UNIVERSITAT DE BARCELONA

Biological interactions and resilience of seagrass ecosystems

Neus Sanmartí Boixeda

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Interaccions biològiques i resiliència dels ecosistemes d'angiospermes marines

Neus Sanmartí Boixeda

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TESI DOCTORAL

Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals
Doctorat en Ecologia, Ciències Ambientals i Fisiologia Vegetal

Biological interactions and resilience of seagrass ecosystems

Interaccions biològiques i resiliència de les angiospermes marines

Memòria presentada per **Neus Sanmartí Boixeda** per optar al Grau de Doctora
per la Universitat de Barcelona

Neus Sanmartí Boixeda
Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals,
Universitat de Barcelona, abril 2020

El director i tutor de la tesi
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Dra. Marta Pérez Vallmitjana
Professora titular
Universitat de Barcelona

A la Bibita

Agraïments

Hola Bibita,

T'escric aquesta carta per dir-te que ja he acabat la tesi. Avui és un dissabte tranquil, el sol encara no ha sortit i estic escoltant una de les teves obres preferides, el concert per a piano i orquestra nº 5 de Beethoven, una connexió directa amb l'emoció. Des d'aquí, et puc assegurar que aquest ha estat un viatge força tranquil, que no vol dir fàcil. Un viatge on l'esforç, la perseverança i la il·lusió han estat la combinació perfecte. Un viatge que només pot acabar en la gratitud. Tu sempre deies que la gratitud és una de les millors virtuts que existeixen, que et fa créixer i expandir-te. Diuen que la gratitud és la memòria del cor, doncs serà que sí, i jo em sento afortunada de tenir tantes raons per estar agraïda. Agraïda a totes aquelles persones que m'han acompanyat fins aquí i que totes, d'una manera o una altra, han contribuït a augmentar la meva resiliència durant el transcurs d'aquesta tesi, i en la majoria de casos, més enllà.

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Advisors' report

Dr. Javier Romero Martinengo and Dra. Marta Pérez Vallmitjana professors at the Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals (Universitat de Barcelona), advisors of the PhD thesis entitled "**Biological interactions and resilience of seagrass ecosystems**",

INFORM, that the research studies developed by Neus Sanmartí Boixeda for her Doctoral Thesis have been organized in four chapters, which correspond to four scientific papers listed below (two published, two intended to be submitted in the next month), plus a general introduction and a general discussion.

and CERTIFY, that the work has been carried out by Neus Sanmartí Boixeda, participating actively in all the tasks: conceiving and setting the objectives, conceiving and performing the analyses, participating actively in the field work, executing the experiments and writing the manuscripts.

Finally, we certify that the co-authors of the publications listed below and that conform this doctoral thesis, will not use these manuscripts in another PhD thesis.

Barcelona, 15th July 2020

Advisor: **Dr. Javier Romero Martinengo** Universitat de Barcelona	Advisor: **Dra. Marta Pérez Vallmitjana** Universitat de Barcelona

List and publication status of the chapters of this thesis

Part of the results of this PhD thesis have been published in indexed international journals:

Chapter 1

Sanmartí, N., Solé, L., Romero, J., Pérez M. (2017). Seagrass-bivalve facilitative interactions: Trait-mediated effects along an environmental gradient. Mar. Env. Res. 133, 99-104. https://doi.org/10.1016/j.marenvres.2017.12.002. Impact factor: 3.10

Chapter 2

Sanmartí, N., Ontoria, Y., M. Ricart, A., Alcoverro, T., Arthur, Pérez M., Romero, J. Habitat facilitation and herbivore behaviour determine the coexistence of a three-species assemblage (*in prep.*)

Chapter 3

Sanmartí, N., Saiz, L., Llagostera, I., Pérez, M., Romero, J. (2014). Tolerance responses to simulated herbivory in the seagrass *Cymodocea nodosa*. Mar. Ecol. Prog. Ser. 517, 159-169. https://doi.org/10.3354/meps11084. Impact factor: 2.619

Chapter 4: Sanmartí, N., Ontoria, Y., M. Ricart, A., Pérez, M., Romero. Recovery of a fast-growing seagrass to small-scale mechanical disturbances: effects of intensity, size and seasonal timing (*in prep.*)

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In addition, the results of this thesis have been presented at international and national conferences (Seagrasses in Europe: Threats, Responses and Management (COST), Portugal, 2014; 11th International Seagrass Biology Workshop, China; 2014; 12th International Seagrass Biology Workshop, Gwynedd, Wales, UK, 2016).

Contents

ABSTRACT

Our world is subjected to a panoply of drivers of change. In this context, the understanding on how our biosphere resists, absorbs or is altered by the changes, appears as a hot question in ecology. In this respect, two ecological concepts appear as essential, resilience and biotic interactions. Resilience is related to how ecosystems persist under stress or suffering disturbances. Interactions among species are to a large part responsible for the delivery of ecosystem functions, and form the architecture of biodiversity. Moreover, a substantial part of ecosystem resilience is founded on species interactions. This thesis is an attempt to shed some light on these issues through the deep exploration of specific case studies in seagrass ecosystems, in particular how seagrasses respond to external drivers (or how resilient they are), how these responses affect species interactions and which mechanisms allow coexistence of species linked by positive and negative interactions. Our approach is based upon field observations and field manipulative experiments.

Chapter 1 shows how an increase of organic matter in sediment weakens the mutualism between the bivalve *Loripes lucinalis* and the seagrass *Cymodocea nodosa*. The mechanism implied is the effect of this increase (and, probably, the resulting anoxia) on seagrass root morphology (plant trait), which results in a lower provision of habitat for the bivalves, whose abundance decreases. The weakening of the mutualism can potentially decrease the resilience of these ecosystems to eutrophication and, therefore, compromise their persistence.

Chapter 2 describes a facilitative cascade in which the seagrass *C. nodosa* favors the abundance of the pen shell *Pinna nobilis,* which positively affects the sea urchin *Paracentrotus lividus*, which in turn consumes the seagrass. We suggest that the persistence of this three-species assemblage rests on the very local impact of sea urchins on the seagrass, likely driven by behavioural and denso-dependent processes.

Chapter 3 and 4 show that fast-growing species such as *C. nodosa* are highly resilient to stress or disturbances when affecting only the aboveground parts, recovering fast (within two weeks) from a single event of disturbance. *C. nodosa* shows several mechanisms of tolerance, such as compensatory growth, reallocation of internal resources and enhancement of the formation of new modules, when coping to repeated defoliation simulating herbivory. However, when the

belowground parts are lost by disturbances, recovery is highly delayed up to two years and is dependent on the characteristics of the disturbance such as size and timing.

Overall, this research has contributed to increase our understanding on how ecosystems respond to changes and how species interactions are maintained and disrupted. We have shown that environmental changes can alter the functioning of seagrass ecosystems at least in two directions. Firstly, by altering fundamental biological interactions, such as the seagrass-lucinid mutualism and, secondly, by affecting the resilience of ecosystems dominated by a foundation species, which promote species coexistence. Advances in the two complementary and interlinked directions will be crucial to better manage and preserve ecosystems and prevent their potential collapse under the increasing human-induced change the world is submitted to.

RESUM

El nostre món està sotmès a un ampli ventall de forces que tendeixen a provocar canvis. En aquest context, entendre com la biosfera resisteix, absorbeix o és alterada per aquestes forces resulta una qüestió candent, especialment per l'ecologia. Al respecte, dos conceptes ecològics esdevenen essencials: la resiliència i les interaccions biològiques. La resiliència és la capacitat de persistència o recuperació que tenen els ecosistemes sotmesos a estrès o pertorbacions. Les interaccions entre espècies (efectes de l'existència d'una espècie sobre la *fitness* d'una altra) contribueixen al manteniment de les funcions ecosistèmiques i, en un cert sentit, constitueixen l'arquitectura de la biodiversitat. A més, la resiliència dels ecosistemes depèn , en gran part, d'aquestes interaccions. Aquesta tesi és un intent d'aprofundir en els aspectes esmentats a través d'una sèrie de casos d'estudi en ecosistemes d'angiospermes marines. Concretament, el que fem és estudiar com els ecosistemes d'angiospermes marines responen a les forces causants de canvis, com aquestes respostes vénen mitjançades per canvis en la interacció entre espècies, i provar d'esbrinar els mecanismes que permeten la coexistència d'espècies que es troben vinculades per interaccions positives i negatives. La nostra aproximació es basa tant en observacions com en experiments en el camp.

El Capítol 1 mostra com un increment de matèria orgànica en el sediment debilita el mutualisme entre el bivalve *Loripes lucinalis* i l'angiosperma marina *Cymodocea nodosa*. El mecanisme implicat que es proposa per explicar-ho està relacionat amb la plasticitat morfològica de la planta. Així, un increment en la matèria orgànica del sediment (i, probablement, l'anòxia que se'n segueix), fa que la planta modifiqui la morfologia de les seves arrels, que esdevenen molt menys ramificades i fan disminuir per tant la disponibilitat d'hàbitat per als bivalves. Una debilitació del mutualisme pot, potencialment, disminuir la resiliència d'aquests ecosistemes a l'eutrofització i, per tant, comprometre la seva persistència.

El Capítol 2 descriu una cascada de facilitació en la qual l'angiosperma marina *C. nodosa* afavoreix l'abundància del gran bivalve *Pinna nobilis*, que ajuda a incrementar l'abundància de la garota *Paracentrotus lividus*, que al seu torn consumeix l'angiosperma. Suggerim que la persistència d'aquest sistema de tres espècies, aparentment inestable (tres interaccions concatenades circularment, dues de

positives i una de negativa) es basa en què la interacció negativa (l'efecte de les garotes sobre l'angiosperma) té un abast molt limitat, probablement degut tant al seu comportament alimentari com a les defenses de la planta enfront de l'herbivorisme.

Els Capítols 3 i 4 mostren que les espècies de creixement ràpid, com ara *C. nodosa*, són altament resilients a l'estrès o a les pertorbacions quan aquestes afecten només les parts aèries de les plantes (defoliació parcial o total), recuperant-se ràpidament (dues setmanes) després d'una pertorbació puntual en el temps. *C. nodosa* mostra diversos mecanismes de tolerància a la defoliació, com ara el creixement compensatori, la reassignació de recursos interns i l'increment en la taxa de formació de nous mòduls. Tanmateix, quan les pertorbacions provoquen la pèrdua de les parts subterrànies (rizomes i arrels), la recuperació és molt més lenta, i triga fins a dos anys. A més, aquesta recuperació depèn de les característiques de la pertorbació com ara la mida de l'àrea afectada i l'època de l'any en què es produeix.

En general, aquesta tesi ha contribuït a comprendre millor les respostes dels ecosistemes als canvis. Hem pogut documentar alguns processos que permeten la coexistència entre espècies, així com mecanismes de resiliència específics que esdevenen ecosistèmics quan es manifesten en espècies fundadores d'hàbitat. També hem demostrat com els canvis, més enllà d'afectar espècies individuals més o menys emblemàtiques, poden provocar alteracions de formes més subtils, com ara erosionant la seva resiliència mitjançant la modificació d'interaccions biològiques. Els avenços en totes aquestes direccions complementàries i interrelacionades són crucials per a gestionar i preservar els ecosistemes i evitar el seu possible col·lapse.

GENERAL INTRODUCTION AND OBJECTIVES

Biological interactions

No man is an island
Devotions upon Emergent Occasions
John Donne (1624)

We could rephrase this first verse of Donne's poetry by saying that no organism is an island. In effect, organisms in nature do not exist in isolation, but participating in complex networks of biotic interactions (communities), interacting in turn with the abiotic scenario (ecosystems). This simple fact is the basis of the science of Ecology, for which the concept of interaction is probably the main pillar upon which ecological knowledge is built. Interactions of single organisms or species with their abiotic (or biotic) environment have been studied by ecologists for a long time (autecology). However, this view lies in the periphery of Ecology, while the study of multispecific ensembles is closer to its core (Margalef, 1974). The essentials of such ensembles are probably the interactions among organisms, which govern the assemblage of species within the ecosystem, playing a key role in the maintenance of biodiversity (Bascompte et al., 2006), constituting its *architecture* (Bascompte and Jordano, 2007) and determining the resilience of the whole (Ives & Carpenter 2007). Their importance was acknowledged from the very beginning of Ecology, in fact from the coining of its name by Haeckel in 1866: "*By ecology we mean the body of knowledge concerning the economy of nature—the investigation of the total relations of the animal both to its inorganic and to its organic environment; including above all, its friendly and inimical relations with those animals and plants with which it comes directly or indirectly into contact—in a word ecology is the study of all those complex interrelations referred to by Darwin as the conditions of the struggle for existence*". Even in this early definition an explicit mention of two kinds of interaction between species, *friendly* and *inimical*, was underlined. Such distinction between what we now call negative (harmful for at least one of the participants) and positive (beneficial for at least one of the participants, neither being harmed) interactions has profound consequences for the ecological theatre and the evolutionary play (in words of Hutchinson, 1965).

Negative interactions

Probably under the influence of Darwin's legacy, who privileged competition and predation as the two main interactions shaping evolution of species, ecologists have traditionally focused on negative interactions between two competing or trophically linked species (Paine, 1966; Chesson, 2000; Silvertown, 2004). On the one hand, competition occurs among organisms of the same or different species for a common limiting resource (e.g. light, food, space). Competition can determine population structure and distribution through intra-specific competition (Connell et al., 1961), lead to evolutionary changes by promoting speciation (Brännström et al., 2012) or regulating local species diversity through competitive exclusion (Harding, 1960). While the competitive exclusion principle has been a central tenet in ecology, the real world is complex and rarely attaches to simple principles, and competing species can also coexist in nature through several mechanisms such as incomplete competition, environmental disturbance, predation or by intransitive competition (Gallien et al., 2017) among others (Chesson, 2000; HilleRisLambers et al., 2012). On the other hand, trophic interactions (predation) are not only crucial for the energy flow and nutrient cycling through ecosystems, but also for shaping the distribution and abundance of organisms in fundamental ways, being, in addition, major evolutionary drivers. For predator-prey interactions, the coexistence of both intervening species relies on the strength of the density-dependent relationships between the consumer and the resource (Kawatsu and Kondoh, 2018), as well as on other factors such as spatial heterogeneity and prey defense mechanisms. Specifically, a crucial negative interaction is herbivory, because it strongly influences primary producers (Cyr and Pace, 1993; Callaway et al., 2003; Wood et al., 2016), and its effects can therefore be pervasive, modifying communities by suppressing plant biomass, affecting plant performance, mediating competitive processes between plants or increasing biodiversity, among others. Given the structural role of many primary producers, all this can have cascading effects to the whole community.

Positive interactions

However, the ecological theatre is not only a place dominated by hunger and fear (Bruno et al., 2003). The publication in 1916 of a pioneer paper on the role of positive interactions (or facilitation, see Box 1 for definition) as the main biotic mechanism of plant community succession in stressful environments (Clements, 1916), was the first attempt to incorporate facilitation in community ecology. However, it was not until the late-nineties when arose a growing recognition of the fact that facilitation is at least an equally important driver as predation or competition of the abundance of species and the structure of ecosystems (Bruno et al., 2003, Callaway, 2007, Altieri et al., 2007; Bulleri et al., 2016). The evidence of facilitation in experiments conducted in severe conditions from marine intertidal and terrestrial environments (Bertness and Callaway, 1994, Bruno and Bertness, 2000) resulted in its incorporation into ecological theory, challenging some ecological paradigms and predictions based solely on negative interactions (Mulder et al., 2001; Bruno et al., 2003). Since then, the interest in facilitation has never ceased to grow (Michalet and Pugnaire, 2016). By increasing access to resources, providing protection from stress, predation and competition and improving dispersal, facilitation can enhance biodiversity and resilience of ecosystems (Michalet et al., 2006; Angelini et al., 2016).

A particular case of facilitation is mutualism, in which both species derive benefit from the interaction. Probably its soundest example is the mutualism between pollinators and plants, wherein pollinators increase the reproductive success of plants by selectively transporting the pollen and obtaining in turn high-quality food, for example in the form of nectar (Bascompte and Jordano, 2007). Other well-known mutualisms are those developed between mycorrhizal fungi and plants, wherein fungi facilitate nutrient uptake by the plant in exchange of carbon in the form of sugars (Smith and Read, 2008), or dinoflagellates and reef-building corals wherein the zooxanthellae provide nutrition to the corals in exchange for inorganic resources (nutrients and CO_2) from coral metabolism (Muscatine and Porter, 1977).

Indirect interactions

Indirect interactions, either negative or positive, appear when the effects of a given species on another propagate to a third one or further, or, following Wootton (1994), when the effect of one species on another is mediated by the presence of a third. The importance of indirect interactions has been confirmed in multiple studies embracing terrestrial and aquatic habitats, from the seminal studies on keystone predation by Paine (1966) to more recent works on cordgrass facilitation by Altieri et al. (2007, Fig. 1) or on bivalve facilitation by Thomsen et al. (2016). Overall, the complexity and diversity of indirect interactions is overwhelming. Maybe the most basic ones are those called "cascades", implying the concatenation of a set of interactions of the same nature. As for instance, consumptive effects of predators on prey populations in top-down trophic cascades have an emergent positive indirect effect on basal resources (Carpenter et al., 1985; Menge, 1995; Pace et al., 1999). Likewise, habitat facilitation of a basal species on a secondary habitat-forming one has positive indirect effects on a third (focal) species. In both cases, these cascades of interactions may typically depend on the density (density-mediated indirect interactions – DMIIs; Abrams et al. 1996) of the species involved and not simply on its presence or absence (Irving and Bertness, 2009, Bishop et al., 2012, 2013). Additionally, other indirect, non-cascading interactions in which different kinds of interactions co-occur, may be equally important in their ecosystem-level consequences. For instance, species can change the environmental context in which two other species interact (environment-mediated interaction modifications; Wootton, 1993) (e.g. macrophytes reducing predation pressure on prey by providing hiding places; Crowder and Cooper, 1982). Analogously, some species can modify the traits (morphological, physiological, behavioural…) of others (trait-mediated indirect interactions - TMIIs; Abrams et al., 1996) (e.g. decreasing of grazing pressure on macrophytes in response to a predatory threat; Pessarrodona et al., 2019).

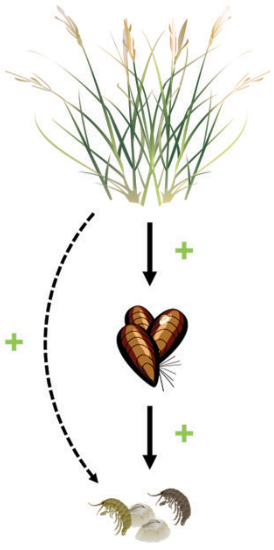

Fig. 1. Example of habitat cascade in intertidal cordgrass bed communities from Altieri et al., 2007. Cordgrass, which can establish and persist without the aid of other foundation species, facilitates a dense assemblage of inhabitants (e.g. mussels, snails, seaweeds) through their roots and rhizomes that stabilize substrate and a dense canopy that baffles waves and provides shade. Within the cordgrass bed community, ribbed mussels provide a hard substrate and crevice space for other species (e.g. amphipods, barnacles) by providing crevice space and hard substrate. Symbols courtesy of the Integration and Application Network (ian.umces.edu/symbols/), University of Maryland Center for Environmental Studies.

Foundation species

The case of foundation species (i.e. those creating habitats, determining environmental conditions that support many associated species and modulating local-scale ecosystem processes, Jones et al., 1994) is of specific interest, as is clearly a positive interaction but also triggers indirect interactions with many more species than any other. They have strong effects on community structure and provide multiple ecosystem functions and services (Thomsen et al. 2010; Ramus et al., 2017; Ellison et al., 2019). Trees, kelps, corals and seagrasses, for instance, are spatially dominant organisms whose biogenic structure promotes species co-existence through the protection against physical stress, the alteration of resources availability, and the creation of fine-scale, complex matrices in which smaller organisms find refuge from predators and competitors (Bulleri, 2009; Angelini et al., 2011). Foundation species are important links in trophic and non-trophic interactions networks, whose outcome can be really complex since it depends not only on the features of each binary (direct) interaction, but also on how they work together, potentially generating stabilizing or destabilizing feedbacks. Indeed, the sign and strength of these feedbacks may determine the system behaviour and, importantly, whether it is persistent or not. For instance, in marine ecosystems, seagrasses provide shelter for mesograzers, which can mitigate the negative impacts of relatively high nutrient loads by consuming macroalgae and/or epiphytes overgrowing seagrass leaves (Maxwell et al., 2016).

While the importance of interactions in the functioning of ecosystems is undisputable (and indeed undisputed), the complexity of the field is huge, and the research advances in this area, often going the experimental approach well beyond than the theoretical one, are currently limited by the lack of knowledge on several aspects related to: (i) the effect of biotic and abiotic drivers of change on the strength of species interactions; (ii) the mechanisms allowing the persistence of species assemblages, particularly when facilitation and trophic interactions are involved and, (iii) the empirical research based on observation and experimentation to support the rapid theoretical advances. Some of these gaps will be addressed in the present thesis.

Resilience of ecosystems

Gaining knowledge on how ecosystems respond to environmental changes (understood here as modifications in the conditions external to the ecosystem itself) has become nowadays one of the biggest challenges in ecology at the light of the accelerated changes our world is submitted to. Such response encompasses from very slight to deep alterations in ecosystem properties and processes, including the shift to an alternative state (Scheffer et al., 2001), often called degraded for being usually poorer in terms of biodiversity and ecosystem services and functions.

The response of ecosystems to environmental changes is complex, and usually involves both a certain capacity to absorb them without great alteration in their functions and structure, and a capacity to recover from a modified state when the action of external drivers ceases. Both aspects are embraced by the term "resilience", which has recently become a sort of hot-term* in the ecological literature, although it was introduced in the earlies 70 by Holling (1973). Yet resilience has been redefined and interpreted many times since then (e.g. see Gibbs, 2009). The concept of resilience can be broadly splitted into two meanings, more or less corresponding to the two capacities outlined above, ecological resilience and engineering resilience (Fig. 2) (Angeler et al., 2018). Thus, ecological resilience is commonly defined as the amount of stress or disturbance that a system can absorb before changing to another state. This include the ability of a system to maintain its functioning, structure, and identity through self-regulating feedbacks often driven by interactions between organisms or between organisms and the abiotic environment (see the seagrasses-mesograzers example mentioned in the previous section) (Scheffer et al., 2001; Scheffer and Carpenter, 2003). Sometimes ecological resilience is also termed "resistance" (Waider and Willig, 2012).

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[*] The term resilience has been used in the title of articles from the field experimental sciences 17430 times in 2017-2019, 9464 times in 2014-2016, 4381 in 2011-2013 and 2025 in 2008-2010. As a reference, the term was used only 14 times in the title of articles published between 1982 and 1984.

In turn, engineering resilience is the time required for a system to return to the original state (or dynamics) following stress or disturbance cessation (e.g. Pimm, 1984; Holling, 1996); sometimes this is also termed "recovery" (Holling, 1996). Overall, both concepts (ecological and engineering resilience) can be included in the general notion of the persistence of natural ecosystems in the face of environmental changes, either due to natural or anthropogenic causes.

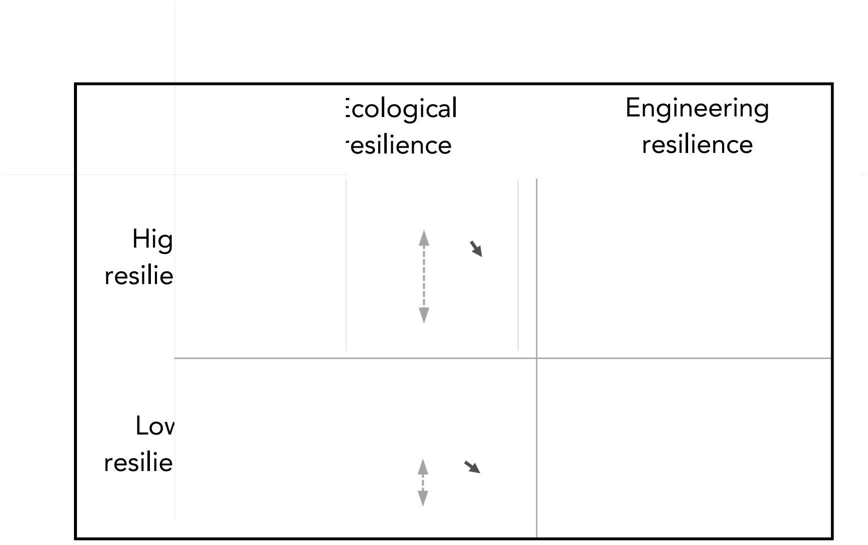


Fig. 2. Conceptual representation of resilience with the ball-in-cup analogy commonly used in ecology. Potential states of ecosystems (e.g. diversity, biomass, etc.) are represented by the blue line, with the position of the ball on this line representing the state of the system at any given time (state A, normal; state B, altered). In the left panel, ecological resilience is represented by the height of the hill: the higher the hill, the higher the push on the ball is needed to leave state A, cross the top and reach the other valley (state B). In the right panel, the engineering resilience is represented by the slope of the valley side, the steeper the slope, the quicker the system will recover from a disturbance returning to state A from state B. Adapted by van Nes and Scheffer (2007).

The external influences an ecosystem can be submitted to are very diverse, depending on their origin (physical, chemical or biological), their intensity or their frequency, among others, being the response of the ecosystems equally diverse. Without going into the detail, for the purposes of this thesis it is relevant to distinguish between agents that influence ecosystems in a more or less continuous way (stress, as for example chronic pollution, warming or most cases of herbivory) and those that are discrete or pulsed disturbances, such as storms, flows, oil accidents, catastrophic herbivory or heat waves (see Box1 for definitions).

Despite important advances made in the understanding on how ecosystems respond to environmental change, there is still major uncertainty in key issues, specially about the mechanisms that provide insurance against changes. Thus, there is still much to explore, particularly in areas related to: (i) the mechanisms that regulates the maintenance of the desired state of ecosystems; (ii) the effects of different disturbances on recovery rates and on recovery pathways and, (iii) how to increase the resilience of ecosystems. This thesis aims to contribute to shed light on some of these aspects.

Biological interactions, resilience and changing world

In the first two sections of this introduction we have outlined the importance of two essential ecological concepts: species interactions and resilience. Both concepts are closely related, as species interactions are at least as important as individual species traits in providing resilience. Or, said in other works, ecosystem deterioration, often viewed as a direct consequence of stressors or disturbances on the performances or survival of individual species, probably also relies on their consequences on species interactions. Stressors and disturbances can alter the interaction strength of coexisting species (as shown in the case of competition or predator–prey interactions, Rall et al., 2010), shift the types of interactions (for example along a stress gradient, from competition to facilitation, He et al., 2013), and even breakdown species interactions (e.g. mutualism, Memmot et al., 2007). Besides, in a recent work, Valiente-Banuet et al. (2015) have shown that along a gradient of environmental deterioration, the loss of ecological interactions precedes the loss of species; or, in other words, interactions breakdown affects ecosystem function, including resilience, at a faster rate than species extinctions.

Despite there is a growing concern that losses in species interactions may lower ecological resilience to and/or recovery from disturbances (Dell et al., 2019), robust experimental support is scarce, albeit needed to better understand the processes and mechanisms of resilience of ecosystems and ultimately to make better management decisions. This is especially important for those ecosystems highly vulnerable and threatened such as coastal ecosystems dominated by foundation species, such as salt-marshes, mangroves, coral reefs and seagrasses.

Seagrass ecosystems

Seagrass meadows are among the most productive and at the same time the most heavily impacted costal ecosystems (Orth et al., 2006; Waycott et al., 2009). Seagrasses, as foundation species, promote biotic interactions both trophic and non-trophic, which makes those meadows excellent models to test some community ecology sound questions on biological interactions and resilience to environmental change.

One of the main focus of the research on biological interactions in seagrass meadows has been herbivory, especially since the role of herbivores on the control of seagrass abundance and productivity was recognized to be greater than previously thought (Valentine and Heck, 1999). The study of seagrass-herbivore interactions has been conducted through two main approaches. The first is oriented to elucidate the effects of grazing on seagrass community, which primarily depend on: (i) the herbivore size, which encompasses several orders of magnitudes from mesograsers (e.g. amphipods, gastropods and isopods), through macrograzers (e.g. sea urchins and fish), to megagrazers (e.g. green turtles and manatees and dugongs), (ii) and the feeding strategy, which includes scraping leaf surface, shredding or biting the seagrass leaves, cropping leaves and removing the whole plants. Each herbivore group has a different impact on meadow function (productivity, canopy structure, induction of plant defenses, structure of species assemblages within the meadow…). For instance, herbivory can have positive effects on seagrasses by enhancing productivity as shown in the mesograzers example scraping the epiphyte cover (Reynolds et al., 2014) or, at larger scales, by facilitating the dispersion of propagules and seeds, thus providing mechanisms for meadow recovery (Tol et al., 2017). However, herbivory can have negative or even dramatic consequences for seagrasses ecosystems when an overgrazing episode drives the meadow to a degraded undesirable state dominated by bare sand or a few macroalgae (Peterson et al., 2002; Alcoverro and Mariani, 2002). Moreover, herbivory can also be at the basis of indirect interactions, for example by allogenic engineering, as shown by herbivore fishes increasing predation risk to sea urchins by severely reducing leaf canopy (Pagès et al., 2012). The second approach to seagrass-herbivore interactions has dealt with the defense of seagrasses to herbivory. Seagrasses, similarly to terrestrial grasses, are able to respond to herbivory through different mechanisms broadly categorized into two main strategies: those allowing plants to withstand stress (in this case herbivory), usually called tolerance strategy (Agrawal, 2000), and those reducing the probability or severity of stress, which is called resistance strategy (Fritz and Simms, 1992). The former attenuates the negative effects of herbivores by minimizing the loss in plant fitness through changes in growth, photosynthetic rate or resource (i.e. nutrients, carbon reserves) allocation pattern (Vergés et al., 2008; Sanmartí et al. 2015), among others. Resistance responses are generally based on changes either in physical or chemical properties of plant tissues (e.g. toughness, production of secondary metabolites) making them less palatable and/or attractive and/or nutritious (Karban and Baldwing, 1997).

As foundation species, seagrasses offer not only foraging grounds for herbivores (or detritivores) but also a suitable habitat, including substrate where to attach for sessile species, shelter against hydrodynamics and protection from predators. Some of the species positively influenced by seagrasses can in turn positively influence the plant. This is the case of suspension-feeding bivalves, with higher survival rates in seagrass meadows than elsewhere which, in turn, enhance seagrass growth by fertilizing pore waters with detritus (Reusch and Williams, 1998). Thus, both bivalves and seagrasses have survival and/or growth rates higher when co-occurring than when growing alone. Research in seagrasses has also shown that positive interactions among habitat-forming species, as the example above, can result in a facilitation cascade whereby primary habitat-forming species, the seagrasses, facilitate secondary habitat-forming species, the bivalves, increasing the biodiversity of associated species (Zhang and Silliman, 2019).

Yet, biological interactions in seagrass meadows, either predatory or facilitative, are crucial for their resilience to stress and disturbances. For instance, the negative effects of eutrophication can be alleviated by active burrowers by enhancing sediment oxygenation (Lee et al., 2018), by mesograzers removing epiphyte biomass (Verhoeven et al., 2012) or by infaunal lucinid bivalves reducing the sulfide (toxic for seagrasses) through its endosymbionts gill bacteria (van der Heide et al., 2012). However, when stress is higher than a certain threshold, seagrass meadows, as other ecosystem (Scheffer et al., 2001) collapses, suffering a critical transition to an alternative state, most commonly bare sediment (Giesen et al., 1990). When (or if) stress decreases, the recovery of the system shows hysteresis (Adams et al., 2016) as it implies the rebuilding of the clonal structure and, specifically, of the rhizome biomass. This is also the case when the system recovers from discrete, acute disturbances.

Apart from the potential effects of legacies from chronic or acute stressors (pollutants, low redox potential, destabilization of the sediments: Ralph et al., 2016; Short and Wyllie-Echeverria, 1996; Cabaço et al., 2005), recovery implies a process of recolonization, whose pace primarily depends on the biological traits of the species concerned and on their growth strategy (i.e. shoot growth, rhizome extension, clonal dispersal and sexual reproduction) (Rasheed, 2004; Kenworthy et al., 2002; Berkovic et al., 2014; McMahon et al., 2014). For instance, high shoot turnover, high rhizome elongation rates and high investment in sexual reproduction, typical strategies of fast-growing species (i.e. opportunistic and colonizing species sensu Kilminster et al. 2015), may speed up recovery (O'Brien et al., 2017).

Much progress has been made over the last decades in improving our understanding on how the drivers of change (eutrophication, warming, pollution, floods, storms, mechanical impacts or dredging: Waycott et al. 2009; Unsworth et al. 2019) affect seagrass ecosystems. However, more research is still required in order to better preserve, manage, and restore these highly valuable ecosystems, and some major gaps still remain. Among them, it is worthy to underline those related to: (i) the mechanisms that underpin resilience, both ecological and engineering, in seagrass ecosystems, (ii) the role of biological interactions in conferring resilience to seagrass ecosystems and, (iii) the importance of facilitation cascades as mechanisms to allow species coexistence within seagrass meadows. In the present thesis we will try to provide insights into some of these points.

Objectives and structure of the thesis

Overall, this thesis is an attempt at approaching seagrass community ecology from two complementary but not independent perspectives, biological interactions and resilience, in order to gain knowledge on the processes and mechanisms, which are at the basis of the persistence of seagrass ecosystems. In this introduction, we have tried to identify some gaps related to these two topics. Some of these gaps will be addressed in the following chapters.

The thesis is structured in four main chapters, each one with its own focus and responding to different questions. **Chapters 1** and **2** are mainly oriented to the biotic interactions in seagrasses, while **Chapters 3** and **4** are aimed at understanding the resilience (ecological and engineering) of seagrass ecosystems. All these questions are summarized in Fig. 3 and explained in more detail below.

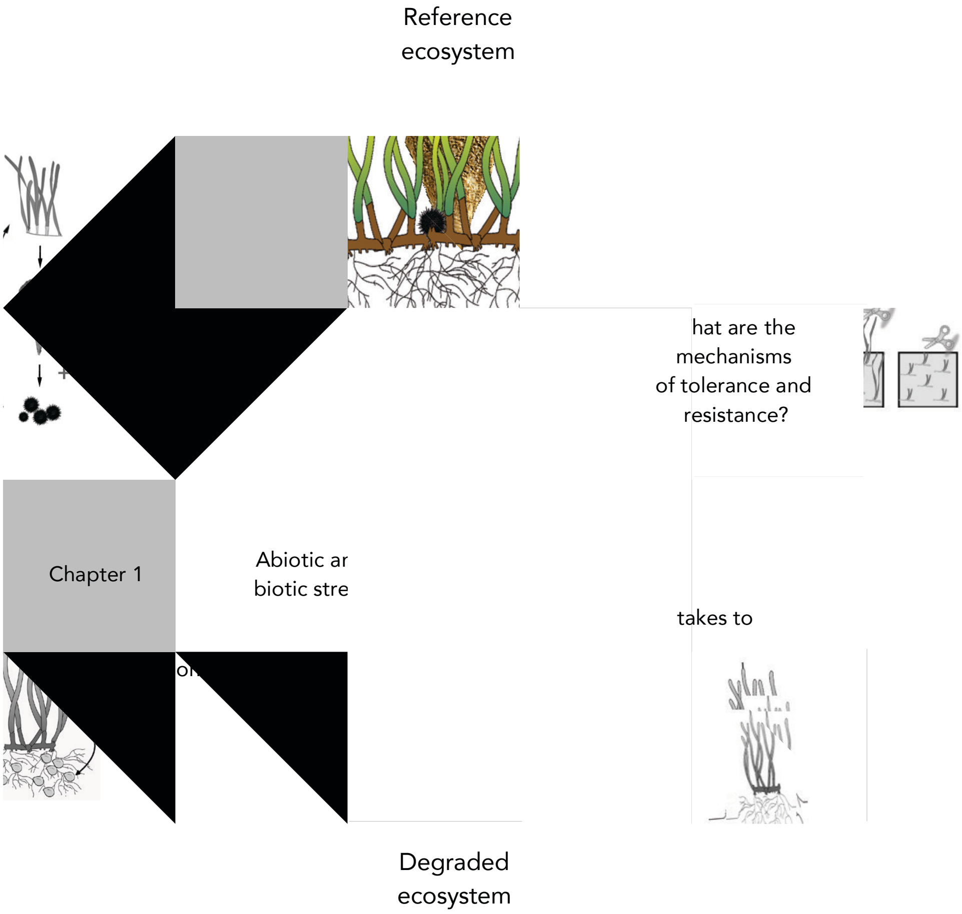


Fig. 3. Diagram of the main objectives of this thesis, summarizing the main questions addressed. See text.

- **Chapter 1.** *Seagrass-bivalve facilitative interactions: Trait-mediated effects along an environmental gradient.* We aim here at assessing how a gradient of sediment organic enrichment affects the mutualism between the seagrass *Cymodocea nodosa* and the lucinid bivalve *Loripes lucinalis*. Given that this mutualism has been postulated as fundamental for seagrass persistence (van der Heide et al., 2012), it is crucial to understand how it is affected by human pressures such as eutrophication. We hypothesize higher lucinid abundances in organic-richer sediments under the assumption of a facilitation enhanced by increased sulfide content in the sediment.

- **Chapter 2.** *Habitat facilitation and herbivore behaviour determine the coexistence of a three-species assemblage*. We first describe a facilitation cascade composed of a foundation species (the seagrass *Cymodocea nodosa*) as a basal species, a filter feeder (the giant bivalve *Pinna nobilis*) as an intermediate species, and a key herbivore (the sea urchin *Paracentrotus lividus*) as a focal species, and evaluate experimentally some of the mechanisms involved in their direct and indirect interactions. Given that the focal species is a consumer of the basal one, we explore the possible processes allowing the coexistence and persistence of this three species system.

- **Chapter 3.** *Tolerance responses to simulated herbivory in the seagrass* Cymodocea nodosa. We examine specific mechanisms of tolerance and resistance of the seagrass *C. nodosa* to different levels of biotic stress (herbivory). Shedding light on the mechanisms underlying plant defense strategies against herbivory may help us to better understand seagrass resilience against this, stressors, and probably others. We hypothesize that the plant responses will be based on the modification of various traits, such as growth, recruitment or translocation of nutrients.

- **Chapter 4.** *Recovery of a fast-growing seagrass to small-scale mechanical disturbances: effects of intensity, size and seasonal timing.* In this chapter we assess the recovery capacity of *C. nodosa* to different mechanical disturbances, characterized by intensity, size of the affected area and seasonal timing. Expanding our understanding on how seagrasses respond to disturbances may help us to improve management practices. We hypothesize that the recovery capacity of *C. nodosa* will decrease with increasing disturbance intensity and size and that the recovery of seagrass will be faster in plots disturbed in spring compared to those disturbed in autumn.

The results obtained from these chapters are mainly based on field observations (**Chapter 1** and **2**) and field manipulative experiments (**Chapter 2, 3** and **4**).

BOX 1. GLOSSARY

Coexistence - Species living together, interacting and persisting in space and time.

Disturbance - Discrete event in time and space that can drastically change the structure of an ecosystem, community, or population as well as resource availability and/or the physical environment (Pickett and White, 1985).

Facilitation - Facilitation between two species which is beneficial for at least one of them, neither being harmed.

Facilitation cascade - Sequences of direct positive effects between species.

Foundation species - A species that provide structure, change local biotic and abiotic conditions, having a large, positive effect on other species in a community (Dayton, 1972; Bruno and Bertness, 2001). They are also called "habitat forming species", and correspond to the "autogenic engineering species" defined by Jones et al. (1997).

Habitat cascade – Sequences of direct positive effects between species in which those positive effects are in the form of biogenic formation or modification of habitat. (Thomsen et al., 2010).

Persistence - Continuity through time of an ecological state.

Resilience - The ability of an ecosystem to absorb stress or disturbances maintaining its functioning, structure, and identity (**ecological resilience or resistance**) and/or the ability to recover to its original state (or dynamics) when the action of external drivers ceases (**engineering resilience or recovery**) (e.g. Holling, 1996; Pimm, 1984).

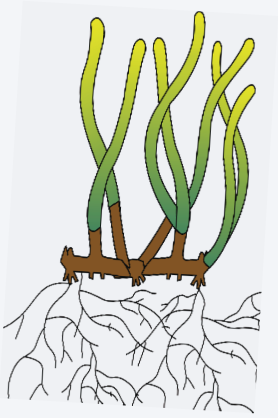
Mutualism - Reciprocally positive interactions between pairs of species.

Stability - Referred to ecosystems, term encompassing different concepts, such as resilience (both ecological and engineering) and persistence (Grimm et al., 1992).

Stress – In Ecology, progressive change in ecological conditions negatively affecting the structure and function of ecosystems (Turner et al., 2003).

BOX 2. SPECIES IN THIS THESIS

Cymodocea nodosa (Ucria) Ascherson**,** also known as little Neptune grass. This small, fast-growing seagrass, has a wide ecological range, strong seasonality, and is widely distributed across a broad variety of shallow Mediterranean environments, from coastal lagoons (0.5 m) to open coastal areas (up to 30 m or more) and extends into the Atlantic, from the south of the Iberian Peninsula to the Canary Islands and Mauritania (Green and Short, 2003). It forms extensive meadows both monospecific or mixed with other macrophytes such as *Zostera noltii, Posidonia oceanica* or *Caulerpa prolifera*. *C. nodosa* meadows are highly productive, rich and diverse. Although it is negatively affected by several stressors and disturbances (i.e., eutrophication, trawling), it is able to resist highly eutrophic conditions, and is not considered to be threatened.

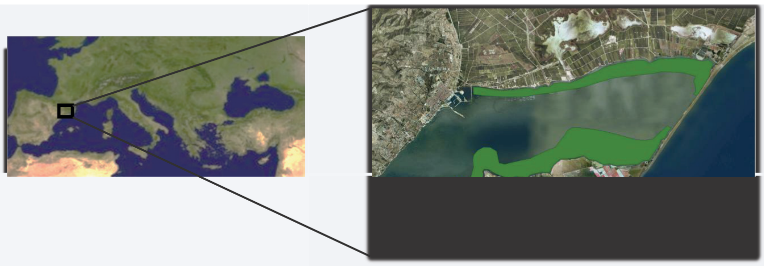


Loripes lucinalis (Lamarck, 1818) is a small bivalve from the family Lucinidae that inhabits reduced sediments from the intertidal zone to 150 m depth. *L. lucinalis* is a suspension filter-feeder, also able to use metabolites released by its chemoautotrophic sulfide-oxidizing bacterial symbionts located in specialized gill cells, the bacteriocytes (Herry et al., 1989). Bivalves belonging to the family Lucinidae are among the most abundant and diverse of the infaunal mollusks living in the sediments of seagrass meadows where they form a fundamental mutualistic relationship with seagrasses. The association between lucinid bivalves and seagrasses is strong enough that the shells of dead lucinid bivalves are used to identify and date relict seagrass meadows (Bretsky, 1978; Barnes, 1996)

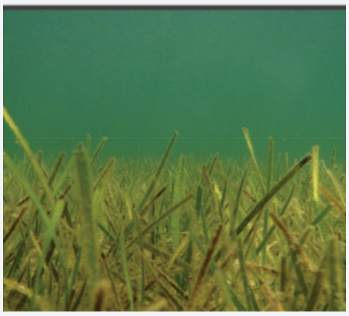
Pinna nobilis (Linnaeus, 1758), also known as pen shell or fan mussel, is the largest bivalve in the Mediterranean reaching a size of up to 120 cm. This Mediterranean endemic is a long-lived species occurring at depths ranging between near the surface and 60 m. It lives with the acute part of the shell partially buried and anchored with its developed byssus filaments (García-March, 2005), most often within seagrass meadows. While the populations of *P. nobilis* have been greatly reduced during the past few decades as a result of a multitude of threats (e.g. fishing, decorative purposes, trawling…), more recently their populations had expanded, mostly in marine protected areas. However, a mass mortality event (detected in 2016) caused, seemingly, by two agents (haplosporidian parasite and a mycobacteria: Darriba, 2017, Carella et al., 2019), is currently reducing drastically their populations all over the Mediterranean. *P. nobilis* is afforded strict protection as endangered and protected species under the European Council Directive 92/43/EEC (EEC, 1992) and recently listed as "Critically Endangered" by the IUCN Red List of Threatened Species.

Paracentrotus lividus (Lamark, 1816) or purple sea urchin is an Atlanto-Mediterranean species inhabiting rocks, boulders and seagrass meadows in shallow waters down to 20 m depth. *P. lividus* is one of the main herbivores of macroalgae and seagrasses in the Mediterranean that is capable of severely overgrazing benthic vegetation (Sala et al., 1998, Guidetti, 2006; Ruíz et al., 2009) triggering macroalgal communities to shift to barren states. This species has been used as an animal model in developmental biology, and to understand regime-shifts dynamics. *P. lividus* is also a valuable resource for the commercial value of its gonads with a high international demand causing the depletion of wild stocks in several countries (Boudouresque and Verlaque, 2001).

BOX 3. STUDY AREA



All the field work in this thesis h [illegible] on the southern side of the Ebre river delta, northwestern Mediterranean. This is a semi-enclosed and relatively shallow bay (maximum depth of 6 m) covering an area of 50 km^2. The bay receives nutrient- and organic matter-rich freshwater discharges from rice crop fields, entering the bay through the northern shore, while the southern shore is influenced by marine waters from the open sea. This creates a marked gradient of nutrient and organic matter concentrations that has been documented in previous works (Mascaró et al., 2014; Oliva et al., 2012; Pérez et al., 1994). The study system is an extensive, rich, diverse, and well-developed series of meadows of *Cymodocea nodosa* thriving on the sandy platforms surrounding the bay (from 0 to 2 m). These meadows have a great ecological interest since they are associated with a wide variety of species, some of them charismatic and highly protected, such as *Pinna nobilis*. Artisanal fisheries and recreational activities produce some of the major pressures (i.e. propelling) on the southern meadows, while the northern one is submitted to strong eutrophication. All of this, together with the relative simplicity of such systems (with few trophic links), makes them an excellent model to test community ecology sound questions on biological interactions and resilience.



CHAPTER 1

Seagrass-bivalve facilitative interactions: trait-mediated effects along an environmental gradient

Abstract[1]

Facilitative interactions are important forces in shaping community structure and function, and understanding how they respond to environmental changes has become an increasing concern in ecology. Lucinid bivalves play a significant role in seagrass meadows, through a mutualism in which the seagrass provides habitat and oxygen via the roots, while the bivalves and their associated bacteria eliminate sulfides from pore water, improving thus plant performance. In this study, we evaluated how this mutualism is modified along a gradient of organic matter content in the sediment, in a coastal bay dominated by *Cymodocea nodosa* meadows. We used a correlative approach, seeking statistical association between sediment organic matter content, lucinid abundance, and plant traits. Lucinid abundance was higher in vegetated that in bare areas. In vegetated areas, lucinid abundance decreased as organic matter content in the sediment increased, decrease seemingly associated to modification in plant traits, i.e. root abundance and morphology. In organic-rich sediments, roots are less abundant and less branched, reducing the potential habitat for lucinids and suggesting a weakening of the interaction. This finding contributes to our understanding of how facilitative interactions can be modified along human disturbance gradients, and how disturbances can reduce resilience of seagrasses through this modification.

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[1] See original publication in Sanmartí et al. (2017)

Introduction

Biological interactions are crucial drivers in shaping the biosphere. Positive interactions (i.e. those benefiting at least one of the species involved and not detrimental to the other), are recognized as playing a critical role in ecosystem function and structure (Brooker et al., 2008; Bruno et al., 2003, Mcintire and Fajardo, 2014). Facilitative interactions are widespread, and their occurrence and strength has been shown to be highly context-sensitive (Eckert et al., 2010, Tylianakis et al., 2008) since environment can alter the biological, ecological, or behavioural traits of the species involved (e.g., Kiers et al., 2010, Schöb et al., 2013), and therefore the outcome of their interaction. Moreover, the behavioural, physiological, and biological traits on which facilitative interactions depend are not built-in software, and can undergo evolutionary modifications as a consequence of their strong influence on fitness. Overall, the nature and strength of these interactions and their effects at the ecosystem level are highly variable in space and time. For instance, facilitative interactions have been hypothesized to increase their frequency, intensity, and importance with increasing environmental stress (stress-gradient hypothesis: Bertness and Callaway, 1994, He and Bertness, 2014), although the generality of this hypothesis remains controversial (He and Bertness, 2014, Maestre et al., 2009, Michalet et al., 2014).

Environmental changes, and specifically those caused by human activities, can alter facilitative interactions with potential consequences for ecosystem function. For example, it has been shown that global change may disrupt critical mutualisms by causing shifts in the phenology of the species involved, as for instance plants and their pollinators (Memmott et al., 2007). Such trait-mediated interactions (TMI) triggered by environmental changes have been shown to exert a major influence on modulating community structure and function in a wide range of ecosystems (Callaway et al., 2003, Coldren, 2013).

In marine ecosystems, positive interactions have been revealed as essential functional components (Bulleri, 2009), however, while in the terrestrial environment the stress-gradient hypothesis (see above) has played a pivotal role in focusing research, much less attention has been paid to it in the marine realm (Bulleri, 2009). Thus, gaining knowledge on how environmental drivers modulate facilitation and its implications at the ecosystem level is essential in order to understand how species fitness, ecological functions, and the associated provision of goods and services will shift in a changing world. This increases in importance when the ecosystem is highly vulnerable to human pressures such as coastal ecosystems (Ruiz-Halpern et al., 2008) and when the species involved are foundation or keystone species (Jones et al., 2008, Kwiatkowski et al., 2015).

Seagrasses are foundation species inhabiting subtidal, usually soft bottom habitats (Green and Short, 2003) that host a huge biodiversity, while providing a number of valuable goods and services. As with other coastal water ecosystems (e.g., kelp forests, mangroves, and coral reefs), in recent decades they have undergone significant deterioration caused by human activities. How this has affected their structure and function has been extensively documented, although most of the studies published to date have been aimed at assessing the direct effects of shifts in environmental factors such as light, nutrients, organic matter, and sedimentation on the plant and associated organisms (Cabaço et al., 2008, Cardoso et al., 2008, Daudi et al., 2012). Less frequently, research has focused on evaluating how human impacts alter biotic interactions that are important for meadow resilience, especially top-down effects (de Paz et al., 2008, Tomas et al., 2015, Van Der Heide et al., 2007). However, the role played by positive interactions (other than the obvious habitat provision by the seagrass), and how they are affected by human impacts have very rarely been examined (but see Maxwell et al., 2016). Nevertheless, such positive interactions can play important roles in seagrass ecosystem persistence and resilience. The best example is probably a complex mutualism involving bacteria, lucinid bivalves, and seagrasses (van der Heide et al., 2012) in which seagrasses provide lucinids with a suitable habitat (the rhizosphere) and oxygen through root radial diffusion, while the bivalves protects the plants against sulfide toxicity by oxidizing pore water sulfide, an oxidation which is mediated by the chemoautotrophic bacteria inhabiting their gills (Pedersen et al., 1998, Reynolds et al., 2007). This mutualism has been postulated as fundamental for seagrass persistence (van der Heide et al., 2012), because seagrass sediment pore water is rich in sulfide, due to the high activity of sulfate-reducing bacteria (Koch et al., 2007). In support of this notion, it has recently been demonstrated that disruption of the mutualism due to drought has worsened the deleterious effects of desiccation on seagrasses (de Fouw et al., 2016).

Eutrophication is one of the most pervasive impacts of human activities worldwide, and the increase in sediment organic matter content is one of its main consequences, involving lower level of oxygen in sediment pore water and, consequently, higher levels of sulfide production (Lamers et al., 2013). While it is clear that sulfide effects can be dampened by the activity of the lucinids and their associated bacteria, the possible modification of the mutualism by the increase in sediment organic matter content remains unexplored.

The aim of our study was to help fill this gap by assessing how seagrass-lucinid mutualism responds to increases in organic matter content of sediment. Such increases can trigger a number of responses, affecting plant traits (e.g., modifying shoot and root morphology and production) (Fertig et al., 2013, Pérez et al., 1994), environmental conditions (e.g., light reaching the plant; Ralph et al., 2007), and sulfide concentration in pore water (Govers et al., 2014), which can potentially affect the outcome of the mutualism. Thus, we studied the association between the seagrass *Cymodocea nodosa* (Ucria) Ascherson and the abundance of the lucinid bivalve *Loripes lucinalis* (Lamarck, 1818) (synonym *Loripes lacteus*, sensu Poli, 1791) along a gradient of organic matter content in sediment, considering the abundance of lucinids as an indicator of the importance of the seagrass-bivalves interaction. We first assessed whether or not the presence of the seagrass facilitated the presence of lucinids, by comparing bivalve densities between seagrass meadows and bare sediments. Second, we explored the variability in lucinid abundance along a gradient of organic matter content in vegetated sediments, hypothesizing higher lucinid abundances in organic-richer sediments under the assumption of a facilitation enhanced by increased sulfide content in the sediment. Third, we explored the association between the abundance of lucinids and a variety of plant traits (e.g. root biomass, root architecture) to gain insight on the influence of such traits on bivalve population.

Material and methods

Study site

The study was conducted in Alfacs bay, situated on the southern side of the Ebro river delta (NW Mediterranean) (Fig. 1). This bay is relatively shallow (maximum depth of 6 m) and has a surface area of 50 km^2. Large *C. nodosa* meadows grow all around the bay, at depths between 0.5 and 1.5 m (Oliva et al., 2012). The area receives nutrient- and organic matter-rich freshwater discharges from rice crop fields, which enter the bay on its northern shore, while the southern shore is influenced by marine waters from the open sea. This generates a marked gradient of eutrophication that has been documented in previous studies based on data of a number of environmental factors (nutrient concentration in water, organic matter content in the sediment, N and P content in seagrass leaves, P in sediment, etc.; see Mascaró et al., 2014, Oliva et al., 2012, Pérez et al., 1994).

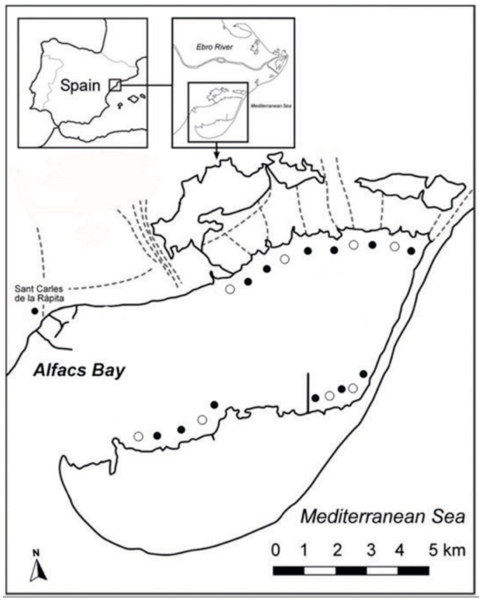

Fig. 1. Sampling locations in Alfacs bay covering the organic matter gradient generated by the freshwater organic-rich inputs from rice fields. Dashed lines represent drainage channel rice fields. Black spots represent samplings in seagrass meadows and bare sediment (plants, lucinids and sediment). White spots represent the additional samples in seagrass meadows (plants and lucinids).

Sampling design

To obtain data from conditions encompassing the wider possible range of sediment organic matter content, twelve sampling points at a constant depth (0.5–0.7 m) were selected around the bay (Fig. 1). At each point, we took two samples: one in the seagrass meadow (including above and belowground seagrass parts, plus bivalves and other fauna) and one in bare sediment (including bivalves and other fauna). This was done using a hand-held core sampler (large core thereinafter, 15 cm diameter) pushed to a sediment depth of 30 cm. To estimate the sediment organic matter content, at each one of these twelve points, two surface (0–3 cm) sediment samples were taken with hand cores (small cores thereinafter, 4 cm in diameter), one close to the sample in vegetated areas and the other close to the sample in bare areas. Additionally, we took eight additional large core samples in vegetated points along the gradient and at the same depth, to better assess plant traits variability and gain statistical power. Immediately after collection, samples from large cores were rinsed, sieved through a 5 mm mesh to remove sediment and transported chilled to the laboratory. Sampling was conducted in June 2013, since June corresponds to the maximum growth period of *C. nodosa* (Mascaró, 2011) and is close to one of the two annual peaks of reproduction of *L. lucinalis* (Johnson and Le Pennec, 1994).

Sampling processing

In the laboratory, live lucinid bivalves from each large core sample were separated from the rest and counted to evaluate their abundance (in terms of density, individuals m^{-2}). In addition to this, in samples obtained in seagrass meadows, shoots were counted to estimate shoot density, and plants were sorted into leaves, rhizomes, and roots. These fractions were then rinsed, dried (70° for 24 h) and weighed. We used the root weight ratio (RWR, Reynolds and D'Antonio, 1996) to express the relative abundance of root versus leaves. The RWR was calculated as the biomass of roots divided by the sum of the biomass of roots plus the biomass of leaves. To assess differences in root morphology and branching (viz. root architecture), five roots from each sample were selected at random before drying, and the maximum length of each root was measured and the number of primary and secondary ramifications counted (Fig. 2). Then, an index of root branching (BI) was calculated as the total number of ramifications divided by the maximum root length. Sediment organic matter content was determined as loss on ignition (LOI450) in samples from small cores. Dried sediment samples (70 °C until constant weight) were combusted for 4 h at 450 °C. Organic matter was expressed as percentage of total weight.

Fig. 2. Images of roots and lucinids during the sampling processing. a) highly branched roots from oligotrophic areas, (b) poorly branched root from eutrophic and c) lucinids.

Statistical analysis

The significance of the differences in lucinid density between habitats (*C. nodosa* and bare sediment) was assessed using a linear model with habitat as a categorical variable (two levels, seagrass and bare sediment) and organic matter content as continuous variable. Data were square-root transformed to meet the assumptions of normality and homogeneity of variances using the customary tests (Shapiro-Wilks test for normality, Bartlett test for homoscedasticity). Association between plant traits (root biomass, root weight ratio, root branching index, and shoot density), organic matter content in the sediment, and lucinid abundance were assessed by linear correlation analyses using Pearson's correlation. All statistical calculations were conducted using the software program R (R Development Core team 2015) with RStudio (version 0.98.1062).

Results

Live individuals of *L. lucinalis* were found in all sampled sites, with their abundances displaying a high variability, from 57 to 1981 ind m^{-2}. Lucinid density was much higher (ca. 5-fold) in vegetated (889 ± 225 ind m^{-2}) than in bare sediments (172 ± 80 ind $m^{-2)}$ (Fig. 3). These differences were highly significant, following the linear model applied ($p < 0.001$). In contrast, and taking all the samples together (from both seagrass and bare areas), organic matter did not influence lucinid abundance ($p = 0.396$). The abundance of lucinids showed a negative correlation with the organic matter content in vegetated sediments (Fig 4a), but showed no correlation in bare ones (Fig. 4b).

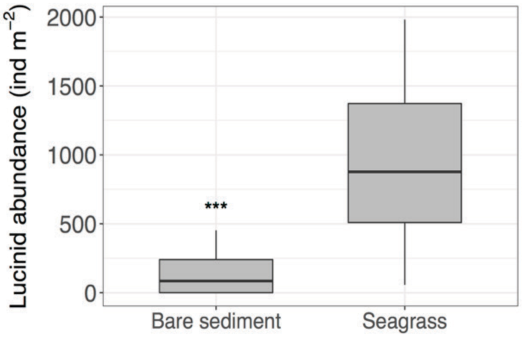

Fig. 3. Abundance of lucinids (ind m^{-2}) in bare sediments (n = 12) and in seagrass meadows (n = 12). Asterisk denote significant differences ($p < 0.001$).

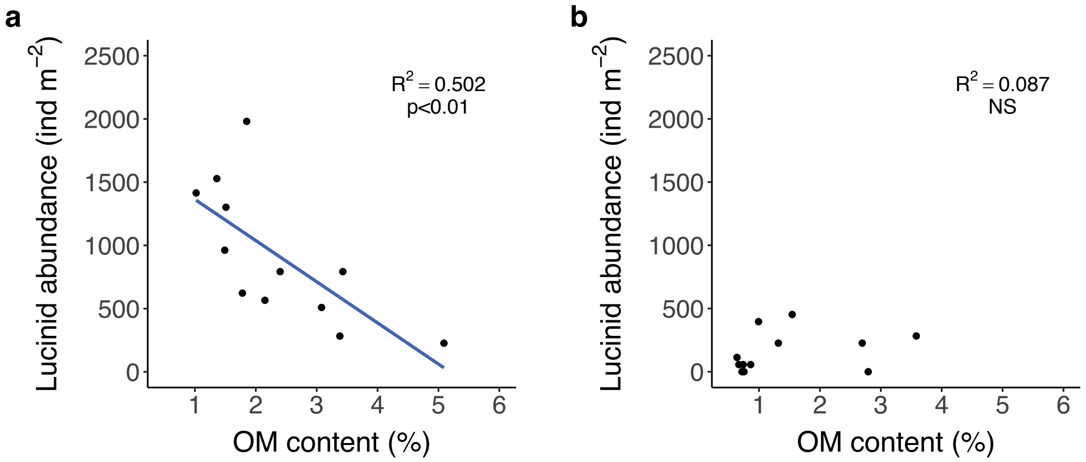

Fig. 4. Statistical association, assessed using linear correlation, between the sediment organic matter content and the abundance of lucinid bivalves (n = 12) in (a) seagrass meadow and (b) bare sediment. R^2 and p-values are reported in the plots.

Seagrass traits varied along the sediment organic matter content gradient. The relative biomass allocation to roots, as indicated by the RWR, clearly decreased with increasing sediment organic matter (Fig. 5a). This paralleled the significant changes evidenced along the gradient in root morphology (as indicated by the branching index, BI), with poorly branched roots in organic-rich sediments and densely branched roots in organic-poor ones (Fig. 5b). These changes in root morphology were visually conspicuous, with roots tending to be fibrous (highly branched and without a distinct main axis) in organic-poor sediments, shifting to monopodial roots (a main vertical axis with limited branching) in organic-rich ones. Root biomass was not correlated with sediment organic matter content (Fig. 5c), while shoot density was (Fig. 5d).

Lucinid abundance was significantly and positively correlated to root traits, including root biomass (Fig. 6a), root weight ratio (Fig. 6b), and the root branching index (Fig. 6c). However, it showed no correlation with shoot density (Fig. 6d).

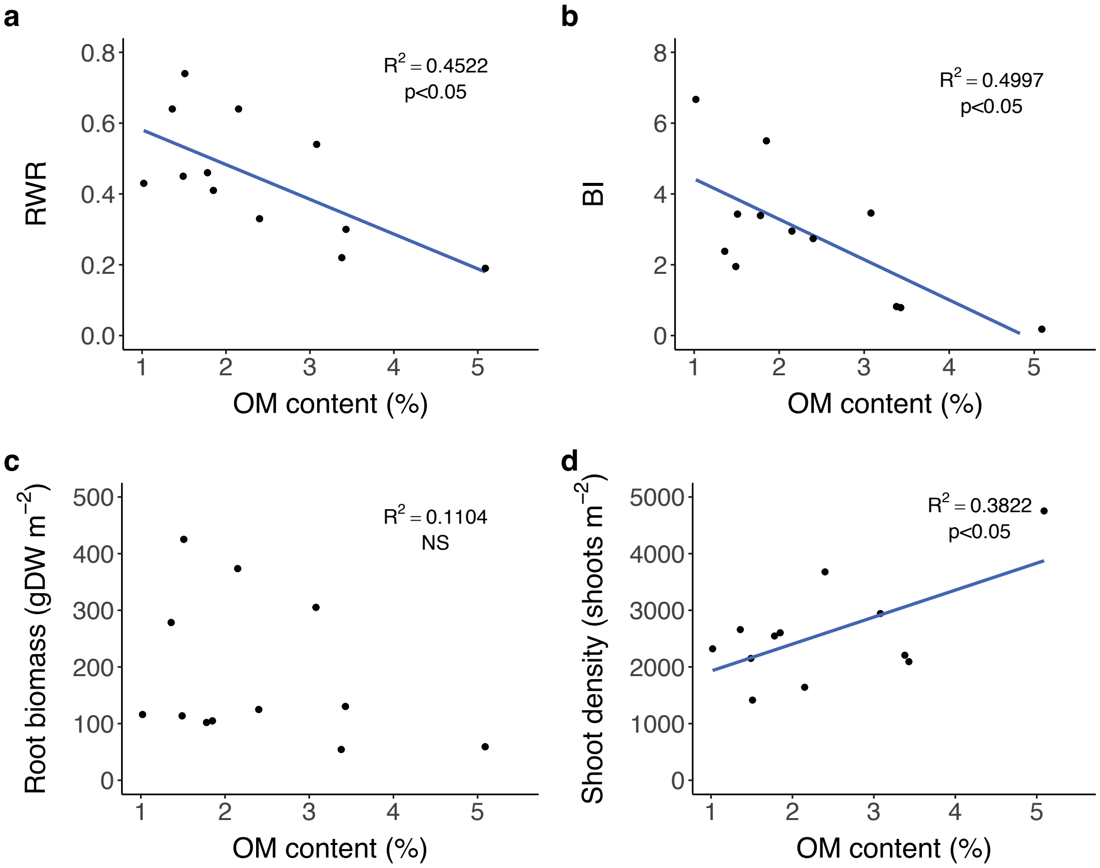

Fig. 5. Statistical association between the organic matter content and plant variables (n = 12) assessed using linear correlation. (a) root weight ratio (RWR), (b) root branching index (BI), (c) root biomass, and (d) shoot density. R^2 and p-values are reported in the plots.

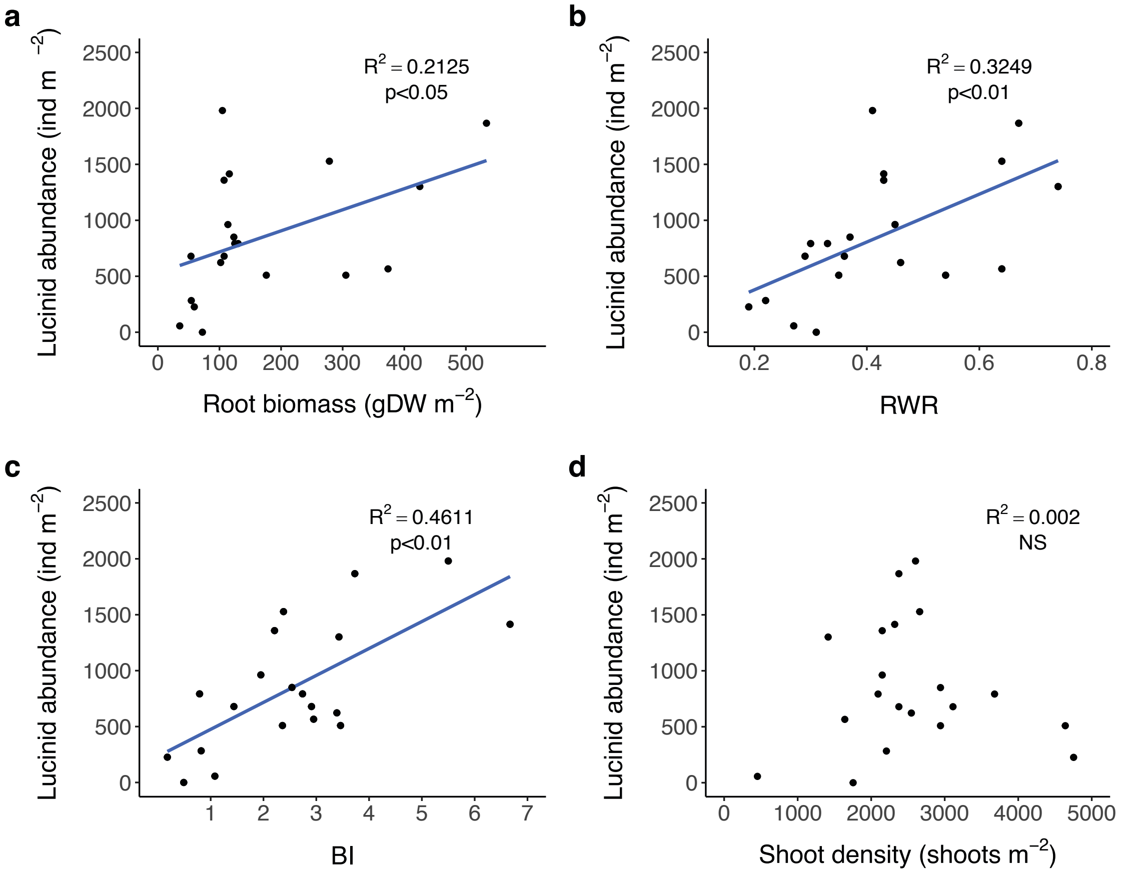

Fig. 6. Statistical association between the abundance of lucinid bivalves and plant variables (n = 20), assessed using linear correlation. (a) root biomass, (b) root weight ratio (RWR), (c) root branching index (BI), and (d) shoot density. R^2 and p-values are reported in the plots.

Discussion

Our results show that an increase in sediment organic matter content is associated to a decline in the abundance of *Loripes lucinalis* (lucinid bivalve) in the *Cymodocea nodosa* meadows studied, which potentially may weaken the mutualism between the two species. This decrease appears to be statistically associated with changes in plant traits, especially in roots, which are less abundant and developed (poorly branched) in the most organic-rich sediments (Fig. 7). Albeit not totally conclusive, this suggests an indirect and negative effect of the sediment organic matter content (and, probably, of eutrophication) on the outcome of the facilitative interaction between the seagrass and the bivalves through the modification of plant traits.

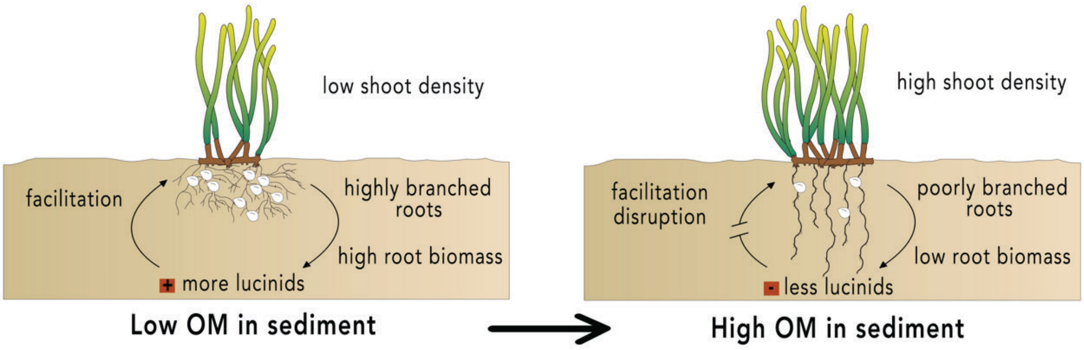

Fig. 7. Effect of organic matter in sediment in root-architecture of *C. nodosa* and on the abundance of *L. lucinalis*. In low organic matter in sediments, roots are highly branched and developed, which may facilitate habitat, protection and oxygen. Under high organic matter conditions, roots are poorly branched, which may negatively affect the abundance of lucinids.

Seagrasses present substantial phenotypic plasticity in morphological and physiological traits, which becomes evident under stress conditions or following disturbance events (Maxwell et al., 2014) and may have a strong influence on modulating species interactions. As a fast growing species, *C. nodosa* displays a high variability in morphological, physiological, and reproductive traits in response to environmental stress (Mascaró et al., 2009; Oliva et al., 2014; Sandoval-Gil et al., 2012). In particular, according to previous studies (Oliva et al., 2007; Pérez et al., 1994), root morphology and architecture change from oligotrophic to eutrophic sites, from thinner and densely branched to thicker and monopodial roots, probably as an adaptive response to optimize biomass allocation and exploit the nutrient pools partitioned between the water column and the sediment (Romero et al., 2006; Kiswara et al., 2009). Given the link between lucinids and seagrass roots, this response can influence the facilitative interplay between the plant and the bivalves.

It is well known that seagrass roots can facilitate the presence of lucinid bivalves, either through habitat provision (the roots themselves) or through resource facilitation (radial oxygen release) (Reynolds et al., 2007; van der Heide et al., 2012). This has been also shown for other infaunal species (Hemminga and Duarte, 2000; Fredriksen et al., 2010), finding habitat, food, and oxygen in or close to the rhizosphere. Indeed, we found that lucinids were much more abundant in areas with *C. nodosa* vegetation, relative to bare ones. Moreover, in vegetated areas, lucinid abundance was positively associated with the root biomass, the relative abundance of roots (relative to shoots, RWR), and the branching index (BI), but not with the shoot density, suggesting that plant root traits are the major drivers of lucinid abundance in the *C. nodosa* meadows studied.

Our data do not allow us to completely rule out the existence of effects other than those mediated by plant trait modifications, such as direct effects of organic matter content in the sediments (inducing oxygen exhaustion) or other variables associated to the gradient (salinity, sediment grain size). However, these confounding effects are highly unlikely to occur. Thus, on the one hand, the maximum values of sediment organic matter content reported here are similar to or lower than most published values for sediments hosting healthy lucinid populations (Como et al., 2007; de Paz et al., 2008). On the other hand, the effect of variables changing along the transect (direct effect of organic matter, salinity, grain size) would have affected both to bare and vegetated sediments populations, whereas our data only indicate a decrease in bivalve abundance in the latter. Moreover, the sediment grain size is quite homogenous across the bay (medium-fine sand; from 181 to 298 μm Ø), and the correlation between the grain size obtained in previous works (Romero et al., 2010) and the abundance of lucinids from this work is not significant ($n = 12$, $r = 0.461$, $p = 0.154$). Accordingly, and in spite of the correlative nature of our study, we conclude that the decline in lucinid abundance along the organic matter gradient in vegetated sediments is an indirect effect mediated by the modification of plant traits.

As facilitation plays a major role in the structuring and the functioning of marine ecosystems (Bertness, 1989; Bertness, 1991; Hacker and Bertness; 1995, Bruno, 2000; Bulleri, 2009; among others), understanding how the strength of these facilitative interactions is altered by increasing levels of stress is critical to assess the resilience of the affected systems. In this respect, and according to the stress-gradient hypothesis, facilitative interactions are more frequent with increasing levels of stress (Bertness and Callaway, 1994; Crotty and Bertness, 2011; He et al., 2013). However, our results do not conform to this general pattern. Two opposite influences seem to act simultaneously on our *C. nodosa* meadows. On the one hand, the increase in sediment organic matter increases pore water concentration of sulfide, which constitutes a resource for the symbiotic lucinid bacteria. This, together with higher food availability for bivalves in organic-rich sediments (van der Geest et al., 2014), would eventually lead to increases in lucinid abundance. On the other hand, the modification of *C. nodosa* root traits reduces habitat and resource provision (oxygen) to the lucinid population. Apparently, and in line with our results, the latter influence overrules the former, yielding as a net result a clear decline in lucinid abundance and, consequently, the possible disruption of the facilitative mechanism. Given the importance attributed to the seagrass-lucinid interaction for the persistence of seagrass meadows (van der Heide et al., 2012), the weakening of the interaction can represent a serious threat for these important habitat-forming species, as has been demonstrated for the seagrass *Zostera noltii* (de Fouw et al., 2016). Interestingly, we did not observe signs of deterioration in plant performance or survival (see, for example, the increase in shoot density with organic matter) in the organic-richest sediments, with low lucinid abundances. This suggests that other mechanisms enable this species to resist relatively high levels of organic matter in the sediments (Terrados and Duarte, 1999; Oliva et al., 2012).

In conclusion, our findings shed light on how human disturbances can weaken facilitative interactions indirectly through changes in species traits. The consequence of this for ecosystem function and service provision is still uncertain and further research is required to clarify this question. The disruption of mutualism caused by stress can represent an additional challenge to the resilience of key ecosystems (Kiers et al., 2010). Assessing the importance of facilitative interactions and their susceptibility to environmental change is therefore crucial to better predict marine ecosystem persistence in a changing world.

Acknowledgments

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CHAPTER 2

Habitat facilitation and herbivore behaviour determine the coexistence of a three-species assemblage

Abstract

Species interactions form the basis of the coexistence of species assemblages. Facilitation cascades are powerful drivers shaping ecological communities, but it remains unclear under what conditions they are stable. Here we describe a three-species complex that includes the seagrass *Cymodocea nodosa*, the pen shell, *Pinna nobilis*, and the sea urchin *Paracentrotus lividus*, and try to disentangle the underlying mechanisms that have allowed the coexistence of the three-species assemblage for at least one and a half decades in the study area. The higher abundance of pen shells (early adults and adults) in seagrass meadows than in adjacent bare sand suggested a facilitation of the seagrasses on early adult stages of the pen shell. A strong correlation between the abundances of pen shells and sea urchins suggested a habitat facilitation on sea urchins. Sea urchins completely overgrazed the meadow in the immediate vicinity of pen shells, being this effect attenuated at the meadow scale. What allows this potentially unstable three-species system to persist may rest on the combination of a facilitation cascade, with seagrasses and pen-shell as habitat forming species, and the behavioural reluctance of sea urchins to move far from pen-shells, making their impact strictly local. In addition, other potential stabilizing feedback mechanisms related to the productivity of seagrass (plant mechanisms to resist herbivory) and to the mechanisms limiting the abundance of pen shells might be acting. These findings contribute to our understanding of how species coexistence can persist, and how the strength of species interactions and potential feedback mechanisms should be considered when addressing stability of species assemblages. These results also highlight the fundamental role of habitat forming species in promoting species coexistence and their potential cascade effects at the ecosystem level.

Introduction

Species interact. Whether the outcomes of these interactions promote or hinder coexistence, and under what conditions species interactions show stability (in the sense of both persistence and resilience; Grimm et al., 1992; Thébault and Fontaine, 2010), is critical to determine how communities are assembled (Tilman, 1982; Chesson, 2000; Lortie et al., 2004). In unpacking mechanisms of coexistence, both theoretical and empirical research have typically focused on negative interactions between two competing or trophically linked species (Chesson, 2000; Silvertown, 2004). Adding one more actor rapidly increases the complexity of the system, making it more difficult to tease apart the mechanisms of coexistence in multiway interactions. May's work predicts that a random association of species with randomly assigned interactions would lead to increasing instability as new species are added to the mix (May, 1973). Yet, real world assemblages are characteristically multi-specific, interacting in dizzyingly complex ways, negative, positive and neutral. But these assemblages are not just random associations of species, and destabilizing interactions may be dampened by countervailing interactions, by population and behavioural feedbacks, or by the environmental stress regime.

Competition and predation are the two classic negative interactions pivotal to many ecological and evolutionary processes. In competitor guilds, coexistence is typically maintained either by environmental disturbance, predation or by intransitive competition – all situations where the strength of the interaction is inherently weak, or mediated by an external factor (Gallien et al., 2017). In contrast, for trophic interactors like predators and prey, coexistence rests on the strength of the density-dependent relationships between the consumer and the resource (Kawatsu and Kondoh, 2018) – here, the influence of the environment or sudden stochastic events could be critical in determining the trajectory of the system (Karacoç et al., 2018). Herbivory is a special case of the consumer-resource interaction because it strongly influences primary producers (Cyr and Pace, 1993; Callaway et al., 2003; Wood et al., 2016), which often constitute the habitat itself. Its effects can therefore be pervasive, modifying communities by suppressing plant biomass, affecting plant performance, mediating competitive processes between plants or increasing biodiversity, among others. When herbivory affects habitat-forming species, it can allogenically engineer (*sensu* Jones et al., 1994) the habitat, producing strong cascading effects (Huntzinger et al., 2008; Parsons et al., 2013). Consequently, any change in herbivore abundance or behaviour can ripple through the ecosystem in dramatic ways. To highlight just two textbook examples - sea urchin outbreaks can cause underwater vegetation collapse (Filbee-Dexter and Scheibling, 2014) and predator-avoidance behavioural changes in elk herbivory can strongly influence forest trees (Ripple and Beschta, 2007).

The dominance of competitive and trophic interactions notwithstanding, positive interactions like facilitation (in which at least one species obtains some benefit from the interaction and no one is negatively affected) are increasingly being recognized as equally important drivers shaping ecological interactions networks (Bruno et al., 2003, Callaway et al., 2002, Altieri et al., 2007; Bulleri et al., 2016). The diversity of facilitative interactions is overwhelming and has been reviewed elsewhere (see Michalet and Pugnaire, 2016). Habitat facilitation is perhaps one of the most conspicuous; organisms are constantly creating and transforming their environments, creating new habitats and becoming habitats themselves for a host of other species (Jones et al., 1997, Bruno and Bertness, 2001). Among these, foundation species – forest trees, grasses, scleractinian coral, mangrove, seagrasses and others – support high diversities within the habitats they create by enhancing living space, providing food resources, and creating refugia for prey and ambush sites for predators (Bruno and Bertness, 2001; Angelini et al., 2011). The effects of foundation species cascade through the ecosystem, and, in some instances, involve multiple foundation species in which a primary habitat-forming species (basal species) facilitates a secondary habitat-former (intermediate species) - commonly embedded within, attached around or to the basal species. This, in turn facilitates a third or more species (focal species), thus enhancing the overall diversity of the system (Thomsen et al., 2010; Angelini and Silliman, 2014). Whether these habitat facilitation cascades can persist through time is not immediately obvious. Their persistence through time will depend heavily on the abundance and the identity of the foundation species involved, the nature and strength of the interaction, and the identity of the focal species that are finally benefited (Irving and Bertness, 2009; Bishop et al., 2012, 2013).

In natural communities, the outcome of co-occurring positive, and negative biotic interactions depends not only on the features of each individual interaction, but also on how they work together, including potential interactive effects and feedbacks (both amplifying and dampening). This is necessarily complex and dynamic, strongly influencing community resilience and ultimately, its stability and persistence (Barabás et al., 2017; Landi et al., 2018). Identifying interactions and feedbacks in real world ecosystems is often difficult given the tremendous diversity of potentially interacting species that coexist in an assemblage. A large number of empirically documented interactions are based on simple two-species examples, which, beyond its indisputable interest, represents a clear oversimplification. Working with three interacting species adds realism (for example, allowing the incorporation of indirect interactions), but also substantially increases complexity. To date, most attempts to investigate how three or more species interact are from a theoretical perspective, which has developed rapidly over the last decade (Levine et al., 2017). Although there are a number of empirical studies supporting theoretical approaches (Angelini and Silliman, 2014; Bishop et al., 2012; Thomsen et al., 2018), more empirical research scaling up from the individual-level interactions to the community and the ecosystem scale processes is still needed.

Here we explore the direct and indirect interactions of a three-species assemblage in a shallow coastal ecosystem dominated by seagrasses. Seagrasses, as habitat-forming species, commonly serve as initiators or intermediate species of trophic and non-trophic interactions that can involve both trait- and density-mediated indirect interactions (e.g. changes in biological traits of seagrass can alter its function as habitat provider; Pagès et al., 2012). By creating new habitat, seagrasses can also facilitate other habitat-forming species by adding structural complexity to the system and increasing the number of interactions and feedbacks (Maxwell et al., 2016). All of this, together with the relative simplicity of such systems (with few trophic links), makes them an excellent model to address the role of interactions in determining coexistence and stability. Specifically, we explore the interactions of a three-species assemblage within a facilitation cascade composed of a foundation species as a basal species (seagrass), a filter feeder as an intermediate species (giant pen shell) and a key herbivore as a focal species (sea urchin) combining observational and experimental approaches. We hypothesize that the coexistence and persistence of this three species system is maintained by a combination of habitat facilitation and weak herbivory interactions. To determine this, we examined: i) the effects of seagrass meadows on pen shell recruitment and subsequent survival in early life stages, ii) the effects of pen shells on the abundance of sea urchins, and iii) the impact of herbivory by sea urchins on seagrass and their potential feedbacks. We used a series of comparative, correlative and field (manipulative) experimental approaches to understand the conditions under which this facilitation cascade persists.

Material and methods

Study system

The study was conducted in Alfacs Bay, a semi-enclosed shallow coastal bay in the North Western Mediterranean. The bay has a maximum depth of 6 m, a surface area of 50 Km^2 and its shallower sandy bottoms (between 0.5 and 1.5 m depth) are covered with vast expanses of seagrass meadows (Pérez and Camp, 1986, Oliva et al., 2012) where pen shell and sea-urchins naturally coexist with great abundances, particularly in the southern shore of the bay. Seagrass meadows are dominated by the species *Cymodocea nodosa* (Ucria) Ascherson (1869), and can be monospecific, or mixed with the green algae *Caulerpa prolifera* (Forsskål) Lamouroux (1809) (Mascaró et al., 2009). The endemic pen shell *Pinna nobilis* (Linnaeus, 1758) grows largely within the *C. nodosa* meadows, although individuals are sometimes found growing in bare sandy areas (Prado et al., 2014). *P. nobilis*, the largest bivalve in the Mediterranean (up to 120 cm), is a long-lived species (maximum reported age of 27 years; García-March and Márquez-Aliaga, 2007) and can act as a habitat-forming species (Rabaoui et al., 2015). *P. nobilis* is afforded strict protection as an endangered and protected species under the European Council Directive 92/43/EEC (EEC, 1992). The sea urchin *Paracentrotus lividus* (Lamark, 1816), is one of the main seagrass herbivores in the Mediterranean (Prado et al., 2007), and is capable of severely overgrazing benthic vegetation (Sala et al., 1998; Ruíz et al., 2001; Ling et al., 2009). The sea urchin *P. lividus* typically inhabits rocky areas or other hard anthropogenic substrates when present (i.e. mussel and oyster farms, harbor jetties and piers). However, in the study area where hard substrates are very scarce, sea urchins are mostly found within seagrass meadows in association with the pen shell, aggregated near the base of the shells or attached on the shells, in groups of two to seven individuals (Fig. 1). Pens shells with sea urchins show areas without seagrass surrounding the pen shell (i.e. grazing halos). The association between the seagrass, pen shell and sea urchin has been naturally present in the bay for at least one and a half decade (authors pers. obs).

Fig. 1. Images of the seagrass-pen shell-sea urchin system in *C. nodosa* meadows with the grazing halo surrounding the pen shell.

Description of facilitative interactions

To assess the effect of the seagrass on the pen shell, we compared the number and size of alive pen shells inside seagrass meadows and in adjacent bare sand. A total of 74 transects (50 m long x 2 m wide) were randomly placed in the two habitats at a depth of 0.5-1.0 m (n=54 in seagrass, and n=20 in bare sand. For every pen shell encountered (new recruits, juveniles and adults), we measured its maximum dorso-ventral shell height (W) and calculated the total shell height (Ht, in cm) according to the following equation $Ht = 1.29 \times W^{1.24}$ as reported by Garcia-March and Nardo (2006).

To assess the effect of the pen shell on the sea-urchin we counted all pen shells and sea urchins encountered in the transects in seagrass meadows. Most sea urchins were attached to the pen shells. We measured their test diameter (without spines) using a caliper. Secondary habitat forming species like pen shells may leave a structural legacy whose influence on associated communities and interactions may continue to accrue long after the individual dies (Hastings et al., 2007). To evaluate this possibility, in a subset of these transects (n=21) we measured the number of alive and dead pen shells – due either to natural mortality or propeller damage. In each pen shell, alive or dead, we counted the number of sea urchins attached directly to them. To assess if this potential facilitation was due merely to the structure or to other characteristics associated with the living pen shell, we compared the number of sea urchins associated with live pen shells to those associated with dead individuals. All surveys were conducted in 2012, 2013 and 2017, before a large disease-related pen shell mass mortality depleted *P. nobilis* populations in the study area (Darriba, 2017; Vázquez-Luis et al., 2017).

Assessment of sea urchin effects on seagrass

Around pen shells within seagrass meadows, we measured the area (length x width, excluding the pen shell) of grazing halos (i.e. areas without seagrass surrounding the pen shell) when present, in pen shells with and without sea urchins (n=47 each). A subset of these (n=25 with sea urchins and n=25 without sea urchins) were selected for determining seagrass structural traits. In each pen shell, we took two seagrass samples (quadrats of 15x15 cm), one at 0 m (from the halo edge or from the bivalve in case of absence of halo) and another at 5 m (Fig.2). In each quadrat we measured the shoot density, the length of the longest leaf (maximum leaf length) and the number of herbivore bite marks of sea urchins in leaves per shoot. Pen shells were selected randomly and all of them were found at a water depth of 0.5-1 m.

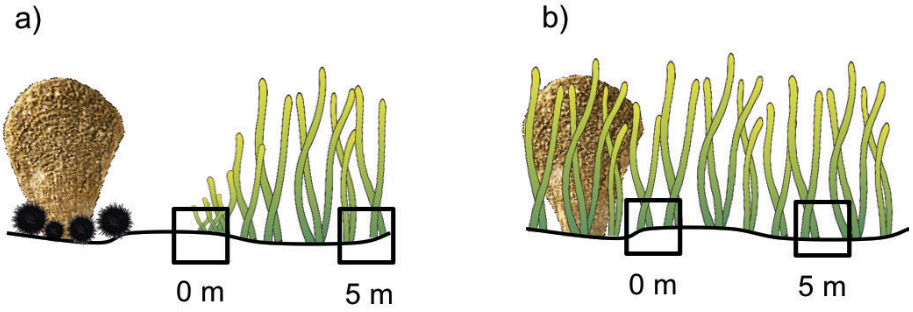

Fig. 2. Design of the field sampling to address the impact of the sea urchins (associated with pen shells) on the seagrass. a) Pen shells with sea urchins and grazing halos, b) pen shells with neither sea urchins nor grazing halos. We took 15x15 cm quadrats of seagrass at 0 m (from the halo edge or from the bivalve in case of absence of halo) and at 5 m to measure seagrass structural traits: shoot density, the length of the longest leaf and herbivory bite marks in leaves. Diagram produced using the Integration and Application Network (IAN), University of Maryland Center for Environmental Science, Cambridge, Maryland.

In addition, we conducted a manipulative field experiment in August 2017 in which 16 pen shells (8 with sea urchins and grazing halos, and 8 without sea urchins or grazing halos) were tagged. We performed urchin translocations by adding 5 individuals (diameter between 4 and 6 cm) to the base of pen shells without sea urchins and, in parallel, removing all individuals from those with sea urchins present (Fig. 3). Additionally, as controls, we tagged 8 pen shells with sea urchins and 8 without sea urchins; these were not manipulated. The surface of the grazing halo was measured in all pen shell individuals at time 0, 30 and 75 days. To avoid any influence of size, all the tagged pen shells had a similar height (ca. 30 cm unburied height). The experiment was conducted at a water depth of 0.5-0.8 m. All surveys were conducted in 2013, 2014 and 2017, while the experiment was conducted in 2017.

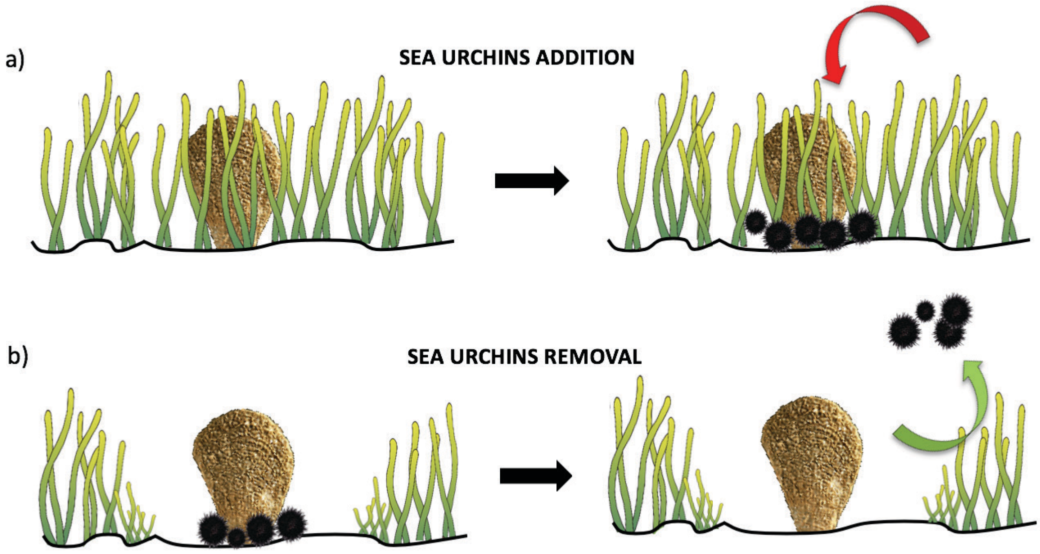

Fig. 3. Experimental design to address the impact of the sea urchins (associated with pen shells) on the seagrass. We tagged 16 pen shells; 8 with neither sea urchins nor grazing halo and 8 with sea urchins and halo. In the former we added 5 individuals at the base of the pen shell (a) whereas in the latter we removed all individuals naturally present (n=4-6) (b). Diagram produced using the Integration and Application Network (IAN), University of Maryland Center for Environmental Science, Cambridge, Maryland.

Statistical analysis

The effect of the seagrass on the abundance of pen shell was assessed using a generalized linear model (GLM) with habitat as fixed factor (2 levels: seagrass and bare sand) and a negative binomial error distribution. The difference in mean sizes of the bivalves between both habitats (bare sand and seagrass) was analyzed with a Wilcoxon's rank paired test, since parametric assumptions were not met. The relationship between the abundance of pen shells and sea urchins was analyzed with a Pearson's pairwise correlation coefficient (r). The effect of the state of the pen shell (alive or dead) on sea urchins' abundance was analyzed with a GLM with the state of the pen shell as fixed factor (2 levels: dead or alive) and a gaussian error distribution. The effect of the sea urchins on the seagrass was measured considering the presence of grazing halos around the pen shells as a binary response (presence vs absence). We used GLM with a binomial error distribution and a logit link function. The effects of sea urchins on canopy height and shoot density of seagrass were analyzed with a GLM and a gaussian distribution with two fixed factors: 1) sea urchins with 2 levels: presence and absence and, 2) distance with 2 levels: close; 0 m and far; 5 m. The effect of sea urchins on seagrass in terms of herbivory bite marks was analyzed with a GLM and adjusted to a negative binomial distribution. The effect of time on the size of the halo after adding and removing sea urchins from the base of pen shells was analyzed with a GLM with two fixed factors and a gaussian distribution: 1) treatment with two levels: add and remove and, 2) time with 3 levels: 0, 30 and 75 days. For all tests, assumption of normality and homogeneity were checked graphically. When necessary, data were transformed to achieve normality. All analyses were performed using the R statistical Software (R development core team, 2020), and the function glm.nb from the MASS package (Venables and Ripley, 2002) was used for GLM with a negative binomial distribution.

Results

Facilitative interactions

The abundance of pen shells was ca. 7-fold higher in seagrass meadows (20 ± 2.6 ind 100 m^{-2}) than in bare sand (3 ± 1.1 ind 100 m^{-2}) (Fig. 4a, Table 1). Additionally, the mean size of pen shells was significantly lower in seagrass meadows (40 ± 0.3 cm, from less than 20 cm to more than 60 cm, estimated total longitude) than in bare sand (54 ± 2 cm), where sizes less than 35 cm were virtually absent (Fig. 4b, Table 1).

The abundances of pen shells and sea urchins were strongly correlated (R^2=0.66) (Fig. 5). Sea urchins, both juveniles and adults, were found mostly at the base of the pen shells but occasionally also attached to the valves. Urchins were very rarely found away from pen shells (less than 1 individual per transect).

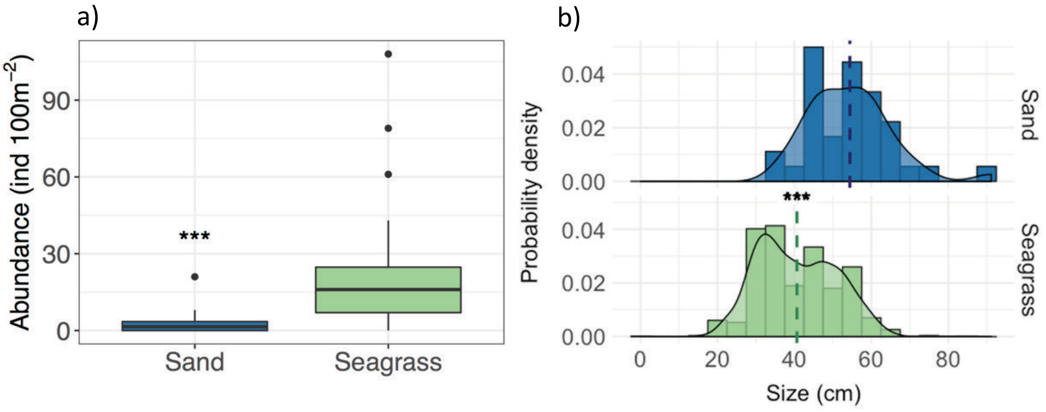

Fig. 4. a) Abundance of pen shells individuals in bare sand (*n=20* transects) and seagrass (*n=54* transects); b) distribution of sizes (total shell height; Ht) of pen shells represented with probability density function in sand and seagrass. Dashed lines represent mean values of size in sand (blue) and seagrass (green). Lines inside the box indicate the median value. Asterisks denote significant differences ($p<0.01$).

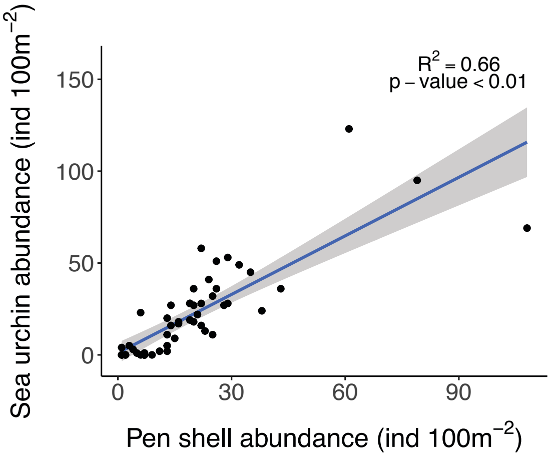

Fig. 5. Relationship between the abundance of *P. nobilis* and *P. lividus* within the seagrass meadow (n=54).

The state of the pen shells (dead vs alive) did not influence the abundance of sea urchins (Fig. 6, Table 1), with mean abundances of 1.8 ± 0.4 and 1.7 ± 0.2 individuals per pen shell respectively in the subsample analyzed.

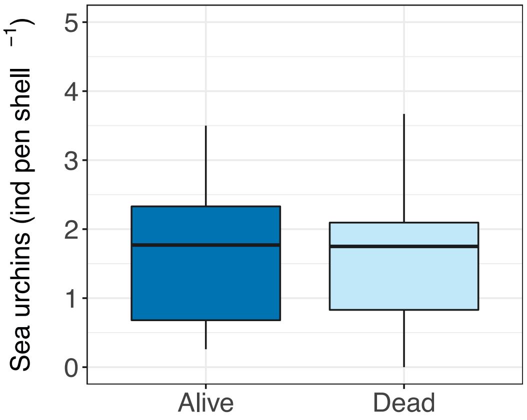

Fig. 6. Counts of sea urchins in alive (n=21) and dead (n=17) individuals of pen shells. There were no significant differences between abundances. Lines inside the box indicate the median value.

Effect of the sea urchin on seagrass

Field observations

The presence of sea urchins around pen shells determined the presence of grazing halos, and significantly affected bite marks on seagrass leaves and maximum seagrass leaf length. Pen shells without sea urchins did not present grazing halos. In contrast, a clear halo, devoid of any seagrass shoots, was observed around the base of all pen shells with sea urchins, with a mean surface area (including the area occupied by the pen shell itself) of 550 ± 66.3 cm² (Fig. 7, Table 1).

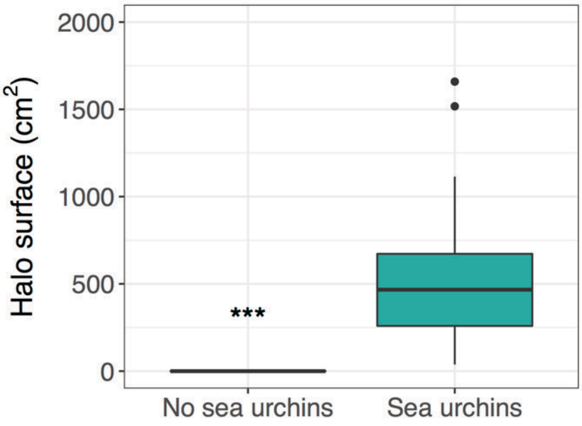

Fig. 7. Grazing halo surface around pen shells with and without sea urchins (n=47). Asterisks denote significant differences (p<0.01). Line inside the box indicates the median value.

Moreover, the presence of sea urchins also increased the frequency of bite marks on seagrass leaves and decreased the maximum leaf length at the edge of the halo, compared to values found 5 m away from the bivalve. No differences were found in shoot density (Fig. 8a, Table 1). In contrast, none of these traits (bite marks, maximum leaf length) varied between 0 m and 5 m around pen shells without sea urchins (Fig. 8b, c, Table 1). Additionally, these traits showed no differences among stands at 5 m from the pen shell with sea urchins and 0 and 5 m from the shell without sea urchins (Fig. 8b, c, Table 1).

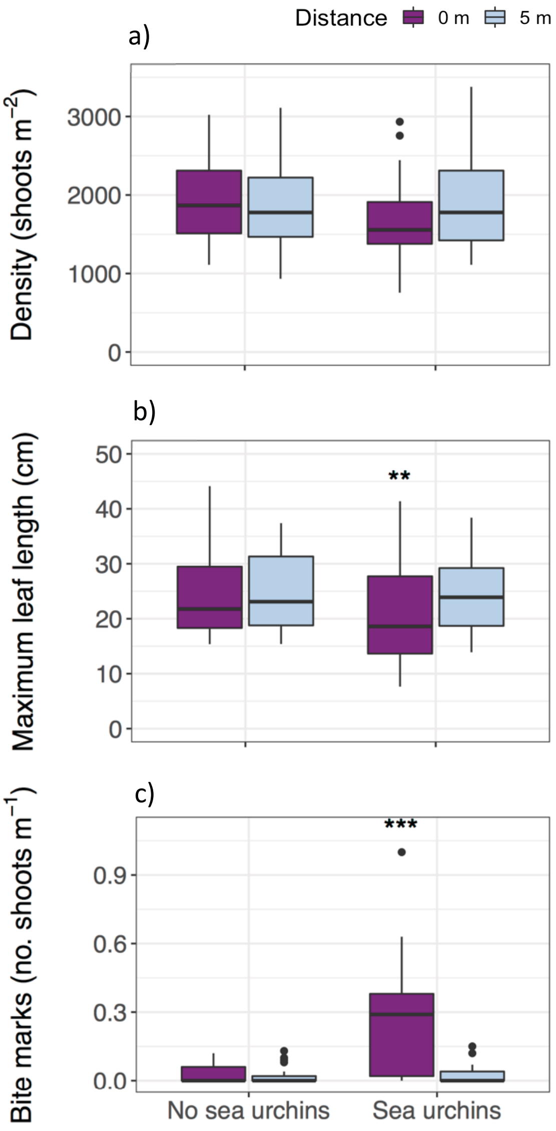

Fig. 8. Effect of the sea urchins on the seagrass surrounding the pen shells at 0 and 5 m from pen shells (*n=25*) with and without sea urchins (see Figure 1). (a) Shoot density, (b) maximum leaf length and (c) herbivory marks on the seagrass. Lines inside the box indicate the median value. Asterisks denote significant differences ($p<0.001$ $^{(***)}$and $p<0.01^{(**)}$).

Experimental manipulation

Our field manipulative experiment confirmed the impact of sea urchins on seagrass. Grazing halos developed rapidly with the addition of 5 urchins at the base of the bivalve, extending to 560 ± 83 cm^2 in 75 days (Fig. 9, S1, Table 1). In contrast, following the removal of urchins from the pen shells, the grazing halo rapidly decreased due to recolonization by the seagrass; the halo surface reduced by 90% on average within 75 days. Controls, both with or without sea urchins and halos, did not show any change during the experiment.

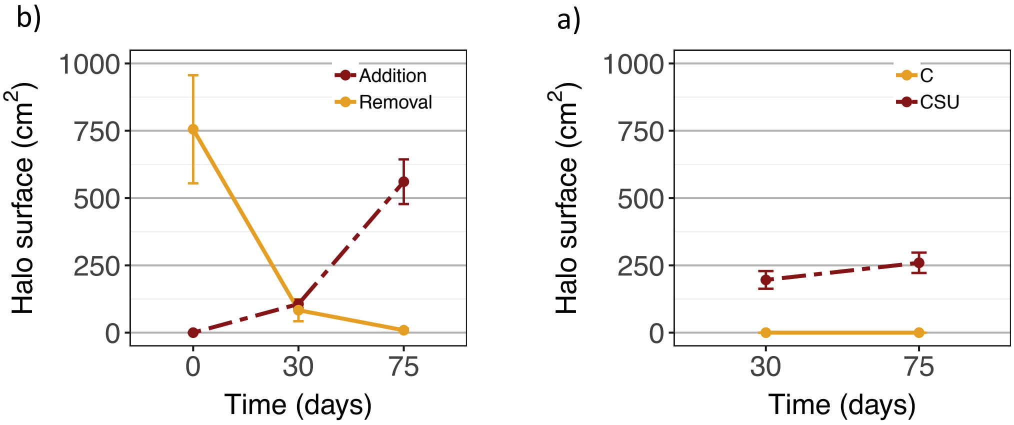

Fig. 9. Effect of sea urchins on *C. nodosa* grazing halos (mean ± SE, *n=8*) in a) translocation experiments and b) controls. Addition: sea urchins added to the base of the pen shells. Removal: sea urchins removed from the pen shells (see Fig. 3). Control sea urchins: controls with sea urchins naturally present on the pen shells. Controls no sea urchins: controls without sea urchins naturally present on the pen shells.

Table 1. Summary of the different analyses performed. Model: type of model used in R (either linear, generalized linear with binomial and with negative binomial distribution or non-parametric Wilcoxson). Trans.: type of transformation applied to the response variable. Df: degrees of freedom. Significance codes $p < 0.001$ ‘***’, $p < 0.01$ ‘**’, $p < 0.05$ ‘*’, $p > 0.05$.

Response variable	**Model**	**Transf.**	**Effect**	**Sum squares**	**df**	***p*-value**
P. nobilis abundance	glm Negative Binomial	–	Habitat	–	1	1.49e-11 ***
P. nobilis size	Wilcoxon	–	Habitat	–		1.3e-7***
P. lividus abundance	Linear	–	State	0.071	1	0.8
			Residuals	56.553	36	
Grazing halo surface	glm Binomial	–	Presence of sea urchins	–	2	4.09e-08 ***
C. nodosa bite marks	glm Negative Binomial	–	Presence of sea urchins	–	1	4.87e-05 ***
		–	Distance	–	1	1.99e-05 ***
C. nodosa canopy	Linear	–	Presence of sea urchins	181.5	1	0.0166 *
			Distance	2130.7	1	5.1e-15 ***
			Residuals	9290.6	297	
C. nodosa density	Linear	log	Presence of sea urchins	0.194	1	0.1725
			Distance	0.143	1	0.2422
			Residuals	10.029	97	
Grazing halo	Linear	–	Time	665131	2	0.0106 *
			Treat	43603	1	0.4192
			Time:Treat	3459643	2	3.75e-08 ***
			Residuals	2751022	42	

Discussion

The three-species system we describe is characterized by a facilitation cascade in which a basal species, the seagrass, promotes the abundance of an intermediate species, the pen shell, which in turn is positively associated to the abundance of a focal species, the key herbivore *P. lividus* (Fig. 10). Likely, the positive effects of seagrass on pen shells are linked to the attachment and protection of early adult stage of the bivalve, while the effect of the pen shells on sea urchins is closely related to habitat or substrate provision. Because the sea urchin has a negative influence on the basal species (the seagrass), this three-species system is potentially unstable (Fig. 9). The persistence of the system for at least one and a half a decades (authors pers. obs.) presumably hinges on the very circumscribed impact of sea urchins on seagrass (less than 0.1 m^2 around the pen shells they are attached) as well on other potential stabilizing feedback mechanisms related to the productivity of seagrass and the abundance of pen shells. While facilitative cascades can promote diversity by providing habitat amelioration, structure and food resources this may result unstable when basal or intermediate species are involved in negative interactions, as seen in this study where basal species is consumed by the focus species. In this case, several mechanism or stabilizing feedbacks need to be present in order to maintain coexistence.

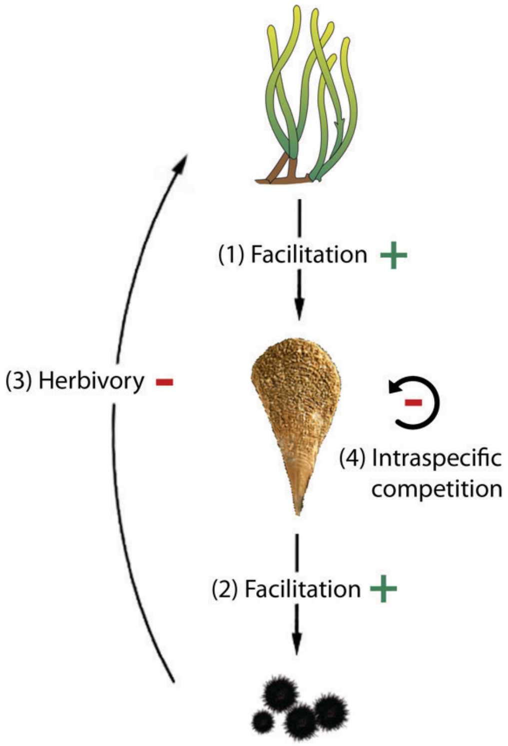

Fig. 10. Conceptual diagram illustrating the interactions and potential feedbacks between *C. nodosa, P. nobilis* and *P. lividus.* The seagrass facilitates the pen shell by providing habitat mostly in early phases (1). The pen shell faciliates the sea urchin also by providing habitat and shelter to young and adult individuals (2). The sea urchin negatively impacts on the seagrass by grazing the meadow in the vicinity of the pen shells (3). The coexistence of this three-species system hinges on the combination of facilitative interactions and the limited impact of sea urchins (limited movement to a small radius around the pen shell and plant responses) on the seagrass. In addition, a possible intraspecific competition in pen shells can limit their density (4).

Unraveling the interactions of the three-species assemblage, our findings show a tight positive association between pen shell abundance and seagrass habitat, indicating a facilitative interaction. The fact that no individuals less than 35 cm (total height) were found outside the seagrass meadow suggests that this facilitation occurs mostly in early stages of development and growth. Bivalves need stable structures to attach their byssus threads to when they first settle, and the structure of the seagrass rhizophere may be an ideal substrate for pen shells to establish (Bologna and Heck, 2000). Moreover, seagrass canopy represents a hide for the most vulnerable small sizes, thus reducing predation, as it does for a range of other seagrass associates (Aucoin and Himmelman, 2011; Heck and Orth, 2006). Once settled though, the pen shell appears less dependent on the meadow for the remainder of its life. Adults in our study, coped quite well in bare sand, albeit at much lower densities. Their presence in bare sandy areas indicates either that they originated in rare successful recruitment events or that these areas probably once hosted seagrass meadows that subsequently disappeared. It is known that shallow *C. nodosa* meadows in this area are characteristically dynamic and may have been lost to a range of abiotic factors such as dune migration (Marbà and Duarte, 1995). Although tantalizing, it is unlikely that urchin overgrazing, as a result of pen shell facilitation, could have led to meadow loss given how little urchins move away from their shelters and the relative sparseness of pen shells in open sandy environments.

Pen shells (including *Atrina* spp and *Pinna* spp) are known secondary habitat species, enhancing structure and increasing macrofaunal diversity in their vicinity (Munguia, 2007; Rabaoui et al., 2015). In this study, pen shells facilitate sea urchins by providing habitat for young and adult individuals, which are virtually absent in the seagrass meadow (except around shells). Several mechanisms may be involved in this facilitation. Physical structure and shell ornaments provide additional space for sea urchins to settle and recruit since they may require a stable hard substrate to attach (Tomas et al., 2004). Additionally, juvenile urchins may find predation refuge on pen shells through at least two strategies: one, by being partially buried in the detrital material that accumulates at the base of bivalves (pers. obs.) and two, by finding shelter under the canopy of spines of conspecific adults (Clemente et al., 2013, Ouréns et al., 2014). Moreover, pen shells also commonly facilitate several other epibiotic communities, which may provide food for sea urchins (unpublished data). More still, detritus accumulation at the basis of pen shells, and organisms entangled to its valves can be additional food sources for sea urchins (Camps et al., 2020).

Sea urchins had strong, albeit local, negative impacts on seagrass abundance, completely overgrazing the meadow in the immediate vicinity of pen shells and resulting in barren halos surrounding the bivalves. These results were confirmed by the manipulative experiments, which exclude any potential mechanical effect on the seagrass by the bivalve. That *P. lividus* can feed voraciously on seagrass and other macrophytes is well documented (Prado et al., 2007; Tomas et al., 2005; Boada et al., 2017). In our study system, however, the impact of herbivory was limited to the immediate vicinity of the bivalve resulting in a strong local impact, lethal for the plant only within ca. 10-15 cm around the pen shell, detectable at >10-15 cm and non-detectable at 5 m distance. Considering that approximately 60% of pen shells sampled had sea urchins in their valves, the area of seagrass completely grazed (ca. 8 m^2) in addition to areas with some grazing impact (ca. 25–95 m^2), still accounts for ca. 1% of the sampled meadow (1200 m^2 sampled in 2017) being overgrazed, and 2–7% moderately grazed. Overall, sea urchins density recorded (0.22 ± 0.08 individuals m^{-2}) are still far from being able to cause a serious impact on this meadow (Ruíz et al., 2009; Boada et al., in prep). Thus, although the strength of the interaction between the sea urchin and the seagrass was strong at the vicinity of the pen shells it attenuated rapidly away from them and was relatively weak relative to the entire ecosystem.

Far from being an ecological curiosity, facilitation cascades may be the primary mechanism of habitat creation in dynamic environments. However, it is not clear at all that facilitation will ensure coexistence in multi-species systems when consumptive interactions are involved. In this seagrass-bivalve-urchin assemblage, if the impact of the herbivore extended beyond the local spatial scale reported and resulted in overgrazing of the entire meadow, the system would have destabilized, destroying the basal species, and, in turn, reducing or eliminating both the intermediate and focal species. Thus, the persistence of the assemblage pivots on the restricted impact that sea urchins have on seagrass. Several factors conspire to enable this. For one, *C. nodosa* has a high tolerance to grazing, compensating with overgrowth, and reallocation of nutrients and carbohydrates (Sanmartí et al., 2014). For another, *P. lividus* individuals restrict their movement to a small radius around the pen shell. They have a high habitat dependency quite like urchins inhabiting rocky reefs, which also show strong fidelity to refuge sites (Johnson et al., 2005). Presumably, such movement limitation may be determined by the availability of food resources on the pen shell (epiphytes, detritus, see above, Camps et al., 2020) or close to it (the seagrass) or by the risk of predation (Pessarrodona et al., 2019). In addition, in these shallow meadows, the probability of being dislodged by waves may also keep urchins close to the safety of their resident pen shells. A final factor contributing to low levels of herbivory at meadow-scales is that pen shell populations were relatively sparse. Given how strongly *P. nobilis* facilitated sea urchins, at higher densities of the pen shell, levels of herbivory could well increase past the capacity of seagrass to sustain it. *P. nobilis* is known to show a clumped distribution (Soo and Todd, 2014), and if the distance between individual pen shells allowed for free movement of urchins between them, the system could well see local extinction of seagrass around the pen shells. What determines the sparse distribution of pen shells in our meadows remains to be investigated, but it is also critical to coexistence in this study system. Predation on early phases (Aucoin and Himmelman, 2011) and resource limitation in areas with high densities (e.g., Peterson, 1982) could be well shaping this distribution.

There are certainly other mechanisms that may affect the stability of the three-species assemblage like changing environmental conditions, which could directly or indirectly influence the interacting species. For instance, eutrophic environments negatively affect the survival of pen shells (Alomar et al., 2015). In the northern shore of Alfacs Bay, where nutrient-rich water from rice fields enter the bay, eutrophic conditions increase organic matter and anoxia in the sediment, resulting in an almost complete absence of pen shells and sea urchins in the meadow (Prado et al., 2014). The decline of *P. nobilis* has also been observed on the Tunisian coast as an effect of marine pollution (Rabaoui et al., 2010). On the other hand, mechanical disturbance such as boat propellers directly affect the survival of seagrass and pen shells (Vázquez-Luis et al. 2015). Finally, biotic factors such as competition or parasitism may also affect the stability of the system. For example, infections by the haplosporidian parasite in Western Mediterranean (Darriba, 2017; Vázquez-Luis et al., 2017) and by mycobacteria in Tyrrhenian Sea (Italy) (Carella et al., 2019) have resulted in large mortality events. Although in the short term, the state of the pen shell does not influence the abundance of sea urchins (and consequently the seagrass-pen shell-sea urchin assemblage), it can, in the long term, result in indirect effects that may be difficult to predict.

Our case-study shows how a relatively simple three species system masks considerable complexity in its interactions. Coexistence in a three species system implying both facilitation cascades and consumptive interactions is not guaranteed, but can exist under a specific set of contextual conditions peculiar to each system (tolerance, feeding behaviour, resource availability, among others) that act as stabilizing feedbacks. The complexity of our simple three-species interaction network highlights the need for more empirical studies to develop theoretical advancements to explore not only the establishment of diverse assemblage but also their maintenance. In this sense, species that create habitat and shape ecological interactions networks have to be recognized not just for their fundamental role in promoting species coexistence, but also for their potential cascade effects at ecosystem level.

Acknowledgments

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CHAPTER 3

Tolerance responses to simulated herbivory in the seagrass *Cymodocea nodosa*

Abstract[1]

Herbivory causes both direct and indirect damage to plants, with negative consequences for plant performance and fitness. Plants have thus evolved strategies to counteract or mitigate such negative effects. The strategies used by aquatic plants to cope with herbivore pressure are of key importance to better understand ecological and evolutionary processes. However, little is known about such strategies. To help fill this gap, and to better understand induced responses to herbivory in aquatic plants, we simulated grazing at various intensities in the seagrass *Cymodocea nodosa* for ca. 4 mo, and measured plant responses in terms of shoot density, aboveground biomass, leaf growth, total nitrogen and carbon content in tissues, total non-structural carbohydrates in rhizomes and total phenolic content in leaves. Most of these plant attributes showed changes under both low and high simulated herbivory at the end of the experiment, indicating that *C. nodosa* is able to change a suite of plant traits to compensate for biomass losses. At least 3 tolerance strategies were involved in this process: growth compensation and overcompensation, increased nitrogen content (either from uptake or through reclamation from rhizome pools) and remobilization of carbohydrates stored in the rhizomes. Phenolic content decreased in the low-intensity treatment but was similar to control plants in the high-intensity herbivore treatment, indicating the role of phenolic compounds in the tolerance response.

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[1] See original publication in Sanmartí et al. (2014)

Introduction

Herbivory is probably one of the most pervasive and influential interactions in the biosphere. Beyond its role in trophic fluxes, herbivory has profound effects on vegetation structure, composition and productivity, and has probably been a strong evolutionary driver since the dawn of life (McNaughton, 2001). Herbivory seriously affects plant performance and fitness in different ways (e.g. by reducing photosynthetic surfaces, injuring parts of key importance such as meristems, removing flowers or seeds), to the point that it is probably among the main forces shaping both plant and herbivore evolution and co-evolution (Rausher, 2001). The long evolutionary history of plant−herbivore interactions is reflected in the large panoply of adaptive mechanisms and strategies displayed by plants to avoid consumption by herbivores or to mitigate its consequences (e.g. Karban and Myers, 1989; Karban and Baldwin, 1997; Bingham and Agrawal, 2010).

Such mechanisms fall into 2 broad categories, constitutive (a constant trait) and inducible (a trait expressed in the presence of herbivores; Karban et al., 1997). In turn, they are based on 2 defense strategies: those reducing the probability or severity of herbivore attack (resistance strategy), and those allowing plants to withstand grazing (tolerance strategy; Agrawal, 2000). Resistance-induced responses are generally based on changes in the properties of plant tissues, making them less palatable and/or attractive to herbivores, or reducing their performance. This is often achieved through the production of secondary metabolites that act as repellents, toxins or agents that reduce plant digestibility (Lattanzio et al., 2006; Wu and Baldwin, 2010), although changes in tissue toughness or in other mechanical properties are also common (Lucas et al., 2000). Tolerance responses attenuate the negative effects of herbivores, by minimizing the loss in plant fitness after herbivore attack, and their nature varies with plant type, developmental status and the part of the plant damaged. A suite of tolerance responses following natural or simulated herbivory has been described, including compensatory growth, increased photosynthetic rate, increased branching, changes in nutrient allocation pattern and increased capacity to shunt carbon reserves from belowground organs to shoots after damage (Strauss and Agrawal 1999; Tiffin 2000). Indeed, several studies have shown that in many plants, primary production can be maintained (compensatory growth) or stimulated (overcompensatory growth) in response to grazing (Gadd et al., 2001), illustrating some of the potential positive effects of herbivory on grazed plants

(Agrawal, 2000; Ruiz et al., 2008). Both tolerance and resistance strategies entail costs and benefits. Different and at times controversial hypotheses have been proposed about their relationship (Restif and Koella, 2004). Apparently, tolerance and resistance are not mutually exclusive and may coexist in plant populations, although trade-offs between them may appear (Mauricio et al., 1997; Leimu and Koricheva, 2006).

Herbivory is considered to be stronger in aquatic systems than in terrestrial ones (Cyr and Pace, 1993). Although less studied than in their terrestrial counterparts, the mechanisms of defense against herbivores are also widespread among aquatic plants (e.g. Toth and Pavia, 2007; Miler and Straile, 2010; Morrison and Hay, 2011). The presence of secondary metabolites deterring grazing in tissues of aquatic macrophytes seems to be an important strategy to protect against consumers that is found in producers from different taxonomic groups (i.e. micro- and macroalgae, angiosperms) and environments (McClintock and Baker, 2001; Pohnert, 2004; Prusak et al., 2005). The tolerance strategy, in contrast, seems to be less common, and this may be because it rarely occurs among algae (but see, for instance, Wai and Williams, 2005). In macro- algae, the lack of a complex morphological and functional organization such as that of higher plants may prevent the existence of tolerance responses. In contrast, angiosperms and, specifically, marine angiosperms (i.e. seagrasses) possess the same functional traits (basal meristems, clonal integration, storage organs; Marbà et al., 2006) that favor tolerance in terrestrial plants. Indeed, compensatory growth has been demonstrated in seagrasses as a response to defoliation (Tomasko and Dawes, 1989, Valentine et al., 1997, Moran and Bjorndal, 2005; Vergés et al., 2008).

Seagrasses are considered to be among the most important components of marine submersed vegetation for the goods they produce and the services they provide (Barbier et al., 2011). Their extensive meadows constitute a key habitat in the littoral system, and are relevant to the global carbon cycle. Recent evidence has proved that grazing in seagrasses is by far more important than previously thought (Heck and Valentine, 2006, Valentine and Duffy, 2006), affecting their population dynamics, composition, distribution and production (Valentine and Heck, 1999, Tomas et al., 2004, Moran and Bjorndal, 2005). For these reasons, seagrasses are excellent model species to explore mechanisms of defense against grazing. However, the responses of sea- grasses to the high herbivory pressure they may suffer have, to date, scarcely been explored. On the one hand, it is known that seagrasses produce secondary metabolites, such as phenolic compounds (Steele et al., 2005; Grignon-Dubois et al., 2012; Ragupathi Raja Kannan et al., 2012), and there is evidence that some of these metabolites, such as condensed tannins, are induced following simulated herbivory (Arnold et al., 2008). However, the precise resistance-induced metabolites produced in response to herbivore attack remain in general poorly known, and it seems that the total phenolic content is not a good predictor of induced defense (Vergés et al., 2007; Steele and Valentine, 2012; Sieg and Kubanek, 2013). On the other hand, there is evidence that seagrasses can tolerate grazing by means of both intensifying recruitment of new shoots (Valentine et al., 1997) and compensatory growth of existing shoots (Tomasko and Dawes, 1989; Moran and Bjorndal, 2005). This compensatory growth could be achieved in part by the use of carbon reserves stored in the belowground organs (Eklöf et al., 2008), and supported by increased nitrogen (N) metabolism (N resorption or uptake; Valentine et al. 2004, Alcoverro and Mariani, 2005). These studies have provided insights into the defense strategies against herbivores in marine plants. However, most were conducted in tropical species, especially *Thalassia testudinum*, thus narrowing the generality of the findings. Despite recent studies (Vergés et al., 2008; Burnell et al., 2013) demonstrating the existence of compensatory growth in the temperate genus Posidonia, our knowledge of seagrass–herbivore interactions, which have both ecological and evolutionary importance, remains poor.

The aim of the present study was thus to assess phenotypic changes in the seagrass *Cymodocea nodosa* caused by simulated macroherbivore attacks to detect possible tolerance responses. *C. nodosa* is a small, fast-growing species with a wide ecological range and high phenotypic plasticity (Pérez and Romero, 1994; Marbà et al., 1996; Cancemi et al., 2002; Mascaró et al., 2009), which is subjected to relatively high levels of herbivory (Cebrián et al., 1996), and can be temporally overgrazed in some coastal lagoons (Fernandez et al., 2012). In this study, we attempt to expand the knowledge of tolerance responses of seagrasses to herbivory, and assess their generality or specificity. Our approach was based on a field experiment consisting of repeatedly clipping the seagrass leaves and measuring subsequent plant responses in terms of changes in density, biomass, leaf growth, carbon and nitrogen content in tissues and total non-structural carbohydrates (TNC). In addition, we measured the total phenolic content in leaves to explore whether or not they participate in the tolerance response rather than in defense mechanisms, as suggested by Vergés et al. (2008).

Material and methods

Study system

The study was carried out in the southern bay (Alfacs Bay) of the Ebro river delta (northeastern coast of Spain; 40° 35′ N, 0° 41′ E), where extensive shallow meadows of *Cymodocea nodosa* develop in the sandy platforms (<1.5 m depth) surrounding the bay (Pérez and Romero, 1994). The study site selected was on the southern shore, where meadows have a good ecological status and are away from the influence of the freshwater entering the bay on its northern shore (Oliva et al., 2012). At this site, shoots show fast turn-over (average shoot life span: 2–4 yr) and reach a maximum density (around 2500–3000 shoots m^{-2}) in May–June (Mascaró et al., 2014). Herbivory is supposed to be low within the bay (Cebrián et al., 1996), although scattered populations of sea urchins (*Paracentrotus lividus*) have been detected (authors′ pers. obs.). The experimental site was chosen to be at a distance from these populations (>500 m), so as to ascertain low natural herbivory pressure throughout the experiment. This made it unnecessary to deploy cages to protect plots against grazing.

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Experimental design

We simulated low and high grazing pressure by macroherbivores by repeatedly clipping the leaves of *C. nodosa* during spring–summer 2010. After 4 mo, we sampled plants to measure several plant response variables (see next section). This period was chosen because it is the period of maximum activity of both plants and macroherbivores in the NW Mediterranean (Prado et al., 2007, Mascaró et al., 2014). The leaf clipping procedure is aimed at mimicking the feeding behaviour of the 2 main macroherbivores in the NW Mediterranean (the sea urchin *P. lividus* and the sparid fish *Sarpa salpa*), as both feed on leaf tips, thus removing the distant part of the leaf blades (Prado et al., 2007). The same approach (i.e. simulating herbivory in stands with low natural levels of grazing) has been used previously and results considered representative of the potential response of the species when grazed (Vergés et al., 2008).

We established 9 plots of 1 m^2 in a *C. nodosa* meadow at 1 m depth, spaced at least 2 m apart. Three treatments, i.e. control, low herbivory (LH) and high herbivory (HH), were randomly assigned to each plot. In the HH treatment plots, the leaf canopy was cut to 10 cm height above sediment level, which corresponds to removal of about 75% of leaf biomass. In the LH treatment plots, only the leaf tips were cut, corresponding to a leaf biomass removal of less than 5% (Fig. 1a, b). In the control plots, the leaf canopy was left unmodified, thus remaining at its normal height (ca. 40 cm above sediment level). The experiment was run from April to late July 2010. During this period, leaves within each plot were clipped periodically. Maintenance (clipping) visits were made every 2 wk, except during the maximum growth period (June and July) when clipping was performed weekly, resulting in a total of 10 clipping events between the start of the experiment and the July sampling. All clipped blades were removed from the plots to avoid any artifact derived from detritus accumulation. At the end of the experimental period, a series of response variables (see next section) were measured. Additional samples were taken for analysis of TNC and phenolic content in October 2010. These samples coincided with the seasonal maximum carbohydrate content (Mascaró et al., 2014). Between July and October, and to maintain the experimental conditions, further clipping visits were made every 2 wk.

Measurement of plant response variables

Biomass and shoot density

At the end of the period of maximum growth (end of July), samples of *C. nodosa* were collected from the central part of each plot using a 16 cm diameter corer. Each sample was thoroughly rinsed in situ with seawater until sediment had been completely removed, and stored in plastic bags that were refrigerated for transport. In the laboratory, all shoots were counted, and the leaves were separated from rhizomes and roots, dried at 70°C for 48 h (until constant weight) and weighed, thus obtaining shoot density (shoots m^{-2}) and leaf biomass (g DW m^{-2}, where DW = dry weight), with $n = 3$ per treatment. Sub-samples of each fraction were kept for biochemical analysis.

Leaf growth and leaf number per shoot

Leaf growth was measured using a modified Zieman method (Pérez and Romero, 1994) (Fig. 1c). On 20 July 2010, 15 shoots were marked in each plot by punching a hole just above the ligule of the outermost leaf using a hypodermic needle. All marked shoots were collected 9 d later. In the laboratory, the leaves from each one of these shoots were separated, the number recorded, and each leaf divided into 'new' and 'old' tissue (i.e. tissue formed during or before the marking, respectively), dried at 70°C for 48 h (until constant weight) and weighed. Leaf growth was expressed in mg DW $shoot^{-1}$ d^{-1}, and relative growth rate (RGR; d^{-1}) was calculated as shoot leaf growth divided per shoot biomass. Both variables thus had 15 subsamples per plot and 3 replicates per treatment.

Fig. 1. a) Experimental plots and sample processing of *C. nodosa*. a) plot of high intensity treatment, b) leaf clipping of shoot, and c) seagrass leaves with punched holes to measure shoot growth.

Tissue biochemical analysis

Dried leaves, rhizomes and roots from the core samples were ground to a fine powder. The carbon and nitrogen content in all tissues was measured in subsamples using a Carlo-Erba elemental auto-analyzer (Scientific and Technical Services of the University of Barcelona). TNC (sucrose plus starch) content was measured in rhizomes, using a modified method from Alcoverro et al. (1999). Ground samples were dissolved in 96% (v/v) ethanol, sonicated for 5 min and heated at 80°C for 15 min to extract soluble carbohydrates. This process was repeated 3 times. Starch was extracted from the remaining ethanol-insoluble pellet by dissolving it in 0.1 N NaOH at room temperature overnight. Sucrose content was determined using a resorcinol assay standardized to sucrose, and starch content was analyzed by spectrophotometry using an anthrone assay with sucrose as a standard. TNC content was the sum of the 2 fractions.

The total phenolic content of leaves was analyzed using a modified Folin-Ciocalteau method (Bolser et al., 1998). Each sample was extracted in 1 ml 50% methanol at 4°C for 24 h. Then, 0.1 ml of the supernatant was added to 7.9 ml distilled water, mixed and combined with 0.5 ml Folin-Ciocalteau reagent. After 2 min, 1.5 ml NaCO3 solution was added to the sample. Two hours were allowed for color development, and absorbance was spectrophotometrically measured at 765 nm and compared with that of a standard curve for gallic acid. Although samples for phenolic analysis were taken in both July and October, the samples from July were lost due to technical problems in the analytical procedure.

Statistical analysis

For the variables shoot density and leaf biomass, the significance of differences among treatments (3 levels: control, LH and HH) was assessed using 1-way ANOVA. For the remaining variables, differences between treatments were analyzed using a 2-way nested univariate ANOVA, considering treatment as a fixed effect and plot (3 levels) as a random effect nested within treatment. To test for between-treatment differences for each variable, we used Tukey's HSD post hoc tests. All variables were individually checked for normality, homogeneity of variance and outliers using first exploratory data analysis procedures (e.g. QQ plots), and parametric tests (Lilliefors and Shapiro-Wilks tests for normality, Cochran test for homoscedasticity) for assessing whether or not the ANOVA assumptions were met. No outliers were removed. Where necessary, data were transformed to achieve normality, as indicated in the 'Results'.

Results

Most of the plant traits investigated responded to the simulated herbivory (Fig. 2). Shoot density increased significantly with clipping intensity (Table 1, Fig. 2a), and was, at the end of the experimental period, > 50 % higher in the HH treatment plots than in the control plots, while LH treatment plots displayed intermediate values. Leaf growth was significantly higher in LH plots (ca. 20% higher) than in control and HH plots (Table 2, Fig. 2c), while the relative growth rate increased significantly in both the LH and HH treatments (Table 2, Fig. 2d). The average number of leaves per shoot increased slightly but significantly in the HH treatment (Table 2, Fig. 2e), relative to the other 2 treatments. In addition, it should be noted that no bite marks made by herbivores were observed in the sampled leaves, thus confirming the low levels of herbivory at the experimental site, and the absence of interferences between natural and simulated herbivory.

In terms of biochemical traits (Figs. 2–5), the HH treatment caused an overall reduction (relative to the control) in nitrogen content, significantly affecting leaves, rhizomes and roots (Table 2, Fig. 3a–c). In contrast, nitrogen content was higher in the leaves of plants from plots subjected to LH, relative to control plots (Table 2, Fig. 3a). Carbon content in leaves and rhizomes tended to be lower in the HH treatment than in the other treatments (Table 2, Fig. 3d, e). Overall, these changes resulted in increased C:N ratios in all 3 organs in the HH treatment (Table 2, Fig. 4a–c). TNC content in rhizomes measured in July in the HH treatment was 50% lower than in the control (Table 2, Fig. 5a). The TNC content increased more than 2-fold from July to October, when the differences among treatments disappeared (Table 2, Fig. 5b). The responses of sucrose and starch were similar. The total phenolic concentration in leaves collected in October was significantly lower (40%) in the LH treatment, relative to both the control and HH treatments (Table 2, Fig. 6).

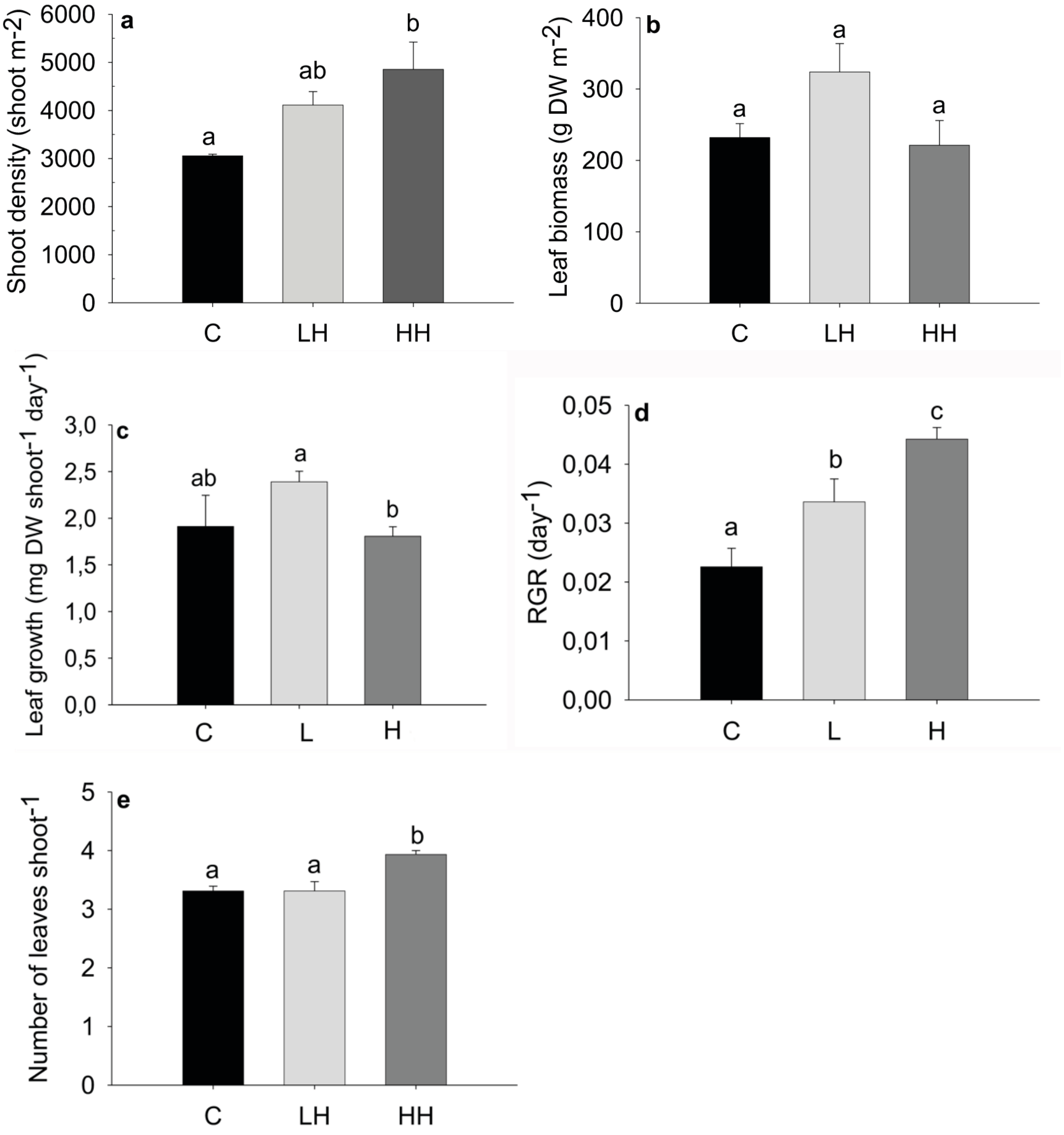

Fig. 2. a) Shoot density, b) leaf biomass, c) leaf growth, d) relative growth rate (RGR) and e) number of leaves per shoot of *Cymodocea nodosa* subjected to simulated herbivory treatments: control (C), low (LH) and high (HH) intensity. Bars labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p \leq 0.05$). Error bars represent standard error of the mean. DW = dry weight.

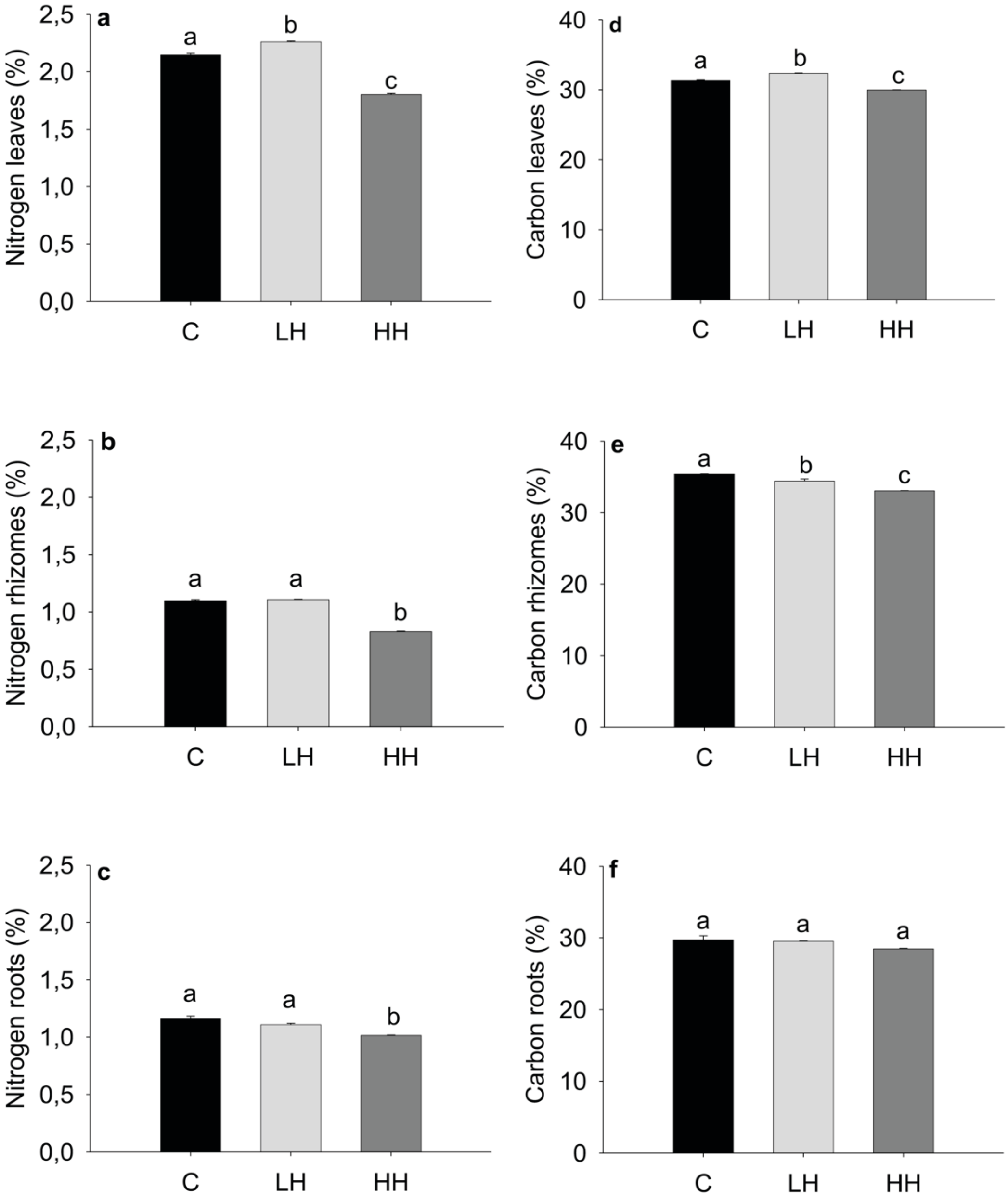

Fig. 3. Content of nitrogen (N) and carbon (C) in different tissues of different simulated herbivory treatments. Bars labelled with the same lower-case letter do not show significant differences according to Tukey HSD test (P≤0.05).

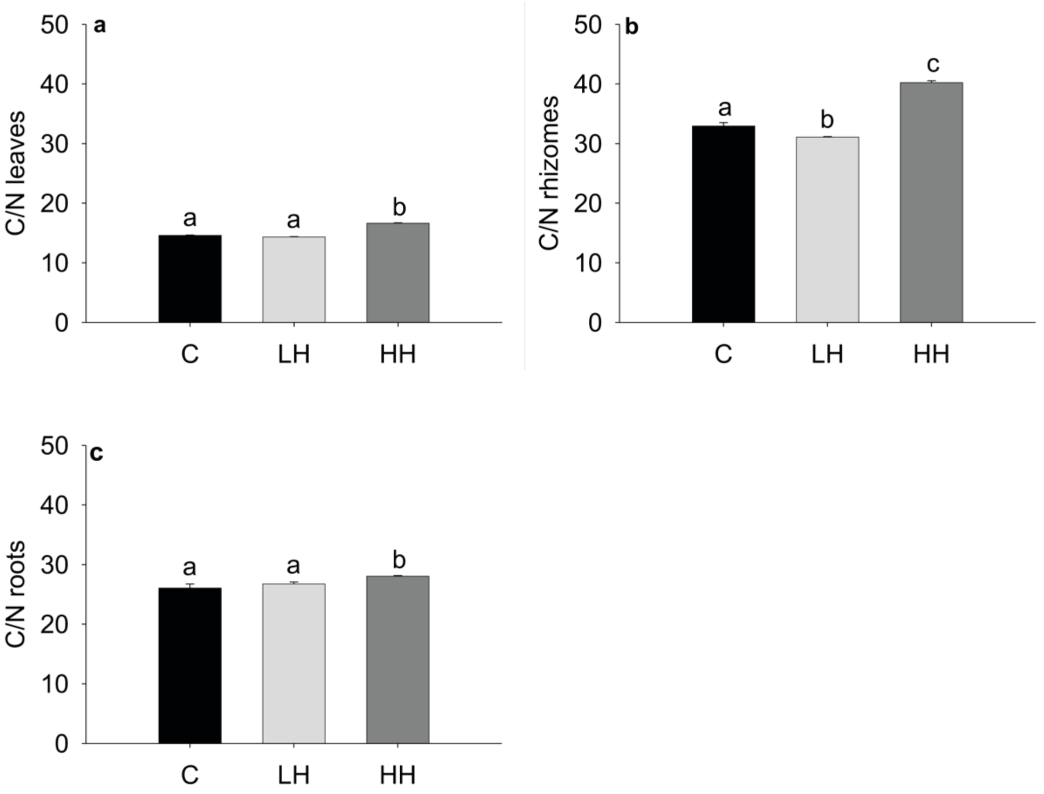

Fig. 4. Content of C/N in different tissues of different simulated herbivory treatments. Bars labelled with the same lower-case letter do not show significant differences according to Tukey HSD test (P≤0.05)

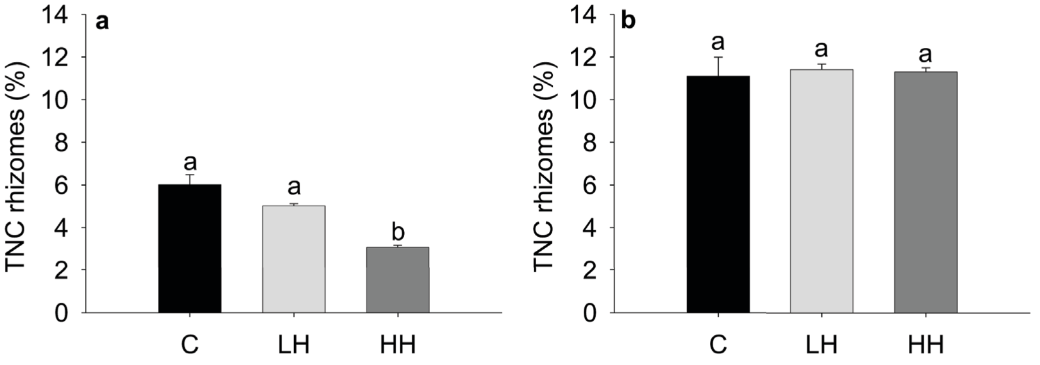

Fig. 5. Content of TNC of different simulated herbivory treatments and measured in July (a) and October (b). Bars labelled with the same lower-case letter do not show significant differences according to Tukey HSD test (P≤0.05)

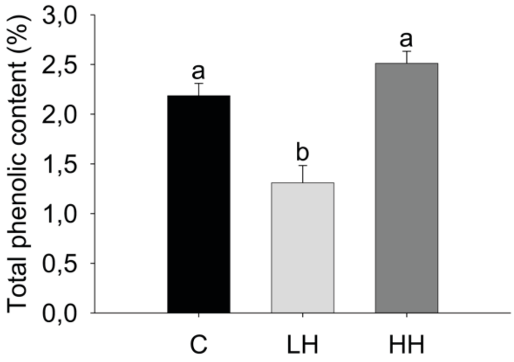

Fig. 6. Content of total phenolics leaves of different simulated herbivory treatments. Bars labelled with the same lower-case letter do not show significant differences according to Tukey HSD test (P≤0.05).

Discussion

This study demonstrates that simulated herbivory causes several responses in the seagrass *Cymodocea nodosa*. While some of these responses seem a simple and direct consequence of defoliation, others appear to attenuate the detrimental effects of consumer damage and are thus suggestive of adaptive tolerance responses. After a 4 mo defoliation period, a suite of plant trait changes, including changes in leaf growth, shoot recruitment, nutrient content and carbohydrate content, were observed, all of them suggestive of nutrient reallocation and mobilization of carbon reserves that either compensated or overcompensated for biomass losses.

Despite the repeated and massive defoliation to which it was submitted in the HH treatment, leaf biomass of *C. nodosa* in HH-treated and control plots at the end of the experimental period were very similar. HH-treated plants compensated for defoliation by the addition of new modules (leaves and shoots), while leaf growth remained similar to that found in control plants. Under the much more benign defoliation performed in LH plots, the response was slightly different. In LH-treated plants, besides the addition of new modules (only shoots), we also found a compensatory leaf growth, that, in the long term, could have led to an overcompensatory biomass response (Belsky, 1986). These compensatory mechanisms described above have been reported in the tropical seagrass *Thalassia testudinum* (Valentine et al., 1997; Moran and Bjorndal, 2005), and in the temperate species *Posidonia oceanica* (Vergés et al., 2008) and *P. sinuosa* (Burnell et al., 2013). Overcompensation is a common response to damage in terrestrial, freshwater and marine plants (e.g. Oba et al., 2000; Li et al., 2010), and is considered more common in fast- than in slow-growing species (Coley et al., 1985; Haukioja and Koricheva, 2000; but see Soti and Volin, 2010). This is consistent with the characterization of *C. nodosa* as a fast-growing and plastic species (Pérez et al., 1994; Mascaró et al., 2009). However, the slow-growing *P. oceanica* has also shown overcompensation for leaf growth (Vergés et al., 2008) but not for shoot recruitment. In this respect, it should be noted that the observed variability in growth compensatory responses of plants is often attributed to extrinsic factors such as nutrients (Li et al., 2010), light availability and damage frequency and intensity (Eklöf et al., 2008).

Our observations stress the importance of nutrients in plant–herbivore interactions. In effect, the observed compensatory growth of *C. nodosa* seems, to some extent, to be related to changes in nutrient content. Our results show that modest defoliation (LH treatment) caused an increase in N concentration in leaves, possibly accounting for the increase in leaf elongation. This increase is more likely to be due to uptake stimulation than to reserve mobilization, as the N concentration in roots and rhizomes remained unaltered. In contrast, intense defoliation (HH treatment) caused an overall reduction in N content (in leaves, rhizomes and roots). Although N uptake stimulation due to defoliation is a common response elsewhere (Jaramillo and Detling, 1988; McNaughton et al., 1996), such stimulation did not occur or was unable to compensate for the N losses in *C. nodosa*. The decrease in N content not only in leaves, but also in rhizomes and roots, suggests a mobilization of nutrients from the belowground organs to the aboveground parts to maintain leaf growth rates and to support the production of new modules (leaves and shoots), resulting in a dilution into the new biomass of N pools and increasing aboveground primary production (Valentine et al., 1997). Incidentally, this depletion of N may have consequences for the palatability of the tissues, as the intense defoliation caused a decline in the nutritional quality of leaves and rhizomes by increasing C:N ratios. Plant quality (often expressed as C:N ratio) has been shown to play a central role in determining herbivore feeding patterns in marine habitats (Cebrián and Duarte, 1998; Barile et al., 2004; Duarte et al., 2011), and some authors have suggested that a low leaf N concentration can act as a plant defense against grazing (Augner, 1995). To what extent this reduction in plant nutritional quality is an adaptive response or a mere consequence of nitrogen loss and dilution, as explained above, remains unclear. In any case, it should be noted that nutrient availability may play an important role in determining the capacity of a plant to compensate for tissue loss (Hay et al., 2011), as has been demonstrated, among others, by Li et al. (2010), who reported that individuals of *Vallisneria spiralis* growing in nutrient-rich habitats were better able to compensate for damage than those plants growing in nutrient-limited habitats, where they were unable to acquire the necessary amount of nutrients to replenish biomass loss.

Nevertheless, the compensatory responses reported are not only facilitated by N mobilization or uptake, but also by the use of carbohydrate reserves. It has been shown that *C. nodosa*, like other seagrasses, has the capacity to store carbohydrates, building up reserves in late summer, and translocating these reserves to support shoot growth from early spring to mid-summer (Mascaró et al., 2014). The depletion of TNC after clipping, by 16 % (LH) and 50 % (HH) relative to controls, suggests that carbohydrate mobilization took place in response to defoliation, and part of the compensatory leaf growth (LH treatment) and the addition of new modules (LH, only shoots; and HH, leaves and shoots) was supported by these reserves. Indeed, mobilization of carbohydrate reserves appears to play a major role in the ability of plants to withstand disturbances involving the loss of aboveground tissue (Rodgers et al., 1995; Brun et al., 2003; Eklöf et al., 2008; Ruiz et al., 2009). However, the magnitude of the contribution of carbohydrates to re-growth may depend on both storage capacity and physiological integration of the plant. It should be emphasized that, despite the significant depletion of TNC in July following defoliation (especially in the HH treatment), TNC recovered, and the values in October, which were much higher than in July (ca. 2-fold, in agreement with the seasonality of the plant; Mascaró et al., 2014), were very similar among treatments.

While our results clearly indicate the ability of *C. nodosa* to develop diverse induced tolerance responses against both low and high simulated herbivory, the results for total phenolic content, which were either lower than (in the LH treatment) or equal to (in the HH treatment) control values, corroborated previous findings that this variable is not a good indicator of defense mechanisms (Sieg and Kubanek, 2013). In this respect, it should be acknowledged that total phenolic content is uninformative about the deterrent capacity of a given tissue, as phenolic compounds participate in a huge number of plant functions besides deterrence (e.g. antioxidant: Hodzic et al., 2009; antimicrobial: Vergeer and Develi, 1997; anti-fungal: Jensen et al., 1998). However, the fact that plants from the LH treatments had a 40% lower total phenolic content than controls, whereas plants from the HH treatment had similar values to controls, suggests a negative relationship between phenolic content and leaf growth. This underlines the role of phenolic compounds as primary metabolites, particularly in cell wall construction during plant growth (Abdulrazzak et al., 2006). Part of the compensatory leaf growth found in plants from the LH plots could thus have been achieved using carbon from the phenolic pool, as suggested by Vergés et al. (2008) based on results very similar to ours obtained in *P. oceanica.*

In conclusion, under low levels of defoliation, leaf losses seem to act as a stimulating cue, triggering overcompensatory responses, apparently using internal carbon sources, to which re-use of phenolic compounds seems to contribute, and external N sources. In contrast, under high levels of defoliation, leaf elongation rates are maintained, while the number of leaves and shoots increases; this compensatory response seems to be supported, at least in part, by internal carbon sources (i.e. carbohydrates supplied by the rhizome reserves) and internal N sources (i.e. N remobilization from belowground organs). At the same time, the C:N ratio increases, potentially lowering the nutritional quality of leaves. All these mechanisms contribute to the tolerance of *C. nodosa* to grazing, reducing the negative effects of herbivore consumption on plant fitness.

Acknowledgements

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CHAPTER 4

Recovery of a fast-growing seagrass from small-scale mechanical disturbances: effects of intensity, size and seasonal timing

Abstract

Seagrass ecosystems are key yet threatened coastal habitat commonly exposed to a wide range of disturbances. The ability of these ecosystems to recover will to a large extent depend on the plant traits and on the characteristics of the disturbance. We studied the effects of small-scale mechanical disturbances on recovery of the fast-growing species *Cymodocea nodosa*. We investigated the effects of the intensity (shoot and entire plant removed), size of the area disturbed (0.04 m^2, 0.25 m^2 and 1 m^2) and seasonal timing (spring and autumn) of disturbance. We monitored recovery by measuring shoot density and canopy height over 27 month and, at the end of each experiment, we also measured different plant traits. *C. nodosa* recovered canopy height and shoot density in 1 mo by shoot regrowth when only shoots were removed. In contrast, it took up to 10-25 mo when the entire plant (shoots, rhizomes and roots) was removed. Unexpectedly, small (0.04 m^2) disturbances took longer to recover than large ones (1 m^2), probably due to limited light availability or to the high accumulation of detritus. *C. nodosa* plots disturbed in autumn took 9 mo longer to recover than those disturbed in spring. After the 27-month recovery period, some traits such as rhizome biomass, did not fully recovered. These results show the relatively high resilience of this seagrass to mechanical disturbance, and highlight the role of the belowground parts for this resilience. Given that rhizome biomass is the only plant trait not having fully recovered after 27 months, our results warn against the negative effect on the long term of repeated mechanical disturbances.

Introduction

The recovery capacity of ecosystems, that is, their ability to return to their original state following a disturbance (Holling, 1973) is one of their most relevant dynamic attributes, and, therefore, an important topic in community ecology research (Schindler et al., 1991; Cole et al., 2014). Disturbances are discrete events that can drastically change community structure, resource availability and/or the physical environment (Pickett and White, 1985). Moderate disturbances play fundamental roles in ecosystem function as important drivers of patch dynamics (Kuuluvainen and Aakala, 2011) or diversity changes (Svensson et al., 2009), triggering successional mechanisms (Chang and Turner, 2019) and framing evolutionary processes (Benmayor et al., 2007). However, high-intensity disturbances can seriously alter ecosystem integrity in the long term, with concomitant losses in ecological functions and services. Although there is a large array of natural disturbances, including storms, floods, hurricanes and many others, the ever-increasing and pervasive effects of human activities with consequences for the natural environment have not only increased the panoply of disturbance types and/or increased the intensity and frequency of existing ones, but they have also altered the natural mechanisms of recovery (Anderson-Teixeira et al., 2013). Under this scenario, which is expected to further worsen in the coming decades, it seems crucial not only to increase our ability to predict the consequences of different kinds of disturbances on ecosystems, but also to deepen our understanding of their capacity to recover, including the mechanisms and the time scales involved. To date, the former aspect has been explored much more than the latter.

This general framework seems particularly relevant for coastal environments, where multiple and often interacting, superimposed pressures elicit significant losses in integrity losses and the degradation of coastal ecosystems (Orth et al., 2006; Waycott et al., 2009; Unsworth et al., 2015). Seagrass meadows, well known for their biological and ecological relevance (Green and Short, 2003), are an example of this. Seagrass meadows provide key ecosystem services including shoreline stabilisation, nutrient cycling, provision of food and habitat for other organisms, carbon sequestration and trophic transfer to adjacent habitats (Hemminga and Duarte, 2000; Barbier et al., 2011; Ricart et al., 2015). Despite some conservation efforts, seagrasses are continuing to decline globally (Waycott et al., 2009; Short et al., 2011; but see de los Santos et al., 2019), due to a number of reasons, such as the detioration of water quality, changes in sedimentary regime and mechanical disturbances (Short and Wyllie-Echeverria, 1996; Erftemeijer and Lewis, 2006; Unsworth et al., 2015).

Mechanical disturbances are among the most destructive forces acting on seagrasses (Duarte et al., 2004). These can occur at a huge range of spatial scales (from hundreds of kilometres to a few square centimetres, Gera et al., 2014; Unsworth et al., 2017), and can be caused by natural factors, either biotic (e.g., grazing, bioturbation) or abiotic (e.g., wave action, storms), and also by human activities (e.g., boat propellers, anchoring or trawling). All this can break clonal networks, remove seagrass biomass, alter the seagrass canopy, eliminate the seedbank, modify the physical and chemical properties of the substrate and destabilize the sediments (Short and Wyllie-Echeverria, 1996; Cabaço et al., 2005), with profound consequences on plant performance from both subindividual and individual level to population level, and ultimately on the integrity and function of the whole system.

Irrespective of their origin, mechanical disturbances are mostly discrete events, potentially allowing enough time for ecosystem recovery. This recovery can be achieved by different mechanisms, including vegetative growth (shoot growth and rhizome extension) (Rasheed, 2004; Kenworthy et al., 2002), clonal dispersal (fragments) and sexual reproduction (seeds) (Berkovic et al., 2014). The mechanisms involved in recovery and its time scale will primarily depend on the biological traits of the species concerned and on the characteristics of the disturbance occurred (Barrett et al., 2008; O'Brien et al., 2017). For instance, plant traits such as high shoot turnover, high rates of rhizome elongation and high investment in sexual reproduction may promote recovery. These traits are typical of small fast-growing species with colonising and opportunistic strategies (*sensu* Kilminster et al., 2015), which recover faster than large, slow-growing and persistent species (Duarte et al., 1997; Kilminster et al., 2015). Moreover, recovery time may vary with the intensity and the frequency of the disturbance, which determine the extent and spatial pattern of habitat that is lost (Peterson et al., 1987; Barrett et al., 2008). At this respect, recovery can be greatly delayed if the disturbance increases the fragmentation of the meadow (Unsworth et al., 2015). In addition, the morphological and physiological traits relevant for recovery vary significantly during the year in species showing high seasonality (Alcoverro et al., 2001; Duarte, 1989; Laugier et al., 1999), making the time of the year at which the disturbance occurs a key aspect of recovery. For instance, seagrasses suffering disturbance at the end of the growing season showed delayed recovery than those disturbed at the beginning (Soissons et al., 2016). Despite their relevance for seagrass management and conservation, all these issues remain poorly explored.

A deep understanding of the mechanisms underlying seagrass recovery from mechanical disturbances is essential not only for predicting its dynamics under different disturbances and environmental scenarios, but also for preventing potential shifts to undesirable alternative stable states (Connell et al., 2017). Despite recent advances in the field (Smith et al. 2016; Soissons et al., 2016; Macreadie et al., 2017), there is still scarce information that is restricted to only a few species and a few types of mechanical disturbances. This limits our ability to predict the long-term consequences of disturbances on seagrass ecosystems, which ultimately can result in inappropriated management practices.

This study aimed to fill these gaps in research by exploring the recovery of the seagrass *Cymodocea nodosa* (Ucria) Ascherson from small-scale mechanical disturbances. *C. nodosa* is a small fast-growing species with a wide ecological range and a high phenotypic plasticity (Pérez et al., 1994; Cancemi et al., 2002; Mascaró et al., 2009). In the temperate areas it inhabits, it shows strong seasonality, with maximum growth in late spring and a quiescent phase in late autumn and winter (Mascaró et al. 2014). Although the effects of different types of environmental alterations on this species have been investigated (Marbà and Duarte, 1994; Malta et al., 2006; Pagès et al., 2010; Sandoval-Gil et al., 2012; Pérez et al., 1994; Ontoria et al., 2019), there are very few studies on the trajectories of *C. nodosa* meadows once (or in the case) these alterations have ceased (Cabaço et al., 2010). We used manipulative field experiments simulating small-scale mechanical disturbances to investigate how recovery of *C. nodosa* proceeds and, specifically, how it varied with: 1) the intensity of disturbance (shoot removal vs. entire plant removed, shoots and rhizomes), 2) the size of the area disturbed (from 0.04 to 1 m^2) and, 3) the seasonality of the disturbance (spring vs. autumn). We assess recovery by monitoring canopy height and shoot density of *C. nodosa* over 27 months and by measuring some physiological, morphological and structural plant traits at the end of the experiments.

Material and methods

Study system

The study was conducted in Alfacs Bay, northwestern Mediterranean. This is a semi-enclosed and relatively shallow bay (maximum depth of 6 m) with extensive *C. nodosa* meadows thriving on marginal sandy platforms (<1.5 m depth; Oliva et al., 2012). The experimental plots were set up on the southern shore of the bay, where healthy meadows develop and reaching maximum density (around 2500–3000 shoots m^{-2}) in May–June (Mascaró et al., 2014).

Experimental design and monitoring

We conducted three experiments to assess the recovery patterns of *C. nodosa* from small-scale mechanical disturbances. Experiment 1 was designed to determine how the intensity of the disturbance (i.e., only a part of the plant or the entire plant removed) affected recovery. We used three treatments applied in June 2015 to plots of 0.25 m^2 surface: low intensity (50 % of the shoots removed, vertical and horizontal rhizomes left), moderate intensity (100% of the shoots removed, vertical and horizontal rhizomes left) and high intensity (entire plant removed, shoots, rhizomes and roots). Experiment 2 was aimed at evaluating the effects of the size of the disturbed area on recovery. We used three treatments applied in June 2015 to plots of different size: small (0.04 m^2), medium (0.25 m^2) and large (1 m^2), in which the entire plant (shoots, rhizomes and roots) was removed as in the high intensity treatment of experiment 1 (Fig. 1a, b). Finally, in Experiment 3, we assessed the influence of seasonality (i.e. the time of the year when the disturbance occurred) on recovery. To this end, we used two treatments applied to plots of 0.25 m^2: spring (treatment applied in June 2015) and autumn (treatment applied in October 2015), in which the entire plant was removed as in the high intensity treatment of Experiment 1. In all cases, we maintained undisturbed plots as procedural controls. Each treatment (including controls) was replicated (n = 3) in randomly placed plots that were separated by at least 5 m from one another. The whole set of experiments was replicated at two sites within our study area (distance > 2 km). The experimental setup was deployed between 0.6-0.8 meters deep.

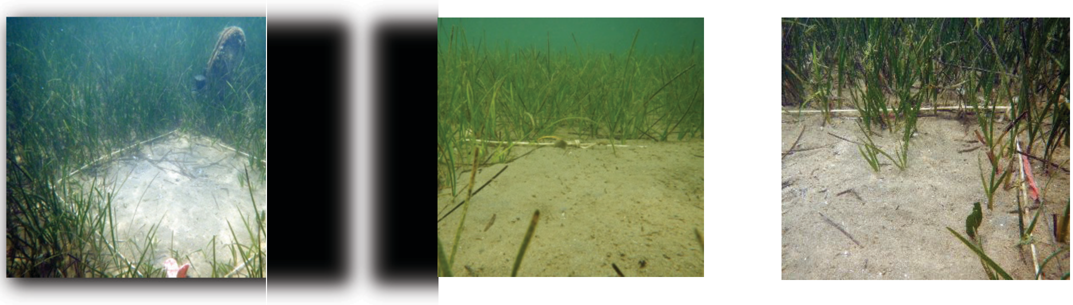

Fig. 1. Experimental plots of large experimental treatment (1 m^2) in *C. nodosa* meadows (a, b) and runners colonizing the disturbed area (c).

To assess recovery at the stand level, we measured canopy height, with a plastic ruler (ignoring the tallest 20% of the leaves; Duarte et al., 2001) and shoot density (counting the number of shoots in the whole plot or in a permanent quadrat measuring 20 x 20 cm when there were more that 200 shoots). Monitoring was more frequent during the first 3 months (maximum growth period, May-June) than in the rest, and concluded after a total of 15 monitoring events (September 2017), except for testing the effects of disturbances in autumn on recovery of Experiment 3 (beginning in October 2015 and ending after 8 monitoring events in September 2017), and investigation of the low- and moderate-intensity disturbances in Experiment 1, which ended in September 2015 after complete recovery had been achieved, with a total of 6 monitoring events. Other relevant details such as the presence of seedlings or accumulation of detritus were also monitored. To assess recovery at the sub-individual and individual plant level, we measured traits from samples (including shoots and rhizomes) collected with a 15 cm diameter core, at 3 mo (for the low and moderate-intensity treatments of Experiment 1, September 2015) and 27 mo (for the rest of the experiments, September 2017) after the disturbances. At the time of collection, all the treated plots showed complete recovery in shoot density and canopy height (i.e., lack of significant differences with control plots, see below).

Measurements of plant traits in the laboratory

In the laboratory, all plant samples were sorted into leaves and rhizomes. We then measured the shoot surface (cm^2) and the number of leaves per shoot in a subsample of 15 shoots chosen at random from each sample, as well as the number of apical shoots and the internodal distance (cm) in all the rhizomes from each sample. We then dried the material at 70°C for 48 h (until constant weight) and weighed it to obtain shoot and rhizome biomass. Subsamples were kept for biochemical analysis: nitrogen content in leaves and rhizomes and total non-structural carbohydrates (NSC) in rhizomes. The nitrogen content of leaves and rhizomes was determined using a Carlo-Erba Elemental Autoanalyzer (Scientific and Technical Services of the University of Barcelona). Total NSC (soluble sugars and starch) amounts were measured in rhizomes using the anthrone assay described in Marín-Guirao et al. (2013), which is based on the method of Invers et al. (2004) and Yemm and Willis (1954).

Statistical analysis

In all the analyses, the plots were considered the experimental units (n=3) (averaging the values when more than one measure per plot was taken). To determine the time of recovery for each experiment and each sampling time, we compared canopy height and shoot density (dependent variables) in plots from each treatment against control plots of the same experiment and time, using two-way ANOVA (treatment and site, site was considered random, with two levels). Recovery was considered achieved when the treated and control plots did not show differences in any of the two dependent variables.

To assess differences in *C. nodosa* traits among the treatments at the end of the experiments, we used again two-way ANOVA again, with treatment (fixed) and site (random) as independent variables. For Experiment 1, we assessed the differences in plant traits after 3 mo for control, low- and moderate-intensity plots, and at the end of the experiment (27 mo) for control and high-intensity plots. Whenever the ANOVA detected significant differences, we applied multiple range contrast tests (Tukey's HSD post-hoc test) to determine specific treatment differences. All variables were individually checked for normality, homogeneity of variance and outliers, and using exploratory data analysis procedures (e.g., QQ plots), and parametric tests (Shapiro-Wilks test for normality and Bartlett's test for homoscedasticity). When necessary, data were transformed to achieve normality. All analyses were performed using the R statistical software (R Core Team, 2017).

Results

Experiment 1: intensity of disturbance

Plots subjected to low- and moderate-intensity disturbances (50 % and 100 % of the shoots removed, respectively, and rhizomes left) recovered much faster (within 1 mo) than those subjected to submitted to high-intensity disturbances (entire plant removed), which took 4 and 12 mo to reach the control values for canopy height and shoot density, respectively (Fig. 2; Table 1; Table S1). Three months after the disturbance, plants from the plots subjected to low- and moderate-disturbances showed no significant differences relative to controls in any of the traits measured (Fig. 3; Fig. S2; Table S4), thus indicating that complete recovery. Twenty-seven months after the high-intensity disturbance (removal of shoots plus rhizomes), the only difference between the treated and control plots was in rhizome biomass, which was 40% lower in the treated plots (Fig. 3d; Table S4).

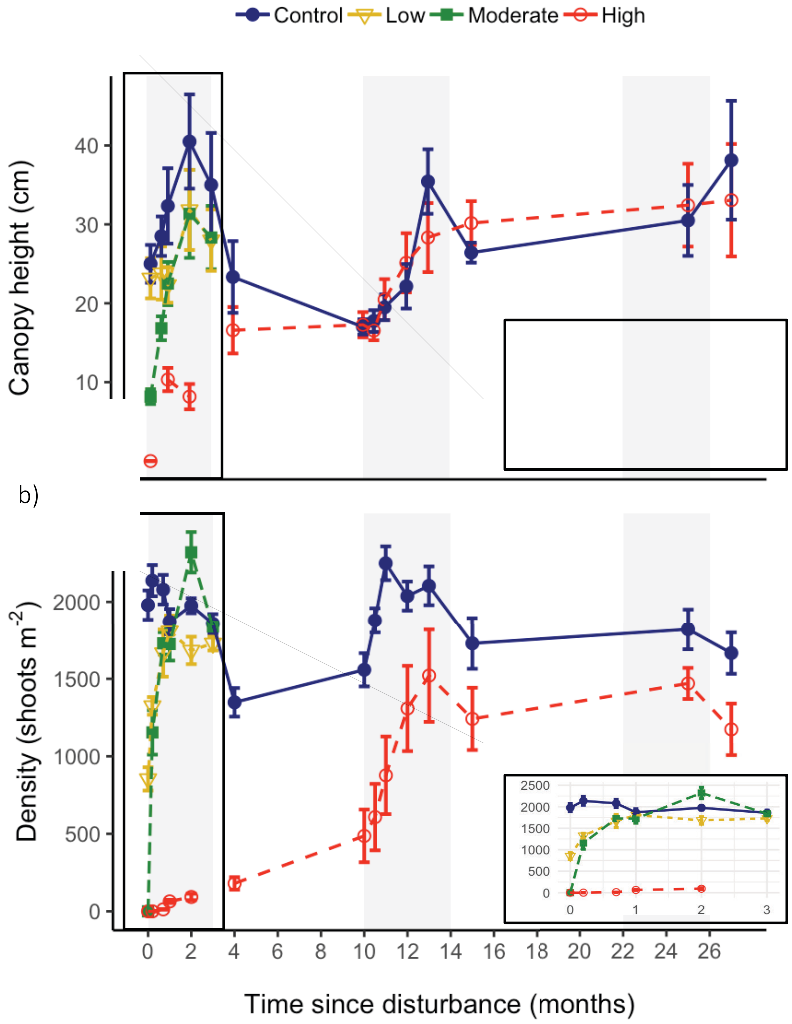

Fig. 2. a) Canopy height (mean ± SE, *n*=3) and b) shoot density (mean ± SE, *n*=3) of *Cymodocea nodosa* over the 27 mo of duration of the experiment in the different experimental intensity treatments (control, low, moderate and high). Grey areas represent spring and summer months and white areas fall and winter months. The first three months are amplified for the sake of clarity.

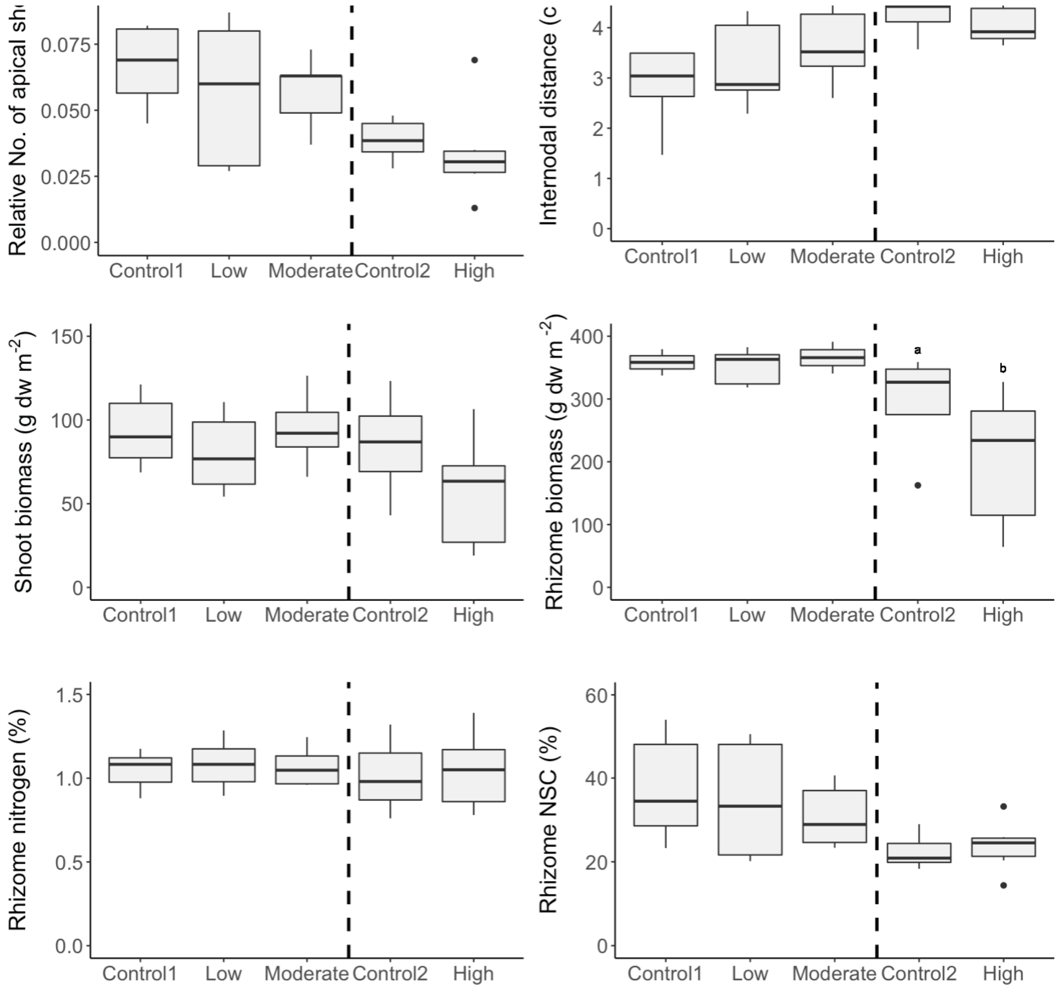

Fig. 3. Boxplots representing different plant traits: a) relative number of apical shoots, b) internodal distance, c) shoot biomass, d) rhizome biomass, e) nitrogen content in rhizomes and f) non-structural carbohydrates (NSC) content in rhizomes of *Cymodocea nodosa* after 3 mo (for low and moderate intensity treatments), and after 27 mo (for high intensity treatments) since experimental disturbances were applied (*n*=3). Plots labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Symbols (•) represent outliers.

Experiment 2: size of disturbance

Canopy height and shoot density recovered faster in large- (1 m^2) and medium-sized (0.25 m^2) plots than in small-sized (0.04 m^2) plots. Canopy height inn large- and medium-sized plots took 10 and 4 mo to reach the control values, respectively, while shoot density took 13 and 12 mo, respectively (Fig. 4a, b; Table 1). Canopy height and shoot density in small-sized plots reached control values after 10 and 25 mo after the disturbance, respectively. At the end of the experiment (27 months), there were no differences in plant traits between the disturbed plots and the control plots (Fig. 4; Fig. S3; Table S4), with the exception of shoot biomass which was lower in the small-sized plots than in controls, and rhizome biomass, which was lower in all the treated plots compared to control (74% in the large-sized plots, 40% in the medium-sized plots and 61% in the small-sized plots) (Fig. 5c; Table S4).

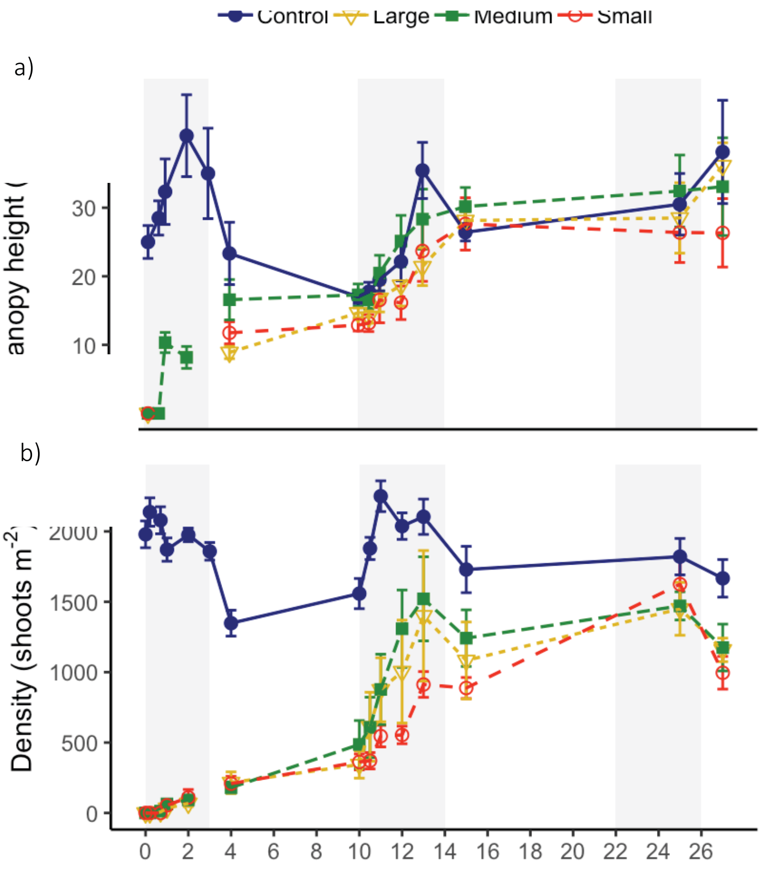

Fig. 4. a) Canopy height (mean ± SE, *n*=3) and b) shoot density (mean ± SE, *n*=3) of *Cymodocea nodosa* over the 27 mo of duration of the experiment in the different size experimental treatments (control, large, medium and small). Grey areas represent spring and summer months and white areas fall and winter months.

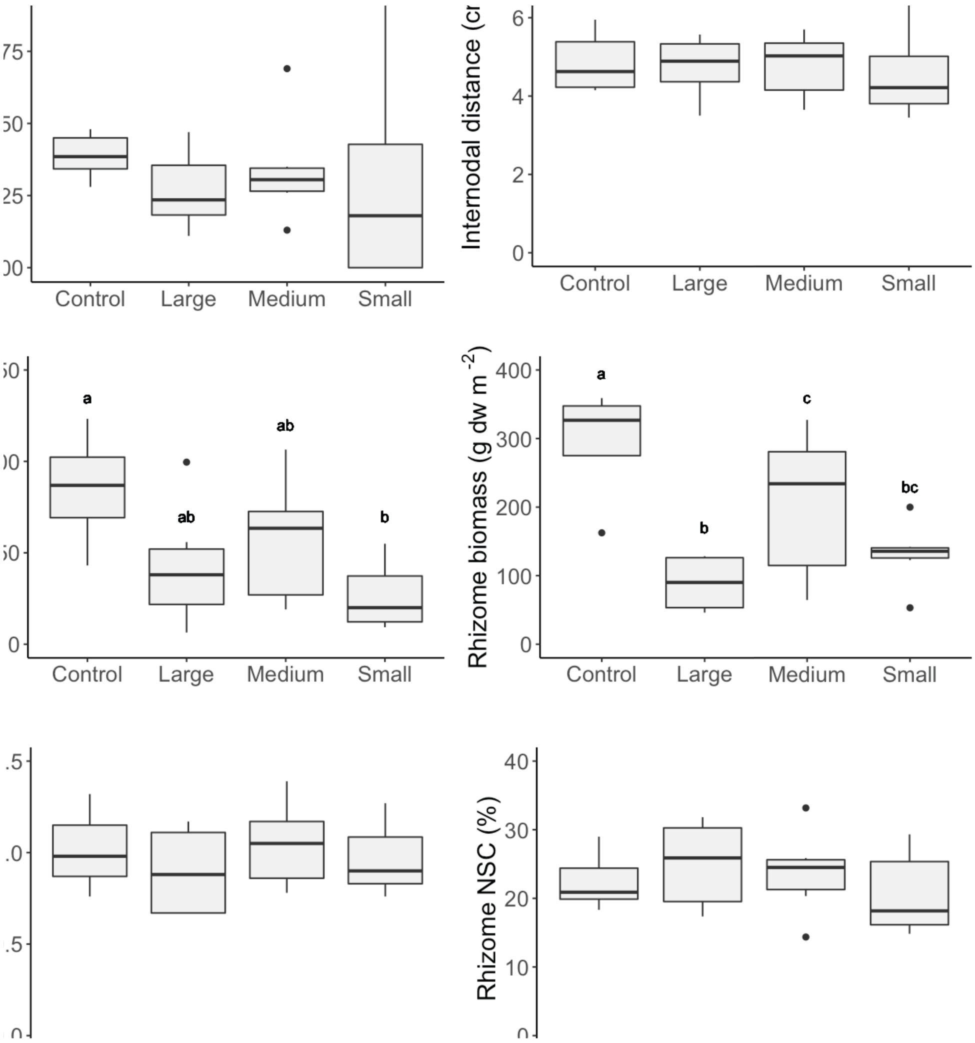

Fig. 5. Boxplots representing different plant traits: a) relative number of apical shoots, b) internodal distance, c) shoot biomass, d) rhizome biomass, e) nitrogen content in rhizomes and f) non-structural carbohydrates (NSC) content in rhizomes of *Cymodocea nodosa* at the end (27 mo) of the size experiment (*n*=3). Plots labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Symbols (•) represent outliers.

Experiment 3: seasonal timing of disturbance

The season in which the disturbance occurred did affect the recovery of *C. nodosa*, with the plots disturbed in spring those taking the shortest time to recover. Canopy height and shoot density in these plots took 4 and 12 mo to reach the control levels, respectively, while those in the plots disturbed in autumn took 7 and 21 mo (Fig. 6a, b; Table 1). Among the plant traits measured 27 mo after disturbance in disturbed plots, there were significant differences with controls only in the rhizome biomass (40% and 55% lower in plots disturbed in spring and autumn, respectively, than in control; Fig. 7d; Table S4). No significant differences were found in the other plant traits except for the nitrogen content of rhizomes, which was 29% lower in plots disturbed in autumn compared to controls (Fig. 7e; Table S4).

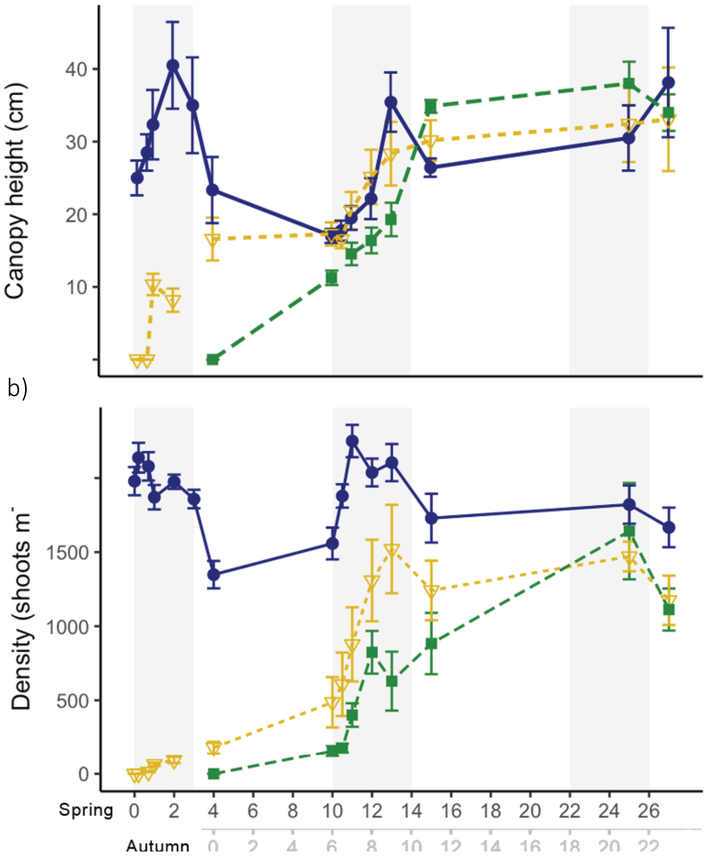

Fig. 6. a) Canopy height (mean ± SE, n=3) and b) shoot density (mean ± SE, n=3) of *C. nodosa* over the 27 mo of duration of the experiment, in spring- and autumn-disturbed plots. Grey areas represent spring and summer months and white areas fall and winter months. Black x-axis indicates the time since disturbance of spring treatment while grey x-axis corresponds to autumn treatment.

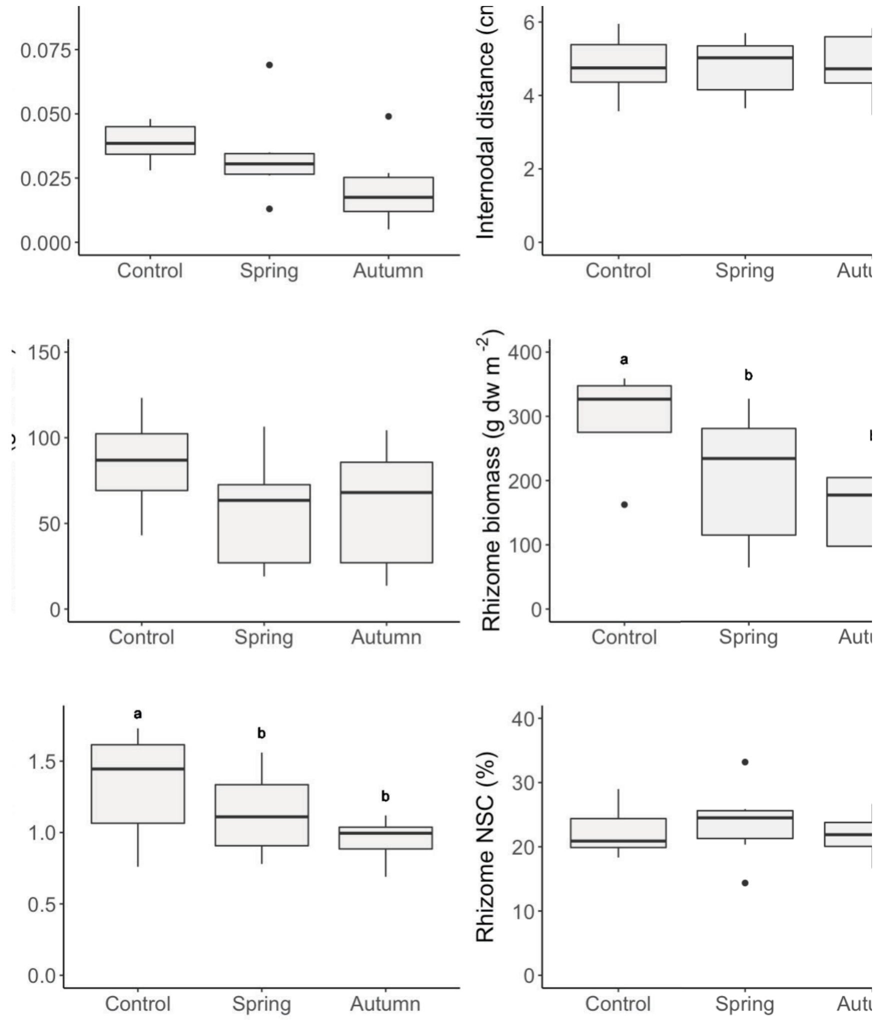

Fig. 7. Boxplots representing different plant traits: a) apical shoots, Relative number of apical shoots, b) internodal distance, c) shoot biomass, d) rhizome biomass, e) nitrogen content in rhizomes and f) non-structural carbohydrates (NSC) content in rhizomes of *Cymodocea nodosa* at the end of the experiment (*n*=3), 27 mo since disturbance. Plots labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Symbols (•) represent outliers.

Additional observations

During the 27 mo of repeated visits to the experimental areas, we observed that in most of the plots submitted to treatments in which the entire plant was removed (Experiment 1, high intensity treatment, and experiment 2 and experiment 3) new runners from the surrounding intact meadow had entered the cleared area a few weeks after the experimental disturbance. At the end of the experiments, we also found a reduced number of seedlings in the disturbed plots, which were more abundant in the control plots probably because the seeds were removed when extracting the rhizomes from the disturbed plots. Finally, we also observed an accumulation of detritus in the disturbed plots, which was especially high in the small-sized plots (0.04 m2) of Experiment 2.

Table 1. Recovery times (months) of canopy height and shoot density after disturbance in the different treatments for all experiments (intensity, size and seasonal timing). Recovery is considered when values of the treatment were no significantly different from the control ($p > 0.05$).

Type of disturbance	Treatment	Canopy height	Shoot density
		(Time since disturbance, mo)	
INTENSITY	Low	1	1
	Moderate	1	1
	High	4	12
SIZE	Small	10	25
	Medium	4	12
	Large	10	13
SEASONAL TIMING	Spring	4	12
	Autumn	7	21

Discussion

The seagrass *Cymodocea nodosa* showed a relatively high capacity to recover from small-scale mechanical disturbances, at least in terms of aboveground meadow integrity (shoot density and canopy height). This recovery was especially fast (a few weeks after the disturbance) when the rhizomes were not damaged. However, when the disturbances affected the entire plants (including rhizomes and roots), the recovery took longer from one to two years. In the first case, the main recovery mechanism involved seemed to be shoot regrowth, probably fueled by local mobilization of resources stored in the rhizomes or by resources conveyed through the intact rhizomes from nearby unaltered meadow. In the second case, recovery was driven by clonal propagation based on horizontal rhizome growth from surrounding plants, and only to a minor extent, by germinating seeds. The recovery time was also affected by the size of the area disturbed (paradoxically, slower for small-sized areas (0.04 m^2) than in medium-sized and large areas, up to 0.25 m^2) and the season when the disturbance occurred (faster for plant disturbed in spring). Once complete recovery achieved in terms of canopy height and shoot density, most of the plant traits measured were similar to undisturbed plots, with the exception of rhizome biomass, which remained below the control values. This represents a kind of legacy of the disturbance, potentially affecting the ecological functions and, specially, their capacity to recover after repeated disturbances.

When only the shoots were removed (50 % or 100%), *C. nodosa* recovered quickly and after 1 mo there were no differences between control and disturbed plots for shoot density and canopy height. This fast recovery, as indicated above, was based on the capacity of shoot regrowth as no evidences of horizontal growth of rhizomes were observed. As in other clonal plants (Ferraro and Oesterheld, 2002), regrowth can be achieved by using the resources from both the rhizomes and the ramets from the neighboring intact parts of the meadow. The mobilisation of resources stored in rhizomes and the translocation of resources over up to 50 cm through horizontal axis have already been demonstrated in this species (Sanmartí et al., 2014: Terrados et al., 1997). However, unexpectedly, we did not find depleted resources (nitrogen, carbohydrates) in rhizomes 3 mo after the disturbance. This was probably due to the time that had elapsed (2 mo) between complete recovery and sampling, which was enough to rebuild the rhizome pools.

When the entire plant was removed (high-intensity disturbance), clonal integration was lost and recovery primarily relied on vegetative growth (Rasheed et al., 2004; Macreadie et al., 2014), that is, horizontal rhizome elongation and branching and, consequently, recovery took much longer, up to one year for shoot density. This recovery time is within the range reported by similar studies applying small-scale mechanical disturbances, spanning from a few months for *Halophila decipiens* to several years, as is the case for *Thalassia testudinum* (Kenworthy et al., 2002; Rasheed et al., 2004; Smith et al., 2016). A short time after the disturbance (three weeks), we observed a few shoots in the denuded plots that had originated from the linear, fast growing horizontal rhizomes (runners) of plant from the surrounding intact meadow. These rhizomes were able to elongate up to 20 cm during this period (direct field observations, consistent with the previous findings of Duarte and Sand-Jensen, (1990)). However, rebuilding the rhizome structure did not result, however, in any change in the architecture of the plant, as the internodal distance and the number of apical shoots did not differ between the disturbed plots and controls plants 27 mo after the disturbance. Furthermore, there were no changes in the other plant traits (number of leaves per shoot and leaf surface) or the resources stored in the rhizomes (nitrogen and carbon reserves). Interestingly, despite the full recovery of the stand and most of the plant features, rhizome biomass was still lower in all high-intensity disturbed plots compared to control at the end of the experiment. Since the life-span of rhizomes is up to 5-7 years (Pérez et al., 2001), the rebuilding of rhizome biomass and, consequently, the recovery of the full integrity of the meadow, might not have been achieved during the period of our study. Given the importance of rhizomes in the quick recovery of *C. nodosa* from low- and moderate-intensity mechanical disturbances, the finding of an incomplete rhizome recovery suggests a significantly reduced recovery capacity in seagrasses exposed to a heavy disturbance affecting the rhizomes.

The effects of the size of the disturbed area are not obvious. Kenworthy et al. (2002), who studied the effect of the size of the disturbance (0.25 m^2, 1.0 m^2 and 2.25 m^2) on the recovery of three different seagrass species (*Thalassia testudinum, Syringodium filiforme* and *Halodule wrighgtii*), found no significant effects of size. Unexpectedly, we observed that recovery took longer (25 months) in small plots (0.04 m^2) than in large (0.25 and 1 m^2) ones (12-13 months). We suggest two alternative explanations for this finding. The first one concerns the decrease in available light in the small plots due to the shading caused by the surrounding standing canopies (which are up to 30 cm in height). This limited availability of light can inhibit lateral shoot development (Olesen and Sand-Jensen, 1994), delaying recovery. A second explanation is the accumulation of detritus, which was much higher in the small plots than in the large ones probably because they acted as traps for the fallen leaves and other litter circulating within and outside the seagrass meadow (Agawin and Duarte, 2002). This accumulation of detritus could act as a barrier for seagrass recolonisation by reducing the light available at the sediment surface or by stimulating the production of H_2S at the sediment-water interface (Kenworthy et al., 2002). Moreover, 27 mo after the disturbance, shoot biomass was still lower in small disturbed plots than in controls and shoots slightly smaller (in terms of the average leaf surface area per shoot) providing further support to our explanations, as both light limitation or an excess of sulphide can reduce leaf growth (Ralph et al., 2007; Holmer et al., 2005). From here, and tentatively, we conclude that disturbances that create several small gaps (0.04 m^2) might be worse (in the sense of longer recovery periods) than those that create fewer but larger gaps (1 m^2), which has implications for impact assessments and management. Although we did not perform an exhaustive search and counting of seedlings, we did observe seedlings in only large plots in very small quantities. Although merely speculative, this observation suggests that the importance of sexual propagation increases with the size of the affected area (Paulo et al., 2019).

The season in which the disturbance occurred also affected the recovery time of *C. nodosa* when the entire plant was removed. Given the strong seasonality of the species (Pérez and Romero, 1992; Mascaró et al., 2014), this is by no means surprising. Recovery was faster when the disturbance occurred at the onset of the period of maximum plant activity (spring-summer) than in autumn, when plant growth decreases and natural shoot mortality peaks. Similarly, in other temperate areas, seagrasses subjected to disturbances at the end of the growing season show delayed recovery than those disturbed at the beginning. This seasonal influence has also been found in species from other temperate areas (Soissons et al., 2016). *C. nodosa* shows high rates of leaf growth and shoot recruitment during late spring, driven by the maximum amount of incident light and the mobilisation of carbohydrate reserves (Mascaró et al., 2014). At this time of the year, recovery proceeds faster. When the unfavourable season begins (autumn), the relatively high number of shoots that remain in place promotes further recovery when the new growing season starts. By contrast, in autumn, when shoot growth and recruitment are drastically reduced, recovery is extremely slow and plots remain depauperate until the next growing season.

Understanding the recovery patterns of seagrasses is important for managers, especially in those locations where the frequency of disturbances is high (e.g. human activities, severe storms). For instance, seagrass meadows in locations where recreational boating is popular are subjected to small-scale but repeated mechanical disturbances caused by propellers, anchoring or other associated activities (Dunton and Schonberg, 2002; Kirsch et al., 2005). The consequences of this can be critical, as we show in this study, if the complete recovery of the damaged seagrass takes up to 2 years with the accumulative effects over time preventing full recovery before the next disturbance. Moreover, small-scale mechanical disturbances, which tend to reduce above- and below-ground biomass, may increase habitat fragmentation and potentially cause a permanent loss of habitat, loss of sedimentary carbon (Ricart et al., 2015), changes in the faunal community (Githaiga et al., 2019), and even increase the opportunities available for invasive species to colonise (McKenzie et al., 2014). Indeed, all of this could reduce the resilience of seagrass ecosystems to other stressors (e.g., storms, pollution or temperature change; Fonseca and Bell, 1998). For instance, disturbed meadows with significant reduced below-ground biomass will be more vulnerable and will suffer greater delay in recovery when experience a large-scale disturbance event (such as severe storms) than do undisturbed meadows. Also, if these large-scale disturbances are strong enough to eliminate a great amount of below-ground biomass, the recovery of the meadow will be seriously compromised.

To the best of our knowledge, this is the first study to explicitly examine the recovery patterns of *C. nodosa* from small-scale mechanical disturbances. Our results, together with those of previous studies (Rasheed, 1999; Macreadie et al., 2014), show that fast-growing species, such as *C. nodosa,* are highly resilient to this kind of disturbances, especially when the rhizomes are preserved. Even in the case of total defoliation, such as that caused by severe overgrazing event (Fernández et al., 2012), aboveground meadow integrity is rebuild in less than a month. This puts forward the great importance of the below-ground parts of the meadow in its persistence. Furthermore, differences in recovery among different sized areas subjected to disturbances and in relation to the season when the disturbance occurs should be considered when preserving seagrass meadows and preventing their potential collapse under human-mediated changes.

Acknowledgements

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GENERAL DISCUSSION

We are living in a changing world where species interactions and ecosystem resilience appear as key aspects for the understanding on how our biosphere resists, absorbs or is altered by the changes. While resilience helps to explain how ecosystems endure modifications in their environment, species interactions not only are the main mechanism behind ecosystem functions, but also the architecture of biodiversity (Bascompte and Jordano, 2007).

This thesis has attempted to shed some light on these issues by a series of snapshots on several case studies, showing how seagrasses respond to external drivers (stress and disturbance), how these changes affect species interactions, and what maintain species coexistence in a facilitative cascade.

Species interactions: the art of living together

Millions of species inhabit the Earth. Subsets of species live together, sharing space and resources, and thus interacting in a variety of ways. Positive interactions have become a major research focus in recent years. We deal with such interactions and with how they can be eroded (**Chapter 1**) or maintained (**Chapter 2**). By merging the results of the first two chapters, it becomes apparent that foundation species such as seagrasses, play a major role in promoting species interactions, both directly as shown by the association with the lucinid bivalves (**Chapter 1**) and indirectly, as demonstrated by the facilitative cascade involving the pen shell and the sea urchin (**Chapter 2**). From the results obtained in **Chapter 1** we provide evidences on the variability in mutualistic interactions, especially along disturbance gradients. An increase in disturbance can weaken and even disrupt mutualisms, as demonstrated before for plant-pollinator, coral reefs and plant-mycorrhiza relationships (Hoegh-Guldberg et al. 2007, Kiers et al. 2010, Burkle et al. 2013). In our case study, an increase in eutrophication (and concomitant increase of organic matter in sediment) induces morphological changes in a relevant plant trait (root morphology) which results in a lower provision of habitats for lucinids, weakening thus the mutualism. The same effect has been shown for *C. nodosa*-lucinids association along a light gradient (Almendro, 2019). The weakening or disruption of this mutualism, which is accepted as a stabilizing feedback mechanism (de Fouw et al., 2018), can accelerate seagrass meadows degradation (de Fouw et al., 2016). Results in **Chapter 1** suggest that there is a response of the plant to eutrophication provoking two effects in opposite directions: a change in plant traits allowing a better performance in reduced sediments (Pérez et al. 1994) and a decrease in the lucinid population, increasing its vulnerability to sulphide. Overall, it would seem that *C. nodosa* survival relies more on its phenotypic plasticity than on the mutualism with the lucinid bivalves and the benefits from changing root morphology seem to override the costs of losing or weakening the mutualism. In any case, it should not be discarded that the weakening of the mutualism of *C. nodosa* in eutrophic zones could also result in physiological or biochemical changes (Holmer and Bondgaard, 2001), which could reduce the sulfide tolerance threshold and even the resilience to further stressors or disturbances. This remains an open question that merits further investigation.

In **Chapter 2** we describe a habitat facilitation cascade with a potentially destabilizing feedback in which a weak effect of grazing, in part mediated by the mechanisms of plant response to herbivory (**Chapter 3)** and the carrying capacity of the pen shells population, explains the persistence of the three species system. Facilitation cascades have pervasive, indirect effects on marine ecosystems, and their role on enhancing local biodiversity and ecosystem functioning has already been demonstrated (Thomsen et al., 2010). Besides, feedbacks by the species facilitated on the facilitating ones can mediate the performance and resilience of the foundation species themselves (Derksen-Hooijberg et al., 2019). Interestingly, the three-species system we have explored (*C. nodosa-P. nobilis-P. lividus*) is absent from the northern, eutrophicated shore of the Alfacs bay (2-3 km apart from our study area), despite the existence of dense *C. nodosa* meadows. This puts forward the notion of the vulnerability of such complex interactions and, by analogy, how vulnerable is species coexistence and, ultimately, biodiversity.

It has to be reminded that, currently, pen shells populations are being dramatically depleted in the Western Mediterranean, mostly due by an infection by an haplosporidium protozoan and, more recently, by a mycobacteria as well (Darriba, 2017; Vázquez-Luis et al., 2017; Carella et al., 2019), to the point that the species has been extirpated from most locations of the Spanish coast. The mortality has been less dramatic in the Alfacs bay, but if this mortality proceeds here as in other sites, despite the legacy of dead pen shell can maintain for some time the association with sea urchins (or even create new associations, see the increase of the abundance of horned blennies in dead pen shells; Macreadie et al., 2014), in the long term the seagrass-pen shell-sea urchin assemblage will obviously be lost.

From the first two chapters of the thesis (**Chapter 1** and **Chapter 2**), and apart from what is reported in the chapters themselves, we have shown how much can be learnt from ecosystems where a relatively simple structure helps to explore facilitative interactions, together with grazing and the effects of environmental factors.

Resilience thinking

Resilience of ecosystems depends on several factors, including propagule availability, connectivity to undisturbed areas, feedbacks, biotic interactions and species traits, among others. In ecosystems reliant on a foundation species, which provide habitat for the entire community, resilience of the ecosystem is closely dependent on the resilience of the foundation species itself, and then on its traits and on its capacity to withstand or to recover from stress or disturbance.

The resilience of seagrass species relies, to a great extent on a wide array of morphological and life history traits, but also on their plasticity. Phenotypic plasticity has been already invoked in **Chapter 1**. In the case study reported there, it consisted in the capacity of the plant to modify root morphology to counteract eutrophication effects. In **Chapters 3** and **4** we describe other mechanisms involved in seagrass resilience (and therefore, in seagrass ecosystem resilience), such as growth plasticity. When a grazer attack takes place, growth is stimulated. This is usually called compensatory growth, a response mechanism commonly found in plants, both terrestrial and aquatic, that mitigates leaf biomass loss due to herbivores. *C. nodosa* was able to compensate for leaf biomass losses maintaining or enhancing growth, or promoting the onset of new modules (leaves and shoots, **Chapter 3**). The responses were slightly different depending upon the intensity of the defoliation. Thus, while low levels of herbivory triggered overcompensatory growth responses, apparently using internal carbon sources and external nitrogen sources, high levels of herbivory resulted in the maintenance of leaf elongation and the increase in the rate of formation of new leaves and new shoots, supported in part by internal carbon sources (i.e. carbohydrates supplied by the rhizome reserves) and internal nitrogen sources (i.e. nitrogen remobilization from belowground organs). Even in the case of extreme defoliation (as in **Chapter 4**), the capacity of shoot regrowth was high, based on internal resources or in the transport through the horizontal rhizomes from shoots thriving in intact meadow areas. Only when rhizomes and roots were eliminated, such as it would be the case of megaherbivores (such as dugongs, Preen, 1995;

manatees, Lefebvre et al., 1999 and in extreme cases sea turtles; Christianen et al., 2014) performing destructive grazing, seagrass recovery would slow down, relying mainly on clonal propagation based on horizontal rhizome growth from the surrounding (**Chapter 4**), but also on sexual reproduction as seen in other species, especially when they have to recover from disturbances having denuded large areas (Olesen et al., 2004). An additional lesson to be learnt from this is about the central role of below-ground biomass in seagrass resilience, and the positive consequences of its persistence both for ecological resilience (for example, to grazing) and for engineering resilience (for example, to mechanical disturbances).

The finding indicating that 1 m^2 of denuded seagrass meadow would take longer to recover when concentrated in a single patch than when distributed in several small patches (**Chapter 4**) is, to some extent, counterintuitive, and we explain it by detritus accumulation or shading effects in the smaller gaps. Although this issue would not be discussed longer here, it is suggestive of the importance of gap size in the recovery time. This should be considered when addressing issues related to mechanical damages of meadows, or in the case of destructive grazing such as that caused by megaherbivores (see above). We have no data assessing the influence of size in the case of intense defoliation but maintaining rhizome integrity, as would result from grazing by other herbivores consuming only leaves, such as fishes and sea urchins. This consumption can be very variable in space and time (Tomás et al., 2005), creating mowed patches from <1 m^2 to >300 m^2. We contend that, given the importance for recovery not only of the resources stored in rhizomes but also of the translocation of resources from surrounding meadow (from up to 50 cm; Terrados et al., 1997), if large areas are defoliated it has to be assumed that recovery time would take much longer than that reported in our small-scale experiments. If this is true, this massive defoliation would open the opportunity for colonization by fast-growing species such as *Caulerpa prolifera* as seen in grazed gaps around the pen shells (**Chapter 2**), curtailing ecosystem functions (see Pagès et al., 2012) and, potentially, would make the meadow less resilient to other stressors.

Interestingly, however, seagrass resilience to intense grazing (such as that described in Tomás et al., 2005) is enhanced by the approximate coupling between the period of maximum activity of seagrasses with the period of maximum activity of macroherbivores in the Mediterranean (Prado et al., 2007; Mascaró et al., 2014). Conversely, other disturbances typically occur in autumn and winter (e.g. storms), when plant growth clearly decreases and natural shoot mortality peaks (Mascaró et al., 2014). This, following our results, will result in longer recovery times (**Chapter 4**). Indeed, meadow persistence will not be compromised provided that the window between successive disturbances is long enough to allow meadow recovery. Nonetheless, and at this respect, the expected increase in extreme storms (Romero and Emanuel, 2017) represents an additional threat on these ecosystems (Gera et al., 2014).

Resilience and biological interactions

Our contribution to the knowledge of seagrass resilience comes from the results of **Chapters 3** and **4,** where we highlight the importance, at this respect, of biological traits and its plasticity. However, we have failed to provide new insights on how biological interactions, particularly facilitation, contribute to seagrass resilience. In **Chapter 1**, for instance, we have seen that the mutualism with the lucinid bivalves does not seem to be crucial for the persistence of *C. nodosa* under eutrophic conditions (see the first section of this discussion), which mainly relies on changes in species straits. In **Chapter 2**, although not measured quantitatively in our study, pen shells seemed to promote species diversity by facilitating not only sea urchins, but also a variety of organisms attached to them (algae, fouling invertebrates, fishes...) (Zhang and Siliman, 2019). It seems unlikely that this increase in biodiversity is critical to sustain seagrass ecosystems, although it would be worth further exploring the issue, in line with previous findings. In effect, Zhang and Silliman (2019) failed to detect any effect of pen clams on seagrass functions, but they hypothesize that this effect could be enhanced in scenarios where secondary foundation species specifically increase the diversity of key functional groups such as epiphyte grazers, and/or when bivalves are infaunal rather than epifaunal. Given the relatively low diversity of species and interactions of seagrass meadows, compared to other habitats such as coral reefs, more efforts should be done in understanding the role of biological interaction on resilience. This understanding will surely provide valuable information for management and restauration, as for example in Gagnon et al. (2020), where it is shown that co-restauration of species promotes the recovery of biodiversity and ecosystem services.

Future research

Throughout this thesis, we have explored specific questions related to biological interactions and resilience in seagrasses, providing some answers to questions addressed in the introduction. Nevertheless, the list of questions that remain open has extended more than decreased. Some of them are listed below in no particular order.

The generality of the seagrass-lucinids mutualism

In **Chapter 1** we have seen that organic enrichment of seagrass sediments indirectly weakens the mutualism seagrass-lucinids. This was, to some extent, surprising, as this mutualism protects plants against the toxic effects of sulfide (van der Geest et al., 2020). Although the correlative nature of our work calls the need for caution, our findings open the debate about the generality and importance of seagrass-lucinid interaction. Thus, more studies widening the biogeographic and environmental focus of the limited studies available up to now should shed some light to such debate, contributing to respond to questions about the generality of the mutualism, its real relevance in seagrass resilience to eutrophication and its sensitivity to environmental gradients. Moreover, very little is known about the relationship between lucinids and their gill bacteria.

How common are the feedbacks between focal and basal species?

The feedback of the focal species (in our case, sea urchin) on the foundation species reported in **Chapter 2,** has also been described in other systems such as salt marshes (Derksen-Hooijberg et al., 2019). Whether (or when) the overall result of these kind of interactions is stabilizing or destabilizing for the foundation (basal) species, and therefore for the whole ecosystem, remains to be clarified under a general theoretical framework. In order to implement such framework, we need more experimental evidence across different ecosystems. Beyond such experimental approach, the implementation of theoretical models would allow to explore such complex interactions and predict, at least qualitatively, their outcome.

How do multiple drivers affect species interactions?

In natural systems, stressors and disturbances rarely occur in isolation. Although there is a raising awareness about the threat from multiple drivers on individual, usually foundation species (Ontoria et al., 2020), this approach ignores for the most a fundamental characteristic of communities, that is, the links among the different species through diverse biotic interactions to form the interaction networks. During the elaboration of this thesis, we have observed in the field a number of drivers of change in action or emerging, such as eutrophication, mechanical disturbances by boat propellers, diseases (mycobacteria and haplosporidian protozoa on *Pinna nobilis*) and the outbreak of populations of invaders (the blue crab, *Callinectes sapidus*, whose populations boomed in 2018-2019), among others. The question is thus how these drivers (or others) and their cumulative and potentially synergistic effects will impact not only the key species but, more importantly, the species interactions. In our case, and as mere examples: would the blue crab disrupt the mutualism by preying on lucinids? Would the risk of predation depend on the level of eutrophication? Would the blue crab destabilize the coexistence of the three species assemblage by preying on sea urchins? Beyond our specific study case, this kind of questions are worthy to be addressed.

What’s the effect of positive interactions on resilience?

We still know relatively little about the role of positive interactions on the resilience of ecosystems, including facilitation cascades between ecosystems engineers (Angelini et al., 2016). Although ecological theory and perspectives on conservation hypothesize that positive interactions promote ecosystem resistance to and recovery from intensifying climatic stress (He et al., 2013), empirical evidence to confirm these predictions is scarce. Thus, a robust experimental approach is needed to better understand the processes and mechanisms underlying resilience, which in turn will help to make better management decisions. Moreover, the inclusion of all these notions in restoration programs should be considered (Renzi et al., 2019).

What is the role of other macrophytes in relation to some of the questions of this thesis?

Although we have focused on four main species to address the different questions addressed, other species can also play important roles. This is the case of the green seaweed *Caulerpa prolifera*, which in most of our study sites coexists with *C. nodosa* forming mixed meadows. While the seagrass lives close to the surface (0-2 m depth), the seaweed coexists in this bathymetric zone but extends deeper, up to 5 m. In the area of coexistence, whether these two species compete or not is far from being established. On the one hand, *C. prolifera,* which tolerate dim light conditions (Tuya et al., 2016), can survive within *C. nodosa* canopies despite shading caused by the long lives of the seagrass. On the other hand, *C. prolifera* seems better defended against herbivores than *C. nodosa* (Erickson et al., 2006; but see del Río et al., 2016). However, other interactions are unknown, as for instance a potential interference via lucinids mutualism or maybe a facilitation of one species on the other, via herbivore deterrence or others. Under the increasing environmental change scenario, it is worth to explore if the coexistence of both species increases, decreases or is neutral for ecosystem resilience.

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The only constant in life is change. This is a major challenge not only when attempting, at a better understanding of the complex dynamics of natural processes, but also when developing strategies of conservation and management. The interaction of humans and nature is, in fact, a specific case of coexistence, which needs, on the one hand, a deep knowledge of the biosphere, its mechanisms and its changes and, on the other hand a serious consideration of human attitudes and values based on sound ethical principles. At this respect, and leaving for an instant the strict academic framework, I would finish my thesis with the words of Gus Speth (environmental lawyer and environmentalist): "*I used to think that top environmental problems were biodiversity loss, ecosystem collapse and climate change. I thought that thirty years of good science could address these problems. I was wrong. The top environmental problems are selfishness, greed and apathy, and to deal with these we need a cultural and spiritual transformation and we scientists don't know how to do that…*". Me, as a scientist, I do not know how to do it either, but during the elaboration of this thesis and after the hard work behind it, I have found enough reasons to convince me that good science addressing the right questions will help us to push in that direction.

MAIN CONCLUSIONS

Chapter 1. *Seagrass-bivalve facilitative interactions: Trait-mediated effects along an environmental gradient*

An increase of organic matter in sediment (and, probably, of eutrophication), indirectly weakens the seagrass-lucinid mutualism through the modification of some plant traits. In organic-enriched sediments roots change their morphology, becoming less branched and lowering their biomass. This represents a reduction in the habitat for the bivalves, thus decreasing their abundance. The weakening of this mutualism can potentially decrease the resilience of these ecosystems to eutrophication and, therefore, compromise their persistence. Therefore, assessing the susceptibility of this and other biotic interactions to environmental change is crucial to better predict ecosystems persistence in an ever-changing world.

Chapter 2. *Habitat facilitation and herbivore behaviour determine the coexistence of a three-species assemblage.*

The facilitative cascade in which the seagrass *C. nodosa* favors the abundance of the pen shell *Pinna nobilis,* which in turn positively affects the sea urchin *Paracentrotus lividus* would seem an unstable 3-species system, given that the sea urchin consumes the seagrass. Its persistence, however, presumably hinges on the limited impact of the sea urchin on the seagrass, which in turn relies on the resilience of the seagrass to grazing, on the feeding behaviour limiting the effect of the sea urchin to the surroundings of the pen shell and on denso-dependent processes affecting both the pen shell and the sea urchin. More empirical studies are needed to develop theoretical advancements to explore not only the establishment of diverse assemblages but also the mechanisms behind their maintenance and persistence.

Chapter 3. *Tolerance responses to simulated herbivory in the seagrass* Cymodocea nodosa.

C. nodosa is highly resilient to herbivory and show slightly different responses depending upon the intensity of defoliation. Low levels of herbivory trigger overcompensatory growth responses, apparently using internal carbon sources and external N sources while high levels, result in the maintenance of leaf elongation and the increase in the rate of formation of new leaves and new shoots, supported in part by internal carbon sources (i.e. carbohydrates supplied by the rhizome reserves) and internal N sources (i.e. N remobilization from belowground organs). All these mechanisms contribute to the tolerance of *C. nodosa* to grazing, reducing the negative effects of herbivore consumption on plant performance and fitness.

Chapter 4. *Recovery of a fast-growing seagrass to small-scale mechanical disturbances: effects of intensity, size and seasonal timing.*

C. nodosa has a relatively high capacity to recover from small-scale mechanical disturbances, which depends on the intensity, the size of the area affected, and the season when disturbance occurs. When rhizomes are not removed, the recovery, driven by shoot regrowth, is very quick (few weeks after disturbance). In contrast, when the entire plant is affected, recovery, driven by clonal growth, takes longer (from 1 to 2 years). Paradoxically, small gaps (0.04 m^2) recover more slowly than intermediate and large ones (up to 0.25 m^2) probably due to a shading effect or to a high accumulation of detritus, and plots disturbed in autumn recover slower than those disturbed in late spring. The lack of a full recovery of the rhizome biomass after 2 years of disturbance represents a kind of legacy of the disturbance, potentially affecting the ecological functions but, above all, the capacity of recovery by regrowth if further disturbances occur.

Approaching the complexity of ecosystems from different and complementary perspectives, such as those addressing biological interactions (positive and negative) and resilience, helps us to a deeper understanding of the ecological dynamics in response to environmental change. Thus, integrating the knowledge from such perspectives becomes a difficult but essential challenge to manage and preserve ecosystems effectively.

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SUPPLEMENTARY MATERIAL

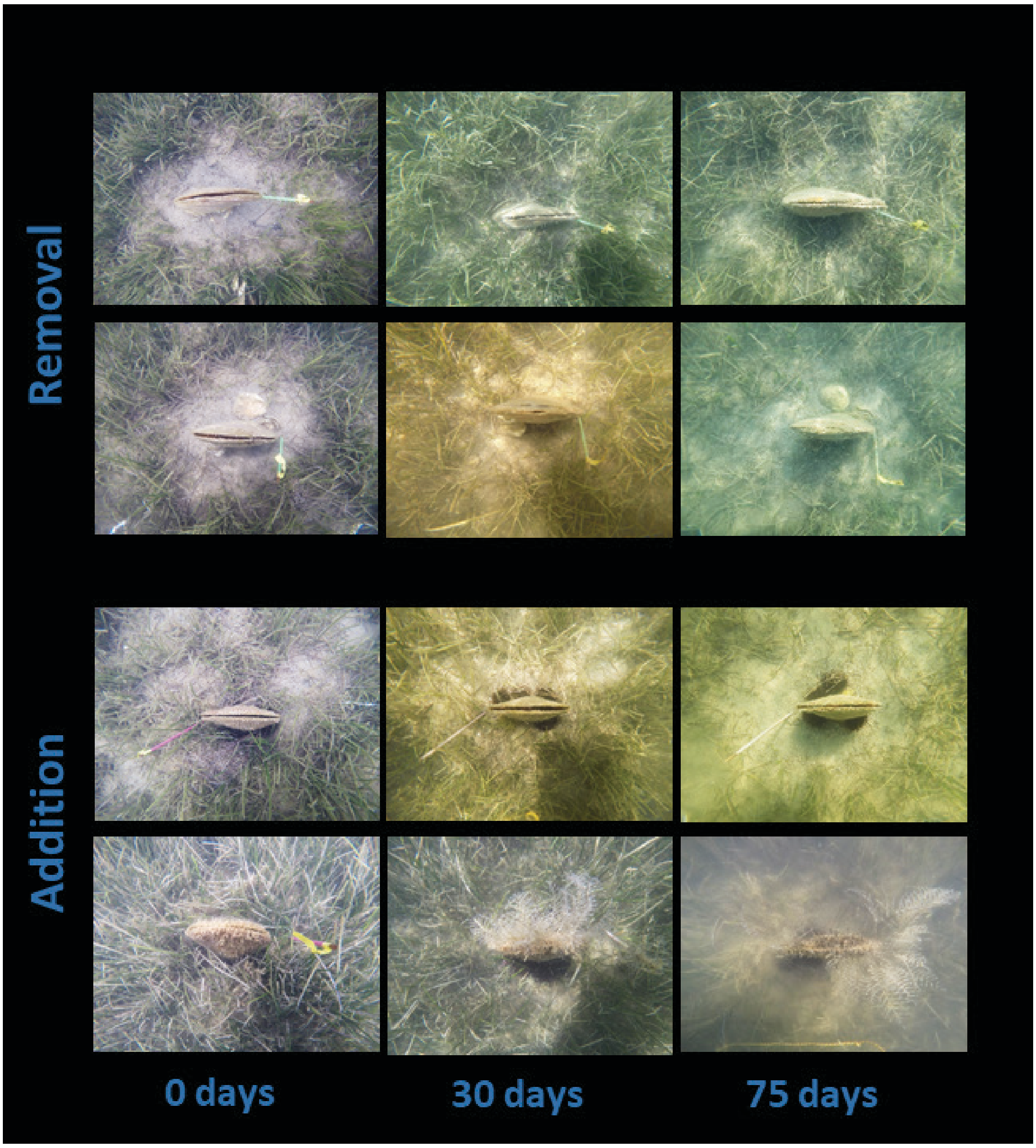

Fig. S1. View of the effect of sea urchins on *C. nodosa* grazing halos in the experiments of translocation at 0, 30 and 75 days. When the sea urchins were removed from the pen shells, the grazing halos reduced its area significantly after 75 days, however, when the sea urchins are added to the base of the pen shells, the grazing halos area increases. Two examples of each treatment are showed.

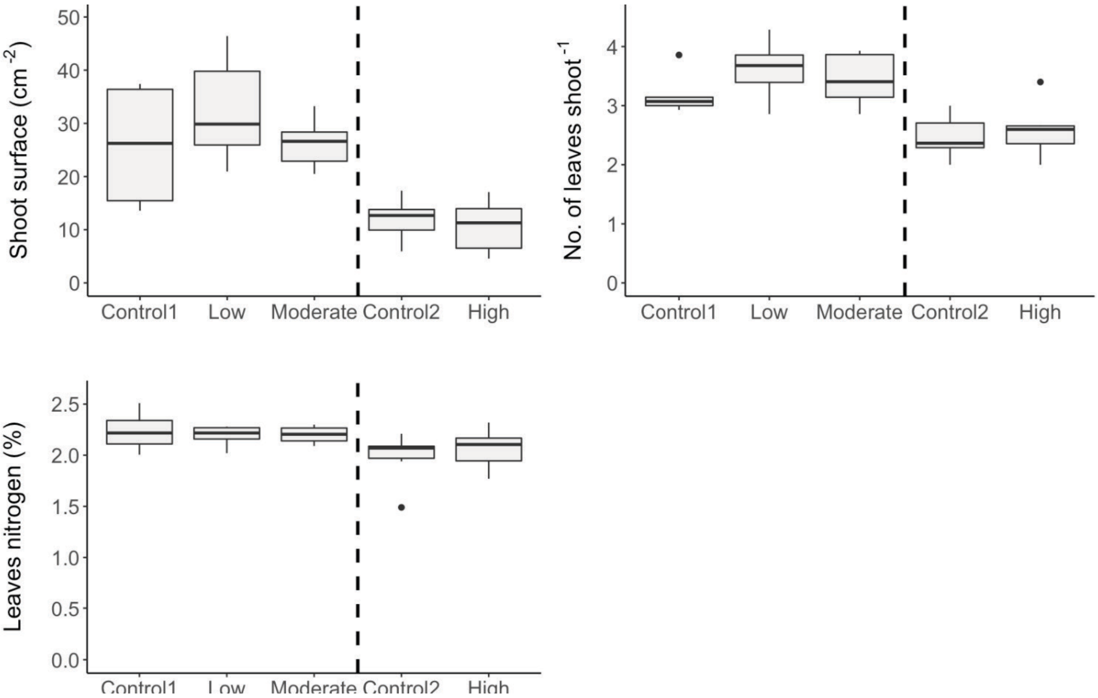

Fig. S2. Boxplots representing different plant traits: a) shoot surface, b) number of leaves shoot^{-1} and c) nitrogen content in leaves of *Cymodocea nodosa* after 3 mo (for low and moderate intensity treatments) and 27 mo (for high intensity treatments) since experimental disturbances were applied (*n*=3). Plots labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Symbols (•) represent outliers.

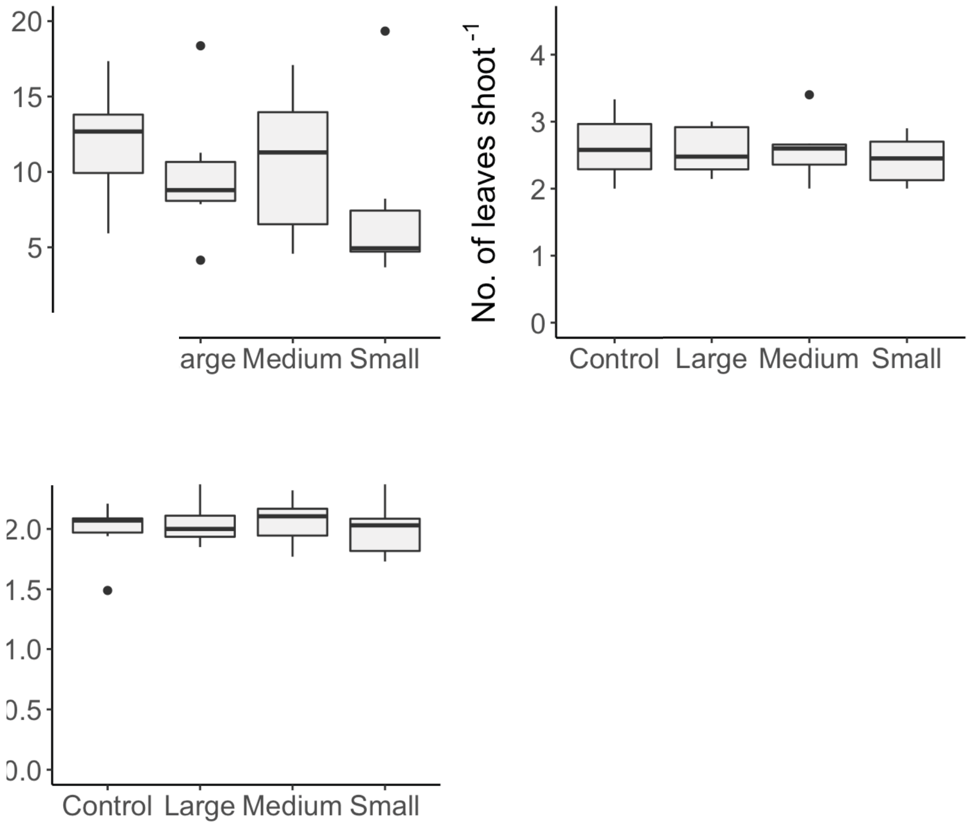

Fig. S3. Boxplots representing different plant traits: a) shoot surface, b) number of leaves shoot $^{-1}$ and c) nitrogen content in leaves of *Cymodocea nodosa* at the end of the experiment (*n*=3), 27 mo since disturbance. Plots labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Symbols (•) represent outliers.

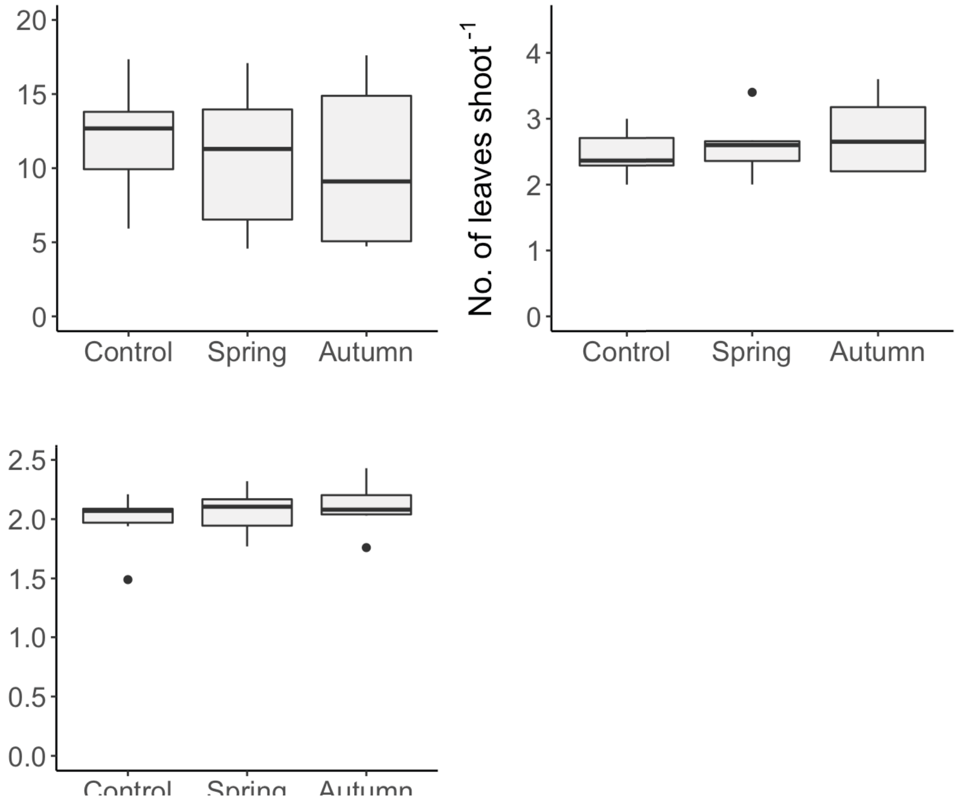

Fig. S4. Boxplots representing different plant traits: a) shoot surface, b) number of leaves shoot $^{-1}$ and c) nitrogen content in leaves of *Cymodocea nodosa* at the end of the experiment (*n*=3), 27 mo since disturbance. Plots labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Symbols (•) represent outliers.

Table S1. Two-way ANOVA assessing differences in canopy height and shoot density of *Cymodocea nodosa* of the intensity disturbance experiment at different times. From 1 to 2 mo we used 4 treatments, control, low, high, very high. From 4 to 27 mo we use 2 treatments, control and very high. (*) Asterisks indicate significant differences ($p < 0.05$). ^{a,b} and ^{c} Data sin, log and sqrt transformed to satisfy parametric test assumptions.

INTENSITY DISTURBANCE EXPERIMENT

Time of disturbance	Seagrass trait	df	MS	F	*P-value*
1 mo	*Canopy height*				
	Treatment	3	486.4	13.44	< 0.001 *
	Residual	19	36.20		
	Site residual	1	442		
	Shoot density				
	Treatment	3	4549192	117.155	< 0.001 *
	Residual	19	38831		
	Site residual	1	131868		
2 mo	*Canopy height*				
	Treatment	3	1150.8	20.49	< 0.001 *
	Residual	19	56.2		
	Site residual	1	1785.4		
	Shoot density				
	Treatment	3	5824601	156.903	< 0.001 *
	Residual	19	37122		
	Site residual	1	128042		
4 mo	*Canopy height*				
	Treatment	1	136.69	1.752	0.218
	Residual	19	78.01		
	Site residual	1	178.64		
	Shoot density ^{b}				
	Treatment	1	14.249	59.823	< 0.001 *
	Residual	9	0.238		
	Site residual	1	0.691		
10 mo	*Canopy height*				
	Treatment	1	0.224	0.024	0.880
	Residual	9	9.231		
	Site residual	1	20.909		
	Shoot density				
	Treatment	1	3446480	47.256	< 0.001 *
	Residual	9	72932		
	Site residual	1	555130		
11 mo	*Canopy height*				
	Treatment	1	2.86	0.271	0.615
	Residual	9	10.55		
	Site residual	1	186.76		
	Shoot density				

		Treatment	1	5651269	27.400	< 0.001 *
		Residual	9	206250		
		Site residual	1	379852		
12 mo	*Canopy height*					
		Treatment	1	26.7	1.512	0.250
		Residual	9	17.7		
		Site residual	1	500.5		
	Shoot density					
		Treatment	1	1221025	6.453	0.0347 *
		Residual	8	189215		
		Site residual	1	1088534		
13 mo	*Canopy height*					
		Treatment	1	151.2	2.745	0.132
		Residual	9	55.1		
		Site residual	1	579.6		
	Shoot density					
		Treatment	1	1020833	3.525	0.093
		Residual	9	289630		
		Site residual	1	541875		
15 mo	*Canopy height*					
		Treatment	1	42.19	2.158	0.176
		Residual	9	19.55		
		Site residual	1	103.84		
	Shoot density					
		Treatment	1	712969	3.345	0.101
		Residual	9	213154		
		Site residual	1	105469		
25 mo	*Canopy height*					
		Treatment	1	11.2	0.157	0.702
		Residual	9	71.6		
		Site residual	1	787.3		
	Shoot density					
		Treatment	1	367500	4.656	0.06
		Residual	9	78935		
		Site residual	1	91875		
27 mo	*Canopy height*					
		Treatment	1	23.13	0.223	0.805
		Residual	8	103.83		
		Site residual	1	4.44		
	Shoot density					
		Treatment	1	725208	4.805	0.056
		Residual	9	150926		
		Site residual	1	7500		

Table S2. Two-way ANOVA assessing differences in canopy height and shoot density of *Cymodocea nodosa* of the size disturbance experiment at different times. (*) Asterisks indicate significant differences ($p < 0.05$). [a,b] and [c] Data sin, log and sqrt transformed to satisfy parametric test assumptions.

SIZE DISTURBANCE EXPERIMENT

Time of disturbance	Seagrass trait	df	MS	F	*P-value*
1 mo	*Canopy height*				
	Treatment	1	1452.0	25.741	< 0.001 *
	Residual	9	56.4		
	Site residual	1	243.0		
	Shoot density [a]				
	Treatment	3	0.739	3.406	0.038 *
	Residual	19	0.217		
	Site residual	1	0.297		
2 mo	*Canopy height*				
	Treatment	1	3136.3	38.25	< 0.001 *
	Residual	9	82.0		
	Site residual	1	408.3		
	Shoot density [b]				
	Treatment	3	0.7394	3.406	0.03 *
	Residual	19	0.217		
	Site residual	1	0.297		
4 mo	*Canopy height* [b]				
	Treatment	3	0.933	7.474	< 0.001 *
	Residual	19	0.125		
	Site residual	1	0.136		
	Shoot density [b]				
	Treatment	3	7.166	21.58	< 0.001 *
	Residual	19	0.332		
	Site residual	1	6.188		
10 mo	*Canopy height* [c]				
	Treatment	3	0.4183	3.89	0.025 *
	Residual	19	0.1075		
	Site residual	1	0.3001		
	Shoot density [c]				
	Treatment	3	639.1	30.56	< 0.001 *
	Residual	19	20.9		
	Site residual	1	404.6		
11 mo	*Canopy height*				
	Treatment	3	27.24	1.652	0.213
	Residual	18	16.5		
	Site residual	1	308.4		
	Shoot density [c]				
	Treatment	3	689.5	17.247	< 0.001 *

	Residual	19	40.0		
	Site residual	1	343.3		
12 mo	*Canopy height*				
	Treatment	3	93.1	3.826	0.027 *
	Residual	19	24.3		
	Site residual	1	651.0		
	Shoot density				
	Treatment	3	1910521	8.735	0.001 *
	Residual	18	218722		
	Site residual	1	3143649		
13 mo	*Canopy height*				
	Treatment	3	229.9	3.809	0.03 *
	Residual	19	60.4		
	Site residual	1	748.2		
	Shoot density				
	Treatment	3	1439262	3.431	0.04 *
	Residual	19	419874		
	Site residual	1	1884401		
15 mo	*Canopy height*				
	Treatment	3	14.65	0.390	0.761
	Residual	19	37.54		
	Site residual	1	51.92		
	Shoot density				
	Treatment	3	776007	3.885	0.0254 *
	Residual	19	199779		
	Site residual	1	633750		
25 mo	*Canopy height* [b]				
	Treatment	3	0.046	0.485	0.697
	Residual	19	0.09		
	Site residual	1	0.978		
	Shoot density				
	Treatment	3	176597	1.371	0.282
	Residual	19	128805		
	Site residual	1	303750		
27 mo	*Canopy height*				
	Treatment	3	127.9	0.964	0.439
	Residual	13	1726.2		
	Site residual	1	19.07		
	Shoot density				
	Treatment	3	504470	4.845	0.011 *
	Residual	19	124116		
	Site residual	1	234		

Table S3. Two-way ANOVA assessing differences in canopy height and shoot density of *Cymodocea nodosa* of the seasonal timing disturbance experiment at different times. (*) Asterisks indicate significant differences ($p < 0.05$). [a, b] and [c] Data sin, log and squared transformed to satisfy parametric test assumptions.

SEASONAL TIMING DISTURBANCE EXPERIMENT

Time of disturbance	Seagrass trait	df	MS	F	*P-value*
1 mo	*Canopy height*				
	Treatment	1	1452	25.741	< 0.001 *
	Residual	9	56.4		
	Site residual	1	243		
	Shoot density				
	Treatment	1	9793940	437.562	< 0.001 *
	Residual	9	22383		
	Site residual	1	6120		
2 mo	*Canopy height*				
	Treatment	1	3136.3	38.25	< 0.001 *
	Residual	9	82.0		
	Site residual	1	408.3		
	Shoot density [a]				
	Treatment	1	4.811	20.284	0.001 *
	Residual	9	0.237		
	Site residual	1	0.038		
4 mo	*Canopy height* [c]				
	Treatment	2	38.94	68.895	< 0.001 *
	Residual	14	0.57		
	Site residual	1	1.26		
	Shoot density [c]				
	Treatment	2	2068.3	263.141	< 0.001 *
	Residual	14	7.9		
	Site residual	1	20.5		
10 mo	*Canopy height*				
	Treatment	2	69.73	7.888	0.005 *
	Residual	14	8.84		
	Site residual	1	10.12		
	Shoot density				
	Treatment	2	3224596	56.742	< 0.001 *
	Residual	14	56829		
	Site residual	1	452200		
11 mo	*Canopy height*				
	Treatment	2	60.76	5.293	0.019*
	Residual	14	11.48		

		Site residual	1	192.93		
	Shoot density					
		Treatment	2	5531160	38.699	< 0.001 *
		Residual	14	142928		
		Site residual	1	424120		
12 mo	*Canopy height*					
		Treatment	2	118.5	8.177	0.004 *
		Residual	14	14.5		
		Site residual	1	550.2		
	Shoot density					
		Treatment	2	1767100	10.873	0.002*
		Residual	12	162521		
		Site residual	1	1048576		
13 mo	*Canopy height*					
		Treatment	2	393.3	9.562	0.002 *
		Residual	14	41.1		
		Site residual	1	659.1		
	Shoot density					
		Treatment	2	3310972	13.000	< 0.001 *
		Residual	14	254692		
		Site residual	1	770868		
15 mo	*Canopy height*					
		Treatment	2	107.2	6.413	0.01 *
		Residual	14	16.72		
		Site residual	1	69.23		
	Shoot density					
		Treatment	2	1081493	4.848	0.025 *
		Residual	14	223100		
		Site residual	1	185035		
25 mo	*Canopy height*					
		Treatment	2	91.0	1.335	0.295
		Residual	14	68.2		
		Site residual	1	747.6		
	Shoot density					
		Treatment	2	183785	0.718	0.505
		Residual	14	255838		
		Site residual	1	390139		
27 mo	*Canopy height*					
		Treatment	2	25.34	0.244	0.789
		Residual	8	103.83		
		Site residual	1	0.013		
	Shoot density					
		Treatment	2	552743	4.331	0.034 *
		Residual	14	127624		
		Site residual	1	185035		

Table S4. Two-way ANOVA assessing differences in plant traits of *Cymodocea nodosa* after 27 mo of disturbance of all experiments. (*) Asterisks indicate significant differences ($p < 0.05$). Models include fixed effects (each seagrass trait) and site as a random effect (n=3).

Type of disturbance	Seagrass Traits	df	MS	F	*p*-Value
INTENSITY	*Apical shoots*				
	Treatment	2	470.9	0.671	0.527
	Residual	14	702.2		
	Site residual	1	19.53		
	Internodal distance				
	Treatment	2	0.195	0.432	0.658
	Residual	14	0.453		
	Site residual	1	18.12		
	Shoot biomass				
	Treatment	2	30.93	2.703	0.102
	Residual	14	11.44		
	Site residual	1	25.53		
	Rhizome biomass				
	Treatment	2	219	2.332	0.134
	Residual	14	93.83		
	Site residual	1	4675		
	Rhizome nitrogen				
	Treatment	2	0.002	0.169	0.846
	Residual	14	0.01		
	Site residual	1	0.1013		
	Rhizome NSC				
	Treatment	2	70.5	2.664	0.105
	Residual	14	26.45		
	Site residual	1	1801		
	Shoot surface				
	Treatment	2	82.4	3.051	0.079
	Residual	14	27.02		
	Site residual	1	942.5		
	Number of leaves				
	Treatment	2	0.295	1.797	0.202
	Residual	14	0.164		
	Site residual	1	0.558		

	Leaves nitrogen				
	Treatment	2	0.003	0.226	0.8002
	Residual	14	0.013		
	Site residual	1	0.087		
SIZE	*Apical shoots*				
	Treatment	3	813.3	1.367	0.283
	Residual	19	595.1		
	Site residual	1	861.0		
	Internodal distance				
	Treatment	3	0.074	0.242	0.866
	Residual	19	0.307		
	Site residual	1	10.854		
	Shoot biomass				
	Treatment	3	4470	3.973	0.024 *
	Residual	19	1125		
	Site residual	1	2719		
	Rhizome biomass				
	Treatment	3	51565	13.18	< 0.001 *
	Residual	18	3913		
	Site residual	1	50553		
	Rhizome nitrogen				
	Treatment	3	0.159	3.208	0.046 *
	Residual	19	0.049		
	Site residual	1	2.362		
	Rhizome NSC				
	Treatment	3	21.36	0.913	0.454
	Residual	19	23.41		
	Site residual	1	222.71		
	Shoot surface				
	Treatment	3	19.91	0.851	0.483
	Residual	19	23.40		
	Site residual	1	48.73		
	Number of leaves				
	Treatment	3	0.043	0.904	0.457
	Residual	19	0.048		
	Site residual	1	2.934		
	Leaves nitrogen				
	Treatment	3	0.009	0.198	0.897
	Residual	19	0.047		
	Site residual	1	0.082		

SEASONAL TIMING	*Apical shoots*				
	Treatment	2	2020.4	9.859	0.002 *
	Residual	14	204.9		
	Site residual	1	262.6		
	Internodal distance				
	Treatment	2	0.001	0.005	0.995
	Residual	14	0.212		
	Site residual	1	8.412		
	Shoot biomass				
	Treatment	2	965	0.658	0.533
	Residual	14	1467		
	Site residual	1	3517		
	Rhizome biomass				
	Treatment	2	40242	12.46	< 0.001 *
	Residual	14	3231		
	Site residual	1	78213		
	Rhizome nitrogen				
	Treatment	2	0.219	6.80	< 0.001 *
	Residual	14	0.032		
	Site residual	1	0.88		
	Rhizome NSC				
	Treatment	2	6.242	0.268	0.768
	Residual	14	23.26		
	Site residual	1	13.01		
	Shoot surface				
	Treatment	2	5.13	0.215	0.809
	Residual	14	23.9		
	Site residual	1	37.57		
	Number of leaves				
	Treatment	2	0.119	1.409	0.277
	Residual	14	0.084		
	Site residual	1	2.552		
	Leaves nitrogen				
	Treatment	2	0.024	0.535	0.597
	Residual	14	0.045		
	Site residual	1	0.13		

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Seagrass-bivalve facilitative interactions: Trait-mediated effects along an environmental gradient

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ABSTRACT

Facilitative interactions are important forces in shaping community structure and function, and understanding how they respond to environmental changes has become an increasing concern in ecology. Lucinid bivalves play a significant role in seagrass meadows, through a mutualism in which the seagrass provides habitat and oxygen via the roots, while the bivalves and their associated bacteria eliminate sulfides from pore water, improving thus plant performance. In this study, we evaluated how this mutualism is modified along a gradient of organic matter content in the sediment, in a coastal bay dominated by *Cymodocea nodosa* meadows. We used a correlative approach, seeking statistical association between sediment organic matter content, lucinid abundance, and plant traits. Lucinid abundance was higher in vegetated that in bare areas. In vegetated areas, lucinid abundance decreased as organic matter content in the sediment increased, decrease seemingly associated to modification in plant traits, i.e. root abundance and morphology. In organic-rich sediments, roots are less abundant and less branched, reducing the potential habitat for lucinids and suggesting a weakening of the interaction. This finding contributes to our understanding of how facilitative interactions can be modified along human disturbance gradients, and how disturbances can reduce resilience of seagrasses through this modification.

1. Introduction

Biological interactions are crucial drivers in shaping the biosphere. Positive interactions (i.e., those benefiting at least one of the species involved and not detrimental to the other), are recognized as playing a critical role in ecosystem function and structure (Brooker et al., 2008; Bruno et al., 2003; Mcintire and Fajardo, 2014).

Facilitative interactions are widespread, and their occurrence and strength has been shown to be highly context-sensitive (Eckert et al., 2010; Tylianakis et al., 2008) since environment can alter the biological, ecological, or behavioral traits of the species involved (e.g., Kiers et al., 2010; Schöb et al., 2013), and therefore the outcome of their interaction. Moreover, the behavioral, physiological, and biological traits on which facilitative interactions depend are not built-in software, and can undergo evolutionary modifications as a consequence of their strong influence on fitness. Overall, the nature and strength of these interactions and their effects at the ecosystem level are highly variable in space and time. For instance, facilitative interactions have been hypothesized to increase their frequency, intensity, and importance with increasing environmental stress (stress-gradient hypothesis: Bertness and Callaway, 1994; He and Bertness, 2014), although the generality of this hypothesis remains controversial (He and Bertness, 2014; Maestre et al., 2009; Michalet et al., 2014).

Environmental changes, and specifically those caused by human activities, can alter facilitative interactions with potential consequences for ecosystem function. For example, it has been shown that global change may disrupt critical mutualisms by causing shifts in the phenology of the species involved, as for instance plants and their pollinators (Memmott et al., 2007). Such trait-mediated interactions (TMI) triggered by environmental changes have been shown to exert a major influence on modulating community structure and function in a wide range of ecosystems (Callaway et al., 2003; Coldren, 2013).

In marine ecosystems, positive interactions have been revealed as essential functional components (Bulleri, 2009), however, while in the terrestrial environment the stress-gradient hypothesis (see above) has played a pivotal role in focusing research, much less attention has been paid to it in the marine realm (Bulleri, 2009). Thus, gaining knowledge on how environmental drivers modulate facilitation and its implications at the ecosystem level is essential in order to understand how species fitness, ecological functions, and the associated provision of goods and services will shift in a changing world. This increases in importance when the ecosystem is highly vulnerable to human pressures such as coastal ecosystems (Ruiz-Halpern et al., 2008) and when the species involved are foundation or keystone species (Jones et al., 2008;

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Kwiatkowski et al., 2015).

Seagrasses are foundation species inhabiting subtidal, usually soft bottom habitats (Green and Short, 2003) that host a huge biodiversity, while providing a number of valuable goods and services. As with other coastal water ecosystems (e.g., kelp forests, mangroves, and coral reefs), in recent decades they have undergone significant deterioration caused by human activities. How this has affected their structure and function has been extensively documented, although most of the studies published to date have been aimed at assessing the direct effects of shifts in environmental factors such as light, nutrients, organic matter, and sedimentation on the plant and associated organisms (Cabaço et al., 2008; Cardoso et al., 2008; Daudi et al., 2012). Less frequently, research has focused on evaluating how human impacts alter biotic interactions that are important for meadow resilience, especially top-down effects (de Paz et al., 2008; Tomas et al., 2015; Van Der Heide et al., 2007). However, the role played by positive interactions (other than the obvious habitat provision by the seagrass), and how they are affected by human impacts have very rarely been examined (but see Maxwell et al., 2016).

Nevertheless, such positive interactions can play important roles in seagrass ecosystem persistence and resilience. The best example is probably a complex mutualism involving bacteria, lucinid bivalves, and seagrasses (van der Heide et al., 2012) in which seagrasses provide lucinids with a suitable habitat (the rhizosphere) and oxygen through root radial diffusion, while the bivalves protects the plants against sulfide toxicity by oxidizing pore water sulfide, an oxidation which is mediated by the chemoautotrophic bacteria inhabiting their gills (Pedersen et al., 1998; Reynolds et al., 2007). This mutualism has been postulated as fundamental for seagrass persistence (van der Heide et al., 2012), because seagrass sediment pore water is rich in sulfide, due to the high activity of sulfate-reducing bacteria (Koch et al., 2007). In support of this notion, it has recently been demonstrated that disruption of the mutualism due to drought has worsened the deleterious effects of desiccation on seagrasses (de Fouw et al., 2016).

Eutrophication is one of the most pervasive impacts of human activities worldwide, and the increase in sediment organic matter content is one of its main consequences, involving lower level of oxygen in sediment pore water and, consequently, higher levels of sulfide production (Lamers et al., 2013). While it is clear that sulfide effects can be dampened by the activity of the lucinids and their associated bacteria, the possible modification of the mutualism by the increase in sediment organic matter content remains unexplored.

The aim of our study was to help fill this gap by assessing how seagrass-lucinid mutualism responds to increases in organic matter content of sediment. Such increases can trigger a number of responses, affecting plant traits (e.g., modifying shoot and root morphology and production) (Fertig et al., 2013; Pérez et al., 1994), environmental conditions (e.g., light reaching the plant) (Ralph et al., 2007), and sulfide concentration in pore water (Govers et al., 2014), which can potentially affect the outcome of the mutualism.

Thus, we studied the association between the seagrass *Cymodocea nodosa* (Ucria) Ascherson and the abundance of the lucinid bivalve *Loripes lucinalis* (Lamarck, 1818) (synonym *Loripes lacteus*, sensu Poli, 1791) along a gradient of organic matter content in sediment, considering the abundance of lucinids as an indicator of the importance of the seagrass-bivalves interaction. We first assessed whether or not the presence of the seagrass facilitated the presence of lucinids, by comparing bivalve densities between seagrass meadows and bare sediments. Second, we explored the variability in lucinid abundance along a gradient of organic matter content in vegetated sediments, hypothesizing higher lucinid abundances in organic-richer sediments under the assumption of a facilitation enhanced by increased sulfide content in the sediment. Third, we explored the association between the abundance of lucinids and a variety of plant traits (e.g. root biomass, root architecture) to gain insight on the influence of such traits on bivalve population.

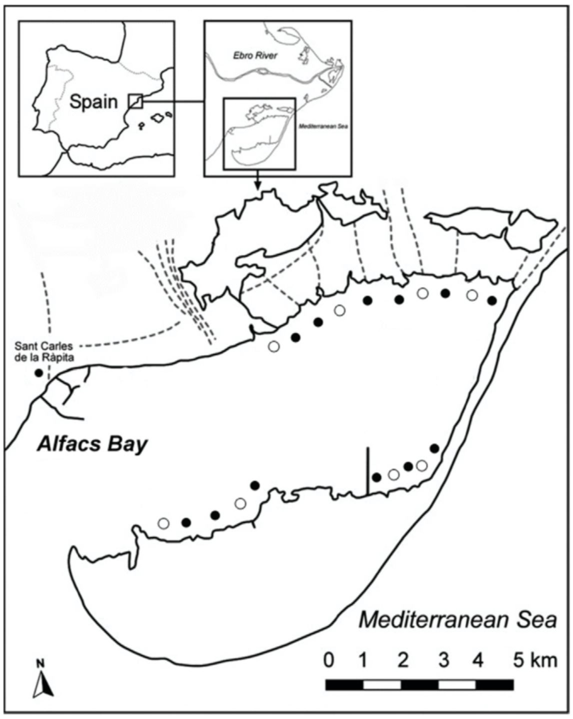

Fig. 1. Sampling locations in Alfacs bay covering the organic matter gradient generated by the freshwater organic-rich inputs from rice fields. Dashed lines represent drainage channels from rice fields. Black spots represent samplings in seagrass meadows and bare sediment (plants, lucinids and sediment). White spots represent the additional samples in seagrass meadows (plants and lucinids).

2. Materials and methods

2.1. Study site

The study was conducted in Alfacs bay, situated on the southern side of the Ebro river delta (NW Mediterranean) (Fig. 1). This bay is relatively shallow (maximum depth of 6 m) and has a surface area of 50 km^2. Large *C. nodosa* meadows grow all around the bay, at depths between 0.5 and 1.5 m (Oliva et al., 2012). The area receives nutrient- and organic matter-rich freshwater discharges from rice crop fields, which enter the bay on its northern shore, while the southern shore is influenced by marine waters from the open sea. This generates a marked gradient of eutrophication that has been documented in previous studies based on data of a number of environmental factors (nutrient concentration in water, organic matter content in the sediment, N and P content in seagrass leaves, P in sediment, etc.; see Mascaró et al., 2014; Oliva et al., 2012; Pérez et al., 1994).

2.2. Sampling design

To obtain data from conditions encompassing the wider possible range of sediment organic matter content, twelve sampling points at a constant depth (0.5–0.7 m) were selected around the bay (Fig. 1). At each point, we took two samples: one in the seagrass meadow (including above and belowground seagrass parts, plus bivalves and other fauna) and one in bare sediment (including bivalves and other fauna). This was done using a hand-held core sampler (large core thereinafter, 15 cm diameter) pushed to a sediment depth of 30 cm. To estimate the sediment organic matter content, at each one of these twelve points, two surface (0–3 cm) sediment samples were taken with hand cores (small cores thereinafter, 4 cm in diameter), one close to the sample in vegetated areas and the other close to the sample in bare areas. Additionally, we took eight additional large core samples in vegetated points along the gradient and at the same depth, to better assess plant traits variability and gain statistical power. Immediately after collection, samples from large cores were rinsed, sieved through a 5 mm mesh to remove sediment and transported chilled to the laboratory. Sampling was conducted in June 2013, since June corresponds to the maximum growth period of *C. nodosa* (Mascaró, 2011) and is close to one of the two annual peaks of reproduction of *L. lucinalis* (Johnson and Le Pennec, 1994).

2.3. Sample processing

In the laboratory, live lucinid bivalves from each large core sample were separated from the rest and counted to evaluate their abundance (in terms of density, individuals m^{-2}). In addition to this, in samples obtained in seagrass meadows, shoots were counted to estimate shoot density, and plants were sorted into leaves, rhizomes, and roots. These fractions were then rinsed, dried (70° for 24 h) and weighed. We used the root weight ratio (RWR, Reynolds and D'Antonio, 1996) to express the relative abundance of root versus leaves. The RWR was calculated as the biomass of roots divided by the sum of the biomass of roots plus the biomass of leaves. To assess differences in root morphology and branching (viz. root architecture), five roots from each sample were selected at random before drying, and the maximum length of each root was measured and the number of primary and secondary ramifications counted. Then, an index of root branching (BI) was calculated as the total number of ramifications divided by the maximum root length.

Sediment organic matter content was determined as loss on ignition (LOI450) in samples from small cores. Dried sediment samples (70 °C until constant weight) were combusted for 4 h at 450 °C. Organic matter was expressed as percentage of total weight.

2.4. Statistical analysis

The significance of the differences in lucinid density between habitats (*C. nodosa* and bare sediment) was assessed using a linear model with habitat as a categorical variable (two levels, seagrass and bare sediment) and organic matter content as continuous variable. Data were square-root transformed to meet the assumptions of normality and homogeneity of variances using the customary tests (Shapiro-Wilks test for normality, Bartlett test for homoscedasticity). Association between plant traits (root biomass, root weight ratio, root branching index, and shoot density), organic matter content in the sediment, and lucinid abundance were assessed by linear correlation analyses using Pearson's correlation. All statistical calculations were conducted using the software program R (R Development Core team 2015) with RStudio (version 0.98.1062).

3. Results

Live individuals of *L. lucinalis* were found in all sampled sites, with their abundances displaying a high variability, from 57 to 1981 ind m^{-2}. Lucinid density was much higher (ca. 5-fold) in vegetated (889 ± 225 ind m^{-2}) than in bare sediments (172 ± 80 ind m^{-2}) (Fig. 2). These differences were highly significant, following the linear model applied ($p < 0.001$). In contrast, and taking all the samples together (from both seagrass and bare areas), organic matter did not influence lucinid abundance ($p = 0.396$).

The abundance of lucinids showed a negative correlation with the organic matter content in vegetated sediments (Fig. 3a), but showed no correlation in bare ones (Fig. 3b).

Seagrass traits varied along the sediment organic matter content gradient. The relative biomass allocation to roots, as indicated by the RWR, clearly decreased with increasing sediment organic matter (Fig. 4a). This paralleled the significant changes evidenced along the gradient in root morphology (as indicated by the branching index, BI), with poorly branched roots in organic-rich sediments and densely branched roots in organic-poor ones (Fig. 4b). These changes in root morphology were visually conspicuous, with roots tending to be fibrous (highly branched and without a distinct main axis) in organic-poor sediments, shifting to monopodial roots (a main vertical axis with limited branching) in organic-rich ones. Root biomass was not correlated with sediment organic matter content (Fig. 4c), while shoot density was (Fig. 4d).

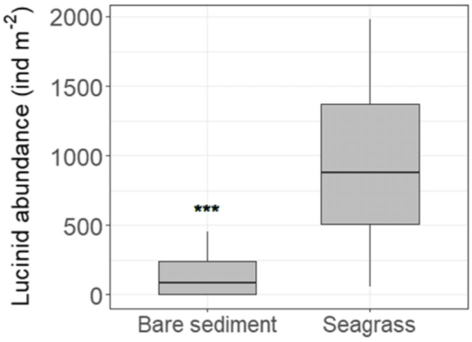

Fig. 2. Abundance of lucinids (ind m^{-2}) in bare sediments (n = 12) and in seagrass meadows (n = 12). Asterisk denote significant differences ($p < 0.001$).

Lucinid abundance was significantly and positively correlated to root traits, including root biomass (Fig. 5a), root weight ratio (Fig. 5b), and the root branching index (Fig. 5c). However, it showed no correlation with shoot density (Fig. 5d).

4. Discussion

Our results show that an increase in sediment organic matter content is associated to a decline in the abundance of *Loripes lucinalis* (lucinid bivalve) in the *Cymodocea nodosa* meadows studied, which potentially may weaken the mutualism between the two species. This decrease appears to be statistically associated with changes in plant traits, especially in roots, which are less abundant and developed (poorly branched) in the most organic-rich sediments. Albeit not totally conclusive, this suggests an indirect and negative effect of the sediment organic matter content (and, probably, of eutrophication) on the outcome of the facilitative interaction between the seagrass and the bivalves through the modification of plant traits.

Seagrasses present substantial phenotypic plasticity in morphological and physiological traits, which becomes evident under stress conditions or following disturbance events (Maxwell et al., 2014) and may have a strong influence on modulating species interactions. As a fast growing species, *C. nodosa* displays a high variability in morphological, physiological, and reproductive traits in response to environmental stress (Mascaró et al., 2009; Oliva et al., 2014; Sandoval-Gil et al., 2012). In particular, according to previous studies (Oliva et al., 2007; Pérez et al., 1994), root morphology and architecture change from oligotrophic to eutrophic sites, from thinner and densely branched to thicker and monopodial roots, probably as an adaptive response to optimize biomass allocation and exploit the nutrient pools partitioned between the water column and the sediment (Romero et al., 2006; Kiswara et al., 2009). Given the link between lucinids and seagrass roots, this response can influence the facilitative interplay between the plant and the bivalves.

It is well known that seagrass roots can facilitate the presence of lucinid bivalves, either through habitat provision (the roots themselves) or through resource facilitation (radial oxygen release) (Reynolds et al., 2007; van der Heide et al., 2012). This has been also shown for other

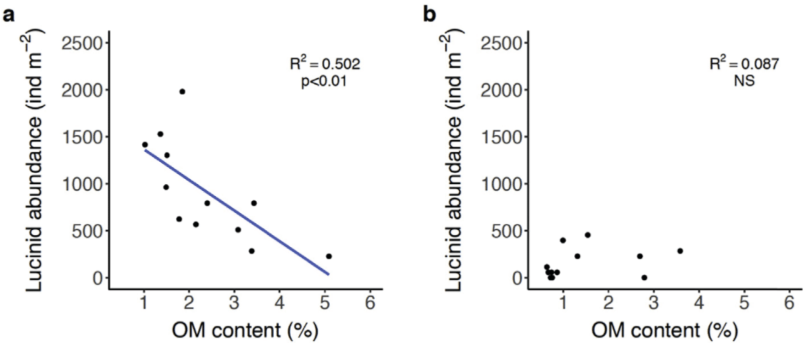

Fig. 3. Statistical association, assessed using linear correlation, between the sediment organic matter content and the abundance of lucinid bivalves (n = 12) in (a) seagrass meadow and (b) bare sediment. R^2 and p-values are reported in the plots.

infaunal species (Hemminga and Duarte, 2000; Fredriksen et al., 2010), finding habitat, food, and oxygen in or close to the rhizosphere. Indeed, we found that lucinids were much more abundant in areas with *C. nodosa* vegetation, relative to bare ones. Moreover, in vegetated areas, lucinid abundance was positively associated with the root biomass, the relative abundance of roots (relative to shoots, RWR), and the branching index (BI), but not with the shoot density, suggesting that plant root traits are the major drivers of lucinid abundance in the *C. nodosa* meadows studied.

Our data do not allow us to completely rule out the existence of effects other than those mediated by plant trait modifications, such as direct effects of organic matter content in the sediments (inducing oxygen exhaustion) or other variables associated to the gradient (salinity, sediment grain size). However, these confounding effects are highly unlikely to occur. Thus, on the one hand, the maximum values of sediment organic matter content reported here are similar to or lower than most published values for sediments hosting healthy lucinid populations (Como et al., 2007; de Paz et al., 2008). On the other hand, the effect of variables changing along the transect (direct effect of organic matter, salinity, grain size) would have affected both to bare and vegetated sediments populations, whereas our data only indicate a decrease in bivalve abundance in the latter. Moreover, the sediment grain size is quite homogenous across the bay (medium-fine sand; from 181 to 298 µm Ø), and the correlation between the grain size obtained in previous works (Romero et al., 2010) and the abundance of lucinids from this work is not significant (n = 12, r = 0.461, p = 0.154). Accordingly, and in spite of the correlative nature of our study, we conclude that the decline in lucinid abundance along the organic matter gradient in vegetated sediments is an indirect effect mediated by the modification of plant traits.

As facilitation plays a major role in the structuring and the functioning of marine ecosystems (Bertness, 1989, 1991; Hacker and Bertness, 1995; Bruno, 2000; Bulleri, 2009; among others), understanding how the strength of these facilitative interactions is altered by increasing levels of stress is critical to assess the resilience of the affected systems. In this respect, and according to the stress-gradient hypothesis, facilitative interactions are more frequent with increasing levels of stress (Bertness and Callaway, 1994; Crotty and Bertness,

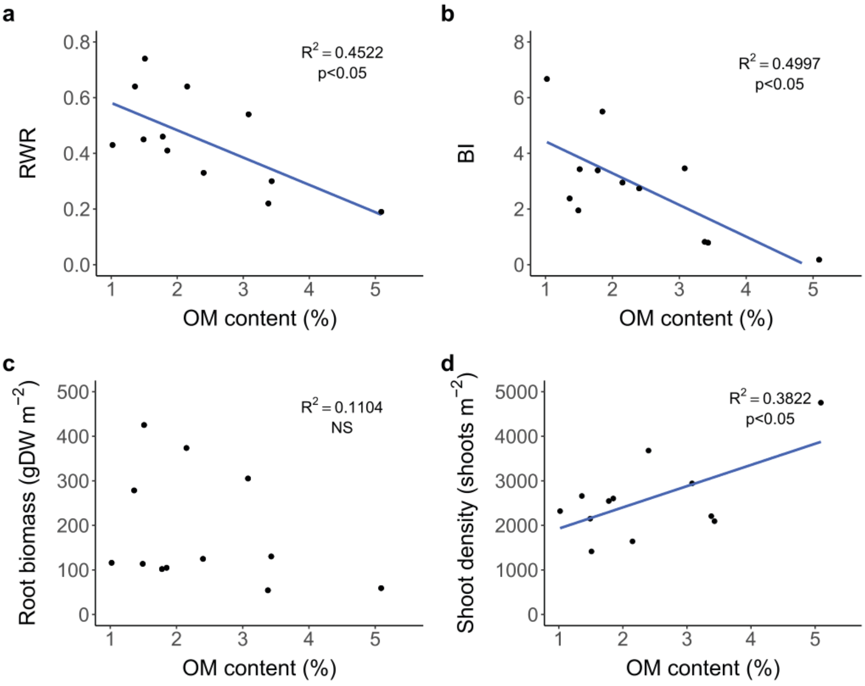

Fig. 4. Statistical association between the organic matter content and plant variables (n = 12) assessed using linear correlation. (a) root weight ratio (RWR), (b) root branching index (BI), (c) root biomass, and (d) shoot density. R^2 and p-values are reported in the plots.

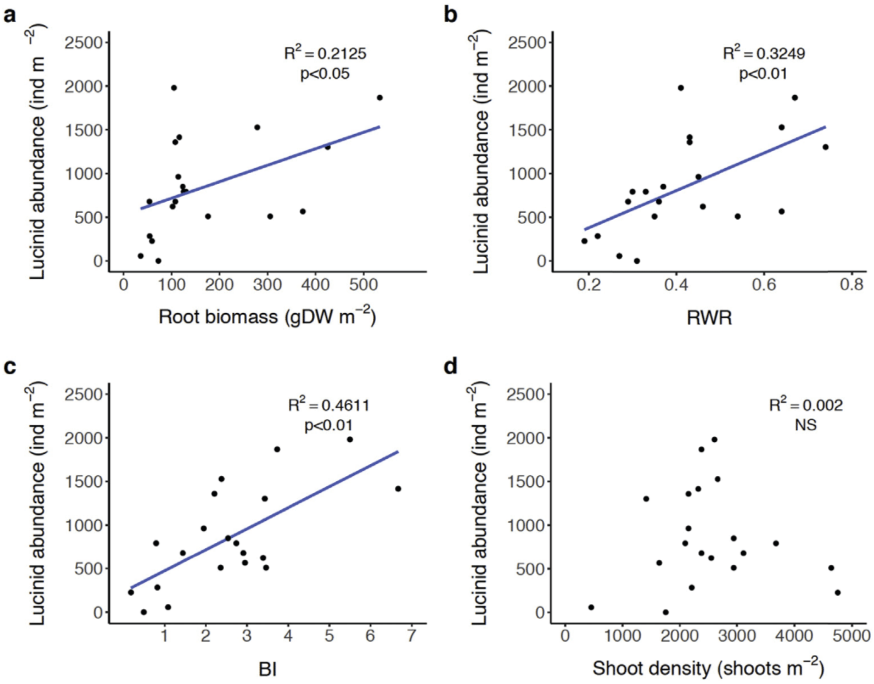

Fig. 5. Statistical association between the abundance of lucinid bivalves and plant variables (n = 20), assessed using linear correlation. (a) root biomass, (b) root weight ratio (RWR), (c) root branching index (BI), and (d) shoot density. R^2 and p-values are reported in the plots.

2011; He et al., 2013). However, our results do not conform to this general pattern. Two opposite influences seem to act simultaneously on our *C. nodosa* meadows. On the one hand, the increase in sediment organic matter increases pore water concentration of sulfide, which constitutes a resource for the symbiotic lucinid bacteria. This, together with higher food availability for bivalves in organic-rich sediments (van der Geest et al., 2014), would eventually lead to increases in lucinid abundance. On the other hand, the modification of *C. nodosa* root traits reduces habitat and resource provision (oxygen) to the lucinid population. Apparently, and in line with our results, the latter influence overrules the former, yielding as a net result a clear decline in lucinid abundance and, consequently, the possible disruption of the facilitative mechanism. Given the importance attributed to the seagrass-lucinid interaction for the persistence of seagrass meadows (van der Heide et al., 2012), the weakening of the interaction can represent a serious threat for these important habitat-forming species, as has been demonstrated for the seagrass *Zostera noltii* (de Fouw et al., 2016). Interestingly, we did not observe signs of deterioration in plant performance or survival (see, for example, the increase in shoot density with organic matter) in the organic-richest sediments, with low lucinid abundances. This suggests that other mechanisms enable this species to resist relatively high levels of organic matter in the sediments (Terrados and Duarte, 1999; Oliva et al., 2012). In conclusion, our findings shed light on how human disturbances can weaken facilitative interactions indirectly through changes in species traits. The consequence of this for ecosystem function and service provision is still uncertain and further research is required to clarify this question. The disruption of mutualism caused by stress can represent an additional challenge to the resilience of key ecosystems (Kiers et al., 2010). Assessing the importance of facilitative interactions and their susceptibility to environmental change is therefore crucial to better predict marine ecosystem persistence in a changing world.

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Tolerance responses to simulated herbivory in the seagrass *Cymodocea nodosa*

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ABSTRACT: Herbivory causes both direct and indirect damage to plants, with negative consequences for plant performance and fitness. Plants have thus evolved strategies to counteract or mitigate such negative effects. The strategies used by aquatic plants to cope with herbivore pressure are of key importance to better understand ecological and evolutionary processes. However, little is known about such strategies. To help fill this gap, and to better understand induced responses to herbivory in aquatic plants, we simulated grazing at various intensities in the seagrass *Cymodocea nodosa* for ca. 4 mo, and measured plant responses in terms of shoot density, aboveground biomass, leaf growth, total nitrogen and carbon content in tissues, total non-structural carbohydrates in rhizomes and total phenolic content in leaves. Most of these plant attributes showed changes under both low and high simulated herbivory at the end of the experiment, indicating that *C. nodosa* is able to change a suite of plant traits to compensate for biomass losses. At least 3 tolerance strategies were involved in this process: growth compensation and overcompensation, increased nitrogen content (either from uptake or through reclamation from rhizome pools) and remobilization of carbohydrates stored in the rhizomes. Phenolic content decreased in the low-intensity treatment but was similar to control plants in the high-intensity herbivore treatment, indicating the role of phenolic compounds in the tolerance response.

KEY WORDS: Plant–herbivore interactions · Induced responses · Tolerance · Compensatory growth · Phenolic compounds · Mediterranean Sea

INTRODUCTION

Herbivory is probably one of the most pervasive and influential interactions in the biosphere. Beyond its role in trophic fluxes, herbivory has profound effects on vegetation structure, composition and productivity, and has probably been a strong evolutionary driver since the dawn of life (McNaughton 2001). Herbivory seriously affects plant performance and fitness in different ways (e.g. by reducing photosynthetic surfaces, injuring parts of key importance such as meristems, removing flowers or seeds), to the point that it is probably among the main forces shaping both plant and herbivore evolution and co-evolution (Rausher 2001). The long evolutionary history of plant–herbivore interactions is reflected in the large panoply of adaptive mechanisms and strategies displayed by plants to avoid consumption by herbivores or to mitigate its consequences (e.g. Karban & Myers 1989, Karban & Baldwin 1997, Bingham & Agrawal 2010).

Such mechanisms fall into 2 broad categories, constitutive (a constant trait) and inducible (a trait expressed in the presence of herbivores; Karban et al. 1997). In turn, they are based on 2 defense strategies: those reducing the probability or severity of herbivore attack (resistance strategy), and those allowing plants to withstand grazing (tolerance strategy; Agrawal 2000). Resistance-induced responses are generally based on changes in the properties of plant

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tissues, making them less palatable and/or attractive to herbivores, or reducing their performance. This is often achieved through the production of secondary metabolites that act as repellents, toxins or agents that reduce plant digestibility (Lattanzio et al. 2006, Wu & Baldwin 2010), although changes in tissue toughness or in other mechanical properties are also common (Lucas et al. 2000). Tolerance responses attenuate the negative effects of herbivores, by minimizing the loss in plant fitness after herbivore attack, and their nature varies with plant type, developmental status and the part of the plant damaged. A suite of tolerance responses following natural or simulated hervibory has been described, including compensatory growth, increased photosynthetic rate, increased branching, changes in nutrient allocation pattern and increased capacity to shunt carbon reserves from belowground organs to shoots after damage (Strauss & Agrawal 1999, Tiffin 2000). Indeed, several studies have shown that in many plants, primary production can be maintained (compensatory growth) or stimulated (overcompensatory growth) in response to grazing (Gadd et al. 2001), illustrating some of the potential positive effects of herbivory on grazed plants (Agrawal 2000, Ruiz et al. 2008). Both tolerance and resistance strategies entail costs and benefits. Different and at times controversial hypotheses have been proposed about their relationship (Restif & Koella 2004). Apparently, tolerance and resistance are not mutually exclusive and may coexist in plant populations, although trade-offs between them may appear (Mauricio et al. 1997, Leimu & Koricheva 2006).

Herbivory is considered to be stronger in aquatic systems than in terrestrial ones (Cyr & Pace 1993). Although less studied than in their terrestrial counterparts, the mechanisms of defense against herbivores are also widespread among aquatic plants (e.g. Toth & Pavia 2007, Miler & Straile 2010, Morrison & Hay 2011). The presence of secondary metabolites deterring grazing in tissues of aquatic macrophytes seems to be an important strategy to protect against consumers that is found in producers from different taxonomic groups (i.e. micro- and macroalgae, angiosperms) and environments (McClintock & Baker 2001, Pohnert 2004, Prusak et al. 2005). The tolerance strategy, in contrast, seems to be less common, and this may be because it rarely occurs among algae (but see, for instance, Wai & Williams 2005). In macroalgae, the lack of a complex morphological and functional organization such as that of higher plants may prevent the existence of tolerance responses. In contrast, angiosperms and, specifically, marine angiosperms (i.e. seagrasses) possess the same functional traits (basal meristems, clonal integration, storage organs; Marbà et al. 2006) that favor tolerance in terrestrial plants. Indeed, compensatory growth has been demonstrated in seagrasses as a response to defoliation (Tomasko & Dawes 1989, Valentine et al. 1997, Moran & Bjorndal 2005, Vergés et al. 2008).

Seagrasses are considered to be among the most important components of marine submersed vegetation for the goods they produce and the services they provide (Barbier et al. 2011). Their extensive meadows constitute a key habitat in the littoral system, and are relevant to the global carbon cycle. Recent evidence has proved that grazing in seagrasses is by far more important than previously thought (Heck & Valentine 2006, Valentine & Duffy 2006), affecting their population dynamics, composition, distribution and production (Valentine & Heck 1999, Tomas et al. 2004, Moran & Bjorndal 2005). For these reasons, seagrasses are excellent model species to explore mechanisms of defense against grazing. However, the responses of seagrasses to the high herbivory pressure they may suffer have, to date, scarcely been explored. On the one hand, it is known that seagrasses produce secondary metabolites, such as phenolic compounds (Steele et al. 2005, Grignon-Dubois et al. 2012, Ragupathi Raja Kannan et al. 2012), and there is evidence that some of these metabolites, such as condensed tannins, are induced following simulated herbivory (Arnold et al. 2008). However, the precise resistance-induced metabolites produced in response to herbivore attack remain in general poorly known, and it seems that the total phenolic content is not a good predictor of induced defense (Vergés et al. 2007, Steele & Valentine 2012, Sieg & Kubanek 2013). On the other hand, there is evidence that seagrasses can tolerate grazing by means of both intensifying recruitment of new shoots (Valentine et al. 1997) and compensatory growth of existing shoots (Tomasko & Dawes 1989, Moran & Bjorndal 2005). This compensatory growth could be achieved in part by the use of carbon reserves stored in the belowground organs (Eklöf et al. 2008), and supported by increased nitrogen (N) metabolism (N resorption or uptake; Valentine et al. 2004, Alcoverro & Mariani 2005). These studies have provided insights into the defense strategies against herbivores in marine plants. However, most were conducted in tropical species, especially *Thalassia testudinum*, thus narrowing the generality of the findings. Despite recent studies (Vergés et al. 2008, Burnell et al. 2013) demonstrating the existence of compensatory growth in the temperate genus *Posidonia*, our knowledge of seagrass–herbivore interactions, which have both ecological and evolutionary importance, remains poor.

The aim of the present study was thus to assess phenotypic changes in the seagrass *Cymodocea nodosa* caused by simulated macroherbivore attacks to detect possible tolerance responses. *C. nodosa* is a small, fast-growing species with a wide ecological range and high phenotypic plasticity (Pérez & Romero 1994, Marbà et al. 1996, Cancemi et al. 2002, Mascaró et al. 2009), which is subjected to relatively high levels of herbivory (Cebrián et al. 1996), and can be temporally overgrazed in some coastal lagoons (Fernandez et al. 2012). In this study, we attempt to expand the knowledge of tolerance responses of seagrasses to herbivory, and assess their generality or specificity. Our approach was based on a field experiment consisting of repeatedly clipping the seagrass leaves and measuring subsequent plant responses in terms of changes in density, biomass, leaf growth, carbon and nitrogen content in tissues and total non-structural carbohydrates (TNC). In addition, we measured the total phenolic content in leaves to explore whether or not they participate in the tolerance response rather than in defense mechanisms, as suggested by Vergés et al. (2008).

MATERIALS AND METHODS

Study site

The study was carried out in the southern bay (Alfacs Bay) of the Ebro river delta (northeastern coast of Spain; 40° 35′ N, 0° 41′ E), where extensive shallow meadows of *Cymodocea nodosa* develop in the sandy platforms (<1.5 m depth) surrounding the bay (Pérez & Romero 1994). The study site selected was on the southern shore, where meadows have a good ecological status and are away from the influence of the freshwater entering the bay on its northern shore (Oliva et al. 2012). At this site, shoots show fast turnover (average shoot life span: 2–4 yr) and reach a maximum density (around 2500–3000 shoots m^{-2}) in May–June (Mascaró et al. 2014). Herbivory is supposed to be low within the bay (Cebrián et al. 1996), although scattered populations of sea urchins (*Paracentrotus lividus*) have been detected (authors' pers. obs.). The experimental site was chosen to be at a distance from these populations (>500 m), so as to ascertain low natural herbivory pressure throughout the experiment. This made it unnecessary to deploy cages to protect plots against grazing.

Experimental design

We simulated low and high grazing pressure by macroherbivores by repeatedly clipping the leaves of *C. nodosa* during spring–summer 2010. After 4 mo, we sampled plants to measure several plant response variables (see next section). This period was chosen because it is the period of maximum activity of both plants and macroherbivores in the NW Mediterranean (Prado et al. 2007, Mascaró et al. 2014). The leaf clipping procedure is aimed at mimicking the feeding behavior of the 2 main macroherbivores in the NW Mediterranean (the sea urchin *P. lividus* and the sparid fish *Sarpa salpa*), as both feed on leaf tips, thus removing the distant part of the leaf blades (Prado et al. 2007). The same approach (i.e. simulating herbivory in stands with low natural levels of grazing) has been used previously and results considered representative of the potential response of the species when grazed (Vergés et al. 2008).

We established 9 plots of 1 m^2 in a *C. nodosa* meadow at 1 m depth, spaced at least 2 m apart. Three treatments, i.e. control, low herbivory (LH) and high herbivory (HH), were randomly assigned to each plot. In the HH treatment plots, the leaf canopy was cut to 10 cm height above sediment level, which corresponds to removal of about 75 % of leaf biomass. In the LH treatment plots, only the leaf tips were cut, corresponding to a leaf biomass removal of less than 5 %. In the control plots, the leaf canopy was left unmodified, thus remaining at its normal height (ca. 40 cm above sediment level).

The experiment was run from April to late July 2010. During this period, leaves within each plot were clipped periodically. Maintenance (clipping) visits were made every 2 wk, except during the maximum growth period (June and July) when clipping was performed weekly, resulting in a total of 10 clipping events between the start of the experiment and the July sampling. All clipped blades were removed from the plots to avoid any artifact derived from detritus accumulation. At the end of the experimental period, a series of response variables (see next section) were measured. Additional samples were taken for analysis of TNC and phenolic content in October 2010. These samples coincided with the seasonal maximum carbohydrate content (Mascaró et al. 2014). Between July and October, and to maintain the experimental conditions, further clipping visits were made every 2 wk.

Measurement of plant response variables

Biomass and shoot density

At the end of the period of maximum growth (end of July), samples of *C. nodosa* were collected from the central part of each plot using a 16 cm diameter corer. Each sample was thoroughly rinsed *in situ* with seawater until sediment had been completely removed, and stored in plastic bags that were refrigerated for transport. In the laboratory, all shoots were counted, and the leaves were separated from rhizomes and roots, dried at 70°C for 48 h (until constant weight) and weighed, thus obtaining shoot density (shoots m^{-2}) and leaf biomass (g DW m^{-2}, where DW = dry weight), with n = 3 per treatment. Subsamples of each fraction were kept for biochemical analysis.

Leaf growth and leaf number per shoot

Leaf growth was measured using a modified Zieman method (Pérez & Romero 1994). On 20 July 2010, 15 shoots were marked in each plot by punching a hole just above the ligule of the outermost leaf using a hypodermic needle. All marked shoots were collected 9 d later. In the laboratory, the leaves from each one of these shoots were separated, the number recorded, and each leaf divided into 'new' and 'old' tissue (i.e. tissue formed during or before the marking, respectively), dried at 70°C for 48 h (until constant weight) and weighed. Leaf growth was expressed in mg DW $shoot^{-1}$ d^{-1}, and relative growth rate (RGR; d^{-1}) was calculated as shoot leaf growth divided per shoot biomass. Both variables thus had 15 subsamples per plot and 3 replicates per treatment.

Tissue biochemical analysis

Dried leaves, rhizomes and roots from the core samples were ground to a fine powder. The carbon and nitrogen content in all tissues was measured in subsamples using a Carlo-Erba elemental autoanalyzer (Scientific and Technical Services of the University of Barcelona). TNC (sucrose plus starch) content was measured in rhizomes, using a modified method from Alcoverro et al. (1999). Ground samples were dissolved in 96% (v/v) ethanol, sonicated for 5 min and heated at 80°C for 15 min to extract soluble carbohydrates. This process was repeated 3 times. Starch was extracted from the remaining ethanol-insoluble pellet by dissolving it in 0.1 N NaOH at room temperature overnight. Sucrose content was determined using a resorcinol assay standardized to sucrose, and starch content was analyzed by spectrophotometry using an anthrone assay with sucrose as a standard. TNC content was the sum of the 2 fractions.

The total phenolic content of leaves was analyzed using a modified Folin-Ciocalteau method (Bolser et al. 1998). Each sample was extracted in 1 ml 50% methanol at 4°C for 24 h. Then, 0.1 ml of the supernatant was added to 7.9 ml distilled water, mixed and combined with 0.5 ml Folin-Ciocalteau reagent. After 2 min, 1.5 ml $NaCO_3$ solution was added to the sample. Two hours were allowed for color development, and absorbance was spectrophotometrically measured at 765 nm and compared with that of a standard curve for gallic acid. Although samples for phenolic analysis were taken in both July and October, the samples from July were lost due to technical problems in the analytical procedure.

Statistical analysis

For the variables shoot density and leaf biomass, the significance of differences among treatments (3 levels: control, LH and HH) was assessed using 1-way ANOVA. For the remaining variables, differences between treatments were analyzed using a 2-way nested univariate ANOVA, considering treatment as a fixed effect and plot (3 levels) as a random effect nested within treatment. To test for between-treatment differences for each variable, we used Tukey's HSD post hoc tests.

All variables were individually checked for normality, homogeneity of variance and outliers using first exploratory data analysis procedures (e.g. QQ plots), and parametric tests (Lilliefors and Shapiro-Wilks tests for normality, Cochran test for homoscedasticity) for assessing whether or not the ANOVA assumptions were met. No outliers were removed. Where necessary, data were transformed to achieve normality, as indicated in the 'Results'.

RESULTS

Most of the plant traits investigated responded to the simulated herbivory (Fig. 1). Shoot density increased significantly with clipping intensity (Table 1, Fig. 1a), and was, at the end of the experimental period, >50% higher in the HH treatment plots than in the control plots, while LH treatment plots displayed intermediate values. Leaf growth was significantly higher in LH plots (ca. 20% higher) than in control and HH plots (Table 2, Fig. 1c), while the relative growth rate increased significantly in both the LH and HH treatments (Table 2, Fig. 1d). The average number of leaves per shoot increased slightly but significantly in the HH treatment (Table 2, Fig. 1e), relative to the other 2 treatments. In addition, it should be noted that no bite marks made by herbivores were observed in the sampled leaves, thus confirming the low levels of herbivory at the experimental site, and the absence of interferences between natural and simulated herbivory.

Table 1. One-way ANOVA assessing differences in density and leaf biomass of *Cymodocea nodosa* between treatments. **Bold** indicates significant ($p < 0.05$) differences

Source	df	MS	*F*	p
Shoot density				
Treatment	2	2451340	6.111	**0.035**
Error	6	401083		
Leaf biomass				
Treatment	2	9519.5	3.020	0.123
Error	6	3151.4		

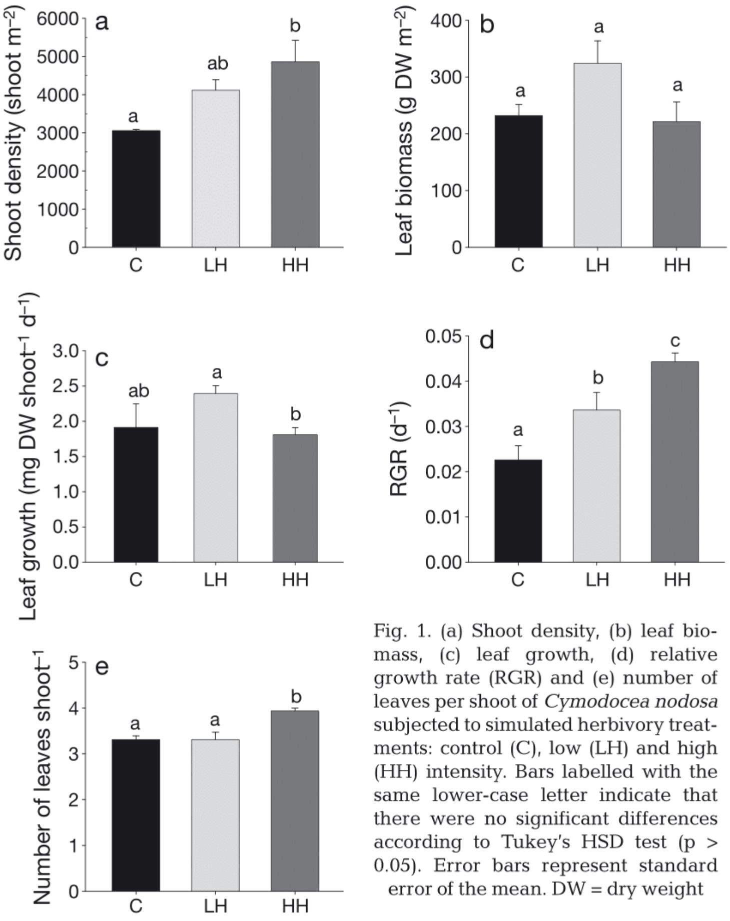

Fig. 1. (a) Shoot density, (b) leaf biomass, (c) leaf growth, (d) relative growth rate (RGR) and (e) number of leaves per shoot of *Cymodocea nodosa* subjected to simulated herbivory treatments: control (C), low (LH) and high (HH) intensity. Bars labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Error bars represent standard error of the mean. DW = dry weight

In terms of biochemical traits (Figs. 2–5), the HH treatment caused an overall reduction (relative to the control) in nitrogen content, significantly affecting leaves, rhizomes and roots (Table 2, Fig. 2a–c). In contrast, nitrogen content was higher in the leaves of plants from plots subjected to LH, relative to control plots (Table 2, Fig. 2a). Carbon content in leaves and rhizomes tended to be lower in the HH treatment than in the other treatments (Table 2, Fig. 2d,e). Overall, these changes resulted in increased C:N ratios in all 3 organs in the HH treatment (Table 2, Fig. 3a–c). TNC content in rhizomes measured in July in the HH treatment was 50% lower than in the control (Table 2, Fig. 4a). The TNC content increased more than 2-fold from July to October, when the differences among treatments disappeared (Table 2, Fig. 4b). The responses of sucrose and starch were similar. The total phenolic concentration in leaves collected in October was significantly lower (40%) in the LH treatment, relative to both the control and HH treatments (Table 2, Fig. 5).

DISCUSSION

This study demonstrates that simulated herbivory causes several responses in the seagrass *Cymodocea nodosa*. While some of these responses seem a simple and direct consequence of defoliation, others appear to attenuate the detrimental effects of consumer damage and are thus suggestive of adaptive tolerance responses. After a 4 mo defoliation period, a suite of plant trait changes, including changes in leaf growth, shoot recruitment, nutrient content and carbohydrate content, were observed, all of them suggestive of nutrient reallocation and mobilization of carbon reserves that either compensated or overcompensated for biomass losses.

Table 2. Two-way nested ANOVA assessing differences between treatments and plots on different traits of *Cymodocea nodosa.* **Bold** indicates significant ($p < 0.05$) differences. TNC = total non-structural carbohydrates

Source	df	MS	*F*	p
Leaf growth[a]				
Treatment	2	0.077	4.838	**0.009**
Plot		0.037	2.347	**0.035**
Error	126	0.016		
Leaf relative growth rate				
Treatment	2	0.002	32.858	**<0.001**
Plot	6	0.000	2.508	**0.025**
Error	126	0.000		
Number of leaves per shoot				
Treatment	2	5.807	10.595	**<0.001**
Plot	6	0.548	1.780	0.108
Error	126	0.308		
Nitrogen content in leaves				
Treatment	2	0.513	119.72	**<0.001**
Plot	6	0.016	3.94	**0.010**
Error	18	0.004		
Nitrogen content in rhizomes				
Treatment	2	0.226	104.42	**<0.001**
Plot	6	0.040	18.69	**<0.001**
Error	18	0.002		
Nitrogen content in roots				
Treatment	2	0.048	6.745	**0.006**
Plot	6	0.032	4.475	**0.006**
Error	18	0.007		
Carbon content in leaves				
Treatment	2	12.78	100.1	**<0.001**
Plot	6	0.94	7.3	**<0.001**
Error	18	0.13		
Carbon content in rhizomes				
Treatment	2	12.42	27.51	**<0.001**
Plot	6	1.16	2.58	0.055
Error	18	0.45		
Carbon content in roots				
Treatment	2	4.12	1.88	0.180
Plot	6	4.17	1.9	0.135
Error	18	2.19		
C:N ratio (leaves)				
Treatment	2	14.349	133.56	**<0.001**
Plot	6	0.493	4.59	**0.005**
Error	18	0.107		
C:N ratio (rhizomes)				
Treatment	2	209.70	50.582	**<0.001**
Plot	6	42.38	10.223	**<0.001**
Error	18	4.15		
C:N ratio (roots)				
Treatment	2	0.048	6.745	**0.006**
Plot	6	0.032	4.475	**0.006**
Error	18	0.007		
TNC content in rhizomes (July)				
Treatment	2	15.721	42.72	**<0.001**
Plot	6	0.734	1.997	0.126
Error	16	0.368		
TNC content in rhizomes (October)[b]				
Treatment	2	1.212	0.599	0.560
Plot	6	4.818	2.379	0.071
Error	16	2.025		
Phenolic content in leaves				
Treatment	2	3.478	65.933	**<0.001**
Plot	6	0.565	10.711	**<0.001**
Error	18	0.052		

[a] Data log $(x + 1)$ transformed to satisfy parametric test assumptions

[b] Data $1/x$ transformed to satisfy parametric test assumptions

Despite the repeated and massive defoliation to which it was submitted in the HH treatment, leaf biomass of *C. nodosa* in HH-treated and control plots at the end of the experimental period were very similar. HH-treated plants compensated for defoliation by the addition of new modules (leaves and shoots), while leaf growth remained similar to that found in control plants. Under the much more benign defoliation performed in LH plots, the response was slightly different. In LH-treated plants, besides the addition of new modules (only shoots), we also found a compensatory leaf growth, that, in the long term, could have led to an overcompensatory biomass response (Belsky 1986). These compensatory mechanisms described above have been reported in the tropical seagrass *Thalassia testudinum* (Valentine et al. 1997, Moran & Bjorndal 2005), and in the temperate species *Posidonia oceanica* (Vergés et al. 2008) and *P. sinuosa* (Burnell et al. 2013). Overcompensation is a common response to damage in terrestrial, freshwater and marine plants (e.g. Oba et al. 2000, Li et al. 2010), and is considered more common in fast- than in slow-growing species (Coley et al. 1985, Haukioja & Koricheva 2000; but see Soti & Volin 2010). This is consistent with the characterization of *C. nodosa* as a fast-growing and plastic species (Pérez et al. 1994, Mascaró et al. 2009). However, the slow-growing *P. oceanica* has also shown overcompensation for leaf growth (Vergés et al. 2008) but not for shoot recruitment. In this respect, it should be noted that the observed variability in growth compensatory responses of plants is often attributed to extrinsic factors such as nutrients (Li et al. 2010), light availability and damage frequency and intensity (Eklöf et al. 2008).

Our observations stress the importance of nutrients in plant–herbivore interactions. In effect, the observed compensatory growth of *C. nodosa* seems, to some extent, to be related to changes in nutrient content. Our results show that modest defoliation (LH treatment) caused an increase in N concentration in leaves, possibly accounting for the increase in leaf elongation. This increase is more likely to be due to uptake stimulation than to reserve mobilization, as the N concentration in roots and rhizomes remained unaltered. In contrast, intense defoliation (HH treatment) caused an overall reduction in N content (in leaves, rhizomes and roots). Although N uptake stimulation due to defoliation is a common response elsewhere (Jaramillo & Detling 1988, McNaughton et al. 1996), such stimulation did not occur or was unable to compensate for the N losses in *C. nodosa*. The decrease in N content not only in leaves, but also in rhi-

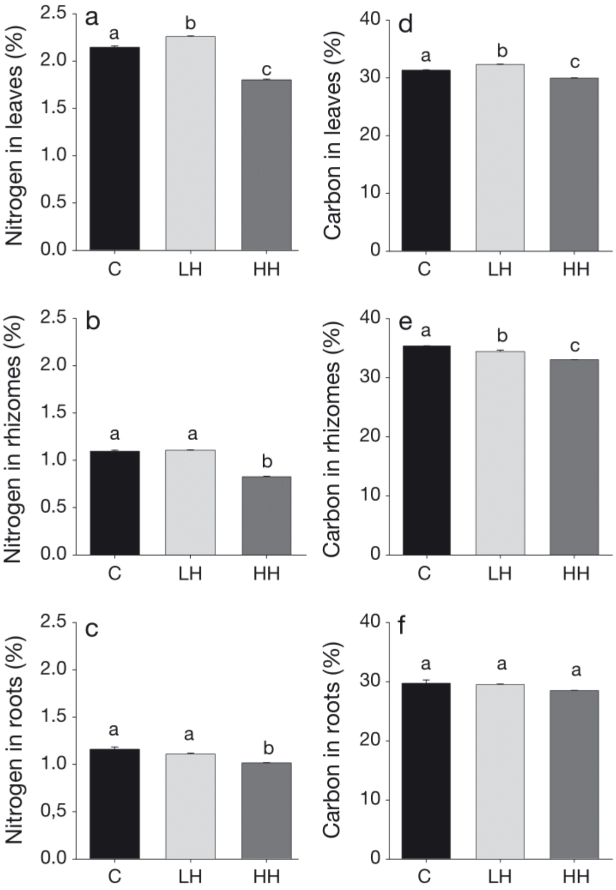

Fig. 2. (a–c) Nitrogen and (d–f) carbon content in leaves, rhizomes and roots of *Cymodocea nodosa* subjected to simulated herbivory treatments: control (C), low (LH) and high (HH) intensity. Bars labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Error bars represent standard error of the mean

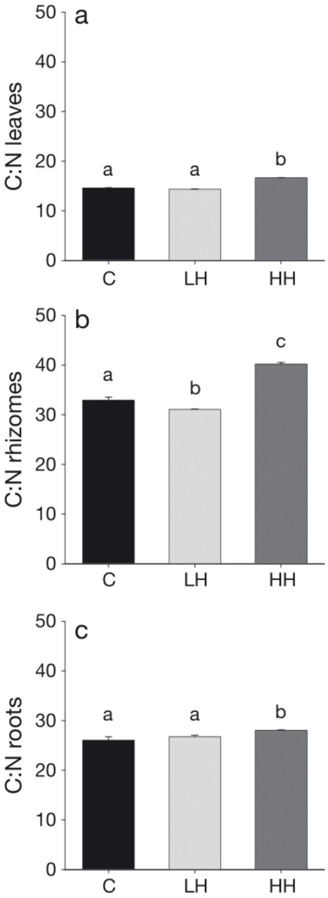

Fig. 3. C:N ratio in (a) leaves, (b) rhizomes and (c) roots of *Cymodocea nodosa* subjected to simulated herbivory treatments: control (C), low (LH) and high (HH) intensity. Bars labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test ($p > 0.05$). Error bars represent standard error of the mean

zomes and roots, suggests a mobilization of nutrients from the belowground organs to the aboveground parts to maintain leaf growth rates and to support the production of new modules (leaves and shoots), resulting in a dilution into the new biomass of N pools and increasing aboveground primary production (Valentine et al. 1997). Incidentally, this depletion of N may have consequences for the palatability of the tissues, as the intense defoliation caused a decline in the nutritional quality of leaves and rhizomes by increasing C:N ratios. Plant quality (often expressed as C:N ratio) has been shown to play a central role in determining herbivore feeding patterns in marine habitats (Cebrián & Duarte 1998, Barile et al. 2004, Duarte et al. 2011), and some authors have suggested that a low leaf N concentration can act as a plant defense against grazing (Augner 1995). To what extent this reduction in plant nutritional quality is an adaptive response or a mere consequence of nitrogen loss and dilution, as explained above, remains unclear. In any case, it should be noted that nutrient availability may play an important role in determining the capacity of a plant to compensate for tissue loss (Hay et al. 2011), as has been demonstrated, among others, by Li et al. (2010), who reported that individuals of *Vallisneria spiralis* growing in nutrient-rich habitats were better able to compensate for damage than those plants growing in nutrient-limited habitats, where they were unable to acquire the necessary amount of nutrients to replenish biomass loss.

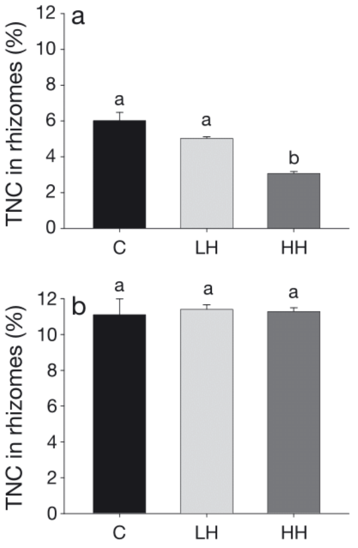

Fig. 4. Total non-structural carbohydrates (TNC) content in rhizomes measured in (a) July and (b) October in *Cymodocea nodosa* subjected to simulated herbivory treatments: control (C), low (LH) and high (HH) intensity. Bars labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test (p > 0.05). Error bars represent standard error of the mean

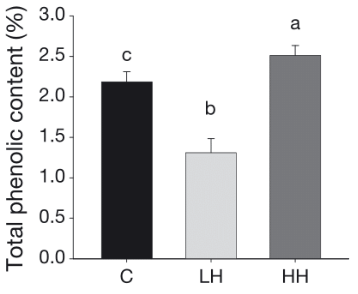

Fig. 5. Total phenolic content in leaves of *Cymodocea nodosa* measured in October subjected to simulated herbivory treatments: control (C), low (LH) and high (HH) intensity. Bars labelled with the same lower-case letter indicate that there were no significant differences according to Tukey's HSD test (p > 0.05). Error bars represent standard error of the mean

Nevertheless, the compensatory responses reported are not only facilitated by N mobilization or uptake, but also by the use of carbohydrate reserves. It has been shown that *C. nodosa*, like other seagrasses, has the capacity to store carbohydrates, building up reserves in late summer, and translocating these reserves to support shoot growth from early spring to mid-summer (Mascaró et al. 2014). The depletion of TNC after clipping, by 16 % (LH) and 50 % (HH) relative to controls, suggests that carbohydrate mobilization took place in response to defoliation, and part of the compensatory leaf growth (LH treatment) and the addition of new modules (LH, only shoots; and HH, leaves and shoots) was supported by these reserves. Indeed, mobilization of carbohydrate reserves appears to play a major role in the ability of plants to withstand disturbances involving the loss of aboveground tissue (Rodgers et al. 1995, Brun et al. 2003, Eklöf et al. 2008, Ruiz et al. 2009). However, the magnitude of the contribution of carbohydrates to regrowth may depend on both storage capacity and physiological integration of the plant. It should be emphasized that, despite the significant depletion of TNC in July following defoliation (especially in the HH treatment), TNC recovered, and the values in October, which were much higher than in July (ca. 2-fold, in agreement with the seasonality of the plant; Mascaró et al. 2014), were very similar among treatments.

While our results clearly indicate the ability of *C. nodosa* to develop diverse induced tolerance responses against both low and high simulated herbivory, the results for total phenolic content, which were either lower than (in the LH treatment) or equal to (in the HH treatment) control values, corroborated previous findings that this variable is not a good indicator of defense mechanisms (Sieg & Kubanek 2013). In this respect, it should be acknowledged that total phenolic content is uninformative about the deterrent capacity of a given tissue, as phenolic compounds participate in a huge number of plant functions besides deterrence (e.g. antioxidant: Hodzic et al. 2009; antimicrobial: Vergeer & Develi 1997; antifungal: Jensen et al. 1998). However, the fact that plants from the LH treatments had a 40 % lower total phenolic content than controls, whereas plants from the HH treatment had similar values to controls, suggests a negative relationship between phenolic content and leaf growth. This underlines the role of phenolic compounds as primary metabolites, particularly in cell wall construction during plant growth (Abdulrazzak et al. 2006). Part of the compensatory leaf growth found in plants from the LH plots could thus have been achieved using carbon from the phenolic pool, as suggested by Vergés et al. (2008) based on results very similar to ours obtained in *P. oceanica.*

In conclusion, under low levels of defoliation, leaf losses seem to act as a stimulating cue, triggering overcompensatory responses, apparently using internal carbon sources, to which re-use of phenolic compounds seems to contribute, and external N sources. In contrast, under high levels of defoliation, leaf elongation rates are maintained, while the number of leaves and shoots increases; this compensatory response seems to be supported, at least in part, by internal carbon sources (i.e. carbohydrates supplied by the rhizome reserves) and internal N sources (i.e. N remobilization from belowground organs). At the same time, the C:N ratio increases, potentially lowering the nutritional quality of leaves. All these mechanisms contribute to the tolerance of *C. nodosa* to grazing, reducing the negative effects of herbivore consumption on plant fitness.

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