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Nested biogeochemical interactions in seagrass ecosystems

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Abstract

Seagrass meadows are globally distributed coastal ecosystems that engineer complex habitats for a plethora of lifeforms colonizing the plant surfaces and the sediment. These lifeforms are not limited to multicellular life: microbial communities are found associated to both the plant and the faunal community at several degrees of metabolic integration, creating with their hosts more complex forms of individuality called holobionts or metaorganisms. The currency of these "trades" is often the exchange of nutrients or an enhanced access to basal resources that otherwise would limit ecosystem diversity and functioning. Thus, seagrass meadows are not only habitat-forming (autogenic) ecosystem engineers, but also allogenic ecosystem engineers that modify the biophysical environment. Of primary importance among seagrass-associated animals are macroinvertebrates, which link benthic primary production to higher-level consumers. However, little research to date has been conducted to study plant-invertebrate-microbe associations in seagrass meadows and their role in biogeochemical cycling of key nutrients, such as carbon (C) and nitrogen (N).

Therefore, in chapter 1 of my thesis I summarize the state of the art on invertebrate-microbe associations as biogeochemical engineers in seagrass sediments. Further, to explore our current collective knowledge on potentially relevant but neglected plant-invertebrate-microbe associations, I build a bipartite network of associations consisting in marine invertebrate genera from seagrass ecosystems and microbe taxa from the NCBI database. This analysis provides a snapshot of the diversity of invertebrate-microbe associations from Mediterranean seagrass ecosystems, showing how microbial taxa can be key links connecting diverse invertebrate bioturbation habits and clear clustering depending on the benthic position of epifauna vs infauna.

Deciphering microbial communities associated with seagrasses is paramount not only for the study of their biodiversity, but also for any prospective management and conservation plan facing climate change. As I uncover in chapter 2, the phyllosphere microbial community structure of *P. oceanica* naturally growing within CO_2 vents of Ischia (Italy) is largely unaffected by the reduced pH mimicking future ocean acidification (OA). Conversely, key nitrogen transformation rates accelerated, with particularly high rates of N_2 fixation but also increases in potential nitrification, denitrification and anammox, highlighting that plasticity of the *P. oceanica* microbiome may be key to the resilience of these ecosystems to OA.

I build upon these multipartite interactions under environmental stress in chapter 3. Using mesocosms, we tested the facultative mutualism between the chemosymbiotic lucinid clam *Loripes*

orbiculatus and the seagrass *Cymodocea nodosa* on sediments from a highly polluted area, and found that the interaction between clams and plants benefitted both organisms and promoted plant growth irrespective of the sediment typ. In particular, *C. nodosa* had higher leaf growth, leaf surface, and leaf biomass when associated with the clams, consolidating the notion that nested plant-invertebrate-microbe associations promote ecosystem functioning.

Chapter 4 delves into a different and less explored plant-invertebrate-microbe association, that between the cyanosponge *Chondrilla nucula* and the seagrass *P. oceanica*. Commonly growing on hard substrates, *C. nucula* in the area of Bacoli (Italy) is found as epibiont of *P. oceanica* surrounding the upper portion of the rhizome. I argue that this association can be described as a facultative mutualism by i) quantifying the benthic distribution of the sponge within the seagrass meadow, verifying mutual (spatial) dependence, and ii) by quantifying net fluxes of organic and inorganic nutrients in incubations with the sponge and the plant (alone or in association), which indicate that the plant and the sponge holobionts may benefit from each other's metabolism.

This thesis provides novel insights into the field of symbiosis research using a holistic approach spanning from ecology to biogeochemistry and microbiology, yielding results of potential interest for innovative seagrass conservation and restoration protocols.

Keywords: *Posidonia oceanica*, *Cymodocea nodosa*, *Loripes orbiculatus*, *Chondrilla nucula*, microbial community, facilitative interactions, biogeochemical cycling, environmental stress.

Ego is the enemy of what you want and of what you have:

Of mastering a craft.

Of real creative insight.

Of working well with others.

Of building loyalty and support.

Of longevity. Of repeating and retaining your success.

It repulses advantages and opportunities.

It's a magnet for enemies and errors.

It is Scylla and Charybdis.

Ryan Holiday

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General introduction

Seagrasses and their importance

Seagrass beds are formed by monospecific or multispecific aggregations of marine angiosperms that transitioned to the marine habitat at least four different times, making them a polyphyletic, ecological group within the order Alismatales (Papenbrock, 2012; Dilipan et al., 2018). Seagrasses have a tropical and subtropical distribution; the global area covered by seagrass meadows have been estimated between 160,387 – 325,178 Km^2 (Unsworth et al., 2019; McKebzie et al., 2020).

In the Mediterranean, the most representative species is the endemic *Posidonia oceanica*. This species covers around 1% of the total surface of the Mediterranean (Pasqualini et al., 1998; Marbà et al., 2014) shares several morphological features that are common among seagrasses, including ribbon-shaped leaves that are devoid of stomata and covered by a cuticle. The leaf bundles connect to the rhizomes through the leaf sheath that partially encloses the rhizome. The leaf sheath besides serving as the insertion point for the leaves also protects the basal meristem and in the *Posidonia* genus, it is particularly lignified. The rhizome is the elongated stem-like structure that clonally propagates and connects the leaf bundles (or ramets) and from which, the plant roots adventitiously emerge (Hemminga & Duarte, 2000).

Besides the asexual propagation of the plant, seagrasses also reproduce sexually. Flowers develop from meristems positioned in the upper side of the rhizome, opposite to the root-producing meristems (Hemminga & Duarte, 2000). These structures are fundamental for the expansion of the meadow, as the pollen is transported passively by the water currents, but also through the interaction with marine invertebrates that can act as pollinators (McMahon et al., 2014; van Tussenbroek et al., 2016).

The complexity of *P. oceanica* beds is related to the diversity of features that provide heterogeneity to the seascape. The leaves decrease the current speed and increase sedimentation rates while prevent their resuspension. This accumulation of trapped sediment leads to an gradual uplift of the seabed, corresponding vertical growth of *P. oceanica* rhizomes and creates a characteristic structural peat-like matrix from the sediments, leaf sheaths, and the network of roots and rhizomes, the so-called "matte" (Boudouresque et al., 2016). The age of the matte can be as old as 6000 yr BP and it is characterized by slow rates of decomposition of plant belowground detritus after an initial rapid decay

of labile organic matter (Romero et al., 1992). This also has a key relevence from the biogeochemical point of view. The persistence of the matte combined with the high productivity of *P. oceanica* meadows, makes this matrix one of the most important (if not the most important) carbon sinks of the Mediterranean Sea, with an estimated total carbon stock between 711 to 1,067 million Mg C, equivalent to 1 to 3 years of CO_2 emission by all Mediterranean countries (Monnier et al., 2020).

Besides the storage of these large amounts of biomass as “blue carbon” (Macready et al., 2021), several other ecosystem services are attritutable to seagrass meadows, benefiting approximately 40% of the world's human population that lives within 100 km of the sea (Cohen et al., 1997; Orth et al., 2006). The aforementioned sediment entrapment is and wave energy reduction by the leaves protects the coasts against erosion. Seagrass ecosystems also attract tourists and recreational users who engage in activities like snorkeling, contributing significantly to local economies. The meadows are also biodiversity hotspots, providing habitat, forage and nursery grounds to a plethora of species. A more extensive list of goods and services perceivable but less frequently studied and quatified include the provision of pharmaceutical and genetic resources, provision of insulation materials, water purification (Nordlund et al., 2016; do Amaral Camara Lima et al., 2023).

Despite their importance, seagrass ecosystems are under constant degradation. The total seagrass cover continues to decrease in recent decades due to a combination of local factors, such as boat anchoring, dredging, increased nutrient and pollutant input; and also global factors such as sea temperature increase (Orth et al., 2006). The global seagrass loss has been estimated around 19-29% and despite this loss ocurring non-linearly, the losses still outweight the recovery and stabilization trends (Waycott et al., 2009; Dunic et al., 2021); raising the urgency to considering new methods or enhacing the existing strategies for seagrass ecosystem restoration and management.

Motivation of the study

"Life finds a way"
(Spielberg, 1993)

Life is hard and living alone can be even harder. This figurative generalization serves us to describe a deceitfully simple observation in nature: No single life form exists in absolute isolation; every organism must deal -to different degrees- with others, as competitors, hunters, prey, collaborators, and anywhere in between.

The collective term that biologists have coined for such a wide range of interactions is *symbiosis*, literally meaning, living together. More precisely, scientist describe use *symbionts* to describe species that interact in a gradient from harm to benefit, that can be variable in space and time; however most of the time, we can identify specific within this gradients as we can see on figure 1.1. Historically, some authors have used this term as a synonym for beneficial interactions, however, in the present work I will adhere to the definition by A.B.Frank, which further classify symbioses into several other types, and that is recovering acceptance among ecologists (Frank, 1877; Martin & Schwab, 2012, 2013; Tipton, Darcy & Hynson, 2019).

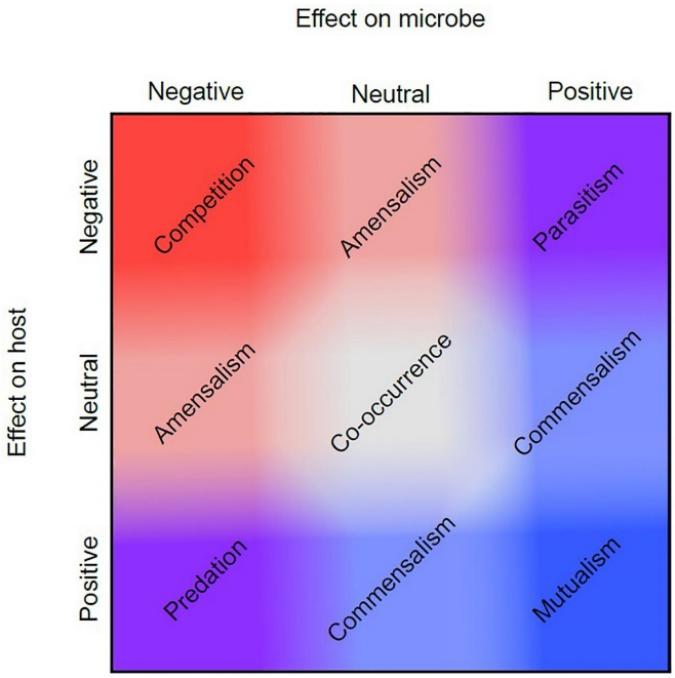

Figure 1.1-Symbiotic relationship gradients. Taken from Tipton et al. (2019).

Interactions between visible, tangible animals and plants are somewhat intuitive to understand, but an additional layer of complexity arises when we understand that we live in a microbial world (McFall-Ngai et al., 2013). What might seem a solitary organism is often in reality the host to a plethora of unicellular symbionts, in turn also colonizing body surfaces, competing, cooperating, and functioning as a downscaled ecosystem.

The above mentioned variability in space and time result in some microbial communities being only transient or facultative symbionts, without exerting a major influence on the host ecology or without being required for the persistence of the host. Conversely, others are obligate, intimately linked through biochemical exchanges that are fundamental to the survival of both species regardless of the

environmental conditions (van der Heide et al., 2020 and references therein). Because different marine taxa are heavily dependent on their microbiome, the terms holobiont and metaorganisms started gaining popularity to describe (practically interchangeably) these associations (Dittami et al., 2021), however, the level of complexity involved makes a challenge to push forward the concept to a more formal framework. Nevertheless, some promising ideas are being laid down, redefining the principles that conceive individuality as a continuous property, emergent at multiple levels of organization, and hence, potentially nested; to establish a common framework that can be applied to organisms, colonies and in between (Krakauer et al., 2020).

The wonders and challenges of understanding these levels of integration are a consequence of the many diverse forms of integration that take place within the holobiont; the microbial residents can influence the host reproduction, its immune repertoire, its development and behavior (McFall-Ngai et al., 2013). In more general terms, these levels if integrations can be seen primarily an integration of different metabolisms, that of the host and that of the collective microbiome. A central body of work is being developed around sponges as a key taxon coupling benthos and the water column by ingesting dissolved organic matter and releasing particulate organic matter that is available to higher trophic levels (de Goeij et al., 2013). This is in part thanks to the complex microbiome associated with poriferans, but the impacts of these symbioses extend beyond trophic transfer. Thanks to the availability of constant water fluxes and the presence of oxic and anoxic compartments, the resident microbiome is also engaging in nutrient transformations, particularly nitrogen (Maldonado, Ribes & van Duyl, 2012; Pita et al., 2018). Of course, there is little reason to assume that sponges are the only holobiont that can affect the biogeochemistry of its surroundings.

The coexistence and interaction of more than one holobiont give rise to complex interaction networks that can lead to range of outputs, from competition, to facilitation between highly distant taxa; in seagrass ecosystems, the accumulation of sulfide in the sediment leads to phytotoxicity but it is also a rich source of electron donors for sulfide-oxidizing bacteria, that in turn will get the benefits of becoming associated with several families of infaunal bivalves (Gagnon et al., 2020). Other, more active organisms also diversify the biogeochemical exchanges within the sediment; polychaetes, oligochaetes, small decapods create galleries and burrows that increase oxygenated water penetration, hence increasing the surfaces for redox exchanges. Despite these studied processes, there is still plenty of work to explore the role of the holobiont in driving fluxes of matter and energy in its own ecosystem.

The availability of open data describing the microbial communities associated with many taxa creates the opportunity to build potential host-symbiont networks that help us to explore patterns amongst

interactions or the redundancy of associations among different hosts and environments. Additionally, considering extensive datasets allow us also to inspect interactions beyond bacteria within this hypothetical network. A preliminary example of this extended microbiome has been provided for corals (Bonacolta et al., 2023), for which a plethora of data exist with regard to their mutualistic association with Symbiodinaceae.

Since symbiosis depends on space and time, changes in environmental conditions, either as natural fluctuations or as the presence of a stressor can also shape these interactions. The study of specific stressors interacting with seagrass hosts and their microbiome are often regarded as a priority topic (Trevathan-Tackett et al., 2019; Nguyen et al., 2021), and while sea surface temperature and nutrient input are frequently studied stressors, ocean acidification effects have been less commonly explored.

Objectives

This thesis addresses these gaps in knowledge through an investigation that addresses the following specific aims:

1) To review current knowledge about invertebrate-microbe associations as biogeochemical engineers of soft-bottom coastal ecosystems through a meta-analysis.

2) To characterize the seagrass phyllosphere microbiome, and to quantify its role in N cycling, in response to ocean acidification.

3) To disentangle nested biogeochemical interactions of a conspicuous invertebrate-microbe-plant association in response to sediment pollution.

4) To explore the ecology of a peculiar invertebrate-microbe-plant association and its effects on nutrient fluxes.

Approach

Most of the fieldwork was carried out in the Gulf of Naples, Italy. The area gives access to a diversity of scenarios that can be used to study hypotheses of different nature, such as (i) the eastern coast of the Ischia Island that offers seagrass beds (i.e. *P. oceanica* meadows) naturally exposed to CO_2 vents, allowing us to compare the effects of the presence and absence of this environmental factor. (ii) The Bagnoli area, that includes a Site of National Interest, selected as a target for restoration efforts after stopping heavy industrial activities in the early 1990 and that has lost a significant cover of the seagrass habitats. (iii) Finally, at Miseno Cape we can find *P. oceanica* beds where the sponge *C. nuculla* exhibits an association pattern that makes it ideal to test hypothesis regarding the potential benefits of growing together. We used a combination of underwater surveys and manipulative experiments in closed

incubation chambers for respirometry, net nutrient fluxes and stable isotope tracing to quantify N2 fixation, nitrification potential, and anammox and denitrification potential. We measured the epiphytic microbial community through 16s rRNA metabarcoding. In addition, we used manipulative experiments in mesocosms to test the effect of the *Loripes-Cymodocea* mutualism on the physiology of the plant when growing on polluted sediment.

About the structure of this thesis

In the present dissertation, I explored different aspects of animal-microbe symbioses occurring on marine seagrasses, with the aim of expanding our knowledge on the role of these associations in nutrient cycling. The first chapter presents a narrative and quantitative review exploring the state of the art in seagrass-associated invertebrate-soft-bottom symbioses, with a focus on epi- and infaunal bioturbators and their reported microbial symbionts, to study association patterns within a symbiosis network. The structure of this chapter follows a narrative review, where there is no specific hypothesis tested.

Chapter II is an evaluation of the variation of the microbiome and key nitrogen cycling processes on *P. oceanica* leaves naturally exposed to CO_2 vents. In this study, we tested whether *P. oceanica* beds growing under CO2 vents exposure show differences in their associated microbial community structure and/or in microbial transformation rates, specifically N fixation, nitrification, and denitrification.

The third chapter deals with the exploration of a mutualism between the chemosymbiotic bivalve *Loripes orbiculatus* and the seagrass *Cymodocea nodosa* as a potential tool to improve seagrass restoration efforts in heavily polluted sediments. We tested whether the interaction between *C. nodosa* and *L. orbiculatus* is beneficial to the organisms involved when colonizing polluted sediments.

Lastly, chapter IV delves deeper into a relatively unexplored association between *P. oceanica* and the sponge *Chondrilla nucula* to disentangle potentially mutualistic benefits. We tested whether an association between *P. oceanica* and the sponge *C. nucula* results in different biogeochemical fluxes of C and N at the community level.

List of publications

This content is also under preparation to be submitted to- or already published as a peer reviewed articles, with author contribution indicated below:

Publication 1:

Montilla LM, Piredda R, Marzocchi U, Bonaglia S, Bartoli M, Zilius M, Cardini U. Plant-invertebrate-microbe associations as biogeochemical engineers of soft-bottom coastal ecosystems. In preparation for FEMS Microbiology Ecology.

The concept of this publication was developed by UC. The metanalysis were carried out by LMM, AA, and RP. The manuscript was written by LMM and UC, with contributions and critical revision from all authors.

Publication 2:

Berlinghof J#, **Montilla LM**#, Peiffer F, Quero GM, Meador TB, Margiotta F, Abagnale M, Wild C, Marzocchi U, Cardini U. Accelerated nitrogen cycling on seagrass leaves in a high-CO2 world. In preparation for Communications Biology. (# shared first authorship).

The concept of this publication was developed by UM, CW and UC. Experiments were performed by FP, GMQ, UM and UC. Lab work was conducted by FP, LMM, TBM, FM, MA. The manuscript was written by JB and LMM, with critical revision from all authors.

Publication 3:

Cardini U#, Marín-Guirao L#, **Montilla LM,** Marzocchi U, Chiavarini S, Rimauro J, Quero GM, Petersen JM, Procaccini G (2022) Nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning in contaminated sediments. Frontiers in Plant Science 13:918675. Doi: 10.3389/fpls.2022.918675. (# shared first authorship).

UC, LM-G, and GP conceived the ideas and designed methodology. UC, LM-G, JP, and GP contributed to the sampling of biological material. UC, LM-G, GP, and GMQ performed the experiment and collected the data. UC, LM-G, LMM, UM, SC, and JR analyzed the data. UC and LM-G led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Publication 4:

Montilla LM, Guarcini E, Gallucci L, Berlinghof J, Furia M, Meador T, Margiotta F, Olivé I, Fraschetti S, Cardini U. A facultative sponge-seagrass mutualism in the Thyrrenian Sea. In preparation for Marine Ecology Progress Series.

The concept of this publication was developed by LMM and UC. Experiments were performed by LMM, EG, LG, JB, IO and UC. Lab work was conducted by LMM, EG, JB, MF, TM and FM. LMM analyzed the data. The manuscript was written by LMM, with critical revision from all authors.

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Chapter 1:
Plant-invertebrate-microbe associations as biogeochemical engineers of soft-bottom coastal ecosystems

Abstract

Healthy seagrass meadows are essential to sustain biodiversity and functioning of marine coastal systems. In addition, marine plants may mitigate the impacts of global changes, such as seawater warming and acidification (i.e., climate rescuing effect). Thus, preserving and restoring these ecosystems is recognized as a promising nature-based solution to the climate-driven biodiversity crisis. At the same time, increasing evidence suggests that associations with invertebrates and their microbial community are vital to sustain seagrass ecosystems functioning and survival. Nonetheless, such species interactions received sparse attention in these systems and are often not included in conservation and restoration plans. Here we build on current understanding of how shallow-water plant-invertebrate-microbe associations contribute to biogeochemical cycling and ecosystem functioning to highlight emerging avenues of research. To explore our current collective knowledge on potentially relevant but neglected plant-invertebrate-microbe associations, we build a bipartite network of associations consisting in marine invertebrate genera from seagrass ecosystems and microbe taxa from the NCBI database. Finally, we consider how environmental stressors arising from human activities may threaten these relationships and explore the potential implications for ecosystem resilience and restoration.

Keywords: coastal ecosystems; bioturbation; holobiont.

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Introduction

Approximately 40% of the world's human population lives within 100 km of the sea, where shallow-water ecosystems provide essential ecosystem services such as coastal protection and food security (Cohen et al., 1997). By and large, these coastal areas are sediment systems and cover less than 20% of the total ocean area, but approximately 90% of the total organic matter is buried here (Hedges & Keil, 1995). Many of these sediment areas are wetland ecosystems, such as mangroves, tidal marshes, and seagrass meadows, which effectively take up CO_2 through photosynthesis and store significant amounts of carbon (thus also called blue carbon ecosystems, Figure 2.1), being thus recognized for their role in mitigating climate change.

Among blue carbon ecosystems, seagrasses are fundamental for the ecology and the economy of the coastal zone. These key foundation species form habitats, sustain biodiversity and provide highly valuable ecosystem services, such as improvement of water quality and carbon sequestration, thus sustaining the economic and social welfare of coastal communities (Savva et al., 2018). Moreover, with the ability to increase seawater pH through their photosynthetic activity and metabolism (Hendriks et al., 2014), seagrasses may contribute to mitigate the expected impacts of ocean acidification on associated calcifying species. However, seagrass meadows are among the world's most threatened ecosystems, with human activities impacting the oceans in general, and the coastal zone in particular, threatening their stability and driving them towards the brink of regime shifts, i.e. the transition to alternative states characterized by less diversified and less productive biotic communities (Serrano et al., 2016).

The notion that biodiversity is key for productivity, resource use, and stability of seagrass ecosystems is not new (e.g. Duffy, 2006). However, an increasing number of studies is finding that specific beneficial associations (i.e., facultative mutualisms) with invertebrates and their related microbial communities may be a critical prerequisite for their persistence under stressful conditions (e.g., van der Heide et al., 2012; Valdez et al., 2020; Gagnon et al., 2020; Malkin & Cardini, 2021). For example, at a local scale, bivalves and sponges may increase the performance of the seagrasses and associated calcified species by reducing the concentration of chemical pollutants. Both bivalves and sponges and/or their microbiome have been shown to accumulate organic and inorganic pollutants, acting as biofilters (Bauvais et al., 2015; Mayer-Pinto et al., 2020; Strehse & Maser, 2020). Bivalves of the family Lucinidae and their microbial chemosynthetic symbionts contribute to the removal of sulfide (toxic to the plant) from sediments and thus enhance seagrass growth (van der Heide et al. 2012).

Additionally, a role of the lucinid bivalve *Loripes orbiculatus* in N provisioning to the plant was recently proposed, given the ability of the symbionts to also fix atmospheric N_2 (Petersen et al., 2016; Cardini et al., 2019). Although yet to be confirmed, there is some evidence suggesting that ammonium excretion by sponges and their symbionts could be an important source of N to the associated plant during shortage periods (Archer, Stoner & Layman, 2015, but see also Chapter 4, this thesis).

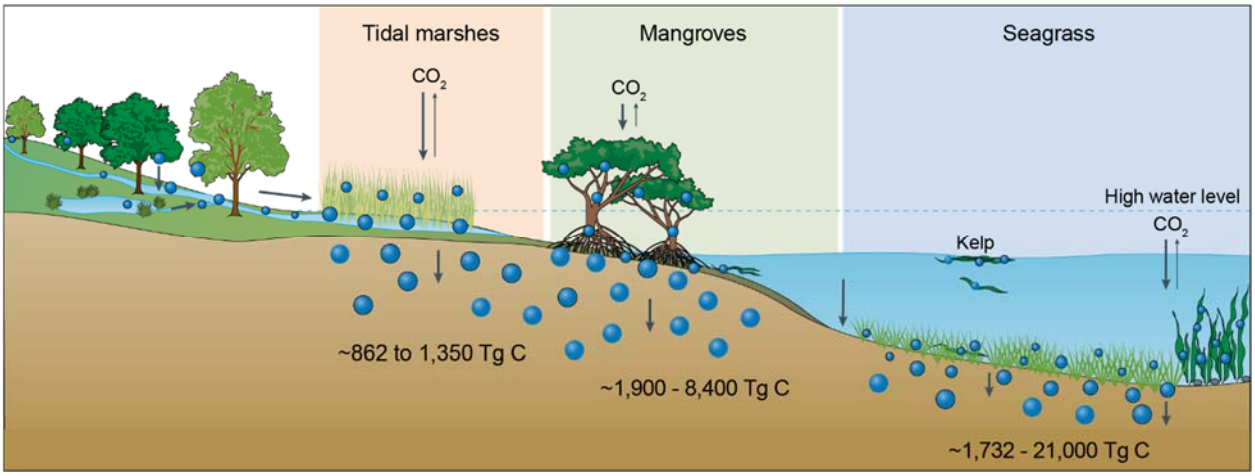

Figure 2.1 "Blue carbon" ecosystems such as salt mangroves, marshes, seagrass meadows and kelp forests are extremely effective at fixing CO2 through photosynthesis and storing carbon in their soil. Adapted from Macreadie et al. (2021) with estimates of soil carbon.

A third essential component of seagrass-invertebrate systems is, thus, the microbial community, either free-living or associated with the plant/animal host. Microbes, and particularly prokaryotes, are the most metabolically diverse organisms and possess a wide range of capabilities that are absent from or only rudimentarily present in eukaryotes (Schlegel & Jannasch, 2006). For example, a diverse group of prokaryotes has evolved to degrade hydrocarbons, using them as a source of carbon and energy, (Prince, Amande & McGenity, 2019). Prokaryotes are also well known to sequester metals and other ions intracellularly in various forms (Edwards & Bazylinski, 2008). Therefore, their ubiquity in nature and their association with macro-organisms to form holobionts (i.e. the assemblage of the host and its microbiota, forming an ecological unit, see Tarquinio et al., 2019 for seagrasses; Petersen & Osvatic, 2018 for invertebrates) is of utmost relevance for effective conservation of vegetated coastal ecosystems. Thanks to the expanded metabolic repertoire of the holobiont and to nested ecosystem interactions (Figure 2.2), plant-invertebrate-microbe associations may have the potential to withstand environmental perturbations preserving ecosystem functioning and associated ecosystem services delivery, e.g. carbon storage capacity.

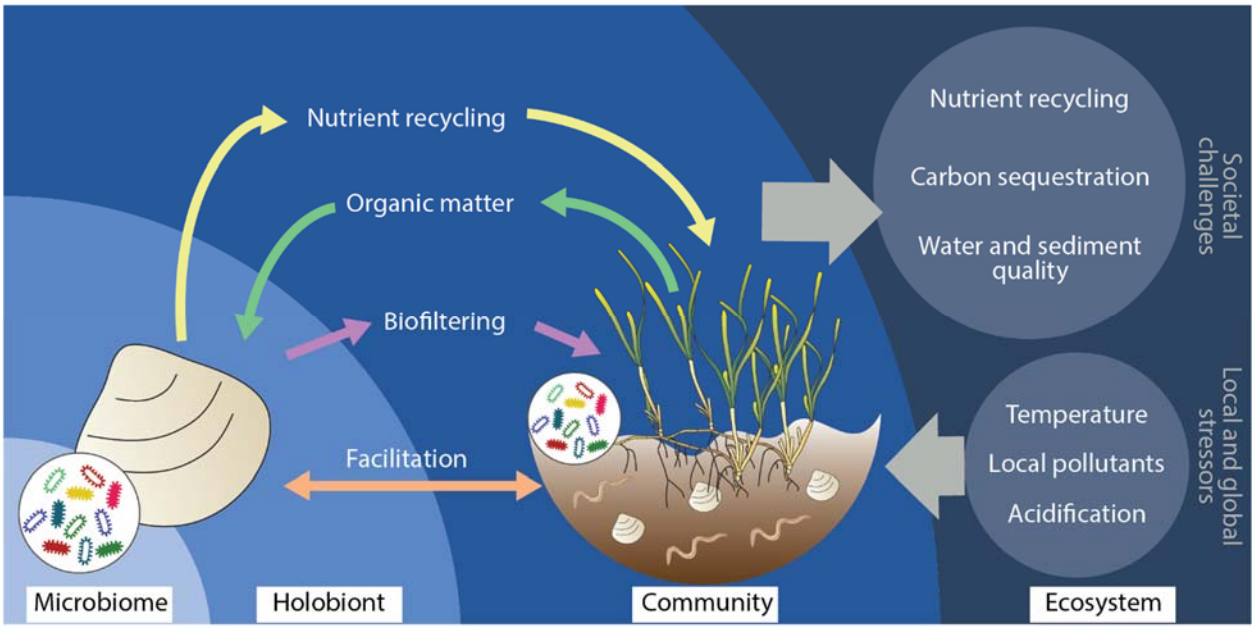

Figure 2.2 The concept of nested ecosystems applied to plant-invertebrate-microbe associations. Indicated are key processes that can be mediated by the microbiome or by the animal/plant host, and will have cascading effects on community functioning in seagrass-invertebrate systems, and on key societal challenges. Seagrasses provide key functions within the nested ecosystem, the most prominent being provision of organic matter. Local and global stressors act at multiple scales, potentially altering microbiome, holobiont, community and thus ecosystem functioning. Adapted from Pita et al. 2018.

In our paper, we will build on the current understanding of the diverse ecological roles of plant-invertebrate-microbe associations in shallow-water ecosystem functioning and their capacity to promote niche specialization and diversity of higher trophic levels to highlight emerging avenues of research. We will argue that plant-invertebrate-microbe associations act as ecosystem engineers that play a key role in sediment carbon, nitrogen, and sulfur cycles due to their ability to generate and transform inorganic and organic materials. Further, we will consider how environmental stressors arising from human activities may threaten plant-invertebrate-microbe associations and the potential implications for ecosystem resilience and restoration. For example, their capacity to detoxify sulfide-rich sediments holds great promise for completely novel restoration strategies that consider symbiotic interactions, aimed at protecting biological diversity by letting nature 'pick the winners' (Webster et al., 2017). Just as modern bioengineers are now discovering the power of endosymbioses for inspiring new biotechnological innovations (Puri, Butardo & Sumer, 2021), ecologists should consider the fundamental role of plant-invertebrate-microbe associations in the functioning and resilience of blue carbon ecosystems.

The biogeochemical engineers of coastal sediments

The water-sediment interface is a very dynamic microenvironment; it is the place where physical, chemical, and biological gradients interact, with important repercussions on the whole ecosystem. Water permeability and particle adsorption can be affected by multiple factors such as sediment composition, wave action, current velocity, temperature (Glud, 2008), therefore influencing oxygen flux to deeper layers of the sediment and the exchange of organic and inorganic dissolved matter. Nevertheless, these passive exchanges are limited to the first millimeters of sediment. The presence of bioturbating invertebrates adds an additional layer of complexity to this dynamic as their perturbations intrinsically have important implications on biogeochemistry such as the modification of sediment texture, bio-irrigation and dispersal of solid particles (Meysman, Middelburg & Heip, 2006). Burrowing macrofauna alter redox conditions and increase coupling between aerobic and anaerobic horizons by constructing and ventilating burrows, and by transporting electron acceptors and metabolic end products (Kristensen, 2001; Nielsen et al., 2004; Kristensen et al., 2012; Bonaglia et al., 2019). Therefore, bioturbating invertebrates stimulate an array of reactions and processes, structuring sediment microbial communities and regulating benthic functioning. At the same time, an increasing body of literature testifies that these organisms are themselves holobionts, which select and host unique microbiomes that significantly contribute to carbon, sulphur and nitrogen (N) biogeochemical cycling (König et al., 2016; Cardini et al., 2019; Zilius et al., 2020; Marzocchi et al., 2021).

With the rework of sediments for the construction of burrows and galleries, bioturbators create new influxes of O_2 that oxidize surrounding sediment, evidenced often by its light coloration in contrast with reduced sediment; these new oxygenated spaces are additional niches for nutrient cycling and increased microbial activity. Lucinid bivalves are one example of infauna that promotes this type of irrigation through their inhalant tubes; in seagrass sediments, incoming O_2 is not only used by the bivalves but it is also required by their symbionts, that oxidize sulfide and use the obtained chemical energy to fix CO_2 and generate sugars, part of which are also passed to the host. Alternatively, if there is enough availability of heavy metal ions, other forms of sulfides can be present, like FeS, which can also be 'mined' by the lucinids (Dando, Ridgway & Spiro, 1994; Reynolds, Berg & Zieman, 2007; van der Heide et al., 2012).

Polychaetes display variation in the architecture of their burrows, which, as mentioned early, leads to a series of effects in the surrounding sediment. One intertaxa comparison (Mermillod-Blondin et al., 2004) showed that the polychaete *Hediste diversicolor* had a larger effect on bacterial abundance in the

sediment, and oxygen, nitrate and phosphate fluxes from the sediment to the water column, in comparison with the bivalve (*Cerastoerma edule* and the amphipod *Corophium volutator)*. Similar comparisons showed an effect over nitrification and sulfide oxidation within the better ventilated burrow of *H. diversicolor* in contrast with the more anoxic environment of the *Marenzelleria viridis* polychaete burrow (Vasquez-Cardenas et al., 2016). In other cases, aggregations of polychaetes like *Lanice conchilega* were associated with more diverse denitrifying microbial communities (Yazdani Foshtomi et al., 2018).

In terms of their microbial endosymbionts, (Dale et al., 2019a) proposed that the hindgut of this polychaete works as an 'incubator' for groups of ammonia-oxidizing archaea with the potential implication that this could contribute to the diversity of the microbial assemblage of the sediment through their excretions; additionally, their mucous layer stimulates the proliferation of nitrifier (Dale et al., 2019b).

Similarly, Axiidean and Gebiidean shrimps are two groups of widely recognized bioturbators that build galleries under the sediment and feed through filtration or ingestion of detritus; their role as bioengineers and structuring agents of microbial communities has been widely studied (Papaspyrou et al., 2005; Demiri et al., 2009; Bertics et al., 2010; Laverock et al., 2010; Pillay, 2019). Some examples include 3-fold increases in denitrification rates inside burrows of *Upogebia deltaura* (Howe, Rees & Widdicombe, 2004) and *Trypaea australiensis* (Webb & Eyre, 2004), and even more pronounced effects in nitrification, denitrification, and ammonification when compared with sediments lacking *Upogebia* shrimps (Jordan et al., 2009; D'Andrea & DeWitt, 2009).

A different situation develops when the body size of the invertebrates is too small to create actual burrows. Some sediment meiofauna is also found in symbiosis with chemosynthetic prokaryotes, and they can also exert an influence on their surroundings, with the difference that they overcome diffusion limitations either by movement or by water flow and advection. Several groups actively move throughout the oxic-anoxic gradients in the sediment with their symbionts using a variety of electron donors and acceptors available in the anoxic zone. When O2 depletes with depth, different groups of prokaryotes make use of other energetically favored reduced compounds of N (such as ammonium and nitrate), S (e.g. sulfide), and C (as methane) (Nealson, 1997; Burgin et al., 2011), and some form chemosynthetic symbiosis with a range of meiofaunal organisms. Some examples of this are the use of sulfide e.g. *Paracatenula* and *Astomonema sp.* (Ott et al., 1991; Gruber-Vodicka et al., 2011; Ott et al., 2023) or thiosulphate, e.g. *Inanidrilus leukodermatus* (Giere et al., 1988). For stilbonematid nematodes like

Stilbonema sp., there is evidence that their symbionts are also able to use nitrate as the electron acceptor, increasing the concentration of nitrite in their surroundings (Hentschel et al., 1999).

Among sediment meiofauna again is present the continuum between strict mutualistic symbioses -with a single symbiont phylotype linked to a single host species (Zimmermann et al., 2016; Schuelke et al., 2018; Jäckle et a., 2019; Seah et al., 2019, Ott et al., 2023)- to loose associations with a variable and transient microbiome (Hammer, Sanders & Fierer, 2019). While there is evidence linking meiofaunal community abundance with variation in sediment denitrification rates (Bonaglia et al., 2014), it is still unclear how much these processes are the result of physical modification by these organisms of their habitat or conversely can be attributed to the holobiont. In this direction, some recent work has been done to partition the role of different organisms and the effect bioturbation vs the effect of the invertebrate-microbe association in driving benthic nitrogen cycling processes (Figure 2.3, Zilius et al., 2021).

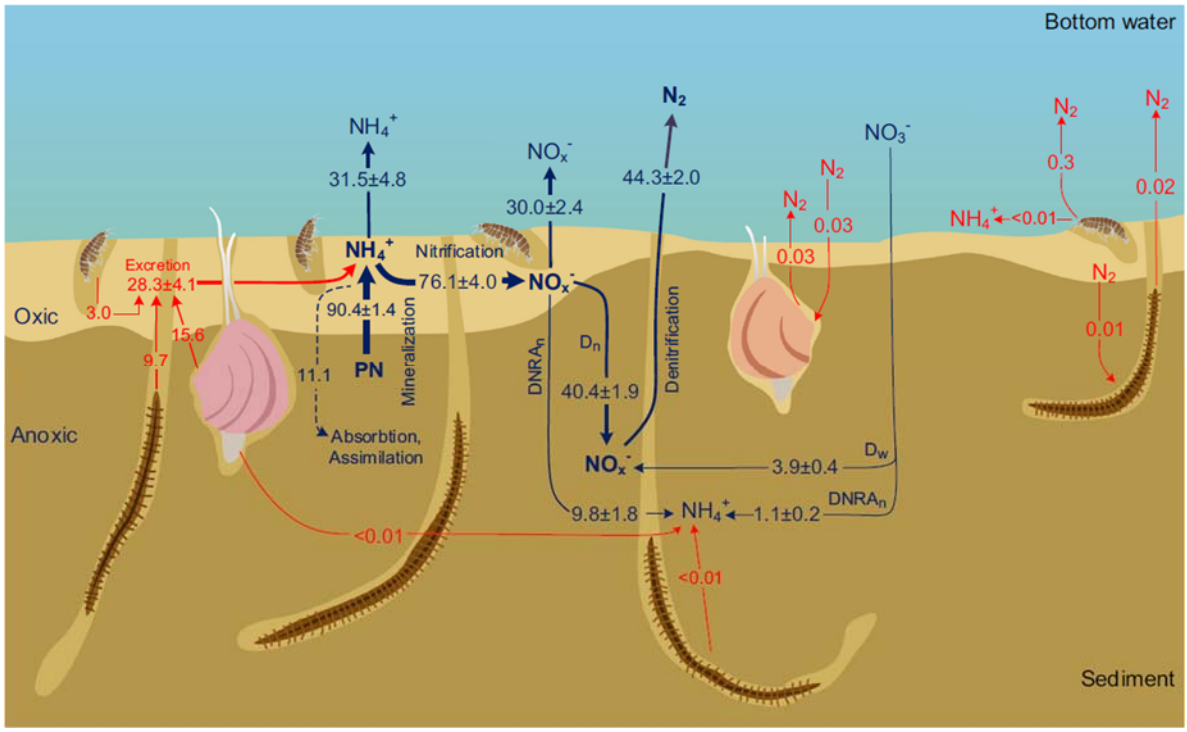

Figure 2.3 Example of partitioning of benthic nitrogen cycle processes among three common macrofauna holobionts from the Baltic Sea (Monoporeia affinis, Marenzelleria spp., and Limecola balthica).These species represent dominant macrofauna from an oligotrophic estuarine habitat, with different lifestyles that have associated different types of bioturbation.Their biomass and the coupling of their role in N transformation have a significant role in N cycling. From Zilius et al. (2021).

Invertebrate-microbe associations are ubiquitous in soft-bottom coastal ecosystems

The relevance of chemosynthetic symbioses with marine invertebrates has been increasingly recognized as a type of interaction with profound effects in the dynamics of marine sediments, particularly because they play a fundamental role in the exchange of nutrients between their hosts and the surrounding habitat (Beinart, 2019; Trevathan-Tackett et al., 2019; Wilkins et al., 2019). Examples of these symbioses are ubiquitous in soft-bottom coastal ecosystems, including but far from limited to mudflats (Dufour, 2018), mangrove sediments (Lim et al., 2019), mangrove peats and sediments, and sunken driftwood (Lechene et al., 2007; Charles et al., 2018; Lim et al., 2019). The prevalence of these chemosynthetic companions has allowed their invertebrate hosts to succeed in otherwise hostile environments (Kopac & Klassen, 2016).

While these chemosynthetic symbioses are examples of strict mutualistic symbioses where the two partners cannot live without one another, the notion of complex associations as a generality should not be surprising from an evolutionary perspective. Since the early 20th century there were been formal proposals of animal and plant cells having their origin as the result of endosymbiosis (Kowallik & Martin, 2021), but the proposal was not extensively accepted until decades later, when Lynn Margulis (then Lynn Sagan) published in 1967 an article championing an endosymbiotic origin of mitochondria and plastids from bacterial ancestors (Sagan, 1967), hypothesis now widely accepted. One of the main consequences of the establishment of symbioses is the acquisition of physiological traits previously lost (because their high metabolic cost, e.g. animals lacking the capacity to synthesize certain amino acids and enzymatic cofactors as could be explained through relaxed selection or the Black Queen hypothesis). Alternatively, they could also be traits that simply were never available to the host, e.g. nitrogen fixation and/or chemosynthetic pathways as described above (Moran, 2007; Lahti et al., 2009; Morris, Lenski & Zinser, 2012; Douglas, 2014; Chomicki, Kiers & Renner, 2020).

From the microbe point of view, being part of these associations represents the opportunity to overcome several difficulties associated with being a small unicellular organism. A most prominent one is that prokaryotes need to position themselves where all they need is at reach. This is not a trivial task as the distribution of nutrients and electron acceptors/donors varies largely in the environment both in space and in time. This urge is even more pressing in the sediment, where cell motility is constrained and mass transport of molecules is generally limited to diffusion. Microorganisms can thus benefit a great deal by associating with invertebrates inhabiting this environment. Distinct functional groups can, in fact,

provide access to otherwise unavailable resources. Filter feeders, for instance, enhance the water flow (and thus the transport of O_2) to centimeters depth favoring aerobic prokaryotes in highly reduced sediment. Stilbonematid nematodes shuttle between the oxic and sulphidic zone of the sediment enabling the ectosymbiotic sulfide oxidizing bacteria to experience the alternate presence of both electron acceptor and donor (Ott et al., 1991).

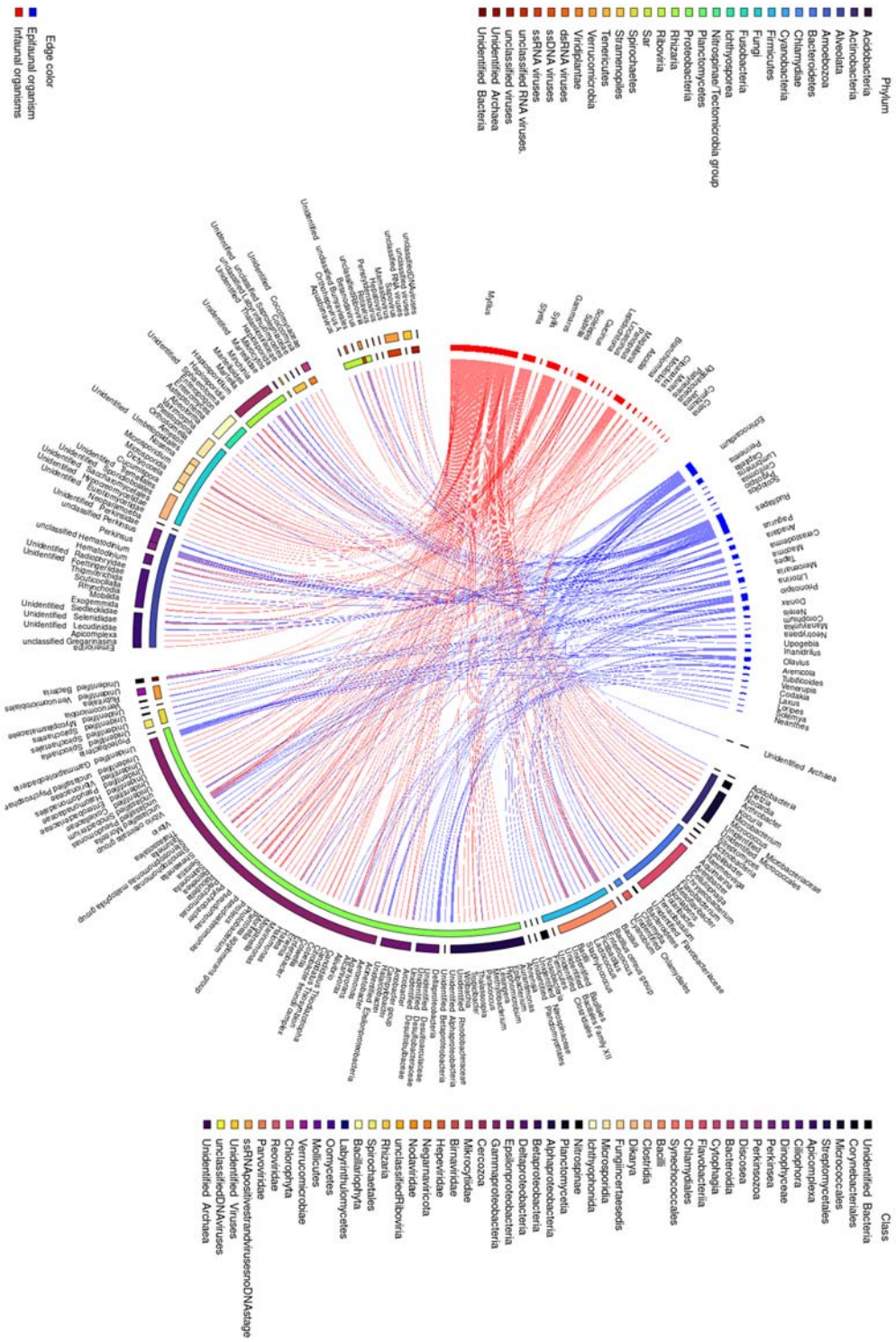

Figure 2.4 Bipartite network of invertebrate-microbe associations from Mediterranean seagrass beds. Red and blue segments represent infaunal and epifaunal bioturbators respectively. The innermost segments represent microbial phyla, emconpassing the outermost segments represent microbial phyla, encompassing the outermost segments that represent microbial classes. The width of the edge is the relative abundance of NCBI entries for microbial phylotypes found associated to epifaunal and infaunal invertebrates.

To explore and interrogate the available data on invertebrate-microbe associations from Mediterranean seagrass beds, we built a bipartite network of invertebrate-microbe associations from seagrass beds based on a list of 275 seagrass-associated invertebrate genera (Figure 2.4). This list was compiled from recent catalogs for the Mediterranean Sea and used to query and download the metadata data available in the NCBI nucleotide database. The search query was built to retrieve specific entries that had the invertebrate genus as part of the field "host".

We used the R packages *rentrez*, *reutils*, and *biofiles* (Schöfl, 2016, 2017; Winter, 2017; R Core team, 2021) to retrieve, clean, and format the data. This relationship was represented as a bipartite network, where the nodes represented the different hosts and microbial taxa, and the links were the co-presence in the records. The final layout was produced using the package *circlize* (Gu et al., 2014). Finally, to explore preferentially connected groups we detected overlapping communities using the *linkcomm* package (Ahn, Bagrow & Lehmann, 2010; Kalinka & Tomancak, 2011). We chose this kind of communities because they can share nodes among multiple groups, allowing the possibility of exploring interconnected clusters.

While only 28% of the genera were represented in the molecular database, indicating the wide scope for further research into this topic, we obtained a network with 112 nodes representing 53 host genera and 59 microbial groups. Segregating the hosts according to their environmental position (infauna vs epifauna) highlighted a widespread association of infauna with gammaproteobacteria, while largely pathogenic eukaryotic microbes were predominantly associated with epifauna, reflecting the contrasting lifestyles of these host groups. At the same time, community analyses on the network allowed to preliminarily discriminate gradients from intimate (one host and few microbes) to loose (diverse) associations. The overlapping community structure detection algorithm generated 17 overlapping communities, ranging from three large communities with 83, 32 and 31 taxa, to several smaller groups with fewer than 10 members. The supplemental material available in https://github.com/luismmontilla/biomass details the methods and includes high-resolution versions of the figures.

As could be expected, the communities showed a gradient of diversity and frequency of association (Hammer, Sanders & Fierer, 2019). Undoubtedly, the phylum Protebacteria was among the main protagonists of the dataset; Gammaprotebacteria and Alphaproteobacteria were not only the most commonly found bacterial phylotypes, but also the ones appearing in most studies and/or datasets, i.e. appearing in the main network as the one with the most links and most communities; they were also ones

of the most widely connected nodes, being associated with 10% of the hosts. This should not be too surprising, since the presence of this group has been usually found in chemosynthetic symbiosis research (Petersen & Yuen, 2021).

Among the key nodes connecting these two bacterial phyla were precisely *Inanidrilus* and *Olavius*. However, alpha and gammaproteobacteira also linked different infaunal and epifaunal habits, such as the bioturbators *Prionospio* and *Sabella*, as well as less active hosts like the bivalve *Anadara* and the tunicate *Ascidia*. The second largest community was centered on the bivalves of the genus *Mytilus*, which was connected to numerous potentially pathogenic symbionts, widely studied probably as a direct consequence of the commercial interest of this host. In fact, some of these symbionts were also shared with another community formed around the similarly commercially exploited bivalve *Ostrea*. Another emerging pattern was the number of epifaunal invertebrates, particularly crustaceans, often associated with single-celled eukaryotes. E.g. Microsporidians, apicomplexans, and the genus *Perkinsea* (this one being a frequent parasite) were relevant enough to be form their own clusters.

These results are but a first step to have a wider insight into the diversity of microbe-invertebrate symbioses from soft bottom ecosystems, that can be further improved, for example, including data from additional, more extensive databases. What is evident is that the fauna from these ecosystems can exhibit remarkable gradients of integration and variability in terms of their microbiome, and understanding how these connections exist can allow researchers to scale up their inferences to ecosystem levels e.g. refining budget models that can be used in the context of ecosystem management, of services like nutrient recycling (Kahiluoto et al., 2014) or understanding ecological patterns in terms of the microbial symbiont niche.

The influence of invertebrate-microbe associations across ecosystem scales

Seagrasses are an iconic example of nested ecosystem, where multiple holobionts engage in synergistic cooperation that can have an important role in biogeochemical cycling. However, we still have limited understanding of the activity and biogeochemical cycling driven by holobionts in seagrass ecosystems. Recent research shows that the persistence and functioning of habitat-forming seagrasses under adverse environmental conditions is sustained by positive interactions with species that ameliorate local conditions or reduce nutrient limitation, which are often neglected in conservation plans. Among many examples, plant–bivalve interactions have been suggested to facilitate foundation species such as

seagrasses, possibly helping to increase the success of restoration efforts (see Meysick et al., 2020; Gagnon et al., 2020). In *Posidonia oceanica* meadows, Cardini, et al. (2019) showed that chemosymbiotic bivalves provide nitrogen (N) to the seagrass ecosystem when this nutrient is more limiting. Similarly, sponges have been shown to facilitate primary producers in seagrass systems (Archer et al., 2021). However, studies that mechanistically test specific interactions for their capacity to improve resistance of the whole consortium of organisms to both local and global anthropogenic stressors are lacking. The magnitude and integration of these associations have deep implications, both for benthic-pelagic coupling and aboveground-belowground interactions (Figure 2.5).

Symbiont hosts can be seen as the habitat of microbial communities whose interactions influence the holobiont as well as the interactions within their own community through cascading effects (McFall-Ngai et al., 2013). For example, this idea of ecosystems nestedness has been used to describe the interactions of poriferans with their microbiome and how they have a key role in structuring the benthic community by providing hosts with predation deterrents and allelopathic compounds. At the same time, the sponge and its associated microbiome play a relevant role in the trophic functioning of the ecosystem, by making dissolved organic matter available with their excretions and through the cycling inorganic nitrogen and phosphorous (Pita et al., 2018).

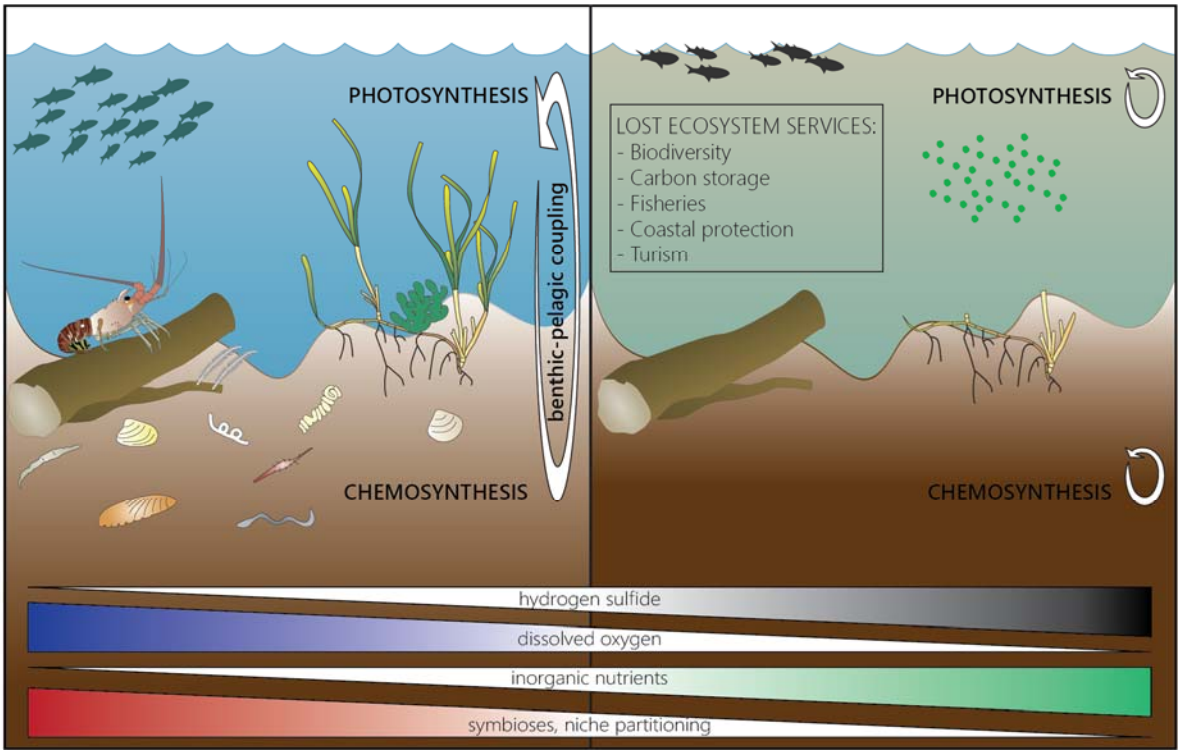

Figure 2.5 Benthic-pelagic coupling and aboveground-belowground interactions as an example of key functions that plant-invertebrate-microbe associations contribute to the ecosystem. In the scenario on the left, associations on/in the benthos

allow cycling of organic matter and nutrients from the sediment back into the system (e.g. through bioturbation, bioirrigation, food provisioning, C, N and S cycling). On the right, excessive nutrient loadings cause breakdown of associations and persistence primarily of free-living microbes and few resistant invertebrates that cannot provide the same functions, with the consequent loss of key ecosystem services.

Seagrass environments are another prime example for such a network of interactions. E.g., paleontological evidence suggest that the apparition of seagrasses contributed to the diversification of early lucinid bivalves and also to their persistence after mass extinction events (Stanley, 2014 and references therein). Lucinids and other families of bivalves are well-described hosts of sulfide-oxidizing bacteria, which contribute to the productivity of the seagrass bed by decreasing the concentration of sulfide in the areas surrounding the rhizomes. At the same time, the persistence of the seagrass generates a continuous supply of organic material to the sulfate-reducing bacteria and archaea of the sediment that will generate sulfide, maintaining the trophic connectivity of the system and structuring microbial communities with the resulting biogeochemical gradients (e.g. Green-García & Engel, 2012; van der Geest et al., 2020).

These bivalves are a core example of interconnectivity and exchange between the deeper anoxic sediment layers and the surface, and also between the living compartments found throughout them: the sulfide they take-up comes in part from the degradation of the seagrass biomass, which incorporated nutrients from the water column. The bivalve excretion products in the form of ammonia are released to the sediment ready to be consumed by ammonium oxidizers and potentially, also by the plant (Cardini et al. 2019). The sediment is not a homogenous matrix, and variations in the conditions under which seagrass systems persists lead to different scale-dependent patterns in the ways that the parties interact. E.g. the nutritional flexibility of lucinids mentioned above can include the digestion of the microbial symbionts (König, Le Guyader & Gros, 2015). This trophic change in the lucinids can be correlated with the spatial variation of sulfide availability that occurs at the seagrass bed edge, generating in turn a structured population of bivalves in the sediment (Rossi et al., 2013) and similar variations in nutrient concentrations in the water column have been attributed to lower levels of lucinids in *Cymodocea nodosa* beds (Sanmartí et al., 2018).

Predation also contributes to the transference of energy between the benthos and the water column. Sea bird predators control the populations of coexisting bivalves in the sediment. Experimentally excluding the predator and competidors lead to a drop in the sulfide concentration as the lucinids rely more heavily on chemoautotrophy (Gils et al., 2012). At the edge and more prominently outside the seagrass patch, lucindids and other infaunal invertebrates are more susceptible to predation, removing

their biomass and bioturbating the sediment; the balance between different competing bivalves driving as consequence changes in the geochemical characteristics of the sediment.

While lucinid bivalves act as sessile hubs of nutrional and ecological exchanges, a range of burrowing shrimps taxa create additional surfaces for redox reactions. Firstly, these openings into the sediment create new influxes of O_2 and nutrient-rich water from the surface; Detritivorous burrowing shrimps translocate seagrass leaves from the surface, increasing the amount of particulate and dissolved organic matter centimeters within the sediment; this matter is not only ingested, but also used as lining of their burrow walls, facilitating the presence of richer microbial and meiofaunal communities within the larger burrow volume (Koller, Dworschak & Abed-Navandi, 2006; Vonk et al., 2008; Kneer, Asmus & Vonk, 2008; Pinn & Atkinson, 2010; Laverock et al., 2013). This vertical diversification of the microbial community is directly translated to spatial and temporal variation in nutrient cycling processes, e.g. marker genes for nitrification have been detected almost seven times more abundantly inside the burrows compared to the to the surface (Laverock et al., 2014). Similarly, ammonium oxidation rates were five times higher inside the burrows (Laverock et al., 2013).

These groups also might also be hosts of nested interactions; although there is evidence of a gut microbiome (Harris, Seiderer & Lucas, 1991; Pinn et al., 1999), their role in the nutrient dynamics has not been extensively explored; it is possible that the gut of *Pestarella tyrrhena* harbours sulfur-oxidizing bacteria which perhaps may contribute to the nutrition of the host (Demiri et al., 2009). Nevertheless, the overall magnitude of nutrient cycling specifically attributable to invertebrate-bacterial associations in seagrass or seagrass associated sediments, be it from infauna or bioturbators remains poorly characterized.

The implications for ecosystem functioning and restoration

The benefits of nested ecosystems that integrate plant-invertebrate-microbe associations as the ones mentioned above are significant both in terms of ecosystem functioning as well as for the provision of services, and the role of biological interactions for any restoration effort are becoming part of the mainstream scientific knowledge (e.g. Braeckman et al., 2014; Ermgassen et al., 2020; Gagnon et al., 2020). However, facilitative associations (a positive interaction where one of the parties enhances the establishment and/or permanence of another organism; see Bronstein 2009 for an extended discussion on the topic) also have downsides, particularly in terms of susceptibility of the whole ecosystem to collapse if key mutualisms are disrupted. For example, an increase in nutrient input and/or water temperature beyond certain thresholds can trigger shifts from a stable state of a seagrass bed to a stable

bare sediment in some cases (Carr et al., 2010) (Figure 2.6). Seagrass stress can lead to mortality and sulfide accumulation beyond the levels that lucinid sulfide-oxidizing bacteria can oxidize, destabilizing the whole seagrass ecosystem in time (Folmer et al., 2012; de Fouw et al., 2018).

Similar disruptions have been hypothesized to occur in other ecosystems as sea temperatures keep rising; mangrove sediments from the Red Sea are naturally nitrogen-limited as a consequence of the interaction between seasonal high temperature, which impairs nitrogenase activity, and intensive crab bioturbation, that decreases the population of nitrifying cyanobacteria in the sediment . Other examples of phase shifts in marine ecosystems are reviewed in (Nyström et al., 2012).

As these ecosystems experience such transformations, the goods and services that they provide are affected or lost as a direct consequence. We therefore argue that the early detection of such coupled threats must become a priority task in coastal ecology. This also poses a significant challenge, as the condition of the ecosystem can be sustained by different feedback mechanisms that create a relatively stable state until the stressors effect is intensive enough to overcome the ecosystem resilience. Hence, the identification of underlying feedback mechanisms should be a fundamental part of any monitoring or management program, to appropriately target any management action (Maxwell et al., 2017). Examples of this perspective in seagrasses include targeting symbiotic relationships to increase lucinid biomass, which reinforce a positive loop that sustains seagrass productivity, as well as suppressing antagonistic bioengineering feedbacks that undermine restoration success (Suykerbuyk et al., 2012; Maxwell et al., 2017). This necessarily requires the use of an adaptive management approach that can implement the most appropriate strategy depending on the available evidence (Lindenmayer & Likens, 2009).

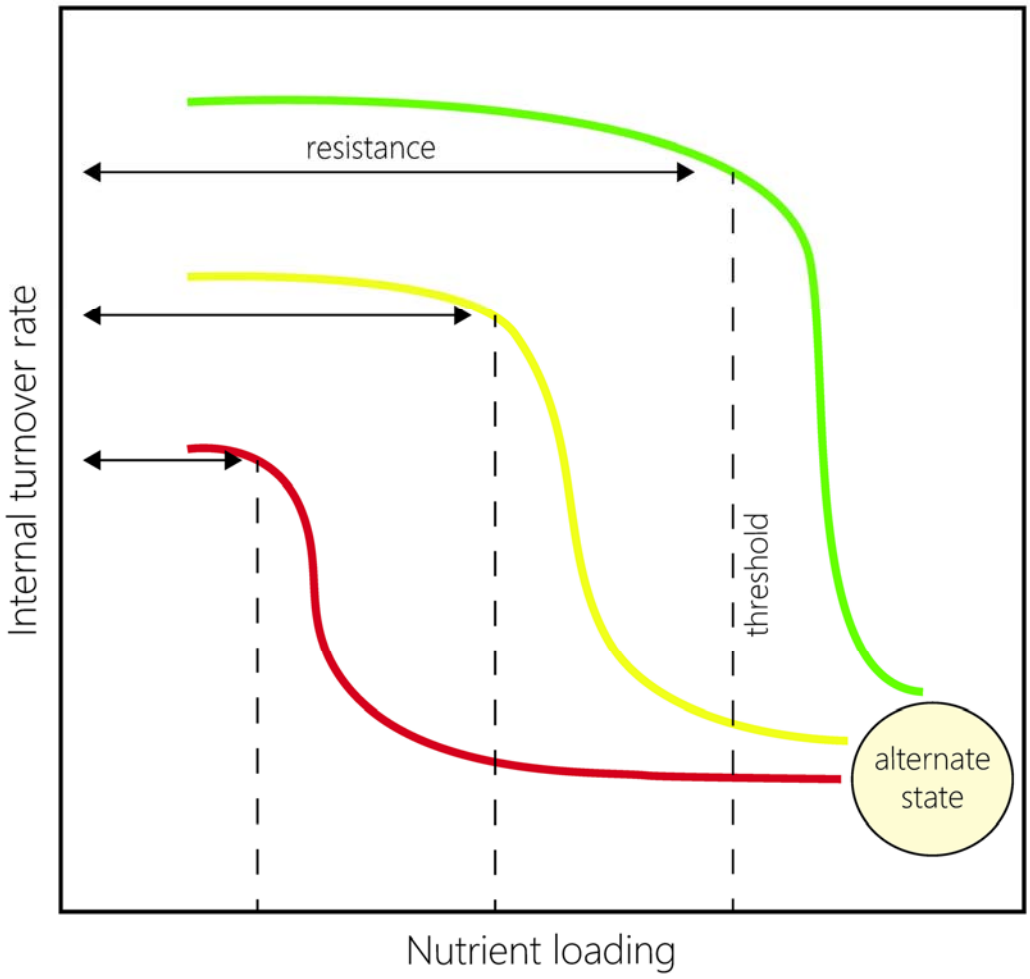

Figure 2.6 Internal turnover rate vs Benthic inorganic nutrient flux/Water column total nutrient mass, as a simple way to express the interaction between the water column and sediment nutrient cycles) in sediments under increasing nutrient loadings. Hypothetical scenarios when both invertebrate-microbe associations and macrophytes are present (green), when only one of the two players is present (yellow), and when both are absent from the system (red).

Considering the importance of the microbial component for ecosystem health, it can be inferred that an additional challenge lies in the identification of variables that can help in monitoring efforts. Sims et al., (2013) proposed a framework for the inclusion of key microbial indicators to assess wetlands condition in combination with physico-chemical variables, e.g. Ammonia-Oxidizing archaea to bacteria ratio, number of relevant functional groups such as methanogens, methanotrophs and/or number of SRB. Other ideas include a microbial assemblage-derived index to infer the degree of stress on the ecosystem, tested on coastal and estuarine areas (Aylagas et al., 2017; Clark et al., 2020); or more straightforward comparisons of the microbial assemblage together with morphological and physiological traits of seagrasses and mangrove sediments (Cao et al., 2011; Mejia et al., 2016). In all cases, all share the pivotal

idea that the inclusion of the microbial component within multivariate evaluations will help to gain a more holistic perspective of the ecosystem condition.

Any of these microbial indicators can be included in the wide range of tools to assess and compare tipping points in marine ecosystems (Andersen et al., 2009; Eslami-Andergoli et al., 2015; Biggs, Peterson & Rocha, 2018). Their application to gain insights about microbial community dynamics, e.g. via time series also have precedents (Faust et al., 2015), which would allow the creation and curation of highly relevant datasets to understand the role of microbes in marine nutrient cycling. Consequently, the goal of using microbial proxies to monitor thresholds of change in coastal ecosystems is feasible, but a major new challenge lies ahead - the design of monitoring programs that consider as many potential indicators as possible (as listed in Eslami-Andergoli et al., 2015) to increase the likelihood to successfully detect potential threats.

Concluding remarks and future directions

The current possibilities to combine multidisciplinary approaches to study marine holobionts is significantly enhancing our understanding of the ecosystems at multiple scales. From the individual hosts, as the carriers microbial communities as variable as can be expected from true ecosystems, to the landscape level where the collective effect of bioturbators over the nutrient exchanges can exert tangible differences. This knowledge is pivotal if we want to have access to new approaches to mitigate the decline and restore the impacts that our society inexorably exerts on coastal ecosystems.

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Chapter 2:
Accelerated nitrogen cycling on seagrass leaves in a high-CO_2 world

Abstract

Seagrass meadows form productive ecosystems in coastal areas worldwide, where they are increasingly exposed to ocean acidification (OA). Efficient nitrogen (N) cycling and uptake are essential to sustain plant productivity, but quantification of processes with an evaluation of the effects of OA are missing. Here we show that complete N cycling occurs on leaves of the seagrass *Posidonia oceanica* in the Mediterranean Sea, with OA affecting both N gains and losses while the prokaryotic community structure remained largely unaffected. N_2 fixation in the light was 3.7-fold higher under OA, possibly sustaining the larger plant N uptake. Contrary to expectations, nitrification potential was only detected under OA. Concurrently, nitrate reduction accelerated, with significant production of $^{29}N_2$ suggesting the occurrence of both denitrification and anammox. Marine plants are bound to adapt to OA if they are to persist; our work shows that plasticity of their N cycling microbiome plays a key role.

Keywords: *Posidonia oceanica*; ocean acidification; holobiont

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Introduction

Seagrass meadows form highly productive ecosystems worldwide, often occurring in nutrient-limited coastal areas (Hemminga & Duarte, 2000). They are one of the most ecologically and economically valuable ecosystems on Earth (Björk et al., 2008). Providing habitat, breeding ground, and food for a wide variety of organisms, they are regarded as "hotspots" for biodiversity (Hyman et al., 2019). Furthermore, they have 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 with its effective CO_2 uptake and large carbon burial (Duarte et al., 2013a) and may even act as a buffer to ocean acidification (OA), temporarily increasing seawater pH thanks to its daylight photosynthesis (Duarte et al., 2013b; Hendriks et al., 2014). This is relevant since the Mediterranean Sea has a higher capacity to assimilate anthropogenic CO_2 than other oceans due to its active overturning circulation and higher temperature and is thus expected to have faster rates of OA (Lacoue-Labarthe et al., 2016).

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

The impact of OA on the biogeochemical cycling of elements other than carbon, such as nitrogen (N), received far less attention. N is an essential nutrient for all living organisms and can be a limiting factor for primary production in marine seagrasses (Hemminga, Harrison & Van Lent, 1991), with its availability depending on diverse N transformation processes that are carried out by a complex network of metabolically versatile microorganisms (Kuypers, Marchant & Kartal, 2018). Seawater pH affects N speciation and concentrations, influencing metabolic processes and N transformations (Wyatt et al., 2010; Wannicke et al., 2018). N_2 fixation is likely to increase under OA (Hutchins, Mulholland & Fu, 2009), even though this response might be species-specific (Eichner, Rost & Kranz, 2014). Autotrophic

microbial nitrification is known to be highly sensitive to pH, and nitrification in the open ocean was found to be significantly reduced with OA (Beman et al., 2011). Dissimilatory nitrate reduction processes (denitrification, anammox, dissimilatory nitrate reduction to ammonium – DNRA), being modular and involving many different bacterial groups often found in low-pH environments, are thought to be less affected by OA, with rates showing contrasting results under lower seawater pH (Wannicke et al., 2018).

Many N cycling microorganisms can be found in close association with *P. oceanica*, together forming a holobiont (Ugarelli et al., 2017; Tarquinio et al., 2019). Nitrogen-fixing bacteria (i.e., diazotrophs) can transform atmospheric dinitrogen (N_2) into bioavailable molecules that can be used by the plant host. N_2 fixation by associated diazotrophic microorganisms can be crucial in providing ammonium needed for seagrass photosynthesis and growth, when N availability is limited (Cardini et al., 2018; Mohr et al., 2021). Diazotrophic bacteria have been detected in the rhizosphere of *P. oceanica* (Garcias-Bonet et al., 2016) with high reported rates of root-associated N_2 fixation (Lehnen et al., 2016). Similarly to many land plants that associate with diazotrophs, a recent study shows that *P. oceanica* lives in symbiosis with a N_2-fixing gammaproteobacterium in its roots, providing N in exchange for sugars, which can fully sustain plant biomass production in its primary growth season (Mohr et al., 2021). Other than this root-symbiosis, N_2 fixation has been shown to occur associated with all parts of *P. oceanica*, above and belowground (Agawin, Ferriol & Sintes, 2019) potentially involving Na^+-dependent high affinity cellular transporters (Rubio et al., 2018). While seagrasses compete with nitrifiers for ammonia/ammonium in surface sediments and overlying water, vegetated coastal sediments show substantially higher nitrification potential (Lin et al., 2021).

Overall, while 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 missing. We hypothesize that seagrass leaves could also be suitable sites for nitrification, the two-step microbial oxidation of NH_4^+ to NO_2^- and then to NO_3^-. 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 tissue. Moreover, anoxic parts within thick biofilms on the leaf surface could be a potential microhabitat for N loss pathways, such as denitrification (Noisette et al., 2020; Brodersen & Kühl, 2022) or anaerobic ammonium oxidation (anammox) carried out by Planctomycetes that can dominate the microbiome of *P. oceanica* leaves (Kohn et .al., 2020).

Here, we investigated the effects of long-term natural OA on the epiphytic prokaryotic community of *P. oceanica* leaves and quantified rates of the key N cycling processes by the plant phyllosphere. We tested the effects of pH and the presence/absence of epiphytes in multifactorial laboratory incubations,

using N stable isotope tracers to quantify the main microbe-mediated N transformation processes, i.e., N_2 fixation (dinitrogen gas to ammonia, adding to the ecosystem bioavailable N pool), nitrification potential (aerobic ammonium oxidation to nitrite and nitrate, recycling N within the ecosystem), and anammox and denitrification potential (dinitrogen gas production from anaerobic ammonium oxidation and/or nitrate reduction, constituting the main pathways of ecosystem N loss). We combined these measurements with 16s rRNA gene amplicon sequencing for exploring the diversity of the prokaryotic community. We found accelerated N cycling on seagrass leaves acclimated to natural OA, with increased rates of microbial N_2 fixation, nitrification potential, and anammox and denitrification potential despite a largely unaffected prokaryotic community (i.e., the diversity and composition taken together remained virtually the same among the pH treatments). Further, we found an increased net uptake of NH_4^+ and NO_3^-, primarily attributed to the plant and the epiphytes, respectively. Plasticity of the phyllosphere N cycling microbiome is therefore a crucial factor in regulating seagrass functioning in a high-CO_2 world.

Methods

Study area

The study area is located at the Castello Aragonese islet on the North-Eastern coast of the island of Ischia (Tyrrhenian Sea, Italy). This site is characterized by the presence of submarine CO_2 vents of volcanic origin that naturally originate a gradient in CO_2 concentrations and pH without affecting the surrounding water temperature or salinity (Hall-Spencer et al. 2008; Foo et al. 2018). Around the islet, meadows of *P. oceanica* occur at 0.5 - 3 m depth, also reaching into venting zones with low pH. We selected two sites characterized by different pH regimes (“vent pH” and “ambient pH”) at approximately 3 m water depth (Table 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 islet (40°43'54.8"N 13°57'47.1"E; figure 3.1).

Figure 3.1- Location of the sampling points in the study area. The top marker indicates the location of the meadow at natural pH and the bottom marker indicates the location of the madows exposed to the vents.

Sampling

Microbial samples were collected in October 2019 at the vent and ambient site described above. Before disturbing the plants, we collected 5 L of seawater from the water column above the plants. Whole seagrass plants were collected, cut into pieces, washed with sterile NaCl solution [0.8 % m/v], and transferred into 15 mL falcon tubes with tweezers. For the transport, the falcon tubes were stored on dry ice and in the laboratory at -20°C. In the laboratory, the seawater was immediately filtered on a 0.2 µm filter and the filters were stored at -20°C until further analysis.

For the incubation experiments, shoots of *P. oceanica* were collected at each site at three days in September 2019 and directly transported into the nearby laboratory. Sections of the central part of the leaf with 3cm in length were cut off, selecting leaves with homogenous epiphyte coverage, and avoiding highly grazed and senescent parts of the plant as described in Berlinghof et al. (2022). For half of the incubations, epiphytes were carefully removed with a scalpel, taking particular care not to damage the plant tissue. Leaf sections, either from the vent pH or the ambient pH site, with epiphytes present (n = 4) or removed (n = 3), were used for incubations in the dark and in the light.

Molecular Analyses of the Prokaryotic Communities

DNA extraction

DNA was extracted using the Qiagen DNeasy Powersoil Kit: first, we cut at least 1 g of the central portion of the leaf and the entirety of the filters into small pieces. These were put into 2 mL vials with 600 µL of sterile NaCl solution [0.8 % m/v] and were vortexed three times for 30 s. The solution was

transferred to the powerbead columns and then processed following the manufacturer's instructions with the addition of two incubation cycles of vortexing for 2 min followed by heating the samples at 70°C for 5 min. The extraction quality was quantified using a microvolume spectrophotometer (Thermo Scientific NanoDrop 2000c).

Sequencing and preprocessing of the data

Illumina MiSeq sequencing 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). We removed the primers from the raw sequence data using cutadapt v2.8 (Martin, 2011) and processed the fastq files using the R package *DADA2* (Callahan et al., 2016; R Core team, 2021). Quality filtering and denoising of the trimmed fastq files were performed using the following parameters: "truncLen = c(200, 200), maxEE = c(2, 2), truncQ = 2, ndmaxN = 0). Then, we assigned the taxonomic categories to the amplicon sequence variants (ASV) using the SILVA v138 database (Quast et al., 2013). The use of ASV instead of the operational taxonomic units has been recommended based on their improved resolution and biological consistency (Callahan, McMurdie & Holmes, 2017). The full pipeline is openly available in the research compendium accompanying this paper at https://github.com/luismmontilla/embrace. Sequences are available in the NCBI SRA database as the BioProject ID PRJNA824287.

Statistical Analyses

The ASV matrix was analyzed as a compositional dataset, as appropriately detailed in other works (Fernandes et al., 2014; 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. Then, we tested the null hypothesis of no effect of the factors described above on the *P. oceanica* prokaryote community through a permutation-based multivariate analysis of variance derived from a Euclidean distance matrix. We performed this test using the *vegan* package for R (Anderson, 2001; Oksanen et al., 2020). Additionally, we performed a differential abundance analysis of the ASV 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).

Nitrogen cycling incubations

Dinitrogen fixation

To determine rates of N_2 fixation, we used the $^{15}N_2$-enriched seawater addition method (Mohr et al., 2010; Klawonn et al., 2015). Stock solutions of 0.22 µm filtered and $^{15}N_2$-enriched water of the two

study sites (vent and ambient pH) were prepared and gently transferred into 24 mL glass vials to minimize gas exchange with the atmosphere. After that, seagrass leaves with (n=4) and without epiphytes (n=3) were added to the vials. Additionally, vials with 0.22 µm filtered but non-enriched site water containing leaves with epiphytes served as controls (n=3). The vials were incubated on a shaker (Stuart orbital shaker SSL1; 30 rpm), with vials for dark incubations covered with aluminum foil, while vials for light incubations were incubated upside down with the transparent bottom exposed to the light source. The incubations were conducted in a temperature-controlled room at 22°C. After an incubation time of T0 = 0 h, T1 = 5 h, and T2 = 9.5 h light/ 8.5 h dark, a part of the vials was opened for sampling. At the beginning and end of the incubation, oxygen concentrations in the incubation vials were measured using a fiber-optic oxygen sensor (FireStingO2, PyroScience), and pH was measured using a pH meter (Multi 3430, WTW). The opened vials were not further incubated, but separate vials were used for each sampling time.

For tissue analysis, epiphytes and seagrass leaves were separately transferred into Eppendorf tubes and freeze-dried for 72 h. Subsequently, they were homogenized by mortar and pestle and weighed into tin cups to determine carbon (% C), nitrogen content (% N), and ^{15}N incorporation. Samples of the incubation water were transferred into 12 mL exetainers (Labco Ltd) and fixed with 200 µL of 7M $ZnCl_2$ for $^{29}N_2$ and $^{30}N_2$ analyses to calculate atom% excess of the medium. Additionally, samples for the analysis of dissolved nutrient concentrations (DIN: NH_4^+, NO_2^-, NO_x^-, and DIP: PO_4^-) were transferred into 20 ml HDPE vials and stored upright at -20°C until further analysis.

The 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 with an elemental analyzer (Flash EA1112, Thermo Scientific) at Aarhus University (Denmark). $^{15}N_2$ incorporation rates were calculated as in Montoya et al. (1996).

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

$$N_2 incorporation = \frac{atom\%(^{15}N_{excess})}{atom\%(^{15}N_{medium})}$$

$^{15}N_{sample}$ 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 higher 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 reached 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).

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 analyser (Flowsys, SYSTEA SpA.). NO_3^- concentrations were calculated as the difference between NO_x^- and NO_2^-.

Nitrification potential

Nitrification potential was determined using stock solutions of 0.22 µm filtered water of the vent and ambient pH site enriched with $^{15}NH_4^+$ with a final concentration of 20 µM. The incubation was carried out as described above with sampling times at T0 = 0h, T1 = 2h, T2 = 5h, and T3 = 9 h light/ 8 h dark. Water samples were 0.22 µm filtered, transferred into 15 mL polypropylene tubes, and stored at -20°C for the analysis of NO_3^- production. At each sampling time, samples were also collected for NO_3^- concentration measurements.

Isotopic samples for $^{15}NO_3^-$ production were analyzed by isotopic ratio mass spectrometry following the Ti(III) reduction method described by Altabet et al. (2019). Sample aliquots for nitrification analysis (3 mL) were acidified by adding 10 µL 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 vial, such that the final concentration of USGS-32 was 0.1 ppm NO3-N. NO_3^- was then converted to nitrous oxide (N_2O) for stable N isotope analysis via the Ti(III) reduction method (Altabet et al., 2019). Briefly, after combining the sample with the standard, the exetainer headspace was flushed with argon for 2 minutes and then amended with 150 µL zinc-treated 30% $TiCl_3$; the final reaction volume was 6.15 mL. The exetainers were immediately capped with a gas-tight, pierceable, chlorobutyl rubber septum. The Ti(III)-treated samples were left > 12 h at room temperature 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, 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 the carrier gas. The sample was then introduced to a MAT 253PLUS IRMS via a Conflo interface (ThermoScientific). $\delta^{15}N$ values were determined relative to the N_2O working gas, then corrected for linearity according to the peak height relationship and 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 via

the concurrent analysis of international standards USGS32, USGS34, and USGS35. 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 ^{15}N-ammonia oxidation rates ($^{15}R_{ox}$) were determined based on the accumulation of ^{15}N in the oxidized pool relative to the initial at% enrichment in the NH_4^+ pool and divided by the incubation time. Rates were calculated using an equation modified from Beman et al. (2011):

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

$$R_{ox} = atom\%(^{15}N_{excess}) \times \quad \text{(VIII)}$$

$^{15}N_t$ 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 higher 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 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 represents the incubation time (h).

Anammox and denitrification potential

To determine potential anammox and denitrification rates, stock solutions of 0.22 µm filtered water of the two study sites (vent and ambient pH) were enriched with $^{15}NO_3^-$ with a final concentration of 10 µM. The incubation was carried out as for the N_2 fixation experiment (see "*Dinitrogen fixation"*), with sampling times at T0 = 0 h, T1 = 2 h, T2 = 5 h and T3 = 8 h light/ 8h dark. Vials with 0.22 µm filtered but non-enriched site water without leaves served as controls (n=3). Samples of the incubation water were transferred into 12 mL exetainers (Labco Ltd) 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-isotopic ratio mass spectrometry (GC-IRMS, Thermo Delta V Plus, Thermo Scientific) following the protocol described by De Brabandere et al. (2015). Production rates of ^{15}N-enriched N_2 gas were calculated from the difference in $^{29}N_2$ and $^{30}N_2$ concentrations between T1 (2 h) and T2 (5 h), because we have seen a lag phase from T0 to T1. Because changes in $^{29}N_2$ and $^{30}N_2$ concentrations were very small, 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. These calculations involve assumptions, such as random isotope pairing (Thamdrup & Dalsgaard, 2002), which would further increase the uncertainty of the calculated rates given the small

concentration changes. $^{29}N_2$ and $^{30}N_2$ production rates were normalized per seagrass leaf area (cm^2) and corrected for the rates in control incubations without organisms.

Statistical Analyses of the N cycling incubations

We tested for normality and homogeneity of variances before each analysis using Shapiro-Wilk's and Levene's tests and removed outliers when 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, nitrification potential ($^{15}R_{ox}$), $^{29}N_2$ 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 (version 4.1.2) using the packages *car* and *emmeans* (R Core team 2021).

Results

Prokaryote community structure

A small group of phyla dominated across the samples, indistinctly from the pH regime or the compartment (Fig. 3.2). In decreasing order of contribution, Proteobacteria accounted for over 50% of the ASV, followed by Bacteroidota (17 - 24%); Cyanobacteria (2 - 14%), and Verrucomicrobiota (3 - 4%). Among all the conditions, the composition pattern of the taxonomic classes was also consistent, with Alphaproteobacteria and Gammaproteobacteria as the sole representatives of Proteobacteria. The rest of the phyla were represented basically by a single class: Bacteroidia, Cyanobacteriia, and Verrucomicrobia, respectively.

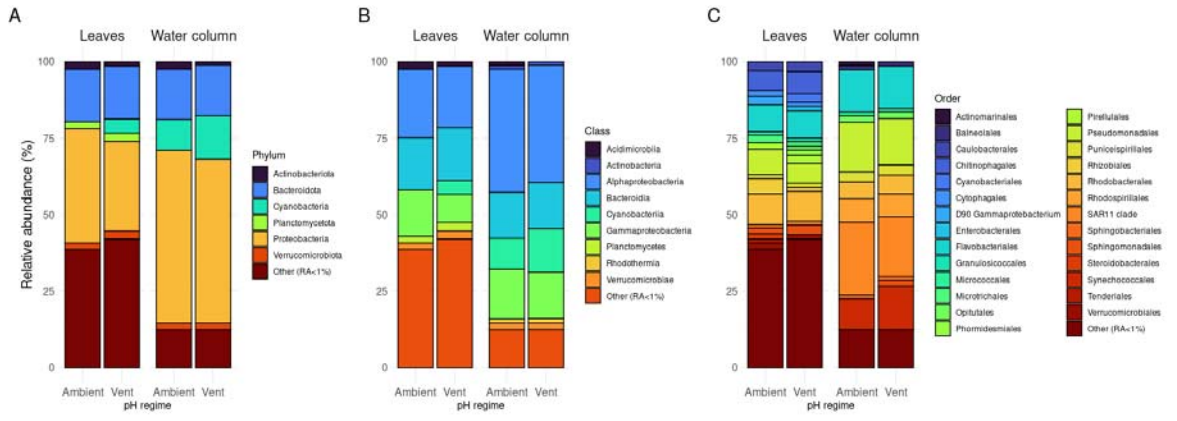

Figure 3.2- ASV relative abundance on leaves and water column samples from both pH regimes, showing the dominant ASV taxonomic phyla, classes, and orders.

We further examined the data to look for patterns in relevant N transformation-related groups. We found one order of magnitude more nitrifiers in the leaves compared to the water column; these were the bacteria families Nitrosomonadaceae, Nitrospinaceae, Nitrospiraceae and the archaea family Nitrosopumilales. While the proportion was similar between pH regimes in the leaves, in the water column from the vent sites, the dominant nitrifiers were archaeal (Fig. 3.3). In particular, all the archaea sequences belonged to Candidatus Nitrosopumilus, while the bacteria sequences included six different phylotypes, of which we could identify the genera Nitrososspira and Nitrospira.

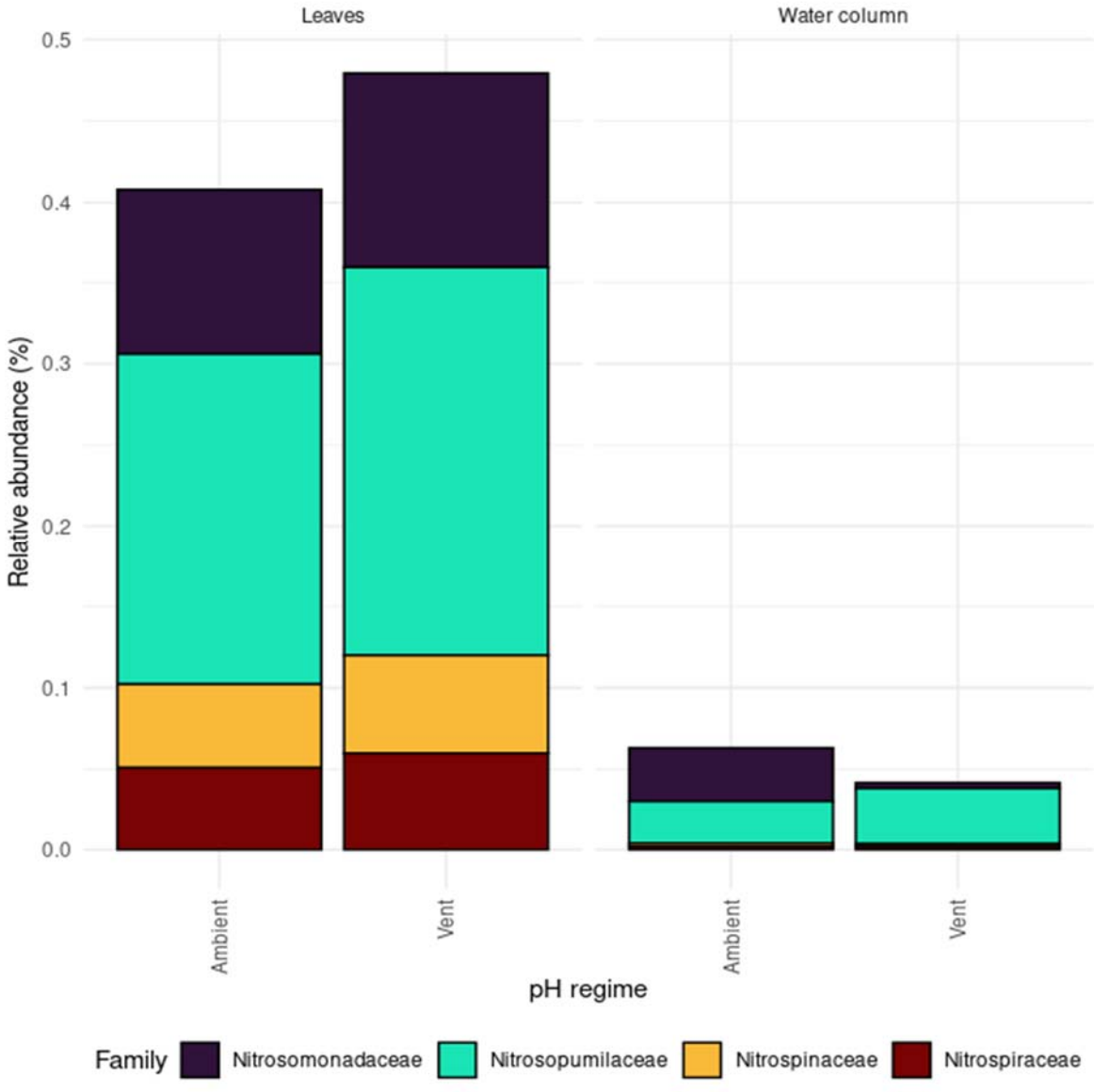

Figure 3.3 - ASV relative abundance of nitrifiying taxa.

The pH regimes did not affect the prokaryote community structure; instead, 70% of the variation was explained by the sampled compartment (plant leaf or water column) with no interaction between

these two factors (Suppl. Table 1). The principal coordinates analysis showed this lack of effect of the pH regimes (Suppl. Fig. 1) and though there seemed to be a variation explained by the pH on the water column samples, the result of the Permanova does not support this as an important effect.

Through the differential abundance analysis, we identified the group of ASV contributing to the variation between compartments. In general, ASV assigned to Alpha-, Gammaproteobacteria, Bacteroidota, and Verrucomicrobiota dominated the abundance in the samples. In the leaf, the most distinctive groups (either because they were rare taxa only present in this compartment or because they were particularly abundant) were the classes Blastocatellia (Acidobacteriota), Polyangia (Myxococcota), Desulfobulbia (Desulfobacterota), Vampirivibriona (Cyanobacteria), Deinococci (Deinococcota), Clostridia (Firmicutes), Nitrososphaeria (Crenarchaeota), Campylobacteria (Campylobacterota), and Bdellovibrionia (Bdellovibrionota). On the other hand, the exclusive taxa from the water column belonged to the classes Dadabacteriia (Dadabacteria) and Kiritimatiellae (Verrucomicrobiota) (Fig. 3.4).

Nitrogen cycling

Dinitrogen fixation

Epiphytic $\delta^{15}N$ increased from 8.29 to 12.59 in light incubations ($F_{1,28} = 48.42$, $p < 0.001$, $R^2 = 0.63$) and from 8.29 to 8.33 in dark incubations ($F_{1,27} = 27.64$, $p < 0.001$, $R^2 = 0.52$), regardless of the pH (Suppl. Fig. 2). In contrast, leaf sections of *P. oceanica* showed no increase in $\delta^{15}N$ over time (Suppl. Fig. 3). We detected epiphytic $^{15}N_2$ incorporation in the light incubations, ranging from 35.43 ± 13.26 nmol N g DW^{-1} h^{-1} (mean ± SE) at the ambient site to 131.08 ± 32.73 nmol N g DW^{-1} h^{-1} at the vent site. $^{15}N_2$ incorporation was increased 3.7-fold at the vent site ($F_{1,12} = 7.20$, $p = 0.020$, $R^2 = 0.68$). In the dark incubations, we observed no significant $^{15}N_2$ incorporation. The N_2 fixation rates in the incubations on leaf dry weight basis in Fig. 3a correspond to 0.020 mmol N m^{-2} d^{-1} at the vent site and to 0.014 mmol N m^{-2} d^{-1} at the ambient site, calculated by considering the areal leaf biomass of *P. oceanica* in the study sites and a 12:12 daylight/night cycle.

Nitrification potential

The overall increase in $\delta^{15}N\text{-}NO_3^-$ over time in incubations with leaf sections with epiphytes in light and dark incubations was not significant due to high variability among the samples (Suppl. Fig. 4). However, we found significant (>2.5 SD) ^{15}N-ammonia oxidation rates ($^{15}R_{ox}$) at the vent site when epiphytes were present (Fig. 3.5b), ranging from 1.49 ± 0.14 pmol NH_4^+ cm^{-2} d^{-1} in the dark to 2.77 ± 0.07 pmol NH_4^+ cm^{-2} d^{-1} in the light. $^{15}R_{ox}$ was 1.86-fold higher in the light ($F_{1,4} = 63.48$, $p = 0.001$, $R^2 = 0.99$). In contrast, we found no significant $^{15}R_{ox}$ in incubations with epiphytes from the ambient site.

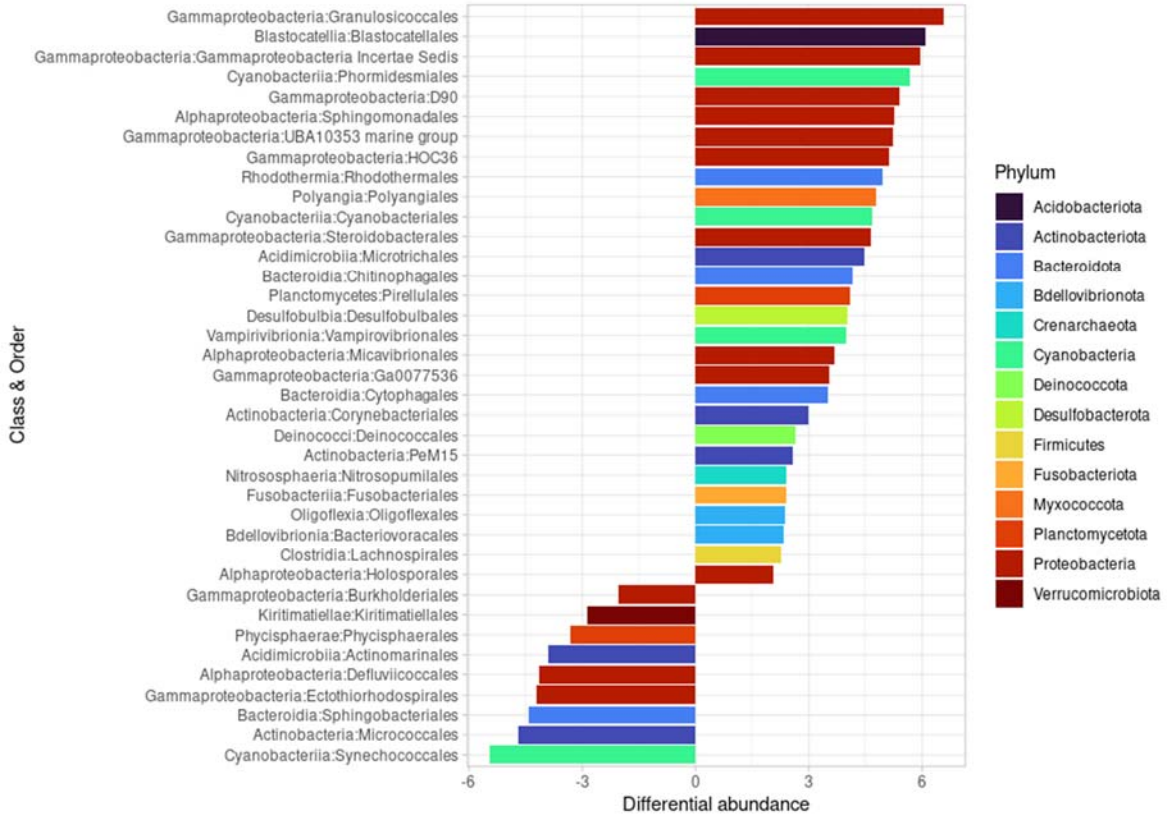

Figure 3.4 - Differential taxonomic class order abundance in leaf and water column samples. Positive values mean differential abundance in the leaves and negative values in the water column.

Anammox and denitrification potential

Production rates of $^{29}N_2$ ranged from 0.05 ± 0.01 nmol N cm^{-2} d^{-1} at the ambient site in the dark to 0.17 ± 0.05 nmol N cm^{-2} d^{-1} at the vent site in the light (Fig. .4c). $^{29}N_2$ production was 2.34-fold 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. Significant production rate of $^{30}N_2$ was only detected at the vent site in the light with 0.45 ± 0.08 nmol N cm^{-2} d^{-1} (Fig. 3.5d).

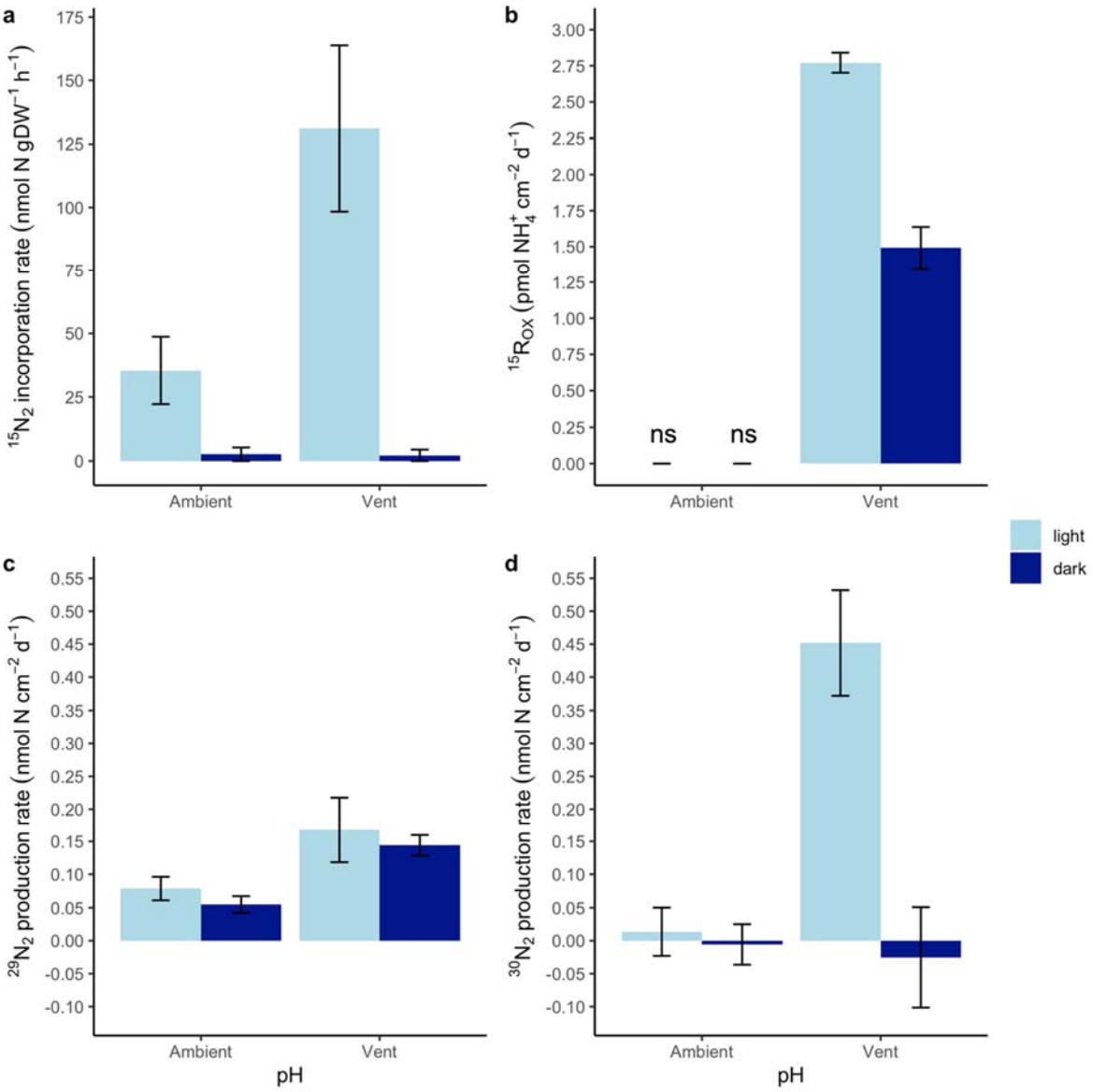

Figure 3.5 - Nitrogen transformations in light and dark incubations from the ambient and vent site. Epiphytic 15N2 incorporation rates (a; n = 4), potential 15N-ammonia oxidation rates (15ROX) in incubations with leaf sections with epiphytes (b; n=2), 29N2 (c) and 30N2 production rate (d) in incubations with leaf sections with epiphytes (n = 4). Error bars indicate mean ± SE, ns indicates enrichment was not significant.

Nutrient fluxes

Ammonium

Net fluxes of NH_4^+ in the light varied from -0.38 ± 0.01 µmol cm^{-2} h^{-1} (mean ± SE) in incubations with leaf sections from the vent site without epiphytes to -0.29 ± 0.04 µmol cm^{-2} h^{-1} in incubations with leaf sections from the ambient site with epiphytes (Fig. 3.6a). In the dark, net fluxes of NH_4^+ varied from -0.35 ± 0.01 µmol cm^{-2} h^{-1} in incubations with leaf sections from the vent site without epiphytes to -0.29 ± 0.01 µmol cm^{-2} h^{-1} in incubations with leaf sections from the ambient site with epiphytes (Fig. 3.6b).

NH_4^+ fluxes were increased by 26% at the vent site in light incubations ($F_{1,10} = 18.22$, $p = 0.002$, $R^2 = 0.58$) and by 21% in dark incubations ($F_{1,10} = 25.25$, $p < 0.001$, $R^2 = 0.66$). By contrast, the presence/absence of epiphytes did not affect NH_4^+ uptake rates.

Nitrate

Net fluxes of NO_3^- in the light varied from -0.036 ± 0.010 μmol cm^{-2} h^{-1} in incubations with leaf sections from the vent site with epiphytes to -0.013 ± 0.001 μmol cm^{-2} h^{-1} in incubations with leaf sections from the ambient site without epiphytes (Fig. 3.6c). In the dark, net fluxes of NO_3^- in the light varied from -0.019 ± 0.018 μmol cm^{-2} h^{-1} in incubations with leaf sections from the vent site with epiphytes to 0.007 ± 0.013 μmol cm^{-2} h^{-1} incubations with leaf sections from the ambient site without epiphytes (Fig. 3.6d). We observed a pattern of higher NO_3^- consumption in incubations where epiphytes were present and at the vent site, but the effects of site or treatment on NO_3^- fluxes in light or dark incubations were not significant.

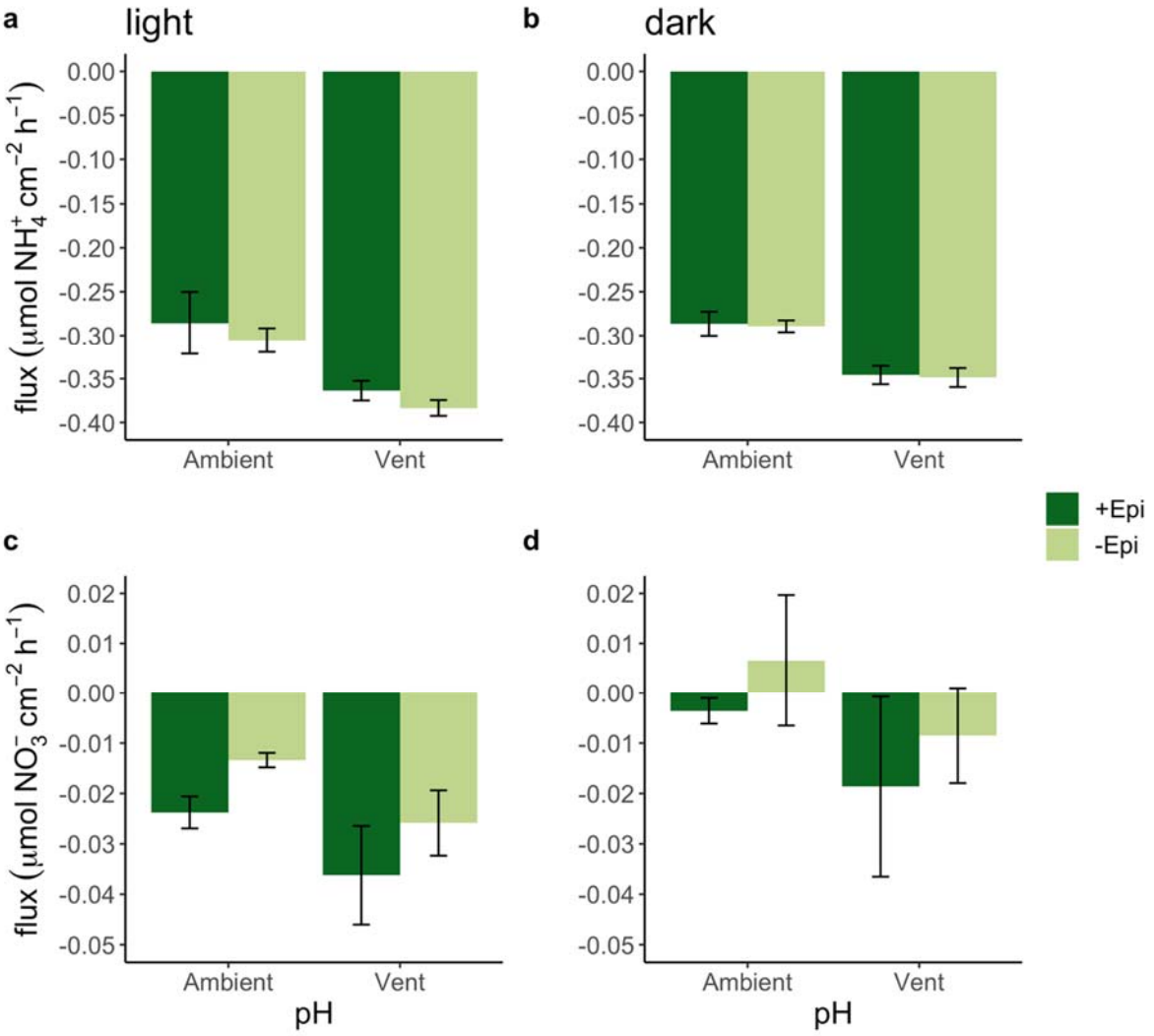

Figure 3.6 - Fluxes of NH4+ (a, b) and NO3- (c, d) during light (a, c) and dark (b, d) incubations with leaf sections from the ambient and vent site with (+Epi, n=4) and without epiphytes (-Epi, n=3). Error bars indicate mean ± SE.

C:N ratios

The C:N molar ratio of seagrass leaf sections ranged from 37.78 ± 2.41 (mean ± SE) at the ambient site to 40.35 ± 3.91 at the vent site (Suppl. Fig. 5a). C:N ratios of epiphytes ranged from 24.66 ± 1.93 at the ambient site to 17.37 ± 2.48 at the vent site (Suppl. Fig. 5b). While the C:N ratio of leaf sections was not affected by pH, it was 42% higher in epiphytes from the ambient site.

Discussion

Nitrogen cycling in the *P. oceanica* phyllosphere

In this study, we show that all key N cycling processes occur in the phyllosphere of *P. oceanica*. Our estimates for N_2 fixation rates associated with *P. oceanica* leaves are in the same order of magnitude

as rates found by Agawin et al. (2016). With values up to 131.08 nmol N g DW^{-1} h^{-1} at the vent site (Fig. 2.4a), they are comparable to those measured in *P. oceanica* roots (Lehnen et al., 2016; Mohr et al., 2021). The daily N budget provided by the seagrass phyllosphere is 0.020 mmol N m^{-2} d^{-1} at the vent site and 0.014 mmol N m^{-2} d^{-1} at the ambient site and comparable to rates estimated by Agawin et al. (2017) in summer incubations with benthic chambers.

N_2 fixation rates were higher under light conditions. This, together with the high rates measured, indicates a diazotrophic community dominated by species that can handle O_2 production from daytime photosynthesis, which would otherwise irreversibly inhibit the enzyme nitrogenase. We found Cyanobacteria accounting for 2 – 14% of the ASVs on seagrass leaves, however interestingly none of the ASV we identified belonged to the orders Nostocales or Stigonmatales, which are the usually expected heterocystous cyanobacteria. This is not necessarily contradictory evidence, considering that we found sequences for *Schizothrix* and *Trichodesmius* in the leaves from both pH regimes*;* these two are able to sustain N_2 fixation in the light (Bergman et al., 2013; Berrendero et al., 2016). Though unexpected, some of the prokaryotes we found are known contributors to N fixation in the dark. For instance, a strain of *Acaryochloris,* genus typically not recognized as a diazotroph, have been detected to be able to undergo N fixation possibly as a consequence of horizontal gene transfer (Pfreundt et al., 2012; Miller et al., 2022); as a side note, it also seems to benefit from the presence of seagrasses in bivalves exposed to OA (Garner et al., 2022). Similarly, *Chroococcidiopsis* and *Synechococcus* are prokaryotes with the potential to fix under appropriate conditions such as dark conditions (Huang et al., 1999; Banerjee & Verma, 2009). Interestingly, *Synechococcus* seems to be a key member microbial biofilms under certain conditions, being able to establish mutualisms with other members of the microbial community to establish a self-sufficient N cycle (Zhang et al., 2021). It was also the dominant cyanobacterial ASV in the seawater samples. These groups are probably the largest contributors to the measured N_2 fixation activity on leaves and in the seawater, respectively.

Fixed N in the form of NH_4^+ can be recycled and converted into NO_3^- via nitrification. However, the plant competes for N with nitrifiers since NH_4^+ is typically readily taken up by *P. oceanica* (Lepoint et al., 2002). Notwithstanding, we found potential nitrification rates ($^{15}R_{OX}$) associated with the seagrass phyllosphere. While rates were low (2.77 ± 0.07 pmol NH_4^+ cm^{-2} d^{-1}), it is significant that potential nitrification was only detected at the vent site in the light when epiphytes were present (Fig. 2.5b). Ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) can be found in seagrass sediments and other seagrass-associated environments (Ling et al., 2018; Zheng et al., 2019; Lin et al., 2021), promoting rates of nitrification potential of up to 250 nmol cm^{-2} d^{-1} in *P. australis* sediments

(Camillini, 2020). We found the families Nitrosomonadaceae, Nitrospiraceae, Nitrospinaceae (AOB), and Nitrosopumilales (AOA) in the phyllosphere of *P. oceanica*. Especially Nitrosopumilales, but also species within the other families, show a high affinity for NH_3 (Lin et al., 2021; Jung et al., 2022) and could therefore compete with the seagrass.

Interestingly, we found higher $^{15}R_{OX}$ in light incubations, though bacterial nitrification is classically thought to be inhibited by light (Ward, 2008). However, nitrification by AOA seems to be less affected by light, meaning that nitrification can occur throughout the water column and even in well-lit surface waters (Zehr & Kudela, 2010).

While nitrification by AOA and AOB is an aerobic process, NH_4^+ can also be oxidized anaerobically, referred to as "anammox". This N loss pathway is estimated to contribute 29% of the global marine N loss from sediments and anoxic water columns (Voss et al., 2013). In our experiments, the addition of $^{15}NO_3^-$ resulted in higher production of $^{29}N_2$ compared to $^{30}N_2$ in most treatments (Fig. 3.5c, d). The increase in the isotope fraction of $^{29}N_2$ and the oligotrophic environmental setting of the sampling site point to anammox dominating over denitrification in our incubations. In fact, when $^{15}NO_3^-$ is added, N_2 produced through anaerobic ammonium oxidation consists of one nitrogen atom from NO_3^- and one from NH_4^+, resulting in only $^{28}N_2$ and $^{29}N_2$ being produced. Conversely, denitrification is assumed to produce $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ through random isotope pairing leading to a binomial distribution of the three isotopes (Nielsen, 1992; Risgaard-Petersen et al., 2003). We observed $^{29}N_2$ production up to 0.22 nmol N cm-2 d-1 and $^{30}N_2$ production up to 0.53 nmol N cm-2 d-1 in our incubations when epiphytes were present. These rates are in the same order of magnitude as denitrification and anammox rates (0.10 and 0.43 nmol N cm-2 d-1, respectively) found in seagrass sediments by (Salk et al., 2017). The sediments of seagrass meadows are considered as important regions for N loss by the conversion of bioavailable to gaseous N (Garcias-Bonet et al., 2018).

Planctomycetes, which utilize anammox and are a group that has been considered a potentially underrated member of the seagrass microbiome, were also found in our microbial communities. In *P. oceanica* samples from Corsica, they were among the most abundant members of the leaf microbiome (Kohn et al., 2020). More generally, this group has been described as a common member of marine biofilms and being a frequent symbiont of marine macrophytes and animals; it has also been found across environmental gradients and is able to fix nitrogen and anaerobically oxidate ammonia (Ikenaga et al., 2010; Kaboré, Godreuil & Drancourt, 2020).

Biofilms on the surface of seagrasses can also be suitable microhabitats for denitrification due the formation of an anoxic microlayer (Noisette et al., 2020; Brodersen & Kühl, 2022). While $^{30}N_2$

production in most of our incubations was not significant, we found high $^{30}N_2$ production rates in light incubations with leaf sections with epiphytes from the vent site. Here, we also found the highest N_2 fixation and $^{15}R_{OX}$, which could sustain the denitrification process. The presence of denitrification proceses have immediate consequences for the plant health, as there can be accumulation of toxic intermediates such as nitric oxide (NO) in this microlayer (Noisette et al., 2020); ultimately, the loss of N through this proces can also be linked emissions of nitrous oxide (N_2O) (Rosentreter et al., 2021).

Effects of ocean acidification

Our results show that OA accelerates key N transformation processes associated with the *P. oceanica* phyllosphere, while the prokaryotic community structure remains largely unaffected. In other words, the leaves microbiome can thrive under lower pH condition while maintaining its diversity and composition; however, key N transformation reactions undergo higher rates compared with *P. oceanica* beds from non-vent sites. We found daylight N_2 fixation significantly higher on leaves acclimated to low pH (Fig. 3.5a). 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* (Liu et al., 2010; Kroeker et al., 2013; Wannicke et al., 2018). Agawin et al. (2021) showed that the *NifH* gene expression (as proxy for nitrogenase activity, the enzyme required for N_2 fixation) of the unicellular N_2-fixing cyanobacterial phylotype UCYN-C associated with the *P. oceanica* phyllosphere was enhanced under elevated CO_2. A widely accepted explanation for the positive influence of increased CO_2 concentrations on some diazotrophs is their ability to reallocate energy from the downregulation of carbon concentrating mechanisms towards N_2 fixation (Kroeker et al., 2013; Wannicke et al., 2018).

P. oceanica can assimilate fixed N as NH_4^+ or NO_3^- (Lepoint et al., 2002). We observed NH_4^+ uptake rates to be significantly increased at the vent site, but unaffected by the presence or absence of epiphytes (Fig. 4 a, b), indicating that *P. oceanica* may adapt to an increased N demand due to higher productivity under OA. This is in line with Ravaglioli et al. (2017), who found overexpression of nitrogen transporter genes after nutrient addition at low pH, suggesting increased nutrient uptake by the seagrass. As discussed above, the high demand for N under OA could be sustained by the enhanced microbial N_2 fixation on its leaves. Conversely, NO_3^- uptake rates (Fig. 4 c, d) seem to be more affected by the presence of epiphytes than by the pH. This indicates that the plant host and its epiphytes might prefer different nitrogen sources; while *P. oceanica* shows higher affinity for NH_4^+ (see also Touchette & Burkholder, 2000), epiphytes seem to favor NO_3^- uptake.

A reduced pH is also expected to negatively affect the microbial conversion of NH_4^+ to NO_3^- as first step of nitrification (Beman et al., 2011; Kitidis et al., 2011; Laverock et al., 2013) probably because

NH_3 (which becomes less abundant with decreasing pH) is preferred than NH_4^+ as substrate by the ammonia mono-oxygenase enzyme (Suzuki, Dular & Kwok, 1974). However, we found increased $^{15}R_{OX}$ under OA conditions in our incubations (Fig. 3b). Hutchins et al. (2009) speculated that increasing CO_2 levels might lead to higher autotrophic nitrification rates through a reduction of CO_2 limitation. Furthermore, Fulweiler et al. (2011) found that a diverse nitrifier community, as it can be found in estuarine and coastal sediments, can adapt to a wider range of pH values. In turn, higher nitrification may result in higher coupled nitrification-denitrification, which removes bioavailable N from the water column and can partially mitigate eutrophication in coastal waters (Lamontagne et al., 2002). Although our potential nitrification rates cannot be extrapolated to other systems, they support the hypothesis that nitrification will be less affected by OA in coastal benthic ecosystems than in the open ocean.

$^{29}N_2$ production in most of our incubations indicates that anammox played an important role as N loss pathway on seagrass leaves, and that the process was likely little affected by seawater pH (Fig. 3c). At the same time, higher N_2 fixation and potential nitrification by leaves epiphytes under OA conditions in the light fueled a concomitant increase in denitrification potential (Fig. 3d, $^{30}N_2$ production). Ocean acidification is not expected to have major direct consequences on denitrification and anammox since both processes occur in anaerobic environments already experiencing elevated CO_2 concentrations and low pH values (Hutchins, Mulholland & Fu, 2009; Wannicke et al., 2018). However, on *P. oceanica* leaves under high CO_2 conditions, an increase in both C (Berlinghof et al. 2022) and N_2 fixation, as well as nitrification, may have favored the creation of anoxic microniches on the leaf biofilm with organic C and oxidized nitrogen compounds for metabolism by denitrifying bacteria (Hutchins, Mulholland & Fu, 2009).

While N cycling accelerated on the *P. oceanica* phyllosphere under OA, the prokaryotic community structure remained mostly unaffected. Similarly, Banister et al. (2021) found the leaf-associated microbiome of *Cymodocea nodosa* to be stable across pH gradients in a comparable Mediterranean CO_2 vent site. 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 the interaction with its plant host (Crump et al., 2018), as seen also on other seagrass species on different compartments of the plant, including the rhizomes (Cucio et al., 2016), or even also regulated by the interaction with other symbionts of the plants, as the epiphytic community is (O'Connor et al., 2022). On the other hand, our biogeochemical measurements imply the presence of coupled metabolisms between the seagrass and its microbiome contributing to plant health and adaptation in a high-CO_2 world

and potentially other stressors whose presence are expected to persist such as increased temperatures and nutrient enichment (Szitenberg et al. 2022).

The increased N_2 fixation under low pH resulted in a lower epiphytic C:N ratio (Suppl. Fig. 5). This can also have up-scale implications in the seagrass associated macrofaunal assemblage. A low C:N represents higher nutritional quality and is associated with higher protein and lower starch content (Scartazza et al., 2017), combined with a lower abundance of calcifying epiphytes (which are of low nutritional value) at vent sites (Berlinghof et al., 2022). These characteristics can favor grazing by herbivores, as shown by several studies (Apostolaki et al., 2014; Ricevuto, Vizzini & Gambi, 2015; Scartazza et al., 2017; Mecca et al., 2020; Martínez-Crego et al., 2020; Mirasole et al., 2021). Our results here can be complemented in future trials by incorporating direct visualization of the incorporation of N, e.g. including high-resolution mass spectrometry and incubations amended with labelled aminoacids (NanoSIP as in Tarquinio et al., 2018; Mohr et al., 2021). Besides inorganic nitrogen forms, organic nitrogen can also be exchanged by the symbionts and the plant host; N fixed on the phyllosphere can be converted to aminoacids such as glutamate, phenylalanine, or leucine, that can also be provided to the plant in exchange of sugars and non-protein amino acid such as γ-aminobutyric acid (Mohr et al., 2021). The contribution of this prokaryote-derived nitrogen might not be the main contribution to the plant nutrition, but it could represent a secondary source of N in addition to the dissolved species in the water column, with the additional advantage that the microbial communities can be more resilient and resistant when facing environmental change such as decreased pH (see next section) or increased water temperature (Agawin et al., 2021; García-Márquez et al., 2022).

Conclusion

All major N cycling processes occur on *P. oceanica* leaves, with OA accelerating N cycling while the prokaryotic community structure remains largely unaffected. Under OA, high rates of microbial daylight N_2 fixation on the *P. oceanica* phyllosphere can sustain an increased N demand of the plant host, with nitrification competing with the plant and providing substrate for denitrification and coupled DNRA-anammox in anoxic microniches. Adaptation of marine plants to environmental changes is fundamental to their survival; we show that the plasticity of their N cycling microbiome is a key factor in regulating seagrass function on a changing planet.

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Chapter 3:
Nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning in contaminated sediments

Abstract

In seagrass sediments, lucinid bivalves and their chemoautotrophic bacterial symbionts consume H_2S, relying indirectly on the plant productivity for presence of the reduced chemical. Additionally, a role of lucinid bivalves in N provisioning to the plant (through N_2 fixation by the symbionts) was hypothesized. Thus, lucinids may contribute to sediment detoxification and plant fitness. Seagrasses are subject to ever increasing human pressure in coastal environments. Here, disentangling nested interactions between chemosynthetic lucinid bivalves and seagrass exposed to pollution may help to understand seagrass ecosystem dynamics and to develop successful seagrass restoration programs that consider the roles of animal-microbe symbioses. We evaluated the capacity of lucinid bivalves (*Loripes orbiculatus*) to promote nutrient cycling and seagrass (*Cymodocea nodosa*) growth during a 6-week mesocosm experiment. A fully crossed design was used to test for the effect of sediment contamination (metals, nutrients and hydrocarbons) on plant and bivalve (alone or interacting) fitness, assessed by mortality, growth and photosynthetic efficiency, and for the effect of their nested interaction on sediment biogeochemistry. Plants performed better in the contaminated sediment, where a larger pool of dissolved nitrogen combined with the presence of other trace elements allowed for an improved photosynthetic efficiency. In fact, porewater nitrogen accumulated during the experiment in the controls, while it was consumed in the contaminated sediment. This trend was accentuated when lucinids were present. Concurrently, the interaction between clams and plants benefitted both organisms and promoted plant growth irrespective of the sediment type. In particular, the interaction with lucinid clams resulted in higher aboveground biomass of *C. nodosa* in terms of leaf growth, leaf surface and leaf biomass. Our results consolidate the notion that nested interactions involving animal-microbe associations promote ecosystem functioning, and potentially help designing unconventional seagrass restoration strategies that exploit chemosynthetic symbioses.

Keywords: ecological facilitation, ecosystem restoration, nature-based solutions, chemosynthetic symbioses, *Loripes orbiculatus*, *Cymodocea nodosa*, Bagnoli-Coroglio, sediment contamination

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Introduction

Seagrasses are habitat-forming marine plants that build the foundation of biodiversity hotspots in coastal marine environments (Heck et al., 2008). However, seagrass ecosystems are under threat due to a variety of human activities, such as coastal exploitation, eutrophication, and climate change (Orth et al., 2006). In many locations, seagrass meadows are becoming fragmented or have already completely disappeared, and have been replaced by bare sediments or by opportunistic macrophytes (Montefalcone et al., 2015). Thus, the restoration of seagrass habitats is often an environmental and economic imperative given that seagrasses provide key ecosystem functions and services (Reynolds et al., 2016), and has recently been recognized as a key action to address the causes of climate change and to mitigate associated effects (Gattuso et al., 2018). However, over the years many seagrass restoration and/or transplantation programs have been costly and unsuccessful (Cunha et al., 2012; van Katwijk et al., 2016). Possibly, this is because these programs did not take into account factors such as the genetic features of donor populations (Pazzaglia et al., 2021) or the important role of positive species interactions in effectively contributing to ecosystem functioning (Cardinale, Palmer & Collins, 2002; Bulleri et al., 2018; Valdez et al., 2020; Gagnon et al., 2021; Malkin & Cardini, 2021; Zhang et al., 2021).

Increasing evidence supports the notion that nested interactions involving animal-microbe associations (also called holobionts) fundamentally contribute to the functioning of diverse marine ecosystems (Pita et al., 2018). The most iconic example is that of coral reefs, where a symbiosis between an animal and a microalgal symbiont forms the basis of some of the most diverse ecosystems on Earth (Muscatine & Porter, 1977). Seagrasses are themselves holobionts associating with a diverse community of microbes, which grow on their leaves as epiphytes or inhabit their rhizosphere (Tarquinio et al., 2019). These microbes play fundamental roles in the overall ecosystem functioning. For example, leaf epiphytes were shown to contribute significantly to the plant N needs, by fixing atmospheric N_2 or converting dissolved organic nitrogen (DON) compounds into bioavailable inorganic forms (DIN) (Agawin et al., 2016; Cardini et al., 2018; Tarquinio et al., 2018). Similarly, sulfate reducing bacteria (SRB) and other microorganisms in the seagrass rhizosphere significantly contribute to the mineralization of organic N and phosphorus (P), and to anaerobic N_2 fixation (Welsh, 2000). Recently, Scholz et al. (2021) demonstrated the widespread relationship of cable bacteria growing in association with the root rhizosphere of aquatic plants and seagrasses. Critically, these bacteria can efficiently remove sulfide from sediments and are likely beneficial for the plant (Malkin & Cardini, 2021; Scholz et al., 2021). Other significant positive effects of microorganisms on seagrasses are for example the production of

phytohormones, or defense against pathogens or toxic compounds (see Tarquinio et al. (2019) for a review).

Symbioses between macro- or meiofauna and microbes are ubiquitous and highly diverse in seagrass sediments. Sediment microorganisms can benefit a great deal by associating with invertebrates inhabiting this environment. The invertebrate host can provide the microbial symbionts with access to resources that may be unavailable, such as nutrients, or electron donors and acceptors that may not be available simultaneously in the sediment environment (Beinart, 2019). One prominent example of symbioses inhabiting seagrass sediments is the lucinid clams, where a bivalve host associates with sulfur-oxidizing bacteria that are hosted in the animal gills (Taylor & Glover, 2006). This holobiont was suggested to form a positive nested interaction with seagrasses (van der Heide et al., 2012). In this example of a nested ecosystem, the clam and its microbial symbionts are suggested to contribute to the removal of sulfide (toxic to the plant) from the sediments and thus to enhance seagrass growth (Chin et al., 2021). Additionally, a role of the lucinid clam *Loripes orbiculatus* in N provisioning to the seagrass ecosystem was recently proposed, given the ability of the symbionts to also fix atmospheric N_2 (Petersen et al., 2016; Cardini et al., 2019).

Seagrasses create the conditions for biodiversity hotspots through their role as habitat-forming species; at the same time, efforts that incorporate biodiversity as a means for restoration of this important ecosystem may be more successful (Williams et al., 2017). Therefore, in this study we aimed to test the importance of nested interactions between the plant (*Cymodocea nodosa*), the lucinid clams (*Loripes orbiculatus*), and their symbionts, in enhancing seagrass performance and growth in natural vs contaminated sediments. By means of a mesocosm experiment, we explored the possibility of exploiting nested interactions for successful seagrass restoration strategies based on the incorporation of sulfide-oxidizing bacteria-bearing bivalves on plots where seagrasses are transplanted, hypothetically enhancing the survavility and establishment of the plant. We used a fully crossed design to examine the effect of sediment contamination (metals, nutrients and hydrocarbons) on plant and bivalve (alone or interacting) fitness, assessed by mortality, growth and photosynthetic efficiency, and for the effect of their nested interaction on sediment biogeochemistry. We hypothesized that the interaction between *Cymodocea nodosa* and *Loripes orbiculatus* may benefit both organisms in colonizing contaminated sediments and may provide a potential restoration strategy that exploit nested interactions involving microbial symbioses as a nature-based solution in coastal polluted areas.

Methods

Collection of sediments, plants and lucinids

Collection of sediments, plants and lucinids was carried out at the end of May 2018. Control sediment was collected north of the Gulf of Napoli, at Cape Miseno (40°47'5.75"N - 14° 4'36.79"E), while polluted sediments were collected within the bay of Bagnoli-Coroglio (40°48'22.10"N - 14° 9'44.59"E), a coastal area impacted by industrial contamination of hydrocarbons and heavy metals (Morroni et al., 2020). At each site, 150 L of surface sediment (max depth 10 cm) were collected between 5 and 10 m depth by divers using a hand-drag, and immediately transported to the laboratories of the Stazione Zoologica Anton Dohrn (SZN) in Napoli, Italy. *Cymodocea nodosa* plants were collected at Cape Miseno at approximately 8 m depth. Large fragments of the species were gently uprooted by divers and transported in coolers to the SZN facilities (within 2 h) to be subsequently introduced in the aquaria for plant acclimation. Specimens of *Loripes orbiculatus* were collected by scuba diving in the bay of Fetovaia, Livorno (Italy) from sediments adjacent to a *Posidonia oceanica* meadow (42°43'48"N 10°9'23"E) at approximately 7 m depth. The bivalves were moved to the HYDRA Institute for Marine Sciences in Fetovaia, prepared for transport in water-tight containers with a good quantity of their surrounding sediment, seawater, and a headspace for gas exchange, and transported to the SZN within 24 h from sampling.

Experimental setup

C. nodosa fragments of similar size, composed of 1 apical shoot and 8-10 connected vertical shoots, were selected for the experiment. Fragments were fixed to a plastic square mesh (mesh size: 4cm) with cable ties to be transplanted into 6L plastic pots (20 x 30 x 15 cm). Three to four *C. nodosa* fragments were fixed to each plastic square mesh to reproduce the plant density of the meadow at the collection site (513 ± 14 shoots m-2). The plastic square mesh was fixed to the top of the pots, and thereafter sediment was carefully poured to allow roots to maintain their vertical position within the sediment. Half of the pots were filled with control sediment and the other half with polluted sediment. Thereafter, 50 lucinid bivalves of similar size (13.2 ± 1.3 mm shell length), equivalent to a realistic density of approximately 830 individuals m^2 (see van der Geest et al., 2020) were transferred onto the sediment of half of the pots to obtain a crossed design. Lucinid clams were left undisturbed and burrowed in the sediment within 8 hours. The pots, filled with either polluted or control "Sediment" (factor 1; 2 levels), were thus reconstructed to recreate four types of "Community" (factor 2; 4 levels): only sediment (S), sediment + plant (P), sediment + lucinids (L), sediment + plant + lucinids (PL). See. Fig. 4.1 for a graphical

representation of the experimental design. Four pots, one for each level of the factor Community, were allocated inside each of the six 500-L experimental tanks (n=3 for each sediment type). See Ruocco et al. (2019) for a description of the aquarium system. The resulting experimental setup was let to acclimate for one week under the environmental conditions present in situ during sampling (temperature: 24.5 °C; salinity: 37.5 psu; maximum noon irradiance: 275 ± 15 µmol m-2 s-1; 12:12 h light:dark photoperiod). The same conditions were kept during the entire duration of the experiment, which lasted 6 weeks (42 days) in total.

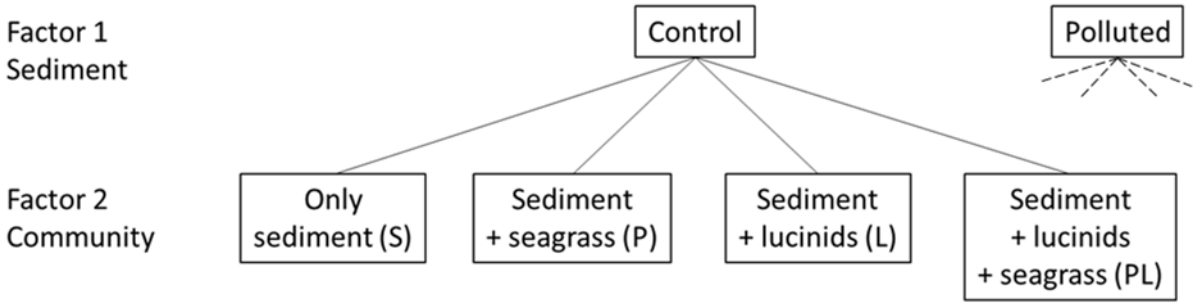

Figure 4.1 - Orthogonal experimental design used in the mesocosm experiment with levels of the factors Sediment (Control and Polluted) and Community (S, P, L, PL).

Chemical characterization of sediments

Sediments chemical characterization was performed as already reported (Armiento et al., 2020; Morroni et al., 2020) by Salvatore Chiavarini and Juri Rimauro at the Division Protection and Enhancement of the Natural Capital - Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) in Rome, Italy. Briefly, total organic carbon (TOC) was determined by a Leco CNS 2000 elemental analysis apparatus. Granulometric size distribution determinations were performed on a Micromeritics SediGraph 5100 X-ray particle size analyser. Major and trace elements were determined by a PerkinElmer Optima 2000DV ICP-OES and an Agilent 7800 ICP-MS, after mineralization by a microwave-assisted acid digestion (Ethos Easy, Milestone). Hg was determined by Automatic solid/liquid Mercury Analyser (FKV AMA-254). PAHs were analyzed according to EPA 8270D method with an Agilent 7890A-5975C GC-MS system, after extraction according to EPA 3545a method by an Accelerated Solvent Extractor (Dionex ASE 200) and silica gel clean-up (EPA 3630). Hydrocarbons in the C12-C40 range were determined by GC-FID on an Agilent 7820a system after Dionex ASE 200 extraction and Florisil clean-up. Sediment redox potential was characterized in each experimental pot at 5 and 10 cm below the sediment-water interface at the end of the experiment. Five measurements were taken in each pot at each of the selected sediment depths, by

inserting a Crison Pt electrode, connected to a portable pH meter (Crison model 507), into the sediments. The electrode was calibrated with a redox standard solution (Crison 468 mV at 25°C) and redox measurements were referred to the standard hydrogen electrode (207 mV) as described in APHA (1992).

Porewater nutrients

Porewater was collected using metered stainless steel lances (Cardini et al., 2019) at the start and at the end of the experiment. Seawater was retrieved from above the sediment (seawater control), and at 2 and 10 cm below the sediment-water interface. Two 30 ml seawater samples were retrieved from each depth and experimental pot. One sample was filtered onto 0.22 µm PES membrane filters (Merck Millipore), preserved frozen at -20 °C and analyzed for nitrogen oxides (NOx) as the sum of nitrate (NO3-) and nitrite (NO2-), ammonium (NH4+), and orthophosphate (PO43-) concentrations on a Continuous Flow Autoanalyzer (Flowsys, Systea) at the SZN laboratories. The other sample was filtered using an acid-washed 50 mL polycarbonate syringe through a pre-combusted 0.7 µm GF/F filter directly into acid-washed 30 ml HDPE sample bottles (Cardini et al., 2015). The sample was then immediately acidified with 80 µl of 18.5% HCl and stored in the dark at 4 ºC until analysis at the SZN by the high-temperature catalytic oxidation method on a TOC-L Analyser with a total nitrogen (TN) unit (Shimadzu) for DOC and DON (as the difference of TN and dissolved inorganic nitrogen) quantification. No differences between 'Community' levels were detected at the start of the experiment, and t0 data were thus pooled in one group and compared against the 'Community' levels at the end of the experiment. Further, no differences were detected between sediment depths, and samples were thus pooled within the respective 'Sediment' and 'Community' level.

Plant photophysiology

All measurements of plant photophysiology, as well as analyses of plant morphology, growth and mortality (following section) were conducted by Lazaro Marín-Guirao and Gabriele Procaccini at the Stazione Zoologica Anton Dohrn. A diving-PAM fluorometer (Walz, Germany) was used to characterize the functioning of the photosynthetic apparatus at the level of photosystem II (PSII). Chlorophyll fluorescence measurements were taken in two randomly selected *C. nodosa* shoots per experimental pot following Marín-Guirao et al. (2013). Briefly, basal (F_0) and maximum fluorescence (F_m) were measured on whole-night adapted plants by the saturation pulse method to calculate the maximum quantum yield of PSII [$(F_v/F_m = (F_m - F_0)/F_m$]. Subsequently, rapid light curves (RLC) were generated on the same shoots after 4 h of illumination in experimental tanks to estimate maximum relative electron transport rates (rel-ETR_{max}). Each RLC was composed of 20 s exposure to 9 incremental irradiances. The curve

fitting method developed by Jassby and Platt (1976) was used for calculating rel-ETR_{max}. Non-photochemical quenching was calculated as $NPQ = (F_m - F_m')/F_m'$; where F_m' is the maximum fluorescence of light-adapted leaves obtained from the RLCs. Measurements taken within each pot were averaged to be used as independent replicates ($n = 3$).

Plant morphology, growth, and mortality

Seagrass growth was measured as leaf elongation and rhizome growth. Leaf elongation was determined by marking the leaves of 5 randomly selected shoots with a needle three weeks after the beginning of the experiment (Zieman, 1974). Marked shoots were collected at the end of the experiment to measure the surface area of newly formed leaf tissues (cm^2 $shoot^{-1}$ day^{-1}). Total leaf biomass, the number of leaves and the percentage of the necrotic leaf surface were also determined on marked shoots. Rhizome growth was determined by marking the apical shoot of each plant fragment with plastic ties at the beginning of the experiment. Plant fragments were harvested at the end of the experiment and the newly produced tissues divided into leaves, rhizomes and roots before being dried and weighed to estimate their biomass. Measurements taken within each pot were averaged to be used as independent replicates (n=3). Finally, all shoots in each experimental pot were counted at the beginning and at the end of the experiment, and the differences normalized to initial shoot number and expressed as percentage of net shoot change.

Lucinid clam mortality and tissue analyses

All *L. orbiculatus* clams were counted at the end of the experiment for determining their mortality rate in the different experimental pots. The rate was expressed as $\%mortality = (\#lucinids_{t0} - \#dead\ lucinids_{tf}) \cdot \#lucinids_{t0}{}^{-1} \cdot 100$. Additionally, ten clams at T0 and four clams from each pot at the end of the experiment were selected randomly, measured for their shell length and dissected for tissue analyses. Symbiont-bearing (gill) tissue and non-symbiotic (host) tissue (i.e., the remaining tissue after removal of the gills) were separated and stored at -20 °C to determine the natural 13C/12C and 15N/14N ratios of the gill and host tissues as in Cardini et al. (2019). Frozen tissues were freeze-dried for 48 h, ground to fine powder and weighed into tin capsules. Samples were analyzed for C% and N% and for δ13C and δ15N by continuous flow isotope ratio mass spectrometry (IRMS, Isoprime, GV Instruments Ltd) coupled with an elemental analyser (Costech Instruments).

Data analysis

Differences in sediment inorganic and organic nutrient concentrations, plant (photochemistry, morphology and growth) and lucinid clam (mortality) were tested using PERMANOVA tests (Anderson, 2001) with "Sediment" and "Community" as fixed factors. The test for Redox potential additionally included the factor "Depth". The analysis was conducted using the Euclidean distance as coefficient of dissimilarity on previously normalized data. Type 3 (partial) sum of squares was used with unrestricted permutation of raw data (9999 permutations). These analyses were run using the PERMANOVA tool included in the PRIMER 6+ package. A principal component analysis (PCA) was also performed to explore overall plant responses (photochemistry, morphology and growth) to "Sediment" and "Community" experimental treatments. The isotopic niche spaces of symbionts and hosts were compared among experimental pots analysing the Bayesian standard ellipse areas (SEA_B) with the SIBER R package (Jackson et al., 2011; R Core team, 2021). The isotopic niche concept builds upon the idea that variation in particular ratios of heavy to light stable isotopes –frequently N and C– are found in the tissue of organisms after accumulating through the food web via primary production or consumption; a biplot of these isotopic signals are a proxy of dietary niche and ultimately, they are used to infer the trophic niche of organisms and/or communities (Jackson et al., 2011).

Results

Sediment geochemistry and porewater nutrients

Both sediments had a similar grain size distribution, characteristic of sandy sediments (Table 4.1). However, the organic content of the two sediment types differed significantly in both their TOC and DON content, as well as for their DOC:DON ratios (Table 4.1).

Table 4.1 - Sediment organic content and grain size distribution (n=6).

Parameter	Control	Polluted	*P* value
TOC (%)	0.02 ± 0.01	0.36 ± 0.06	< 0.01
DOC (µM)	174.57 ± 22.02	166.39 ± 15.99	
DON (µM)	9.65 ± 2.18	35.28 ± 6.53	< 0.01
DOC:DON	18.51 ± 2.53	4.82 ± 0.79	< 0.01
Gravel: > 2 mm (%)	0.30	0.30	
Sand: 2 > 0,063 mm (%)	99.60	99.40	
Silt: < 0,063 mm (%)	0.10	0.30	

Abundant elements showed similar concentrations in sediments from both sites (Supp. Table 2). However, P and Fe were significantly more concentrated in polluted sediments (Table 2). Further, heavy metals and metalloids were significantly more concentrated in polluted sediments (Table 2), with some vastly exceeding environmental quality standards, such as arsenic (As), cadmium (Cd) and lead (Pb). Similarly, hydrocarbon concentrations were high in polluted sediment, with the EPA's 16 priority pollutant polycyclic aromatic hydrocarbons exceeding by 5-fold the environmental quality standard (Table 4.2).

Table 4.2 - Concentration of key elements and hydrocarbons in the sediment, and Environmental Quality Standard expressed as an Annual Average values (EQS-AA) according to the IT law 260/2010. Numbers in bold indicate values exceeding the EQS-AA

Element (ppm)	**Control**	**Polluted**	**EQS-AA**
Fe	20458 ± 155	102466 ± 2794	
P	504 ± 10	4336 ± 68	
As	21.3 ± 0.6	**75.2 ± 2.9**	12 ± 20%
Cd	0.148 ± 0.011	**0.76 ± 0.04**	0.3 ± 20%
Cr	13.8 ± 2.4	30.9 ± 1.1	50 ± 20%
Cu	6.34 ± 0.40	157 ± 9	
Hg	0.009 ± 0.001	0.220 ± 0.019	0.3 ± 20%
Ni	7.84 ± 1.30	13.6 ± 1.4	30 ± 20%
Pb	31 ± 1	**281 ± 15**	30 ± 20%
Hydrocarbons (ppb)	**Control**	**Polluted**	
Σ PAHs (16 priority pollutants EPA)	40,5	**4450,4**	800 ± 20%
Heavy hydrocarbons (C>12)	18.5 ± 3.7	155.5 ± 28	

Redox potential (Supp. Fig. 7, Supp. Table 3) significantly differed between the two analyzed sediment depths ($p<0.001$) and among communities ($p<0.01$) but was similar between control and polluted sediments. Sediment redox potential at 5 cm depth was significantly higher in all treatments, almost the double that at 10 cm depth. Regardless of depth, the redox potential in the PL community was significantly higher than that in the S community, both in the control ($p<0.05$) and in the polluted sediment ($p<0.01$). In the control sediment, lower redox values were found associated with the S community, with values significantly different from the L community ($p<0.05$). In the polluted sediment, lower redox potentials were associated with the L community, which significantly differed from the redox conditions of the PL community ($p<0.05$). Across all treatments, the highest mean redox potential was found in PL communities of polluted sediments (102.7 ± 13.7 mV), while the lowest was found in L communities of polluted sediments (26.5 ± 27.3 mV).

Inorganic and organic nutrient concentrations were stable in the aquaria seawater during the experiment (Supp. Table 4), while they generally increased in the control sediment porewater, regardless of the 'Community' level (Supp. Fig. 9; Supp. Table 3). The same trend was observed in the polluted sediment porewater for PO43- and DOC, although DOC increased significantly more in the P and PL community compared to the other treatments (Supp. Fig. 9). Conversely, we detected a significant decrease in NH4+ and DON concentrations in the polluted sediment, particularly in the PL community (Supp. Fig. 9).

Plant photophysiology

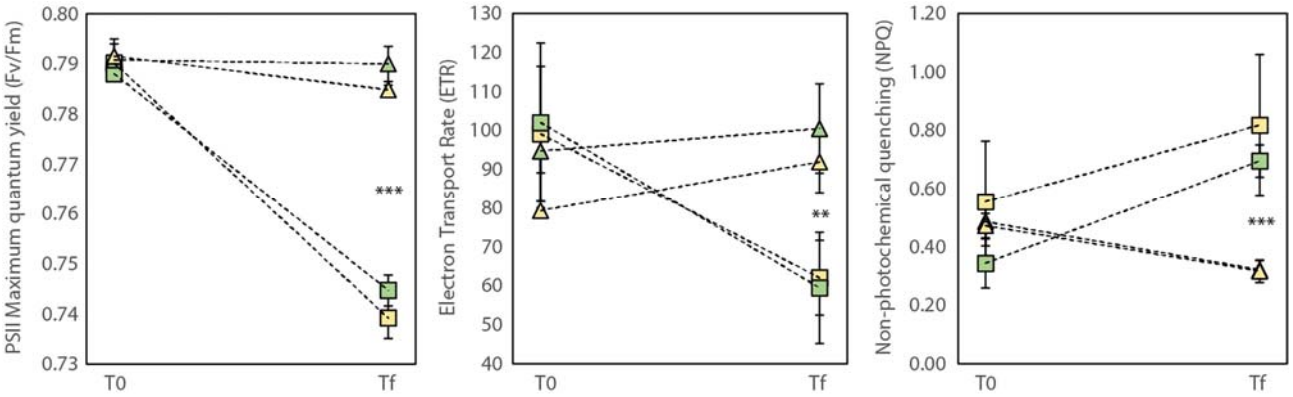

*Figure 4.2 - Evolution of chlorophyll a fluorescence parameters. A) Maximum quantum yield of PSII (Fv/Fm), B) Electron transport rate (rel-ETR), C) Non-photochemical quenching (NPQ) at the beginning (T0) and end (Tf) of the experiment (± SE, n = 3). Colours indicatte absence (yellow) or presence (green) of the interaction with lucinid clams. Symbols are used to indicate control (quadrats) or polluted (triangles) sediment. Asterisks indicate significant differences in the factor "Sediment" at Tf (**, p<0.01; ***, p<0,001); see Table S4 for the statistics.*

At the end of the experiment, the photochemical efficiency of plants growing in polluted sediments was significantly higher than the efficiency of plants in control sediments (Fig. 4.2; Supp. Table 7). These plants also showed significantly higher values of electron transport rate (rel-ETR) but lower values of non-photochemical quenching (NPQ) (Figure 4.2; Supp. Table 7). There was no indication of an effect of the community type on the photophysiology of *C. nodosa*.

Plant morphology, growth and mortality

The total leaf surface area of *C. nodosa* interacting with *L. orbiculatus* was significantly higher irrespective of sediment pollution (Figure 4.3, Supp. Table 6). These plants also showed a trend of higher leaf elongation and leaf biomass compared to plants growing in the absence of lucinid clams, but differences were deemed not significant by the statistical test (Figure 3.2, Supp. Fig. 10, Supp. Table 6). New apical growth was measured for the root, rhizome, and leaf portion of the plant. Neither rhizome nor leaf apical growth showed significant differences among the experimental pots (Fig. S5, Supp. Table

7).

However, there was a significant effect of the sediment type, regardless of the interaction with lucinids, on the growth of the apical roots (Figure 4.3, Supp. Table 7). At the same time, there was a significant increase of necrotic tissue in *C. nodosa* from the polluted compared to the control sediment, regardless of the presence of the clams, while all plots showed a positive net shoot change during the course of the experiment regardless of the experimental treatment (Supp. Fig 10, Supp. Table 6).

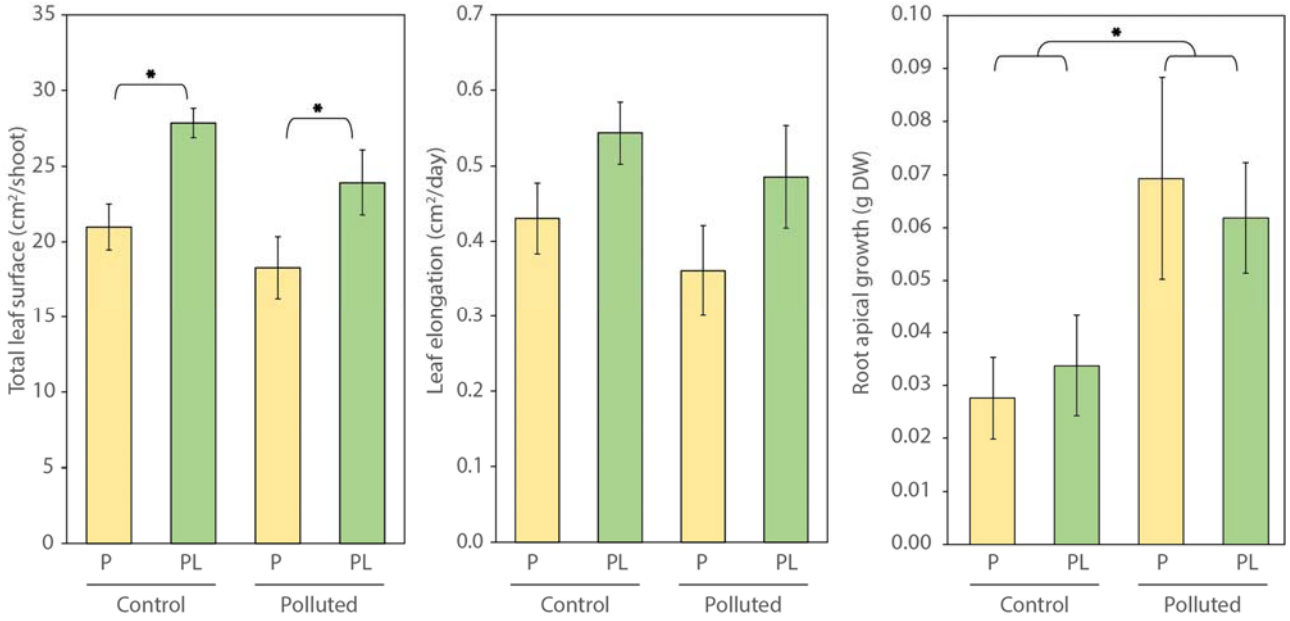

Figure 4.3 - Plant morphology and growth. A) Total leaf surface, B) Leaf elongation, C) Root apical growth of C. nodosa at the end of the experiment (± SE, n = 3). Levels of the factor "Community" are identified with letters as indicated in the methods. Colours indicate absence (yellow) or presence (green) of the interaction with lucinid clams. Asterisks (, p<0.05) indicate significant differences; see Tables S5, S6 for the statistics.*

In a principal component analysis (Figure 4.4), sediment type (control vs polluted) segregated samples along axis 1 (37.9% of total variance), which was mainly correlated with photochemical parameters and the newly produced tissues by apical growth. Conversely, the community type (P vs PL)

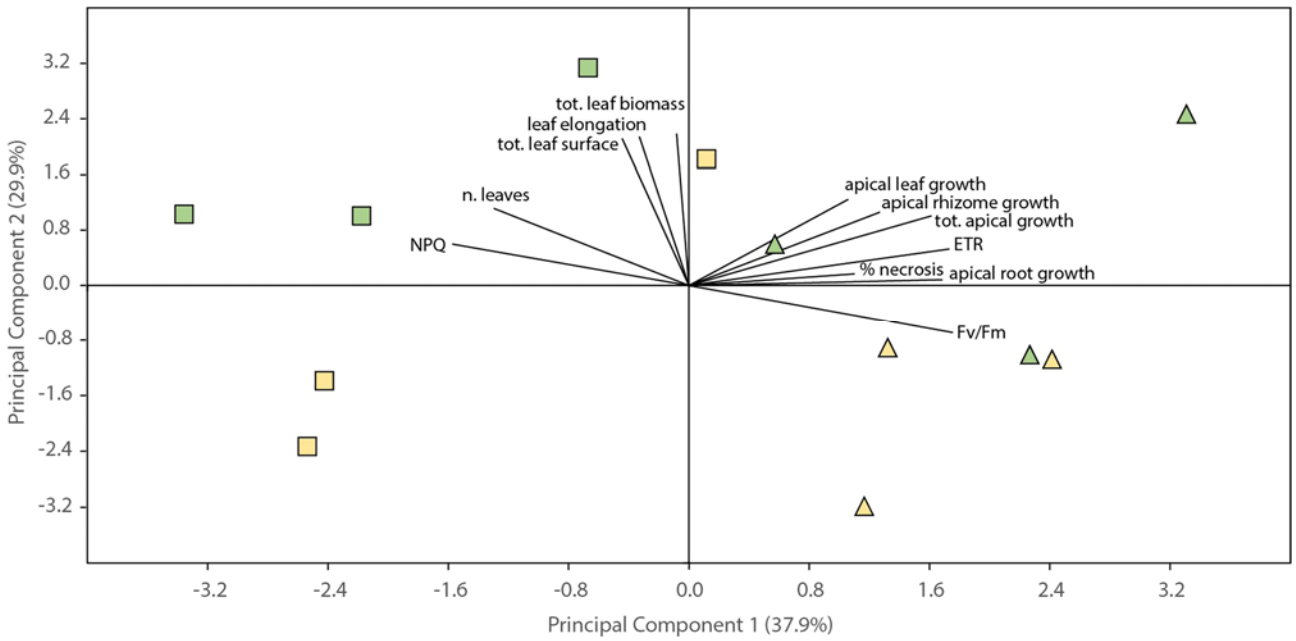

Figure 4.4 - Principal component analysis testing for multivariate changes in plant variables among treatment levels. Colours indicate absence (yellow) or presence (green) of the interaction with lucinid clams. Symbols are used to indicate control (quadrats) or polluted (triangles) sediment. Note that the per cent variation explained by the principal components is indicated in the graph and refers to the fraction of the total variance explained by each axis or principal component (i.e., PC1 & PC2).

segregated samples along axis 2 (29.9% of total variance). This axis was mainly correlated with leaf growth and shoot size (in terms of both leaf surface and leaf biomass).

Lucinid clams

L. orbiculatus showed significantly more mortality in the polluted sediment (Figure 4.5, Supp. Table 7) compared to the control sediment ($p<0.05$), approaching a value of 10% where the plant was not present. The isotopic niche of *L. orbiculatus* sampled at the beginning of the experiment showed a differentiation between symbiont-free (rest) and symbiont-hosting (gill) lucinid clam tissues, with the latter having more negative δ13C and δ15N values and in SEA_B (Figure 4.6). At the end of the experiment, the same pattern was generally maintained in all treatments. SEA_B of *L. orbiculatus* tissues overlapped significantly between the beginning and the end of the experiment, except for symbiont-free (rest) tissues in the L treatment of the polluted sediment (Figure 4.6). These samples also showed the largest SEA_B (Figure 4.6). The interaction of *L. orbiculatus* with the plant also caused larger SEA_B, regardless of the sediment type (Figure 4.6).

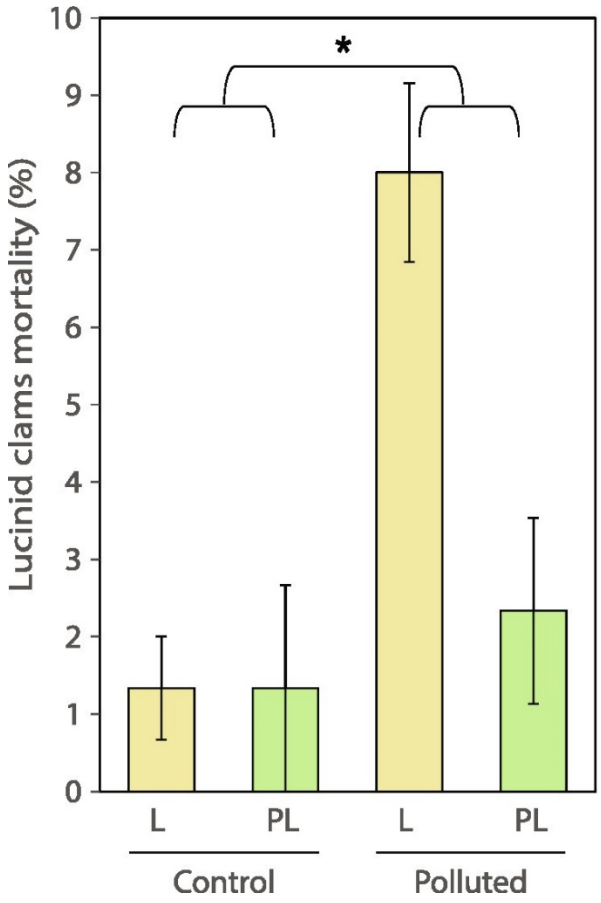

*Figure 4.5- Lucinid clams mortality (±SE, n = 3). Levels of the factor "Community" are identified with letters as indicated in the methods. Colors indicate absence (yellow) or presence (green) of the interaction with the plant. Asterisks (*p < 0.05) indicate significant differences*

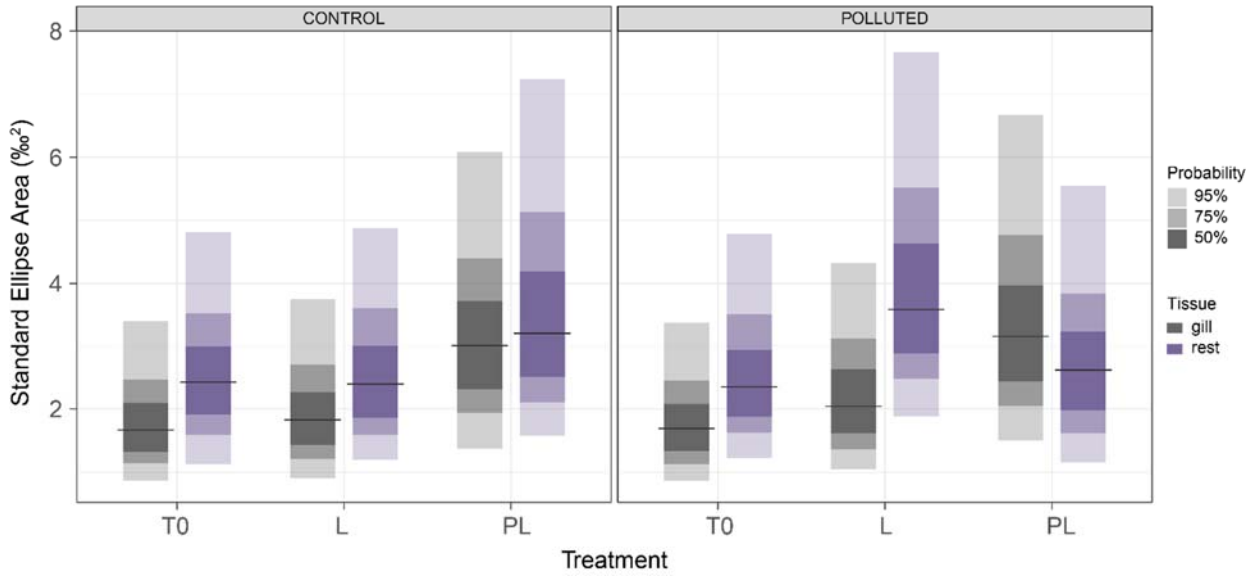

Figure 4.6- Distribution of Bayesian ellipses (SEAB) showing the isotopic niche width (as a proxy of trophic specialization) and its uncertainty, color-coded in purple for symbiont-free (rest) and grey for symbiont-hosting (gill) lucinid clam tissues. Black lines represent the mode while the shaded boxes represent the 50%, 75% and 95% credible intervals from dark to light.

Discussion

Here, we found evidence that nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning, and that these interactions can play a role in the capacity of the mutualistic consortium to resist stress and grow in polluted sediments.

C. nodosa is tolerant to pollution

Our study confirms that *C. nodosa* is highly plastic at the morphological level. This species can in fact rapidly change the plant architecture modifying the ratio of above- vs below-ground biomass depending on environmental conditions and resource availability (Perez et al., 1994; Marín-Guirao et al., 2018). Our study demonstrates that this plant is resistant to high doses of pollution deriving from the massive industrial contamination by trace metals and hydrocarbons found in the Bagnoli area (Armiento et al., 2020; Morroni et al., 2020).

In our experiment *C. nodosa* plants exposed to pollution showed increased photosynthetic efficiency and apical growth. Nutrients in *C. nodosa* are mainly taken up through the root system, with leaf uptake dominant only when seawater concentrations suddenly increase after a nutrient pulse (Alexandre & Santos, 2020). In particular, ammonium is the preferential nitrogen source for *C nodosa*, while amino acids can also represent a large fraction of this species' N demand (Alexandre and Santos, 2020). In our study, higher availability of nutrients (and particularly of ammonium and dissolved organic nitrogen) in polluted sediments likely contributed to increased uptake rates and stimulation of plant roots growth.

Other important macronutrients (P) and as well as micronutrients/trace metals (e.g. Fe, Cu, Mn, and Zn) that are used as co-factors in photosynthesis were significantly more abundant in polluted sediments, possibly explaining the more efficient use of light for *C. nodosa* plants. At the same time, our lower NPQ in plants exposed to pollution suggests that *C. nodosa* activated responses at the physiological level that enhance acclimation and plant tolerance to stress (Marín-Guirao et al., 2013). Indeed, a recent study showed for NPQ a biphasic dose-response pattern typical of hormesis in *C. nodosa* exposed to ZnO nanoparticles (Malea et al., 2019).

In a different study, *C. nodosa* meadows growing on mining impacted sediments were more dense and lush than meadows on control sediments (Marín-Guirao et al., 2005), further demonstrating the capacity of *C. nodosa* to tolerate stress from heavy metals. Many of these metals are accumulated by the plant if bioavailable and not sulfide-bound. Unfortunately, neither metal accumulation in plant tissues

nor sulfide concentrations were quantified in this study, making it impossible to speculate whether the plant was able to cope with pollution because of their low bioavailability or despite their accumulation. Notwithstanding, it appears clear that *C. nodosa* is resistant to stress deriving from heavy metal and hydrocarbon contamination, at least when this is accompanied by a significant increase in macro and micronutrient availability that boost plant growth.

L. orbiculatus response depends on seagrass presence

The lucinid bivalve *L. orbiculatus* was susceptible to pollution, as indicated by the significantly higher mortality rate of clams burrowing in polluted sediment, particularly for those animals maintained in the absence of the plant. Further, in this study we explored the response of the *L. orbiculatus* chemosynthetic symbiosis to pollution and interaction with *C. nodosa* by quantifying the isotopic niche width of the symbiotic vs non-symbiotic clam tissues.

The isotopic niche has become an established concept in ecology because stable isotope ratios in consumer tissues are tightly linked to those in their diet (Jackson et al., 2011) offering a potentially powerful way to investigate ecological niches and trophic interactions (Yeakel et al., 2016). Recently, the method was used to also look at trophic interactions in chemosynthetic symbioses (Cardini et al., 2019). Stress-induced variability in physiological status can induce changes in isotopic niche width. For example, greater isotopic niche estimates were derived for the deposit-feeding amphipod *Monoporeia affinis* exposed to sediments contaminated with polychlorinated biphenyls, heavy metals, chlorophenols and polycyclic aromatic hydrocarbons (Karlson et al., 2018). Similarly, in our study we obtained greater isotopic niche estimates for non-symbiotic tissues of *L. orbiculatus* exposed to the polluted sediments. This is consistent with the increase in stress-induced clam mortality and decay. On the other hand, the symbiotic (gill) tissues of clams exposed to polluted sediments showed isotopic niche comparable to the control group, suggesting that the microbial partners (hosted in the bivalve gills) may remain little affected in those individuals that overcome the stressful conditions.

In our study, we found that *L. orbiculatus* exposed to polluted sediments had larger isotopic niche estimates in their non-symbiotic tissues. This finding aligns with the observed increase in stress-induced mortality for these clams, likely caused by the decrease in redox potential due to clam mortality and decay. On the other hand, the symbiotic (gill) tissues of clams exposed to polluted sediments exhibited similar isotopic niche widths compared to the control group. This suggests that the microbial partners hosted in the bivalve gills may remain relatively unaffected in those clams that manage to cope with the stressful conditions.

Nested interactions promote ecosystem functioning

The association between lucinid bivalves and seagrass was suggested to function as a tripartite mutualism (van der Heide et al., 2012). In this system, the plant provides organic matter, which is respired by sulfate-reducing bacteria in anoxic sediments leading to formation of hydrogen sulfide, the energy source needed by the clam's chemosynthetic bacteria. At the same time, oxygen transported from the leaves to the rhizome by partial pressure-differences is partly released to the surrounding sediment, in a process known as radial oxygen loss (Borum et al., 2006), which can in turn facilitate the clam's respiration. In return, the clam symbionts oxidize hydrogen sulfide back to sulfate, preventing a potential build-up of the powerful phytotoxin in sediments (Lamers et al., 2013) and thus, sulphide intrusion into the seagrass with potential to induce plant starvation and mortality (Holmer & Hasler-Sheetal, 2014). This mutualism was recently also verified in a field survey in a temperate lagoon system (van der Geest et al., 2020).

Our experiment seems to confirm these studies and the presence of ecological facilitation, irrespective of sediment type. While a longer duration of the experiment and higher replication would have likely resulted in lower variability and clearer differences among the treatments, the experiment clearly showed an effect of both pollution and the interaction between *L. orbiculatus* and *C. nodosa* on some of the investigated variables (Fig. 4.7). The interaction of plants and lucinids significantly improved sediment oxic conditions as shown by the increase of redox potential. At the same time, *C. nodosa* enriched sediment porewater in dissolved organic carbon, a potential food source for sediment sulfate-reducers. The interaction with lucinid clams further resulted in higher aboveground biomass of *C. nodosa* in terms of leaf growth, leaf surface and leaf biomass, similar to the findings of Van Der Geest et al. (2020) for *L. orbiculatus* and *Zostera noltii* in the Thau lagoon, France.

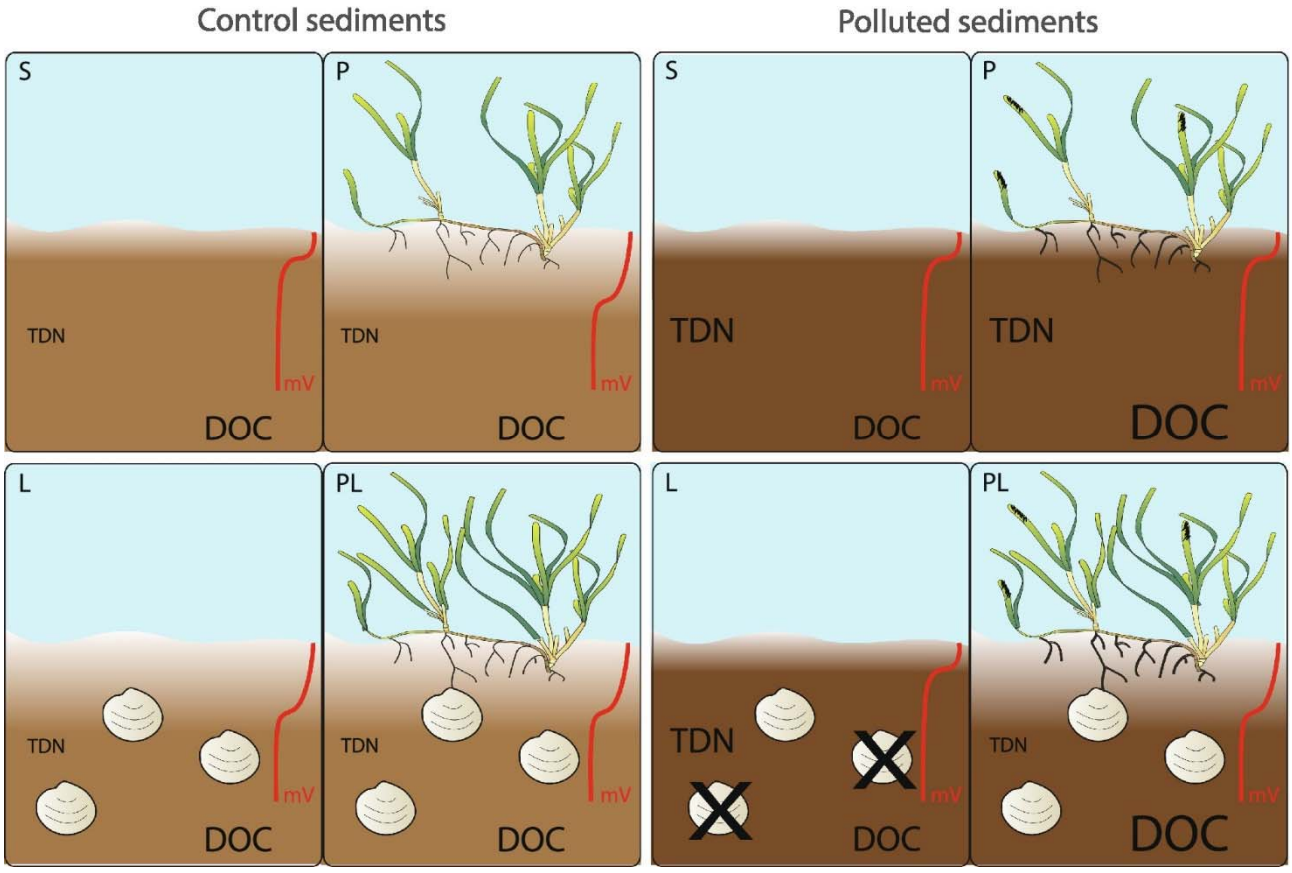

Figure 4.7- Conceptual model of facilitation of the seagrass Cymodocea nodosa by the lucinid clam Loripes orbiculatus, in control (left) vs. polluted (right) sediments. (S) represents pots with only sediment; (P) are pots with seagrass; (L) are pots with lucinids; (PL) represents pots with the plant and lucinids. Presence of lucinids resulted in increased aboveground biomass (more leaves) irrespective of the sediment type. Conversely, root apical growth (thicker roots) increased in polluted sediments, regardless of lucinid clams. Lucinid mortality was high in the polluted sediment (black crosses), but only when the plant was absent, while the percentage of plant necrotic tissue (black markings on leaves) were higher in the polluted sediment regardless of lucinids. The interaction of plants and lucinids significantly improved sediment conditions as shown by the increase in redox potential (mV) and decrease (consumption) of total dissolved nitrogen (TDN) in the polluted sediment, where the plant released large quantities of dissolved organic carbon (DOC). Seagrass symbol credit: Integration and Application Network.

Our study further demonstrates that the interaction between the plant and lucinid clams facilitates the consortium especially in heavily contaminated sediments. In these sediments, the interaction with lucinids turned the plant treatment into a sink for nitrogen (both ammonium and dissolved organic nitrogen), suggesting a more efficient uptake of this nutrient when the plant and the clam are associated. While the specific mechanisms involved are difficult to pinpoint, our results seem to support the notion of a role in nitrogen cycling for *L. orbiculatus* as indicated by Cardini et al. (2019).

Further, we found that *L. orbiculatus* isotopic niche is larger (both for the animal host and for the symbiont) when associated with the plant, regardless of the type of sediment. This was previously linked to the flexible nutritional mutualism of *L. orbiculatus*, in which the clam host and its symbionts cycle

between a looser trophic association and a tight chemoautotrophic partnership, changing nutritional strategy according to the environmental conditions (Cardini et al., 2019). Importantly, the present study further shows how the remarkable flexibility of this chemosynthetic symbiosis allows it to withstand heavy pollution if associated with a seagrass partner, the plant in turn benefitting and building more aboveground biomass in the presence of the clam.

Conclusions

Harnessing positive species interactions as a tool for restoration of degraded systems, or to counteract climate-driven loss of coastal biodiversity, is urgently needed (Bulleri et al., 2018). In particular, plant–bivalve interactions have been suggested to facilitate foundation species such as seagrasses, possibly helping to increase the success of restoration efforts (Gagnon et al., 2020). However, studies that mechanistically test specific interactions for their capacity to improve resistance of the whole consortium of organisms to anthropogenic stress are lacking. In this study, we showed that the interaction between *C. nodosa* and *L. orbiculatus* favors both organisms in colonizing highly polluted sediments from the Bagnoli-Coroglio area, promoting growth and resilience of the foundation species. Thus, co-restoration of *C. nodosa* and *L. orbiculatus* may be used in heavily impacted sites where other options are prone to failure and may improve restoration success by increasing the removal of dissolved nitrogen from the water column while also promoting the production of seagrass biomass, ultimately leading to recovery of associated biodiversity, functioning and ecosystem services.

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Chapter 4:
A facultative sponge-seagrass mutualism in the Thyrrenian Sea

Abstract

Seagrasses are ecosystem engineers that promote biodiversity hotspots. Within seagrass meadows, several animal taxa establish close associations that may result in mutual benefits, but little is known about the effects of these associations on biogeochemical cycling. Here, we study the association between the demosponge *Chondrilla nucula* and the Mediterranean seagrass *Posidonia oceanica*, in a small inlet near Napoli (Italy) where the former grows conspicuously within the seagrass bed. The sponge benthic cover had a non-linear relationship with the plant cover, with maximum *C. nucula* cover corresponding with intermediate *P. oceanica* cover, and asymmetry analyses suggesting codependency of the two organisms at the sampling site. Oxygen fluxes derived from closed-chamber incubations indicated opposite patterns across spring and autumn for the seagrass and the sponge, with the former showing maximum productivity in spring, and the latter showing highest gross photosynthesis in autumn, likely attributable to cyanobacterial symbionts. Conversely, oxygen fluxes were stable when the two organisms were associated, regardless of the two contrasting seasons. Organic and inorganic nutrient fluxes were variable but provided indications that *P. oceanica* benefited from ammonium and nitrate released by *C. nucula*. We conclude that *C. nucula* facilitates *P. oceanica* by providing key nutrients, while the presence of the primary producer may allow the sponge to augment its food intake.

Keywords: *Posidonia oceanica*; *Chondrilla nucula*; holobiont; mutualism

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Introduction

Seagrasses are ecosystems engineers that provide habitat to a plethora of associated lifeforms. A seagrass meadow can host ~60% more species than unvegetated areas, considering only fish and inverterbrate communities (McHenry et al., 2021) and even if we focus only on the epibenthic community, seagrasses provide the habitat to complex communities (Whippo et al., 2018). Many of these inhabitants are more than residents of the meadows; different species establish a wide variety of associations with other animals, microorganisms and/or with the seagrass itself, in what has been defined as a nested ecosystem (Pita et al., 2018). Examples of nested ecosystems in seagrass meadows include sediment macrofauna such as lucinid and thyrasinid bivalves, which host sulfide oxidizing bacteria in their gills that, thanks to their high densities and chemosynthetic activity, help avoiding sediment sulfide toxicity favoring plant growth (van der Geest et al., 2020; Chin et al., 2021) and at the same time provide readily available nitrogen to the ecosystem (Cardini et al., 2019).

Poriferans are also common residents inside seagrass meadows, but the range and details of potential mutualisms are yet to be explored. We know however that sponges have the capacity to take up significant amounts of dissolved organic matter (DOM) which they can then recycle to particulate organic matter (POM) to be fed upon by higher trophic levels, in a benthic counterpart of the oceanic microbial loop (de Goeij et al., 2013). For this reason, sponges may benefit from associating with a primary producer such as seagrasses, which are known to release significant amounts of DOM to the surrounding seawater and sediment (Sogin et al., 2022). At the same time, seagrass productivity in oligotrophic systems is often N-limited, and the plant may benefit from the capacity of sponges and their rich microbiomes to mitigate N limitation through processes such as ammonium excretion, but also nitrogen fixation or nitrification (Davy et al., 2002; Jiménez & Ribes, 2007; Fiore et al., 2010; Rix et al., 2015). In the Caribbean, the seagrass *Thalassia testudinum* is found to benefit from dissolved inorganic nutrients provided by the sponge *Halichondria melanadocia* (Archer, Stoner & Layman, 2015), and the sponge putatively benefits from the physical substrate provided by the plant. Further, examples of collaboration among sponge species inside seagrass meadows have been described in the Caribbean, a strategy to potentially deter predators (Wolff, 2008). In the Gulf of California, an association between a sponge and a red algae is suggested to provide mutual structural support as the main currency of the mutualism, leading to the capacity of the partnership to colonize habitats otherwise not suitable to the individual parties (Avila, Carballo & Cruz-Barraza, 2007). In the Mediterranean, the sponge *Crambe crambe* can recruit on and coat the shells of the seagrass-inhabiting bivalve *Arca noae*, with mutual benefits in terms

of substrate availability and survival to predation (Marin & López Belluga, 2005).

In the Mediterranean, a species commonly reported in *Posidonia oceanica* meadows is the demosponge *Chondrilla nucula*, a species known to host an abundant community of prokaryotes mostly belonging to Cyanobacteria, Acidobacteria, Gamma- and Deltaproteobacteria (Thiel et al. 2007) and thus ascribed to the category of high microbial abundance (HMA) sponges within the High-Low microbial abundance (HMA-LMA) dichotomy (Erwin et al., 2015). HMA sponges have been associated with a preferential uptake of dissolved organic matter, in contrast to particulate organic matter in LMA sponges; also, the HMA microbiome offers the host a greater diversity and redundancy in its functional repertoire, enabling coexistence by avoiding overlapping trophic niches (Weisz, Lindquist & Martens, 2008; Morganti et al., 2017; Lesser et al., 2022).

In other congeneric, it has been demonstrated that the Cyanobacterial symbionts can provide part of the sponge nutritional requirements through the translocation of photosynthates or direct ingestion of the algal cells even under low-light regimes (Hudspith et al., 2022). It is also able to actively translocate its body by crawling and/or extending and excising body sections, as well as being described as a frequent epibiont of a range of organisms including macroalgae, crustaceans, molluscans, ascidians, and other poriferans, causing no damage to the supporting organisms (Sidri et al., 2004).

Here we report on the association between *C. nucula* and *P. oceanica* in the area of Bacoli, in the central Tyrrhenian Sea (Italy), where the former can be found growing within many of the seagrass patches of a fragmented *P. oceanica* meadow. The sponge grows at the base of the seagrass shoot, surrounding the upper portion of the rhizome (Figure 1). Despite the availability of other hard substrate around and within the seagrass bed (such as boulders and rubble), the sponge seems to recruit predominantly on the plant, to then expand laterally towards neighbor shoots or standing rhizomes of dead *P. oceanica*.

Both *P. oceanica* and *C. nucula* are species harboring complex microbial communities, with complex body structures that make them a suitable habitat for a variety of microorganisms that can provide key metabolic functions (e.g. phototrophy, nitrogen fixation) and thus contribute to biogeochemical cycling within this nested ecosystem (McFall-Ngai et al., 2013). In our work, we explore whether this association can be described as a facultative mutualism by i) quantifying the benthic distribution of the sponge within the seagrass meadow, to verify mutual (spatial) dependence, and ii) by quantifying net fluxes of organic and inorganic nutrients in incubations with the sponge and the plant (alone or in association), to infer whether the plant and the sponge holobionts may benefit from each other's metabolism.

Methods

Study area

We carried out the experiments in the area of Bacoli (Italy), in the Tyrrhenian sea, in a small bay called Schiachetiello (40.7938 N, 14.0870 E), a marine reserve area inside the "Campi Flegrei" regional park. Here, *P. oceanica* can be found at 0-6m depth in a discontinuous meadow formed by several patches. In most patches, *C. nucula* can be found abundantly growing at the base of the seagrass shoot, surrounding the upper portion of the rhizome (Figure 5.1).

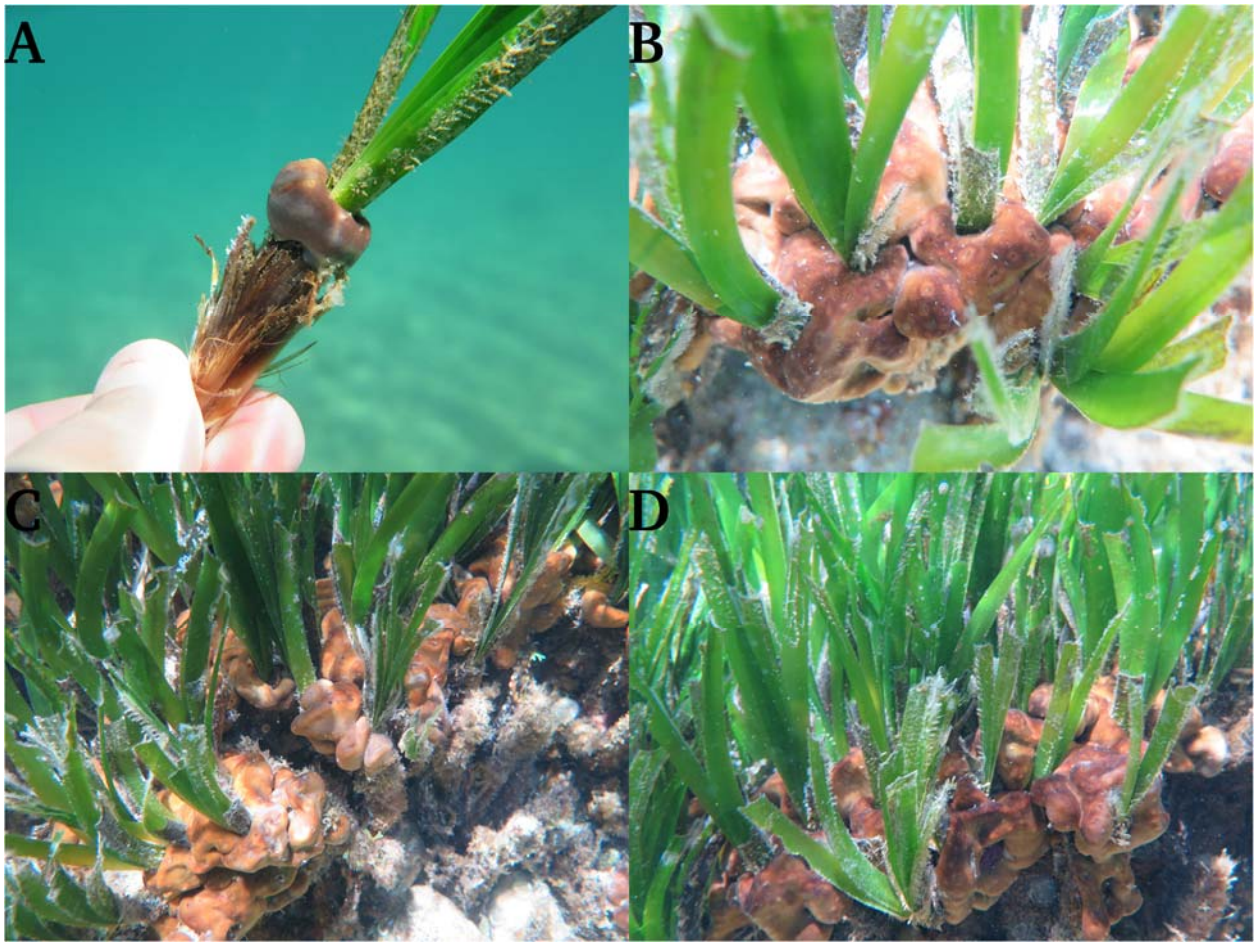

Figure 5.1- A) detail of a P. oceanica shoot bearing a C. nucula bundle around the transition between leaves and the foliar sheath. B) detail of the sponge bundles growing attached to the shoots. C) and D) large C. nucula colonies expanding in between shoots and fusing together. Pictures by U. Cardini.

Cover estimations

We shot video-transects by snorkeling along the longest distance across the shallow patches during November 2021. From each file, we extracted the frames using FFMPEG (https://ffmpeg.org/)

and selected 82 images based on their sharpness, to estimate the cover percentage of the main benthic substrates on quadrats of 0.25 m^2, for a total surveyed area of 20.5 m^2.

O_2 and nutrient fluxes

We collected *P. oceanica* shoots, *C. nucula* bundles, and *C. nucula*-bearing seagrass shoots from shallow beds and performed closed-chamber incubations *in situ* in November 2021 (autumn) and May 2022 (spring). For the November incubation, we used 0.55L chambers for ~3.75h, while in May, we used 1.1L chambers during ~6h incubations. Our design included two fixed factors: Treatment (with three levels: *P. oceanica* alone, *C. nucula* alone, and their association) and Light condition (with two levels, under natural light and in the darkness, Figure 5.2). Additionally, we incubated a set of chambers containing only seawater to be used as controls for quantifying O_2 fluxes. For the November incubation, we used three replicate chambers per combination of treatments (n = 24) and we increased this number to four chambers during the May incubation (n = 32).

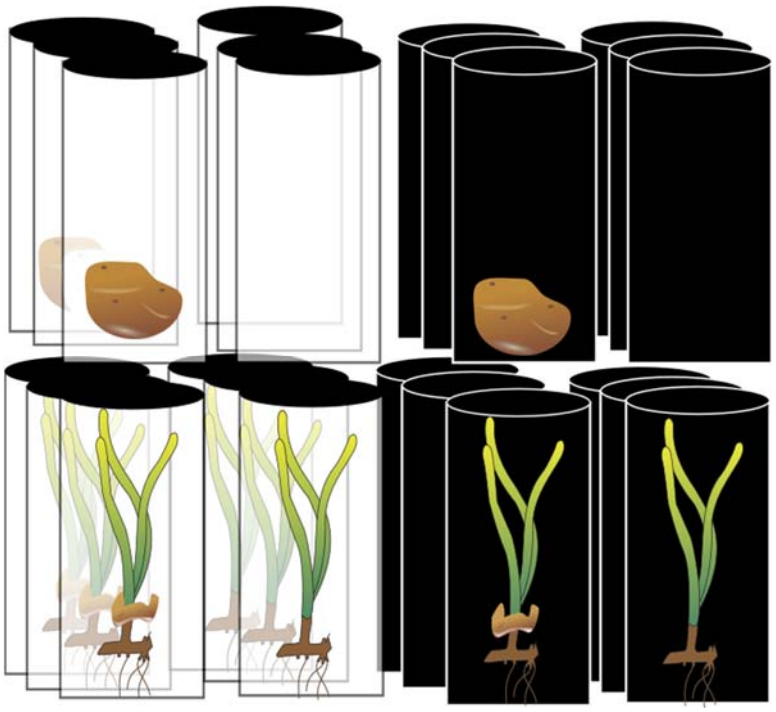

Figure 5.2: Schematic organization of the treatments, showing dark and light chambers with all the combinations of C. nucula, P. oceanica, and the association.

During the incubations, the chambers were held in floating baskets, allowing the chambers to receive full sunlight while also exposing them to gentle wave action that prevented oxygen, temperature or dissolved nutrient stratification within the chambers. HOBO loggers were used to control light intensity within and outside the chambers (on a nearby rock at ca. 0.5 m depth) and to verify that light

levels resembled those *in situ*. Conversely, "dark" chamber crates were placed inside several layers of black polyethylene bags to avoid any exposure to light and incubated parallelly to the "light" chambers.

At the beginning and the end of the incubations, we opened the chambers and measured the O2 concentration with a digital meter (WTW Multi 3430 Set K). At the end, from each incubation chamber, we collected 30mL of the incubation water for the determination of dissolved organic carbon (DOC) and nitrogen (DON), and 20mL for inorganic NH_4^+, PO_4^{3-}, NO_2^-, NO_3^-, and SiO_4^- concentrations. All aliquots for dissolved nutrient analysis were collected with acid-washed syringes; aliquots for DOC/DON were filtered through pre-combusted GF/F glass microfiber filters (0.7µm), immediately acidified with 80µL HCl 6M, and kept refrigerated until analysis. Samples for inorganic nutrients were filtered through cellulose acetate membrane filters (pore size: 0.22µm), frozen in situ, and kept at -20°C until analysis with a continuous flow analyzer (Flowsys, SYSTEA SpA.) Sponge and plant specimens were collected from each chamber and frozen for later dry weight determination. From each chamber, we measured the total seawater volume to calculate the oxygen and nutrient flux rates as the difference between final and initial concentrations, then corrected for controls and normalized to effective volume in the chamber and dry weight of the samples.

Data analysis

Oxygen and nutrient flux rates for each chamber, corrected by the changes observed in the seawater controls, and standardized by the dry weight of the incubated organisms are expressed as µmol g^{-1} h^{-1}. Net photosynthesis (Pn) and dark respiration (Rd) of the specimen were assessed by their O_2 fluxes in the light and in the dark, respectively, and gross photosynthesis (Pg) was calculated ($Pg = Pn + |R|$) from the average of each treatment level.

We analyzed the potential asymmetric dependence between *P. oceanica* and *C. nucula* using the R package *qad* (Griessenberger, Trutschnig & Junker, 2022). This approach stems from the idea that the correlation A~B would be the same as B~A only if the variables are truly independent. Complementarily, we modeled their relationship using a generalized additive model using the R package *mgcv* (Wood, 2011). Finally, we assessed the specific effects of each nutrient through a multivariate linear model with the *mvabund* package (Wang et al., 2012). All the data, supplementary material, and code to reproduce are available at: https://github.com/luismmontilla/ponchos

Results

Seagrass-sponge cover relationship

C. nucula cover didn't change linearly with *P. oceanica* cover (Fig. 5.3), with our model

approaching a cubic curve (edf = 3.7; p = 6.55e-5). The most noticeable effect is the peak of *C. nucula* cover was at intermediate levels of *P. oceanica* cover. Under an independent relationship, the sponge would be present also at low cover of the plant since there is more space available. This asymmetry was also corroborated with the obtained q values (Table 5.1).

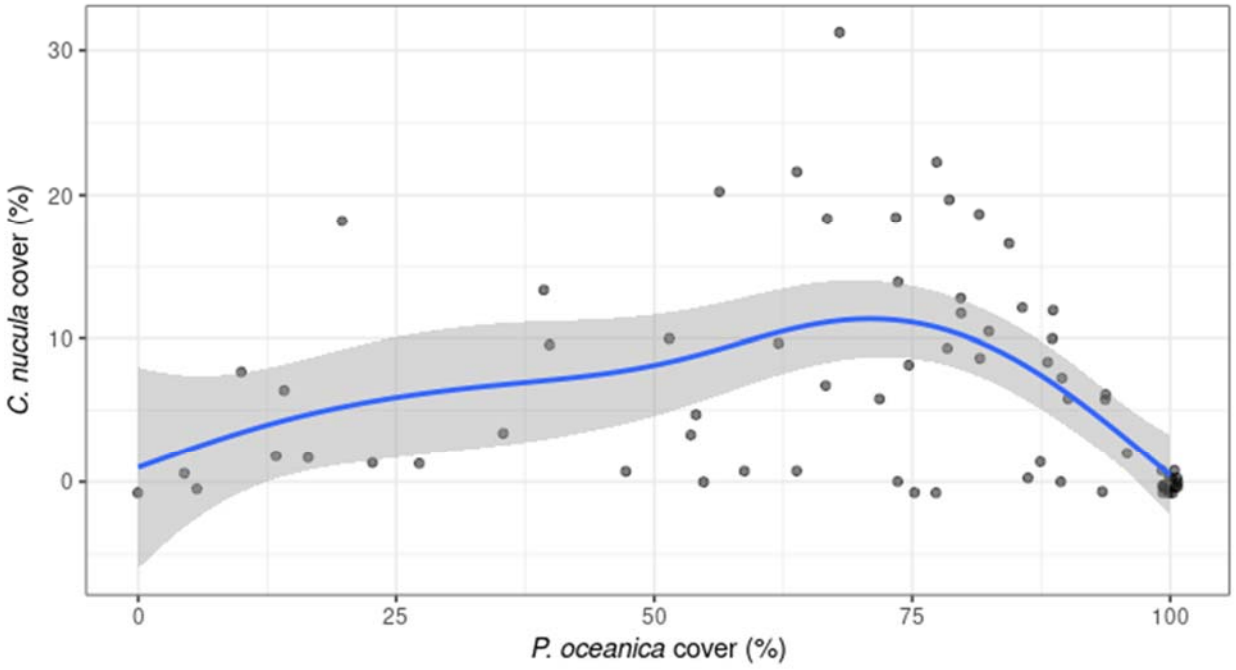

Figure 5.3- Seagrass-sponge cover relationship. The blue line represents the generalized additive model fit and the standard error.

Table 5.1- Coefficients of assymetric dependence between P. oceanica and C. nucula benthic cover.

	coefficient	p-value
q (*Chondrilla-Posidonia*)	0.252	0.007
q (*Posidonia-Chondrilla*)	0.366	0.0001

Respiration, Net- and Gross photosynthesis

The association showed intermediate net photosynthesis rates between the individual members but this was not constant in both seasons (Pseudo-F = 8.5; p = 0.002, Figure 5.4A, Table 4.2). In autumn, the average net photosynthesis of the association and *P. oceanica* alone was among the same range of values (12.0 and 10.3 µmol g^{-1} h^{-1} respectively; p-adj = 0.9; Figure 5.4A, Supp. Table 8). Both the association and *C. nucula* alone maintained similar net photosynthesis magnitudes during both seasons, in contrast with *P. oceanica* alone, that was 70% more productive in spring (20.4 µmol g^{-1} h^{-1}; p-adj = 0.00001; Fig. 4.3A; Supp. Table 8).

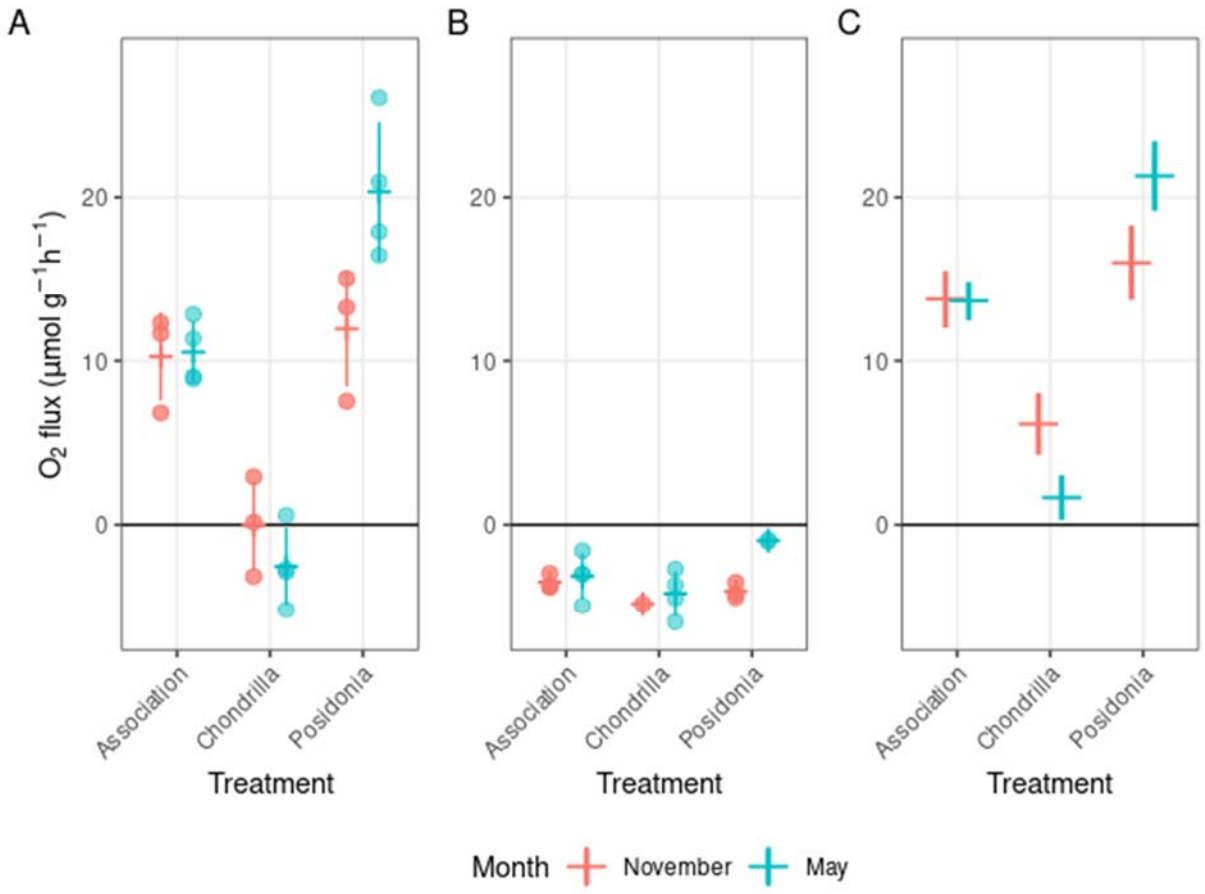

Figure 5.4- Net photosynthesis (A), respiration (B), and gross photosynthesis rates (C). The crossbars represent mean and standard deviations.

A similar pattern happened with the respiration rates. Some small differences (2~4 µmol g^{-1} h^{-1}) were detected in the interaction of both the treatment and the month (Pseudo-F = 4.27; p = 0.03, Figure 5.4B, Table 5.2). In particular, the association showed similar respiration rates for both *C. nucula* and *P. oceanica* on November, and May, however the difference in magnitude between the sponge and plant alone were larger on May (Fig. 5.4B; Supp. Table 9). Gross photosynthesis (Figure 5.4C) indicate that the photosynthetic component of the *C. nucula symbiont* alone is more productive in autumn than in spring. When using these values to estimate P:R ratios, *C. nucula* seemed to be net heterotrophic during May (P:R = 0.4) and having a balanced productivity in November (P:R = 0.99).

Table 5.2- Community production and respiration extrapolations in mmol C m-2 day-1. Values indicate mean ± standard deviation.

	Treatment	Net community production	Community Respiration
Autumn	*P. oceanica*	260.91 ± 29.2	-88.5 ± 29.2

	Association	146.1 ± 47.5	-49.8 ± 47.5
Spring	*P. oceanica*	444.22 ± 29.2	-21.1 ± 29.2
	Association	150.0 ± 47.5	-44.7 ± 47.5

Nutrient flux rates

The multivariate comparison of the nutrient rates in light chambers showed differences depending only on the season (Pseudo-F = 5.37; p = 0.001; table 5.3) and a common pattern in both light and dark incubations is that the variation between *P. oceanica* and *C. nucula* alone is marked and evident, while the samples from the association were spread and in between (Supp. Fig . 13). In autumn, the nutrient rates indicated overall excretion except for the silicate rates from the association. In spring, the rates across treatments were much more variable and only silicates showed net incorporation. Silicates and nitrates fluxes were the main drivers of seasonal variation, with silicates showing an average incorporation in spring 3-fold the excretion in autumn; *C. nucula* alone excreted on average 5x more than *P. oceanica* alone and 2x the intake of the association (Pseudo-F_{SiO4} = 10.24, p_{SiO4} = 0.016; Pseudo-F_{NOx} = 14.75, p_{SiO4} = 0.005; Supp. Table 10). Overall nitrates fluxes were similar magnitudes (but opposite sign) and more specifically, *C. nucula* having an average excretion rate in autumn 2-fold the association and 4-fold *P. oceanica* alone (Figure 5.5).

Table 5.3- Permutation-based analysis of variance of the nutrient flux rates in the treatments, months, and their interaction.

	Source of variation	Degrees of freedom	Sums of squares	Mean Squares	Pseudo-F	R^2	p(>Pseudo-F)
Net productivity	Treatment	2	15.44	7.72	1.66	0.13	0.131
	Month	1	24.97	24.97	5.37	0.21	0.001
	Treatment x Month	2	9.88	4.94	1.06	0.08	0.404
	Residuals	15	69.72	4.65		0.58	
	Total	20	120.00	6.00		1.00	
Dark respiration	Treatment	2	35.82	17.91	5.16	0.30	0.001
	Month	1	17.14	17.14	4.93	0.14	0.002
	Treatment x Month	2	14.92	7.46	2.15	0.12	0.038
	Residuals	15	52.12	3.47		0.43	
	Total	20	120.00	6.00		1.00	

In dark chambers, the interaction of month and the treatment was more relevant (Pseudo-F = 2.15, p = 0.038, table 5.3). Nitrogen oxides fluxes rates changed consistently across treatments, with *C. nucula*

alone having excretion fluxes up to 1.5 µmol g^{-1} h^{-1}, on average 4 times larger than the association, while *P. oceanica* alone had a net minute uptake flux (Pseudo-F_{NOx} = 21.91, p_{NOx} = 0.002; Supp. Table 11, fig 5.6). Ammonium in spring was excreted in the association and *C. nucula* alone up to 0.1 µmol g^{-1} h^{-1} but it remained around zero in *P. oceanica* alone. In contrast, in autumn but the sponge and plant alone had net uptake fluxes, while the association had an average flux to the values during spring (0.028 – 0.019 µmol g^{-1} h^{-1}). Phosphate fluxes were also most dissimilar among treatments (Pseudo-F_{NOx} = 8.41, p_{NOx} = 0.006; Supp. Table 11, fig 5.6); the association excreted phosphates in both seasons at similar average rates but it was much more variable during spring (0.0064±0.0007 vs 0.0067±0.007 µmol g^{-1} h^{-1}). The plant alone also excreted 0.0009 µmol g^{-1} h^{-1} concentrations of phosphate during autumn but had an average uptake 3-fold during spring.

Silicate fluxes were, on average, negative in spring in *P. oceanica* and the association, and positive on *C. nucula*, however these magnitudes were low and spread around zero (-0.03 – 0.005 µmol g^{-1} h^{-1}). The most relevant flux was the *C. nucula* excretion in autumn, which was an order of magnitude higher than the rest of the treatments. Dissolved organic carbon and nitrogen were net excreted across all the treatments in autumn, and more variably, with more extreme values in spring, e.g. up to 9 and 2 µmol g^{-1} h^{-1} respectively. Some incorporation values were detected in the sponge chambers, reaching values of 3 µmol g^{-1} h^{-1} (figure 5.6).

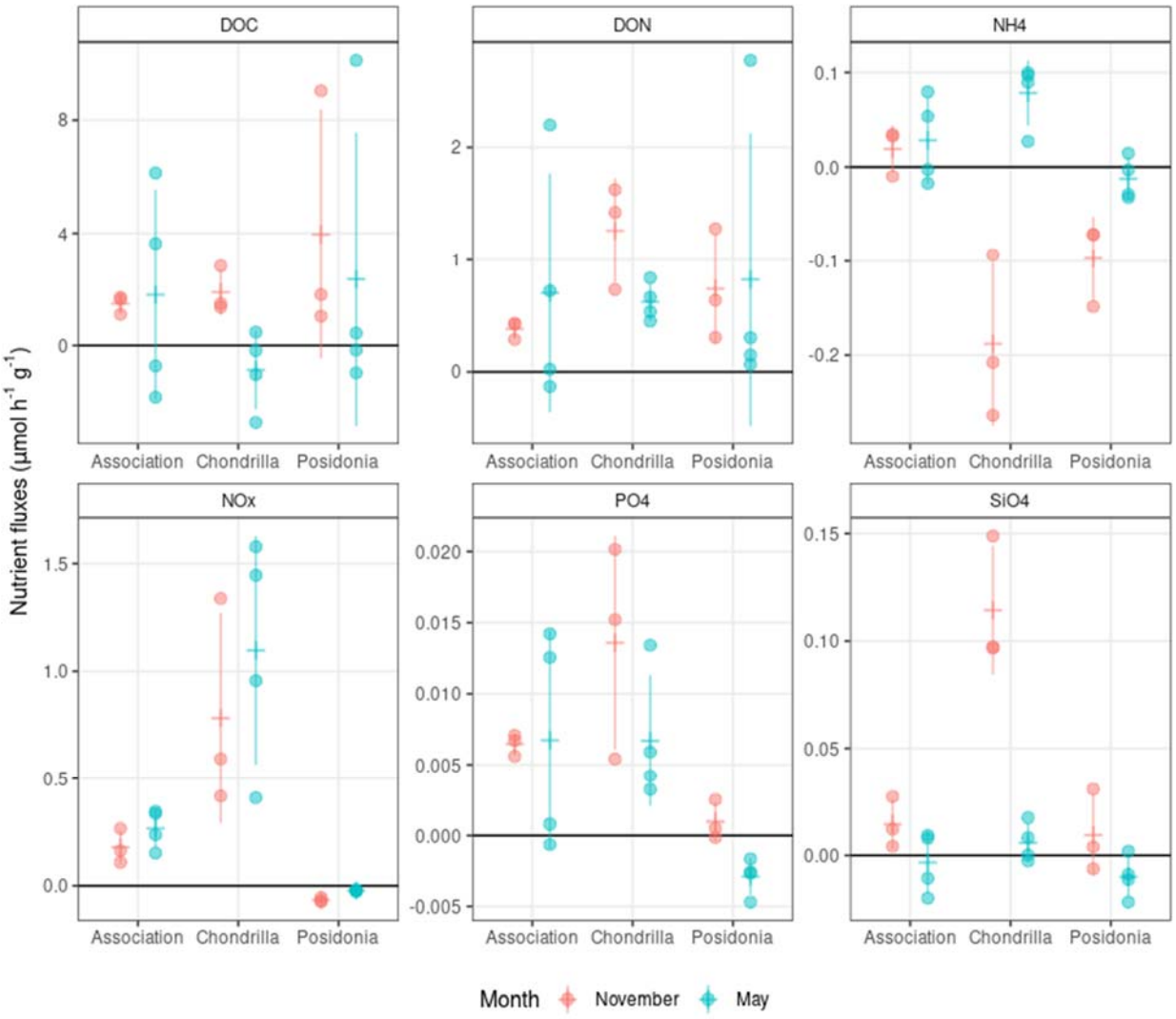

Figure 5.5- Dissolved organic carbon (DOC), nitrogen (DON), ammonium (NH4), nitrates (NOx), phosphate, and silicates (SiO4) fluxes in the light in both seasons. The crossbars represent mean and standard deviations

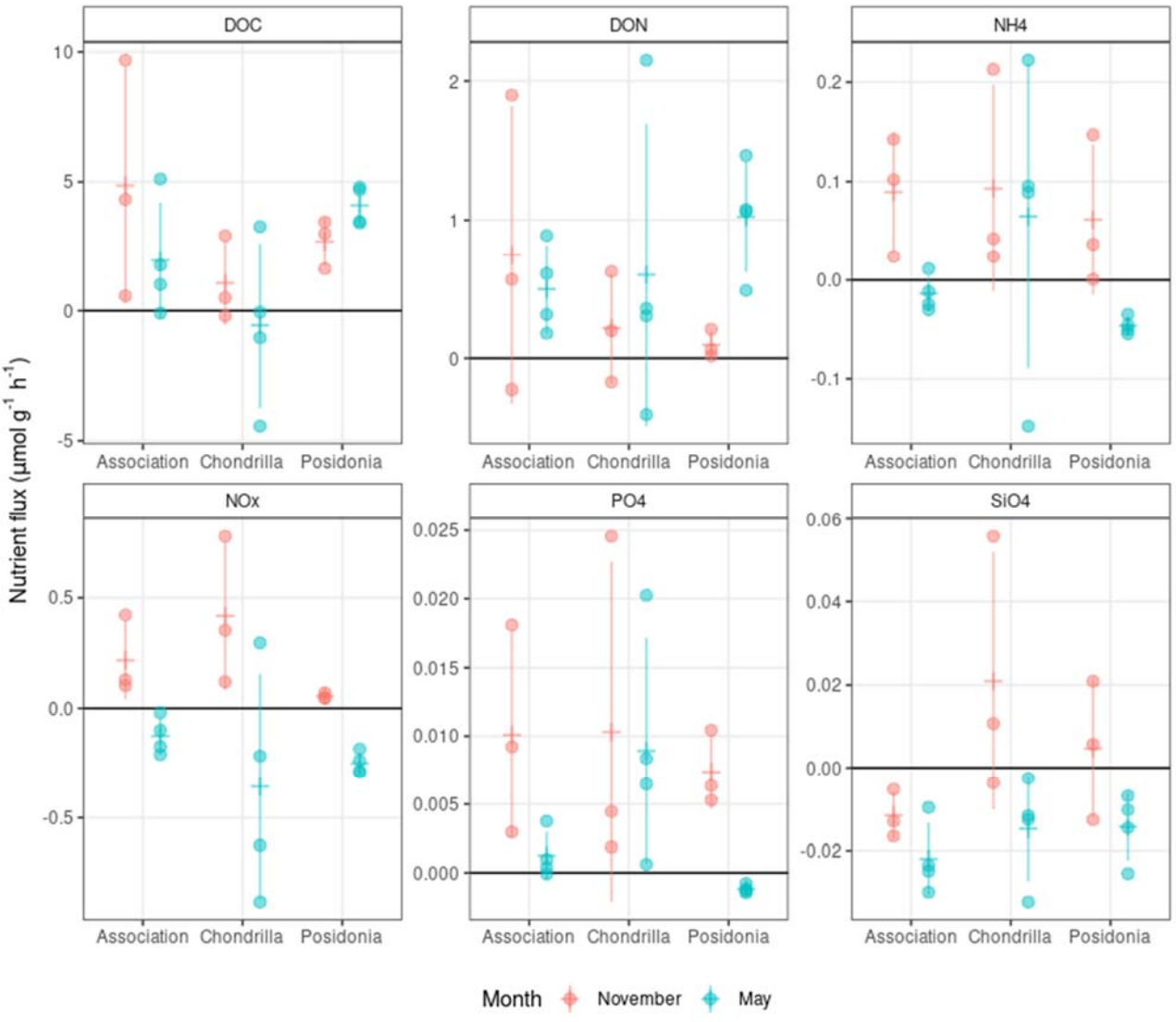

Figure 5.6- Dissolved organic carbon (DOC), nitrogen (DON), ammonium (NH4), nitrates (NOx), phosphate, and silicates (SiO4) fluxes in the dark in both seasons. The crossbars represent mean and standard deviations.

Discussion

Here we report on the distribution and biogeochemistry of the sponge-seagrass association between *C. nucula* and *P. oceanica*, finding evidence supporting the hypothesis of the association as a facultative mutualism.

The effects of the association on benthic cover and primary productivity

C. nucula cover showed its maximum values under intermediate seagrass cover. This suggests that the sponge benefited from presence of the primary producer. This supports the ideas exposed by Archer et al (2015), where intermediate levels of seagrass cover offer substrate to be colonized without

diminishing water currents to a level that could be detrimental for the sponge nutrition. The amount of light that is blocked by the seagrass leaves is also a factor to consider; on different latitudes, cyanobacterial sponges seem to meet more than half their energetic requirements from photosynthesis under ideal conditions (Wilkinson, 1987) however the O_2 fluxes and P:R ratios we obtained don't suggest a particularly intensive photosynthetic activity. On the other hand, different cyanosponges can exhibit different levels of dependence on their photobionts (Thacker, 2005).

Our respirometry results are roughly in line with the range of other *P. oceanica* O_2 fluxes estimations such as those reported by Olivé et al. (2016), Berlinghof et al. (2022), or Koopmans et al. (2020). *C. nucula* alone showed no net photosynthesis but low levels of gross photosynthesis, suggesting that this sponge is also associated with photosynthetic symbionts similarly to its congeneric species. This is unlikely to be enough to sustain the nutritional requirements of the sponge alone since it's P:R ratio is <1; this trend to net heterotrophy has been observed on other congeneric at deeper habitats (Hudspith et al., 2022) and it is expected, considering that cyanosponges are nutritionally flexible, and mixotrophy is not uncommon among different species. Some species have access to a diversity of photosynthetic pigments including phycoerythrins, which might be giving *C. nucula* its brownish color (as reviewed in Usher, 2008), which could also help to sustain sponge productivity during lower light intensity conditions, such as autumn, where we observed a larger gross productivity than in spring.

If we look at gross photosynthesis, the association has a stable gross photosynthesis (C fixation) irrespective of the season, differently from the plant alone. This suggests that the association may have a buffering effect on the heterotrophy/autotrophy continuum of the seagrass community. It is likely that the association with *C. nucula* is providing a local nutritional enhancement to the plant (see next paragraph); other studied seagrass mutualisms also contribute to increase the meadow productivity by decreasing grazing and facilitating the expansion of the meadow (Leemans et al., 2020). In our case, whether the stability we observed in sponge associations translates to a consistent increase in productivity of the meadow remains to be further explored. However, our results let us hypothesize that seagrass beds colonized by the sponge have lower production in respect to the rest of the meadow during more productive seasons but that the increased sponge production during lower light intensity conditions will have a buffering effect and keep overall meadow yearly production stable.

The effect of the association on nutrient cycling

Our results show highly variable DOC and DON fluxes, indicating net release in most treatments and in both seasons. However, we detected net DOC uptake by *C. nucula* in May, both in the light and in the dark. This may suggest that the intermediate DOC and DON fluxes detected in the presence of the

association with the plant are the result of the sponge taking up DOM released by the primary producer (in our case *P. oceanica*), as it has been described in coral reefs for the so-called sponge loop (Rix et al., 2017). In *P. oceanica* alone, if inorganic nitrogen provision is enough to fulfil the plant needs, little or negligible incorporation of DON is expected (Touchette & Burkholder, 2000; Tarquinio et al., 2018). While the plant by itself can excrete high concentrations of labile carbon (Barrón & Duarte, 2009; Sogin et al., 2022), the high variability in DOC and DON fluxes in our chambers may also be the result of excreted products being readily consumed by water column microbes.

The poriferan body can be the stage of several steps of the marine nitrogen cycle. NH_4^+ production is expected as part of animal excretion, but in cyanosponges like *C. nucula*, host-produced NH_4^+ can be expected to be recycled and used by the cyanobacterial symbionts, and ammonia uptake from the water column can be detected in some species (Maldonado, Ribes & van Duyl, 2012), as was the case in our dark sponge incubations in Autumn. Specifically, the switch for *C. nucula* in November between NH_4^+ release in the light and uptake in the dark is interesting, as it may reflect differences in abiotic factors in the internal environment of the sponge resulting from diel cycles in pumping rates (Fiore et al., 2010). As a matter of fact, the respiratory activities of host and microbes create strong gradients of hypoxia when the sponge slows down or interrupts pumping; the resultant internal changes allow different functional groups of symbiotic (e.g. nitrogen-fixing and nitrifying) prokaryotes to be both spatially and temporally separated over a range of physiological conditions that are created by the sponge itself (Fiore et al. 2010). *P. oceanica* alone displayed ammonium uptake in Spring, the plant main growing season, which makes sense as the plant needs nutrients for building biomass and can quickly incorporate ammonium through both its leaves and roots (Lepoint et al., 2002). On the contrary, the sponge mainly displayed NH_4^+ release, and when the plant was associated with the sponge the flux was close to zero. One possibility is that there was no change during the incubation period, but more likely; this net ammonium flux is the result of balanced plant uptake and sponge excretion. For example, in the *Haliclona-Ceratodyction* association, ammonium excretion fulfills the nitrogen requirements of the macroalgal partner with little to no surplus (Davy et al., 2002).

A similar pattern can be seen in nitrate fluxes, with the sponge mostly releasing and the plant mostly taking up this nutrient, and the association displaying intermediate values. Our nitrogen oxides excretion fluxes were higher or in the same magnitudes as what has been detected in previous *C. nucula* dark incubation experiments in the Caribbean, e.g. 0.428 μmol g^{-1} h^{-1} by Corredor et al., (1988) and 1.04-1.65 μmol g^{-1} h^{-1} (Diaz & Ward, 1997) respectively. Our values are also not far from those reported by in situ studies from Australia and the Caribbean for a wide variety of sponge species (Southwell et al., 2008;

Keesing et al., 2013). Positive nitrogen oxides excretion rates may also be coupled to ammonium incorporation in HMA sponges such as *C. nucula*, as ammonium is oxidized by nitrifier symbionts (Subina, Thorat & Gonsalves, 2018). These fluxes can also be decoupled, as measured in the HMA sponge *Chondrosia reniformis*, and they can be a response to pulses of ammonium in the environment, e.g. as could be produced by antropogenic activities (P. Baquiran & Conaco, 2018; Nemoy, Spanier & Angel, 2021).

In terms of phosphate fluxes, release rates were observed for *C. nucula* alone chambers, while *P. oceanica* displayed fluxes close to zero. Campana et al. (2021) also detected net excretion at similar rates to our results in *Chondrilla caribensis*. In general, the few available studies looking at phosphate fluxes by sponges similarly report net PO_4^{3-} release by both LMA and HMA species from the Mediterranean and North-Pacific (Maldonado, Ribes, van Duyl 2012), in the same order of magnitude than rates reported here. On the other hand, *P. oceanica* inorganic phosphates fluxes has been correlated to the distance to areas of eutrophication, with meadows closer to the zone of nutrient production releasing PO_4^- continuously throughout the year and more distant, reference meadows showing net uptake, both in the same magnitude as our (Apostolaki et al., 2010a,b) but significantly lower than the values presented by Barron & Duarte (2009). Despite detecting a small uptake in the plants alone in spring, it should be expected considering that we also obtained larger net productivity in the same season and the opposite for autumn. Once more, the intermediate values in the association chambers may result from the plant incorporating at least a portion of the sponge-excreted nutrients (Archer, Stoner & Layman, 2015; Archer et al., 2021). Further, a role of prokaryotic symbionts is likely, since Cyanobacterial symbionts of marine sponges have been found to efficiently produce and accumulate phosphorous in the form of polyphosphate (poly-P) granules (Zhang et al., 2015).

Organisms with siliceous body parts, including *C. nucula*, incorporate dissolved silicon (DSi, in the form of orthosilicate ions - SiO_4^-) which is used to build siliceous spicules in the case of the sponge, making this group a relevant and underestimated Si sink (Maldonado et al., 2005, 2011; López-Acosta et al., 2018). In our study, we found small silicate fluxes primarily showing slight uptake in all treatments in the light and net zero fluxes in the dark, except for *C. nucula* in November that showed silicate release both in the light and in the dark. Sponge DSi uptake rates are related to growth periods and temporal variation have been linked to factors regulating such process, e.g. predation (López-Acosta et al., 2023). The fact that the largest excretion rates were observed on the sponge alone suggests that the association and *C. nucula* alone have different effects on the local Si dynamic. On one hand, light-associated release can be related to *C. nucula* phototrophic symbiont activity, which leads to higher concentrations of free

radicals that are quenched by ascorbic acid, which in turn can dissolve the siliceous spicules of the sponge (Bavestrello, Bonito & Sarà, 1993; Bavestrello et al., 1995; Cattaneo-Vietti et al., 2004). On the contrary, silicate release when the sponge was associated to the plant was comparatively smaller or close to zero. This also supports the role of the plant as mechanical support, as suggested by authors studying other sponge epibiosis (Vicente et al., 2014), in which the sponge showed a reduced investment in spicules and subsequent decreased cost in spicule synthesis, with the authors suggesting that this could be one of the benefits of growing closely together. Additionally, *C. nucula* from the association are also under prolonged shading in comparison with *C. nucula* alone and its symbionts should have reduced photosyntethic activity, therefore a reduced Si dissolution through the aforementioned process. We suspect that the association might be promoted by other factors besides substrate availability if we draw comparisons with the sponges (and other epibionts) recruiting on other systems such as mangrove roots, where living roots are colonized by richer communities compared with inert or dead root surrogates (Stewart et al., 2022). We could further explore this hypothesis by performing an experiment with artificial seagrass units, testing if *C. nucula* bundles colonize and grow on *P. oceanica* surrogates following the same pattern. Given that both species are most often found alone, the association could be case of a facultative commensalism, potentially unstable under the current environmental conditions of the location and therefore, it could be disrupted if the system undergoes further perturbations (Thompson, 1988; Bronstein, 1994; Hay et al., 2004; Chamberlain, Bronstein & Rudgers, 2014). Along these lines, there is evidence in favor of the sponge-seagrass association in the Caribbean being unstable and nutrient dependent (Archer, Hensel & Layman, 2018). On the other hand, a different possibility is that the association provides stability under further environmental pressures, considering that *C. nucula* seems to thrive under chemical pollution and eutrophication scenarios (Milanese et al., 2003; P. Baquiran & Conaco, 2018).

Conclusions

Our results contribute to the body of research studying symbiotic relationships using a holistic approach, and fortifies the notion that we need to probe multiple scales and processes to better understand the implications of a closely associated set of species. Our results support the hypothesis of a facultative mutualism between *P. oceanica* and *C. nucula*, where the sponge may benefit from carbon released by the plant while providing key inorganic nutrients that can support seagrass productivity. However, there is much more to this, as the sponge is also likely associated with phototrophic symbionts and thus directly contributes to C fixation. This peculiar and conspicuous association deserves further efforts to explore

its potential nutritional coupling as well as its microbial ecology, and to further test its stability across environmental conditions.

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Discussion

This work presents a multidisciplinary approach to advance our current knowledge about microbe-animal symbiosis in seagrass ecosystems. By combining ecological surveys, high throughput sequencing, stable isotope analysis, dissolved nutrient concentrations, and evidence synthesis, we have expanded on what we know about Mediterranean seagrasses as nested ecosystems (Figure 6.1).

The study of symbiosis is ultimately the study of interactions and as such, the use of networks provides an appropriate approach. My survey in chapter I, though conservative, revealed a landscape of invertebrate-microbe symbioses confirming the key role of taxa like the class Gammaprotebacteria, being present sometimes as the most abundant symbiont in chemosynthetic invertebrates, or as a marginal member abundant enough to be consistently in my dataset. The potential of this tool can also be seized to go one step further and model symbiotic microbiomes in terms of their co-occurrence, or their metabolic exchanges as metacommunities, incorporating a wider range of animal hosts that are part of the seagrass ecosystem, such our described interaction between *P. oceanica* and *C. nucula*, potentially shedding additional light on the persistence of this growth form next to the two organisms alone (Toju, 2015; Mendes-Soares et al., 2016; Layeghifard, Hwang & Guttman, 2017; Muller et al., 2018). Other improvements that could help getting more generalized results include expanding the scope of our queries to include short reads derived from sequencing platforms like Illumina as has been used to build similar meta-analyses in other plant symbionts meta-analyses (Toju, 2015; Kivlin et al., 2017).

On the other hand, when used as part of a meta-analysis, this approach certainly provides a larger, more integrated picture of the available collective evidence, with the additional advantage that it can be updated regularly, e.g. through living systematic quantitative reviews; therefore, we can have at hand reach a condensed perspective that can help us to engage with applied practices such as adaptive ecosystem restoration efforts and policy making (Pullin, Knight & Watkinson, 2009; Elliott et al., 2017). Eventually, networks can also be used in the active restoration efforts by monitoring and identifying the most robust set of species that can provide resilience to the newly restored areas (e.g. Pocock, Evans & Memmott, 2012). We limited our metadata to the benthic position of the invertebrates, however, other highly relevant traits that could be included in a more dedicated and extensive analysis could be the bioturbation mode of the host (Queirós et al., 2013), different seagrass species and/or a range of ecoregions.

Understanding these symbiotic interactions is also improving the tools we have available to

minimizing our impact and reducing the cumulative damage that our development is dealing to coastal ecosystems. The results from chapter II, showed that microbial communities of *P. oceanica* leaves remained stable against the long term exposure to a lower pH regime. Moreover, prokaryotes that are able to carry out key N transformations such as N_2 fixation or nitrification were among the key groups. Biogeochemical measurements detected active N_2 fixation as well as nitrification and anammox/denitrification potential, providing evidence of an accelerated N cycling on seagrass leaves in a high CO_2 world. The importance of constantly probing the potential of the ecosystems to naturally persist with minimum to no intervention goes along the lines of realizing that some stressors can have a local impact, but large scale or global effects like sea surface warming or ocean acidification requires vastly different strategies for mitigation. The fact that the microbial community remains stable under low pH scenarios is a hopeful result that also highlights the upmost importance of understanding the microbiome. Along these lines, the incorporation of microbiome-related metrics that can work as early signal of ecosystem deterioration are being developed (Sims et al., 2013; Aylagas et al., 2017), and the use of facilitative interactions (or control of antagonistic species) to improve the efficiency of ecosystem restoration efforts is starting to be applied (Suykerbuyk et al., 2012). Approaches that are more ambitious include conditioning and breeding stress-resistant plants or using probiotics to supply the host with the microbiome members that can enhance its survivability (Marín-Guirao et al., 2019; Peixoto et al., 2021; Pazzaglia et al., 2022). This probably represents the ultimate challenge, as we must find alternatives to continue our development while minimizing our impact and reducing the cumulative damage that coastal ecosystems are withstanding.

In this regard, my thesis work adds to what has been done to scale up research efforts and disentangle the interactions between the plant and other associated holobionts, potentially important for seagrass ecosystem functioning, such as sponges or sediment infauna other than chemosymbiotic clams. To accelerate progress in the study of multi-holobiont interactions we need to expand the scope of our experiments to include not only the macrophyte component and its microbiome, but also invertebrates along the gradient of association. Ultimately, as we aim to capture as much of this information as possible, we need to keep in mind the long term, open availability and integration of our ecological data, embracing the paradigm of ecology as a data intensive discipline. Similarly, to the way molecular databases have transformed the way we engage in molecular biology, a network of resources applying common standards to report and make available environmental and ecological data would revolutionize our capabilities to understand ecosystems. This idea that is not new among ecologists (Michener & Jones, 2012; McFall-Ngai et al., 2013; Duffy et al., 2019) and projects like the National Microbiome Data Collaborative are

spearheading this transition (Wood-Charlson et al., 2020).

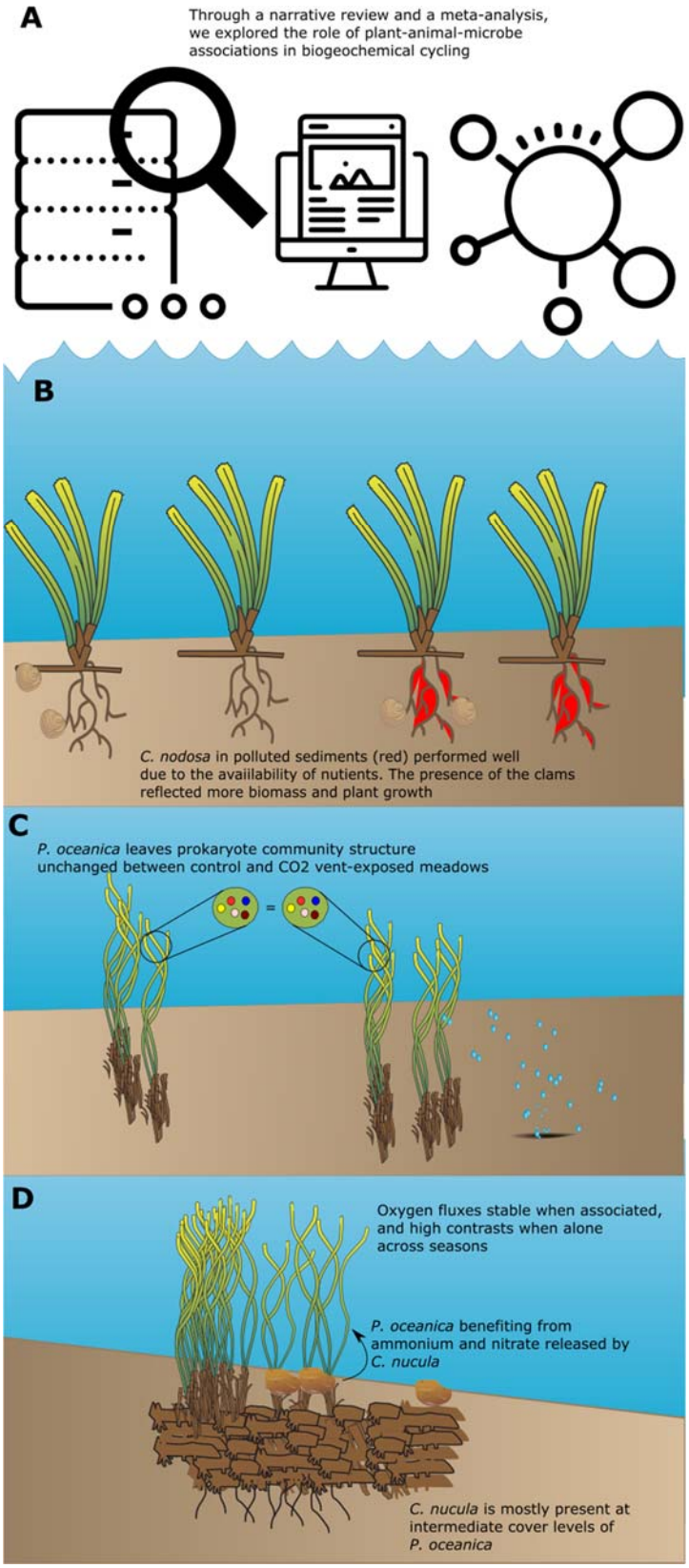

Figure 6.1 - Graphical summary of the four chapters that constitute this thesis

The focus of this dissertation was not only on nested ecosystems *per se* but also on how the nested ecosystem and the holobionts are fundamental gears on the nitrogen cycling machinery (Aoki, McGlathery & Oreska, 2020; Orth et al., 2020). As we continue expanding the exploration of the role of holobionts in nitrogen transformations, we can increase the accuracy of our models predicting how ecosystems cope with the inorganic nitrogen input in shallow coastal marine ecosystems (e.g. Sousa et al., 2012; Banks et al., 2013; An et al., 2021; Booth et al., 2023), especially framing this into the implementation of cost-effective nature based solutions (Onorevole, Thompson & Piehler, 2018; Cheng et al., 2020; Álvarez-Rogel et al., 2020). Our results from the chapters dealing with seagrass species interacting with animals support the notion that taking into account organisms with microbiomes that couple nitrogen recycling and provision (e.g. through microbe-mediated N transformations) should increase the chance of success on these conservation efforts against threats such as increased temperature, extension of hypoxic zones, and ever-increasing nutrient inputs into the coastal zone. The degradation of this ecosystem leads to the evident dissapearence of key ecosystem services, for example, i) loss of C storage capacity with seagrasses becoming a C source to the water column and to the atmosphere. ii) Loss of coupled nitrification-denitrification, with consequent buildup of ammonium and nitrate that can exacerbate coastal eutrophication, further pushing planetary boundaries that are already in the high-risk zone (Steffen et al., 2015). iii) the loss of the associated macro- and microbial (systematic and functional) biodiversity, which in turn, also implies iv) loss of commercial and recreational fisheries production, estimated around 40% for Mediterranean seagrass beds (Jackson et al., 2015) and loss of recreation and tourism value.

This work shows that it is paramount to study marine plants with a holistic approach that include and consider the role of both the associated microbiome and nested interactions with other holobionts, such as sponges and bivalves, which engage in an integrated and synergistic cooperation with the plant and have cascading effects on organism fitness further affecting the biogeochemistry and the functioning of the whole ecosystem.

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Supplemental tables

Supplemental table 1- Permutation-based analysis of variance of the pH regime, compartments and their interaction.

Source of variation	Degrees of freedom	Sum of squares	R2	Pseudo-F	P(>F)
pH regime	1	821.5626	0.0641022	2.013119	0.1885
Compartment	1	8928.5753	0.6966495	21.878170	0.0010
Treatment x Compartment	1	617.6887	0.0481950	1.513556	0.2077
Residual	6	2448.6258	0.1910533		
Total	9	12816.4525	1.0000000		

Supplemental table 2-Concentration of elements and hydrocarbons in the two sediments (control vs polluted)

Element (ppm)	**Control**	**Polluted**
Mg	8,104 ± 336	14,660 ± 570
Ca	59,836 ± 1,000	44,442 ±1,315
Na	21,508 ± 192	21,334 ± 396
K	40,712 ± 371	45,293 ±882
Fe	20,458 ± 155	102,466 ± 2,794
P	504 ± 10	4,336 ± 68
Mn	889 ± 4	1,198 ± 19
Al	64,316 ± 271	72,633 ± 1,668
As	21.3 ± 0.6	75.2 ± 2.9
Cd	0.148 ± 0.011	0.76 ± 0.04
Cr	13.8 ± 2.4	30.9 ± 1.1
Cu	6.34 ± 0.40	157 ± 9
Hg	0.009 ± 0.001	0.220 ± 0.019
Ni	7.84 ± 1.30	13.6 ± 1.4
Pb	31 ± 1	281 ± 15
V	47 ± 1	113 ± 5
Zn	45 ± 2	703 ± 26
Polycyclic Aromatic Hydrocarbons (ppb)	**Control**	**Polluted**
Naphthalene	0.27 ± 0.06	50.3 ± 9.6
Acenaphthylene	< 0.1	59.6 ± 7.8
Acenaphthene	< 0.1	10.6 ± 1.7
Fluorene	< 0.1	17.6 ± 2.1
Pheenanthrene	2.11 ± 0.23	229.8 ± 20.7
Anthracene	0.16 ± 0.02	133.4 ± 14.7
Fluoranthene	3.35 ± 0.30	694.6 ± 48.6
Pyrene	2.8 ± 0.28	546.1 ± 43.7
Benzo[*a*]antracene	17.6 ± 1.9	383.7 ± 34.5
Crysene	2.22 ± 0.33	281.9 ± 36.7
Benzo[*b*]fluoranthene	1.98 ± 0.20	318.0 ± 25.4
Benzo[*k*]fluoranthene	1.13 ± 0.15	157.0 ± 17.3
Benzo[*a*]pyrene	2.48 ± 0.33	551.9 ± 38.6
Indeno[1,2,3-*cd*]pyrene	2.63 ± 0.26	507.9 ± 40.6
Dibenz[*a,h*]anthracene	1.14 ± 0.16	137.4 ± 16.5
Benzo[*ghi*]perylene	2.65 ± 0.29	370.5 ± 33.3
Σ PAH (EPA 16 list)	40.5	4450.4
Benzo[*j*]fluoranthene	1.00 ± 0.11	125.3 ± 11.3
Benzo[*e*]pyrene	2.75 ± 0.28	413.9 ± 33.1
C12-C40 Hydrocarbons	18.5 ± 3.7	155.5 ± 28

*Supplemental table 3- Sediment inorganic and organic nutrient concentrations, and sediment redox potential (Eh). Pair-wise tests for the term 'Sediment x Treatment' for levels pairs of the factor 'Treatment'. See the Methods section for details on the analyses. *, P(perm)<0.05; **, P(perm)<0.01; ***, P(perm)<0.001*

		NH_4^+					NOx					PO_4^{3-}					DOC					DON					Eh			
Sediment	**Community**	**T0**	**S**	**L**	**P**	**PL**	**T0**	**S**	**L**	**P**	**PL**	**T0**	**S**	**L**	**P**	**PL**	**T0**	**S**	**L**	**P**	**PL**	**T0**	**S**	**L**	**P**	**PL**	**S**	**L**	**P**	**PL**
Control	**T0**																													
	S						*					*					**					***								
	L	***					**					*					***					***					*			
	P	***										**					**					***								
	PL	**		*			**					***					***					***					*			
Polluted	**T0**																													
	S	**										*					***					*								
	L	**										*					***													
	P											***					***	*				*								
	PL	***	*		*							*					***	*				**		**			**	*		

Supplemental table 4- Inorganic and organic nutrient concentrations (µM) in the seawater of the experimental aquaria. Results are reported as averages (n = 6) ± standard deviations of T0 and Tfinal, since no significant differences were detected between time intervals.

Treatment	NH_4^+	NO_X	PO_4^{3-}	DOC	DON
Control	1.46 ± 0.31	9.43 ± 2.38	0.13 ± 0.05	203.8 ± 66.5	9.43 ± 3.83
Polluted	1.44 ± 0.19	9.74 ± 3.60	0.17 ± 0.01	238.4 ± 83.0	10.09 ± 3.31

Supplemental table 5- PERMANOVA table of results assessing the effect of "Sediment" and "Community" on photochemical variables derived from chlorophyll a fluorescence measurements.

Variable	Treatment	df	MS	Pseudo-F	P(perm)	P(MC)
Fv/Fm	Sediment	1	6.67	257.73	**0.0025**	**0.0001**
	Community	1	0.00	0.01	0.9186	0.9257
	SexCo	1	0.09	3.65	0.1033	0.0937
rel-ETR	Sediment	1	437.01	8.55	**0.0195**	**0.0175**
	Community	1	11.11	0.22	0.6785	0.6757
	SexCo	1	1.55	0.03	0.9285	0.9294
NPQ	Sediment	1	1220.30	24.11	**0.0026**	**0.0007**
	Community	1	5.17	0.10	0.8624	0.8445
	SexCo	1	4.08	0.08	0.9002	0.8719

Supplemental table 6- PERMANOVA table of results assessing the effect of "Sediment" and "Community" on morphology and growth of the plant.

Variable	Treatment	df	MS	Pseudo-F	P(perm)	P(MC)
Tot leaf surface	Sediment	1	42.88	3.09	0.1359	0.1146
	Community	1	145.26	10.47	**0.0137**	**0.0105**
	SexCo	1	0.55	0.04	0.8994	0.8825
Leaf elongation	Sediment	1	0.008	1.42	0.2837	0.2720
	Community	1	0.024	4.45	0.0737	0.0712
	SexCo	1	0.000	0.03	0.8529	0.8624
Leaf biomass	Sediment	1	0.003	2.52	0.1588	0.1548
	Community	1	0.004	4.22	0.0768	0.0702
	SexCo	1	0.000	0.11	0.7434	0.7412
N. leaves	Sediment	1	0.009	5.01	0.0566	0.0543
	Community	1	0.003	1.48	0.2729	0.2611
	SexCo	1	0.001	0.34	0.6185	0.5808
Necrotic tissue	Sediment	1	5.19	7.69	**0.0264**	**0.0231**
	Community	1	0.92	1.37	0.2811	0.2677
	SexCo	1	0.95	1.41	0.2996	0.2734
Net shoot change	Sediment	1	0.23	0.37	0.5599	0.5581
	Community	1	0.30	0.47	0.5172	0.5141
	SexCo	1	0.27	0.42	0.5401	0.5287

Supplemental table 7- PERMANOVA table of results assessing the effect of "Sediment" and "Community" on the growth of the apical portions of the plant, and on clam mortality

Variable	Treatment	df	MS	Pseudo-F	P(perm)	P(MC)
Apical Leaf growth	Sediment	1	1.0E-03	0.27	0.6068	0.6183
	Community	1	3.5E-03	0.90	0.3635	0.3659
	SexCo	1	5.5E-04	0.14	0.7199	0.7150
Apical rhizome growth	Sediment	1	2.7E-03	3.14	0.1057	0.1189
	Community	1	1.7E-03	1.94	0.2046	0.2059
	SexCo	1	1.2E-03	1.39	0.2901	0.2714
Apical root growth	Sediment	1	2.0E-02	9.02	**0.0207**	**0.0166**
	Community	1	1.8E-05	0.01	0.9336	0.9297
	SexCo	1	6.1E-04	0.28	0.5979	0.6214
Tot apical growth	Sediment	1	1.4E-02	3.01	0.1267	0.1230
	Community	1	4.2E-03	0.87	0.3643	0.3745
	SexCo	1	2.4E-04	0.05	0.8209	0.8340
Clam mortality	Sediment	1	75.00	8.33	**0.0325**	**0.0197**
	Community	1	8.33	0.93	0.3673	0.3569
	SexCo	1	8.33	0.93	0.3779	0.3610

Supplemental table 8- Pairwise permutation based analysis of variance for the interaction of Month and Treatment in light chambers

Combination	Difference	Lower value	Upper value	Adjusted p
May-Chondrilla-May-Association	-13.08	-19.64728	-6.506729	0.0000315
May-Posidonia-May-Association	9.8093142	3.239039	16.37959	0.0013761
November-Association-May-Association	-0.2630314	-6.260845	5.734782	0.9999926
November-Chondrilla-May-Association	-10.5526617	-16.550475	-4.554848	0.0001797
November-Posidonia-May-Association	1.4234572	-4.574356	7.421271	0.9756211
May-Posidonia-May-Chondrilla	22.8863185	16.316043	29.456594	0
November-Association-May-Chondrilla	12.8139729	6.816159	18.811786	0.0000107
November-Chondrilla-May-Chondrilla	2.5243427	-3.473471	8.522156	0.7814375
November-Posidonia-May-Chondrilla	14.5004615	8.502648	20.498275	0.0000014
November-Association-May-Posidonia	-10.0723456	-16.070159	-4.074532	0.0003311
November-Chondrilla-May-Posidonia	-20.3619759	-26.359789	-14.364162	0
November-Posidonia-May-Posidonia	-8.385857	-14.38367	-2.388044	0.0028286
November-Chondrilla-November-Association	-10.2896302	-15.654238	-4.925023	0.0000541
November-Posidonia-November-Association	1.6864886	-3.678119	7.051096	0.922364
November-Posidonia-November-Chondrilla	11.9761189	6.611511	17.340726	0.0000053

Supplemental table 9- Pairwise permutation based analysis of variance for the interaction of Month and Treatment in dark chambers

Combination	Difference	Lower value	Upper value	Adjusted p
May-Chondrilla-May-Association	-1.068332	-3.1103175	0.9736535	0.5887929
May-Posidonia-May-Association	2.1759836	-0.3249277	4.6768948	0.1130386
November-Association-May-Association	-0.36264	-2.2267092	1.5014292	0.989467
November-Chondrilla-May-Association	-3.0347181	-4.8987873	-1.1706489	0.0005604
November-Posidonia-May-Association	-0.9185081	-2.7825773	0.9455611	0.6466931
May-Posidonia-May-Chondrilla	3.2443155	0.7434042	5.7452268	0.0063289
November-Association-May-Chondrilla	0.7056919	-1.1583773	2.5697611	0.8417994
November-Chondrilla-May-Chondrilla	-1.9663861	-3.8304553	-0.1023169	0.0347179
November-Posidonia-May-Chondrilla	0.1498238	-1.7142454	2.013893	0.9998449
November-Association-May-Posidonia	-2.5386236	-4.8965054	-0.1807418	0.0299622
November-Chondrilla-May-Posidonia	-5.2107016	-7.5685834	-2.8528199	0.0000088
November-Posidonia-May-Posidonia	-3.0944917	-5.4523734	-0.7366099	0.0056713
November-Chondrilla-November-Association	-2.672078	-4.3393522	-1.0048039	0.0006752
November-Posidonia-November-Association	-0.5558681	-2.2231423	1.1114061	0.8996529
November-Posidonia-November-Chondrilla	2.1162099	0.4489358	3.7834841	0.0077453

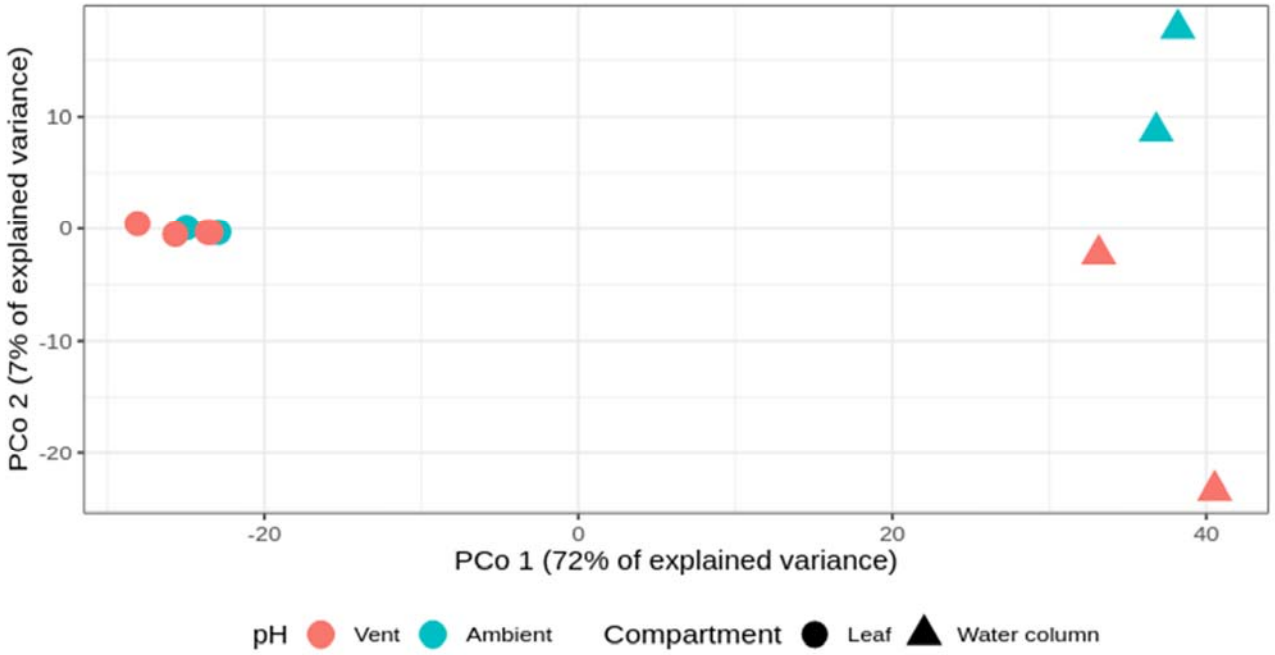
Supplemental table 10- Univariate analysis of variance for each nutrient rate in light chambers.

	NH4		PO4		NOx		SiO4		DOC		DON	
	F	p>F	F	p>F	F	p>F	F	p>F	F	p>F	F	p>F
Treatment	1.242	0.664	1.747	0.599	0.259	0.953	1.633	0.599	3.674	0.193	0.153	0.953
Month	4.633	0.124	4.79	0.124	14.751	0.005	10.243	0.015	0.851	0.381	1.35	0.381
Treatment x Month	0.455	0.72	0.703	0.72	1.574	0.71	1.232	0.72	1.313	0.72	1.279	0.72

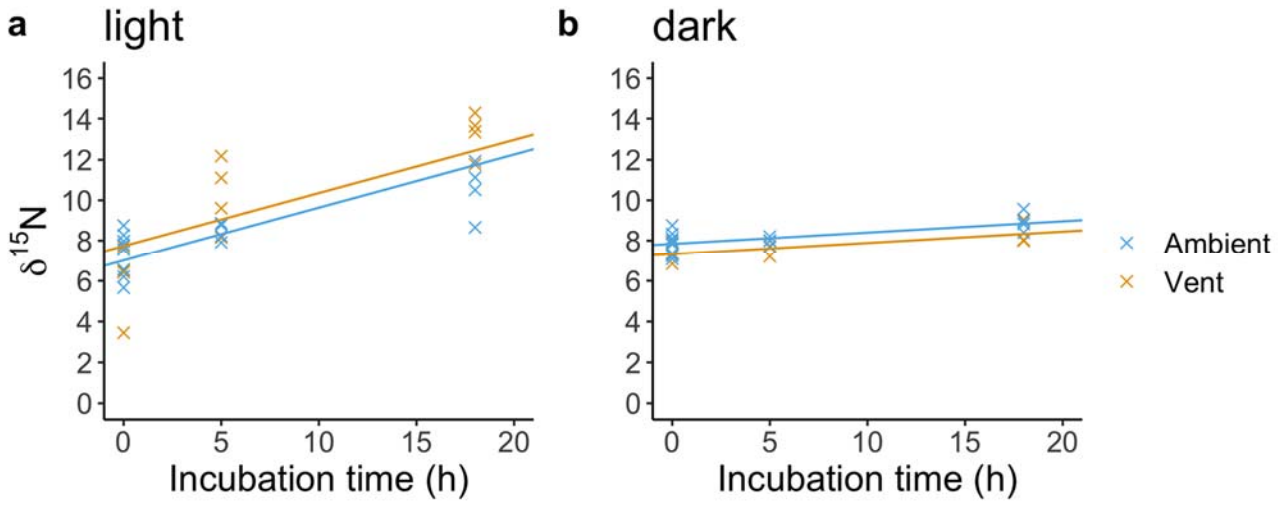
Supplemental table 11- Univariate analysis of variance for each nutrient rate in dark chambers

	NH4		PO4		NOx		SiO4		DOC		DON	
	F	p>F	F	p>F	F	p>F	F	p>F	F	p>F	F	p>F
Treatment	1.158	0.62	8.413	0.014	21.909	0.002	4.357	0.094	1.253	0.62	0.344	0.7
Month	13.78	0.009	2.601	0.367	1.349	0.531	15.709	0.006	0.896	0.531	0.051	0.822
Treatment x Month	14.224	0.003	0.91	0.85	0.399	0.898	17.496	0.002	0.367	0.898	0.674	0.85

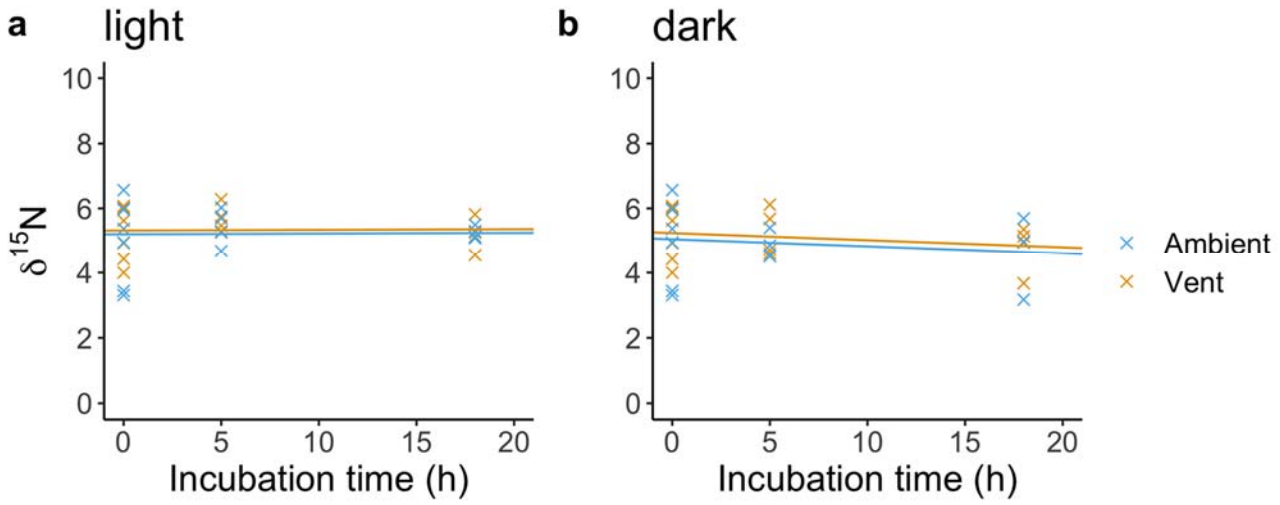
Supplemental figures



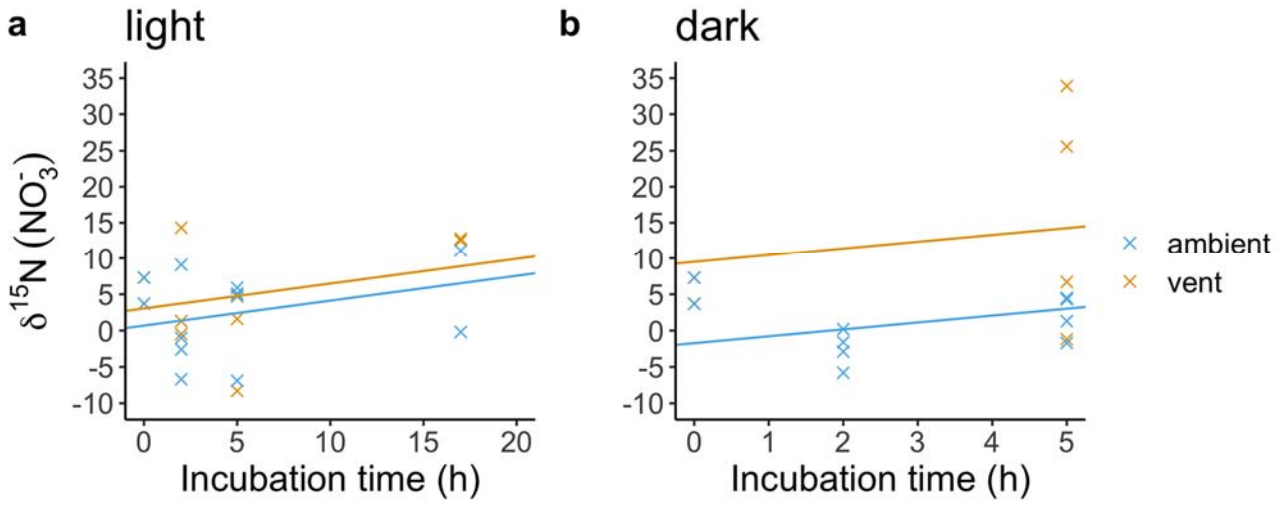
Supplemental figure 1- Principal coordinates analysis of the ASV community depending on pH regime and sample compartment.



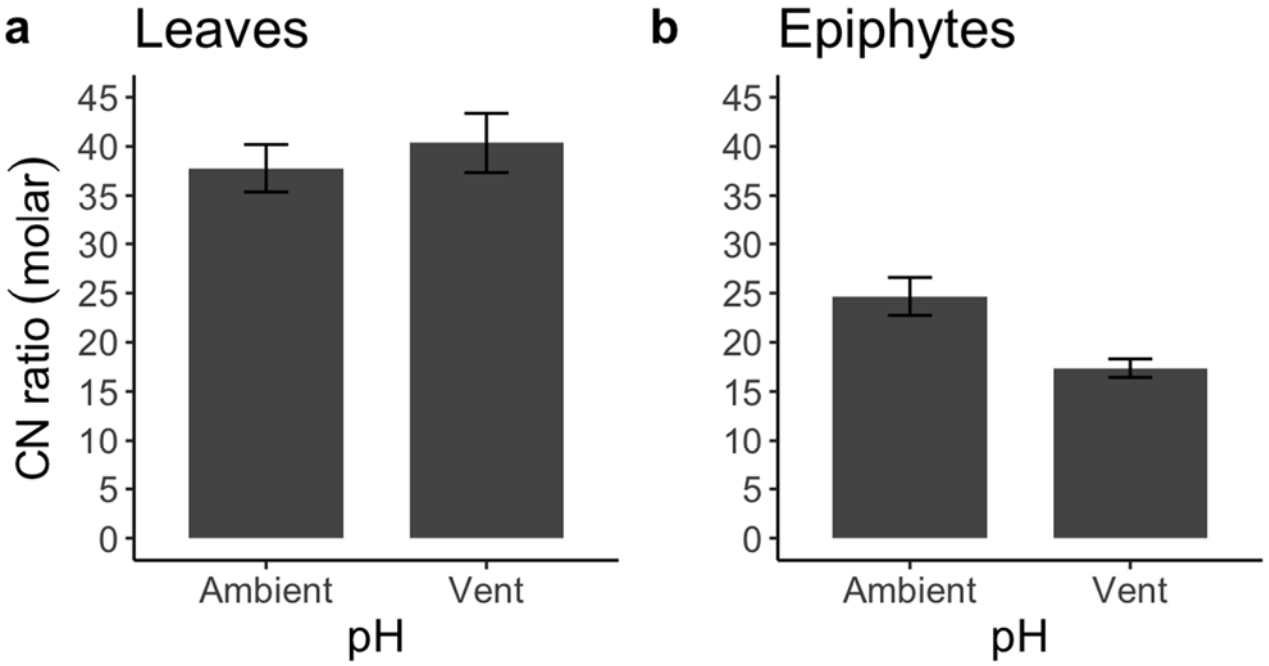
Supplemental figure 2- ^{15}N increase during light (a) and dark (b) incubations in epiphytes from the ambient and the vent site. Lines represent linear regressions.



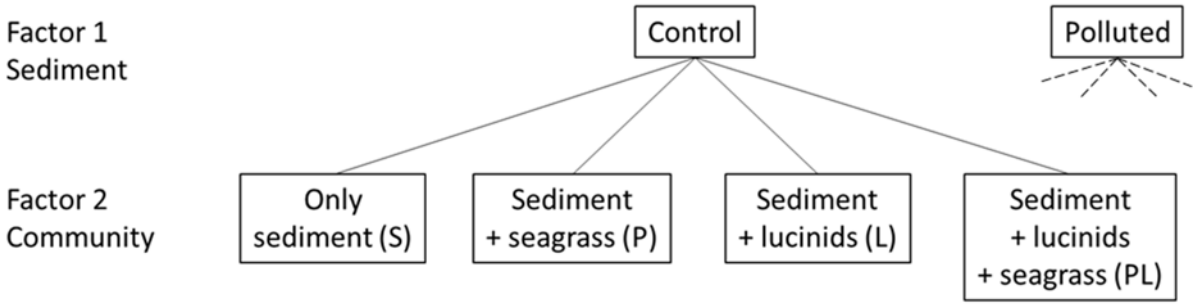
Supplemental figure 3- ^{15}N increase during light (a) and dark (b) incubations in seagrass leaf sections from the ambient and the vent site. Lines represent linear regressions.



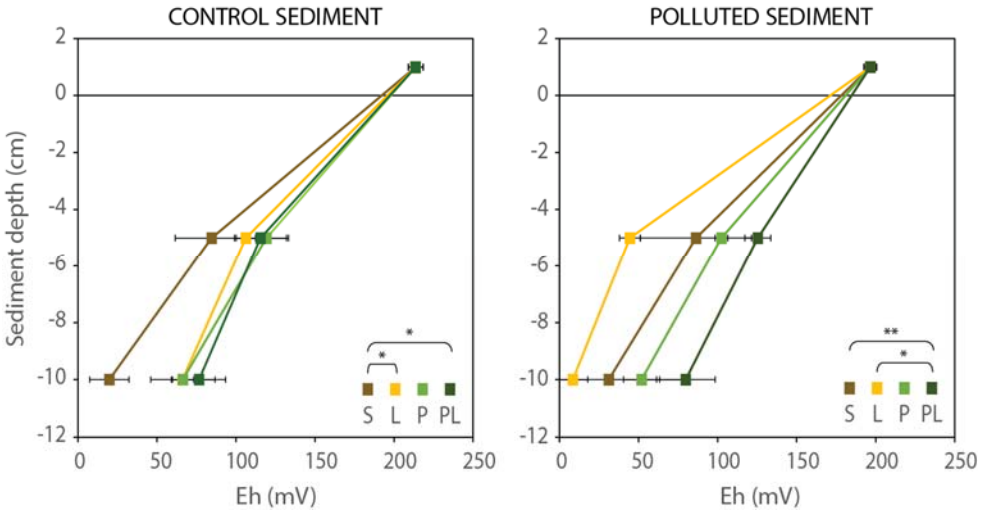
Supplemental figure 4- ^{15}N (NO_3^-) increase during light (a) and dark (b) incubations with seagrass leaf sections with epiphytes. Solid lines represent linear regressions.



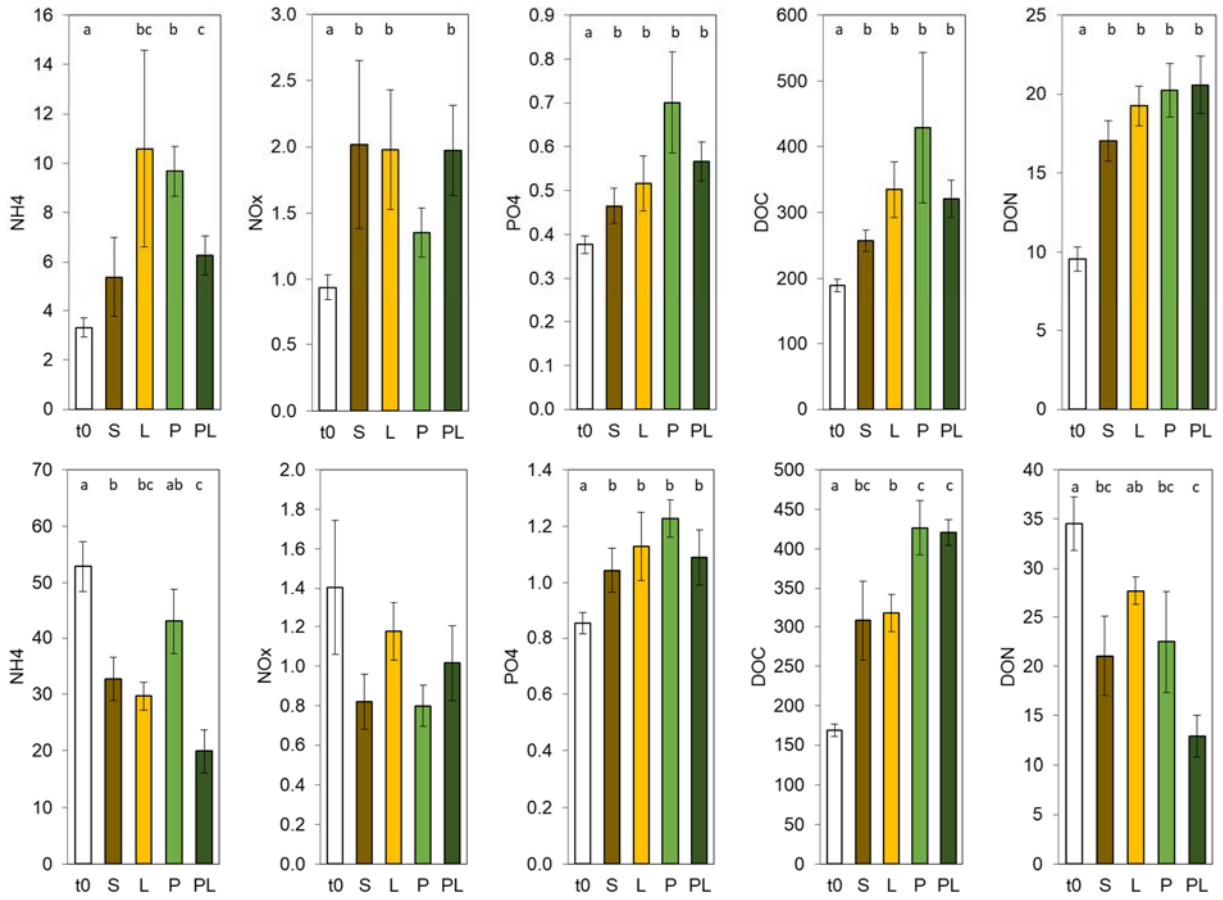
Supplemental figure 5- : C:N ratios of leaf sections (a) and epiphytes (b) from the ambient (n leaves = 14, n epiphytes =8) and vent site (n leaves = 14, n epiphytes =7). Since there were no differences between light and dark incubations, the samples were combined and treated as replicates. Error bars indicate mean ± SE.



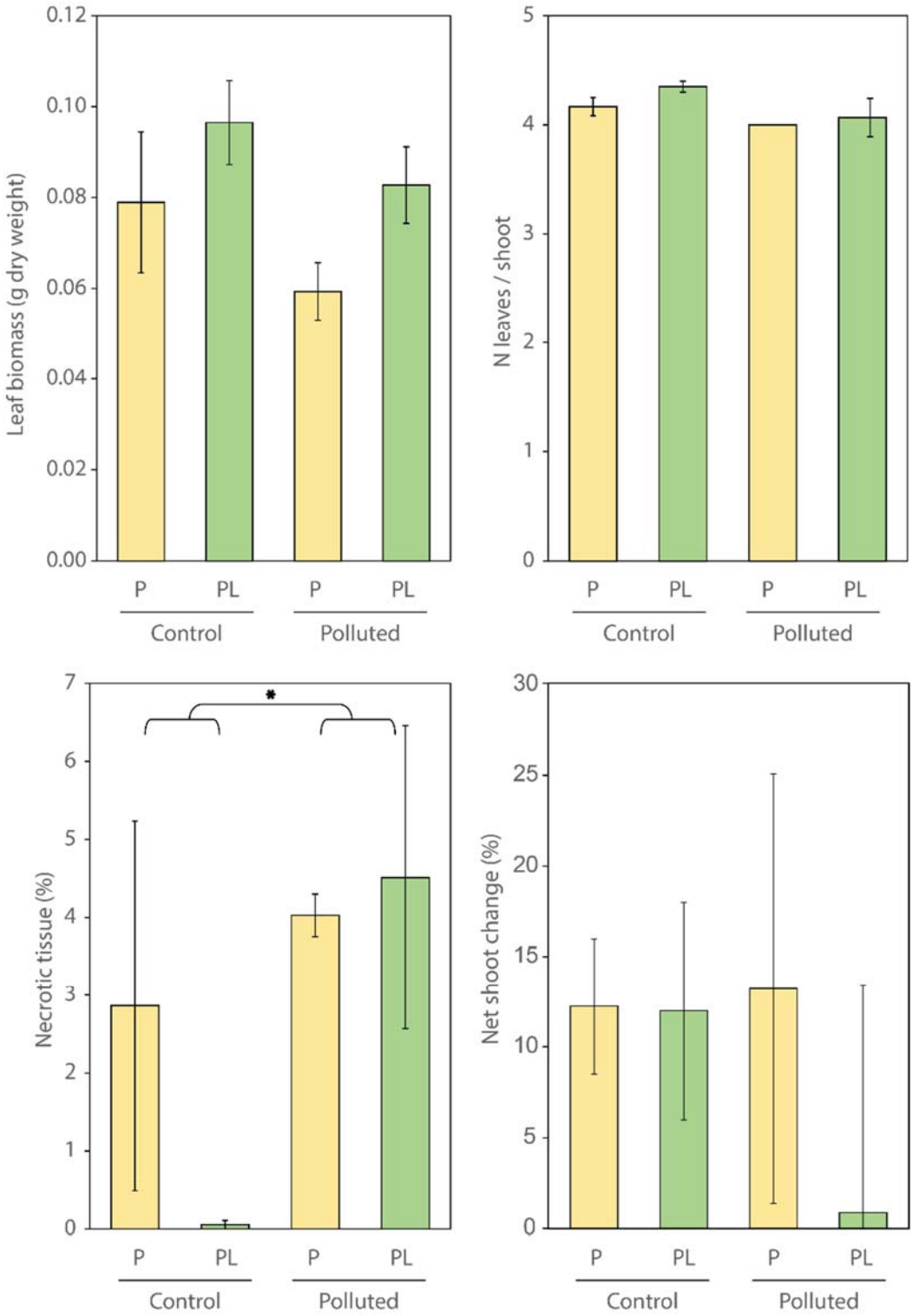
Supplemental figure 6- Orthogonal experimental design used in the mesocosm experiment with levels of the factors Sediment (Control and Polluted) and Community (S, P, L, PL).



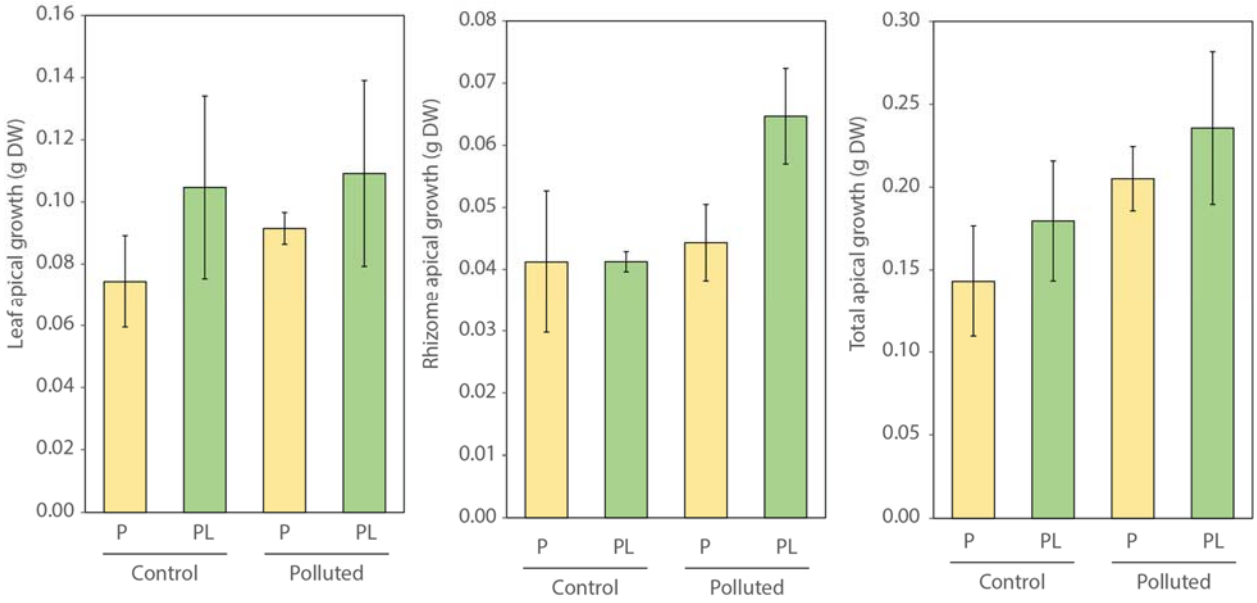
Supplemental figure 7- Supplemental.figure 7: Vertical profiles of sediment redox potential (Eh) in sediment porewater at the end of the experiment in control vs polluted sediments. Letters and colours indicate different "Community" levels as reported in the figure legend. Asterisks (, p<0.05; **, p<0.01) indicate significant differences; see Table S3 for the results of the PERMANOVA pair-wise test.*



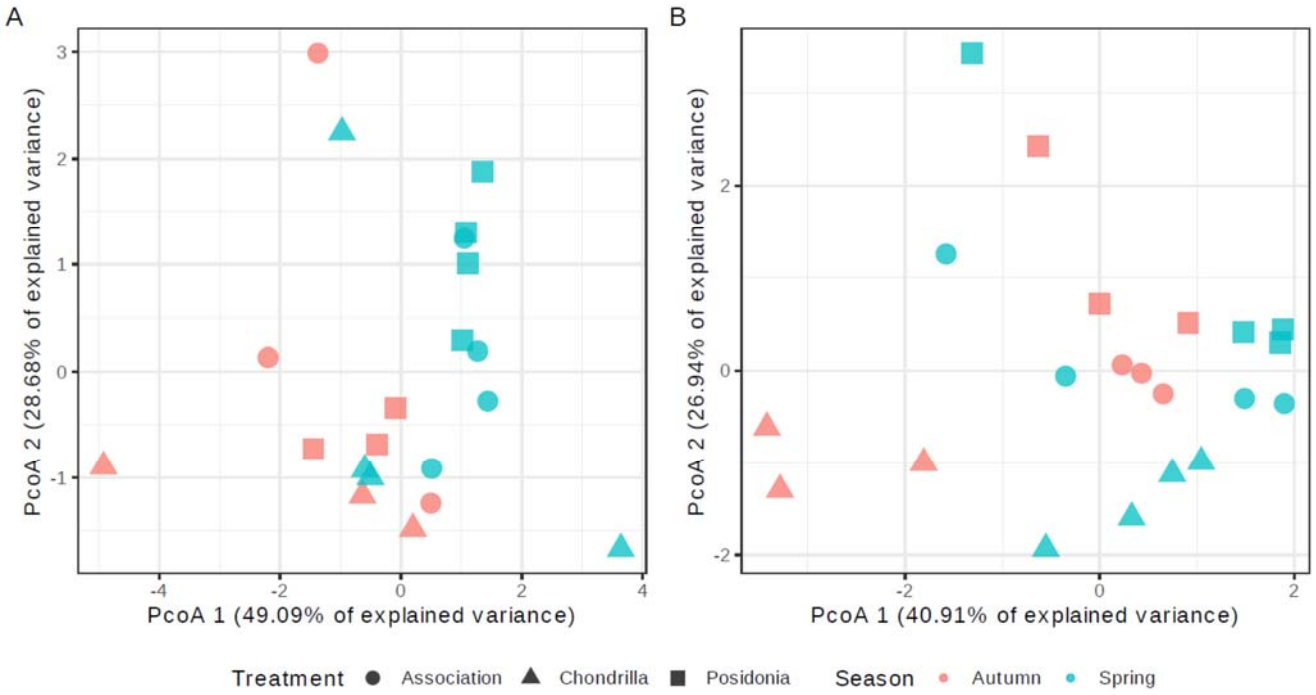
Supplemental figure 8- Sediment inorganic and organic nutrient concentrations (µM) according to the different treatments. Upper and lower panels are for the control and polluted sediments, respectively. Different letters above the bars represent significant differences as indicated in Table S3; same letters indicate no difference between specific treatments; absence of letters indicates that the specific treatment was no different from all the others.



Supplemental figure 9- Plant growth and morphology. A) Leaves biomass, B) Number of leaves per shoot, C) Percentage of necrotic tissue, and D) Net shoot change of C. nodosa at the end of the experiment. Levels of the factor "Community" are identified with letters as indicated in the methods. Colours indicate absence (yellow) or presence (green) of the interaction with lucinid clams. Asterisks (, $p<0.05$) indicate significant differences; see Table S5 for the statistics*



Supplemental figure 10- Newly produced plant biomass. A) Leaf apical growth, B) Rhizome apical growth, C) Total apical growth of C. nodosa at the end of the experiment. Levels of the factor "Community" are identified with letters as indicated in the methods. Colours indicate absence (yellow) or presence (green) of the interaction with lucinid clams. See Table S6 for the statistics.



Supplemental figure 11- Principal coordinate analysis of the nutrient rates in the light (A) and dark (B) chambers.