuged for 30 minutes at 14000 rpm, the supernatant was discarded, the pellet was dried at room temperature for 1 hour and then resuspended in 50 µl of sterile water.

The extracted DNA samples were quantified using a microvolume spectrophotometer (Thermo Scientific NanoDrop 2000c) and stored at -20 °C until processing. PacBio Sequel sequencing of the full 16S rRNA gene was

performed using the 27F (=AGRGTTYGATYMTGGCTCAG) and 1492R (=RGYTACCTTGTTACGACTT) bacteria-specific primers. Additionally, PacBio Sequel sequencing was performed with Arch21Ftrim (=TCCGGTTGATCCYGCCGG) and A1401R (=CRGTGWGTRCAAGGRGCA) as archaea-specific primers. The primers were removed from the raw sequence data and the fastq files were processed using the R package DADA2 v.1.28.0 (Callahan et al., 2016). Quality filtering and denoising of the trimmed fastq files was performed using the following parameters: "minQ=3, minLen=1000, maxLen=1600, maxN=0, rm.phix=FALSE, maxEE=2). Paired-end reads were then merged into amplicon sequence variants (ASVs); chimeric sequences were identified and removed. Prokaryotic taxonomy assignment was performed using the SILVA v 138.1 database.

Bioinformatics and data analysis of the sequencing data

The ASV matrix was analyzed using the R package phyloseq v.1.44.0 (McMurdie & Holmes, 2013). Chloroplast and mitochondrial sequences were removed, the data was transformed to relative abundances and samples were pooled per treatment (sponge alone, sponge from association, water column) to calculate the average relative abundances. We tested the effects of sample type (sponge vs water column) and sponge type (associated vs non-associated) on the microbial community associated with *C. nucula* at genus level in a differential abundance analysis using the R package DESeq2 v.1.40.2 (Love et al., 2014). The dataset was then filtered for nitrifying taxa, and we tested the effects of sample and sponge type using a permutation-based multivariate analysis of variance derived from a Euclidean distance matrix using the vegan package v. 2.6.4 (Oksanen et al., 2020).

Results

Potential nitrification rates (PNR)

We explored the nitrification potential of the seagrass and sponge microbiomes in incubation experiments with amended $^{15}N\text{-}NH_4^+$. We found significant (>2.5 x SD of T_0) potential nitrification rates (PNR) in incubations where the sponge was present but not when only seagrass was present (Fig. 5.1, Table S5.1). PNR were highest in the association, followed by the sponge, although differences between these treatments were only significant in autumn (Fig. 5.1, Table S5.2). In spring, PNR reached 5.31 ± 0.75 nmol g DW^{-1} h^{-1} (mean ± SE) in the association in the light (Fig. 5.1a) and 21.51 ± 6.76 nmol g DW^{-1} h^{-1} in the dark (Fig. 5.1b). In autumn, PNR in the association reached 106.15 ± 16.21 nmol g DW^{-1} h^{-1} in the light (Fig. 5.1c), and 267.25 ± 33.01 nmol g DW^{-1} h^{-1} in dark incubations treatment (Fig. 5.1d). The sponge showed intermediate PNR rates in spring (3.74 ± 1.93 nmol g DW^{-1} h^{-1} in the light and 13.40 ± 3.53 nmol g DW^{-1} h^{-1} in the dark, Fig. 5.1a, b) and in autumn (26.13 ± 6.85 nmol g DW^{-1} h^{-1} in the light and 45.39 ± 14.86 nmol g DW^{-1} h^{-1} in the dark, Fig. 5.1c, d). The seagrass showed PNR close to zero in all incubations (Fig. 5.1). PNR of the sponge and the association was higher in dark incubations than in the light with rates 259 % (sponge) and 305 % (association) higher in spring and 74 % (sponge) and 152 % (association) higher in autumn (Fig. 5.1, Table S5.2). A seasonal effect was particularly evident in the association, where PNR in autumn was one magnitude higher than in spring (Fig. 5.1, Table S5.2).

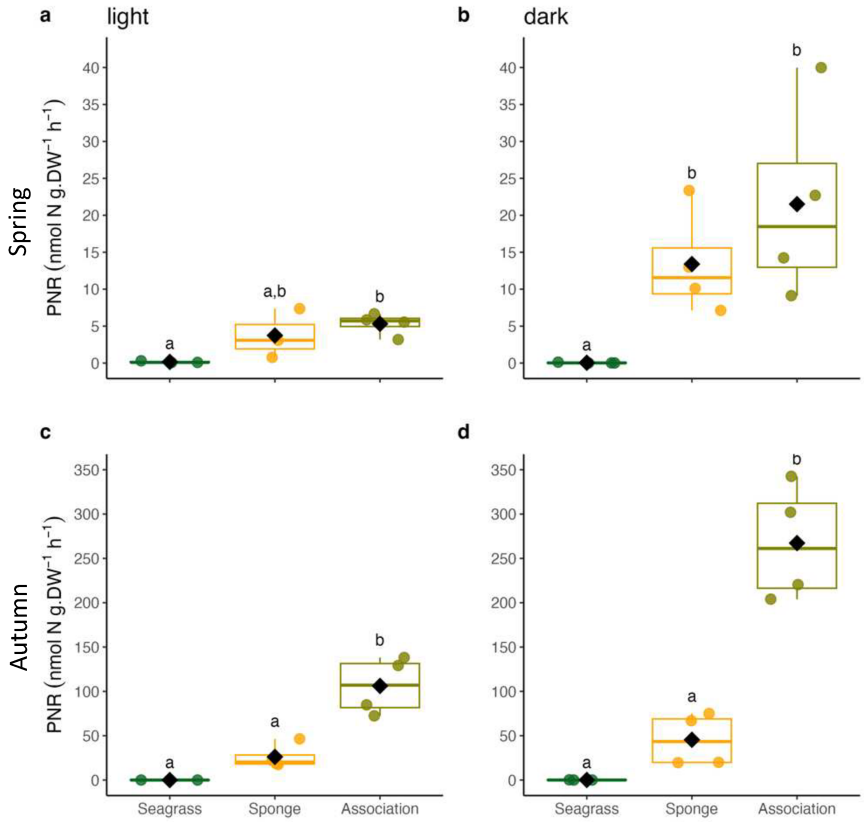

Fig. 5.1. PNR of the seagrass, sponge and the association during light (a, c) and dark (b, d) incubations in spring and autumn. The center line denotes the median value (50th percentile), and the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles. Black squares indicate mean values; letters indicate significant differences between treatments, n=4.

Stable Isotope Analysis

The stable isotope analysis of natural abundance samples in spring, revealed an increase in $\delta^{15}N$ in old *P. oceanica* leaves and epiphytes living associated with the sponge, from 3.8 ± 0.4 ‰ to 5.6 ± 0.2 ‰ and from 5.9 ± 0.3 ‰ to 7.3 ± 0.5 ‰, respectively (Fig. S5.1a, Tables S5.4, S5.5). Conversely, the association type had no effect in autumn, but $\delta^{15}N$ values were generally lower than in spring (Fig. S5.1b, Tables S5.4, S5.6). $\delta^{13}C$ of the seagrass leaves ranged from -14.3 ± 0.2 ‰ in spring and -13.7 ± 0.2‰ in autumn, with no statistical differences between associated and non-associated state (Fig. S5.1c, d, Tables S5.4, S5.7, S5.8). $\delta^{13}C$ values of the sponge *C. nucula* were higher in autumn than in spring (-19.1 ± 0.1 ‰ vs 18.4 ± 0.1 ‰, Fig. S5.1c, d) but showed no differences between associated and non-associated state (Tables S5.4, S5.7, S5.8). Conversely, $\delta^{15}N$ values *C. nucula* were lower in autumn than in spring (6.6 ± 0.2 ‰ vs 5.1 ± 0.1 ‰, Fig. S5.1a, b), but also showing no effects of the association type (Tables S5.4, S5.5, S5.6). Plant epiphytes in spring had $\delta^{13}C$ values similar to those of the sponge (-18.9 ± 0.4 ‰, Fig. S5.1c, d) and in autumn similar to seagrass leaves (-14.3 ± 0.5 ‰, Fig. S5.1c, d, Tables S5.4, S5.7, S5.8). Epiphytes showed an increase in their $\delta^{15}N$ of 1.4 ‰ when the plant was associated with the sponge in spring (from 5.9 ± 0.3 ‰ to 7.3 ± 0.5 ‰, Fig. S5.1a, Table S5.4, S5.5), but not in autumn (Fig. S5.1b, Table S5.4, S5.6).

INORGANIC NUTRIENT FLUXES

Daily net NH_4^+ fluxes in spring showed a trend towards release by the sponge and uptake by the seagrass (14.71 ± 8.38 and -11.75 ± 4.88 µmol g DW^{-1} d^{-1}, respectively, mean ± SE), while the association showed net fluxes close to zero (Fig. 5.2a, Tables S5.9, S5.10). Conversely, in autumn, all community types showed similar uptake rates (-19.68 ± 2.88 µmol g DW^{-1} d^{-1}, Fig. 5.2b, Tables S5.9, S5.10). We observed daily net NO_3^- production by sponges in spring and autumn (17.78 ± 3.49 and 23.59 ± 2.89 µmol g DW^{-1} d^{-1}, respectively, Fig. 5.2c, d, Tables S5.9, S5.10). In spring, the seagrass and the association showed net fluxes close to zero (Fig. 5.2c, d). Conversely, in autumn, the seagrass showed NO_3^- uptake, while we observed intermediate net production in the association (-11.23 ± 3.03 and 9.73 ± 3.03 µmol g DW^{-1} d^{-1}, respectively, Fig. 5.2d, Tables S5.9, S5.10). We observed daily net NO_2^- release in all incubations in spring, highest in the sponge (0.60 ± 0.02 µmol g DW^{-1} d^{-1}), followed by the seagrass (0.28 ± 0.11 µmol g DW^{-1} d^{-1}) and the association (0.19 ± 0.12 µmol g DW^{-1} d^{-1}, Fig. 5.2e, Tables S5.9, S5.10). In autumn, the seagrass and the association showed net NO_2^- fluxes close to zero and the sponge a trend towards NO_2^- release (0.15 ± 0.06 µmol g DW^{-1} d^{-1}), but differences were not significant (Fig. 5.2f, Tables S5.9, S5.10).

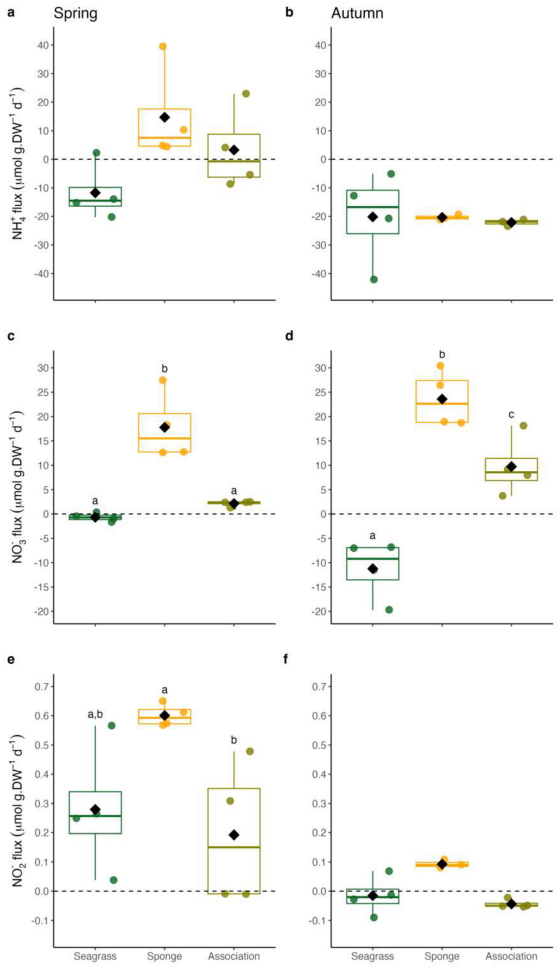

Fig. 5.2. Daily fluxes of NH_4^+ (a, b), NO_3^- (c, d), and NO_2^- (e, f) in incubations with the seagrass, sponge and the association in spring and autumn. Positive values indicate production, negative values indicate uptake. The center line denotes the median value (50th percentile), and the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles. The horizontal dashed line marks the zero-flux threshold. Black squares indicate mean values; letters indicate significant differences between treatments, n=4.

Organic Nutrient Fluxes

The seagrass was a constant source of daily net DOC flux in spring and in autumn (140.05 ± 32.35 and 185.10 ± 36.97 µmol g DW^{-1} d^{-1}, respectively; mean ± SE, Fig. 5.3a, b, Tables S5.11, S5.12). We observed net DOC fluxes close to zero of the sponge in spring and in autumn, while the association showed net fluxes close to zero in spring and uptake in autumn (-40.37 ± 15.35 µmol g DW^{-1} d^{-1}, Fig. 5.3a, b). Conversely, the sponge was a constant source of daily net DON flux in spring and in autumn (38.90 ± 5.36 and 54.42 ± 2.84 µmol g DW^{-1} d^{-1}, respectively, Fig. 5.3c, d, Tables S5.11, S5.12). The seagrass and the association showed similar net DON production in spring (5.84 ± 3.80 and 9.07 ± 5.05 µmol g DW^{-1} d^{-1}, respectively, Fig. 5.3c). In autumn, the seagrass showed DON production (16.16 ± 5.72 µmol g DW^{-1} d^{-1}, Fig. 5.3d) and the association net fluxes close to zero (Fig. 5.3d). Seasons had no significant effect on daily net DOC or DON fluxes (Tables S5.11, S5.12).

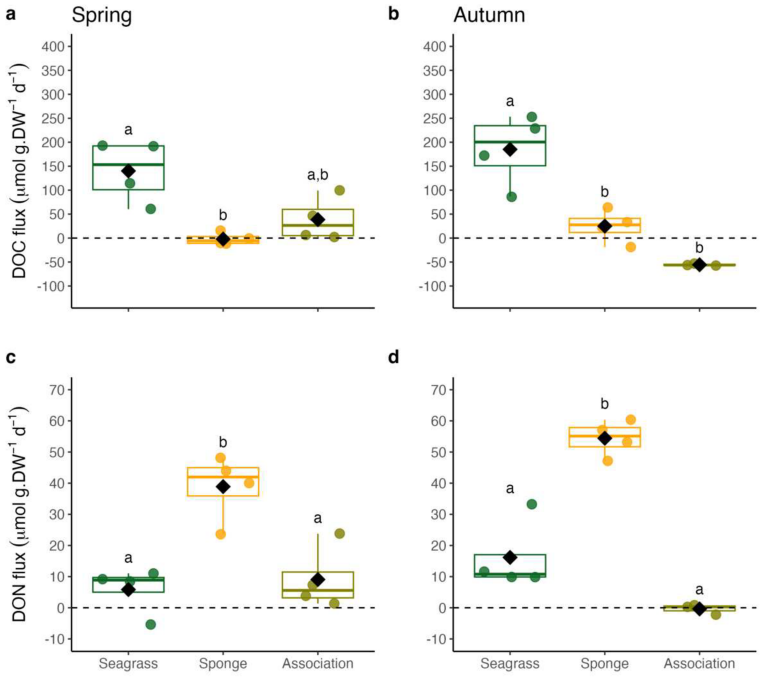

Fig. 5.3. Daily fluxes of DOC (a, b) and DON (c, d) in incubations with the seagrass, sponge and the association in spring and autumn. Positive values indicate production, negative values indicate uptake. The center line denotes the median value (50th percentile), the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles. The horizontal dashed line marks the zero-flux threshold. Black squares indicate mean values; letters indicate significant differences between treatments, n=4.

Primary Production and Respiration Rates

Net community production (NCP) in spring was similar in the seagrass and the association (16.20 ± 2.48 and 19.24 ± 4.38 µmol g DW^{-1} h^{-1}, respectively; mean ± SE, Fig. S5.2a, Tables S5.13, S5.14), followed by the sponge (1.79 ± 0.41 µmol g DW^{-1} h^{-1}, Fig. S5.2a, Tables S5.13, S5.14). Community respiration (CR) in spring was highest (more negative) in the association (-4.63 ± 0.39 µmol g DW^{-1} h^{-1}), followed by the sponge (3.92 ± 0.78 µmol g DW^{-1} h^{-1}), and the seagrass (1.79 ± 0.40 µmol g DW^{-1} h^{-1}, Fig. S5.2b, Tables S5.13, S5.14). We observed a seasonal effect, where NCP in autumn was lower than in spring (Fig. S5.2c, Tables S5.13, S5.14), reaching 14.41 ± 3.35

μmol g DW^{-1} h^{-1} in the seagrass, 10.03 ± 1.35 μmol g DW^{-1} h^{-1} in the association and a net flux close to zero in the sponge. CR in autumn was higher (more negative) in autumn than in spring (Fig. S5.2d, Tables S5.13, S5.14), reaching -15.26 ± 2.22 μmol g DW^{-1} h^{-1} in the sponge, -7.77 ± 0.17 μmol g DW^{-1} h^{-1} in the association, and -5.32 ± 0.85 μmol g DW^{-1} h^{-1} in the seagrass.

Microbial Community Structure

The 16s rRNA gene amplicon sequencing of the sponge-associated microbiome revealed a diverse microbial community (Fig. 5.4) differing between the water column and sponge community, but not between the sponges growing alone or in association with the seagrass (Fig. S5.3).

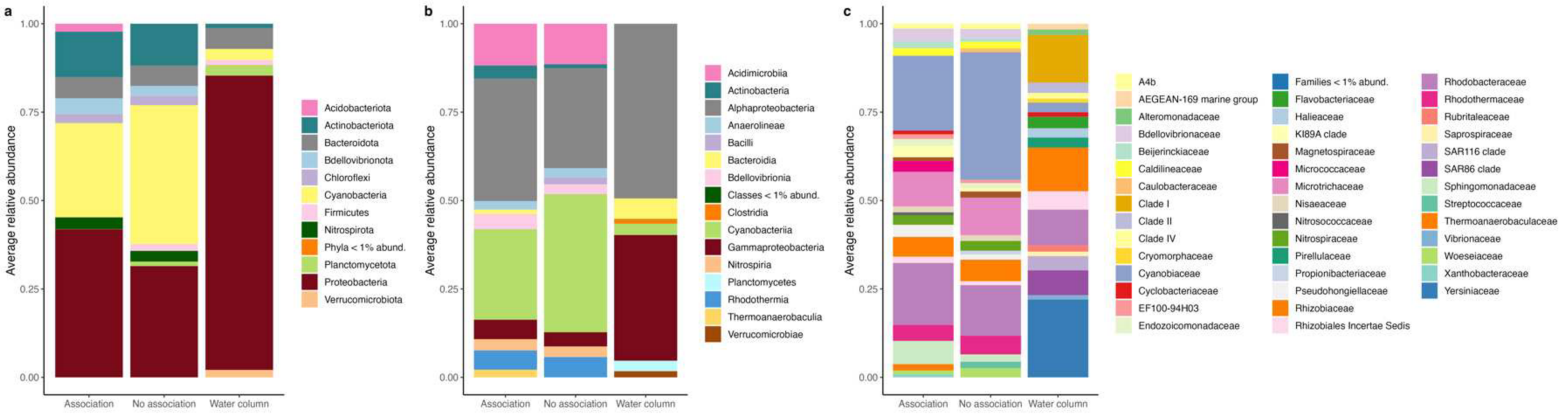

Fig. 5.4. Average relative abundances of bacterial phyla (a), classes (b), and families (c) in *C. nucula* growing in association (n = 8), or growing alone (n = 4), and water column samples (n = 4).

The sponges were dominated by the phyla *Proteobacteria* (40.02 ± 4.26 %) and *Cyanobacteria* (32.17 ± 5.00 %) (Fig. 5.4). Among the predominant classes were *Alphaproteobacteria* (34.99 ± 3.98 %) and *Cyanobacteriia* (32.17 ± 5.00 %). Accordingly, the family of *Cyanobiaceae* accounted for 32.15 ± 4.99 % in sponge communities, followed by *Rhodobacteraceae* (20.52 ± 3.62 %) and *Microtrichaceae* (12.59 ± 1.28 %). The water column was dominated by the phylum of *Proteobacteria* (83.56 ± 0.96 %) with the classes *Alphaproteobacteria* (48.61 ± 4.83 %) and *Gammaproteobacteria* (34.95 ± 4.80 %). The most prevalent families were *Yersiniaceae* (20.75 ± 6.14 %) and Clade I (12.73 ± 2.99 %).

Taxonomic groups in the sponge microbiome with the largest effect detected in the differential abundance analysis (Fig. S5.4) were the *Synechococcus* spongiarum group within the family of *Cyanobiaceae* and *Silicimonas* within the family of *Rhodobacteraceae*, but also *Nitrospira* of the *Nitrospiraceae* family. Groups that had the highest effect on the differential abundance of the water column community were several species of the genus *Serratia* within the family of *Yersiniaceae*.

We found a significantly higher relative abundance of nitrifying families in the sponge communities compared to the water column (Table S5.15). No significant differences were found between the microbial communities of the sponge growing alone or in association. *Nitrospiraceae* were only found in sponge communities (Fig. 5.5)

and accounted for 2.62 ± 0.76 % (mean ± SE). *Nitrososococcaceae* accounted for 0.42 ± 0.12 % of the sponge microbial communities and 0.09 ± 0.07 % in the water column (Fig. 5.5). Within the family of *Nitrososococcaceae* we found the genera Cm1-21 and AqS1, both belonging to AOB. Additionally, in a separate sequencing approach, we found the AOA taxon *Candidatus Nitrosopumilus* present in the sponge microbiome with no differences if the sponge was growing alone or in association with the seagrass.

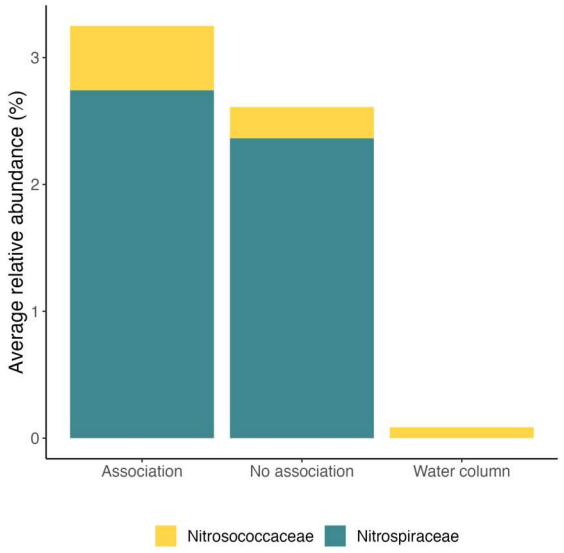

Fig. 5.5. Average relative abundances of nitrifying families in the microbial community of *C. nucula* growing in association with *P. oceanica* (n=8), growing alone (n=4), and of the water column (n=4) in autumn.

DISCUSSION

This study of potential nitrification rates (PNR) and inorganic nutrient fluxes is the first to show that DIN provided by nitrification in the sponge *Chondrilla nucula* can be taken up by the seagrass *Posidonia oceanica*, supporting the N demand of the seagrass holobiont. The 16s rRNA gene amplicon sequencing of the sponge-associated microbiome revealed a diverse microbial community, including microorganisms involved in nitrification. Seagrasses in the Mediterranean Sea are threatened by climate change and anthropogenic impact (Boudouresque et al., 2009). This specific seagrass-sponge association, however, can thrive in an environment with high human pressure. Thus, investigating the biogeochemical and molecular mechanisms involved in its regulation can help to understand if this association can be beneficial for both partners under future environmental conditions.

NITRIFICATION CONTRIBUTES TO N-CYCLING IN THE SEAGRASS-HOLOBIONT

PNR of the *P. oceanica* - *C. nucula* association in dark incubations (21.51 ± 6.76 nmol g DW^{-1} h^{-1} in spring and 267.25 ± 33.01 nmol g DW^{-1} h^{-1} in autumn, Fig. 5.1) are within the range of those reported for other Mediterranean sponges (344 nmol g DW^{-1} h^{-1} in unstimulated incubations, Bayer et al., 2008; 180 - 780 nmol g DW^{-1} h^{-1}, Jiménez & Ribes, 2007), and tropical sponges (30 - 2650 nmol g DW^{-1} h^{-1}, Diaz & Ward, 1997). Reported PNR in surface sediments of seagrass meadows (0.15 - 1.0 µmol g^{-1} d^{-1}, corresponding to 6.25 - 41.67 nmol g^{-1} h^{-1}, Lin et al., 2021) or sandy estuaries (up to 40 nmol g DW^{-1} h-1 (Magalhães et al., 2005) are slightly lower. Since

sponges are frequently found in many benthic environments such as seagrass meadows, the DIN excretion via nitrification can contribute significantly to nitrogen cycling (Diaz & Ward, 1997; Jiménez & Ribes, 2007). We found higher PNR in dark incubations, which is in line with the widely accepted explanation that both parts of the nitrification process (ammonium and nitrite oxidation) are light-inhibited (Guerrero & Jones, 1996; Horrigan et al., 1981).

We observed higher $\delta^{15}N$ values in natural abundance samples of seagrass leaves and epiphytes when the plant was associated with the sponge in spring and of seagrass meristem tissue in autumn (Fig. S5.1). Nitrification is causing negative fractionation of NO_3^- (depleted in $\delta^{15}N$) and positive fractionation of NH_4^+ (enriched in $\delta^{15}N$; Casciotti, 2016; Mariotti et al., 1981). NO_3^- excreted from sponges and produced by nitrification, can therefore have lower $\delta^{15}N$ values than NO_3^- from the water column (Southwell, Popp, et al., 2008). At the same time, fractionation of NH_4^+ during uptake could increase the $\delta^{15}N$ in the residual NH_4^+ pool in the sponge tissue (Hoch et al., 1994). Our higher $\delta^{15}N$ values in plant and epiphyte tissue indicate that $\delta^{15}N$ -enriched NH_4^+ excreted by the sponge was preferential taken up in the association. *P. oceanica* can assimilate N as NH_4^+ or NO_3^- but usually shows a higher affinity for NH_4^+ (Touchette & Burkholder, 2000). Berlinghof et al. (2024) showed that *P. oceanica* prefers NH_4^+ uptake, while its epiphytes may preferentially use NO_3^- as a strategy to avoid competition for N with the plant. The plant could therefore also compete for NH_4^+ with the sponge nitrifiers. We observed NH_4^+ production by the sponge only in spring, indicating that DIN excreted via nitrification might become more important in autumn. We measured high NO_3^- production by the sponge (17.78 ± 3.49 μmol g DW^{-1} d^{-1} in spring and 23.59 ± 2.89 μmol g DW^{-1} d^{-1} in autumn), while the seagrass showed net fluxes close to zero or NO_3^- uptake (Fig. 5.2). Net NO_3^- fluxes in incubations with the seagrass-sponge association were also close to zero or showed NO_3^- production, but lower compared to incubations with the sponge alone. This indicates that sponge-mediated nitrification produces NO_3^- that is taken up by the seagrass holobiont. Whether NO_3^- produced by sponge-associated nitrification benefits the seagrass or rather its associated epiphytes needs to be further investigated.

SEASONAL DIFFERENCES IN PNR AND NUTRIENT FLUXES

We observed PNR to be one order of magnitude higher in autumn than in spring (Fig. 5.1). Potential nitrification rates tend to be higher during the warmer seasons in salt marshes and estuary sediments. However, strong, site-specific variations are often reported (Caffrey et al., 2003; Dollhopf et al., 2005). While environmental conditions, such as temperature, light or water column O_2 concentrations were similar across seasons (Table 5.1), ambient NH_4^+ concentrations were higher in spring (12.74 ± 3.09 μM in spring vs 2.76 ± 1.75 μM in autumn) and would therefore, in contrast to our findings, indicate higher PNR in spring.

P. oceanica can exhibit strong seasonal dynamics, depending on light and temperature, but also on local factors such as nutrient availability (Alcoverro et al., 1995). Metabolism studies show that the main growth phase occurs in spring while in autumn the seagrass is in a senescent phase (Berlinghof et al., 2022; Koopmans et al., 2020; Olivé et al., 2016). Accordingly, uptake rates of NH_4^+ and NO_3^- by *P. oceanica* tend to be highest in spring and early summer (Lepoint et al., 2002; Nayar et al., 2010). At our study site, we observed seasonal morphological differences of the seagrass, indicating higher growth and biomass in spring and less in autumn, when we noticed

high leaf loss and a shorter average leaf length. With *P. oceanica* being in its main growth phase in spring and early summer at our study location, there could have been increased competition for NH_4^+ by the plant, resulting in lower NH_4^+ availability for the nitrifying microbial community of the sponge and thus lower PNR.

POTENTIAL EFFECTS AT THE HOLOBIONT AND ECOSYSTEM LEVEL

We calculated the N demand of *P. oceanica* with the daily C budget based on the O_2 fluxes and the C:N ratios of the holobiont (seagrass + epiphytes, Table 5.2). We further calculated the percentage of daily primary production of the seagrass holobiont that can be supported by sponge-mediated PNR. Based on these assumptions, sponge-mediated PNR can support 8.35 % of the holobiont primary production in spring and even 47.38 % in autumn. Since *P. oceanica* prefers the uptake of NH_4^+ over NO_3^- (Touchette & Burkholder, 2000), while epiphytes potentially prefer NO_3^- (Berlinghof et al., 2024), it appears as if mostly the seagrass epiphytes can benefit from sponge-mediated PNR.

We observed DON production by *C. nucula* in both seasons (Fig. 5.3c, d). Ammonification of DON by seagrass-associated microbes produces NH_4^+ that can enhance the access of the seagrass to inorganic N as shown by Pfister et al., 2023. Thus, DON released by the sponge could further support the N demand of the seagrass holobiont. The sponge on the other side, can take up DOC release by the seagrass (Fig. 5.3a, b). However, the extent of these beneficial processes varies a lot throughout seasons and depends on environmental conditions, such as light or nutrient availability. Further investigations of the benefits for the sponge in this association are therefore needed.

Table 5.2. Nitrogen requirements of the *P. oceanica* holobiont (plant + epiphytes) in spring and autumn.

Season	Daily C budget (µmol g DW^{-1} d^{-1})	CN ratio holobiont	N demand (µmol g DW^{-1} d^{-1})	Daily PNR budget (µmol g DW^{-1} d^{-1})	% support
Spring	57.37	23.3	2.462	0.21	8.35
Autumn	50.36	27.8	1.811	0.86	47.38

MICROBIAL COMMUNITY

We found a diverse microbial community associated with *C. nucula* (Fig. 5.4) that was not affected by the association with *P. oceanica*. *Chondrilla nucula* harbors a distinct and stable bacterial community little affected by ambient seawater in the Mediterranean Sea (Thiel et al., 2007) or the Caribbean (Hill et al., 2006). Among the most prevalent and most distinct groups we found, was the *Synechococcus* spongiarum group (*Cyanobacteria*). These cyanobacterial symbionts are commonly found in marine sponges (Konstantinou et al., 2018; Usher, 2008), and are also reported for *C. nucula* (Usher et al., 2004). Another frequent group in the sponge communities were members of the family *Rhodobacteraceae* (*Alphaproteobacteria*); a family known to have several symbionts capable of fixing C via anoxygenic photosynthesis (Brinkmann et al., 2018) and also previously reported for *C. nucula* (Thiel et al., 2007). Families of the order Sphingomondales (*Alphaproteobacteria*) are known to be associated with marine sponges and have been linked to vitamin B12 synthesis (Thomas et al., 2010). Additionally, it has been demonstrated that the presence of *Sphingomonadaceae* can enhance degradation rates of artificial chemicals (Dai et al., 2022; Oh & Choi, 2019).

As *C. nucula* has been shown to have a high capacity for bioaccumulation of pollutants (Ferrante et al., 2018), this could explain the high differential abundance of *Sphingomonas* in the microbial community of the sponge. Another dominant group was the family *Microtrichaceae*, which is potentially involved in nitrate supply as part of a nitrification-anammox system (Szitenberg et al., 2022).

Among the bacterial groups involved in nitrification (Fig. 5.5), we found the nitrite-oxidizing bacteria (NOB) *Nitrospira* within the family *Nitrospiraceae* (*Nitrospiria*), which is also reported for other species such as the cold-water sponge *Geodia baretti* (Hoffmann et al., 2009), but to our knowledge so far not for *C. nucula*. We also recovered the genera Cm1-21 and AqS1, both ammonia-oxidizing bacteria (AOB) within the family of *Nitrosococcaceae* (*Gammaproteobacteria*) (Hollingsworth et al., 2021; Semedo et al., 2021). We found the ammonia-oxidizing archaea (AOA) *Candidatus Nitrosopumilus*, and studies showed that they are stable associates of many sponge species (Bayer et al., 2008; Hoffmann et al., 2009; Holmes & Blanch, 2007).

Taken together, at the ecosystem level, we could show that the symbiosis between *P. oceanica*, *C. nucula*, and their microbiomes contributes significantly to nitrogen cycling. Since *C. nucula* shows a strong ability to compete for space (Bond & Harris, 1988; Milanese et al., 2003), it can quickly colonize new substrates. With increasing human pressure, that will open more space for the sponge to occupy (for example by boat anchoring in seagrass meadows, as we have seen at our study location), it is therefore essential to further investigate the dynamics of seagrass-sponge-associations and their implications at the ecosystem level as well as its potential as a nature-based solution for seagrass protection measures.

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AUTHOR CONTRIBUTIONS

J.B., L.M.M., C.W., and U.C. designed the study. J.B., L.M.M., and U.C. performed the experiments. T.B.M. performed the mass spectrometry analyses and J.B. and T.B.M. analyzed the results. J.B. and L.G. performed the molecular analyses and J.B. and H.G-V. analyzed the results. J.B. and U.C. wrote the manuscript with contributions from all co-authors.

References

Agawin, N. S. R., Ferriol, P., Cryer, C., Alcon, E., Busquets, A., Sintes, E., Vidal, C., & Moyà, G. (2016). Significant nitrogen fixation activity associated with the phyllosphere of Mediterranean seagrass Posidonia oceanica: First report. *Marine Ecology Progress Series*, *551*, 53–62. https://doi.org/10.3354/MEPS11755

Alcoverro, T., Duarte, C. M., & Romero, J. (1995). Annual growth dynamics of Posidonia oceanica: Contribution of large-scale versus local factors to seasonality. *Marine Ecology Progress Series*, *120*(1/3), 203–210.

Anderson, M. J. (2017). Permutational Multivariate Analysis of Variance (PERMANOVA). In *Wiley StatsRef: Statistics Reference Online* (pp. 1–15). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118445112.stat07841

Archer, S. K., English, P. A., Campanino, F. M., & Layman, C. A. (2021). Sponges facilitate primary producers in a Bahamas seagrass system. *Marine Biology*, *168*(11), 162. https://doi.org/10.1007/s00227-021-03977-x

Archer, S. K., Stoner, E. W., & Layman, C. A. (2015). A complex interaction between a sponge (Halichondria melanadocia) and a seagrass (Thalassia testudinum) in a subtropical coastal ecosystem. *Journal of Experimental Marine Biology and Ecology*, *465*, 33–40. https://doi.org/10.1016/J.JEMBE.2015.01.003

Ávila, E., Ávila-García, A. K., & Cruz-Barraza, J. A. (2015). Temporal and small-scale spatial variations in abundance and biomass of seagrass-dwelling sponges in a tropical estuarine system. *Marine Ecology*, *36*(3), 623–636. https://doi.org/10.1111/maec.12171

Barrón, C., & Duarte, C. M. (2009). Dissolved organic matter release in a Posidonia oceanica meadow. *Marine Ecology Progress Series*, *374*, 75–84. https://doi.org/10.3354/MEPS07715

Bayer, K., Schmitt, S., & Hentschel, U. (2007). Microbial nitrification in Mediterranean sponges: Possible involvement of ammonia-oxidizing Betaproteobacteria. *Porifera Research: Biodiversity, Innovation and Sustainability*, 165–171.

Bayer, K., Schmitt, S., & Hentschel, U. (2008). Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge Aplysina aerophoba. *Environmental Microbiology*, *10*(11), 2942–2955. https://doi.org/10.1111/J.1462-2920.2008.01582.X

Beman, J. M., Chow, C. E., King, A. L., Feng, Y., Fuhrman, J. A., Andersson, A., Bates, N. R., Popp, B. N., & Hutchins, D. A. (2011). Global declines in oceanic nitrification rates as a consequence of ocean acidification. *PNAS*, *108*(1), 208–213. https://doi.org/10.1073/PNAS.1011053108

Beman, J. M., Leilei Shih, J., & Popp, B. N. (2013). Nitrite oxidation in the upper water column and oxygen minimum zone of the eastern tropical North Pacific Ocean. *The ISME Journal*, *7*(11), Article 11. https://doi.org/10.1038/ismej.2013.96

Berlinghof, J., Montilla, L. M., Peiffer, F., Quero, G. M., Marzocchi, U., Meador, T. B., Margiotta, F., Abagnale, M., Wild, C., & Cardini, U. (2024). Accelerated nitrogen cycling on Mediterranean seagrass leaves at volcanic CO2 vents. *Communications Biology*, *7*(341), 1–11. https://doi.org/10.1038/s42003-024-06011-0

Berlinghof, J., Peiffer, F., Marzocchi, U., Munari, M., Quero, G. M., Dennis, L., Wild, C., & Cardini, U. (2022). The role of epiphytes in seagrass productivity under ocean acidification. *Scientific Reports*, *12*(6249). https://doi.org/10.1038/s41598-022-10154-7

Bond, C., & Harris, A. K. (1988). Locomotion of sponges and its physical mechanism. *Journal of Experimental Zoology*, *246*(3), 271–284. https://doi.org/10.1002/jez.1402460307

Boudouresque, C. F., Bernard, G., Pergent, G., Shili, A., & Verlaque, M. (2009). Regression of Mediterranean seagrasses caused by natural processes and anthropogenic disturbances and stress: A critical review. *Botm*, *52*(5), 395–418. https://doi.org/10.1515/BOT.2009.057

Brinkmann, H., Göker, M., Koblížek, M., Wagner-Döbler, I., & Petersen, J. (2018). Horizontal operon transfer, plasmids, and the evolution of photosynthesis in Rhodobacteraceae. *The ISME Journal*, *12*(8), Article 8. https://doi.org/10.1038/s41396-018-0150-9

Caffrey, J. M., Harrington, N., Solem, I., & Ward, B. B. (2003). Biogeochemical processes in a small California estuary. 2. Nitrification activity, community structure and role in nitrogen budgets. *Marine Ecology Progress Series*, *248*, 27–40. https://doi.org/10.3354/meps248027

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cardini, U., Marín-Guirao, L., Montilla, L. M., Marzocchi, U., Chiavarini, S., Rimauro, J., Quero, G. M., Petersen, J. M., & Procaccini, G. (2022). Nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning in contaminated sediments. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.918675

Casciotti, K. L. (2016). Nitrogen and Oxygen Isotopic Studies of the Marine Nitrogen Cycle. *Annual Review of Marine Science*, *8*(Volume 8, 2016), 379–407. https://doi.org/10.1146/annurev-marine-010213-135052

Corredor, J. E., Wilkinson, C. R., Vicente, V. P., Morell, J. M., & Otero, E. (1988). Nitrate release by Caribbean reef sponges. *Limnology and Oceanography*, *33*(1), 114–120. https://doi.org/10.4319/lo.1988.33.1.0114

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R. V., Paruelo, J., Raskin, R. G., Sutton, P., & van den Belt, M. (1997). The value of the world's ecosystem services and natural capital. *Nature*, *387*, 253–260.

Dai, H., Gao, J., Li, D., Wang, Z., Cui, Y., & Zhao, Y. (2022). Family Sphingomonadaceae as the key executor of triclosan degradation in both nitrification and denitrification systems. *Chemical Engineering Journal*, *442*, 136202. https://doi.org/10.1016/j.cej.2022.136202

De Goeij, J. M., Van Oevelen, D., Vermeij, M. J. A., Osinga, R., Middelburg, J. J., De Goeij, A. F. P. M., & Admiraal, W. (2013). Surviving in a Marine Desert: The Sponge Loop Retains Resources Within Coral Reefs. *Science*, *342*(6154), 108–110. https://doi.org/10.1126/science.1241981

Diaz, M. C., & Ward, B. B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series*, *156*, 97–107.

Dollhopf, S. L., Hyun, J.-H., Smith, A. C., Adams, H. J., O'Brien, S., & Kostka, J. E. (2005). Quantification of Ammonia-Oxidizing Bacteria and Factors Controlling Nitrification in Salt Marsh Sediments. *Applied and Environmental Microbiology*, *71*(1), 240–246. https://doi.org/10.1128/AEM.71.1.240-246.2005

Ferrante, M., Vassallo, M., Mazzola, A., Brundo, M. V., Pecoraro, R., Grasso, A., & Copat, C. (2018). In vivo exposure of the marine sponge Chondrilla nucula Schmidt, 1862 to cadmium (Cd), copper (Cu) and lead (Pb) and its potential use for bioremediation purposes. *Chemosphere*, *193*, 1049–1057. https://doi.org/10.1016/j.chemosphere.2017.11.144

Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E., & Oakley, B. B. (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences*, *102*(41), 14683–14688. https://doi.org/10.1073/pnas.0506625102

Freitag, T. E., & Prosser, J. I. (2003). Community Structure of Ammonia-Oxidizing Bacteria within Anoxic Marine Sediments. *Applied and Environmental Microbiology*, *69*(3), 1359–1371. https://doi.org/10.1128/AEM.69.3.1359-1371.2003

Guerrero, M., & Jones, R. (1996). Photoinhibition of marine nitrifying bacteria. I. Wavelength-dependent response. *Marine Ecology Progress Series*, *141*, 183–192. https://doi.org/10.3354/meps141183

Hill, M., Hill, A., Lopez, N., & Harriott, O. (2006). Sponge-specific bacterial symbionts in the Caribbean sponge, Chondrilla nucula (Demospongiae, Chondrosida). *Marine Biology*, *148*, 1221–1230. https://doi.org/10.1007/s00227-005-0164-5

Hoch, M. P., Fogel, M. L., & Kirchman, D. L. (1994). Isotope fractionation during ammonium uptake by marine microbial assemblages. *Geomicrobiology Journal*, *12*(2), 113–127. https://doi.org/10.1080/01490459409377977

Hoer, D. R., Tommerdahl, J. P., Lindquist, N. L., & Martens, C. S. (2018). Dissolved inorganic nitrogen fluxes from common Florida Bay (U.S.A.) sponges. *Limnology and Oceanography*, *63*(6), 2563–2578. https://doi.org/10.1002/lno.10960

Hoffmann, F., Radax, R., Woebken, D., Holtappels, M., Lavik, G., Rapp, H. T., Schläppy, M. L., Schleper, C., & Kuypers, M. M. M. (2009). Complex nitrogen cycling in the sponge Geodia barretti. *Environmental Microbiology*, *11*(9), 2228–2243. https://doi.org/10.1111/J.1462-2920.2009.01944.X

Hollingsworth, A. L., Jones, D. O. B., & Young, C. R. (2021). Spatial Variability of Abyssal Nitrifying Microbes in the North-Eastern Clarion-Clipperton Zone. *Frontiers in Marine Science*, *8*. https://www.frontiersin.org/articles/10.3389/fmars.2021.663420

Holmer, M., Andersen, F. Ø., Nielsen, S. L., & Boschker, H. T. S. (2001). The importance of mineralization based on sulfate reduction for nutrient regeneration in tropical seagrass sediments. *Aquatic Botany*, *71*(1), 1–17. https://doi.org/10.1016/S0304-3770(01)00170-X

Holmes, B., & Blanch, H. (2007). Genus-specific associations of marine sponges with group I crenarchaeotes. *Marine Biology*, *150*(5), 759–772. https://doi.org/10.1007/s00227-006-0361-x

Horrigan, S., Carlucci, A., & Williams, P. (1981). Light inhibition of nitrification in sea-surface films. *Journal of Marine Research*, *39*(3). https://elischolar.library.yale.edu/journal_of_marine_research/1559

Jiménez, E., & Ribes, M. (2007). Sponges as a source of dissolved inorganic nitrogen: Nitrification mediated by temperate sponges. *Limnology and Oceanography*, *52*(3), 948–958. https://doi.org/10.4319/lo.2007.52.3.0948

Konstantinou, D., Gerovasileiou, V., Voultsiadou, E., & Gkelis, S. (2018). Sponges-cyanobacteria associations: Global diversity overview and new data from the Eastern Mediterranean. *PLoS ONE*, *13*(3), 1–22. https://doi.org/10.1371/journal.pone.0195001

Koopmans, D., Holtappels, M., Chennu, A., Weber, M., & De Beer, D. (2020). High Net Primary Production of Mediterranean Seagrass (Posidonia oceanica) Meadows Determined With Aquatic Eddy Covariance. *Frontiers in Marine Science*, *7*, 118. https://doi.org/10.3389/fmars.2020.00118

Lepoint, G., Millet, S., Dauby, P., Gobert, S., & Bouquegneau, J. M. (2002). Annual nitrogen budget of the seagrass Posidonia oceanica as determined by in situ uptake experiments. *Marine Ecology Progress Series*, *237*, 87–96. https://doi.org/10.3354/meps237087

Lin, X., Zheng, P., Zou, S., Sun, F., Zhang, & X., & Gong, J. (2021). Seagrass (Zostera marina) promotes nitrification potential and selects specific ammonia oxidizers in coastal sediments. *Journal of Soils and Sediments*, *21*, 3259–3273. https://doi.org/10.1007/s11368-021-02951-w/Published

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Magalhães, C. M., Joye, S. B., Moreira, R. M., Wiebe, W. J., & Bordalo, A. A. (2005). Effect of salinity and inorganic nitrogen concentrations on nitrification and denitrification rates in intertidal sediments and rocky biofilms of the Douro River estuary, Portugal. *Water Research*, *39*(9), 1783–1794. https://doi.org/10.1016/j.watres.2005.03.008

Maldonado, M., Ribes, M., & Van Duyl, F. C. (2012). Nutrient Fluxes Through Sponges. In *Advances in Marine Biology* (Vol. 62, pp. 113–182). Elsevier. https://doi.org/10.1016/B978-0-12-394283-8.00003-5

Malkin, S., & Cardini, U. (2021). Facilitative interactions on the rise: Cable bacteria associate with diverse aquatic plants. *New Phytologist*, *232*(5), 1897–1900. https://doi.org/10.1111/nph.17664

Mariotti, A., Germon, J. C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., & Tardieux, P. (1981). Experimental determination of nitrogen kinetic isotope fractionation: Some principles; illustration for the denitrification and nitrification processes. *Plant and Soil*, *62*(3), 413–430. https://doi.org/10.1007/BF02374138

Mazzella, V., Dell'Anno, A., Etxebarría, N., González-Gaya, B., Nuzzo, G., Fontana, A., & Núñez-Pons, L. (2024). High microbiome and metabolome diversification in coexisting sponges with different bio-ecological traits. *Communications Biology*, *7*(1), 1–18. https://doi.org/10.1038/s42003-024-06109-5

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Milanese, M., Chelossi, E., Manconi, R., Sarà, A., Sidri, M., & Pronzato, R. (2003). The marine sponge Chondrilla nucula Schmidt, 1862 as an elective candidate for bioremediation in integrated aquaculture. *Biomolecular Engineering*, *20*(4), 363–368. https://doi.org/10.1016/S1389-0344(03)00052-2

Mohr, W., Lehnen, N., Ahmerkamp, S., Marchant, H. K., Graf, J. S., Tschitschko, B., Yilmaz, P., Littmann, S., Gruber-Vodicka, H., Leisch, N., Weber, M., Lott, C., Schubert, C. J., Milucka, J., & Kuypers, M. M. M. (2021). Terrestrial-type nitrogen-fixing symbiosis between seagrass and a marine bacterium. *Nature 2021*, 1–5. https://doi.org/10.1038/s41586-021-04063-4

Nayar, S., Collings, G. J., Miller, D. J., Bryars, S., & Cheshire, A. C. (2010). Uptake and resource allocation of ammonium and nitrate in temperate seagrasses Posidonia and Amphibolis. *Marine Pollution Bulletin*, *60*(9), 1502–1511. https://doi.org/10.1016/J.MARPOLBUL.2010.04.018

Nemoy, P., Spanier, E., & Angel, D. L. (2021). Nitrification Activity of the Sponge Chondrosia reniformis Under Elevated Concentrations of Ammonium. *Frontiers in Marine Science*, *7*(607979). https://doi.org/10.3389/fmars.2020.607979

Nielsen, L. B., Finster, K., Welsh, D. T., Donelly, A., Herbert, R. A., De Wit, R., & Lomstein, B. AA. (2001). Sulphate reduction and nitrogen fixation rates associated with roots, rhizomes and sediments from Zostera noltii and Spartina maritima meadows. *Environmental Microbiology*, *3*(1), 63–71. https://doi.org/10.1046/j.1462-2920.2001.00160.x

Nordlund, L. M., Koch, E. W., Barbier, E. B., & Creed, J. C. (2016). Seagrass Ecosystem Services and Their Variability across Genera and Geographical Regions. *PLOS ONE*, *11*(10), e0163091. https://doi.org/10.1371/journal.pone.0163091

Oh, S., & Choi, D. (2019). Microbial Community Enhances Biodegradation of Bisphenol A Through Selection of Sphingomonadaceae. *Microbial Ecology*, *77*(3), 631–639. https://doi.org/10.1007/s00248-018-1263-4

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O' Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. J. (2020). *vegan: Community ecology package*.

Olivé, I., Silva, J., Costa, M. M., & Santos, R. (2016). Estimating Seagrass Community Metabolism Using Benthic Chambers: The Effect of Incubation Time. *Estuaries and Coasts*, *39*, 138–144. https://doi.org/10.1007/s12237-015-9973-z

Park, S.-J., Park, B.-J., & Rhee, S.-K. (2008). Comparative analysis of archaeal 16S rRNA and amoA genes to estimate the abundance and diversity of ammonia-oxidizing archaea in marine sediments. *Extremophiles*, *12*(4), 605–615. https://doi.org/10.1007/s00792-008-0165-7

Pfister, C. A., Cardini, U., Mirasole, A., Montilla, L. M., Veseli, I., Gattuso, J.-P., & Teixido, N. (2023). Microbial associates of an endemic Mediterranean seagrass enhance the access of the host and the surrounding seawater to inorganic nitrogen under ocean acidification. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-47126-4

Pita, L., Rix, L., Slaby, B. M., Franke, A., & Hentschel, U. (2018). The sponge holobiont in a changing ocean: From microbes to ecosystems. *Microbiome*, *6*(46). https://doi.org/10.1186/s40168-018-0428-1

R Core team. (2023). *R: A language environment for statistical computing*.

Rix, L., de Goeij, J. M., van Oevelen, D., Struck, U., Al-Horani, F. A., Wild, C., & Naumann, M. S. (2017). Differential recycling of coral and algal dissolved organic matter via the sponge loop. *Functional Ecology*, *31*(3), 778–789. https://doi.org/10.1111/1365-2435.12758

Semedo, M., Lopes, E., Baptista, M. S., Oller-Ruiz, A., Gilabert, J., Tomasino, M. P., & Magalhães, C. (2021). Depth Profile of Nitrifying Archaeal and Bacterial Communities in the Remote Oligotrophic Waters of the North Pacific. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.624071

Seymour, J. R., Laverock, B., Nielsen, D. A., Trevathan-Tackett, S. M., & Macreadie, P. I. (2018). The Microbiology of Seagrasses. In A. W. D. Larkum, G. A. Kendrick, & P. J. Ralph (Eds.), *Seagrasses of Australia* (pp. 343–392). Springer International Publishing. https://doi.org/10.1007/978-3-319-71354-0_12

Soest, R. W. M. V., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., Voogd, N. J. D., Santodomingo, N., Vanhoorne, B., Kelly, M., & Hooper, J. N. A. (2012). Global Diversity of Sponges (Porifera). *PLOS ONE*, *7*(4), e35105. https://doi.org/10.1371/journal.pone.0035105

Sogin, E. M., Michellod, D., Gruber-Vodicka, H. R., Bourceau, P., Geier, B., Meier, D. V., Seidel, M., Ahmerkamp, S., Schorn, S., D'Angelo, G., Procaccini, G., Dubilier, N., & Liebeke, M. (2022). Sugars dominate the seagrass rhizosphere. *Nature Ecology and Evolution*, *6*, 866–877. https://doi.org/10.1038/S41559-022-01740-Z

Southwell, M. W., Popp, B. N., & Martens, C. S. (2008). Nitrification controls on fluxes and isotopic composition of nitrate from Florida Keys sponges. *Marine Chemistry*, *108*, 96–108. https://doi.org/10.1016/j.marchem.2007.10.005

Southwell, M. W., Weisz, J. B., Martens, C. S., & Lindquist, N. (2008). In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. *Limnology and Oceanography*, *53*(3), 986–996. https://doi.org/10.4319/lo.2008.53.3.0986

Szitenberg, A., Beca-Carretero, P., Azcárate-García, T., Yergaliyev, T., Alexander-Shani, R., & Winters, G. (2022). Teasing apart the host-related, nutrient-related and temperature-related effects shaping the phenology and microbiome of the tropical seagrass Halophila stipulacea. *Environmental Microbiome*, *17*(1), 18. https://doi.org/10.1186/s40793-022-00412-6

Tarquinio, F., Hyndes, G. A., Laverock, B., Koenders, A., & Säwström, C. (2019). The seagrass holobiont: Understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. *FEMS Microbiology Letters*, *366*(6), 1–15. https://doi.org/10.1093/femsle/fnz057

Taylor, M. W., Schupp, P. J., Dahllöf, I., Kjelleberg, S., & Steinberg, P. D. (2004). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology*, *6*(2), 121–130. https://doi.org/10.1046/j.1462-2920.2003.00545.x

Thiel, V., Leininger, S., Schmaljohann, R., Brümmer, F., & Imhoff, J. F. (2007). Sponge-specific Bacterial Associations of the Mediterranean Sponge Chondrilla nucula (Demospongiae, Tetractinomorpha). *Microb. Ecol.*, *54*, 101–111. https://doi.org/10.1007/s00248-006-9177-y

Thomas, T., Moitinho-Silva, L., Lurgi, M., Björk, J. R., Easson, C., Astudillo-García, C., Olson, J. B., Erwin, P. M., López-Legentil, S., Luter, H., Chaves-Fonnegra, A., Costa, R., Schupp, P. J., Steindler, L., Erpenbeck, D., Gilbert, J., Knight, R., Ackermann, G., Victor Lopez, J., … Webster, N. S. (2016). Diversity, structure and convergent evolution of the global sponge microbiome. *Nature Communications*, *7*(1), Article 1. https://doi.org/10.1038/ncomms11870

Thomas, T., Rusch, D., DeMaere, M. Z., Yung, P. Y., Lewis, M., Halpern, A., Heidelberg, K. B., Egan, S., Steinberg, P. D., & Kjelleberg, S. (2010). Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. *The ISME Journal*, *4*(12), Article 12. https://doi.org/10.1038/ismej.2010.74

Touchette, B. W., & Burkholder, J. A. M. (2000). Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, *250*, 133–167. https://doi.org/10.1016/S0022-0981(00)00195-7

Ugarelli, K., Chakrabarti, S., Laas, P., & Stingl, U. (2017). The Seagrass Holobiont and Its Microbiome. *Microorganisms*, *5*(81). https://doi.org/10.3390/microorganisms5040081

Usher, K. M. (2008). The ecology and phylogeny of cyanobacterial symbionts in sponges. *Marine Ecology*, *29*(2), 178–192. https://doi.org/10.1111/j.1439-0485.2008.00245.x

Usher, K. M., Fromont, J., Sutton, D. C., & Toze, S. (2004). The Biogeography and Phylogeny of Unicellular Cyanobacterial Symbionts in Sponges from Australia and the Mediterranean. *Microb. Ecol.*, *48*, 167–177. https://doi.org/10.1007/s00248-003-1062-3

Ward, B. B. (2008). Nitrification in Marine Systems. In *Nitrogen in the Marine Environment* (pp. 199–261). https://doi.org/10.1016/B978-0-12-372522-6.00005-0

Welsh, D. T. (2000). Nitrogen fixation in seagrass meadows: Regulation, plant-bacteria interactions and significance to primary productivity. *Ecology Letters*, *3*, 58–71.

Wuchter, C., Abbas, B., Coolen, M. J. L., Herfort, L., van Bleijswijk, J., Timmers, P., Strous, M., Teira, E., Herndl, G. J., Middelburg, J. J., Schouten, S., & Sinninghe Damsté, J. S. (2006). Archaeal nitrification in the ocean. *Proceedings of the National Academy of Sciences*, *103*(33), 12317–12322. https://doi.org/10.1073/pnas.0600756103

6. General Discussion

Thesis highlights

- Seagrass epiphytes contribute significantly to the net primary production of the *Posidonia oceanica* holobiont (**Chapter 2**)
- Key nitrogen cycling processes (N_2 fixation, nitrification, denitrification/anammox) occur in the *P. oceanica* phyllosphere (**Chapter 3**)
- Natural ocean acidification accelerates net primary production and key nitrogen cycling processes in the *P. oceanica* phyllosphere, while the prokaryotic community structure remains largely unaffected (**Chapters 2 & 3**)
- *P. oceanica* and *C. nucula* live in facultative mutualism, where the sponge benefits from carbon released by the plant while providing key inorganic nutrients that can support seagrass productivity (**Chapters 4 & 5**)
- *C. nucula* harbors nitrifying microorganisms that can provide nitrogen to the *P. oceanica* holobiont (**Chapter 5**)

Overview

This thesis aims to increase our knowledge of carbon (C) and nitrogen (N) cycling in benthic host-microbe associations under environmental change. Research on seagrass holobionts has gained increasing attention over the last decade, as it is now known that the presence of the seagrass microbiome is critical for the development of the plants, from seed germination to enhanced nutrient availability and defense against pathogens (Seymour et al., 2018; Tarquinio et al., 2019; Ugarelli et al., 2017). These holobionts do not exist in isolation, but are embedded within communities of other organisms that coexist and interact with progressively larger networks of increasingly complex assemblages of microbes, fungi, plants, and animals (Mcfall-Ngai et al., 2013; Pita et al., 2018). This dissertation contributes to a broader understanding of the mechanisms shaping these complex host-microbe interactions, particularly under environmental and anthropogenic stressors. We provide new insights into I) the contribution of the seagrass *Posidonia oceanica* and its epiphytes to primary production under ocean acidification (**Chapter 2**), II) the microbiome associated with the *P. oceanica* phyllosphere and the key nitrogen cycling processes they provide (**Chapter 3**), III) nutrient fluxes and primary productivity in the association between *P. oceanica* and the sponge *Chondrilla nucula* (**Chapter 4**), and IV) nitrification in the *P. oceanica- C. nucula* association (**Chapter 5**).

SYNOPTIC ANSWERS TO RESEARCH QUESTIONS

1 | How does the epiphytic community contribute to seagrass productivity? What are the effects of OA on the productivity of seagrass leaves and their epiphytes? What is the ecological relevance?

Epiphytes contributed significantly to the net primary production (NPP) and gross primary production (GPP) of *P. oceanica* leaves (**Chapter 2**), accounting for 50% of NPP at vent pH and 62% at ambient pH conditions. Despite the significant role of epiphytes in driving GPP and NPP, their presence did not affect respiration (R) in our experiments. *Posidonia oceanica* leaves from vent pH showed 47 $\pm$ 21% (mean $\pm$ SE) higher NPP and 50 $\pm$ 4% R, indicating that the seagrass is indeed carbon-limited under current seawater carbon concentrations. These results are consistent with studies showing increased primary productivity of *P. oceanica* and other seagrass species under decreasing pH (Apostolaki et al., 2014; Cox et al., 2015; Egea et al., 2018; Guilini et al., 2017; Hall-Spencer et al., 2008; Jiang et al., 2010). However, higher seagrass productivity does not necessarily result in an overall increase in meadow growth. *Posidonia oceanica* meadows at vent pH sites have higher shoot density, but shorter leaves compared to ambient pH sites. These morphological changes, reported also in other studies (Guilini et al., 2017; Mecca et al., 2020), are likely due to increased grazing pressure, attributed to the more labile organic composition of the seagrass holobiont (Scartazza et al., 2017) and, as our data indicate, the absence of calcareous epiphytes at vent pH sites. This increased herbivory is prompting the seagrass to allocate energy to shoot recruitment rather than belowground carbon storage.

Epiphytic communities growing at vent pH (OA conditions) differed significantly from communities growing under ambient pH conditions (**Chapter 2**). Overall epiphyte cover was 25% higher under ambient pH. Encrusting red algae decreased from 32% coverage at ambient pH to 12% at vent pH, while non-calcifying hydrozoans increased from 7% to 21%. This shift from coralline to non-calcifying organisms is in line with findings from other studies (Cox et al., 2015; Gravili et al., 2021; Mecca et al., 2020). Epiphytic biomass was significantly higher at ambient pH sites, due to higher coverage and calcium carbonate mass (**Chapter 2**). Furthermore, the epiphytic contribution to NPP was different under altered pH conditions. Net primary production of leaves from the vent pH site was 26% higher with epiphytes present and even 68% with epiphytes removed. The lower epiphytic contribution to NPP in the CO_2 vents was likely a combined result of changes in biomass, community composition, as well as species-specific rates.

In situ, there were no significant differences in net community productivity (NCP) or community respiration (CR), though a trend toward higher autotrophy was observed at one of the investigated sites (**Chapter 2**). This highlights the high complexity of host-epiphyte interactions and their response to environmental changes, such as OA.

2 | What are the rates of key microbial N cycling processes under ambient and OA conditions in the *P. oceanica* phyllosphere and how do these processes contribute to the N demand of the seagrass holobiont? How does OA affect the diversity of the microbial community on *P. oceanica* leaves? What role does the microbial community on *P. oceanica* leaves play in N cycling?

Incubation experiments with ^{15}N stable isotope labeling demonstrated that all key microbial N cycling processes occur in the *P. oceanica* phyllosphere, resulting in a net N gain by the holobiont under ambient and OA conditions (**Chapter 3**). We detected N_2 incorporation rates by epiphytes in light incubations, reaching 0.62 ± 0.15 nmol N cm^{-2} h^{-1} under OA conditions. These rates were comparable to N_2 fixation rates measured *in situ* in minimally disturbed *P. oceanica* meadows (Agawin et al., 2017) and to rates measured by root symbionts of *P. oceanica* under ambient pH (Lehnen et al., 2016; Mohr et al., 2021). Microbial-mediated N-loss pathways (denitrification and anammox) were also observed, with N_2 production rates up to 7.14 ± 2.07 pmol N cm^{-2} h^{-1} in light conditions at the vent site (**Chapter 3**). The daily budgets of total N loss at the vent site were significant, and comparable to N loss rates reported from seagrass sediments by Salk et al. (2017). The presence of Planctomycetes and detectable rates of $^{29}N_2$ production in our incubations suggest that anammox may play an important role as an N loss pathway on seagrass leaves. Nitrification potential was detected only at the vent site, though rates are low, contributing marginally to the N budget. Notably, NO_3^- uptake rates increased significantly (147-270%) in the presence of epiphytes (**Chapter 3**), indicating a potential strategy by epiphytes to reduce competition for N with the plant that prefers NH_4^+ over NO_3^- uptake (Touchette & Burkholder, 2000).

Ocean acidification at natural CO_2 vents accelerates N cycling on *P. oceanica* leaves, tipping the balance decisively in favor of increased N gain. Under OA conditions, daylight N_2 fixation rates were 409% higher (**Chapter 3**); the positive response of N_2 fixation rates to elevated CO_2 concentrations is supported by several studies with planktonic diazotrophs (Kroeker et al., 2013; Liu et al., 2010; Wannicke et al., 2018). NH_4^+ uptake increased by 62-97% and NO_3^- uptake by 330-412% at the vent pH site (**Chapter 3**), probably due to decreased epiphytic load (Apostolaki et al., 2012). Conversely to other studies (Beman et al., 2011; Kitidis et al., 2011), nitrification potential was only detected under OA conditions in our incubations. Increasing CO_2 levels could result in higher autotrophic nitrification rates by reducing CO_2 limitation (Hutchins et al., 2009) and a diverse nitrifier community can adapt to a wider range of pH values (Fulweiler et al., 2011). Although OA is not expected to have direct effects on denitrification and anammox, an increase in both C and N_2 fixation, may have favored the formation of anoxic microniches on the leaf biofilm and generated organic C and oxidized N compounds available for metabolism by denitrifying bacteria.

16S rRNA gene sequencing revealed distinct microbial communities on *P. oceanica* leaves compared to the water column, with no significant differences between ambient and vent pH sites (**Chapter 3**). The leaves were dominated by Proteobacteria, particularly Alphaproteobacteria (20-22%) and Gammaproteobacteria (9-15%). Key groups included Rhodobacterales, known for early colonization and N_2 fixation (Dang et al., 2008; Mejia et al., 2016; Trevathan-Tackett et al., 2020), and Rhizobiales, which include a diversity of N_2-fixing microbes that can form symbiotic relationships with terrestrial plants (Lindström & Mousavi, 2020) and are known for

promoting plant health and growth (Avis et al., 2008). We found Cyanobacteria (2-14%), among which are taxa that can cope with O_2 production from daytime photosynthesis, and thus, sustain N_2 fixation in the light. Planctomycetes accounted for 2% of the microbial leaf community, among them members known for their abilities for N_2 fixation and anammox (Delmont et al., 2018; Jetten et al., 2009; Strous et al., 1999). We found the nitrifying families Nitrosomonadaceae, Nitrospiraceae, Nitrospinaceae (ammonia-oxidizing bacteria; AOB), and Nitrosopumilales (ammonia-oxidizing archaea; AOA) in the phyllosphere of *P. oceanica* (Hutchins & Capone, 2022; Kuypers et al., 2018).

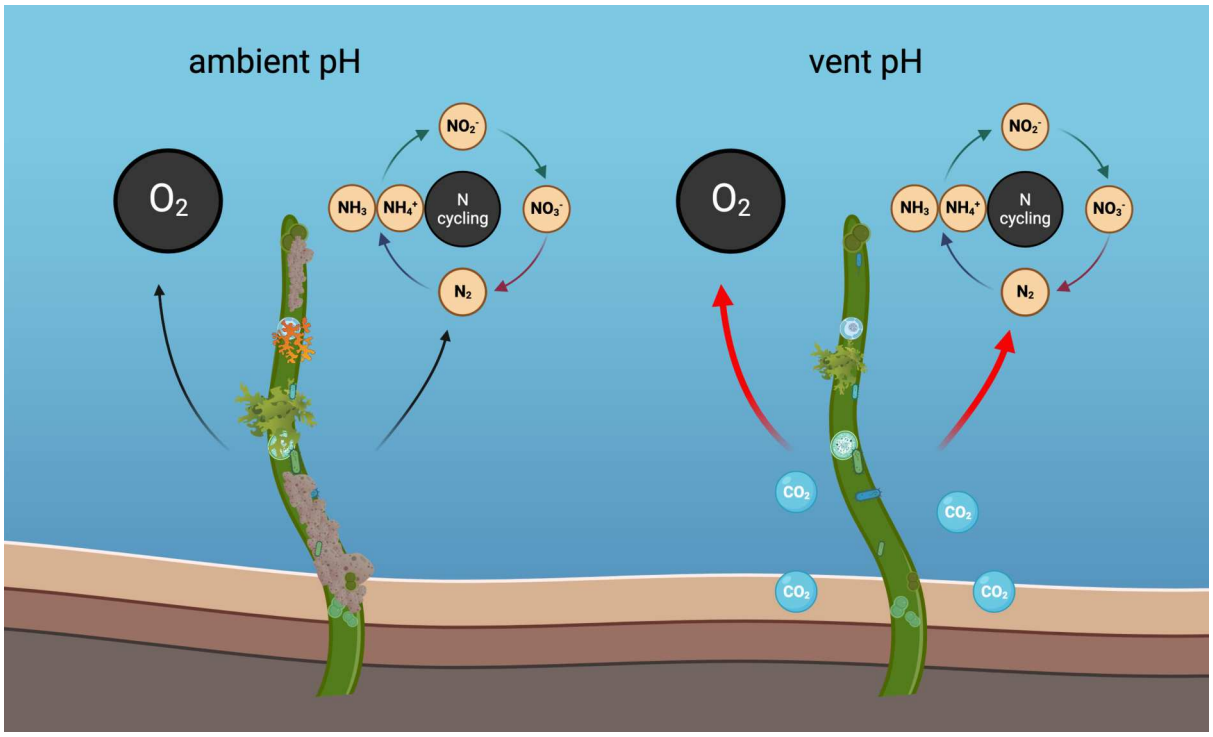

Fig. 6.1. Carbon and nitrogen cycling within the *Posidonia oceanica* holobiont under ambient and vent pH conditions (**Chapters 2 & 3**). Epiphytes contribute significantly to *P. oceanica* productivity (**Chapter 2**) and key N cycling processes (N_2 fixation, nitrification, denitrification/anammox; **Chapter 3**) in the seagrass phyllosphere. OA accelerates holobiont productivity (**Chapter 2**) and N cycling processes (**Chapter 3**). OA affects larger calcifying epiphytes (**Chapter 2**), while the microbial community remains unchanged (**Chapter 3**). Created with BioRender.com.

3 | How does the association between *P. oceanica* and the sponge *Chondrilla nucula* affect primary production, inorganic and organic nutrient fluxes in the seagrass holobiont? What are potential benefits for the seagrass and the sponge?

The association between the seagrass *P. oceanica* and the sponge *C. nucula* reveals a dynamic and reciprocal interaction that influences primary production and nutrient fluxes within the seagrass holobiont (**Chapter 4**). Net primary production (NPP) measurements showed that *C. nucula* was near metabolic balance in autumn but shifted to net heterotrophy in spring. In contrast, *P. oceanica* and its association with the sponge remained autotrophic year-round. The photosynthetic activity of cyanobacterial symbionts in *C. nucula* provided 52% of its respiratory carbon needs in autumn and only 10% in spring (**Chapter 4**). This emphasizes the sponge's reliance on mixotrophy, with dissolved organic matter (DOM) uptake playing a larger role, which is also reported for a

congeneric species in the Caribbean (Hudspith et al., 2022). Our results show high *P. oceanica* productivity in spring and senescence in autumn, aligning with previous studies on seasonal dynamics of the seagrass' productivity (Koopmans et al., 2020; Olivé et al., 2016). However, the presence of *C. nucula* appears to buffer seasonal productivity variations of the seagrass, likely due to increased sponge autotrophy in autumn. Seagrass meadows associated with *C. nucula* may experience reduced primary production during growth periods but benefit from enhanced nutrient recycling during senescence periods, promoting ecosystem stability and long-term resilience (Duffy, 2006; Isbell et al., 2009).

P. oceanica released significant amounts of dissolved organic carbon (DOC; 46% and 33% of NCP in autumn and spring, respectively; **Chapter 4**). Concurrently, we detected net DOC uptake by *C. nucula* during spring and release in autumn, aligning with the sponge's mixotrophic strategy, shifting between heterotrophy in spring and autotrophy in autumn. The sponge, in turn, provided substantial amounts of NH_4^+ and NO_3^-, with NO_3^- fluxes (13.6 - 16.7 μmol g^{-1} day^{-1}) likely driven by nitrification (Jiménez & Ribes, 2007; Schläppy et al., 2010; Southwell et al., 2008). This nutrient exchange benefits the seagrass, contributing 10% to the plant's N demand in spring through NO_3^- uptake and about 13% through NH_4^+ uptake.

Field observations demonstrated a non-linear relationship between their benthic cover, with maximum sponge cover (~10%) occurring at intermediate seagrass densities (~75%; **Chapter 4**). This pattern suggests a spatial dependence of both organisms, favoring a balance of available substrate and resource availability (Archer et al., 2015). While the seagrass appears neutral to the sponge's presence, this neutrality likely reflects a balance between competition for space and benefits from nutrient recycling by the sponge.

4 | What are the potential nitrification rates in the association between *P. oceanica* and *C. nucula*? Does the microbiome of *C. nucula* harbor nitrifying microorganisms and can microbial nitrification contribute to the seagrass holobiont N demand?

We were able to demonstrate that dissolved inorganic nitrogen (DIN) from nitrification in the sponge *C. nucula* can be taken up by the seagrass *P. oceanica*, supporting its N demand (**Chapter 5**). With rates of 21 ± 7 nmol g DW^{-1} h^{-1} in spring and 267 ± 33 nmol g DW^{-1} h^{-1} in autumn, the potential nitrification rates (PNR) of *C. nucula* in association with the seagrass were comparable to other Mediterranean and tropical sponges (Bayer et al., 2008; Diaz & Ward, 1997; Jiménez & Ribes, 2007). Sponge-mediated nitrification supplied NO_3^-, which was utilized by the seagrass holobiont. The sponge also released dissolved organic nitrogen (DON), which can additionally benefit the seagrass, while it can take up dissolved organic carbon (DOC) released by the seagrass (Sogin et al., 2022). PNR was highest in autumn, possibly due to reduced competition for NH_4^+ from the seagrass during this period of lower growth. Microbial-mediated nitrification associated with *C. nucula* can support a substantial proportion of the seagrass holobiont's primary production, 8% in spring and 47% in autumn (**Chapter 5**). The seasonal variation in nutrient dynamics emphasizes the importance of understanding how environmental factors influence these associations.

Stable isotope analyses showed increased $\delta^{15}N$ values in *P. oceanica* and its epiphytes when associated with the sponge (**Chapter 5**). This enrichment was likely driven by the uptake of $\delta^{15}N$-enriched residual NH_4^+ excreted by the sponge, which becomes enriched during nitrification (Casciotti, 2016; Mariotti et al., 1981). While *P. oceanica* can assimilate both NH_4^+ and NO_3^-, it typically favors NH_4^+ (Touchette & Burkholder, 2000). In **Chapter 3**, we have demonstrated that epiphytes may rely more on NO_3^- to reduce competition with the plant. However, sponge-derived NH_4^+ production was only observed in spring (**Chapter 5**), suggesting that NO_3^- produced via nitrification becomes more important in autumn. Contrarily, the sponge showed high NO_3^- production (17.78 ± 3.49 µmol g^{-1} day^{-1} in spring and 23.59 ± 2.89 µmol g^{-1} day^{-1} in autumn), while the seagrass displayed near-zero net flux or uptake. In incubations with the association, NO_3^- fluxes were lower than in sponge-only incubations, indicating sponge-mediated nitrification contributes NO_3^- that is taken up by the seagrass holobiont. Further research is needed to clarify whether sponge-produced NO_3^- benefits *P. oceanica* directly or rather its epiphytes.

We found a distinct and stable microbial community associated with *C. nucula* that is not affected by the association state with *P. oceanica* (**Chapter 5**). This is consistent with other studies that found the microbiome of *C. nucula* not affected by ambient seawater in the Mediterranean Sea (Thiel et al., 2007) or the Caribbean (Hill et al., 2006). Key bacterial groups involved in nitrification were the nitrite-oxidizing bacteria (NOB) *Nitrospira* within the family *Nitrospiraceae* (*Nitrospiria*), and the genera Cm1-21 and AqS1, both ammonia-oxidizing bacteria (AOB) within the family of *Nitrosococcaceae* (*Gammaproteobacteria*) (**Chapter 5**). Additionally, we found the ammonia-oxidizing archaea (AOA) *Candidatus Nitrosopumilus*, which are stable associates of many sponge species (Bayer et al., 2008; Hoffmann et al., 2009; Holmes & Blanch, 2007).

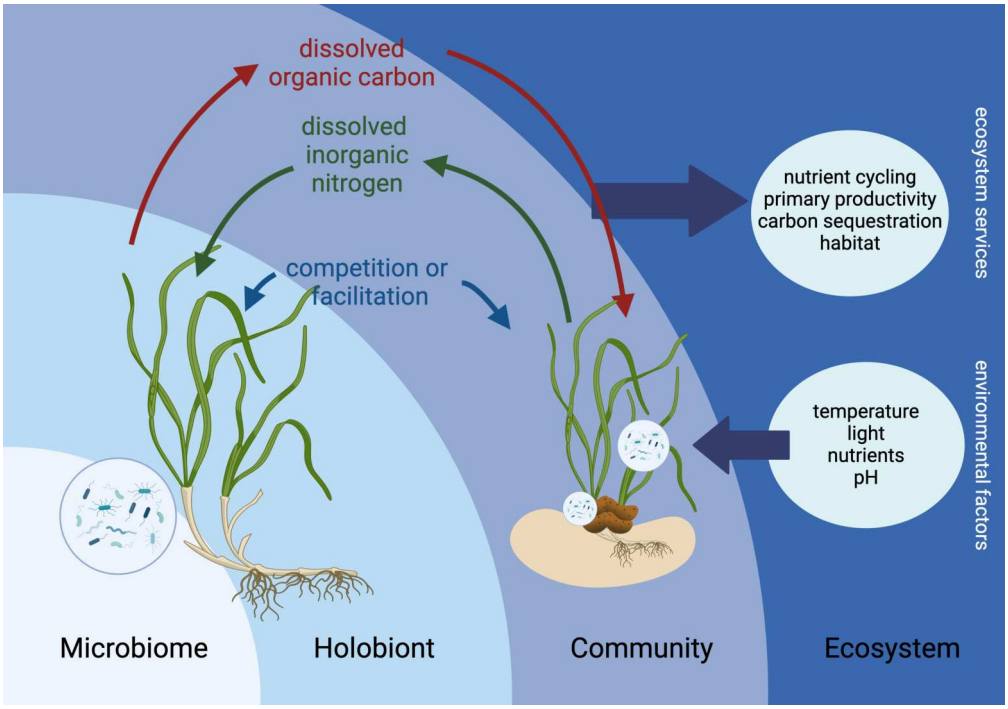

Fig. 6.2. The seagrass-sponge association as an example of a nested ecosystem (**Chapters 4 & 5**). DOC released by the seagrass can benefit the sponge, while the plant benefits from DIN released by the sponge (**Chapter 4**). Seagrass and sponge can compete with or facilitate each other depending on environmental conditions (**Chapter 4**). The seagrass-sponge-microbe community affects nutrient cycling, primary production, and other ecosystem services while it is influenced by environmental factors such as temperature, light, pH, and nutrient concentrations (**Chapter 5**). Adapted from Pita et al. (2018). Created with BioRender.com.

OUTLOOK AND FUTURE RESEARCH DIRECTIONS

This thesis combined research on the physiology, biogeochemistry, and microbial ecology of the *Posidonia oceanica* holobiont under environmental change and on different levels of ecological organization. We could demonstrate that organisms associated with the seagrass plant, be it microorganisms, macro-epiphytes, or larger invertebrates, play a crucial role in the biogeochemical cycling of C and N, and thus the plant's physiology and health. Environmental stressors, such as OA, can tip the balance in favor of either benefits or increased competition for the seagrass host. However, knowledge about biogeochemical cycling within the seagrass holobiont is still limited, and by answering the research questions included in this thesis, several questions for future research emerge:

1 | How does OA affect the *in situ* seagrass community productivity?

We could show that natural CO_2 enrichment increases the productivity of both seagrass leaves and their epiphytic community *ex situ* (**Chapter 2**). However, this was only marginally translated to changes in NCP or CR *in situ*. While laboratory studies provide excellent insights into metabolic processes, these results demonstrate the high complexity of host-epiphyte interactions and their response to environmental changes. Future studies should thus also focus on the benthic metabolism at the community level and especially on the role of seagrass epiphytes. Additionally, experiments throughout the year would provide helpful insights about seasonal patterns that might occur.

2 | How much of the N fixed by epiphytic diazotrophs is transferred to the seagrass host?

The results of **Chapter 3** show N_2 incorporation in epiphyte tissue in our incubation experiments with a $^{15}N_2$ tracer, highlighting the role of the seagrass microbiome in N cycling and thus plant physiology and health. However, in the limited time frame of the experiment, we could not measure a significant transfer of fixed N to the *P. oceanica* plant tissues. Therefore, further research (e.g., using NanoSIMS or longer-term incubations with stable isotopes) should investigate how much of the N_2 fixed by the epiphytic diazotrophs can actually be transferred to the plant host.

3 | How does the sponge *Chondrilla nucula* benefit from the association with *P. oceanica*?

In **Chapter 4**, we demonstrated a reciprocal interaction between *C. nucula* and *P. oceanica*, with nutrient exchange facilitating a facultative mutualism that supports the productivity of the holobiont. While the seagrass benefits from nutrients released by the sponge, the sponge may benefit from DOC released by the seagrass. As shown in the study, the extent of these beneficial processes can vary a lot throughout seasons. Further investigations should therefore focus on the benefits for the sponge throughout the year.

4 | Who benefits from sponge-mediated nitrification in the seagrass holobiont?

In our incubation experiments amended with ^{15}N ammonium in **Chapter 5**, we could measure high nitrification rates mediated by the sponge *C. nucula*. However, we demonstrated in Chapter 3 that while the seagrass host preferentially takes up NH_4^+, its epiphytes may rely more on NO_3^- to reduce competition with the plant. Therefore, whether NO_3^- produced by sponge-associated nitrification benefits the seagrass or rather its associated epiphytes needs to be further investigated by using specifically targeting stable isotope techniques.

REFERENCES

Agawin, N. S. R., Ferriol, P., Sintes, E., & Moyà, G. (2017). Temporal and spatial variability of in situ nitrogen fixation activities associated with the Mediterranean seagrass Posidonia oceanica meadows. *Limnology and Oceanography*, *62*(6), 2575–2592. https://doi.org/10.1002/LNO.10591

Apostolaki, E. T., Vizzini, S., Hendriks, I. E., & Olsen, Y. S. (2014). Seagrass ecosystem response to long-term high CO2 in a Mediterranean volcanic vent. *Marine Environmental Research*, *99*, 9–15. https://doi.org/10.1016/j.marenvres.2014.05.008

Apostolaki, E. T., Vizzini, S., & Karakassis, I. (2012). Leaf vs. Epiphyte nitrogen uptake in a nutrient enriched Mediterranean seagrass (Posidonia oceanica) meadow. *Aquatic Botany*, *96*, 58–62. https://doi.org/10.1016/j.aquabot.2011.09.008

Archer, S. K., Stoner, E. W., & Layman, C. A. (2015). A complex interaction between a sponge (Halichondria melanadocia) and a seagrass (Thalassia testudinum) in a subtropical coastal ecosystem. *Journal of Experimental Marine Biology and Ecology*, *465*, 33–40. https://doi.org/10.1016/J.JEMBE.2015.01.003

Avis, T. J., Gravel, V., Antoun, H., & Tweddell, R. J. (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. *Soil Biology and Biochemistry*, *40*(7), 1733–1740. https://doi.org/10.1016/j.soilbio.2008.02.013

Bayer, K., Schmitt, S., & Hentschel, U. (2008). Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge Aplysina aerophoba. *Environmental Microbiology*, *10*(11), 2942–2955. https://doi.org/10.1111/J.1462-2920.2008.01582.X

Beman, J. M., Chow, C. E., King, A. L., Feng, Y., Fuhrman, J. A., Andersson, A., Bates, N. R., Popp, B. N., & Hutchins, D. A. (2011). Global declines in oceanic nitrification rates as a consequence of ocean acidification. *PNAS*, *108*(1), 208–213. https://doi.org/10.1073/PNAS.1011053108

Casciotti, K. L. (2016). Nitrogen and Oxygen Isotopic Studies of the Marine Nitrogen Cycle. *Annual Review of Marine Science*, *8*(Volume 8, 2016), 379–407. https://doi.org/10.1146/annurev-marine-010213-135052

Cox, T. E., Schenone, S., Delille, J., Diaz-Castaneda, V., Alliouane, S., Gattuso, J.-P., & Gazeau, F. (2015). Effects of ocean acidification on Posidonia oceanica epiphytic community and shoot productivity. *Journal of Ecology*, *103*, 1594–1609. https://doi.org/10.1111/1365-2745.12477

Dang, H., Li, T., Chen, M., & Huang, G. (2008). Cross-ocean distribution of Rhodobacterales bacteria as primary surface colonizers in temperate coastal marine waters. *Applied and Environmental Microbiology*, *74*(1), 52–60. https://doi.org/10.1128/AEM.01400-07

Delmont, T. O., Quince, C., Shaiber, A., Esen, Ö. C., Lee, S. T., Rappé, M. S., McLellan, S. L., Lücker, S., & Eren, A. M. (2018). Nitrogen-fixing populations of Planctomycetes and Proteobacteria are abundant in surface ocean metagenomes. *Nature Microbiology*, *3*(7), 804–813. https://doi.org/10.1038/s41564-018-0176-9

Diaz, M. C., & Ward, B. B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series*, *156*, 97–107.

Duffy, J. E. (2006). Biodiversity and the functioning of seagrass ecosystems. *Marine Ecology Progress Series*, *311*, 233–250. https://doi.org/10.3354/meps311233

Egea, L. G., Jimé Nez-Ramos, R., Herná Ndez, I., Bouma, T. J., & Brun, F. G. (2018). Effects of ocean acidification and hydrodynamic conditions on carbon metabolism and dissolved organic carbon (DOC) fluxes in seagrass populations. *PLoS ONE*, 1–20. https://doi.org/10.1371/journal.pone.0192402

Fulweiler, R. W., Emery, H. E., Heiss, E. M., & Berounsky, V. M. (2011). Assessing the Role of pH in Determining Water Column Nitrification Rates in a Coastal System. *Estuaries and Coasts*, *34*, 1095–1102. https://doi.org/10.1007/s12237-011-9432-4

Gravili, C., Cozzoli, F., & Gambi, M. C. (2021). Epiphytic hydroids on Posidonia oceanica seagrass meadows are winner organisms under future ocean acidification conditions: Evidence from a CO2 vent system (Ischia Island, Italy). *European Zoological Journal*, *88*(1), 472–486. https://doi.org/10.1080/24750263.2021.1899317

Guilini, K., Weber, M., De Beer, D., Schneider, M., Molari, M., Lott, C., Bodnar, W., Mascart, T., De Troch, M., & Vanreusel, A. (2017). Response of Posidonia oceanica seagrass and its epibiont communities to ocean acidification. *PLoS ONE*, *12*(8). https://doi.org/10.1371/journal.pone.0181531

Hall-Spencer, J. M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S. M., Rowley, S. J., Tedesco, D., & Buia, M.-C. (2008). Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature*, *454*. https://doi.org/10.1038/nature07051

Hill, M., Hill, A., Lopez, N., & Harriott, O. (2006). Sponge-specific bacterial symbionts in the Caribbean sponge, Chondrilla nucula (Demospongiae, Chondrosida). *Marine Biology*, *148*, 1221–1230. https://doi.org/10.1007/s00227-005-0164-5

Hoffmann, F., Radax, R., Woebken, D., Holtappels, M., Lavik, G., Rapp, H. T., Schläppy, M. L., Schleper, C., & Kuypers, M. M. M. (2009). Complex nitrogen cycling in the sponge Geodia barretti. *Environmental Microbiology*, *11*(9), 2228–2243. https://doi.org/10.1111/J.1462-2920.2009.01944.X

Holmes, B., & Blanch, H. (2007). Genus-specific associations of marine sponges with group I crenarchaeotes. *Marine Biology*, *150*(5), 759–772. https://doi.org/10.1007/s00227-006-0361-x

Hudspith, M., De Goeij, J. M., Streekstra, M., Kornder, N. A., Bougoure, J., Guagliardo, P., Campana, S., Van Der Wel, N. N., Muyzer, G., & Rix, L. (2022). Harnessing solar power: Photoautotrophy supplements the diet of a low-light dwelling sponge. *ISME*. https://doi.org/10.1038/s41396-022-01254-3

Hutchins, D. A., & Capone, D. G. (2022). The marine nitrogen cycle: New developments and global change. *Nature Reviews Microbiology*, *20*(7), 401–414. https://doi.org/10.1038/S41579-022-00687-Z

Hutchins, D. A., Mulholland, M. R., & Fu, F. (2009). Nutrient Cycles and Marine Microbes in a CO_2-Enriched Ocean. *Oceanography*, *22*(4), 128–145. https://doi.org/10.2307/24861030

Isbell, F. I., Polley, H. W., & Wilsey, B. J. (2009). Biodiversity, productivity and the temporal stability of productivity: Patterns and processes. *Ecology Letters*, *12*(5), 443–451. https://doi.org/10.1111/j.1461-0248.2009.01299.x

Jetten, M. S. M., Niftrik, L. van, Strous, M., Kartal, B., Keltjens, J. T., & Op den Camp, H. J. M. (2009). Biochemistry and molecular biology of anammox bacteria. *Critical Reviews in Biochemistry and Molecular Biology*, *44*(2–3), 65–84. https://doi.org/10.1080/10409230902722783

Jiang, Z. J., Huang, X.-P., & Zhang, J.-P. (2010). Effects of CO 2 Enrichment on Photosynthesis, Growth, and Biochemical Composition of Seagrass Thalassia hemprichii (Ehrenb.) Aschers. *Journal of Integrative Plant Biology*, *52*(10), 904–913. https://doi.org/10.1111/j.1744-7909.2010.00991.x

Jiménez, E., & Ribes, M. (2007). Sponges as a source of dissolved inorganic nitrogen: Nitrification mediated by temperate sponges. *Limnology and Oceanography*, *52*(3), 948–958. https://doi.org/10.4319/lo.2007.52.3.0948

Kitidis, V., Laverock, B., McNeill, L. C., Beesley, A., Cummings, D., Tait, K., Osborn, M. A., & Widdicombe, S. (2011). Impact of ocean acidification on benthic and water column ammonia oxidation. *Geophysical Research Letters*, *38*(L21603). https://doi.org/10.1029/2011GL049095

Koopmans, D., Holtappels, M., Chennu, A., Weber, M., & De Beer, D. (2020). High Net Primary Production of Mediterranean Seagrass (Posidonia oceanica) Meadows Determined With Aquatic Eddy Covariance. *Frontiers in Marine Science*, *7*, 118. https://doi.org/10.3389/fmars.2020.00118

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M., & Gattuso, J.-P. (2013). Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Global Change Biology*, *19*, 1884–1896. https://doi.org/10.1111/gcb.12179

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Microbial Biogeochemistry*, *16*, 263–276. https://doi.org/10.1038/nrmicro.2018.9

Lehnen, N., Marchant, H. K., Schwedt, A., Milucka, J., Lott, C., Weber, M., Dekaezemacker, J., Seah, B. K. B., Hach, P. F., Mohr, W., & Kuypers, M. M. M. (2016). High rates of microbial dinitrogen fixation and sulfate reduction associated with the Mediterranean seagrass Posidonia oceanica. *Systematic and Applied Microbiology*, *39*(7), 476–483. https://doi.org/10.1016/J.SYAPM.2016.08.004

Lindström, K., & Mousavi, S. A. (2020). Effectiveness of nitrogen fixation in rhizobia. *Microbial Biotechnology*, *13*(5), 1314–1335. https://doi.org/10.1111/1751-7915.13517

Liu, J., Weinbauer, M. G., Maier, C., Dai, M., & Gattuso, J. P. (2010). Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. *Aquatic Microbial Ecology*, *61*(3), 291–305. https://doi.org/10.3354/AME01446

Mariotti, A., Germon, J. C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., & Tardieux, P. (1981). Experimental determination of nitrogen kinetic isotope fractionation: Some principles; illustration for the denitrification and nitrification processes. *Plant and Soil*, *62*(3), 413–430. https://doi.org/10.1007/BF02374138

Mcfall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Loso, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *PNAS*, *110*(9), 3229–3236. https://doi.org/10.1073/pnas.1218525110/-/DCSupplemental

Mecca, S., Casoli, E., Ardizzone, G., & Gambi, M. C. (2020). Effects of ocean acidification on phenology and epiphytes of the seagrass Posidonia oceanica at two CO2 vent systems of Ischia (Italy). *Mediterranean Marine Science*, *21*(1), 70–83. https://doi.org/10.12681/MMS.20795

Mejia, A., Rotini, A., Lacasellab, F., Bookman, R., Thaller, M., Shem-Tovd, R., Wintersd, G., & Migliore, L. (2016). Assessing the ecological status of seagrasses using morphology, biochemical descriptors and microbial community analyses. A study in Halophila stipulacea (Forsk.) Aschers meadows in the northern Red Sea. *Ecological Indicators*, *60*, 1150–1163. https://doi.org/10.1016/j.ecolind.2015.09.014

Mohr, W., Lehnen, N., Ahmerkamp, S., Marchant, H. K., Graf, J. S., Tschitschko, B., Yilmaz, P., Littmann, S., Gruber-Vodicka, H., Leisch, N., Weber, M., Lott, C., Schubert, C. J., Milucka, J., & Kuypers, M. M. M. (2021). Terrestrial-type nitrogen-fixing symbiosis between seagrass and a marine bacterium. *Nature 2021*, 1–5. https://doi.org/10.1038/s41586-021-04063-4

Olivé, I., Silva, J., Costa, M. M., & Santos, R. (2016). Estimating Seagrass Community Metabolism Using Benthic Chambers: The Effect of Incubation Time. *Estuaries and Coasts*, *39*, 138–144. https://doi.org/10.1007/s12237-015-9973-z

Pita, L., Rix, L., Slaby, B. M., Franke, A., & Hentschel, U. (2018). The sponge holobiont in a changing ocean: From microbes to ecosystems. *Microbiome*, *6*(46). https://doi.org/10.1186/s40168-018-0428-1

Salk, K. R., Erler, D. V., Eyre, B. D., Carlson-Perret, N., & Ostrom, N. E. (2017). Unexpectedly high degree of anammox and DNRA in seagrass sediments: Description and application of a revised isotope pairing technique. *Geochimica et Cosmochimica Acta*, *211*, 64–78. https://doi.org/10.1016/J.GCA.2017.05.012

Scartazza, A., Moscatello, S., Gavrichkova, O., Buia, M. C., Lauteri, M., Battistelli, A., Lorenti, M., Garrard, S. L., Calfapietra, C., & Brugnoli, E. (2017). Carbon and nitrogen allocation strategy in Posidonia oceanica is altered by seawater acidification. *Science of the Total Environment*, *607*, 954–964. https://doi.org/10.1016/j.scitotenv.2017.06.084

Schläppy, M.-L., Schöttner, S. I., Lavik, G., Kuypers, M. M. M., de Beer, D., & Hoffmann, F. (2010). Evidence of nitrification and denitrification in high and low microbial abundance sponges. *Marine Biology*, *157*(3), 593–602. https://doi.org/10.1007/s00227-009-1344-5

Seymour, J. R., Laverock, B., Nielsen, D. A., Trevathan-Tackett, S. M., & Macreadie, P. I. (2018). The Microbiology of Seagrasses. In A. W. D. Larkum, G. A. Kendrick, & P. J. Ralph (Eds.), *Seagrasses of Australia* (pp. 343–392). Springer International Publishing. https://doi.org/10.1007/978-3-319-71354-0_12

Sogin, E. M., Michellod, D., Gruber-Vodicka, H. R., Bourceau, P., Geier, B., Meier, D. V., Seidel, M., Ahmerkamp, S., Schorn, S., D'Angelo, G., Procaccini, G., Dubilier, N., & Liebeke, M. (2022). Sugars dominate the seagrass rhizosphere. *Nature Ecology and Evolution*, *6*, 866–877. https://doi.org/10.1038/S41559-022-01740-Z

Southwell, M. W., Popp, B. N., & Martens, C. S. (2008). Nitrification controls on fluxes and isotopic composition of nitrate from Florida Keys sponges. *Marine Chemistry*, *108*, 96–108. https://doi.org/10.1016/j.marchem.2007.10.005

Strous, M., Fuerst, J. A., Kramer, Evelien, H. M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K. T., Webb, R., Kuenen, J. G., & Jetten, M. S. M. (1999). Missing lithotroph identified as new planctomycete. *Letters to Nature*, *400*, 446–449.

Tarquinio, F., Hyndes, G. A., Laverock, B., Koenders, A., & Säwström, C. (2019). The seagrass holobiont: Understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. *FEMS Microbiology Letters*, *366*(6), 1–15. https://doi.org/10.1093/femsle/fnz057

Thiel, V., Leininger, S., Schmaljohann, R., Brümmer, F., & Imhoff, J. F. (2007). Sponge-specific Bacterial Associations of the Mediterranean Sponge Chondrilla nucula (Demospongiae, Tetractinomorpha). *Microb. Ecol.*, *54*, 101–111. https://doi.org/10.1007/s00248-006-9177-y

Touchette, B. W., & Burkholder, J. A. M. (2000). Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, *250*, 133–167. https://doi.org/10.1016/S0022-0981(00)00195-7

Trevathan-Tackett, S. M., Allnutt, T. R., Sherman, C. D. H., Richardson, M. F., Crowley, T. M., & Macreadie, P. I. (2020). Spatial variation of bacterial and fungal communities of estuarine seagrass leaf microbiomes. *Aquatic Microbial Ecology*, *84*, 59–74. https://doi.org/10.3354/ame01926

Ugarelli, K., Chakrabarti, S., Laas, P., & Stingl, U. (2017). The Seagrass Holobiont and Its Microbiome. *Microorganisms*, *5*(81). https://doi.org/10.3390/microorganisms5040081

Wannicke, N., Frey, C., Law, C. S., & Voss, M. (2018). The response of the marine nitrogen cycle to ocean acidification. *Global Change Biology*, *24*, 5031–5043. https://doi.org/10.1111/gcb.14424

RESOURCES

- ChatGPT version 3.5, OpenAi: https://www.openai.com/chat (assisting with the text structure)
- DeepL Translate, Deepl SE: https://www.deepl.com/translator (translation of text passages)
- DeepL Write, Deepl SE: https://www.deepl.com/write (rephrasing of text passages)

APPENDIX - SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL TO CHAPTER 2

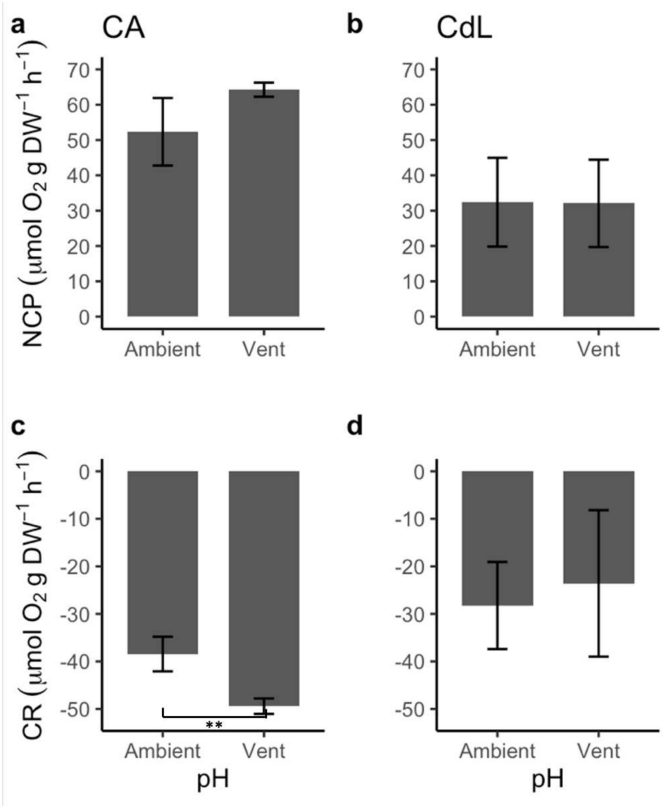

Fig. S2.1. In-situ net community production at Castello Aragonese (CA, a) and Chiane del Lume (CdL, b), and community respiration at CA (c) and CdL (d) at vent and ambient sites, normalized by seagrass leaf biomass (dry weight). Negative values represent oxygen consumption, while positive values show oxygen production. Error bars indicate 95% confidence intervals. Stars show significant differences; number of stars show significance level (* p<0.05, ** p<0.01, *** p<0.001).

Table S2.1. Organic and inorganic nutrient fluxes (mean ± SE) during dark and light incubations at acidified and control pH sites of Chiane del Lume. ANOVA results testing the differences between sites and dark/light incubation are given.

Variable ($\mu M\ h^{-1}$)	Vent pH (mean ± SE)		Ambient pH (mean ± SE)		ANOVA	Sum of squares	*F*-value	*p*-value
	Dark (*n*=4)	Light (*n*=3)	Dark (*n*=7)	Light (*n*=7)				
DOC	21.85 ± 19.28	19.30 ± 13.39	23.05 ± 14.63	20.50 ± 15.01	Site (df=1)	5.9	0.01	ns
					Dark/light (df=1)	27.3	0.03	ns
DON	-1.14 ± 1.46	0.34 ± 1.14	-3.74 ± 2.05	-2.26 ± 1.83	Site (df=1)	31.36	1.57	ns
					Dark/light (df=1)	11.44	0.52	ns
NH_4^+	0.10 ± 0.46	-0.31 ± 0.17	-0.12 ± 0.24	-0.54 ± 0.15	Site (df=1)	0.23	0.69	ns
					Dark/light (df=1)	0.91	2.73	ns
PO_4^-	0.006 ± 0.004	-0.019 ± 0.017	0.007 ± 0.013	-0.018 ± 0.007	Site (df=1)	0.000005	0.01	ns
					Dark/light (df=1)	0.003	4.56	0.047
NO_2^-	0.023 ± 0.028	0.007 ± 0.021	0.019 ± 0.027	0.003 ± 0.012	Site (df=1)	0.00007	0.02	ns
					Dark/light (df=1)	0.001	0.45	ns
NO_3^-	0.17 ± 0.08	0.13 ± 0.08	-0.02 ± 0.19	-0.06 ± 0.07	Site (df=1)	0.16	1.34	ns
					Dark/light (df=1)	0.01	0.07	ns

Table S2.2. Morphological traits (mean ± SE) of *P. oceanica* leaves from acidified and control sites pH at Castello Aragonese (CA) and Chiane del Lume (CdL). ANOVA results testing the differences between sites and station are given.

Variable	Vent pH (mean ± SE)		Ambient pH (mean ± SE)		ANOVA	Sum of Squares	*F*-value	*p*-value
	CA (*n*=4)	CdL (*n*=3)	CA (*n*=4)	CdL (*n*=4)				
Shoot density (m^{-2})	504.13 ± 117.07	307.15 ± 57.50	315.12 ± 58.21	118.13 ± 56.26	Site (df=1)	132705	4.87	<0.05
					Station (df=1)	144120	5.29	<0.05
Leaf length (cm)	9.35 ± 0.21	19.08 ± 0.47	13.90 ± 0.58	23.63 ± 1.06	Site (df=1)	25454	262.08	<0.001
					Station (df=1)	5510	56.74	<0.001
Leaf width (cm)	0.912 ± 0.004	0.904 ± 0.004	1.026 ± 0.005	0.952 ± 0.004	Site (df=1)	1.86	379.73	<0.001
					Station (df=1)	0.36	73.80	<0.001
Leaf area index ($m^2\ m^{-2}$)	3.17 ± 0.43	5.25 ± 0.75	3.01 ± 0.31	5.09 ± 0.10	Site (df=1)	0.10	0.16	<0.001
					Station (df=1)	16.01	27.83	ns

SUPPLEMENTARY MATERIAL TO CHAPTER 3

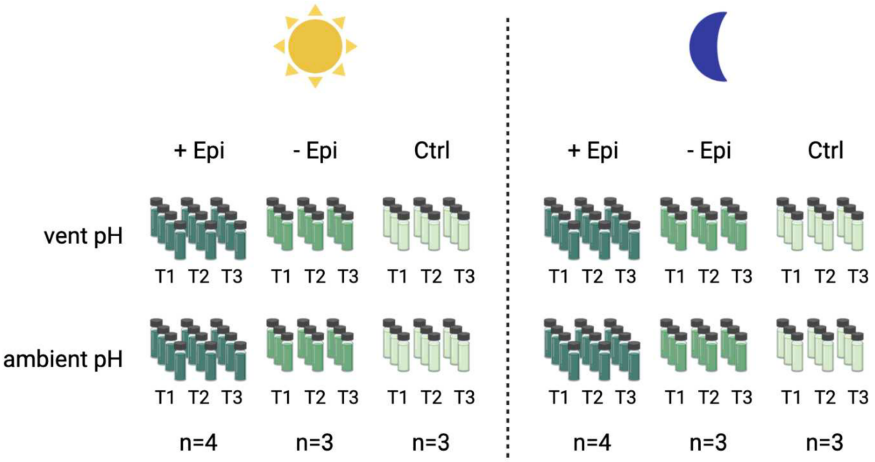

Fig. S3.1. Experimental design of the three isotopic tracer incubation experiments with light and dark incubations, leaves from vent and ambient pH, with epiphytes present or removed, as well as controls. T1, 2, and 3 represent samples that were opened at different timepoints.

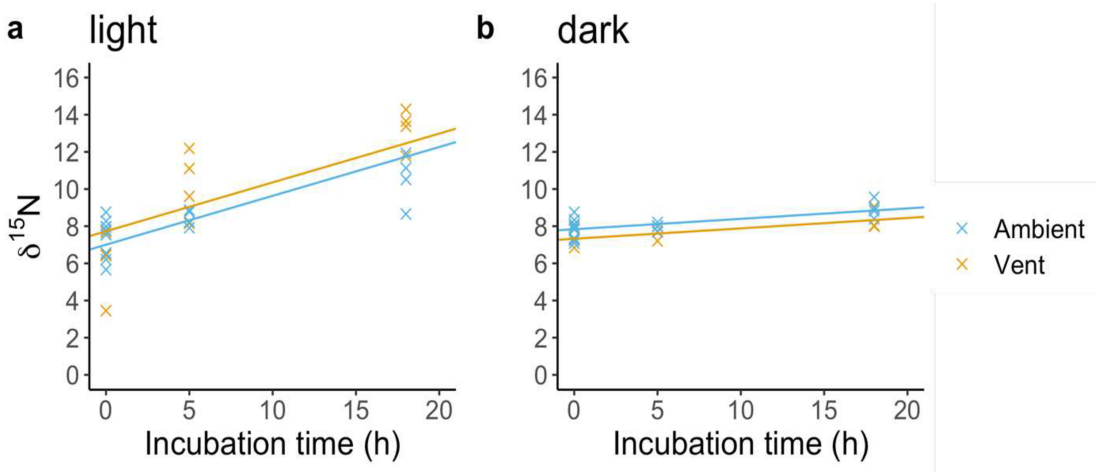

Fig. S3.2. $\delta^{15}N$ increase during light (**a**) and dark (**b**) incubations in epiphytes from the ambient and the vent site. Solid lines represent linear regressions.

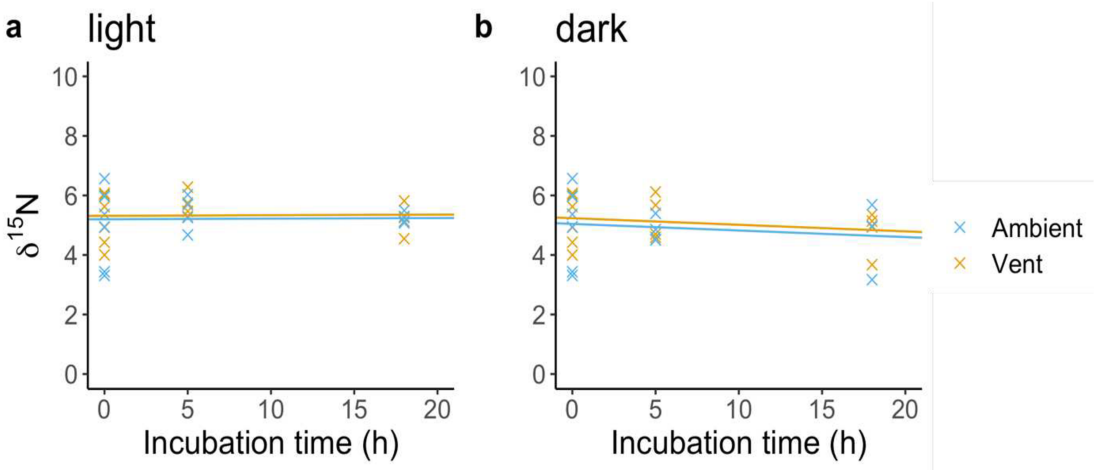

Fig. S3.3. $\delta^{15}N$ increase during light (**a**) and dark (**b**) incubations in seagrass leaf sections from the ambient and the vent site. Solid lines represent linear regressions.

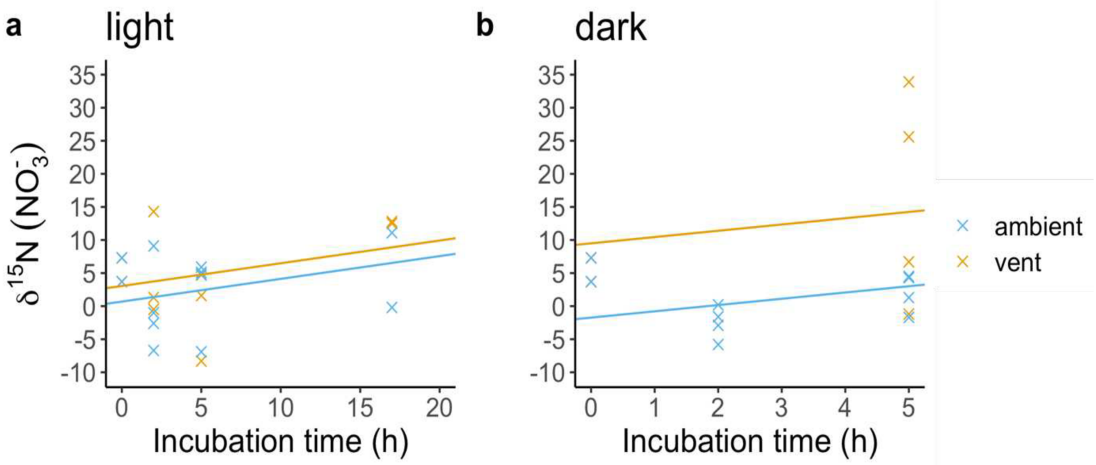

Fig. S3.4. $\delta^{15}N$ (NO_3^-) increase during light (a) and dark (b) incubations with seagrass leaf sections with epiphytes. Solid lines represent linear regressions.

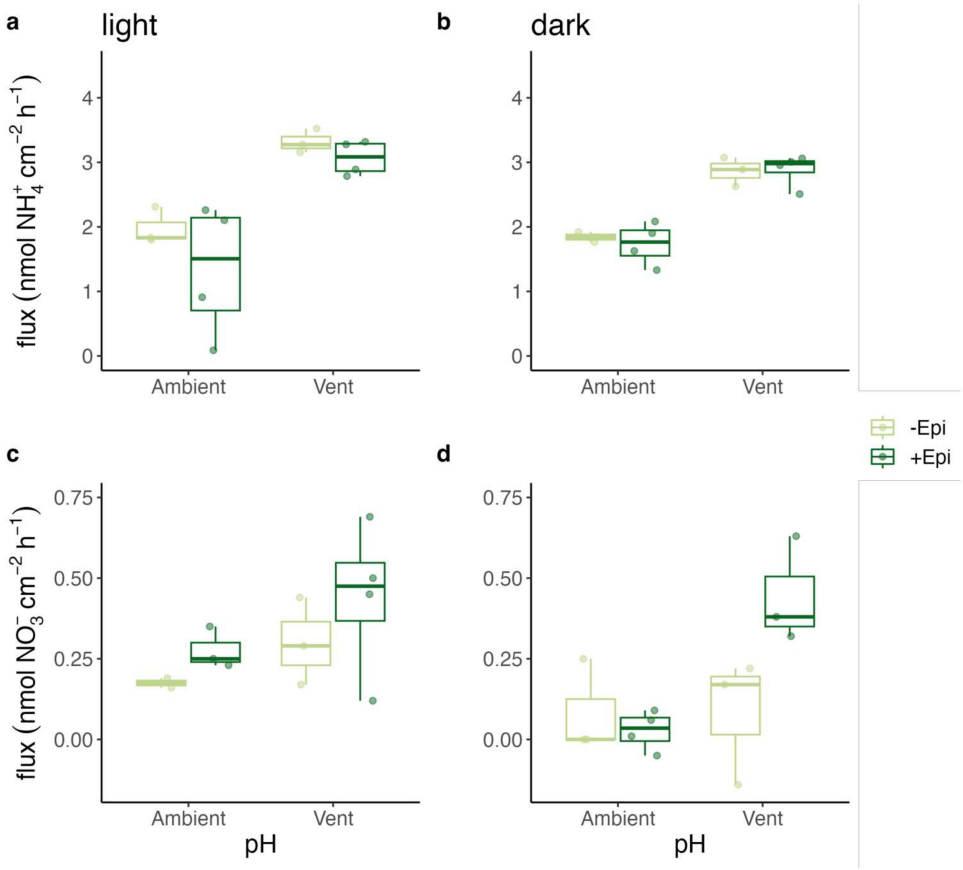

Fig. S3.5. Uptake of NH_4^+ (**a, b**) and NO_3^- (**c, d**) during light (**a, c**) and dark (**b, d**) incubations with leaf sections from the ambient and vent site with (+Epi, n=4) and without epiphytes (-Epi, n=3). The center line denotes the median value (50th percentile), the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles.

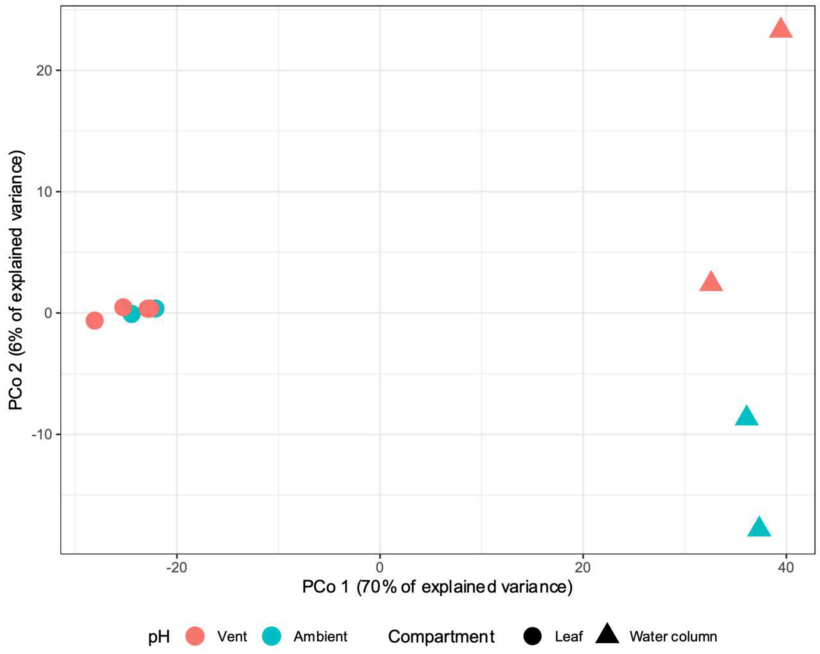

Fig. S3.6. Principal coordinates analysis of the prokaryote community from leaves (circles) and water column (triangles) samples of vent (red) and ambient (blue) pH sites.

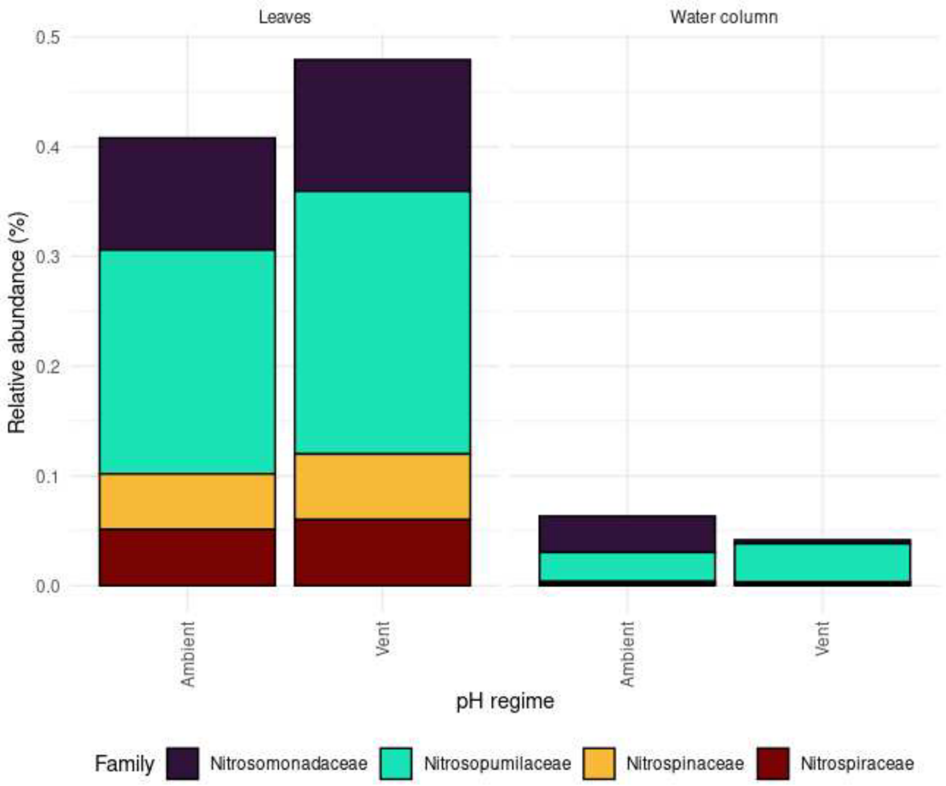

Fig. S3.7. Relative abundances of nitrifying prokaryotic taxa collapsed at the family level on leaves and water column samples from both pH regimes.

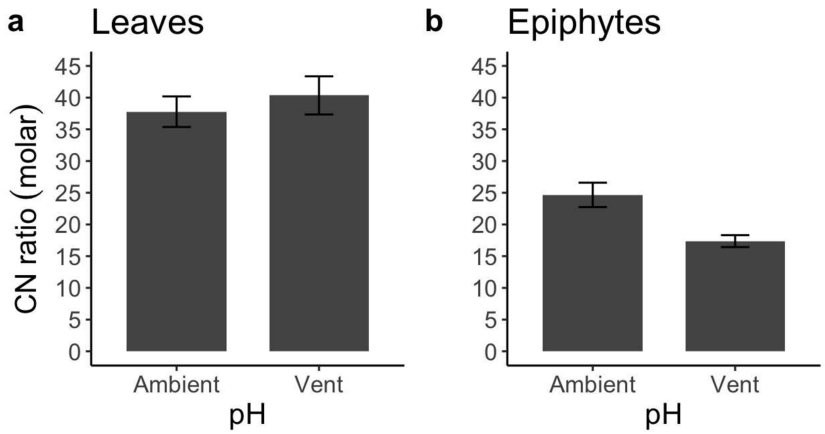

Fig. S3.8. C:N ratios of leaf sections (**a**) and epiphytes (**b**) from the ambient (*n* leaves = 14, *n* epiphytes =8) and vent site (*n* leaves = 14, *n* epiphytes =7). Since there were no differences between light and dark incubations, the samples were combined and treated as replicates. Error bars indicate mean ± SE.

Table S3.1. Permutation-based analysis of variance (PERMANOVA) of the microbial communities associated with *P. oceanica* leaves, water column and pH regime. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Source of variation	Df	SS	R^2	Pseudo-F	P(>F)
pH regime	1	822	0.06	2.01	0.189
Compartment	1	8929	0.70	21.88	**<0.001**
Treatment x Compartment	1	618	0.05	1.51	0.208
Residual	6	2449	0.19		
Total	9	12816	1.00		

Table S3.2. Permutation-based analysis of variance (PERMANOVA) of the nitrifying communities associated with *P. oceanica* leaves, water column and pH regime. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Source of variation	Df	SS	R^2	Pseudo-F	P(>F)
pH regime	1	1.16E-08	0.00	0.78	0.986
Compartment	1	1.44E-06	0.11	9.67	**<0.001**
Treatment x Compartment	1	2.25E-08	0.00	0.15	0.960
Residual	76	1.13E-05	0.89		
Total	79	1.28E-05	1.00		

Table S3.3. Environmental parameters (mean ± SE, n=3) measured at the vent and ambient pH site at Castello Aragonese.

	Ambient pH	Vent pH
T (°C)	23.94 ± 0.05	23.74 ± 0.01
Light (Lux)	10438 ± 872	16631 ± 628
pH	8.07 ± 0.08	7.06 ± 0.37
DO (mg L^{-1})	9.15 ± 0.02	8.26 ± 0.02

Average temperature, light, and DO were continuously measured with data loggers during the sampling time between 11 am and 4 pm of the respective sampling day. PH was measured on 13.09.2019 with a pH logger (*n* ambient = 15, *n* vent = 8).

Table S3.4. $^{29}N_2$ and $^{30}N_2$ concentrations in the denitrification experiment at different incubation timepoints (mean ± SD).

Site	Timepoint	Incubation	Treatment	$^{29}N_2$ concentration (nmol/L)	$^{30}N_2$ concentration (nmol/L)
Vent	T0			0.080 ± 0.015	0.263 ± 0.012
	T1	light	+Epi	0.075 ± 0.008	0.313 ± 0.024
	T1	light	-Epi	0.062 ± 0.006	0.355 ± 0.013
	T1	dark	+Epi	0.057 ± 0.008	0.323 ± 0.056
	T1	dark	-Epi	0.056 ± 0.008	0.249 ± 0.007
	T2	light	control	0.091 ± 0.018	0.019 ± 0.038
	T2	dark	control	0.042 ± 0.051	-0.015 ± 0.054
	T2	light	+Epi	0.144 ± 0.036	0.369 ± 0.059
	T2	light	-Epi	0.105 ± 0.027	0.091 ± 0.021
	T2	dark	+Epi	0.097 ± 0.013	0.069 ± 0.008
	T2	dark	-Epi	0.098 ± 0.010	0.062 ± 0.012
Ambient	T0			0.059 ± 0.015	0.302 ± 0.041
	T1	light	+Epi	0.022 ± 0.080	0.231 ± 0.063
	T1	light	-Epi	0.010 ± 0.114	0.229 ± 0.060
	T1	dark	+Epi	0.025 ± 0.067	0.206 ± 0.062
	T1	dark	-Epi	-0.006 ± 0.096	0.209 ± 0.009
	T2	light	control	0.104 ± 0.013	0.026 ± 0.006
	T2	dark	control	0.097 ± 0.012	0.050 ± 0.023
	T2	light	+Epi	0.122 ± 0.015	0.053 ± 0.037
	T2	light	-Epi	0.072 ± 0.051	0.036 ± 0.043
	T2	dark	+Epi	0.110 ± 0.014	0.024 ± 0.030
	T2	dark	-Epi	0.035 ± 0.048	-0.071 ± 0.104

Table S3.5. Morphological traits (mean ± SE) of *P. oceanica* from ambient and vent pH sites.

	Ambient pH	Vent pH
Shoot density (m^{-2})	527.38 ± 110.90	1130.09 ± 234.24
Leaf density (m^{-2})	4237.85 ± 515.01	7496.29 ± 674.21
Leaf dry weight (g)	0.041 ± 0.005	0.087 ± 0.013

SUPPLEMENTARY MATERIAL TO CHAPTER 4

SUPPLEMENTARY METHODS

To evaluate the reciprocal nutritional benefits between the sponge and the seagrass, we estimated the sponge daily respiratory carbon (C) demand and the seagrass daily nitrogen (N) demand in Fig. 4.6. These estimates were derived from sponge respiration data and plant NCP, respectively, assuming a 24h cycle for respiration and a respiratory/photosynthetic quotient of 1 for C demand, and a 12:12 h light/dark cycle and average plant C:N ratio of 16 for N demand (Fig. S4.3). To quantify the contribution of different C sources to the sponge respiratory C demand, we used the daily sponge DOC uptake and GPP rates to calculate the percentage of C demand met by heterotrophic DOC uptake and photoautotrophic C fixation, respectively. The remainder was attributed to particulate organic carbon (POC) uptake via filter-feeding. This approach was conservative because sponge DOC uptake was lower than seagrass DOC release, although it is possible that sponge DOC uptake (and its contribution to the sponge respiratory C demand) increased when in association with the seagrass. For the seagrass, we used daily plant NH_4^+ and NO_x^- uptake rates to conservatively estimate the percentage of its total daily N demand potentially fulfilled by NH_4^+ and NO_x^- released by the sponge. The remainder was attributed to other N sources. This approach was also conservative because seagrass uptake rates were lower than sponge release, although plant uptake rates (and their contribution to the plant N demand) may have increased when associated with the sponge.

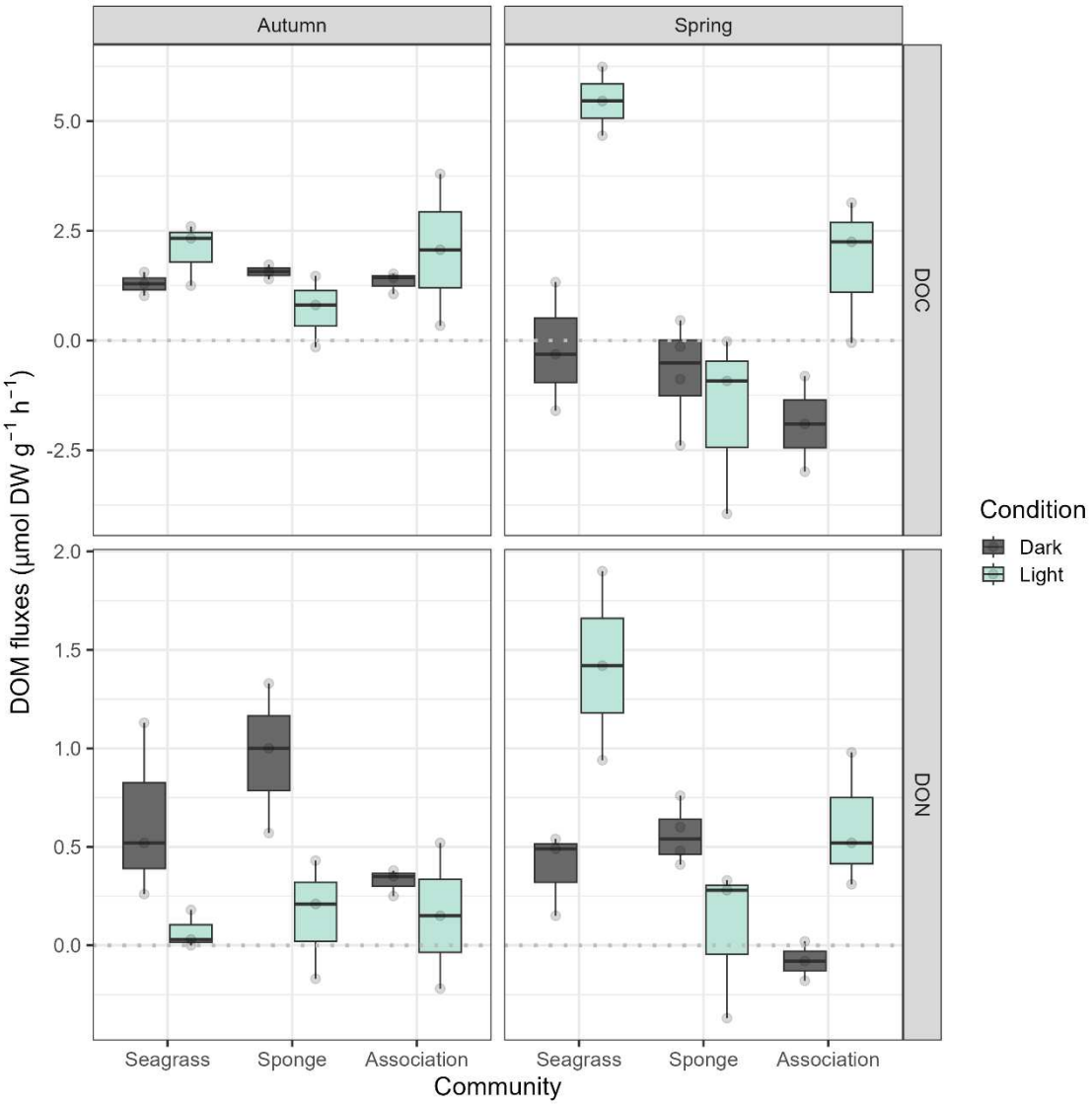

Fig. S4.1. Dissolved organic matter (DOM) fluxes expressed as µmol DW^{-1} g^{-1} h^{-1} for dissolved organic carbon (DOC, top panels) and dissolved organic nitrogen (DON, bottom panels) across three communities—*Posidonia oceanica* (Seagrass), *Chondrilla nucula* (Sponge), and their Association. Fluxes are shown separately for two seasons: Autumn (left) and Spring (right). Each boxplot displays the fluxes under dark (gray) and light (teal) conditions. Positive values represent net release, while negative values indicate uptake. The horizontal dashed line marks the zero-flux threshold. Whiskers denote variability across replicates, and the central line in each box indicates the median flux.

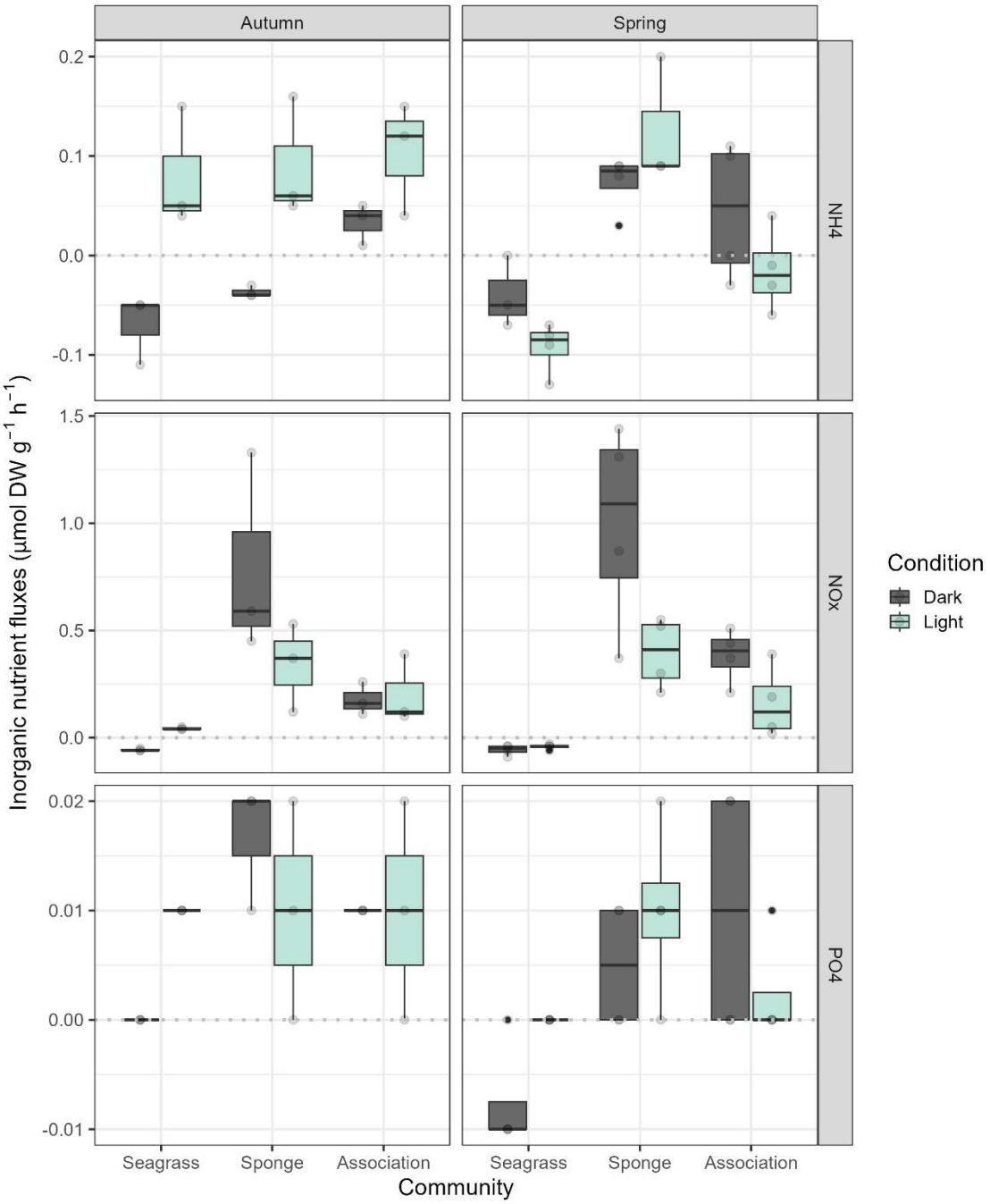

Fig. S4.2. Inorganic nutrient fluxes expressed as µmol DW^{-1} g^{-1} h^{-1} for ammonium (NH_4^+, top panels), nitrate+nitrite (NO_x^-, middle panels), and phosphate (PO_4^{3-}, bottom panels) across three communities—*Posidonia oceanica* (Seagrass), *Chondrilla nucula* (Sponge), and their Association. Fluxes are presented separately for Autumn (left) and Spring (right) seasons under dark (gray) and light (teal) conditions. Positive values represent net release, while negative values indicate uptake. The horizontal dashed line marks the zero-flux threshold. Whiskers display variability across replicates, with the central line in each box representing the median flux.

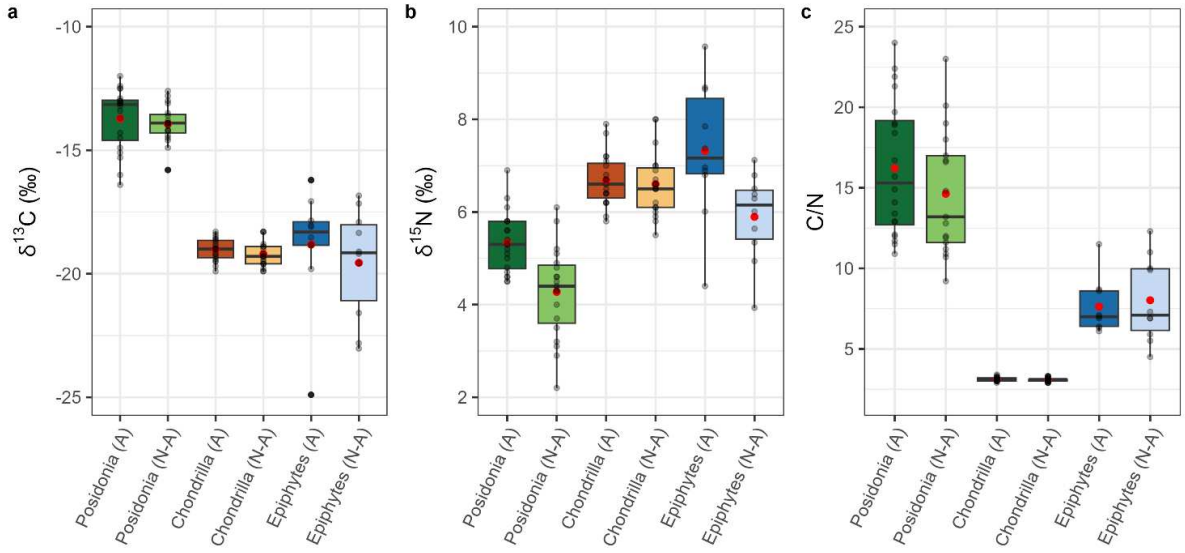

Fig. S4.3. Stable isotope composition and C/N ratios across communities in the association (A) and non-association (N-A) states. (a) $\delta^{13}C$ values (‰), (b) $\delta^{15}N$ values (‰), and (c) C/N ratios for *Posidonia oceanica*, *Chondrilla nucula*, and seagrass epiphytes. Each boxplot shows the distribution of values, with individual data points represented by black dots and the mean indicated by a red dot. Whiskers display the range of variability across replicates.

Table S1. Coefficients of asymmetric dependency between the benthic cover of the seagrass *Posidonia oceanica* and the sponge *Chondrilla nucula*. The coefficient q(X,Y) indicates the strength of the dependency of organism Y on organism X. The asymmetry coefficient quantifies the imbalance between these dependencies, with a non-significant value suggesting that the interaction does not exhibit a strong directional asymmetry.

Coefficients	**q**	**p-value**
q(*C. nucula*, *P. oceanica*)	0.249	**0.021**
q(*P. oceanica, C. nucula*)	0.381	**0.001**
asymmetry	-0.132	0.106

Table S4.2. Permutation-based analysis of variance (PERMANOVA) for net primary production (NPP, µmol O_2 g DW^{-1} h^{-1}), respiration (R, µmol O_2 g DW^{-1} h^{-1}), gross primary production (GPP, µmol O_2 g DW^{-1} h^{-1}) and daily net community production (NCP, µmol O_2 g DW^{-1} d^{-1}) across different *Community* types, *Seasons*, and their interaction. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Variable	Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
Net primary production (NPP)	Community	2	963.6	0.83	63.48	**0.001**
	Season	1	5.3	0.00	0.70	0.389
	Community:Season	2	81.9	0.07	5.40	**0.022**
	Residual	15	113.9	0.10		
	Total	20	1164.6	1.00		
Respiration (R)	Community	2	13.6	0.33	9.20	**0.001**
	Season	1	13.7	0.33	18.51	**0.001**
	Community:Season	2	4.0	0.10	2.68	0.108
	Residual	14	10.4	0.25		
	Total	19	41.7	1.00		
Gross primary production (GPP)	Community	2	2588.8	0.79	208.26	**0.001**
	Season	1	12.7	0.00	2.05	0.159
	Community:Season	2	271.5	0.08	21.84	**0.001**
	Residual	65	404.0	0.12		
	Total	70	3277.0	1.00		
Net community production (NCP)	Community	2	592852	0.84	332.33	**0.001**
	Season	1	14804	0.02	16.60	**0.003**
	Community:Season	2	38754	0.06	21.72	**0.001**
	Residual	65	57978	0.08		
	Total	70	704388	1.00		

Table S4.3. Adjusted p-values from multilevel pairwise comparisons of net primary production (NPP), gross primary production (GPP) and net community production (NCP) between *Community* and *Season*. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

NPP	Seagrass (Autumn)	Seagrass (Spring)	Sponge (Autumn)	Sponge (Spring)	Association (Autumn)	Association (Spring)
Seagrass (Autumn)	x					
Seagrass (Spring)	0.067	x				
Sponge (Autumn)	0.100	**0.034**	x			
Sponge (Spring)	**0.029**	**0.032**	0.136	x		
Association (Autumn)	0.500	**0.030**	0.100	**0.024**	x	
Association (Spring)	0.356	**0.025**	**0.031**	**0.030**	0.855	x

GPP	Seagrass (Autumn)	Seagrass (Spring)	Sponge (Autumn)	Sponge (Spring)	Association (Autumn)	Association (Spring)
Seagrass (Autumn)	x					
Seagrass (Spring)	**0.008**	x				
Sponge (Autumn)	**0.001**	**0.001**	x			
Sponge (Spring)	**0.001**	**0.001**	**0.001**	x		
Association (Autumn)	0.150	**0.001**	**0.002**	**0.001**	x	
Association (Spring)	**0.008**	**0.001**	**0.001**	**0.001**	0.375	x

NCP	Seagrass (Autumn)	Seagrass (Spring)	Sponge (Autumn)	Sponge (Spring)	Association (Autumn)	Association (Spring)
Seagrass (Autumn)	x					
Seagrass (Spring)	**0.001**	x				
Sponge (Autumn)	**0.001**	**0.001**	x			
Sponge (Spring)	**0.001**	**0.001**	0.602	x		
Association (Autumn)	0.419	**0.001**	**0.001**	**0.001**	x	
Association (Spring)	0.320	**0.001**	**0.001**	**0.001**	0.858	x

Table S4.4. PERMANOVA for hourly (µmol g DW^{-1} h^{-1}) and daily (µmol g DW^{-1} d^{-1}) DOC and DON fluxes across different *Community* types, *Seasons*, *Condition* (light vs dark, when present) and their interaction terms. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Variable	Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
Hourly DOC fluxes	Community	2	22.21	0.15	7.61	**0.001**
	Season	1	14.81	0.10	10.15	**0.005**
	Condition	1	14.82	0.10	10.16	**0.004**
	Community:Season	2	12.04	0.08	4.13	**0.040**
	Community:Condition	2	24.93	0.17	8.54	**0.001**
	Season:Condition	1	13.32	0.09	9.13	**0.009**
	Community:Season:Condition	2	9.06	0.06	3.10	0.057
	Residual	24	35.01	0.24		
	Total	35	146.20	1.00		
Hourly DON fluxes	Community	2	0.57	0.08	2.89	0.082
	Season	1	0.04	0.01	0.46	0.513
	Condition	1	0.10	0.01	1.01	0.341
	Community:Season	2	0.64	0.09	3.26	0.053
	Community:Condition	2	1.45	0.19	7.40	**0.003**
	Season:Condition	1	1.75	0.23	17.82	**0.002**
	Community:Season:Condition	2	0.58	0.08	2.97	0.071
	Residual	24	2.36	0.31		
	Total	35	7.50	1.00		
Daily DOC fluxes	Community	2	25459	0.38	38.82	**0.001**
	Season	1	11740	0.17	35.80	**0.001**
	Community:Season	2	14172	0.21	21.61	**0.001**
	Residual	48	15741	0.23		
	Total	53	67112	1.00		
Daily DON fluxes	Community	2	502.44	0.21	11.53	**0.001**
	Season	1	33.01	0.01	1.51	0.225
	Community:Season	2	776.75	0.33	17.82	**0.001**
	Residual	48	1045.98	0.44		
	Total	53	2358.18	1.00		

Table S4.5. Adjusted p-values from multilevel pairwise comparisons of hourly DOC and DON fluxes between *Community* and *Condition*, and daily DOC and DON fluxes between *Community* and *Season*. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

Hourly DOC fluxes	Seagrass (daylight)	Seagrass (dark)	Sponge (daylight)	Sponge (dark)	Association (daylight)	Association (dark)
Seagrass (daylight)	x					
Seagrass (dark)	**0.011**	x				
Sponge (daylight)	**0.008**	0.337	x			
Sponge (dark)	**0.009**	0.710	0.495	x		
Association (daylight)	0.186	0.118	**0.031**	0.078	x	
Association (dark)	**0.017**	0.369	0.871	0.578	0.050	x

Hourly DON fluxes	Seagrass (daylight)	Seagrass (dark)	Sponge (daylight)	Sponge (dark)	Association (daylight)	Association (dark)
Seagrass (daylight)	x					
Seagrass (dark)	0.832	x				
Sponge (daylight)	0.198	0.071	x			
Sponge (dark)	0.707	0.257	**0.001**	x		
Association (daylight)	0.600	0.533	0.229	0.126	x	
Association (dark)	0.216	**0.034**	0.975	**0.004**	0.204	x

Daily DOC fluxes	Seagrass (Autumn)	Seagrass (Spring)	Sponge (Autumn)	Sponge (Spring)	Association (Autumn)	Association (Spring)
Seagrass (Autumn)	x					
Seagrass (Spring)	**0.010**	x				
Sponge (Autumn)	**0.004**	**0.001**	x			
Sponge (Spring)	**0.001**	**0.001**	**0.001**	x		
Association (Autumn)	0.925	**0.048**	0.068	**0.001**	x	
Association (Spring)	**0.001**	**0.001**	**0.002**	**0.020**	**0.002**	x

Daily DON fluxes	Seagrass (Autumn)	Seagrass (Spring)	Sponge (Autumn)	Sponge (Spring)	Association (Autumn)	Association (Spring)
Seagrass (Autumn)	x					
Seagrass (Spring)	**0.001**	x				
Sponge (Autumn)	**0.049**	**0.020**	x			
Sponge (Spring)	0.705	**0.001**	**0.009**	x		
Association (Autumn)	0.193	**0.001**	**0.003**	0.316	x	
Association (Spring)	0.307	**0.001**	**0.009**	0.443	0.753	x

Table S4.6. PERMANOVA for hourly (µmol g DW^{-1} h^{-1}) and daily (µmol g DW^{-1} d^{-1}) NH_4^+, NO_x^-, PO_4^{3-} fluxes (µmol g DW^{-1} h^{-1}) across different *Community* types, *Seasons*, *Condition* (light vs dark, when present) and their interaction terms. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Variable	Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
Hourly NH_4^+ fluxes	Community	2	0.07	0.27	16.38	**0.001**
	Season	1	0.00	0.02	1.93	0.178
	Condition	1	0.01	0.05	6.44	**0.026**
	Community:Season	2	0.04	0.16	9.86	**0.003**
	Community:Condition	2	0.01	0.06	3.41	**0.050**
	Season:Condition	1	0.04	0.18	21.09	**0.001**
	Community:Season:Condition	2	0.01	0.03	1.58	0.233
	Residual	28	0.06	0.23		
	Total	39	0.25	1.00		
Hourly NO_x^- fluxes	Community	2	3.27	0.56	31.06	**0.001**
	Season	1	0.06	0.01	1.22	0.298
	Condition	1	0.36	0.06	6.76	**0.009**
	Community:Season	2	0.08	0.01	0.72	0.509
	Community:Condition	2	0.52	0.09	4.91	**0.013**
	Season:Condition	1	0.05	0.01	0.96	0.347
	Community:Season:Condition	2	0.01	0.00	0.13	0.872
	Residual	28	1.47	0.25		
	Total	39	5.82	1.00		
Hourly PO_4^{3-} fluxes	Community	2	0.001	0.29	9.24	**0.002**
	Season	1	0.000	0.08	4.93	**0.040**
	Condition	1	0.000	0.00	0.00	0.980
	Community:Season	2	0.000	0.04	1.13	0.328
	Community:Condition	2	0.000	0.05	1.62	0.204
	Season:Condition	1	0.000	0.00	0.06	0.797
	Community:Season:Condition	2	0.000	0.09	2.86	0.086
	Residual	28	0.001	0.45		
	Total	39	0.002	1.00		
Daily NH_4^+ fluxes	Community	2	79.80	0.58	86.21	**0.001**
	Season	1	0.89	0.01	1.92	0.189
	Community:Season	2	30.60	0.22	33.06	**0.001**
	Residual	58	26.84	0.19		
	Total	63	138.13	1.00		
Daily NO_x^- fluxes	Community	2	2763.4	0.78	121.04	**0.001**
	Season	1	50.6	0.01	4.43	**0.048**
	Community:Season	2	53.8	0.02	2.35	0.116
	Residual	58	662.1	0.19		
	Total	63	3529.8	1.00		
Daily PO_4^{3-} fluxes	Community	2	0.77	0.48	37.66	**0.001**
	Season	1	0.23	0.14	21.96	**0.001**
	Community:Season	2	0.03	0.02	1.59	0.186
	Residual	58	0.60	0.37		
	Total	63	1.63	1.00		

Table S4.7. Adjusted p-values from multilevel pairwise comparisons of hourly NH_4^+ and NO_x^- fluxes between *Community* and *Condition*, and daily NH_4^+ fluxes between *Community* and *Season*. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

Hourly NH_4^+ fluxes	Seagrass (daylight)	Seagrass (dark)	Sponge (daylight)	Sponge (dark)	Association (daylight)	Association (dark)
Seagrass (daylight)	x					
Seagrass (dark)	0.427	x				
Sponge (daylight)	**0.023**	**0.002**	x			
Sponge (dark)	0.359	**0.019**	**0.035**	x		
Association (daylight)	0.285	**0.018**	0.110	0.802	x	
Association (dark)	0.197	**0.005**	0.059	0.628	0.886	x

Hourly NO_x^- fluxes	Seagrass (daylight)	Seagrass (dark)	Sponge (daylight)	Sponge (dark)	Association (daylight)	Association (dark)
Seagrass (daylight)	x					
Seagrass (dark)	0.051	x				
Sponge (daylight)	**0.002**	**0.005**	x			
Sponge (dark)	**0.001**	**0.004**	**0.037**	x		
Association (daylight)	**0.005**	**0.004**	**0.038**	**0.002**	x	
Association (dark)	**0.002**	**0.001**	0.228	**0.002**	0.192	x

Daily NH_4^+ fluxes	Seagrass (Autumn)	Seagrass (Spring)	Sponge (Autumn)	Sponge (Spring)	Association (Autumn)	Association (Spring)
Seagrass (Autumn)	x					
Seagrass (Spring)	**0.001**	x				
Sponge (Autumn)	**0.002**	**0.001**	x			
Sponge (Spring)	**0.002**	**0.001**	**0.001**	x		
Association (Autumn)	**0.001**	**0.001**	**0.009**	**0.027**	x	
Association (Spring)	0.105	**0.001**	0.368	**0.001**	**0.005**	x

Table S4.8. PERMANOVA for $\delta^{13}C$ and $\delta^{15}N$ values and C:N ratios across different *Sample* types and *Association* types. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Variable	Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
$\delta^{13}C$ values	Sample	2	658.12	0.82	208.75	**0.001**
	Association	1	2.35	0.00	1.49	0.236
	Sample:Association	2	1.15	0.00	0.37	0.700
	Residual	91	143.45	0.18		
	Total	96	805.07	1.00		
$\delta^{15}N$ values	Sample	2	75.99	0.46	50.64	**0.001**
	Association	1	13.83	0.08	18.44	**0.001**
	Sample:Association	2	7.56	0.05	5.04	**0.005**
	Residual	91	68.28	0.41		
	Total	96	165.66	1.00		
C:N ratios	Sample	2	3241.8	0.71	112.31	**0.001**
	Association	1	6.7	0.00	0.46	0.509
	Sample:Association	2	9.4	0.00	0.32	0.692
	Residual	91	1313.4	0.29		
	Total	96	4571.3	1.00		

Table S4.9. Adjusted p-values from multilevel pairwise comparisons of $\delta^{15}N$ values between *Sample* types and *Association* types. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

$\delta^{15}N$ values	*P. oceanica* leaves (associated)	*P. oceanica* leaves (not associated)	*C. nucula* (associated)	*C. nucula* (not associated)	*P. oceanica* epiphytes (associated)	*P. oceanica* epiphytes (not associated)
P. oceanica leaves (associated)	x					
P. oceanica leaves (not associated)	**0.001**	x				
C. nucula (associated)	**0.001**	**0.001**	x			
C. nucula (not associated)	**0.001**	**0.001**	0.734	x		
P. oceanica epiphytes (associated)	**0.001**	**0.001**	0.106	0.104	x	
P. oceanica epiphytes (not associated)	0.064	**0.001**	**0.011**	**0.029**	**0.027**	x

SUPPLEMENTARY MATERIAL TO CHAPTER 5

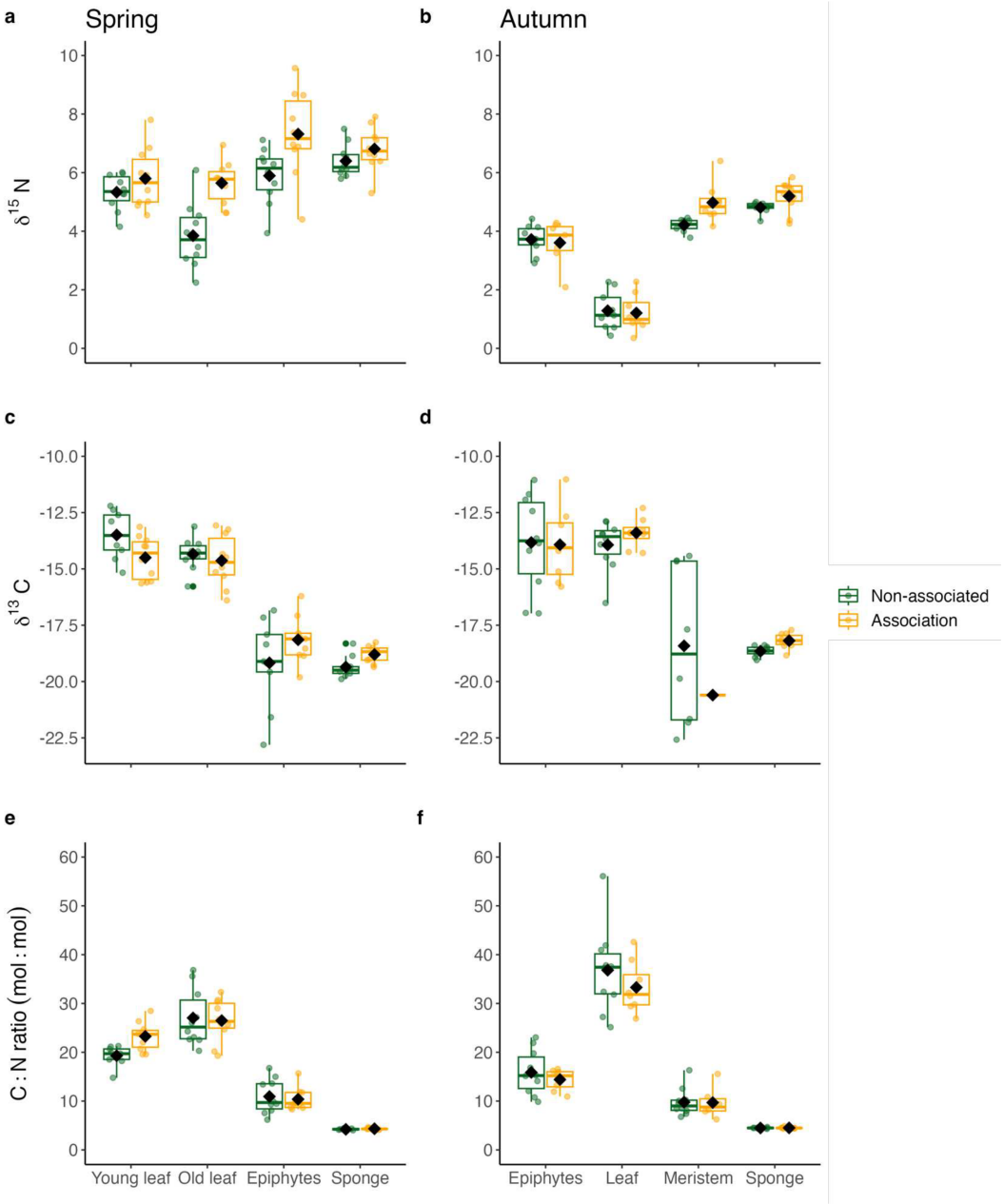

Fig. S5.1. Stable isotope composition and C/N ratios across communities in associated and non-associated states in spring (a, c, e) and autumn (b, d, f). (a, b) $\delta^{15}N$ (‰), (c, d) $\delta^{13}C$ (‰) and (e, f) C:N ratios for young and old *Posidonia oceanica* leaves, seagrass epiphytes and the sponge *Chondrilla nucula*. The center line denotes the median value (50th percentile), the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles. Black squares indicate mean values.

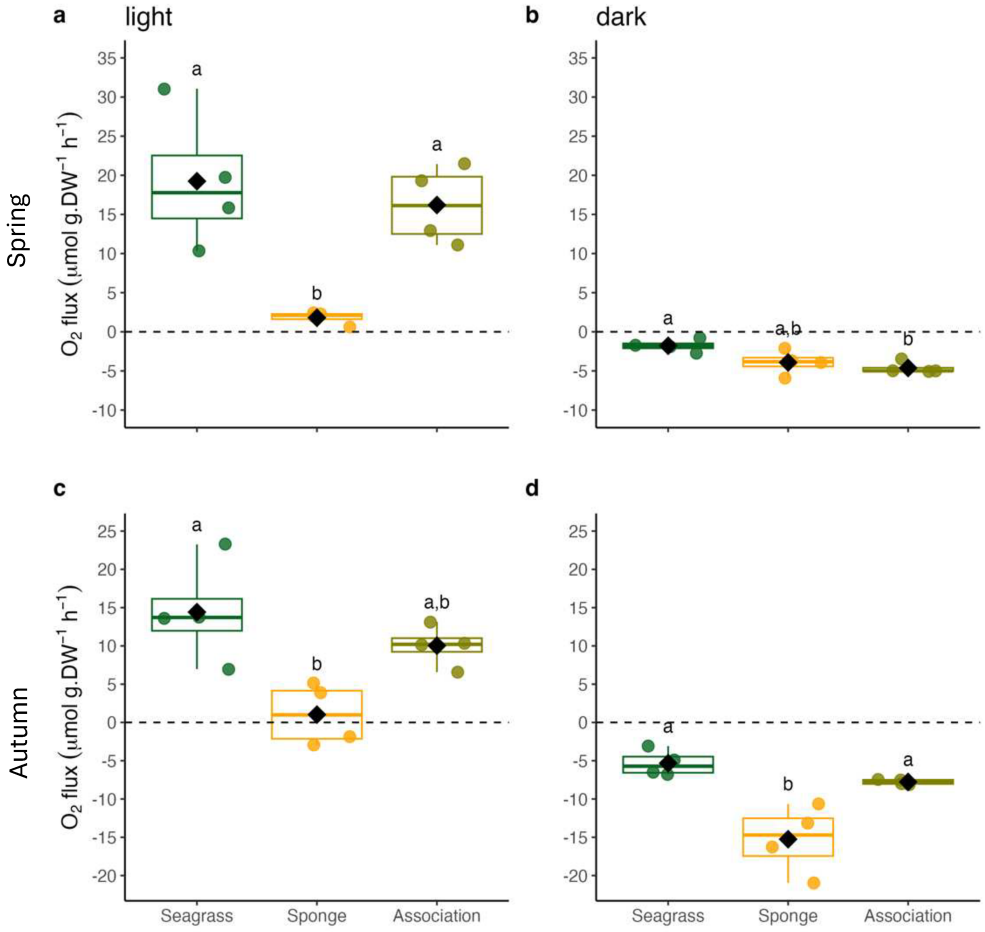

Fig. S5.2. O_2 fluxes of the seagrass, sponge and the association. (a, c) Net community production (NCP) and (c, d) community respiration (CR) in incubations in spring (a, b) and autumn (c, d). Positive values indicate O_2 production, negative values indicate respiration. The center line denotes the median value (50th percentile), the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles. The horizontal dashed line marks the zero-flux threshold. Black squares indicate mean values; letters indicate significant differences between treatments, n=4.

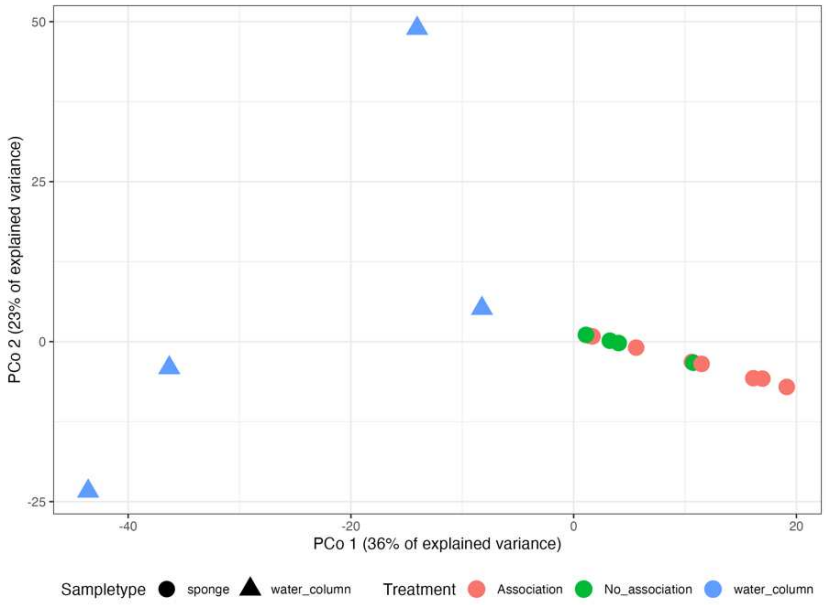

Fig. S5.3. Principal coordinates analysis of the bacterial community from *Chondrilla nucula* growing alone or in association and the water column community.

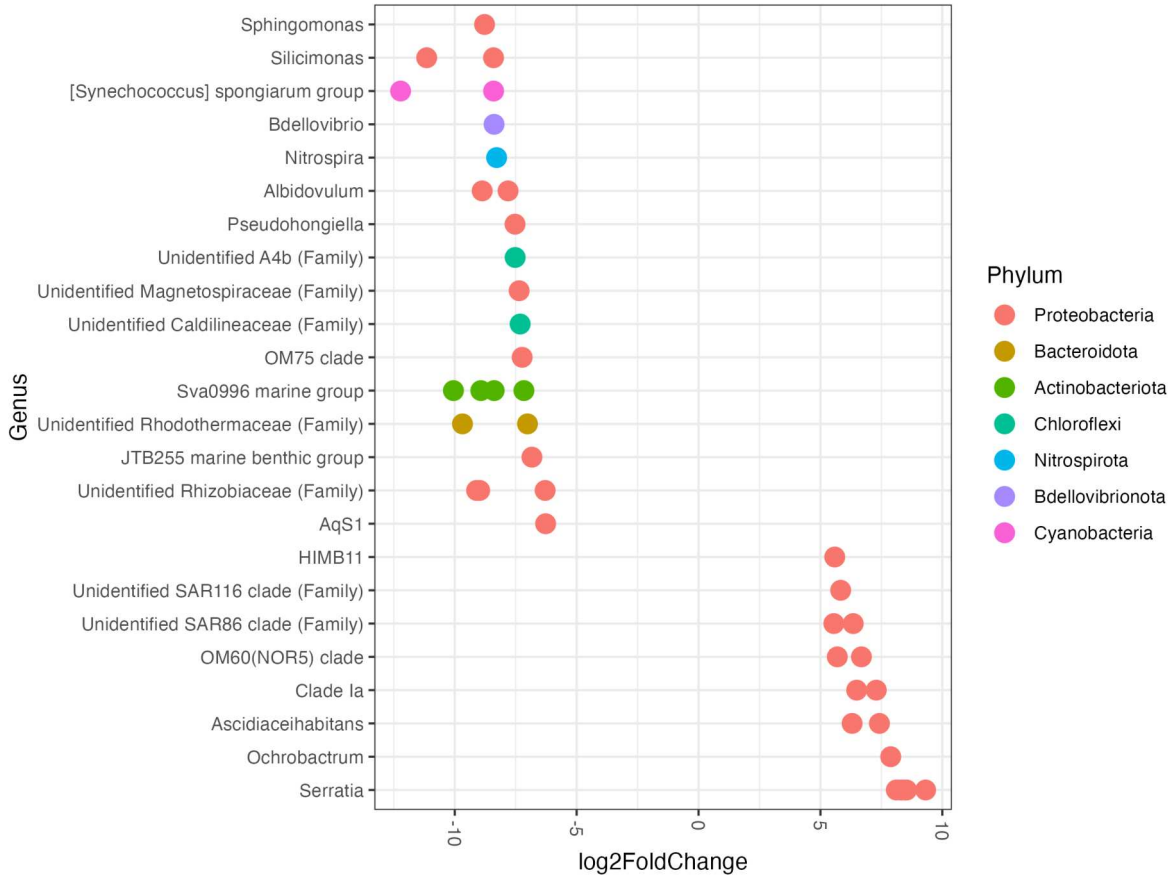

Fig. S5.4. Differential abundances in sponge and water column samples as log2FoldChange. Positive values mean differential abundance is higher in the water column and negative values higher in the sponges.

Table S5.1. Permutation-based analysis of variance (PERMANOVA) for PNR (nmol N g DW^{-1} h^{-1}) on different *community* types, *incubation* types, *seasons*, and their interactions. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Source of variation	**Df**	**SS**	**R^2**	**Pseudo-F**	**P (>F)**
Community	2	79707	0.29	61.26	**0.001**
Season	1	58900	0.21	90.54	**0.001**
Incubation	1	16269	0.06	25.01	**0.001**
Community: Season	2	63357	0.23	48.70	**0.001**
Community: Incubation	2	16468	0.06	12.66	**0.002**
Season: Incubation	1	9010	0.03	13.85	**0.001**
Community: Season: Incubation	2	12072	0.04	9.28	**0.002**
Residual	31	20166	0.07		
Total	42	275949	1.00		

Table S5.2. Adjusted p-values from multilevel pairwise comparisons of PNR between *community* and *season*. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

PNR	Seagrass (spring)	Seagrass (autumn)	Sponge (spring)	Sponge (autumn)	Association (spring)	Association (autumn)
Seagrass (spring)	x					
Seagrass (autumn)	1.000	x				
Sponge (spring)	0.988	0.999	x			
Sponge (autumn)	0.637	0.724	0.857	x		
Association (spring)	0.992	0.995	1.000	0.914	x	
Association (autumn)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	x

Table S5.3. Adjusted p-values from multilevel pairwise comparisons of PNR between *community* and *incubation* type. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

PNR	Seagrass (light)	Seagrass (dark)	Sponge (light)	Sponge (dark)	Association (light)	Association (dark)
Seagrass (light)	x					
Seagrass (dark)	1.000	x				
Sponge (light)	0.998	0.997	x			
Sponge (dark)	0.971	0.956	0.999	x		
Association (light)	0.688	0.595	0.863	0.967	x	
Association (dark)	**0.006**	**0.002**	**0.008**	**0.016**	0.109	x

Table S5.4. Permutation-based analysis of variance (PERMANOVA) of $\delta^{15}N$ (‰), $\delta^{13}C$ (‰), and C:N ratio (mol:mol) on different *tissue* types, *association* types, *seasons*, and their interaction. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Variable	Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
$\delta^{15}N$	Tissue	5	276.89	0.56	88.75	**0.001**
	Association	1	18.50	0.04	29.65	**0.001**
	Season	1	93.24	0.19	149.43	**0.001**
	Tissue: Association	5	9.60	0.02	3.08	**0.018**
	Tissue: Season	1	7.45	0.02	11.95	**0.001**
	Association: Season	1	2.69	0.01	4.32	**0.031**
	Tissue: Association: Season	1	2.63	0.01	4.22	**0.045**
	Residual	136	82.99	0.17		
	Total	151	494.01	1.00		
$\delta^{13}C$	Tissue	5	559.73	0.55	58.59	**0.001**
	Association	1	0.02	0.00	0.01	0.925
	Season	1	131.74	0.13	68.95	**0.001**
	Tissue: Association	5	14.15	0.01	1.48	0.198
	Tissue: Season	1	79.56	0.08	41.64	**0.001**
	Association: Season	1	1.61	0.00	0.84	0.331
	Tissue: Association: Season	1	1.22	0.00	0.64	0.420
	Residual	124	236.93	0.23		
	Total	139	1024.97	1.00		
C:N	Tissue	5	15645.9	0.87	211.15	**0.001**
	Association	1	2.5	0.00	0.17	0.674
	Season	1	104.1	0.01	7.02	**0.010**
	Tissue: Association	5	139.9	0.01	1.89	0.115
	Tissue: Season	1	86.3	0.01	5.82	**0.017**
	Association: Season	1	1.2	0.00	0.08	0.773
	Tissue: Association: Season	1	0.8	0.00	0.05	0.812
	Residual	132	1956.2	0.11		
	Total	147	17936.8	1.00		

Table S5.5. Adjusted p-values from multilevel pairwise comparisons of $\delta^{15}N$ (‰) between *tissue* types and *association* types in spring. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

$\delta^{15}N$	Young leaves (associated)	Young leaves (not associated)	Old leaves (associated)	Old leaves (not associated)	Epiphytes (associated)	Epiphytes (not associated)	Sponge (associated)	Sponge (not associated)
Young leaves (associated)	x							
Young leaves (not associated)	0.955	x						
Old leaves (associated)	1.000	0.996	x					
Old leaves (not associated)	**<0.001**	**0.017**	**0.002**	x				
Epiphytes (associated)	**0.014**	**<0.001**	**0.004**	**<0.001**	x			
Epiphytes (not associated)	1.000	0.887	0.999	**<0.001**	**0.026**	x		
Sponge (associated)	0.267	0.018	0.126	**<0.001**	0.930	0.389	x	
Sponge (not associated)	0.849	0.207	0.638	**<0.001**	0.384	0.932	0.977	x

Table S5.6. Adjusted p-values from multilevel pairwise comparisons of $\delta^{15}N$ (‰) between *tissue* types and *association* types in autumn. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

$\delta^{15}N$	Leaves (associated)	Leaves (not associated)	Epiphytes (associated)	Epiphytes (not associated)	Meristem (associated)	Meristem (not associated)	Sponge (associated)	Sponge (not associated)
Leaves (associated)	x							
Leaves (not associated)	1.000	x						
Epiphytes (associated)	**<0.001**	**<0.001**	x					
Epiphytes (not associated)	**<0.001**	**<0.001**	1.000	x				
Meristem (associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	x			
Meristem (not associated)	**<0.001**	**<0.001**	0.369	0.517	0.092	x		
Sponge (associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.990	**0.005**	x	
Sponge (not associated)	**<0.001**	**<0.001**	**0.002**	**0.002**	0.999	0.333	0.808	x

Table S5.7. Adjusted p-values from multilevel pairwise comparisons of $\delta^{13}C$ (‰) between *tissue* types and *association* types in spring. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

$\delta^{13}C$	Young leaves (associated)	Young leaves (not associated)	Old leaves (associated)	Old leaves (not associated)	Epiphytes (associated)	Epiphytes (not associated)	Sponge (associated)	Sponge (not associated)
Young leaves (associated)	x							
Young leaves (not associated)	0.452	x						
Old leaves (associated)	1.000	0.292	x					
Old leaves (not associated)	1.000	0.664	0.999	x				
Epiphytes (associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	x			
Epiphytes (not associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.461	x		
Sponge (associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.894	0.995	x	
Sponge (not associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.251	1.000	0.952	x

Table S5.8. Adjusted p-values from multilevel pairwise comparisons of $\delta^{13}C$ (‰) between *tissue* types and *association* types in autumn. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

$\delta^{13}C$	Leaves (associated)	Leaves (not associated)	Epiphytes (associated)	Epiphytes (not associated)	Meristem (associated)	Meristem (not associated)	Sponge (associated)	Sponge (not associated)
Leaves (associated)	x							
Leaves (not associated)	0.997	x						
Epiphytes (associated)	0.998	1.000	x					
Epiphytes (not associated)	0.999	1.000	1.000	x				
Meristem (associated)	**0.004**	**0.008**	**0.009**	**0.007**	x			
Meristem (not associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.921	x		
Sponge (associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.867	1.000	x	
Sponge (not associated)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.953	1.000	0.998	x

Table S5.9. Permutation-based analysis of variance (PERMANOVA) of daily NH_4^+, NO_3^- and NO_2^- fluxes (μmol g DW^{-1} d^{-1}) on different *community* types, *seasons*, and their interaction. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Variable	Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
NH_4^+	Community	2	919	0.14	2.94	0.079
	Season	1	2671	0.39	17.09	**0.001**
	Community: Season	2	681	0.10	2.18	**0.142**
	Residual	16	2501	0.37		
	Total	21	6771	1.00		
NO_3^-	Community	2	2848	0.76	54.54	**0.001**
	Season	1	5.4	0.00	0.21	0.651
	Community: Season	2	400	0.11	7.66	**0.003**
	Residual	18	470	0.13		
	Total	23	3724	1.00		
NO_2^-	Community	2	0.39	0.27	9.98	**0.002**
	Season	1	0.65	0.45	33.06	**0.001**
	Community: Season	2	0.07	0.05	1.89	0.184
	Residual	17	0.34	0.23		
	Total	22	1.49	1.00		

Table S5.10. Adjusted p-values from multilevel pairwise comparisons of daily NH_4^+, NO_3^- and NO_2^- fluxes (μmol g DW^{-1} d^{-1}) between *community* and *season*. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

Variable		Seagrass (spring)	Seagrass (autumn)	Sponge (spring)	Sponge (autumn)	Association (spring)	Association (autumn)
NH_4^+	Seagrass (spring)	x					
	Seagrass (autumn)	0.926	x				
	Sponge (spring)	0.077	**0.012**	x			
	Sponge (autumn)	0.941	1.000	**0.021**	x		
	Association (spring)	0.553	0.142	0.782	0.191	x	
	Association (autumn)	0.880	1.000	**0.015**	1.000	0.140	x
NO_3^-	Seagrass (spring)	x					
	Seagrass (autumn)	0.082	x				
	Sponge (spring)	**<0.001**	**<0.001**	x			
	Sponge (autumn)	**<0.001**	**<0.001**	0.604	x		
	Association (spring)	0.968	**0.017**	**0.005**	**<0.001**	x	
	Association (autumn)	0.089	**<0.001**	0.274	**0.013**	0.330	x
NO_2^-	Seagrass (spring)	x					
	Seagrass (autumn)	0.078	x				
	Sponge (spring)	**0.047**	**<0.001**	x			
	Sponge (autumn)	0.525	0.911	**0.002**	x		
	Association (spring)	0.946	0.340	**0.008**	0.934	x	
	Association (autumn)	**0.045**	1.000	**<0.001**	0.798	0.221	x

Table S5.11. Permutation-based analysis of variance (PERMANOVA) of daily DOC and DON fluxes (μmol g DW^{-1} d^{-1}) on different *community* types, *seasons*, and their interaction. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Variable	Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
DOC	Community	2	129813	0.69	28.25	**0.001**
	Season	1	52	0.00	0.02	0.870
	Community: Season	2	20703	0.11	4.51	**0.039**
	Residual	17	39058	0.21		
	Total	22	189626	1.00		
DON	Community	2	8131	0.85	14.33	**0.001**
	Season	1	39	0.00	0.67	0.366
	Community: Season	2	603	0.06	1.15	**0.011**
	Residual	16	763	0.08		
	Total	21	9535	1.00		

Table S5.12. Adjusted p-values from multilevel pairwise comparisons of daily DOC and DON fluxes (μmol g DW^{-1} d^{-1}) between *community* and *season*. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

Variable		Seagrass (spring)	Seagrass (autumn)	Sponge (spring)	Sponge (autumn)	Association (spring)	Association (autumn)
DOC	Seagrass (spring)	x					
	Seagrass (autumn)	0.766	x				
	Sponge (spring)	**0.007**	**<0.001**	x			
	Sponge (autumn)	**0.034**	**0.002**	0.964	x		
	Association (spring)	0.074	**0.005**	0.831	0.998	x	
	Association (autumn)	**<0.001**	**<0.001**	0.690	0.286	0.157	x
DON	Seagrass (spring)	x					
	Seagrass (autumn)	1.000	x				
	Sponge (spring)	**<0.001**	**<0.001**	x			
	Sponge (autumn)	**<0.001**	**<0.001**	0.054	x		
	Association (spring)	1.000	1.000	**<0.001**	**<0.001**	x	
	Association (autumn)	0.500	0.411	**<0.001**	**<0.001**	0.481	x

Table S5.13. Permutation-based analysis of variance (PERMANOVA) of O_2 fluxes (μmol g DW^{-1} d^{-1}) on different *community* types, *incubation* types, *seasons*, and their interactions. The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Source of variation	Df	SS	R^2	Pseudo-F	P (>F)
Community	2	971.4	0.17	29.62	**0.001**
Season	1	295.7	0.05	18.03	**0.001**
Incubation	1	3426.4	0.61	208.98	**0.001**
Community: Season	2	7.5	0.00	0.23	0.791
Community: Incubation	2	211.1	0.04	6.44	**0.005**
Season: Incubation	1	13.0	0.00	0.79	0.387
Community: Season: Incubation	2	109.5	0.02	3.34	**0.049**
Residual	36	590.2	0.10		
Total	47	5624.8	1.00		

Table S5.14. Adjusted p-values from multilevel pairwise comparisons of O_2 fluxes between *community* and *season*. The comparisons are performed using Tukey's honest significant difference (HSD) test. Bold p-values indicate combinations that differ significantly ($p < 0.05$).

O_2	Seagrass (spring)	Seagrass (autumn)	Sponge (spring)	Sponge (autumn)	Association (spring)	Association (autumn)
Seagrass (spring)	x					
Seagrass (autumn)	0.962	x				
Sponge (spring)	0.402	0.878	x			
Sponge (autumn)	**0.036**	0.220	0.840	x		
Association (spring)	0.992	1.000	0.758	0.137	x	
Association (autumn)	0.670	0.984	0.998	0.590	0.940	x

Table S5.15. Permutation-based analysis of variance (PERMANOVA) of the nitrifying community abundance between sample type (sponge vs water column) and association type (associated vs non-associated). The table provides the degrees of freedom (Df), sum of squares (SS), proportion of variance explained (R^2), pseudo-F statistics, and associated p-values (P(>F)) for each source of variation. Bold p-values ($p < 0.05$) indicate which factors contribute to differences in the measured variables.

Source of variation	Df	SS	R^2	Pseudo-F	P(>F)
Sample	1	0.0013	0.11	3.5704	**0.050**
Association	1	0.0001	0.05	0.1492	0.729
Residual	29	0.0106	0.89		
Total	31	0.0120	1.00		