Grazing on the epiphytic community of *Posidonia oceanica* (L.)Delile: An assessment of its relevance as a buffering process of eutrophication effects.

PhD Thesis

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Septiembre 2011

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Tesis Doctoral

Memoria presentada para optar al título de doctor por el Departamento de Biología. Universidad de las Islas Baleares, 2011

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Departamento de Biología de la Universidad de las Islas Baleares
Área de conocimiento: ECOLOGÍA (Código UNESCO 220)
Fecha de defensa: 10 de octubre 2011

Palabras clave: *Posidonia oceanica*, comunidad epifita, epiphyte commmunity, nutrientes, nutrients, top-down-control, bottom-up control, epifauna, grazer community.

Resumen

El incremento de disponibilidad de nutrientes produce cambios en la estructura y funcionamiento de los ecosistemas litorales. La eutrofización en los ecosistemas litorales mediterráneos favorece el predominio de algas epifitas de crecimiento rápido que compiten por la luz y los nutrientes con *Posidonia oceanica.* La herbivoría sobre los epifitos suministra la mayor parte del carbono que asimilan los consumidores primarios y secundarios asociados a la pradera. Esta tesis evalúa la importancia del consumo ejercido por la epifauna asociada a las praderas de *P. oceanica* en revertir los efectos de la eutrofización sobre la biomasa de algas epifitas. Los resultados muestran un

incremento de las tasas de consumo en respuesta a una mayor disponibilidad de biomasa epifita si bien el consumo no es capaz de revertir los efectos del aumento de nutrientes sobre la biomasa epifita. La comunidad íctica tiene un papel marginal en la regulación de la biomasa epifita en la Bahía de Palma.

Agradecimientos

En primer lugar le agradezco a Jorge todos estos años de formación científica y humana así como su paciencia y apoyo incondicional a lo largo de este proyecto. A Beatriz por darme la oportunidad de emprender este doctorado, por aportar su visión experimentada a este trabajo y por su sinceridad tanto en las críticas como en los halagos. A mis compañeros de grupo por estos años de convivencia y aprendizaje juntos. A mis padres, a mi familia y a mis amigos por apoyarme entre bastidores durante estos años; mil gracias por demostrar siempre empatía con las alegrías y decepciones que han acompañado el desarrollo de esta tesis. A Lluís por su inquebrantable “ya no te queda nada” y por evitar que viva dentro de un ordenador. A la Dra. María Cristina Gambi y al Dr. Claudio Vasapollo por darme la oportunidad de compartir con ellos su tiempo, por su buen humor y por enseñarme a reconocer y apreciar la riqueza faunística de las praderas. Al Dr. Emmett Duffy y el equipo del Laboratorio de Biodiversidad Marina (VIMS) por acogerme en su grupo como una más y por compartir su tiempo y conocimientos conmigo. Gracias al Dr. Alejandro Martínez Abraín por sus críticas constructivas, por su vocación de naturalista de ataño, por sus consejos sobre estadística y por su lucidez generalista. Gracias también a la Dra. Rosa García Novoa por sus ánimos y por sus consejos en la redacción de esta tesis. Gracias a Eduardo Infantes y Miquel Pontes por facilitarme buena parte de las imágenes que aparecen en este documento. Al Dr. Gabriel Martorell por ayudarme con el análisis de nutrientes aunque supusiera acudir a la UIB interrumpiendo las vacaciones de Pascua, gracias. Al Dr. Ramón Roselló Mora y a Mercedes Urdiain Asensio por permitirme usar parte del equipamiento de su laboratorio y explicarme todo lo necesario para procesar nuestras muestras, por el mismo motivo gracias al Dr. Miquel Ribas Carbó. Gracias también a Juan Carlos Alonso y a Itziar Álvarez por ayudarme con los análisis de nutrientes. Al Club Náutico S’Arenal por poner a nuestra disposición sus instalaciones facilitando enormemente nuestro trabajo. Al CSIC por convocar el programa de becas predoctorales I3P-FSE que me ha permitido iniciarme en este oficio y a la Dirección general de Pesca por apoyar este proyecto como Ente Promotor-Observador. Al Ministerio de Educación y Ciencia, al VII Programa Marco de la Comisión Europea y al Gobierno de las Islas Baleares por financiar este doctorado a través de los proyectos CTM2005-23775-E, Project Conflict CGL2008-958 y UGIZC. Agradezco haber podido compartir el tiempo de esta etapa formativa con el personal de IMEDEA, muy en especial agradezco a los becarios-precarios por los buenos ratos pasados juntos, las buenas charlas, las comidas en el ágora, los partidos de voley-playa, en resumen por reflejarnos unos en otros durante estos años de convivencia.

A mi familia

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Prólogo

La presente tesis doctoral titulada "Grazing on the epiphytic community of *Posidonia oceanica* (L.) Delile: An assessment of its relevance as a buffering process of eutrophication effects" se presenta en inglés. El documento consta de ocho capítulos, cuatro de los cuales corresponden a artículos aceptados, enviados o en preparación para su publicación en revistas científicas indexadas. Cada uno de los cuatro capítulos centrales de la tesis responde a objetivos, específicos y diferenciados, relacionados con el objetivo general de este proyecto doctoral. Al tratarse de capítulos independientes, si bien relacionados, cada capítulo consta de una sección de introducción al trabajo, material y métodos utilizados, resultados obtenidos y discusión de los resultados.

A fin de contextualizar el trabajo realizado la tesis se inicia con una sección de Introducción General a modo de revisión del conocimiento existente sobre los procesos de interés para el proyecto. Asimismo la tesis cuenta con un capitulo de Discusión y Síntesis General en la parte final del documento. Esta sección engloba y pone en relación los resultados obtenidos en los capítulos centrales del documento y da respuesta al objetivo general de la tesis basándose en los resultados específicos obtenidos en dichos capítulos. Espero que esta estructura clarifique y amenice la lectura del documento.

RESUMEN

Ramoneo sobre la comunidad epifita de *Posidonia oceanica* (L.) Delile: una evaluación de su relevancia como proceso atenuador de los efectos de la eutrofización.

Las praderas de la angiosperma marina *Posidonia oceanica* L. albergan gran número de especies vegetales y animales que contribuyen notablemente a la biodiversidad de los ecosistemas costeros mediterráneos. Sobre las hojas y rizomas de la *Posidonia* se asientan gran variedad de especies epifitas cuya productividad primaria es del mismo orden de magnitud que la de la propia angiosperma. La herbivoría sobre estos organismos parece suministrar la mayor parte del carbono que asimilan los consumidores primarios y secundarios asociados a la pradera, entre los que se encuentran grupos de invertebrados, crustáceos, moluscos, equinodermos y peces que bien durante toda o parte de su vida son consumidores de epífitos y macroalgas.

Por otro lado la tasa de crecimiento de los epífitos es mayor que la de *Posidonia* y por tanto su respuesta a los procesos de eutrofización es más rápida. Esto produce una acumulación de biomasa epifita principalmente sobre las hojas de *Posidonia*; única estructura fotosintetizadora de la planta. El crecimiento excesivo de epifitos limita la adquisición de luz y nutrientes de la angiosperma y puede comprometer su crecimiento y supervivencia.

Este proyecto de tesis tiene el objetivo de esclarecer las interacciones que se están produciendo en las praderas de *P. oceanica* del litoral mallorquín entre disponibilidad de nutrientes, presión de herbivoría, biomasa de epífitos y estado de conservación de las praderas. La hipótesis de trabajo

es que el ramoneo sobre los epifitos que ejercen los invertebrados asociados a la pradera amortigua la acumulación de epifitos sobre las hojas. Para testar esta hipótesis se han desarrollado los siguientes estudios:

Estudio de la variación espacial de la carga epifita, tamaño de los haces y disponibilidad de nutrientes en dos praderas de *Posidonia oceanica* (L.) Delile de la Bahía de Palma.

Con el objetivo de conocer el comportamiento en ausencia de manipulación de las variables más relevantes para el proyecto en la zona de estudio, se evaluaron las diferencias a distintas escalas espaciales de la disponibilidad de nutrientes, la carga de epifitos sobre las hojas y el tamaño de los haces de *Posidonia oceanica* en la Bahía de Palma.

Se desarrolló un diseño muestral anidado de tres niveles que nos permitió evaluar las diferencias en los valores de las variables consideradas a las escalas espaciales de centenas de metros, decenas de metros y metros en dos localidades de la Bahía de Palma: Coll d'en Rebassa y Cap Enderrocat

En ambas praderas los valores de las variables fueron marcadamente heterogéneos a todas las escalas, excepto la carga de epifitos y la disponibilidad de nutrientes, estimada a través del contenido en nutrientes en las hojas, que fueron homogéneos a escala de decenas de metros. Los mayores porcentajes de la varianza espacial total se encuentran entre haces individuales para todas las variables, este hecho es especialmente notable en Coll d’en Rebassa donde concentra más del 65% de la variación para todas las variables.

Evaluación de la respuesta de la carga epifita sobre hojas de P*osidonia oceanica* (L.) Delile frente a la manipulación de la disponibilidad de nutrientes y la presión ejercida por la comunidad íctica.

El objetivo de este trabajo fue evaluar la respuesta de la carga de epifitos frente a una manipulación combinada de la disponibilidad de nutrientes y la presión ejercida por peces herbívoros. Se llevo a cabo un experimento de manipulación *in situ* de la disponibilidad de nutrientes y del acceso de peces a las parcelas entre junio y octubre de 2007. Se eligió un diseño experimental factorial con dos tratamientos: fertilización y exclusión de peces mediante jaulas. A partir del segundo mes desde el comienzo del experimento la carga epifita manifestó un fuerte incremento en las parcelas fertilizadas que se mantuvo los meses posteriores. No hubo cambios significativos en el comportamiento de la biomasa de epifita debida a la exclusión de la comunidad íctica. El tratamiento de exclusión excluye la herbivoría por peces pero también impide el acceso de peces carnívoros y omnívoros lo cual podría haber impulsado el aumento de las poblaciones de invertebrados ramoneadores, presas habituales de los peces y haber incrementado así la presión soportada por los epifitos. Sin embargo la biomasa de epifitos no muestra alteraciones en ningún sentido a causa del tratamiento de exclusión, lo que sugiere un papel reducido de la comunidad íctica en condiciones naturales en la Bahía de Palma.

Evaluación de la respuesta de la comunidad de invertebrados y la carga epifita en las hojas de cuatro praderas *de Posidonia oceanica* (L.) Delile de la Bahía de Palma frente a un aumento de la disponibilidad de nutrientes.

Se efectuó un estudio previo en diez localidades de la Bahía de Palma de las que se seleccionaron dos pares de localidades con cargas de de epifitos contrastantes (muy epifitada vs. poco epifitada). Se llevo a término un experimento de manipulación de la disponibilidad de nutrientes *in situ* en las cuatro praderas de un mes de duración. Se evaluó la respuesta de la comunidad de invertebrados asociados a las praderas, la carga de epifitos sobre la hojas, la disponibilidad de nutrientes (a través del contenido en nutrientes de epifitos y hojas) así como el número y tamaño de las marcas de ramoneo encontradas en las hojas antes y después de la manipulación.

El incremento de nutrientes condujo a un incremento en la biomasa epifítica que a su vez produjo un aumento en abundancia de las poblaciones de invertebrados. El aumento de las poblaciones de consumidores en las parcelas fertilizadas no devolvió la biomasa de epifitos a un nivel semejante al que encontramos en los controles. La composición de la comunidad de invertebrados en el estudio previo mostró claras diferencias entre las localidades con alta biomasa de epifitos y las localidades con baja biomasa de epífitos. Tras la adición de nutrientes las diferencias en composición de la comunidad de epifauna se redujeron entre las parcelas fertilizadas, no así para los controles.

Estimas en acuario de las tasas de consumo de epifitos de los gasterópodos mas frecuentes en las praderas *de Posidonia oceanica* (L.) Delile de la Bahía de Palma.

Se llevaron a cabo medidas en acuario de las tasas de consumo de epifitos de once especies frecuentes de las praderas de *Posidonia oceanica* en la Bahía de Palma. Con el fin de evitar confusión con el ramoneo directo que pudiera existir sobre las hojas de *Posidonia oceanica,* se empleó una malla artificial puesta a colonizar en el campo durante un mes bajo dos tratamientos: disponibilidad ambiental de nutrientes y disponibilidad de nutrientes aumentada mediante fertilización. Las estimas se llevaron a cabo presentando mallas colonizadas a individuos de cada especie durante intervalos de 24 a 72 horas en acuario. Las medidas de la tasa de consumo se realizaron mediante la comparación de contenido en clorofila a sobre la malla antes y después de haber estado expuesta al ramoneador. Las mallas colonizadas con nutrientes añadidos presentaron mayores cargas de epifitos que las colonizadas en condiciones naturales. La presencia de gasterópodos redujo significativamente la carga epifita sobre las mallas. Las tasas de consumo fueron mayores sobre las mallas colonizadas con nutrientes añadidos para la mayor parte de gasterópodos con rádula riphidoglosa, si bien este efecto no se produjo en las demás especies.

Finalmente la síntesis de los resultados parciales ha permitido demostrar que el efecto de los nutrientes sobre la biomasa de epífitos es consistente en verano y que la intensidad de la herbivoría por peces o invertebrados no es capaz de revertir el incremento de la carga epifita.

CHAPTER 1: General Introduction

Grazing on the epiphytic community of *Posidonia oceanica*: An assessment of its relevance as a buffering process of eutrophication effects

Seagrasses are highly productive clonal marine angiosperms that dominate shallow benthic ecosystems in coastal seas from the tropics to temperate latitudes (Green and Short 2003). Seagrass meadows are one of the world's most productive ecosystems with an average annual productivity of 817 g of C m^2, that is, three folds higher than coral reefs (Duarte and Chiscano 1999). Seagrass meadows provide important economic and ecological ecosystem services by serving as habitat to many species and preventing coastal erosion (Gambi et al. 1990; Perkins-Visser et al. 1996), influencing trophic webs by providing food for herbivores and detritivores (Perkins-Visser et al. 1996), and by enhancing the accumulation of particulate and dissolved organic matter in sediments, the abundance of bacteria, and the oxygenation of sediments by diffusion of oxygen through the rhizomes in the coastal and global biogeochemical cycles (Marbà et al. 2006; Duarte and Cebrián 1996). Seagrass themselves are just one component of a highly diverse ecosystem where the epiphytic algal communities contribute to 50% of seagrass meadows productivity (Borowitzka et al. 2006) and play an important trophic role sustaining a wide range of grazing organisms such as fishes and small invertebrates that fuel larger consumers (Valentine and Duffy 2006). Understanding the buffering mechanisms that keep seagrass ecosystems in balance is important to identify the exogenous impacts that can have strong harmful effects on this marine ecosystem.

Bottom-up and top-down controls in ecosystems

The structure and functioning of seagrass ecosystems and any other benthic community is driven by inputs of energy (bottom-up control) and by trophic links (top down controls). Bottom-up control refers to how resource availability (i.e. light or nutrients) regulates the structure, abundance, distribution and/or diversity of the whole community. This model, called productivity model, implies that herbivore populations are limited by the abundance of producers and, in turn, by abiotic factors (Fretwell 1977; Oksanen et al. 1981). Top-down control on the contrary refers to the influence of trophic linkages regulating community structure: in this scenario carnivore pressure will control herbivore populations and herbivore populations will control producer abundance (Hairston et al. 1960). This traditional dichotomy (Power 1992 and references herein) has evolved to a more integrative outlook which considers that community structure results from the balance of both mechanisms (Menge 1992; Power 1992).

The primacy of bottom-up or top-down control in a community will depend on the inherent productivity of the community (Burkepile and Hay 2006 and references herein), on the system hydrodynamics (Schanz et al. 2002), on the consumer community composition (Korpinen et al. 2007; Sieben et al. 2011) and their feeding preferences (Nielsen 2001). The accumulated evidence points out to stronger top-down control and trophic cascade effects in freshwater and marine systems than in terrestrial systems (Shurin et al. 2002). Trophic cascade involves that population changes in a trophic level will affect indirectly the abundance or composition of lower trophic levels by changing the abundance of organisms in trophic levels in-between. Some examples can be found in the Baltic and Mediterranean Seas where the experimental exclusion of carnivorous fishes promoted the reduction of algae cover in rocky bottoms by enhancing grazer activity through a trophic cascade mechanism (Korpinen et al. 2007; Sieben et al. 2011; Hereu et al. 2006). In the Pacific coast nutrient addition in tide pools produced an increase of algal biomass in a wave-sheltered locality only, and herbivore exclusion led to an increase of fleshy algae cover (Nielsen 2001).

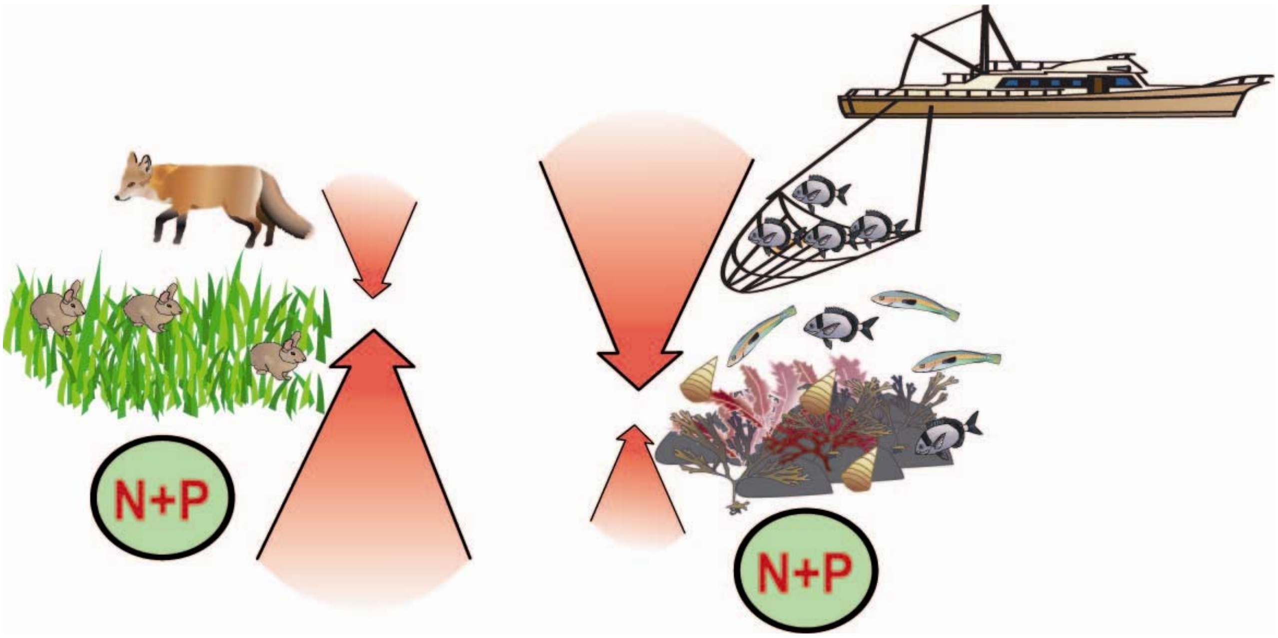


Figure 1. Schematic illustration of bottom-up and top-down control in terrestrial and marine ecosystem.

Why focus on bottom-up and top-down control in seagrass meadows

Seagrass meadows are currently among the most vulnerable ecosystem, experiencing global decline rates of 2-5% per year (Duarte et al. 2008; Waycott et al. 2009), leading to the loss of the functions and the goods and services these ecosystems provide. The causes of this decline are of anthropogenic origin, in particular eutrophication, mechanical destruction from anchoring, trawl fishing, dredging and coastal construction activities, as well as the introduction of exotic species and global change (Duarte 2004). Fisheries have also indirect impacts on seagrasses by removing higher trophic levels from the community and modifying the trophic web structure through a trophic cascade mechanism (Sala et al. 1998; Estes et al. 2011).

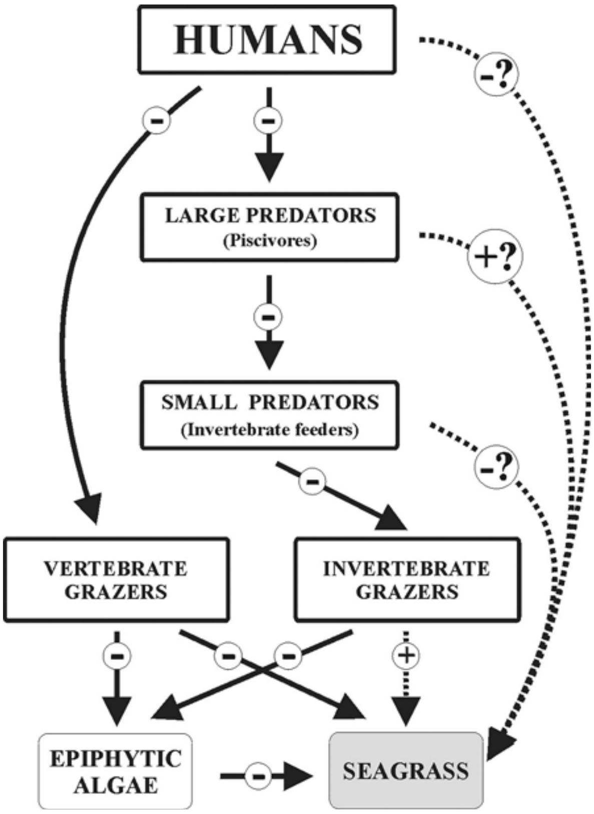


Figure 2. Conceptual framework of seagrass-bed food web. Solid and broken arrows indicate direct and indirect effects, respectively. The possible cascading effects of humans and other predators on seagrasses are indicated with question marks, reflecting the current inadequacy of data to evaluate these potential effects. Note that the hypothesized human impacts include only those mediated through the food web, not those resulting from eutrophication and other disturbances (From Valentine and Duffy 2006).

Eutrophication is possibly the most widespread impact on seagrass ecosystem. The response of marine angiosperms to nutrient addition varies from increased growth to growth inhibition and die-off depending on species and nutrient addition method, intensity, and duration of the nutrient load (Burkholder et al. 2007 and references herein). Increased nutrient availability will stimulate the productivity of seagrass communities by enhancing fast growing competing autotrophs (Nielsen 2001) and might also produce physiological stress of the seagrass due to nitrate or ammonium toxicity. The accumulation of epiphyte biomass on seagrass leaves increases the rates of leaf loss, decrease photosynthesis rates and reduces leaf nutrient uptake (Tomasko and Lapointe 1991; Burkholder et al. 1992; Wear et al. 1999; Cornelisen and Thomas 2004). There is accumulated evidence supporting this bottom-up approach of seagrass decline. Increased productivity of diatoms and filamentous epiphytic algae of epiphyte together with reduced seagrass production has been found in response to increased nutrient availability in the water column in European and American *Zostera marina* beds (Borum 1985; Colleman and Burkholder 1994). Similar response was found in *Syringodium filiforme, Thalassia testudinum* (Wear et al. 1999) and macroalgae in a *Zostera noltii* ecosystem in Southern Portugal (Carbaço et al. 2008).

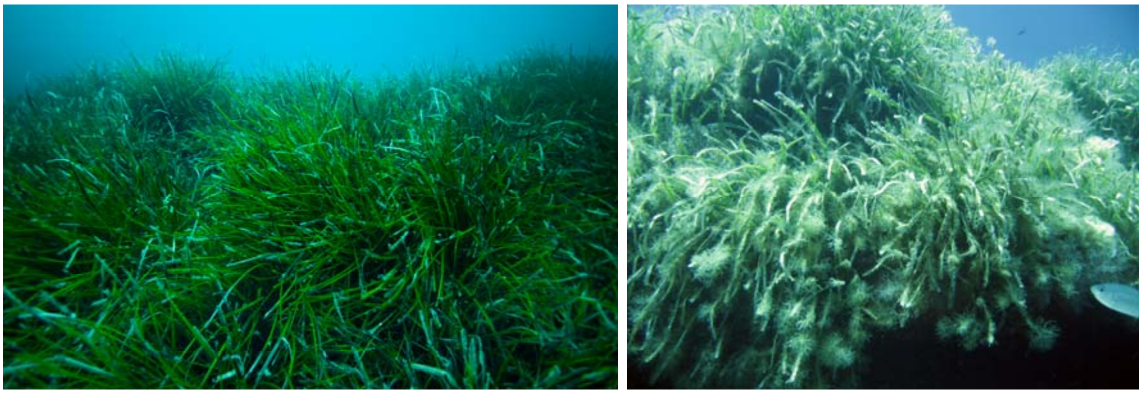


Figure 3. Typical “healthy” seagrass meadow and seagrass covered in high algal (epiphyte) growth (Photo by Eduardo Infantes).

Nutrients, temperature and light seem to regulate the primary production of *Posidonia oceanica* (Alcoverro et al. 1995; 1997). Leaf production in *P. oceanica* is highest in spring when light and nutrient conditions are not limiting and decrease during the summer. The long life-span of *P. oceanica* leaves (202-345days) (Hemminga et al. 1999) allows the development of an abundant and species-rich epiphyte community. Nutrient availability in the water column results in strong increases of epiphyte biomass on *P. oceanica* leaves during summer (Prado et al. 2008a). Thus bottom-up control seemly has a relevant role in Mediterranean meadows.

Traditionally, the approach of bottom-up regulation of epiphyte algae has underestimated the importance of grazers in controlling the growth of algal component in seagrass systems. Grazing is a simultaneous process that may buffer the effects of algal proliferation on seagrass productivity and vitality. In the seagrass meadows of Philippines, grazer populations were able to consume between the 20% and 62% of the epiphyte production (Klumpp et al. 1992). Similar results were found in Baltic and Atlantic *Zostera marina* meadows where grazers reduced epiphyte biomass by over 60% (Borum 1987; Duffy et al. 2001). In a *Zostera noltii* meadow experimental increase of mudsnail density led to a 50% decrease of periphyton total chlorophyll content and enhanced seagrass vitality (25% increase of shoot density and 50% increase of biomass) (Philippart 1995).

The accumulated evidence of consumer regulation in *P. oceanica* meadows is focussed on the macrograzers herbivore fish *Sarpa salpa* and the sea urchin *Paracentrotus lividus. S. salpa* feeds on the leaves and the epiphytes *of P. oceanica* and can slightly reduce epiphyte biomass (Prado et al. 2007; Tomas et al. 2005a). *P. lividus* achieves strong reductions of epiphyte load (60-80% after Tomas et al., 2005) by feeding preferentially on the oldest leaves of the shoots which have higher epiphytic biomass. Although both macrograzers feed simultaneously on leaves and epiphytes, their main nitrogen source comes from epiphytes (Jennings et al. 1997; Tomas et al. 2006). Epiphytes are also the main carbon and nitrogen source for some species of gastropods (Gacia et al. 2009). The role of the invertebrate epifauna community (crustaceans, molluscs, polychaetes) of *P. oceanica* meadows is to a large extent unknown although several are supposed to feed on epiphytes (Mazzella and Russo 1989; Mazzella et al. 1992).

Figure 4. *Smaragdia viridis* on a *Posidonia oceanica* leaf. Radular mark on the leaf is visible in the image (Photo by Inés Castejón).

The regulation of epiphyte biomass by consumer pressure depends on the grazer community species composition and on the resource availability in the system. Grazer population may increase their abundance and consumption rates (i.e. can have both numerical and functional responses) under nutrient enriched conditions due to the better quality and quantity of the food supply (e.g. Jaschinski and Sommer 2011). The reduction of the effects of nutrient enrichment in epiphyte load and seagrass vitality by epiphyte grazers is favoured by high grazer densities and moderate eutrophic scenario (e.g. Jaschinski and Sommer 2008a). A recent meta-analysis showed that the positive effect of algal grazers on seagrasses was comparable in magnitude to the negative effect of water column nutrients on the plant (Hughes et al. 2004).

Figure 5. *Gibbula ardens* on a *Posidonia oceanica* leaf (Photo by Inés Castejón)

The functional diversity of grazer communities also plays a main role in the potential top-down control of epiphyte biomass. In this sense a mesocosms experiment in the York River Estuary found the amphipods *Gammarus, Cymadusa* and *Dulichiella* and the isopod *Idotea* to be more efficient mesograzers over eelgrass epiphytes than *Bittium* and *Erichsonella* (Duffy et al. 2003). In another eelgrass system the gastropods *Littorina* and *Rissoa* were responsible for the major reductions of epiphyte biomass, and *Gammarus* was a low effectiveness mesograzer when compared with the other grazer species in the community (Jaschinski and Sommer 2008b).

Figure 6. *Serranus scriba* in *Posidonia oceanica* meadow (Photo by Eduardo Infantes)

Fisheries drive shifts in fish community structure and promote changes at lower trophic levels in the community (Sala et al. 1998; Pinnegar et al. 2000). An experimental manipulation of mesopredator density was performed in a *Zostera marina* meadow to emulate the effects of top-predator removal. The inclusion of the mesopredator *Callinectes sapidus* decreased grazer abundance and promoted the increase of epiphyte biomass (Douglass et al. 2007). An analogous experiment led to similar results with the inclusion of a gobid in a Swedish *Z. marina* meadow (Moksnes et al. 2008).

The case of the *Posidonia oceanica* meadows

Posidonia oceanica is a Mediterranean endemism that forms widespread monospecific meadows covering about the 23% of the basins between the depths of 0-40 meters (Pascualini et al. 1998). The *P. oceanica* bottoms frequently show substantial spatial heterogeneity with patched distribution and important variations of the plant features at all spatial scales ranging from kilometres to centimetres (Balestri et al. 2003). *P. oceanica* meadows are climax communities adapted to an oligotrophic environment which provide major ecological and economic services like oxygen production, nutrient recycling, shoreline protection, fish nursery and water transparency. The production of *P. oceanica* leaves has been estimated between 162-722 g dry weight m^{-2} $year^{-1}$ in shallow beds (Buia et al. 2000).

The distribution of epiphyte community is not spatially homogeneous along the shoots or meadows, higher biomass and diversity are found on the older parts of the leaves (Alcoverro et al. 2004) and high variability of the composition has been detected at spatial scales ranging from metres to kilometres (Piazzi et al. 2004; Pardi et al. 2006; Balata et al. 2007).

Nowadays the increase of human population along Mediterranean coasts lead to more discharges of waste waters and to the increase of nutrient inputs to the meadows. The rise of nitrogen and phosphorus availability increase fast-growing epiphyte algae biomass and promote the decline of *P. oceanica* competing for light and nutrients as reported in other seagrass systems (Silberstein et al. 1986; Tomasko and Lapoint 1991). While other seagrass species may rapidly recover and even expand their coverage after a regression event, the acutely slow-growth rates of *Posidonia* make the regression irreversible at human scales (Boudouresque et al. 2009). The eutrophication disturbance concurs with the impacts of artisanal and industrial fisheries that have fully exploited or overexploited most demersal stocks of the Mediterranean (Coll et al. 2006). The disturbance of the trophic structure is evident in the gradual reduction of the mean trophic level on the fishing catch in the past 50-year historical series (Pauly et al. 1998). The primacy of resources or trophic control in the regulation of epiphytic biomass will highlight the vulnerability of *P. oceanica* meadows to different kinds of human disturbances.

The role of the invertebrate community in the regulation of the epiphyte biomass is not understood well enough so far. The characterization of the epifaunal community hosted by *P. oceanica* meadows is also necessary to elucidate the strength of the trophic links between grazers and epiphytes.

The aim of this PhD thesis is to assess the relative strength of bottom-up and top-down regulation in the regulation of the epiphytic biomass in *Posidonia oceanica* leaves. Our specific objectives were:

1) To evaluate the spatial variability of epiphyte load and nutrient availability in *P. oceanica* meadows.
2) To assess the importance of grazing as a buffer of the effects of nutrient availability on epiphyte load.
3) To evaluate the effects of fish community in the control of epiphyte biomass.
4) To assess the feeding rates of common species of mesograzers in *P. oceanica* meadows.
5) To elucidate if the mesograzer community of *P. oceanica* meadows is affected by a nutrient-driven increase of epiphyte biomass.

During this thesis I carried out an in situ evaluation of nutrient content in both the leaves and the epiphytes of *P. oceanica*, epiphyte biomass and *P. oceanica* shoot size at different spatial scales across Palma Bay (Majorca, Balearic Islands). I evaluated the spatial distribution and the relationship among those variables in natural conditions. This allowed testing the following hypotheses:

- Epiphyte biomass in *P. oceanica* leaves is determined by nutrient availability in the water column and both variables are correlated in natural conditions.
- The spatial distribution of epiphyte biomass is similar to the spatial distribution of nutrient availability.

Second, I performed an experimental addition of nutrients in the water column combined with the exclusion of the fish community. I evaluated the response of epiphyte biomass, *P. oceanica* shoot size, gastropod grazing marks and fish bites on the leaves and nutrient content in the leaves and in the epiphytes. This allowed testing the following hypotheses:

- Increased nutrients in the water column drive changes on epiphyte biomass.
- Fish community removal modifies the response of epiphyte biomass to nutrient availability.

Third, I performed an experimental addition of nutrients in the water column in localities with initial contrasting epiphyte load. I characterized the invertebrate community composition before and after nutrient enrichment. I evaluated the response of epiphyte biomass, nutrient content in the leaves and in the epiphytes, invertebrate community and *P. oceanica* shoot size. This allowed testing the following hypotheses:

- Increased nutrients in the water column drive changes on epiphyte biomass.
- The composition of the invertebrate community responds to nutrient-driven changes of epiphyte biomass.

- The grazer community modifies the response of epiphyte biomass to nutrient availability.

Fourth, I evaluated the consumption rates of some of the most frequent grazer gastropods of *P. oceanica* meadows under aquarium conditions and whether those rates were affected by nutrient-driven changes of epiphyte biomass. This will allow testing the following hypothesis:

- There are species–specific effects on grazing pressure.
- Feeding rates of grazers increase in a scenario of higher epiphyte biomass.

Study Site

Our study was performed in Palma Bay, located in the southern part of the Majorca Island. Majorca is the main island in the Balearic Archipelago, has 623 km of coastline, 39 harbours, and a total of 14 196 moorings in October 1998 (Morales-Nin et al. 2005).

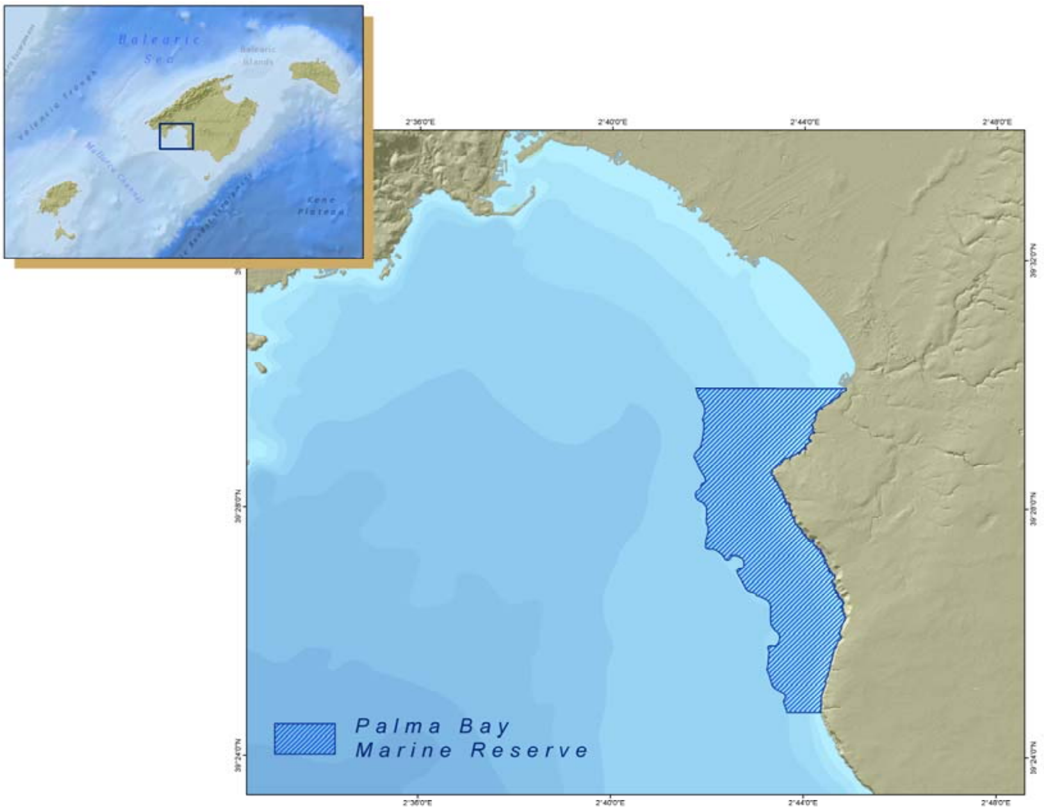
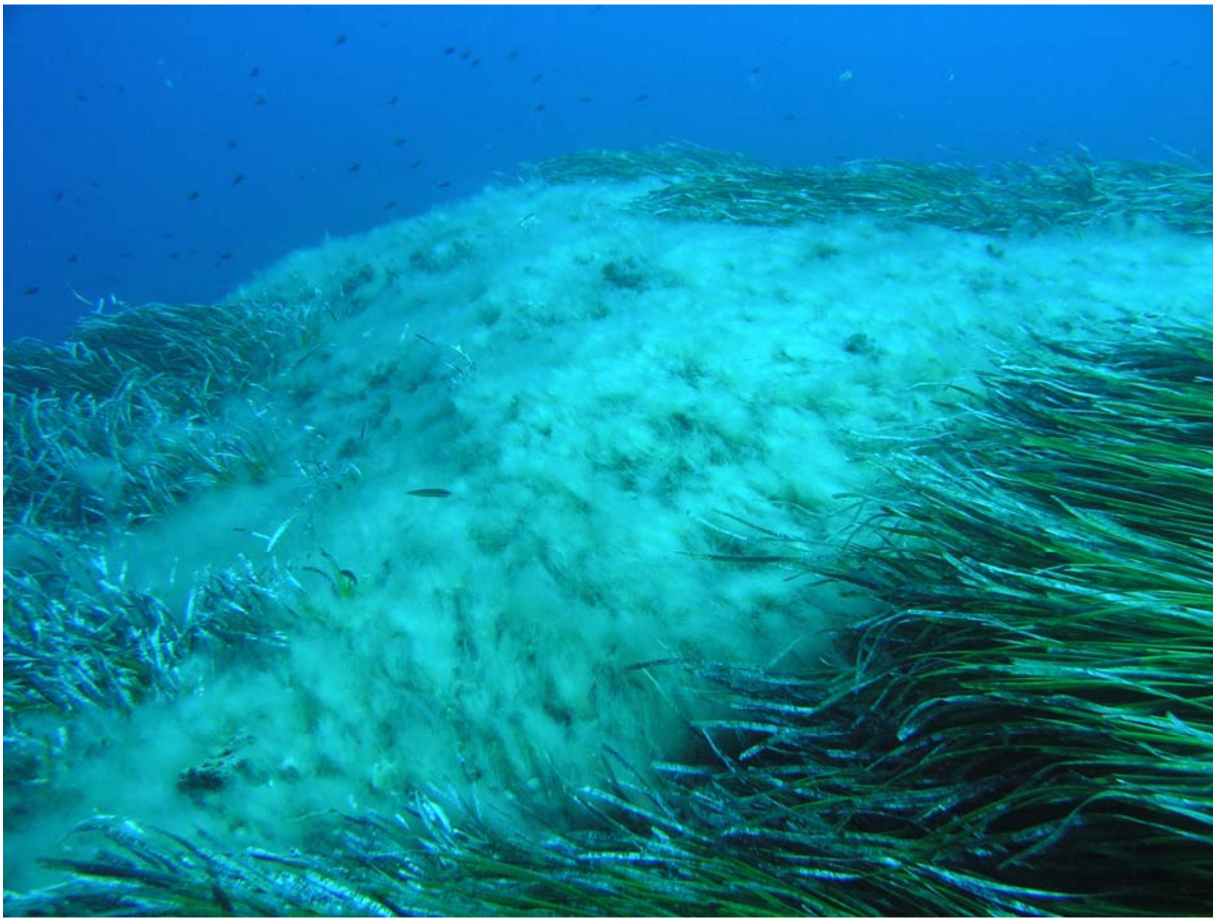


Figure 7. Location Map of the Balearic Islands with the detail of Palma Bay. Marine Reserve of Palma Bay is indicated in the map and isolines are marked in blue tones at 10 m intervals.

The bay of Palma has 489.122 inhabitants (National Institute of Statistics) and is one of the areas with the highest densities of recreational fishers (Morales-Nin et al. 2005). Palma Bay has 50 km of coastline, and covers 220 km^2 of surface with an average slope of 3 % and a maximum depth of 50 m. Bottom habitats of the bay from 0 to 35 m are dominated by seagrass meadows of *P. oceanica* and rocky bottoms. Sediments are mainly composed by carbonates of coarse granulometry (sand and gravels) (Orfila et al. 2011). Mild winds and currents, below 6 m/s and 0.5 cm/s respectively, prevail in Palma Bay. There are punctual and diffuse nutrient inputs in Palma Bay. The punctual sources of nutrients mainly come from torrents discharges, during strong rain events, and wastewater outfalls. Diffuse nutrient sources come from runoff and groundwater. In raining season, punctual and diffuse drainage drive important rises of the nutrient content in the water column of Palma Bay (Basterretxea et al. 2011; Vol. 5; Informe Final Proyecto Playa de Palma; IMEDEA and Consorci Playa de Palma). Marine Reserve is located in the eastern part of Palma Bay, and protects an open water area that expands from the shoreline to the 30 m isobath. This MPA is divided into two management areas with different levels of protection: (1) the Integral Zone where all fishing activity is prohibited, and (2) the Buffer Zone, where both artisanal and recreational fisheries are permitted but with some management regulations (e.g., daily bag limits, minimum hook size and temporal closures) (March et al. 2011).

CHAPTER 2

Patterns of spatial variation of nutrient content, epiphyte load and shoot size of *Posidonia oceanica* meadows



Patterns of spatial variation of nutrient content, epiphyte load and shoot size of *Posidonia oceanica* meadows. Inés Castejón-Silvo, Jorge Terrados. Manuscript accepted in Marine Ecology 2011.

Abstract

Knowledge of patterns of spatial variability of vegetative development, epiphyte load and nutrient availability in seagrass meadows is essential for the adequate design of research and environmental monitoring programmes. Differences in shoot size, epiphyte load and nutrient content of leaves and epiphytes of the Mediterranean endemic seagrass *Posidonia oceanica* at spatial scales ranging from metres to hundreds of metres are evaluated using a hierarchical nested sampling design. The size and epiphyte load of *P. oceanica* shoots and the nitrogen and phosphorus content of leaves and epiphytes were different in most of the spatial scales considered. Sampling efforts concentrated at the metre scale incorporated most of the variability in size, epiphyte load and nutrient content of the leaves and epiphytes of *P. oceanica* shoots. Epiphyte load showed no correlation with nutrient content in the epiphytes or in the leaves. However, epiphyte load and shoot size negatively correlated, which suggests that light penetration in the canopy may be a main determinant of epiphyte load.

Keywords: epiphyte load; nitrogen; phosphorus; *Posidonia oceanica*; seagrass; shoot size; spatial scales.

Introduction

Seagrasses are key components of shallow coastal ecosystems in temperate and tropical coasts (Green and Short 2003). Seagrasses are vulnerable to increases of water turbidity and sediment and nutrient loads in coastal waters (Ralph et al. 2006, 2007) and their presence and status is considered indicative of the quality of coastal waters (Kenworthy et al. 2006). Seagrass beds are characterized by complex above and belowground structures that provide a habitat for numerous sessile and mobile species (Williams and Heck 2001). The epiphytic community that grows on the leaves and rhizomes is an important contributor to seagrass ecosystem productivity (Borowitzka et al. 2006), provides food for a diverse assemblage of fish and invertebrates (Fong et al. 2000; Russell et al. 2007) and plays a major role in nitrogen assimilation and carbonate production (Gacia et al. 2002; Romero et al. 2006; Lepoint et al. 2007).

Epiphyte abundance and species composition are early responders to changes in environmental quality (Frankovich et al. 2006; Giovannetti et al. 2010; Martinez-Crego et al. 2010) and may be indicators of human-induced disturbances (Piazzi et al. 2004; Balata et al. 2008; Balata et al. 2010; Giovannetti et al. 2010; Martinez-Crego et al. 2010). Increased nutrient loadings in the water column promote increases of epiphyte biomass and epiphyte overgrowth has been considered a driver of seagrass loss though the obstruction of light and nutrient flow to the leaves (Silberstein et al. 1986; Tomasko and Lepoint 1991; Frankovich and Fourqurean 1997; Hauxwell et al. 2001; Perez et al. 2008). In oligotrophic coastal systems, epiphyte biomass seems to be less sensitive to increased nutrient loads than the species composition of the epiphyte community (Piazzi et al. 2004; Prado et al. 2008a; Terrados and Pons 2008; Fourqurean et al. 2010).

The status of seagrass meadows and their epiphytic community is usually assessed by extrapolating data from samples at various sites obtained along a depth or disturbance gradient (Mazzella et al. 1989; Perez et al. 2008) or from samples collected at random sites and subsites (Alcoverro et al. 1995; Giovannetti et al. 2010). Knowledge of the spatial variation patterns of the descriptors considered and how those patterns change according to the spatial measurement scale is required to support conclusions obtained by extrapolating data from various sites and attempt to discover the mechanisms behind the patterns. For instance, the epiphytic biomass in multi-species Australian temperate seagrass meadows was homogeneous when samples were separated from decimetres to tens of metres, yet was different when the samples were collected in meadows tens and hundreds of kilometres apart (Moore and Fairweather 2006). The abundance of different functional groups of epiphytic macroalgae in *Zostera marina* L. meadows was not different at the spatial scale of metres, yet was indeed different when the samples were separated by kilometres (Saunders et al. 2003). The wealth of species in the epiphytic macroalgal community increases progressively with an increasing spatial scale (from tens to thousands of metres) in *Posidonia coriacea* (Kuo and Cambridge) (Vanderklift and Lavery 2000) and *Amphibolis griffithii* (J. Black) Den Hartog (Lavery and Vanderklift 2002). These results highlight the complexity provided by multi-scale spatial variability when considering ecosystem traits (Wiens 1989; Levin 1992).

Posidonia oceanica (L.) Delile is the main seagrass species in the Mediterranean Sea, since it covers 23% of all shallow bottoms (depth < 45 m) (Bethoux & Copin-Montegut 1986; Pasqualini et al. 1998; Procaccini et al. 2003). *P. oceanica* forms wide monospecific meadows characterized by a complex topography and patchiness at shallow depths (Mateo et al. 1997; Kendrick et al. 2005). Nutrient availability plays a major role in *P. oceanica* growth (Alcoverro et al. 1995) and epiphyte development (Prado et al. 2008a).

A high variability in the composition of the epiphytic community has been detected at spatial scales ranging from metres to kilometres in *P. oceanica* meadows (Piazzi et al. 2004; Pardi et al. 2006; Balata et al. 2007). In contrast to epiphytes, the patterns of spatial variability of seagrass vegetative development have not been studied as exhaustively, yet high variability in *P. oceanica* meadows has been detected at small spatial scales (from centimetres to hundreds metres) (Balestri et al. 2003; Gobert et al. 2003; Borg et al. 2005). Epiphyte nutrient content is considered an indicator of nutrient availability in the water column (Lin et al. 1996; Perez et al. 2008). The leaf nutrient content is an indicator of the balance between environmental nutrient availability, nutrient storage, nutrient retranslocation and nutrient requirements for seagrass growth (Duarte 1990; Fourqurean et al. 1992; Abal et al. 1994; McClelland and Valiela 1998; Fourqurean et al. 2007; Lepoint et al. 2008). The hypothesis of this study is that the patterns of spatial variability of vegetative features of the plant and epiphyte load are consistent with the spatial variability of nutrient content of the leaves and epiphytes. We expect epiphyte nutrient content to be positively associated with epiphyte load. To that end, we use a hierarchical, nested sampling design to evaluate differences in the size and epiphyte load of *P. oceanica* shoots and in the nitrogen and phosphorus content of the epiphytes and of *P. oceanica* leaves at three spatial scales (metres, tens of metres, and hundreds of metres) and to estimate how total variance is distributed among the different spatial scales in two localities.

Material and methods

The study was conducted in two localities in a *Posidonia oceanica* meadow in Palma Bay (Mallorca, Western Mediterranean), namely Coll d'en Rebassa (39º 32' N, 2º 41' E) and Cap Enderrocat (39º 29' N, 2º 29' E), both located on sandy sediments at depths from 17 to 22 metres, 1-1.5 km from the coastline and six kilometres from each other. *P. oceanica* is a cover dominant in the bottom of Palma Bay between 10 and 30 metres of depth (Fig.1) (Rey and Diaz del Rio 1989). Land-derived nutrients enter Palma Bay at several places (Fig. 1). A hierarchical, nested sampling design was used including three levels or spatial scales: hundreds of metres (site), tens of metres (subsite), and metres (plot) (Fig. 1). Three sites roughly 600 metres from each other were randomly selected in each of the Rebassa and Enderrocat localities. Three subsites approximately 100 metres from each other were randomly selected in each site and three 0.25 square-metre plots were randomly selected. Ten *P. oceanica* orthotropic shoots were randomly collected in each plot by SCUBA divers, placed in individual zip-lock plastic bags and kept frozen until processing. Sampling started on November 7, 2006 and ended on December 12, 2006.

In laboratory, epiphytes were carefully scraped using a razor blade from leaves of each shoot and they were collected in pre-weighed Whatman GF/C fiberglass. Filters and leaves were then dried at 60ºC for 48 h and they were weighed with milligram precision to estimate the mean leaf biomass per shoot (g DW shoot^{-1}) and to calculate the mean epiphyte dry weight per shoot (g DW epiphytes / g DW shoot^{-1}) after subtraction of the pre-determined dry weigh of the filter.

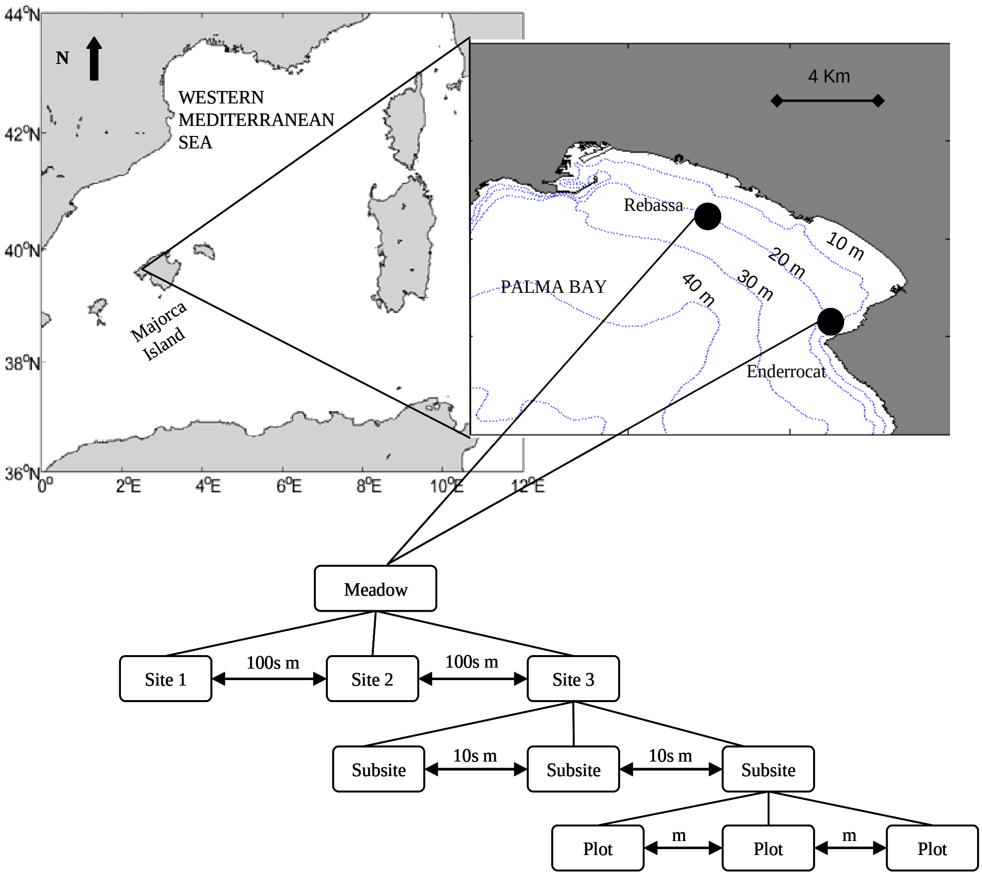


Figure 1. Location of Palma Bay (Majorca, Western Mediterranean) with detail of the two meadows studied (Coll d'en Rebassa, Cap Enderrocat) and the scheme of the hierarchical nested sampling design performed in each of them. Isolines in the Palma Bay panel represent depth contours at 10 m intervals.

Three shoots and three filters with scraped epiphytes from each plot were randomly selected and ground to powder with a stainless steel ball mill (MM200 RETSCH, Haan, Germany). Different aliquots of the ground material were used to determine the total concentration of nitrogen and phosphorus in the leaves of each shoot and in the epiphytes. The total nitrogen content of the leaves was determined using a Heraeus CHN-o-rapid elemental analyser and expressed as the % of DW. The total nitrogen content in the ground filters with scraped epiphytes was analysed using a CHN elemental analyzer (1100 CE Instruments, Elantech, NJ, USA) connected to an Isotope Ratio Mass

Spectrometre (IRMS) Delta-Plus (Thermo). To verify that no nitrogen signal due to filter composition was present, additional ground filters with no epiphytes were analysed. The total nitrogen in epiphytes was expressed as the % of DW after correction for the contribution of filter DW to sample weight. We used certified standard beech leaves (CRM No. 100) as reference material for nitrogen concentration. We tested the accuracy and possible bias between the two nitrogen determination methods by analyzing ten samples of leaves and epiphytes in both analyzers and the differences in the determinations were smaller than 0.01 % DW. The phosphorus content in the leaves and epiphytes was analysed following the protocol described by Fourqurean et al. (1992), using high temperature combustion and addition of Na_2SO_4 and $MgSO_4$. Certified standard beech leaves were used as reference (CRM No. 100). The total phosphorus content in epiphytes was expressed as the % of DW after correction for the contribution of filter DW to sample weight.

Shoot size was described in terms of the average number of leaves per shoot (n shoot^{-1}) and leaf biomass per shoot (g DW shoot^{-1}). The epiphyte load per shoot was calculated as the ratio of epiphyte biomass to leaf biomass (g DW epiphytes / g DW shoot^{-1}).

Statistical analyses were performed using STATISTICA.7 (StatSoft, Inc. 2005). The differences in epiphyte load, shoot size and nutrient content of the leaves and epiphytes in the Rebassa and Enderrocat localities were evaluated using t-tests. Nested ANOVA was performed in each locality to evaluate the differences at each spatial scale. Site, subsite and plot were introduced into the analysis as random effects. The percentage of total variance attributable to each spatial scale was also calculated for all the variables. A non-linear correlation analysis (Spearman rank) was used to evaluate the association between the shoot size, epiphyte load and nutrient content of the shoots and epiphytes in each locality.

Results

Posidonia oceanica leaves showed higher phosphorus content in Rebassa than in Enderrocat (Table 1, Fig. 2). The phosphorus content of the epiphytes was higher in Enderrocat than in Rebassa (Table 1, Fig. 3). There were differences in the size of *P. oceanica* shoots in terms of the number of leaves and biomass, which were lower in Rebassa than in Enderrocat (Table 1, Fig. 2). Enderrocat shoots had lower epiphyte load than Rebassa shoots (Table 1, Fig. 3).

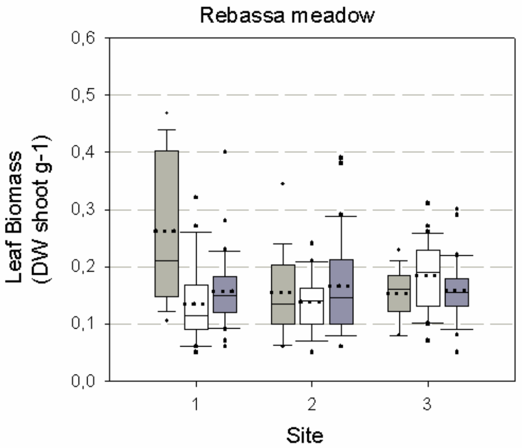
The nitrogen content of the leaves was different at the spatial scales of hundred metres (among sites) and metres (among plots) in both localities (Table 2, Fig. 2). The phosphorus content of *P. oceanica* leaves was also different at all the spatial scales examined in both localities as well. The number of leaves per shoot and leaf biomass were different at almost all spatial scales considered in both localities (Table 2).

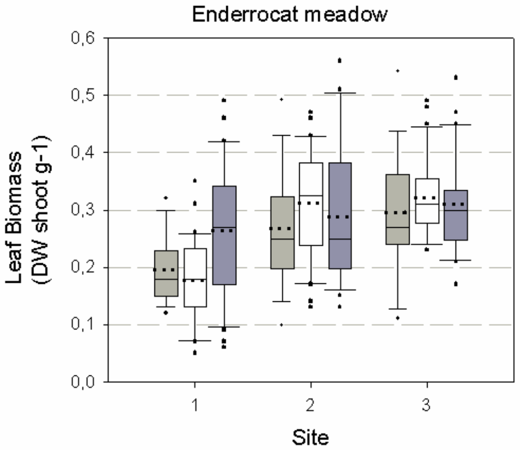
Table 1. Summary of comparisons between Rebassa and Enderrocat for shoot size, epiphyte load, leaf nitrogen and leaf phosphorus content and epiphyte nitrogen and epiphyte phosphorus content of *Posidonia oceanica* shoots in Palma Bay (Majorca, Western Mediterranean) using T-test.

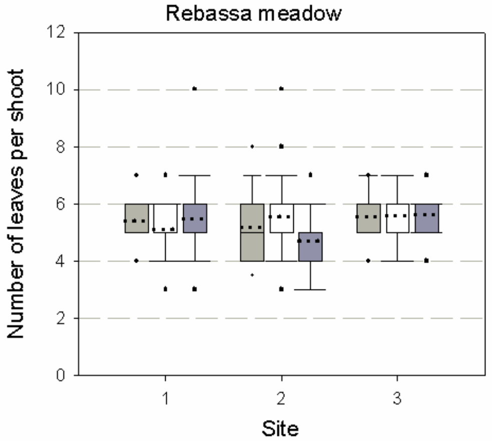
		Rebassa			**Enderrocat**				
Parameter	**df**	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**t-value**	**p**
Leaf nitrogen (%DW)	160	81	1,52	0,253	81	1,53	0,179	-0,441	0,6599
Leaf phosphorous (%DW)	160	81	0,19	0,067	81	0,16	0,033	4,449	**<0,001**
Number of leaves per shoot	538	270	5,4	1,04	270	5,9	1,050	-6,466	**<0,001**
Leaf biomass (g DW shoot^{-1})	538	270	0,17	0,081	270	0,27	0,105	-12,678	**<0,001**
Epiphyte nitrogen (%DW)	160	81	0,64	0,215	81	0,63	0,262	-0,463	0,6440
Epiphyte phosphorus (%DW)	160	81	0,07	0,056	81	0,11	0,067	4,050	**<0,001**
Epiphyte load (g DW/g DW shoot^{-1})	538	270	0,29	0,165	270	0,18	0,077	9,440	**<0,001**

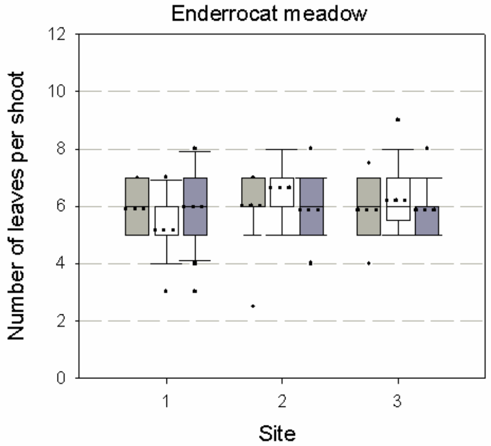
The nitrogen content of the epiphytes was not different at almost any spatial scale with the exception of Rebassa locality which showed differences within subsites (Table 2, Fig. 3). The phosphorus content of *P. oceanica* epiphytes was different at all spatial scales in Enderrocat, but only at the hundreds of metres spatial scale (among sites) in Rebassa. The epiphyte load of the *P. oceanica* leaves was different at the spatial scales of hundreds of metres and metres in Rebassa and Enderrocat (Table 2, Fig. 3).

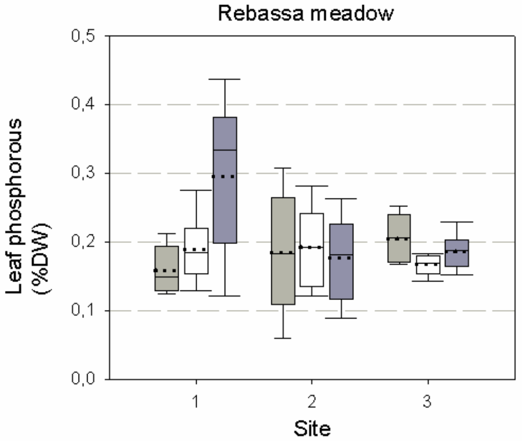
Figure 2 (next page). Shoot size, number of leaves per shoots and nitrogen and phosphorus content in the leaves of *Posidonia oceanica* shoots in two meadows in Palma Bay (Majorca, Western Mediterranean). Sub-sites are indicated by grey tones. The boundary of the box closest to zero indicates the 25th percentile, the solid line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th Whiskers above and below the box indicate 10th and 90th percentiles. Outliers are marked with points and dotted line represents the mean.

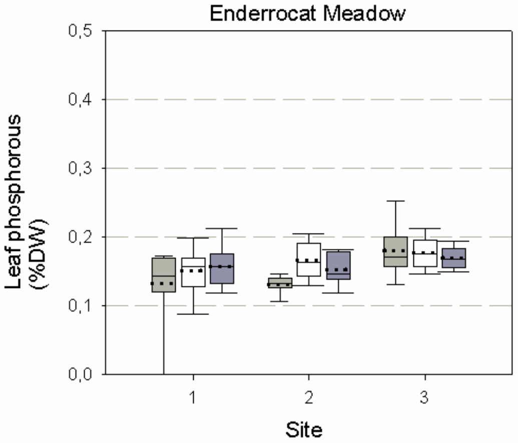

Rebassa meadow

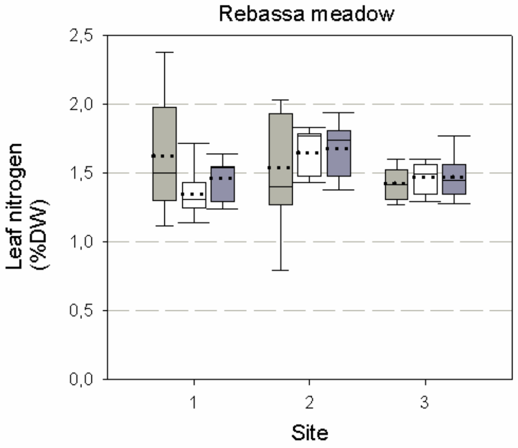

Enderrocat meadow

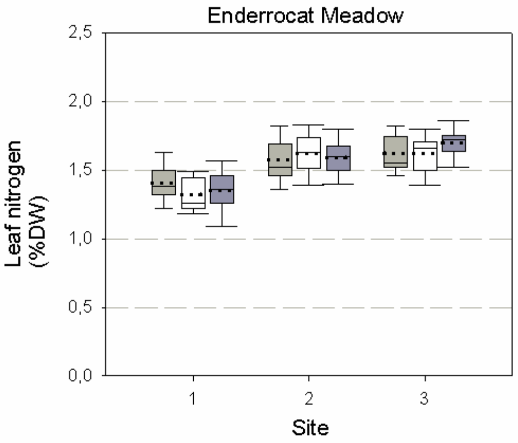

Rebassa meadow


Enderrocat meadow


Rebassa meadow


Enderrocat Meadow


Rebassa meadow


Enderrocat Meadow

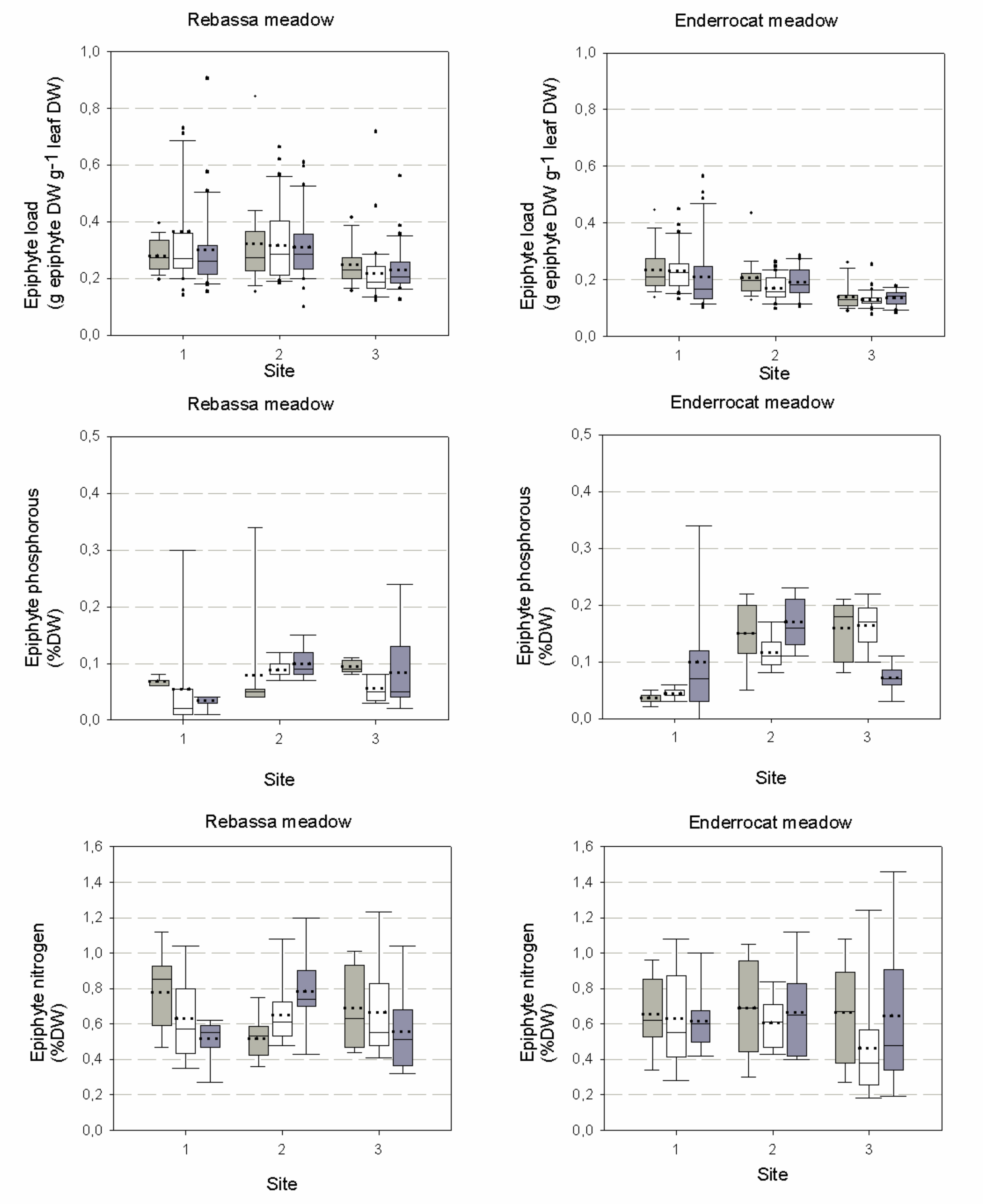


Figure 3. Epiphyte load, epiphyte nitrogen content and epiphyte phosphorus content of *Posidonia oceanica* shoots in two meadows in Palma Bay (Majorca, Western Mediterranean). Sub-sites are indicated by grey tones. Box legend similar to figure 2.

Table 2. Summary of comparisons in each locality between sites, subsites, and plots for leaf nitrogen and phosphorus content, shoot size, epiphyte nitrogen and phosphorus content and epiphyte load of *Posidonia oceanica* shoots in Palma Bay (Majorca, Western Mediterranean) using nested ANOVAs. Significant levels are indicated by: p<0.05* ; p<0.01** ; p<0.001***

		Leaf nitrogen (%DW)			Leaf phosphorus (%DW)			Number of leaves per shoot			Leaf biomass (g DW shoot^{-1})			Epiphyte nitrogen (%DW)			Epiphyte phosphorus (%DW)			Epiphyte load (g DW/g DW shoot^{-1})		
		df	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**
Rebassa	Site	2	0,223	4,782*	2	0,008	3,385*	2	3,226	3,223*	2	0,023	5,133**	2	0,002	0,046	2	0,010	3,758*	2	0,209	8,432***
	Subsite	6	0,075	1,604	6	0,017	7,294***	6	2,574	2,572*	6	0,051	11,169***	6	0,120	3,228**	6	0,002	0,923	6	0,024	0,961
	Plot	18	0,095	2,026*	18	0,007	2,957**	18	1,437	1,436	18	0,018	3,872***	18	0,053	1,421	18	0,004	1,617	18	0,043	1,717*
	Error	54	0,047		54	0,002		243	1,001		243	0,005		54	0,037		54	0,003		243	0,025	
		df	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**	**df**	**MS**	**F**
Enderrocat	Site	2	0,634	51,255***	2	0,007	9,739***	2	5,181	5,684**	2	0,235	35,438***	2	0,028	0,416	2	0,057	30,069***	2	0,189	56,913***
	Subsite	6	0,013	1,050	6	0,002	2,276*	6	3,907	4,287***	6	0,029	4,314***	6	0,043	0,640	6	0,014	7,447***	6	0,005	1,449
	Plot	18	0,030	2,462**	18	0,002	2,185*	18	2,281	2,503***	18	0,040	6,074***	18	0,085	1,256	18	0,003	1,799*	18	0,022	6,537***
	Error	54	0,012		54	0,001		243	0,912		243	0,007		54	0,067		54	0,002		243	0,003	

The variability within plots (among shoots) was the most important contributor to the total variance of all the variables studied (Fig. 4). This was particularly evident in Rebassa. Only in the case of the nitrogen content of the leaves in Enderrocat the variability among sites showed the greatest contribution to total sample variance (Fig. 4).

The nitrogen and phosphorus contents of the *P. oceanica* leaves were positively correlated in both locations (Table 3). The nitrogen content of the leaves did not correlate with shoot size. The phosphorus content of the leaves did not correlate with shoot size in Enderrocat, but was negatively correlated in Rebassa.

The comparison between nutrient contents and epiphyte load showed a negative correlation with epiphyte P only in Enderrocat. Nutrient contents of epiphytes were positively correlated with shoot size with the exception of Enderrocat, where epiphyte P was not correlated to the number of leaves per shoot. The nitrogen and phosphorus content of epiphytes positively correlated in Rebassa, but not in Enderrocat. We found a strong negative correlation between epiphyte load and shoot size both in Rebassa and Enderrocat (Table 3).

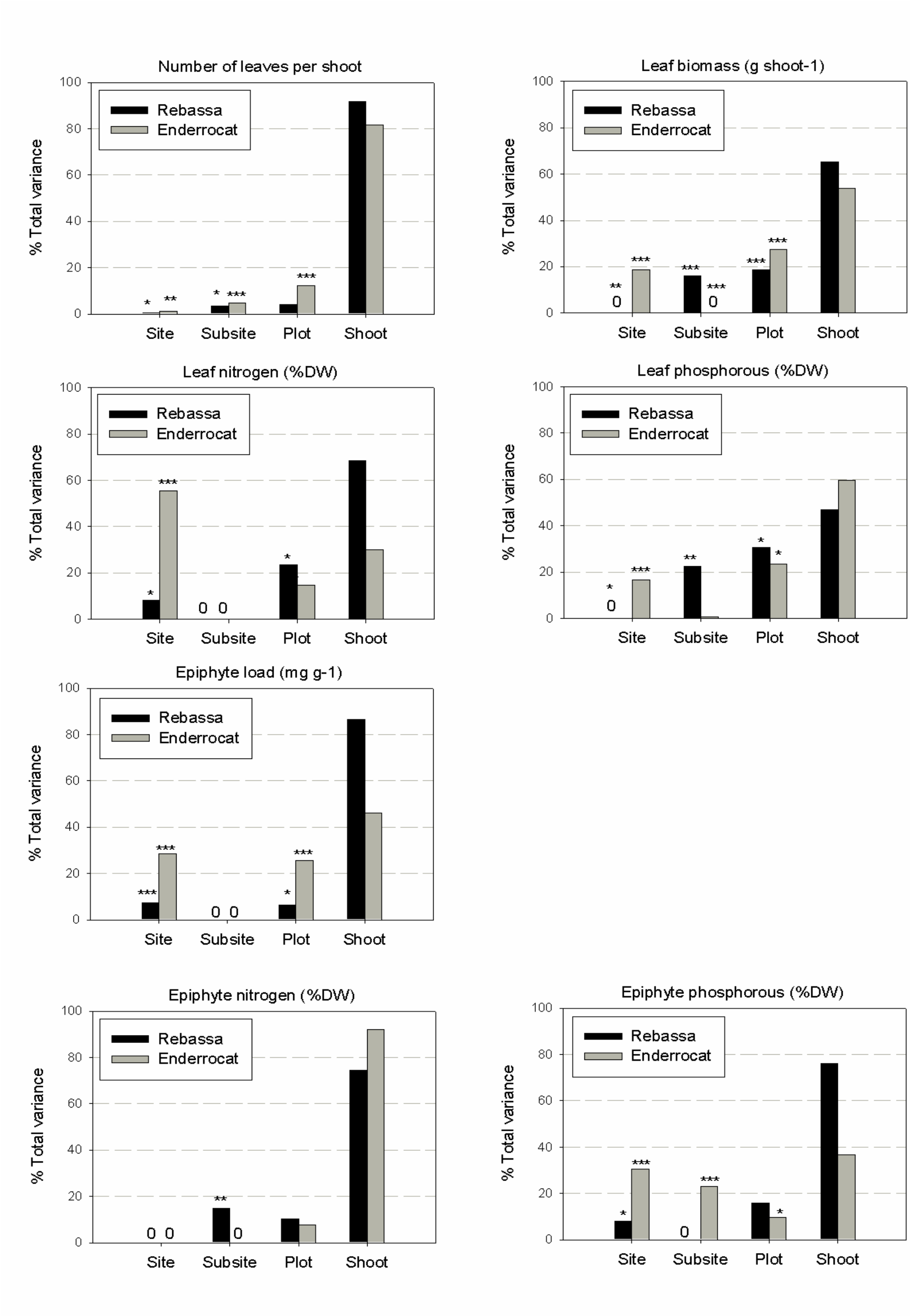


Figure 4. Percentage of total variance attributable to each spatial scale considered in shoot size, epiphyte load, leaf nitrogen and phosphorus content and epiphyte nitrogen and phosphorus content of *Posidonia oceanica* shoots in Palma Bay (Majorca, Western Mediterranean). Significant differences of nested ANOVA are indicated by: $p<0.05$* ; $p<0.01$** ; $p<0.001$***

Table 3. Spearman correlation coefficients among the size of the shoots, epiphyte load and nitrogen and phosphorus content of the leaves and epiphytes for each location. The Spearman correlation coefficients above the main diagonal (marked with gray cells) evaluate the association between the variables in Enderrocat. The Spearman correlation coefficients below the main diagonal evaluate the association between the variables in Rebassa. Significant levels are in bold and indicated by: p<0.05* ; p<0.01**; p<0.001***.

		Enderrocat						
		Leaf N (%DW)	**Leaf P (%DW)**	**Number of leaves per shoot**	**Leaf biomass (g DW shoot^{-1})**	**Epiphyte N (%DW)**	**Epiphyte P (%DW)**	**Epiphyte load (g DW g DW shoot^{-1})**
Rebassa	**Leaf N (%DW)**		**0,399*** **	-0,004	0,205			**-0,306** **
	Leaf P (%DW)	**0,265* **		-0,191	0,055			-0,175
	Number of leaves per shoot	-0,111	**-0,226* **		**0,710*** **	**0,369*** **	0,204	**-0,456*** **
	Leaf biomass (g DW shoot^{-1})	-0,183	**-0,388*** **	**0,647*** **		**0,254* **	**0,459*** **	**-0,777*** **
	Epiphyte N (%DW)			**0,241* **	**0,514*** **		0,174	0,051
	Epiphyte P (%DW)			**0,261* **	**0,298** **	**0,375*** **		**-0,325** **
	Epiphyte load (g DW g DW shoot^{-1})	0,053	0,137	**-0,560*** **	**-0,616*** **	-0,122	-0,15	

Discussion

Our results showed that the nitrogen and phosphorus content of the leaves and epiphytes were highly heterogeneous spatially in *Posidonia oceanica* meadows in autumn. The variability of the nutrient content was concentrated within plots (among shoots), except for the nitrogen content of the leaves in Enderrocat. Our results indicated that epiphyte and leaf nutrient content, particularly phosphorus content, in *P. oceanica* meadows may be quite variable spatially even at the smallest spatial scales (within one square metre). Leaf nitrogen content was similar to previous data obtained in the Western Mediterranean (1.4-2.0 % DW) in the autumn (Alcoverro et al. 1995, 1997, Leoni et al. 2007; Lepoint et al. 2007; Peirano et al. 2001) and slightly lower than the results obtained in winter, spring and summer (Peirano et al. 2001). The nitrogen content of the epiphytes (about 0.6 % DW) was between the lowest values measured in the Western Mediterranean (0,5-1.0 % DW) (Leoni et al. 2007; Lepoint et al. 2007; Perez et al. 2008). The leaf phosphorus content in shoots from Rebassa meadow was similar to previous data in the Western Mediterranean in summer (about 0.19 % DW) (Perez et al. 2008) and lower in Enderrocat (0.16 % DW) (Perez et al. 2008). The epiphyte phosphorus content was higher than earlier measures (about 0.09 % DW) (Perez et al. 2008) in Enderrocat (0.11 % DW) and our results were lower in Rebassa (0.07 % DW).

Size of *P. oceanica* shoots was extremely variable at spatial scales ranging from metres to hundreds of metres. Previous studies have also shown that the vegetative development of *P. oceanica* and the leaf epiphytic community are heterogeneous at a wide range of spatial scales (Ballestri et al. 2003; Gobert et al. 2003; Pardi et al. 2006; Balata et al. 2007). Gobert et al. 2003 found high spatial variability of the leaf area index (m^2 of leaves per m^2 of sediment), leaf biomass per shoot, and number of leaves per shoot in shoots collected throughout a 100 m^2 plot. Balestri et al. (2003) described a high spatial variability of the vegetative development of *P. oceanica* in August, when the shoots reach their maximum size during the year (Ballesteros 1987; Alcoverro et al. 1995). They found that the leaf length and rhizome elongation rate were different at the spatial scales of metres, tens and hundreds of metres and tens of kilometres, while the other variables used to describe the size (i.e., number of leaves) and growth (i.e., number of leaves produced per year) of the shoots and the structure of the meadow (i.e., shoot density, leaf area index, leaf standing crop) were different at least at one of the spatial scales considered. Our study showed that the size of *P. oceanica* shoots is also highly variable spatially in November-December when shoot size is at its annual minimum. Our results also showed that the spatial heterogeneity of the epiphyte load still is significant at the metre and hundreds of metres spatial scales in the autumn when the epiphyte load of *P. oceanica* shoots reaches its annual minimum values (Ballesteros 1987; Romero 1988; Alcoverro et al. 1997a). Hence, the overall evidence points to spatial heterogeneity as a primary feature of both the seagrass and its epiphytes in *P. oceanica* meadows.

The differences among the shoots were the major contributor to total variance of the shoot size, epiphyte load and nutrient content of the leaves and the epiphytes in both localities. Balestri et al. (2003) also found that the variation in the number of standing leaves and leaf features was greater among shoots collected within 0.25 square metres than among shoots separated by tens of metres, hundreds of metres or kilometres. The high variability among shoots separated by only a few cm might be related to a highly heterogeneous physiological status that may result from age differences among shoots (Kraemer et al. 1993; Kraemer et al. 1998) or the different levels of physiological integration between them (Marbà et al. 2002). As regards the epiphytes, previous studies show that the variability of the epiphytic composition, in terms of the percentage of cover of the main epiphytic groups, is concentrated among shoots collected within quadrants (0.25 m^2) (Piazzi et al. 2004; Pardi et al. 2006). In agreement with Piazzi et al. (2004) the design of studies of epiphyte load might consider that replication at tens of metres scale will not provide extra information for epiphyte load. Sampling efforts in upcoming studies might be focussed at the shoot scale, increasing the number of collected shots within plots, to cover most of the variability of shoot size, epiphyte load and nutrient content.

The simultaneous evaluation of shoot size, epiphyte load and nutrient content of leaves and epiphytes allows us to evaluate whether their patterns of spatial variability were associated. This exercise is useful because epiphytic overgrowth due to increased nutrient availability, traditionally measured in terms of the nutrient content of seagrass leaves, is a long established paradigm of seagrass ecology and conservation (Tomasko and Lepoint 1991; Hauxwell et al. 2001, 2003). The nutrient content of seagrass leaves is in fact the result of the balance between the nutrient availability and nutrient requirements of seagrass growth (Duarte 1990; Fourqurean et al. 1992; McClelland and Valiela 1998; Lepoint et al. 2008) and therefore, the relationship between the nutrient content of the leaves and nutrient availability is not univocal (Fourqurean et al. 2007). The nutrient content of epiphytes has been suggested to be an indicator of nutrient availability in the water column (Lin et al. 1996; Perez et al. 2008). High nutrient supply may stimulate the overgrowth of the epiphytic algae (bottom-up process), reducing seagrass access to light and water column nutrients (Sand-Jensen 1977; Silberstein et al. 1986; Tomasko and Lepoint 1991; Frankovich and Fourqurean 1997; Hauxwell et al. 2001). This process has been regarded as the major driver of seagrass decline. However, the spatial variability of epiphyte load and composition might also result from the spatial differences of herbivore pressure (Hughes et al. 2004; Valentine and Duffy 2006; Prado et al. 2007). Nowadays the abundance and composition of the epiphyte community is considered to be a result of the balance between bottom-up and top-down processes (Valentine and Duffy 2006).

We did not find a positive relationship between epiphyte load and nutrient content in the *P. oceanica* epiphytes in Palma Bay, a result consistent with other studies (Lin et al. 1996). Hence, epiphyte biomass is not univocally determined by water column nutrient availability and may also depend on other factors such as grazing pressure, light or hydrodynamics (Borowitzka et al. 2006; Valentine and Duffy 2006). Herbivory and seasonality affecting the epiphytic community development uncouple *P. oceanica* epiphyte response to water nutrient availability (Alcoverro et al. 1997a; Prado et al. 2007).

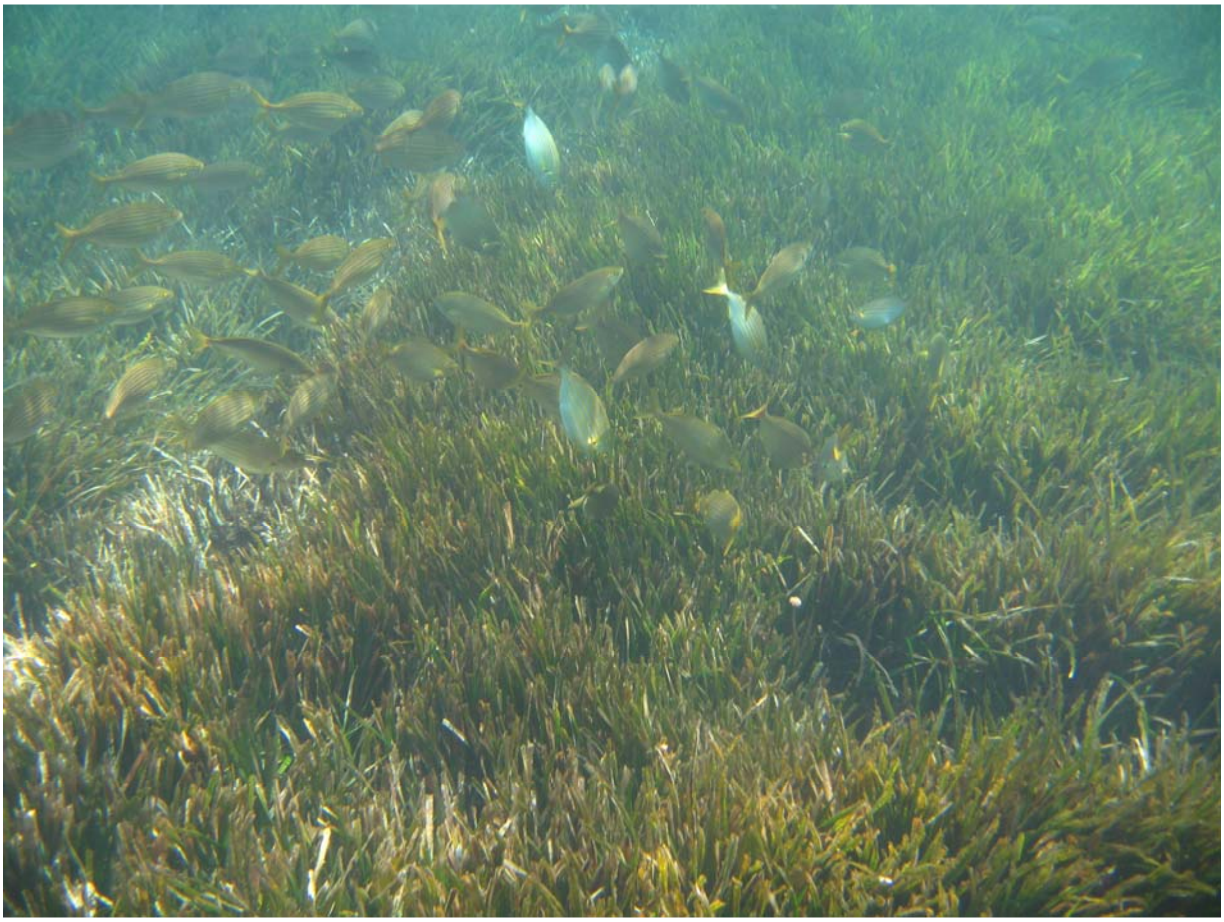
We found a negative correlation between shoot size and epiphyte load in both meadows. Shade from the leaf canopy might be a mechanism that generates this pattern. Smaller shoots might allow a higher penetration of light in the canopy and promote the development of epiphytes. Epiphyte development is known to be constrained by light availability, even when nutrients are in ample supply (Tomasko and Lapointe 1991).

Summary

We found high spatial variability of shoot size, epiphyte load and nutrient availability in terms of the nutrient content of the leaves and epiphytes. The differences were especially concentrated among shoots within one square metre. We concur with other studies that have also shown that both the vegetative development (Balestri et al. 2003) and the epiphyte community (Piazzi et al. 2004; Pardi et al. 2006; Balata et al. 2007) of *Posidonia oceanica* are heterogeneous at several spatial scales and that this heterogeneity should be considered when designing research and environmental monitoring programmes. Replication at tens of metres scale will not provide extra information for epiphyte load (Piazzi et al. 2004, this study) and concentrating sampling efforts at the shoot scale will cover most of the variability of shoot size, epiphyte load and nutrient content.

CHAPTER 3

Epiphyte response to *in situ* manipulation of nutrient availability and fish presence in a *Posidonia oceanica* (L.) Delile system



Epiphyte response to *in situ* manipulation of nutrient availability and fish presence in a *Posidonia oceanica* (L.) Delile system. Inés Castejón-Silvo, Jorge Terrados, Marta Domínguez and Beatriz Morales-Nin.

Abstract

Epiphytes are an important component of the biomass and productivity of *Posidonia oceanica* systems. Nutrient availability in the water column may promote epiphyte biomass through a bottom up mechanism. At the same time, epiphytes represent an essential trophic resource for higher trophic levels of seagrass food webs. *P. oceanica* meadows host a diverse assemblage of fishes that feeds directly on the leaves, on the epiphytes, and on the mesograzers inhabiting the meadows. In this work we experimentally evaluate the overall effect of the fish community and increased water column nutrient availability on seagrass and the associated epiphytes. Our results show that nutrient addition strongly increase epiphyte biomass and reduce shoot size after two months of experiment, while fish exclusion does not modify this effect. Fish exclusion did not have strong effects on *P. oceanica* leaves biomass or epiphyte load. Consumption marks of gastropod herbivores in the leaves were present in 78% of the shoots and only 6% of the shoots showed fish bites. Our results show that grazer activity cannot control epiphytic biomass despite the high frequency of radular marks in the shoots. These results highlight the importance of nutrient impact in oligotrophic meadows where bottom up processes seem to be more relevant in the control of algal growth.

Keywords: epiphyte load; nitrogen; phosphorus; *Posidonia oceanica*; nutrient addition, fish exclusion.

Introduction

Seagrass epiphytes are an important contributor to the biomass and the productivity of seagrass ecosystems (Borowitzka et al. 2006). The epiphytic community grows on the above ground tissues of seagrass and acquires nutrients from the water column. Increases of nutrient availability in the water column may promote increases of epiphyte abundance (Tomasko and Lapointe 1991; Neckles et al. 1993; Short et al. 1995). Epiphyte overgrowth can shade seagrass leaves (Borum 1985; Tomasko and Lapointe 1991; Wear et al. 1999) and have detrimental effects on seagrass health such as higher rates of leaf loss, lower rates of photosynthesis, or reduced rates of nutrient incorporation (Shepherd et al. 1989; Cornelisen and Tomas 2004; Burkholder et al. 2007). The development of the epiphytic community is influenced not only by nutrient supply, but also by the feeding rates and preferences of consumers (Hughes et al. 2004). Epiphytic biomass sustains a diverse assemblage of fishes and invertebrates that feed preferentially on it rather than on seagrass leaves (McGlathery et al. 1995; Peirano et al. 2001; Tomas et al. 2005a; Moksnes et al. 2008; Doropoulos et al. 2009) and epiphyte development in seagrass ecosystems is generally considered to be the result of the balance between nutrient availability and grazer control (Valentine and Duffy 2006). Grazing activity has been found to control epiphyte biomass in eelgrass meadows in natural conditions of nutrient availability and also when extra nutrients are supplied (Williams et al. 1993; Duffy et al. 2001, 2003; Schanz et al. 2002; Douglass et al. 2007).

Posidonia oceanica L. Delile is the most abundant seagrass species in the Mediterranean Sea covering 23% of shallow bottoms (depth < 45 m) (Benthoux and Copin-Montegut 1986; Pasqualini et al. 1998; Procaccini et al. 2003). The lifespan of *P. oceanica* leaves is the longest of seagrasses (from 202 to 345 days, Hemminga et al. 1999) and allows the development of an abundant and species-rich epiphytic community (Antolié 1985; Mazzella et al. 1989; Hemminga et al. 1999; Piazzi et al. 2004; Balata et al. 2007) that reaches a mature stage of colonization in summer, when the community is composed mainly by filamentous macroalgae and sessile fauna (Mazzella et al. 1992; Pardi et al. 2006; Prado et al. 2008a). The response of epiphyte biomass in *P. oceanica* leaves to nutrient addition in the water column occurs especially during summer (Leoni et al. 2006; Prado et al. 2008a).

Posidonia oceanica shoots and the epiphytic community living in their leaves compose a structurally complex habitat for dense mesograzers populations dominated by crustaceans, gastropods and polychaetes (Gambi et al. 1992). These mesograzers and the epiphytes represent an essential food source for higher trophic levels in Mediterranean meadows (Gambi et al. 1992; Mazzella et al. 1992; Buia et al. 2000; Tomas et al. 2006). The *P. oceanica* fish community is dominated in number and biomass by labrids, sparids, serranids, gobids and scorpaenids (Bell and HarmelinVivien 1982; Reñones et al. 1995; Francour 1997; Moranta et al. 2006; Deudero et al. 2008) and omnivory is the most frequent feeding behaviour (Stergiou and Karpouzi 2001; Deudero et al. 2008). The fishes can feed directly on the shoots (Havelange et al. 1997; Tomas et al. 2005a; Prado et al. 2008b), on the epiphytes (Alcoverro et al. 1997a) and on the mesograzers (Jennings et al. 1997; Pinnegar et al. 2000).

Posidonia oceanica leaves are less attractive for consumers than epiphytes probably due to their relatively lower nutritional quality (Alcoverro et al. 1997a; Tomas et al. 2005a). The herbivore fish *Sarpa salpa* and the sea urchin *Paracentrotus lividus* are the main leaf consumers of *P. oceanica* (Cebrián et al. 1996; Prado et al. 2007). *P. lividus* feeds preferentially on the oldest leaves, which have higher epiphytic biomass than the youngest leaves of the shoot (Alcoverro et al. 1997a; Tomas et al. 2005a), and may achieve important reductions of epiphyte load (60-80% after Tomas et al. 2005a). *S. sarpa* feeds on the leaves and the epiphytes *of P. oceanica* and can also reduce epiphyte biomass (Tomas et al. 2005a; Prado et al. 2007). Although both macrograzers feed simultaneously on leaves and epiphytes, their main nitrogen source comes from epiphytes (Jennings et al. 1997; Tomas et al. 2006) what suggests that epiphyte availability may be driving consumption rates (Vergés et al. 2010).

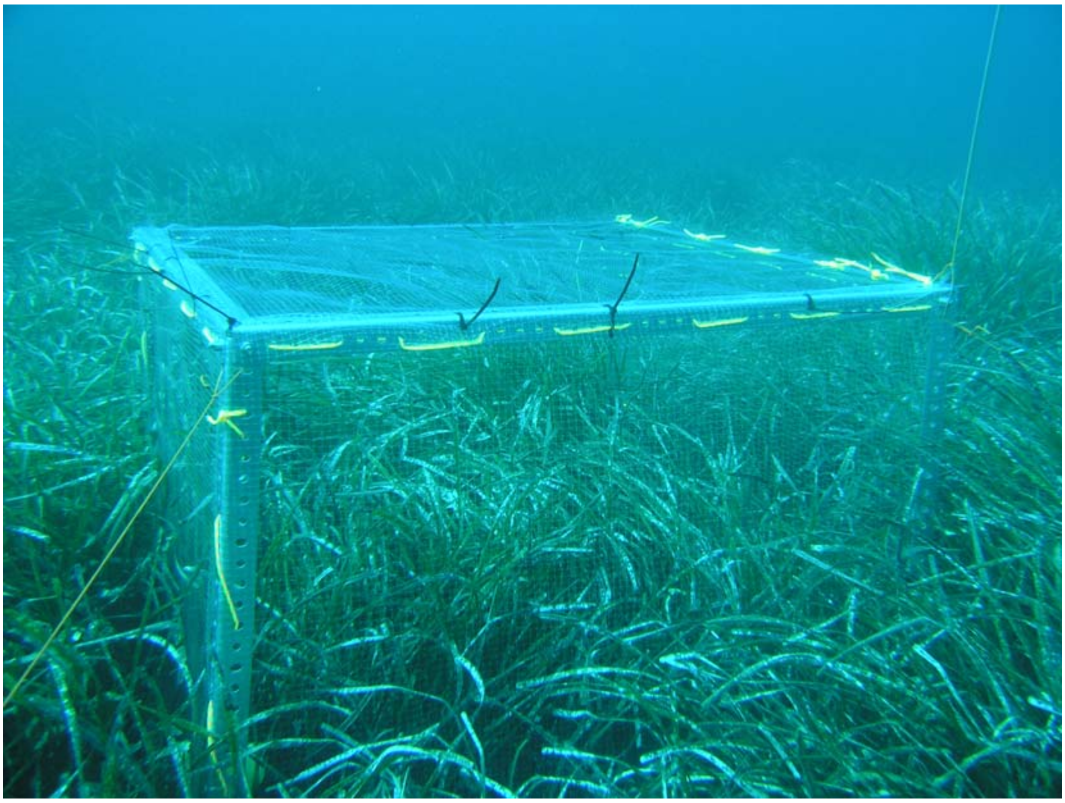
Human activities may affect epiphyte load and composition in *P. oceanica* leaves both by increasing nutrient content in the water column (bottom up processes) and by producing direct and indirect changes in the populations of epiphyte consumers through fishing activity (top down processes) (Pinnegar et al. 2000; Moksnes et al. 2008). The accumulation of epiphytic biomass on *P. oceanica* leaves might be reduced by direct fish feeding pressure on the epiphytes or through indirect effects of the reduction of fish predatory pressure on the epiphyte grazer populations. This work evaluates the role of the fish community associated to a *P. oceanica* meadow in modulating the response of the epiphytic community to an increase of nutrient availability. We assess the overall effect of the fish community in the response of *P. oceanica* epiphytes to an experimental increase in the availability of nutrients in the water column through the exclusion of fish from experimental plots.

Material and methods

The study was performed in the No take Zone of the Marine Protected Area of Palma Bay (39° 28' 11.13" N, 2° 43' 27.84" E), (Majorca, Balearic Islands). Since 1999 fishing and other extractive practices, diving or boat anchoring in the meadows are forbidden in the 2 squared kilometres of the no take zone and allows the preservation of *Posidonia oceanica* meadows. Scientific activities are only allowed under permission.

We designed a factorial experiment with two crossed factors to evaluate the effects of nutrient enrichment and fish exclusion on *P. oceanica* vegetative features, epiphyte load, grazing pressure and nutrient content in the epiphytes and in the leaves. Four treatments were established in the experiment: nutrient enrichment + fish exclusion (Nutrients & Cage); nutrient enrichment + natural fish abundance (Nutrients); no nutrients added + fish exclusion (Cage); no nutrients added + natural fish abundance (Control). Each treatment was replicated three times. In June 2007 twelve plots of 1m^2 (4 treatments * 3 replicates) were delimited in a *P. oceanica* meadow between 10 and 12 meters depth and approximately ten metres apart from each other to avoid influences between them. Treatments were randomly assigned to each plot. The experiment run between June and October 2007 for it is during this time of year when *P. oceanica* epiphytes are known to respond to an increase of nutrient availability (Leoni et al. 2006; Prado et al. 2008a) and when grazing pressure by herbivore fish *Sarpa salpa* is maximal (Tomas et al. 2005b; Prado et al. 2007).

Fish exclusion cages consisted of 1m^3 cubes with 1 cm^2 mesh to avoid fish access but allow the movement of small invertebrates. The net was cleaned monthly to prevent the accumulation of fouling during the study. Paired measurements of light availability inside and outside one of the cages were carried out monthly between June and September with a LiCor LI193SA spherical quantum sensor and LI1400 data logger to evaluate potential shading effects in the cages. Irradiance inside cages at noon was, on average, 80 % of that outside the cages. Sea urchins were not abundant at the study site and their absence inside the plots was checked at the beginning of the experiment. The temperature increased from 23.4 ºC in June to 26.5ºC in August and decrease afterwards to 22.7 ºC in October (sensor and logger: StowAway TidBit Temp Logger, Onsset Co., USA).



Fish exclusion cage (photo by Eduardo Infantes)

Slow-release fertilizer (Osmocote N:P:K 15:9:9 + 3MgO + trace elements) was employed to enrich nutrient content in the water column (Heck et al. 2000; Prado et al. 2008a). Four 250 ml plastic diffusers with OSMOCOTE fertilizer were placed 40 cm above sediment surface in each plot assigned to receive the nutrient addition. OSMOCOTE diffusers were replaced monthly by new ones to ensure continuous nutrient release during the experiment. Nitrogen and phosphorous release was estimated as 43.9 grams and 11.7 grams respectively per plot and month based on weight loss of diffusers.

Seven shoots of *P. oceanica* were collected at random from each plot every month throughout the experiment. The shoots were placed in individual plastic bags and stored frozen at -20º C until processing. After thawing at room temperature, epiphytes in all the leaves of each shoot were scraped using a razor blade and collected in preweighed Whatman GF/C glass fibre filters. Filters were dried (60ºC, 48 h) to determine epiphyte dry weight (g DW). The length and width of each leaf was measured to calculate the total leaf surface of each shoot (cm^2). Leaves with fish bites and radular marks of gastropods were scanned (see Buia et al. 2003 for herbivore bites; Rueda and Salas 2007 for radular marks) and the area of these herbivore marks was measured on the scanned images using ImageJ 1.43 software. The leaves were also dried (60ºC, 48 h) to estimate leaf biomass of each shoot (g DW). Shoot size was described in terms of number of leaves per shoot and leaf biomass per shoot (g DW $shoot^{-1}$). Epiphyte load was expressed as epiphyte biomass per leaf biomass (g DW epiphyte / g DW leaf). Gastropod grazing pressure was expressed as % of leaf surface with radular marks per shoot. Fish grazing pressure was expressed as number of shoots with fish bites.

Nutrient content in epiphytes and leaves were considered as indicators of nutrient availability in the water column and relative nutrient availability for seagrass growth, respectively (McClelland and Valiela 1998; Lepoint et al. 2007; Perez et al. 2008). A subsample of three shoots and three filters with scraped epiphytes was haphazardly selected from each plot and month and set aside for nutrient analysis. Every shoot and filter was ground to powder with a stainless steel ball mill (MM200 RETSCH, Haan, Germany). An aliquot of the ground material was used to determine total concentration of nitrogen in the leaves and in the epiphytes of each shoot using either a Heraeus CHN-o-rapid elemental analyzer or a CHN elemental analyzer (1100 CE Instruments, Elantech, NJ, USA) connected to an Isotope Ratio Mass Spectrometer (IRMS) DeltaPlus (Thermo). No differences between both instruments were found after processing the same samples in both analyzers (n=10, identical results). We analyzed ground filters with no epiphytes to verify that there was not nitrogen signal due to filter composition. Leaf nitrogen content in the leaves was expressed as % of DW. Nitrogen content in epiphytes was expressed as %

of DW after correction for the contribution of filter DW to sample weight. We used beech leaves certified standard (CRM No. 100) as reference material for nitrogen concentration. Phosphorus content in the leaves and epiphytes was analysed following the protocol described by Fourqurean et al. (1992) using beech leaves certified standard (CRM No. 100). Phosphorous content in the leaves was expressed as % of DW. Phosphorous content in epiphytes was expressed as % of DW after correction for the contribution of filter DW to sample weight.

ANOVAs with two factors (nutrient enrichment and fish presence) were performed to evaluate differences of leaf biomass, number of leaves per shoot, epiphyte load, percentage of grazed area and nutrient content of the leaves and epiphytes of *P. oceanica* shoots in each month of the experiment. Repeated measures ANOVA with these two fixed factors were also performed to evaluate if the effects of nutrient enrichment and fish exclusion on leaf biomass, number of leaves per shoot, epiphyte load, percentage of grazed area and nutrient content of the leaves and epiphytes of *P. oceanica* shoots changed during the course of the experiment. ANOVA assumptions were tested by Kolmogorov Smirnov test and Cochran’s test for normality and homogeneity of variances, respectively.

Results

Both nitrogen and phosphorus content of *Posidonia oceanica* leaves increased throughout the experiment. Leaf nitrogen content increased from 1.49 ± 0.03 to 1.66 ± 0.09 % DW and leaf phosphorus content varied from 0.095 ± 0.002 to 0.267 ± 0.01 % DW in June and October respectively (Table 1, Fig. 1). Nitrogen content of the leaves increased after two months of the beginning of the experiment in the plots where nutrients were added while phosphorus content of the leaves did not change during the first two months of the experiment but it increased in September and October in the plots where nutrients were added and fish excluded.

Nutrient content of the epiphytes decreased throughout the experiment from 1.29 ± 0.05 % DW in June to 0.64 ± 0.03 % DW in October, for nitrogen content, and from 0.071 ± 0.002 % DW in June to 0.055 ± 0.003 % DW in October for phosphorus (Table 1, Fig. 1). The nutrient content of the epiphytes was not different among the different treatments, except in the fish exclusion treatment in August.

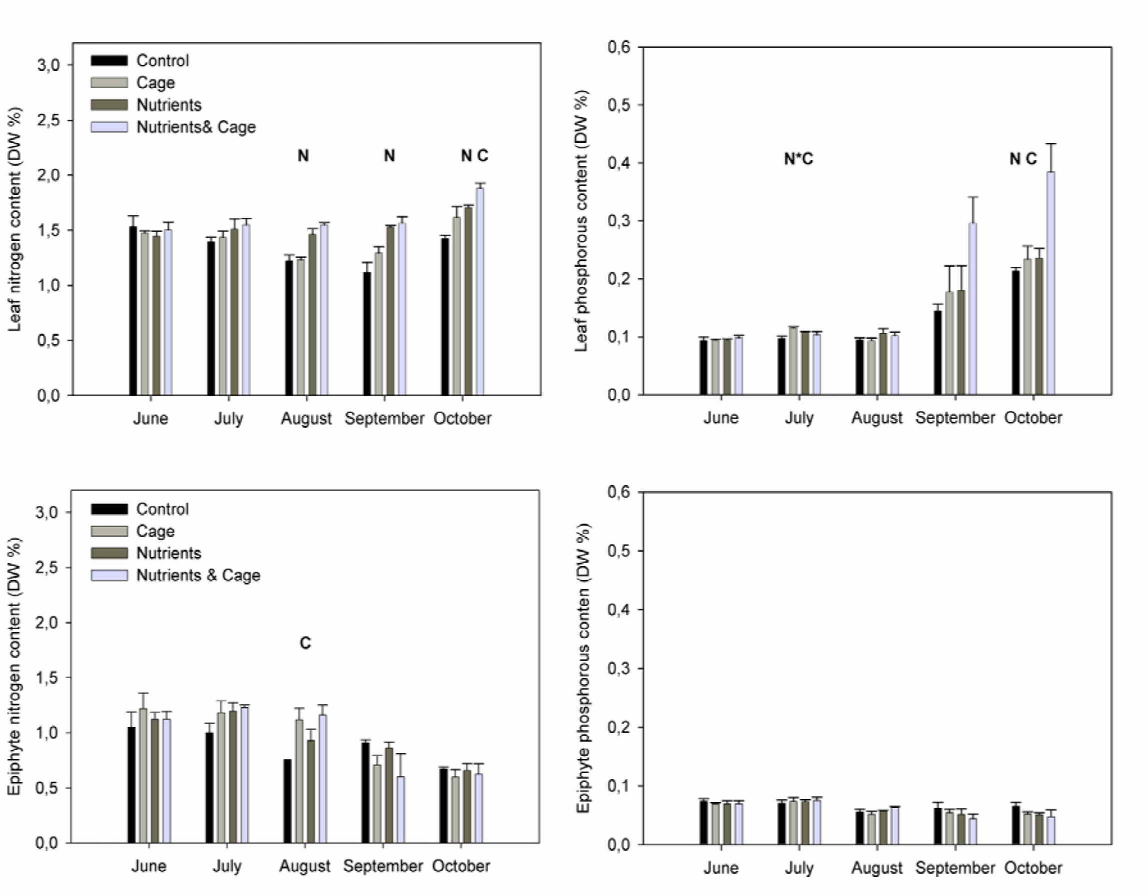


Figure 1. Nitrogen and phosphorous content in the leaves and epiphytes of *Posidonia oceanica* in control and treatment plots along the experiment. Significant effects of nutrient addition (N) , fish exclusion (C) or their interaction (N * C) detected by two-way ANOVAs of data corresponding to each month are indicated above each group of bars. Error bars represent + 1 SE.

The number of leaves per shoot decreased from 4.88 ± 0.09 in June to 3.88 ± 0.15 in August and increased afterwards to 5.52 ± 0.11 in October (Table 2, Fig. 2). Shoots in plots where nutrients were added had lower number of leaves in August, September and October (Fig. 2). The leaf biomass of the shoots decreased from 0.94 ± 0.03 g leaf DW shoot^{-1} in June to 0.31 ± 0.02 g leaf DW shoot^{-1} in October (Table 2, Fig. 2). Leaf biomass was not different among experimental treatments in July but it was reduced by 42 % in August and by 62 % in September in the plots where nutrients were added (Fig. 2).

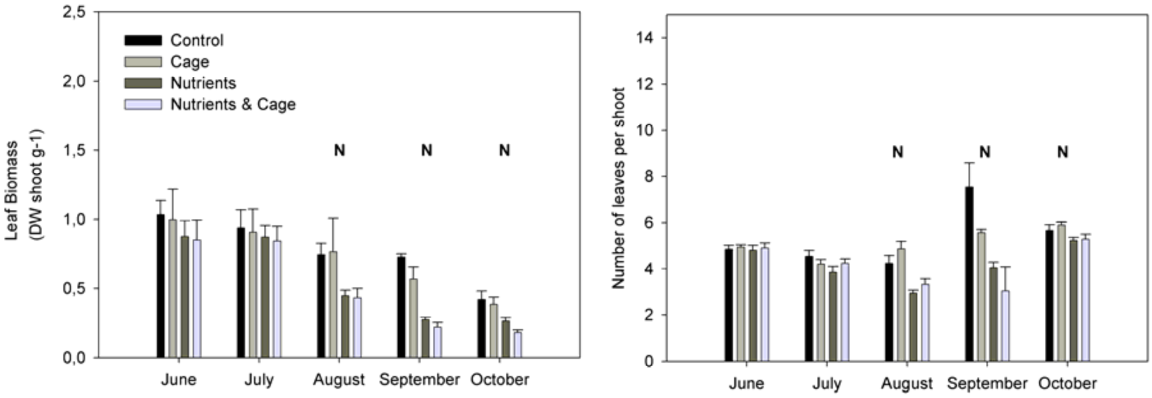


Figure 2. Number of leaves per shoot and leaf biomass (mean ± SE) of *Posidonia oceanica* shoots in control and treatment plots along the experiment. Significant effects of nutrient addition (N), fish exclusion (C) or their interaction (N * C) detected by two-way ANOVAs of data corresponding to each month are indicated above each group of bars. Error bars represent + 1 SE.

Table 1. Repeated-measures ANOVA to assess differences among treatments during the experiment for nitrogen and phosphorous content in *Posidonia oceanica* leaves and epiphytes. ns: non significant.

Variable	Effect	DF	MS	F	p
Leaf nitrogen content (% DW)	Time	4	0.162	17.165	**0.000**
	Time*Nutrients	4	0.069	7.330	**0.000**
	Time*Cage	4	0.016	1.651	ns
	Time*Nut*Cage	4	0.006	0.685	ns
Leaf phosphorous content (% DW)	Time	4	0.071	55.267	**0.000**
	Time*Nutrients	4	0.005	4.303	**0.007**
	Time*Cage	4	0.005	4.267	**0.007**
	Time*Nut*Cage	4	0.003	2.410	ns
Epiphyte nitrogen content (% DW)	Time	4	0.384	15.235	**0.000**
	Time*Nutrients	4	0.020	0.812	ns
	Time*Cage	4	0.085	3.363	ns
	Time*Nut*Cage	4	0.006	0.239	ns
Epiphyte phosphorous content (% DW)	Time	4	0.001	9.357	**0.000**
	Time*Nutrients	4	0.000	1.431	ns
	Time*Cage	4	0.000	0.622	ns
	Time*Nut*Cage	4	0.000	0.180	ns

Epiphyte load on *P. oceanica* shoots increased by 92 % in August, 89 % in September, and 66 % in October in the plots where nutrients were added (Table 2, Fig. 3). The exclusion of fish seemed to reduce the epiphyte load of the shoots in plots where nutrients were added in September and October (significant Time * Nutrient * Cage interaction detected by repeated measures ANOVA) but the two way ANOVA corresponding to each month did not found the effect of fish exclusion to be significant (Fig. 3).

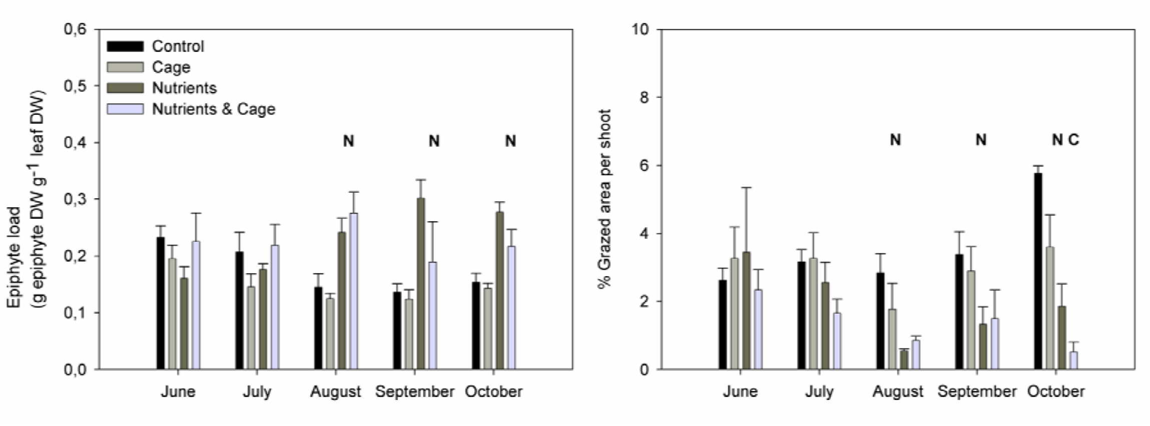


Figure 3. Epiphyte load on *Posidonia oceanica* shoots and percentage of the leaf surface of the shoot with radular marks in control and treatment plots along the experiment. Significant effects of nutrient addition (N), fish exclusion (C) or their interaction (N * C) detected by two-way ANOVAs of data corresponding to each month are indicated above each group of bars. Error bars represent + 1 SE.

Almost 80 % of the collected shoots showed radular marks, only 6% exhibited fish bites. Exclusion of herbivorous fish was effective, as indicated by the progressive decrease in fish bite marks in the “caged” plots (with no marks were found from August onwards) (Table 3). In contrast, shoots with fish bite marks were almost always found in plots where fishes were not excluded. The percentage of leaf surface with radular marks per shoot changed from 2.92 ± 0.49 % in June to 1.50 ± 0.34 % in August and to 2.94 ± 0.64 % in October (Fig. 3, Table 2). Nutrient enrichment led to a reduction of the percentage of leaf surface with radular marks per shoot from August onwards. There was also a reduction in cage plots in October (Fig. 3, Table 2).

Table 2. Repeated-measures ANOVA to assess differences among treatments during the experiment for number of leaves per shoot, leaf biomass, epiphyte load, and the percentage of shoot leaf area with radular marks of *Posidonia oceanica* shoots. ns: non significant.

Variable	**Effect**	**DF**	**MS**	**F**	**p**
Number of leaves per shoot	Time	4	5.388	13.050	**0.000**
	Time*Nutrients	4	4.331	10.489	**0.000**
	Time*Cage	4	1.799	4.358	**0.006**
	Time*Nut*Cage	4	0.235	0.569	ns
DW shoot (g)	Time	4	0.8977	52.9831	**0.0000**
	Time*Nutrients	4	0.0537	3.1701	**0.0266**
	Time*Cage	4	0.0051	0.3012	ns
	Time*Nut*Cage	4	0.0028	0.1632	ns
DW epp /DW shoot (g/g)	Time	4	0.001	0.708	ns
	Time*Nutrients	4	0.012	14.422	**0.000**
	Time*Cage	4	0.003	3.452	**0.019**
	Time*Nut*Cage	4	0.006	7.589	**0.000**
% Grazed area per shoot	Time	4	4.283	2.683	**0.049**
	Time*Nutrients	4	4.669	2.925	**0.036**
	Time*Cage	4	1.314	0.823	ns
	Time*Nut*Cage	4	1.319	0.826	ns

Table 3. Number (mean and standard error) of *Posidonia oceanica* shoots with marks of fish bites found in the samples collected since the beginning of the experiment, in June, to the end of it, in October.

Treatments	June		July		August		September		October	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	1,00		0,33	0,33	1,33	1,33	1,00	1,00	0,33	0,33
Cage	1,33	0,67	0,67	0,33	0,00		0,00		0,00	
Nutrients	1,00	0,58	1,00	0,58	0,00		0,67	0,33	0,00	
Nutrients*Cage	0,00		0,00		0,00		0,00		0,00	

Discussion

Our results show that increase of nutrient availability in the water column during summer promotes the increase of epiphyte biomass in *Posidonia oceanica*. Exclusion of fish does not promote neither epiphyte proliferation nor affects the response of epiphytes to the increase of nutrient availability in the water column. The number of shoots with fish bites and the grazed area per shoot (i.e. radular marks area) did not increase after nutrient enrichment. Neither the higher nutrient content of the leaves nor the higher epiphytic biomass found in nutrient enriched plots led to an increase of grazing by mesograzers or herbivorous fish as has been found in other locations (Cebrián and Duarte 1998; Prado et al. 2010).

Cages effectively prevented the access of herbivore fish as indicated by the disappearance of fish bite marks in *P. oceanica* shoots inside the cages throughout the experiment. The low number of shoots with fish bites marks may indicate low herbivore fish pressure in our study site and may explain the negligible role of direct grazing by herbivore fish in controlling epiphyte biomass in our study site. Previous studies have found that the influence of fish grazing on epiphyte biomass in *P. oceanica* shoots was also low (Alcoverro et al. 1997a; Tomas et al. 2005a; Leoni et al. 2006) even though herbivorous fish may consume up to 40% of *P. oceanica* leaf production (Prado et al. 2007). Sea urchin grazing is considered to have a larger influence than grazing by herbivore fish on the epiphyte biomass of *P. oceanica* shoots (Tomas et al. 2005a) but sea urchins were also very scarce in our study site. Our results indicate that grazing by the two most important macroherbivores in *P. oceanica* meadows, the herbivore fish *Sarpa salpa* and the sea urchin *Paracentrotus lividus*, either on the epiphytes or the leaves is very low in our study site.

It could be also expected that the exclusion of the whole fish community could produce changes on epiphyte load through indirect effects in *P. oceanica* trophic web. A reduction of predation pressure by fish could promote epiphyte consumption by invertebrate grazers and the reduction of epiphyte biomass (Hughes et al. 2004; Moksnes et al. 2008). This trophic link has been demonstrated to be essential in the regulation of epiphytic biomass in other benthic systems (Pinnegar et al. 2000; Valentine and Duffy 2006). Fish mesopredator release in the benthic food webs of the Baltic Sea produced strong rises of macroalgal biomass (50%-75% of increase) by reducing grazer abundance (Sieben et al. 2011a, 2011b).

In *Zostera marina* meadows the manipulation of mesopredator abundance buffers nutrient additions effects on epiphytic algal growth by cascading effects on grazer community (Douglass et al. 2007; Moksnes et al. 2008).

The mesograzer community that inhabits *P. oceanica* leaves is species-rich (>300 ssp of molluscs, polychaetes and amphipods after Gambi et al. 1992) and many of the species are epiphyte consumers (Mazzella et al. 1989; Mazzella and Russo 1989, 1992; Gambi et al. 1992; Gacia et al. 2009). However, our results show that the exclusion of the whole fish community did not produce any effect on epiphyte biomass and did not change its response to nutrient enrichment. Different not mutually exclusive hypothesis may be considered at this point. First, mesograzers might graze on epiphytes but their consumption rates are not enough to control epiphytic biomass. Second, mesograzers do not become more effective epiphyte consumers when released from predation pressure perhaps because the predation they suffer is already low. Third, mesograzers might not be able to detect high food availability patches (nutrient enriched plots) and concentrate on them to exploit that resource. As we do not have data about mesograzer abundance or the predation rates they experienced in the different treatments, we cannot elucidate the mechanism by which the exclusion of fish does not cascade to epiphyte biomass. However radular marks on the *P. oceanica* epidermis provide information about the grazing activity of the gastropod component of the mesograzer community. The area of radular marks per shoots was reduced in nutrient enriched plots while fish exclusion does not have any effect on the gastropods activity. The increase of nutrient availability led to a higher epiphytic biomass and the development of a layer of more palatable filamentous macroalgae over the layer of crustose corallines, bryozoans and hydrozoans (Klumpp et al. 1992; Prado et al. 2008a; Giovanetti et al. 2010) that could explain why leaf damage caused by gastropods (radular marks) is reduced in nutrient enriched plots.

Grazer community has been frequently demonstrated to modulate epiphyte biomass even under experimental nutrient supplies (Williams et al. 1993; Douglas et al. 2007; Jaschinski and Sommer 2008a, Jaschinski et al. 2011). However the strength of these trophic links depends on the system. Top down herbivore control of algal biomass appears to be stronger in tropical benthic systems (Burkepile et al. 2006) and in systems with simple food webs (Sieben et al. 2011a, 2011b). In high productivity temperate benthic systems and in oligotrophic meadows bottom up processes seems to be more relevant in the control of algal growth (Keuskamp 2004; Burkepile et al. 2006; Cardona et al. 2007; Peterson et al. 2007; Poore et al. 2009).

Nutrient addition led to a reduction of *P. oceanica* shoot size after two months of continued treatment. This reduction of shoot size may not be explained by an increase of herbivore pressure because the number of shoots with fish bite marks did not increased in nutrient enriched plots even considering that *P. oceanica* leaves had higher nutrient content and epiphyte load in these plots. Decline in seagrass vitality in response to intensive nutrient enrichment has been previously reported for *P. oceanica* and *Zostera marina* (Burkholder et al. 1992, 1994, 2007; Touchette et al. 2003; Invers et al. 2004; Leoni et al. 2006). Burkholder et al. (1992, 1994, 2007) suggest that seagrass species adapted to very oligotrophic waters do not have an inhibition mechanism to stop nitrate assimilation when the cost of the process starts to reduce carbon reserves and affect plant growth. The direct negative effects of epiphyte shading on seagrass vitality might be a mechanism causing shoot size decrease. Light availability reduction, caused by shading, combined with summer water temperatures and continued nutrient enrichment could have led to stronger effects of nutrient enhancement on *P. oceanica* growth (Leoni et al. 2008). The fact that there are not morphological differences between cage and control treatment, except in the number of leaves per shoots in September, discards that cage induced light attenuation causes smaller shoot size.

Summary

In summary, this work shows that fish herbivory on epiphytes and *P. oceanica* leaves does not represent a main driver of change of epiphyte abundance and leaf biomass in our study site. Our results also show that trophic linkages changes caused by the exclusion of the whole fish community do not modify epiphyte biomass or its response to high nutrient availability. Epiphyte abundance strongly responded to nutrient availability increases and seems to have negative effects on *P. oceanica* shoot size. This work suggests that nutrient availability is the main driver of epiphyte biomass during summer. Further studies are needed to characterize the trophic linkages existing in the community of grazers inhabiting *P. oceanica* meadows and, particularly the responses of the mesograzer community to increased epiphyte biomass.

CHAPTER 4

Invertebrate community, epiphyte load and seagrass response to experimental increase of nutrient availability in *Posidonia oceanica* (L.) Delile systems

Invertebrate community, epiphyte load and seagrass response to experimental increase of nutrient availability in *Posidonia oceanica* (L.) Delile systems. Inés Castejón-Silvo, Marta Domínguez, Jorge Terrados, Fiona Tomas and Beatriz Morales-Nin. Manuscript submitted to Marine Environmental Research.

Abstract

Epiphytes support an abundant and diverse community of resident invertebrates which fuel higher trophic levels of *Posidonia oceanica* food web. Nutrient availability in the water column may promote epiphyte biomass through a bottom-up mechanism. In this work we evaluate the response of seagrass, epiphytes and the invertebrate community to an experimental increase of water column nutrient availability. Nutrient increase was followed by a rise of epiphyte biomass which promoted a global increase of the populations of invertebrates, even of those that are not directly trophic related with epiphytes. The increase of invertebrate populations does not reversed epiphyte biomass to a non-nutrient-enriched situation. Epiphyte abundance strongly responded to nutrient increase and seems to have negative effects on *P. oceanica* shoot size. This work suggests that epiphyte biomass affects the abundance of epifaunal grazers populations in *P. oceanica* systems and that nutrients are the main driver of epiphyte biomass during summer.

Keywords: seagrass, epiphyte load, nutrient addition, epifauna, *Posidonia oceanica*, Western Mediterranean.

Introduction

Productivity and community structure of marine benthic systems is a consequence of the balance between top-down (i.e. consumer-driven) and bottom-up (i.e. resource-driven) processes. Review of evidence through meta-analysis has found that interaction between the effects of nutrient enrichment and consumer pressure is common (Worm et al. 2002) and that the direction, magnitude, and significance of nutrient enrichment and consumer pressure effects is context-dependent. In coastal soft bottoms, coral reefs and temperate rocky reefs, nutrient enrichment and herbivore absence promote the abundance of primary producers but the effects are additive on the first two systems while they are not in the latter (Gruner et al. 2008). In high productivity areas nutrient enrichment significant promotes the abundance of temperate macroalgae and benthic microalgae only when herbivores are absent while, in low productivity areas, the removal of herbivores has insignificant effects on the abundance of primary producers (Burkepile and Hay 2006).

In seagrass systems primary production has been traditionally considered to be bottom-up controlled by nutrient availability (Romero et al. 2006), although growing evidence indicates that top-down control by herbivores is also significant (see review by Valentine and Duffy 2006). Seagrass production combined with that of benthic and epiphytic macroalgae fuel seagrass trophic webs where epiphytes represent an essential food source to the abundant and diverse community of resident invertebrates (Borowitzka et al. 2006). Small grazing and detritivorous crustaceans and molluscs are considered key actors in controlling epiphyte biomass in seagrass meadows and fuelling higher trophic levels of the seagrass food web (Valentine and Duffy 2006). Epiphyte grazers can consume more than half of epiphyte production (Borum 1987; Klumpp et al. 1992) and benefit seagrass growth (Neckles et al. 1993; Philippart 1995; Hays 2005) by reducing light and nutrient competition. A recent meta-analysis (Hughes et al. 2004) found that the relative strength of resource control and consumer pressure on biomass of seagrass epiphytes and the resulting effects on seagrass biomass are of similar magnitude. In addition, the balance between bottom-up and top-down forces may also be context dependent also in seagrass systems. For instance, epiphyte grazers have limited effect on epiphytic biomass in low productivity seagrass meadows, which may be due to the limiting nutrient conditions in these areas (Keuskamp 2004; Peterson et al. 2007).

Posidonia oceanica L. Delile is a species adapted to Mediterranean nutrient poor conditions (Gobert et al. 2006), covering ca. 23% of shallow bottoms (depth < 45 m) (Pasqualini et al. 1998). Increased nutrient inputs can be detrimental for this critical coastal ecosystem that contributes to fisheries production, biodiversity preservation, nutrient cycling, and shoreline protection (Hemminga and Duarte 2000). *Posidonia oceanica* meadows are in decline in several areas as a result of human activities (Boudouresque et al. 2009). Eutrophication and the associated increased nutrient inputs are the most widespread human processes negatively affecting seagrass meadows (Burkholder et al. 2007). Nutrient enrichment of water column promotes the biomass of epiphytic macroalgae in *P. oceanica* leaves (Leoni et al. 2006; Prado et al. 2008a) and high epiphyte biomass is associated to low photosynthetic and growth rates of this seagrass species (Delgado et al. 1999), likely through leaf shading. High nutrient inputs have also been associated to increased herbivore pressure on *P. oceanica* (Ruiz et al. 2009).

Consumer control in *P. oceanica* meadows has traditionally focused on macroherbivores (fish and sea urchins) (i.e. Tomas et al. 2005a; Prado et al. 2007; Vergés et al. 2011) while *P. oceanica* meadows harbour a rich community of invertebrate epifauna (crustaceans, molluscs, polychaetes) which may potentially feed on seagrass and epiphytes (Mazzella and Russo

1989; Mazzella et al. 1992). In fact, two species of gastropods can reduce by half epiphyte biomass when present at maximal field densities in microcosm conditions and epiphytes have been found to be a main carbon and nitrogen source for some species of gastropods (Gacia et al. 2009). Yet, the response of invertebrate epifauna to increased epiphyte biomass and the control that such fauna might exert on epiphyte biomass is unknown to a large extent.

This work aims to evaluate the responses of invertebrate epifauna community, epiphytes and *P. oceanica* features to an increase of nutrient availability in the water column. An experimental nutrient addition was settled in localities with different levels of epiphyte load, because the magnitude of the response of leaf epiphytes to nutrient additions and hence of the epifaunal community might depend on background level of epiphyte biomass and/or nutrient availability (Burkepile and Hay 2006). The results might contribute insights into the responses of a threatened ecosystem to human impact such as eutrophication.

Material and methods

The study was performed in the sublitoral zone of Palma Bay (Majorca, Western Mediterranean) (Fig. 1). In July 2008, ten *Posidonia oceanica* meadows located at a depth of 5 m to 6 m were selected, and five shoots of *P. oceanica* were collected at random in each meadow (locality) to perform an extensive assessment of leaf biomass, epiphyte load and gastropod grazing pressure on shoots, and of the nitrogen content of epiphytes and leaves. The main goal of this assessment was to evaluate if there were differences of epiphyte load among the ten localities chosen. According to the results obtained in this assessment (see details of *P. oceanica* shoot processing below), a subgroup of four localities, two with high epiphyte load (Estancia and Nova, see Fig. 1) and two with low epiphyte load (Enderrocat and Viñas), was chosen to experimentally evaluate the effects of water column nutrient addition on shoot size, epiphyte load, nitrogen (N) and phosphorus (P) content of epiphytes and leaves, and on the abundance of different groups of invertebrate epifauna.

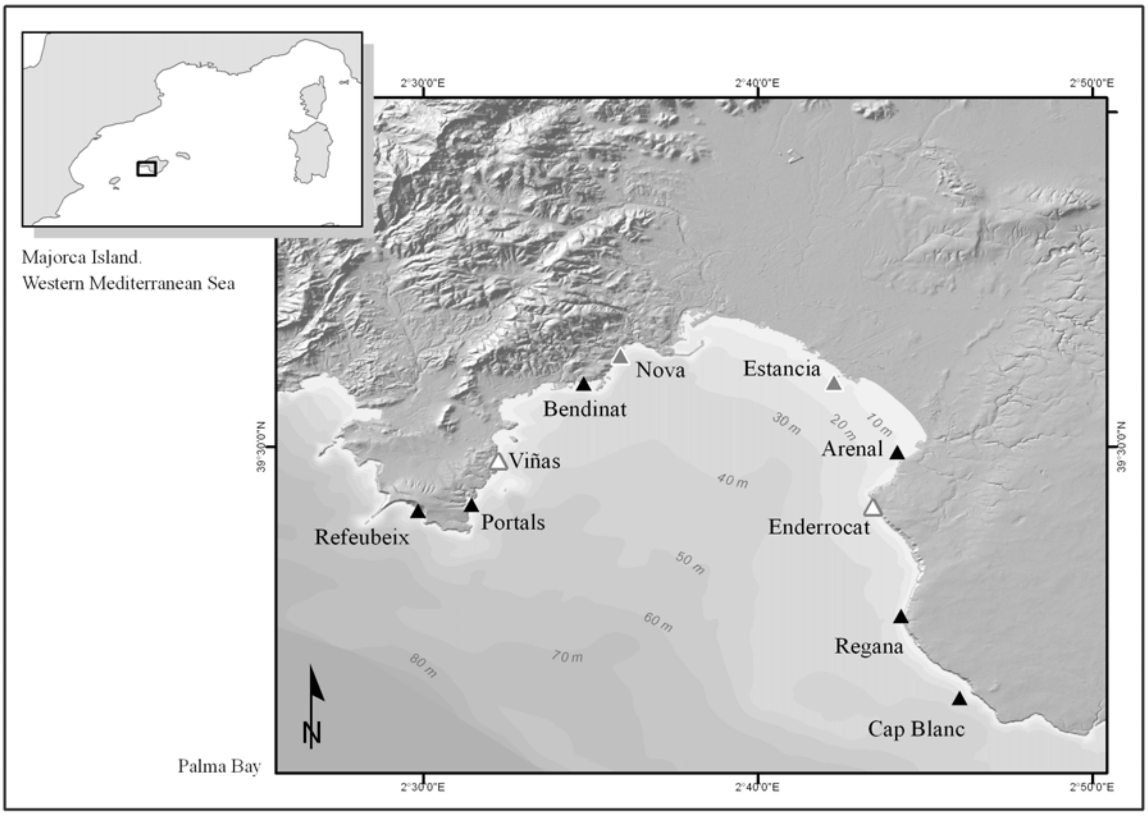


Figure 1. Palma Bay (Majorca, Western Mediterranean) with indication of the ten localities of the study. The four localities where water column nutrient enrichment was performed are indicated by white (low epiphyte load) and gray (high epiphyte load) triangles. Depth in 10 m intervals is represented by gray tones.

The nutrient addition experiment at each locality started in August 2008, when the invertebrate epifauna associated to *P. oceanica* was sampled using an air-lift with an opening mouth of 40x40 cm and a collector bag made of 200x200 µm mesh (see Buia et al. 2003). Three samples were collected in each locality that were sieved with a 500 µm mesh and fixed in 4% buffered formaldehyde in seawater. The main groups of epifaunal invertebrates (amphipods, copepods, gastropods, polychaetes, caprellids, mysids, bivalves, decapods, isopods, cumaceids, ostracods, acari, tanaids, ophiuroids, picnogonids, opisthobranchs, echinoids, asteroids) were sorted in the laboratory using a dissecting scope and abundance was expressed in number of individuals per plot (40x40 cm). The experiments proceeded with the establishment of six 1-m^2 plots in each of the 4 localities using galvanized iron bars at the corners. Three plots were assigned to receive nutrient addition while the other three plots served as controls for this factor. Slow-release fertilizer (Osmocote™ N:P:K 15:9:9 + 3MgO + trace elements) was employed as source of nutrients (Heck et al. 2000 7; Prado et al. 2008a) and they were provided by placing a 250 ml plastic diffuser filled with fertilizer at 40 cm above sediment in each of the assigned plots. Forty-two days after nutrient addition, samples of *P. oceanica* (5 shoots per plot) and of the invertebrate epifaunal community (one 40x40 cm sample per plot) were collected as previously described. We assumed this interval would be enough to detect responses because epiphytic macroalgae respond fast to nutrient enrichment during summer (Leoni et al. 2006, Prado et al. 2008a), and because the mobility of invertebrate epifauna in meadows of other seagrass species is very high (Howard 1985, Virnstein and Curran 1986).

After collection, each *P. oceanica* shoot was placed in an individual plastic bag and stored frozen at -20º C until processing. Epiphytes in all the leaves of each shoot were scraped off using a razor blade and collected in preweighed Whatman GF/C glass fibre filters. Filters were dried (60ºC, 48 h) to determine epiphyte dry weight (g DW). The length and width of each leaf was measured to calculate the total leaf surface of each shoot (cm^2). Leaves with radular marks of gastropods were scanned (see Rueda and Salas 2007) and the area of these herbivore marks was measured on the scanned images using ImageJ 1.43 software. Following the same procedure, leaves with fish bites (sparid *Sarpa salpa,* see Buia et al. 2003) were scanned to quantify the bitten area of each shoot. No sea urchins marks were observed in the shoots. Seagrass leaves were dried (60ºC, 48 h) to quantify the leaf biomass (g DW) of each shoot. Epiphyte load of each *P. oceanica* shoot was expressed as epiphyte biomass per leaf biomass (g DW epiphyte g DW $leaf^{-1}$). Gastropod grazing pressure was expressed as the percentage of leaf surface of each shoot presenting radular marks, and fish herbivory as the percentage of leaf surface of each shoot exhibiting fish bites.

A subsample of three shoots and three filters with epiphytes was haphazardly selected from each plot and month and set aside for nutrient analysis. Every shoot and filter was ground to powder with a stainless steel ball mill (MM200 RETSCH, Haan, Germany). An aliquot of the ground material was used to determine total concentration of N in the leaves and in the epiphytes of each shoot using a CNH elemental analyzer (1100 CE Instruments, Elantech, NJ, USA). Ground filters with no epiphytes were also analyzed to verify that there was not N signal due to filter composition. P content in the leaves and epiphytes was analyzed following the protocol described by Fourqurean et al. (1992) using beech leaves as certified standard (CRM No. 100). N and P content in the leaves was expressed as % of leaf DW, and the N and P content in epiphytes was expressed as % of epiphyte DW after correction for the contribution of filter DW to sample DW.

One way ANOVAs were performed to evaluate differences among the ten localities for leaf biomass, number of leaves per shoot, epiphyte load, percentage of grazed area and N content of the leaves and epiphytes. Spearman rank correlation analysis was used to evaluate association between those variables. The results of the nutrient addition experiments were evaluated using three-way ANOVAs, with epiphyte load (high *versus* low) and nutrients (fertilized *versus* control) as orthogonal and fixed factors, and locality as a random factor nested in the interaction of epiphyte load and nutrient addition. Post-hoc multiple comparisons for significant factors were performed using Tukey test. ANOVA assumptions were tested by Kolmogorov Smirnov test and Cochran's test for normality and homogeneity of variances, respectively, and the variables were transformed when necessary.

Multi-Dimensional scaling (MDS) ordination of samples using Bray Curtis similarities and analysis of similarities (ANOSIM) were performed to analyze the differences in the invertebrate epifaunal communities between high and low epiphyte load localities at the start of the experiment (August), to assess the evolution of the invertebrate epifaunal community between the initiation (August samples) and the end of the experiment in non-fertilized treatments (August samples vs. "control" samples in September), and to compare the invertebrate epifaunal communities of "control" and "fertilized" plots at the end of the experiment in September.

Results

We found significant differences in epiphyte load, leaf biomass, leaf N content and epiphyte N content among the ten localities studied in Palma Bay (Table 1, Fig. 2), while the number of leaves per shoot and the percentage of leaf area grazed by gastropods did not differ (Table 1). Spearman rank correlation analysis indicated a significant positive correlation between epiphyte load and N content in seagrass leaves while negative correlations of epiphyte N content with epiphyte load and of shoot biomass with epiphyte load (Table 2).

Estancia and Nova exhibited the highest epiphyte load of all localities (0.58 ± 0.04 (mean ± SE) g DW epiphyte g DW leaf^{-1}, N = 5, and 0.48 ± 0.1 g DW epiphyte g DW leaf^{-1}, N = 5 respectively) while epiphyte load was lowest in Enderrocat (0.21 ± 0.04 g DW epiphyte g DW leaf^{-1}, N = 5) and Viñas (0.021 ± 0.01 g DW epiphyte g DW leaf^{-1}, N = 5). These were the four localities chosen to perform the nutrient addition experiments.

Table 1. One way ANOVA to evaluate the differences among the ten localities considered in epiphyte load, leaf biomass, number of leaves per shoot, percentage of shoot leaf area with radular and bite marks and nitrogen content in the leaves and in the epiphytes of *Posidonia oceanica* shoots. Significant effects are in bold, ns: non significant.

		Leaf biomass (g DW shoot^{-1})			**Number of leaves per shoot**			**Epiphyte load (g DW/g DW shoot^{-1})**		
	DF	MS	F	p	MS	F	p	MS	F	p
Locality	9	0,277	3,013	**0,008**	0,447	0,912	0,525	0,063	8,746	**0,000**
Error	40	0,092			0,490			0,007		

		% Grazed area per shoot (cm^2/cm^2 shoot)		
	DF	MS	F	p
Locality	9	0,481	1,594	0,150
Error	40	0,301		

		Leaf nitrogen (%DW)				**Epiphyte nitrogen (%DW)**		
	DF	MS	F	p	DF	MS	F	p
Locality	9	0,057	2,920	**0,022**	9	0,271	3,102	**0,046**
Error	40	0,020			10	0,087		

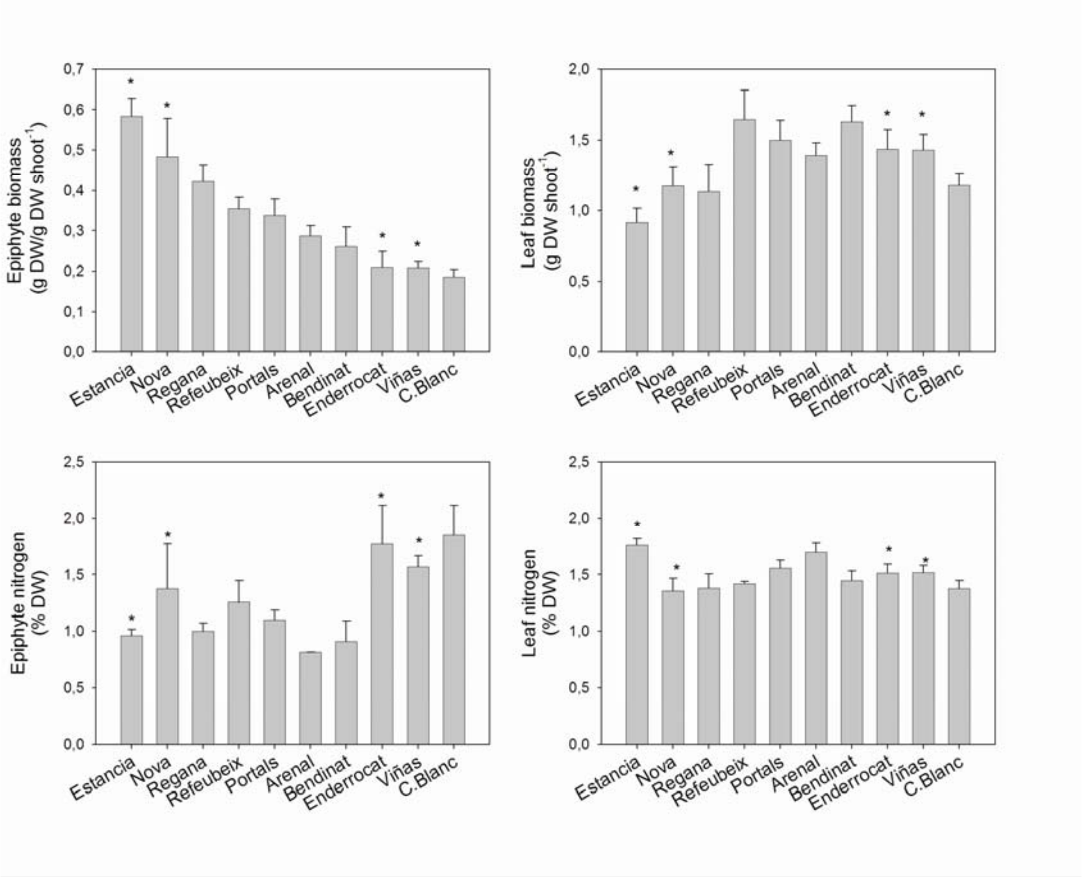


Figure 2. Mean and SE of epiphyte biomass, leaf biomass, number of leaves per shoots, and nitrogen content in leaves and in epiphytes of *Posidonia oceanica* in ten localities in Palma Bay (Majorca, Western Mediterranean). The four localities selected for the nutrient addition are marked with asterisks

Water column nutrient addition led to an increase of epiphyte load both in high and low epiphyte load localities, generally doubling it in fertilized treatments. Leaf biomass was lower in high than in low epiphyte load localities and the addition of nutrients led to a reduction of leaf biomass in all localities (Fig. 3, Table 3). The percentage of leaf area both grazed by gastropods and fish did not differ between high and low epiphyte load localities and was not affected by nutrient additions (Table 3). N content of epiphytes was higher in low than in high epiphyte load localities and it was not affected by nutrient additions (Fig. 3, Table 3). Epiphyte phosphorus content increased by 44% in low epiphyte load localities after nutrient addition while it decreased by 25% in high epiphyte load localities (Fig. 3, Table 3). Nutrient content of seagrass leaves was not affected by nutrient additions. While N content was higher in high epiphyte load localities, P was lower. A significant effect of locality was present in epiphyte and shoot features, as illustrated by the lower percentage of grazed area in Nova than in the rest of localities, the 74% higher leaf biomass in Viñas than in the others localities, or the lower epiphyte P content in Enderrocat (a low epiphyte load site) than in Nova (a high epiphyte load site) (Fig. 3, Tukey).

Table 2. Spearman correlation coefficients among epiphyte load, leaf biomass, number of leaves per shoot, grazed area and nitrogen content of the leaves and epiphytes in the ten localities considered. Significant levels are in bold and indicated by: p<0.05* ; p<0.01** ; p<0.001***

	Epiphyte load (g DW/g DW shoot^{-1})	**Leaf biomass (g DW shoot^{-1})**	**Number of leaves per shoot**	**% Grazed area per shoot**	**Leaf nitrogen (% DW)**	**Epiphyte nitrogen (% DW)**
Epiphyte load (g DW/g DW shoot^{-1})						
Leaf biomass (g DW shoot^{-1})	**-0,509****					
Number of leaves per shoot	-0,033	0,219				
% Grazed area per shoot	0,015	-0,059	-0,071			
Leaf nitrogen (% DW)	**0,390***	-0,048	0,006	0,258		
Epiphyte nitrogen (% DW)	**-0,426***	-0,143	-0,115	0,059	-0,283	

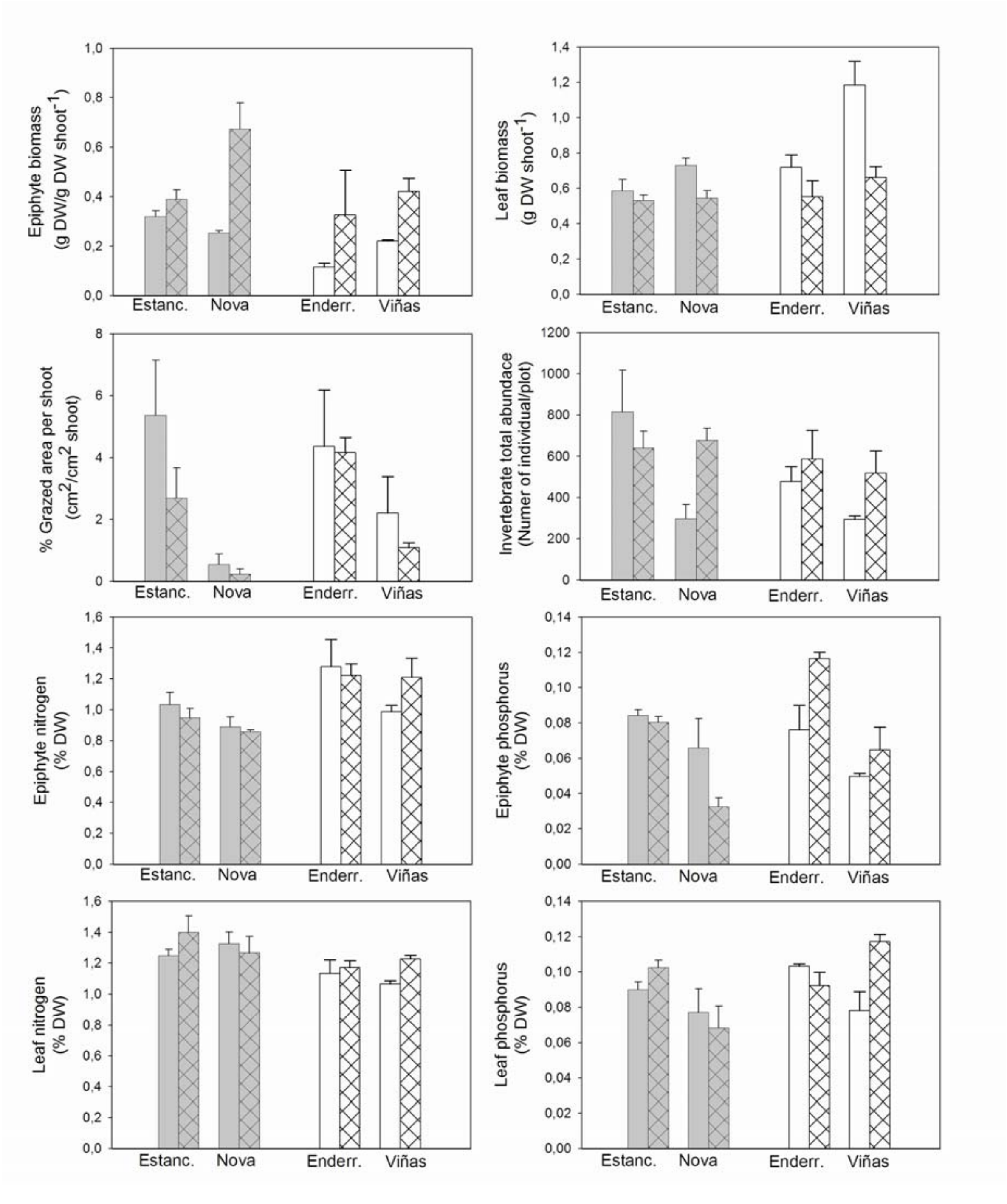


Figure 3. Mean and SE of epiphyte biomass, leaf biomass, percentage of grazer area, total number of invertebrates per plot, epiphyte nitrogen and phosphorus content and leaf nitrogen and phosphorus content of *Posidonia oceanica* shoots in control (blank) and nutrient enriched (striped) plots in September 2008. The white columns correspond to the two low epiphyte load localities and the gray columns correspond to the two high epiphyte load localities.

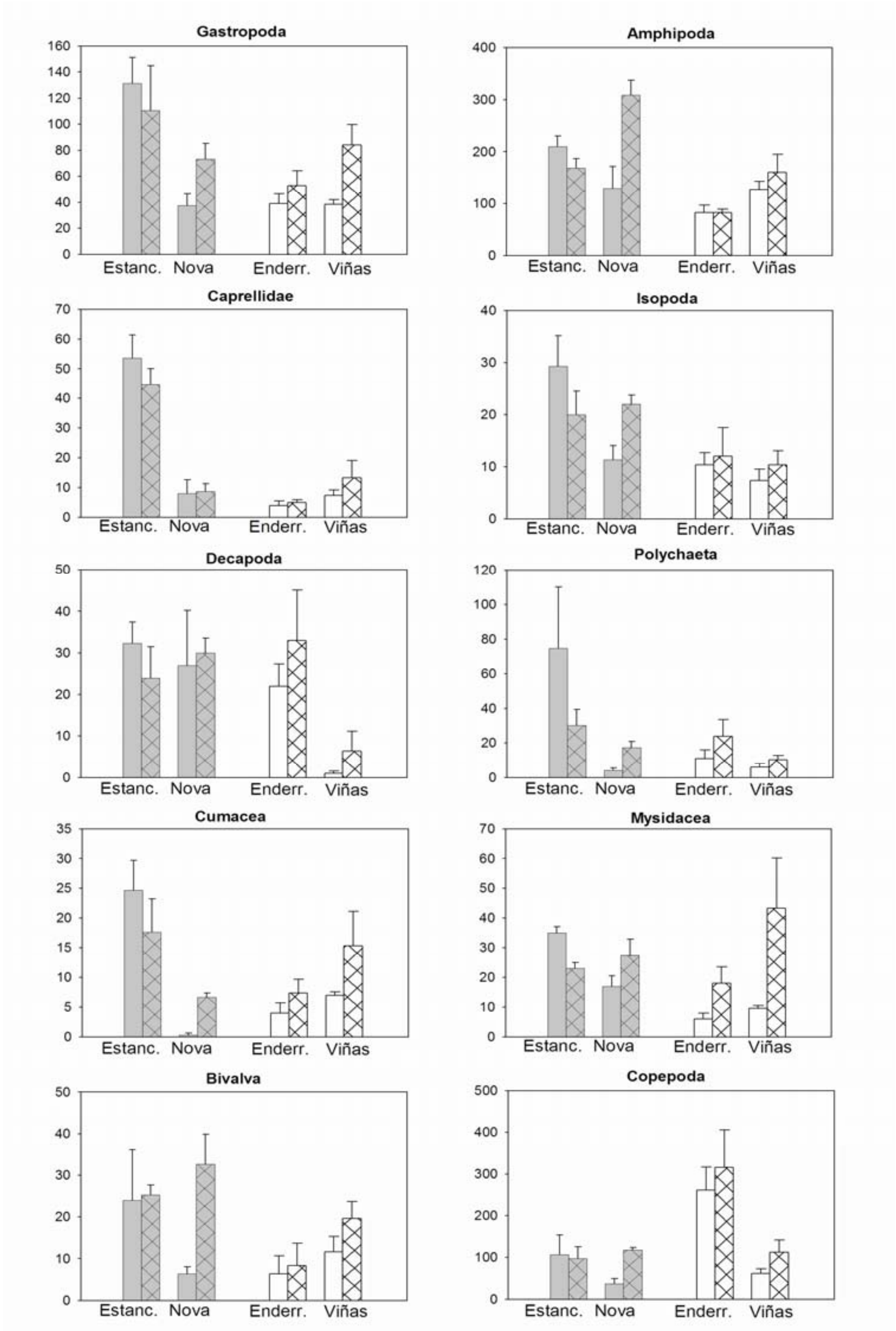


Figure 4. Mean + SE number of individuals per plot for the main groups of *Posidonia oceanica* epifaunal invertebrates found in control (no pattern) and nutrient-enriched (grilled pattern) plots in September 2008. The white columns correspond to the two low epiphyte load localities and the gray columns correspond to the two high epiphyte load localities.

Total abundance of invertebrate epifauna was higher in Estancia than in the other localities but it was not different between high and low epiphyte load localities (Table 4, Fig. 3, Tukey). Although total abundance tended to increase after nutrient additions in Nova, Enderrocat and Viñas, and decrease in Estancia, neither the factor nutrient addition nor its interaction with epiphyte load were significant. Differences in abundance among localities were detected for almost all invertebrate groups (Table 4). Gastropods, caprellids, isopods, polychaetes and cumacea were more abundant in Estancia than in the other localities (Fig. 4, Table 4, Tukey). The abundance of amphipods and mysidacea was higher in high than in low epiphyte load localities and increased after nutrient addition. Similarly, the abundance of cumacea, bibalvia, pycnogonida and ophiuroidea also increased after nutrient addition. There were no differences in the abundance of opisthobranchs, tanaids, echinoids, chaetognaths or fish between any of the treatments (Table 4).

At the beginning of the experiment invertebrate epifaunal communities were clearly different between high and low epiphyte load levels (MDS stress 0.05; significance level ANOSIM < 0,01; Fig. 5a). Epifaunal communities at the initiation and the end of the experiment (control plots, no nutrients added) also aggregated in high versus low epiphyte load level (significance level ANOSIM < 0,05), but the similarity between communities of high and low epiphyte loads increased during the experiment (compare the MDS stress of Fig. 5a vs. 5b). Nutrient additions further reduced the differences between the epifaunal communities found at the beginning of the study between high and low epiphyte load localities (stress 0.1; significance level ANOSIM > 0,05) (Fig. 5c).

Table 3. ANOVA results to assess differences in plant and epiphyte parameters among epiphyte load level (E), locality (L), nutrient addition (N) and their interactions. Significant effects are in bold, ns: non significant.

			Epiphyte load (g DW/g DW shoot-1)			**Leaf biomass (g DW shoot-1)**			**Number of leaves per shoot**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	0,163	54,35	**0,000**	0,218	14,74	**0,001**	8,760	31,52	**0,000**
N	**F**	1	0,138	45,98	**0,000**	0,294	19,89	**0,000**	2,870	10,33	**0,005**
E*N	**F**	1	0,007	2,34	ns	0,062	4,19	ns	0,304	1,09	ns
L(E*N)	**R**	4	0,046	15,24	**0,000**	0,090	6,12	**0,003**	0,417	1,50	0,249

			% Grazed area per shoot (cm2/cm2 shoot)			**% Bitten area per shoot (cm2/cm2 shoot)**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	0,899	3,60	ns	0,001	0,01	ns
N	**F**	1	0,568	2,27	ns	0,112	1,42	ns
E*N	**F**	1	0,127	0,51	ns	0,193	2,44	ns
L(E*N)	**R**	4	1,972	7,89	**0,001**	0,217	2,74	ns

Table 3 (continued).

			Epiphyte nitrogen (% DW)			Epiphyte phosphorus (% DW)		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	0,347	13,55	**0,002**	0,001	2,68	ns
N	**F**	1	0,001	0,02	ns	0,000	0,48	ns
E*N	**F**	1	0,030	1,18	ns	0,003	12,11	**0,003**
L(E*N)	**R**	4	0,043	1,67	ns	0,002	8,50	**0,001**

			Leaf nitrogen (% DW)			Leaf phosphorus (% DW)		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	0,154	10,04	**0,006**	0,001	4,90	**0,042**
N	**F**	1	0,032	2,08	ns	0,000	1,75	ns
E*N	**F**	1	0,004	0,29	ns	0,000	1,06	ns
L(E*N)	**R**	4	0,011	0,75	ns	0,001	4,59	**0,012**

Table 4. ANOVA results to assess differences among the factors: epiphyte load level (E), locality (L) and nutrient (N) addition during the experiment in the number of individual per plot of the different taxons and total abundance of epifauna of *Posidonia oceanica* meadows. Significant effects are in bold, ns: non significant.

			Abundance			**Gastropoda**			**Amphipoda**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	115509,375	3,33	ns	7176,042	8,49	**0,010**	49504,167	26,14	**0,000**
N	**F**	1	108945,375	3,14	ns	2109,375	2,50	ns	10922,667	5,77	**0,029**
E*N	**F**	1	6048,375	0,17	ns	737,042	0,87	ns	4056,000	2,14	ns
L(E*N)	**R**	4	115120,542	3,32	**0,037**	4196,708	4,97	**0,009**	12766,250	6,74	**0,002**

			Caprellidae			**Isopoda**			**Decapoda**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	2730,667	46,31	**0,000**	682,667	16,22	**0,001**	975,375	5,55	**0,032**
N	**F**	1	0,667	0,01	ns	13,500	0,32	ns	45,375	0,26	ns
E*N	**F**	1	88,167	1,49	ns	4,167	0,10	ns	176,042	1,00	ns
L(E*N)	**R**	4	1298,250	22,02	**0,000**	127,417	3,03	**0,049**	456,208	2,60	ns

			Polychaeta			**Cumacea**			**Mysidacea**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	15,640	5,67	**0,030**	0,391	0,53	ns	6,014	5,63	**0,030**
N	**F**	1	1,149	0,42	ns	4,032	5,47	**0,033**	9,358	8,76	**0,009**
E*N	**F**	1	4,013	1,45	ns	0,065	0,09	ns	9,564	8,96	**0,009**
L(E*N)	**R**	4	15,835	5,74	**0,005**	9,404	12,75	**0,000**	3,351	3,14	**0,044**

			Bivalva			**Copepoda**			**Asteroidea**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	672,042	6,30	**0,023**	86,721	11,91	**0,003**	3,375	9,00	**0,008**
N	**F**	1	532,042	4,98	**0,040**	28,968	3,98	ns	0,375	1,00	ns
E*N	**F**	1	117,042	1,10	ns	0,170	0,02	ns	2,042	5,44	**0,033**
L(E*N)	**R**	4	196,042	1,84	ns	50,318	6,91	**0,002**	2,042	5,44	**0,006**

Table 4 (continued)

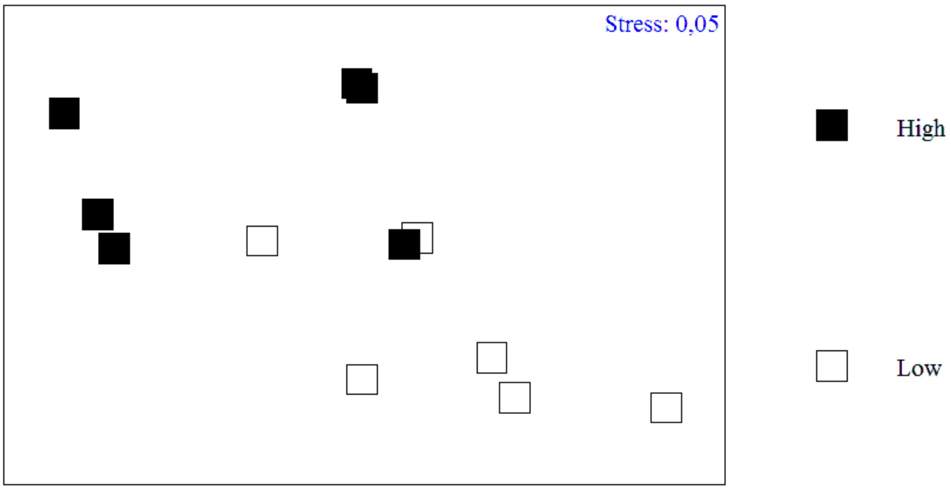
			Ophiuroidea			**Pycnogonida**			**Acaros**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	0,375	0,24	ns	13,500	3,724	ns	10,667	1,68	ns
N	**F**	1	12,042	7,60	**0,014**	20,167	5,563	**0,031**	0,667	0,10	ns
E*N	**F**	1	0,042	0,03	ns	8,167	2,253	ns	13,500	2,13	ns
L(E*N)	**R**	4	6,708	4,24	**0,016**	5,500	1,517	ns	27,917	4,41	**0,014**

			Opisthobranchia			**Tanaidacea**			**Ostracoda**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	16,667	1,45	ns	170,667	3,507	ns	0,554	0,50	ns
N	**F**	1	2,667	0,23	ns	0,167	0,003	ns	3,746	3,37	ns
E*N	**F**	1	0,000	0,00	ns	37,500	0,771	ns	3,389	3,05	ns
L(E*N)	**R**	4	21,500	1,87	ns	54,583	1,122	ns	5,476	4,93	**0,009**

			Echinoidea			**Quetognatos**		
		df	**MS**	**F**	**p**	**MS**	**F**	**p**
E	**F**	1	2,042	1,63	ns	2,042	0,58	ns
N	**F**	1	0,375	0,30	ns	1,042	0,29	ns
E*N	**F**	1	0,042	0,03	ns	0,042	0,01	ns
L(E*N)	**R**	4	1,875	1,50	ns	2,958	0,83	ns

Figure 5 (next page). MDS ordination of the epifaunal community samples. 5a: MDS comparing epifaunal communities at the four localities before the addition of nutrients (August 2008). 5b MDS comparing epifaunal communities at the beginning (August 2008) and the end of the experiment (September 2008) when no nutrients were added and therefore indicating the temporal change of the community. 5c MDS comparing epifaunal communities at the end of the experiment with or without nutrient enrichment.

5a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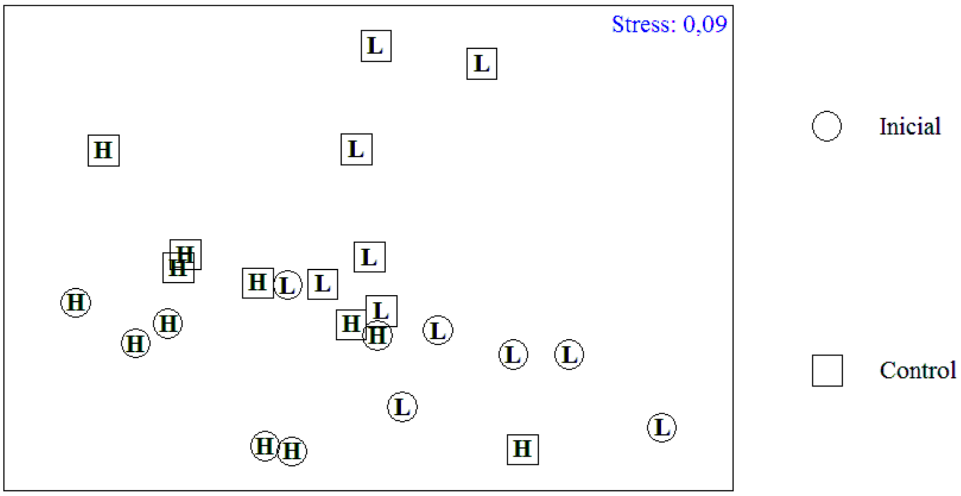
High
Low

5b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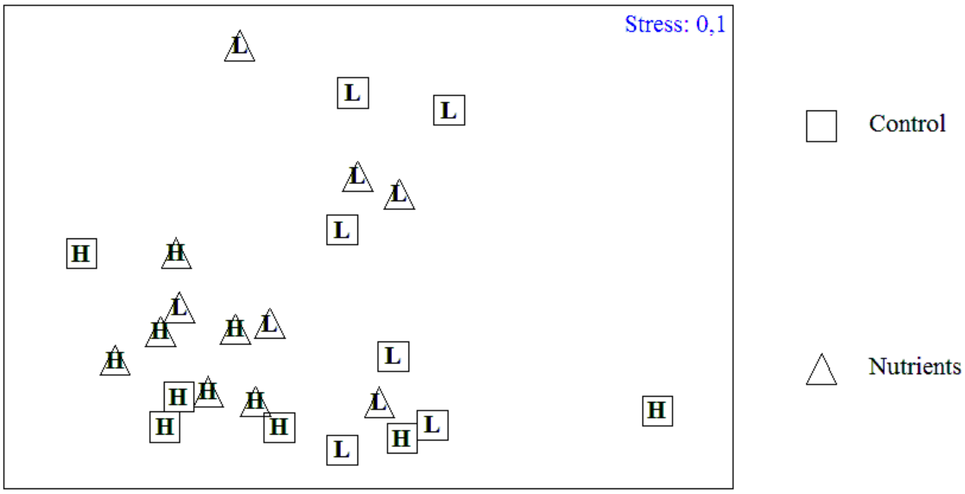
Inicial
Control

5c


Stress: 0,1
Control
Nutrients

Discussion

Our results show that epiphyte biomass was higher in nutrient enriched plots than in controls despite the increase of epifaunal density, suggesting that bottom-up controls have stronger effects in our system.

Epiphytic biomass response was consistent with previous studies (Leoni et al. 2006; Prado et al. 2008a) which show that the strength of nutrient-driven increases of epiphyte biomass in *Posidonia oceanica* is season-dependent and particularly evident in summer. While N content of epiphytes was higher in the high epiphyte load than in the low epiphyte load localities, it did not increase after nutrient addition. On the other hand, epiphyte P content increased in the nutrient enriched plots with low initial epiphyte biomass (Enderrocat and Viñas). These results suggest that the increase of epiphyte biomass observed may result from the alleviation of phosphorus limitation. Alternatively, a reduction of grazer consumption during summer could also enhance epiphyte biomass. Seasonality of seagrass herbivory in *P. oceanica* meadows has been described for fish (increasing in summer; Tomas et al. 2005b; Prado et al. 2007) and sea urchins (low in summer; Peirano et al. 2001; Tomas et al. 2005b), but little is known for most mesograzers (but see Peirano et al. 2001 for information on *Idotea baltica*).

We observed a reduction of seagrass biomass in nutrient-enriched plots (where epiphyte biomass increased) and a negative correlation between epiphyte and seagrass biomass. While seagrass consumption may be enhanced with nutrient fertilization through an increase of tissue food quality (e.g. Goecker et al. 2005; Prado et al. 2010; but see Tomas et al. in press), nutrient enrichment did not appear to increase herbivore pressure on *P. oceanica* shoots in our study, as shown by the lack of response of the percentage of leaf area bitten by fish. Thus lower seagrass biomass is likely a negative competitive effect of epiphytes through a reduction of light or nutrients available to leaves (e.g. Burkholder et al. 2007; Leoni et al. 2008). The lack of effect of nutrient additions on seagrass nutrient content further suggests that relative nutrient availability for *P. oceanica* growth was not greatly altered and point to epiphyte shading as the likely mechanism driving the reduction of shoot biomass (Ruiz and Romero 2003).

The influence of grazers on epiphyte biomass was not evaluated in this study, since experiments did not include a grazer exclusion treatment, but our results suggest that grazing does not offset epiphyte response to increased nutrients and that epiphyte load appears to be mostly controlled by bottom-up forces. However, a stronger top-down control may be occurring in one of the localities studied (Estancia), where the abundance of invertebrates was highest and nutrient addition promoted the lowest rise of epiphyte load. On the other hand, Estancia showed higher epiphyte load in the controls than the rest of localities, which could also suggest that the environmental nutrient supply already fulfils epiphyte requirements.

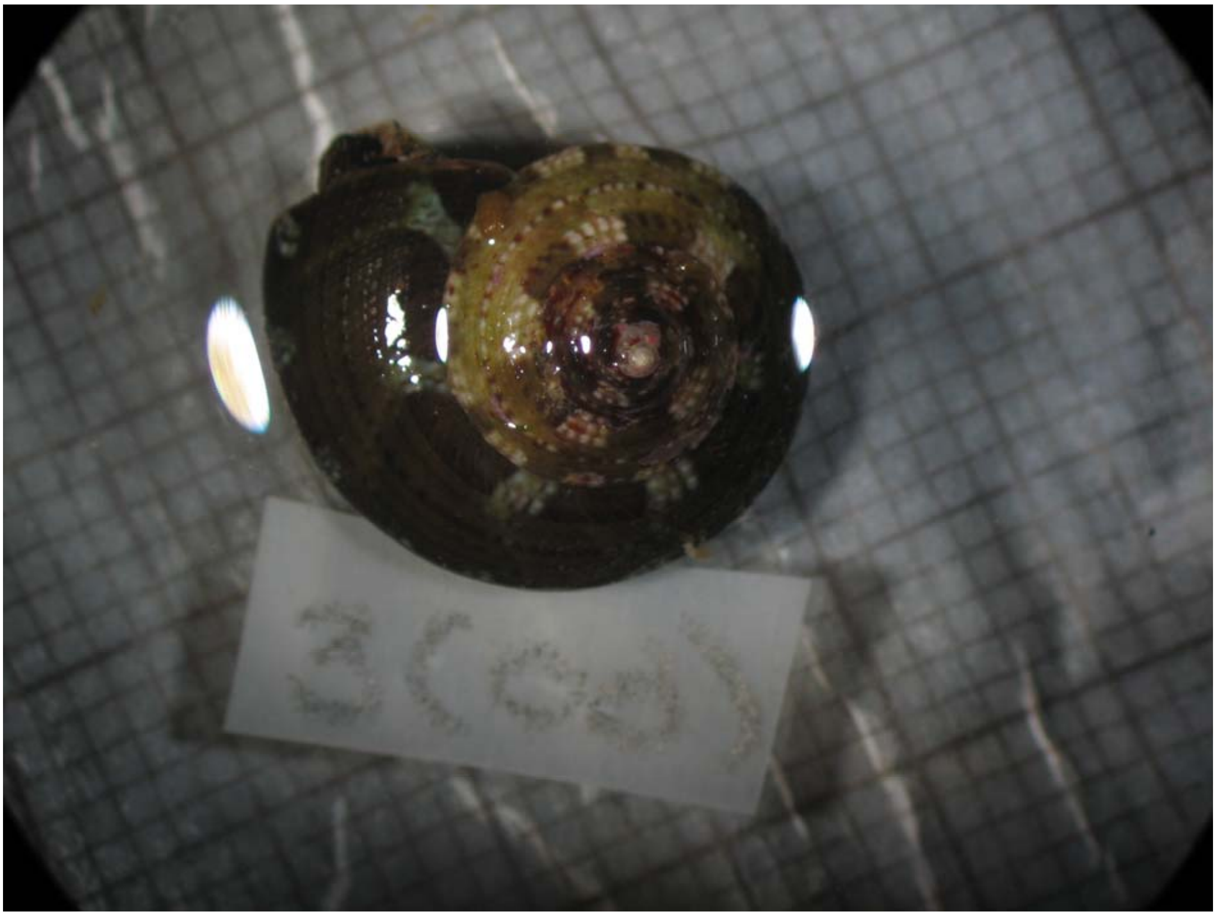
Furthermore, the invertebrate communities also appear to be driven by epiphytes and, ultimately, nutrient additions. For instance, the abundance of most invertebrate groups tended to increase when nutrients were added suggesting that higher accumulation of epiphyte biomass in fertilized treatments stimulated the development of invertebrate populations, even for the non epiphyte-consumer groups. Yet the ANOVA analysis did not detect a significant effect of nutrient for many groups, which is likely a consequence of the higher abundances of Estancia. In fact, when Estancia is excluded from the analysis, the trend of increasing abundance in nutrient enriched treatments turns significant for most of the invertebrate groups (data not shown). Furthermore, the strong differences in invertebrate community composition between low and high epiphyte load localities disappeared when nutrients were added, while they were maintained among unfertilized plots (Fig. 5). In addition, the significance of the factor “locality” for most of the epifaunal groups highlights that local conditions and spatial variability are important in determining epifaunal communities (Mazzella et al. 1989; Vizzini et al. 2006).

Summary

In summary, this work shows that nutrient availability in the water column is the main driver of change of epiphyte biomass in *Posidonia oceanica* shoots. Our results also show that the increase of epiphyte biomass cascades up to a global increase of the populations of invertebrates, even those that are not directly trophic related with epiphytes. However, the increase of invertebrate populations does not reverse epiphyte biomass to a non-nutrient-enriched situation. Epiphyte abundance strongly responded to nutrient availability increases and seems to have negative effects on *P. oceanica* shoot size. This work suggests that epiphyte biomass affects the abundance of the populations of epifaunal grazers in *P. oceanica* systems and that nutrient availability is the main driver of epiphyte biomass during summer. Further studies are needed to characterize the trophic linkages existing in the community of grazers inhabiting *P. oceanica* meadows. The assessment of species-specific feeding characterization of the main grazers in *P. oceanica* meadows and density-dependent effects on the consumption rates over epiphytes abundance should be also considered.

CHAPTER 5

Gastropod grazing on *Posidonia oceanica* early-succesional epiphytic community



Gastropod grazing on *Posidonia oceanica* early-succesional epiphytic community. Inés Castejón-Silvo, Jorge Terrados

Abstract

Nutrient availability in the water column may promote epiphyte biomass through a bottom-up mechanism. Gastropods are an important component of the abundant and diverse community of resident invertebrates that live in *Posidonia oceanica* meadows and feed on epiphytes. Gastropod grazing may increase in response to better quantitative and qualitative epiphyte supply. In this work we evaluate in aquaria conditions the consumption rate of eleven frequent species of gastropods on early successional epiphytic community. We offered two meshes of artificial substrata to the gastropods, one colonized in natural conditions and other colonized with increased-nutrient availability. Gastropods actively consumed on the epiphytes and reduced epiphyte biomass on the meshes. Increased nutrient availability led to a higher colonization of epiphytes in the meshes and promoted the increase of consumption rate for most of the species with riphidoglossan radula.

Keywords: biofilm, nutrient addition, gastropods, *Posidonia oceanica*, consumption rate.

Introduction

Herbivores represent an important component of the faunal communities in seagrass meadows (Mazzella et al. 1992; Valentine and Heck 1999) although the role of herbivory is quite variable between seagrass ecosystems (Cebrián and Duarte 1999). In temperate meadows the small invertebrates, dominated by amphipods, isopods, decapods, and gastropod molluscs, constitute a key component of primary consumers (Orth and van Montfrans 1984; Jernakoff et al. 1996; Heck et al. 2000). This mesograzer community feeds preferentially on epiphytes rather than on seagrass leaves and is responsible for an important part of epiphyte consumption (McGlathery et al. 1995; Peirano et al. 2001; Moksnes et al. 2008; Doropoulos et al. 2009). Epiphytic algae are an important component of primary production and biomass in seagrass meadows and their abundance is the result of the balance between nutrient availability and grazer consumption (Valentine and Duffy 2006). The effects of consumer pressure on epiphyte biomass will depend on the grazer and epiphyte species composition, on the hydrodynamics and on the resource availability in the system (Schanz et al. 2002; Jaschinski et al. 2010; Jaschinski and Sommer 2011). Grazer abundance is lower in wave exposed meadows and seagrass epiphytes support lower grazing pressure than in sheltered meadows (Schanz et al. 2002). Increased nutrients promote the rise of epiphytic algal abundance (i.e. Frankovich et al. 2006; Balata et al. 2010) and may also increase the consumption rates of grazers in response to the higher nutrient quality of food supply (e.g. Jaschinski and Sommer 2011). Species-specific effects of grazers and the influence of nutrient supply on epiphyte biomass have been demonstrated in field approaches, mesocosms and aquaria experiments (Duffy et al. 2001, Hily et al. 2004). Gastropods are an important component of grazer community in seagrass meadows. For instance in situ experiments on the eelgrass (*Zostera marina*) meadows of Baltic Sea showed that the gastropods *Littorina littorea* and *Rissoa membranacea* were more efficient grazers than the isopod *Idotea baltica* under nutrient enriched conditions despite the similar effects of the three species on epiphyte biomass in natural nutrient conditions (Jaschinski and Sommer 2011). The experimental field work of Philippart (1995) showed important reductions of epiphyte biomass under increased densities of the gastropod *Hydrobia ulvae,* similar to the results found in aquaria by Hootsmans and Vermaat (1985). Aquaria assays found highest grazing activity of *Gibbula umbilicalis* and *Jujubinus striatus* when epiphytic algae were most abundant. Moreover both gastropods grazed preferentially on the apical part of the leaves were filamentous algae where present (ectocarpales and ceramiales) (Hily et al. 1999). *J. striatus* showed weight-specific grazing rates positively related with epiphyte biomass; hence this species increases its feeding activity with food availability (Hily et al. 1999). The efficiency and feeding preference of gastropod grazers has been demonstrated to be strongly related with the radular morphological features and with the palatability of available algae (Steneck and Watling 1982).

Posidonia oceanica meadows harbour a rich community of invertebrate epifauna dominated by crustaceans, molluscs and polychaetes that mainly graze on epiphytes (Mazzella and Russo 1989, Mazzella et al. 1992). Gastropods are one of the most abundant components of epiphyte grazer community in Mediterranean meadows (Mazzella and Russo 1989; Gacia et al 2009) and stable isotopes analysis has confirmed their trophic link with epiphytes (Gacia et al. 2009). *P. oceanica* leaves constitute a substrate for the settlement of a diverse community of epiphytic flora and fauna. The long life-span of *P. oceanica* leaves (from 202 to 345 days, Hemminga et al. 1999) allows the development of an abundant and species-rich community of epiphytes with different successional stages according to the leaf age-gradient (Antolié 1985; Mazzella et al. 1989; Hemminga et al. 1999; Piazzi et al. 2004; Balata et al. 2007). Bacterial microorganisms appear in leaves within one or two days of leaf-life and a layer of a rich community of diatoms develops in the next few weeks (Mazzella et al. 1986). At three months of leaf age an encrusting macrophyte layer dominated by red and brown algae is well developed (Mazzella et al. 1986; Mazzella et al. 1992) on which a mature stage of colonization composed mainly by filamentous macroalgae and sessile fauna settles (Mazzella et al. 1992; Pardi et al. 2006; Prado et al. 2008a). Maximum field densities of *J. striatus* and *Bittium reticulatum* stocked in microcosms are able to reduce by half epiphyte biomass on *P. oceanica* leaves (Gacia et al. 2009). Aquaria trial by Mazzella and Russo (1989) showed the feeding preferences of *Gibbula ardens* by bacterial colonies and first stages of algal germination contrary to the feeding behaviour of *Gibbula umbilicalis* that was focussed on encrusting and filamentous algae and ignored bacteria and diatoms. Apart from these works almost nothing is known about the species-specific feeding capabilities of the gastropod component of grazer community in *P. oceanica* meadows. This work aims to assess the species-specific consumption rates of a number of typical *P. oceanica* gastropods on an early successional epiphytic community in laboratory conditions.

Material and methods

Gastropods were collected from *Posidonia oceanica* meadows in the Marine Protected Area of Palma Bay during the summer 2010. Scuba divers collected haphazardly gastropods once a week during May and June from the leaf canopy of *P. oceanica* at 3-4 meters depth. Temperature and salinity were measured every dive to emulate natural conditions in the aquaria. Animals were immediately transported to the laboratory where they were housed in 20 L aquaria (40 cm long * 25cm wide * 20 cm high) together with *P. oceanica* epiphytized leaves for two days to acclimatise to laboratory conditions before experimentation. To assure independence, no individual gastropod was used more than once in feeding assays.

Artificial substrata were used in the feeding assays to reduce differences of epiphyte composition and biomass among experiments and to avoid confounding the herbivory on the epiphytes with the ingestion of epiphytes by chance by herbivores feeding on the leaves. An artificial substratum composed of 250 μm nytex mesh was placed for three weeks before each feeding assay in the meadows to be colonized by epiphytes. One mesh was colonized in environmental nutrient conditions and another in nutrient-enriched conditions. Nutrients were supplied using slow-release fertilizer (Osmocote N:P:K 15:9:9 + 3MgO + trace elements) contained in a 250 ml plastic diffuser placed at some decimetres from the mesh. New meshes and diffuser were placed each week to assure artificial substrata availability to every feeding assay.

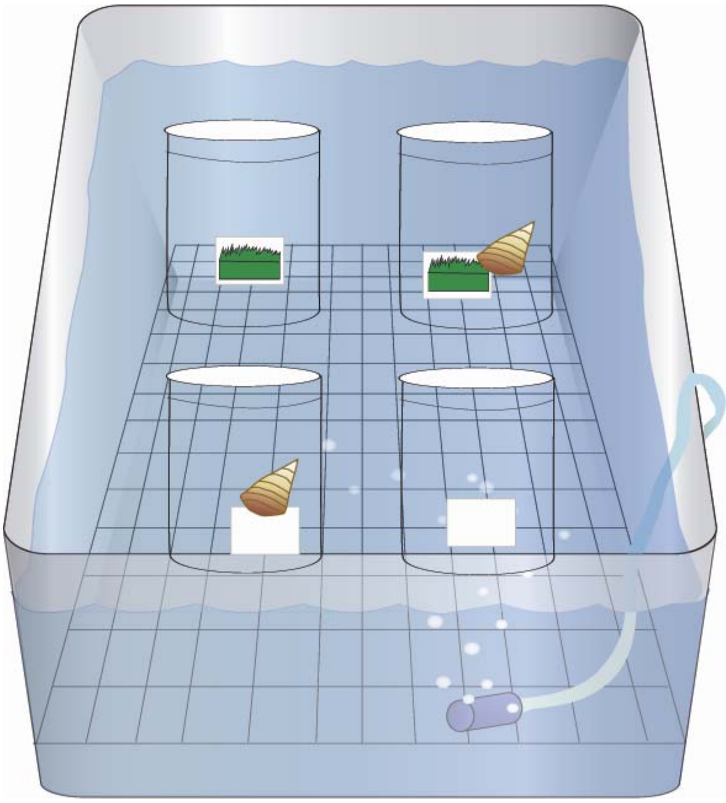


Figure 1. Diagram of an aquarium during the experiment. Inside the aquaria there are four containers one for each treatment. Control contains a mesh colonized without extra-nutrients. Grazer contains a mesh colonized without extra-nutrients with a gastropod; Control-Nutrients contain a mesh colonized with increased nutrients and Grazer-nutrients, contains a mesh colonized with increased nutrients with a gastropod.

In the laboratory the epiphytized meshes were cut in 9 squared centimetres pieces and gastropods were identified and separated in pairs of the same species and similar size. Four containers were placed inside each aquarium one dedicated to each of the four treatments. The control (C) had a piece of epiphytized mesh in environmental nutrient conditions; the nutrient-control (NC) had a piece epiphytized mesh in increased nutrient conditions, grazer (G) enclosed a gastropod with mesh epiphytized in environmental nutrient conditions and nutrient-grazer (NG) enclosed a gastropod with mesh epiphyted in increased nutrient conditions (Fig. 1). All aquaria were housed in a controlled temperature room, aerated using air pumps and cleaned of particulate material with mechanical filters. Daylight fluorescent lights were used to illuminate the aquaria on a 15:9 day/night cycle to match the number of environmental hours of light during the study. Feeding assays lasted between 24 and 72 hours depending on the gastropods size. Epiphytic chlorophyll was used to quantify epiphyte algae biomass. At the end of the assays the meshes were immediately extracted with acetone and cooled at 15 ºC for 24 hours. The subsequent analysis of the acetone extracts was carried out with a Turner Designs bench fluorometer. Epiphyte biomass was expressed as µg Chl a per square centimetre of mesh. The dry weight (DW; 60ºC, 72 h) was determined for every gastropod of the assays. Consumption rates were expressed as the differences in chlorophyll between control and grazer treatments and between nutrient-control and nutrient-grazer treatments normalized by the duration of the assay and the gastropods dry weight. Three extra control and three extra nutrient-control meshes were fixed in acetone at the beginning of every assay to asses the variations in epiphyte biomass during the experiments. After manipulations the exemplars were identified at species taxonomic level and classified in function of their radular type.

Paired T-test was performed to compare the epiphyte biomass established on meshes with and without increased nutrient. Two-way ANOVA was performed to evaluate the overall effects of the type of the epiphytic community (control vs. nutrient-enriched) and gastropod presence on the epiphyte biomass in the meshes at the end of the assays. We performed a two-way factorial ANOVA to assess the differences of consumption rates between species (random factor) and type of epiphytic community (fixed factor). We evaluate the differences of consumption rates for rates between the type of the epiphytic community (control vs nutrient-enriched) using a T-test analysis for each species. Normality and homogeneity of variances assumptions were tested by Kolmogorov Smirnov test and Cochran's test respectively, and the variables were transformed when necessary.

Results

After three weeks of colonization the meshes presented a thick layer of diatoms and the meshes colonized in nutrient-enriched conditions showed higher epiphyte biomass (without nutrients 0.94 ± 0.29 µg Chl a cm^{-2} (mean ± SD) and with nutrients 1.25 ± 0.32 µg Chl a cm^{-2}; t-value = -2.96; $p < 0.01$.

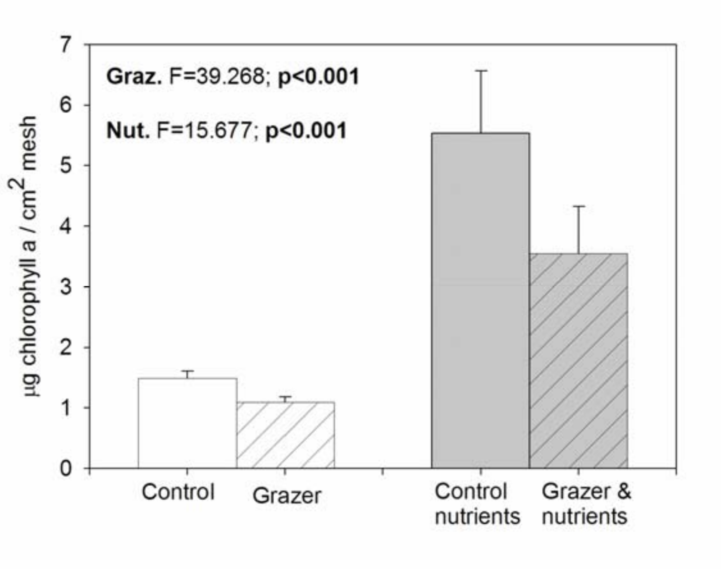


Figure 2. Mean and SE for the epiphyte biomass per square centimetre of mesh in the four treatments (µg chlorophyll a * cm^{-2} mesh): Control, Grazer, Control-nutrients, and Grazer-nutrients. T-test results are showed in the figure.

A total of 234 individuals of gastropods divided in five families and eleven species were collected. Seven species belong to Archaegastropods with rhipidoglossan radula and the other four were Ceanogastropods with taenioglossan radula (Table 1). Gastropod presence reduced epiphyte biomass on the meshes during the assays (Fig. 2).

Table 1. Gastropods species, number of individuals and radular types collected in the *Posidonia oceanica* meadow.

Order	**Family**	**Species**	**Radula type**	**Nº experiments**
Archeogastropoda	Trochidae	Calliostoma zizyphinum (Linnaeus, 1758)	Rhipidoglossan	18
Archeogastropoda	Trochidae	Gibbula ardens (von Salis, 1793)	Rhipidoglossan	23
Archeogastropoda	Trochidae	Jujubinus exasperatus (Pennant, 1777)	Rhipidoglossan	18
Archeogastropoda	Trochidae	Jujubinus striatus (Linnaeus, 1767)	Rhipidoglossan	4
Archeogastropoda	Niritidae	Smaragdia viridis (Linnaeus, 1758)	Rhipidoglossan	3
Archeogastropoda	Trochidae	Tricolia pullus (Linnaeus, 1758)	Rhipidoglossan	19
Archeogastropoda	Trochidae	Tricolia speciosa (von Mühlfeldt, 1824)	Rhipidoglossan	4
Ceanogastropoda	Alvania	Alvania montagui (Payraudeau, 1826)	Taenioglossan	10
Ceanogastropoda	Cerithiidae	Bittium reticulatum (da Costa, 1778)	Taenioglossan	14
Ceanogastropoda	Rissoidae	Rissoa auriscalpium (Linnaeus, 1758)	Taenioglossan	2
Ceanogastropoda	Rissoidae	Rissoa ventricosa Desmarest, 1814	Taenioglossan	2

Consumption rates were higher on the mesh colonized with increased nutrients than in the mesh colonized with natural nutrient availability (Fig. 3, Table 2). The increase of consumption rates when feeding on nutrient-enriched epiphytes varied among species (Table 2) and was especially noticeable for *Calliostoma zizyphinum, Gibbula ardens, Tricolia pullus and Tricolia speciosa* (Fig. 3).

Table 2. Factorial ANOVA results evaluation consumption rate differences among gastropod species and type of epiphytic community. Significant results are marked in bold.

	Consumption rate (µg Chl a * hour^{-1} * g^{-1} DW)			
	df	**MS**	**F**	**p**
species	10	0,20091	2,5719	**<0,001**
nutrients	1	0,62398	7,9878	**<0,001**
species*nutrients	10	0,09266	1,1862	0,301686

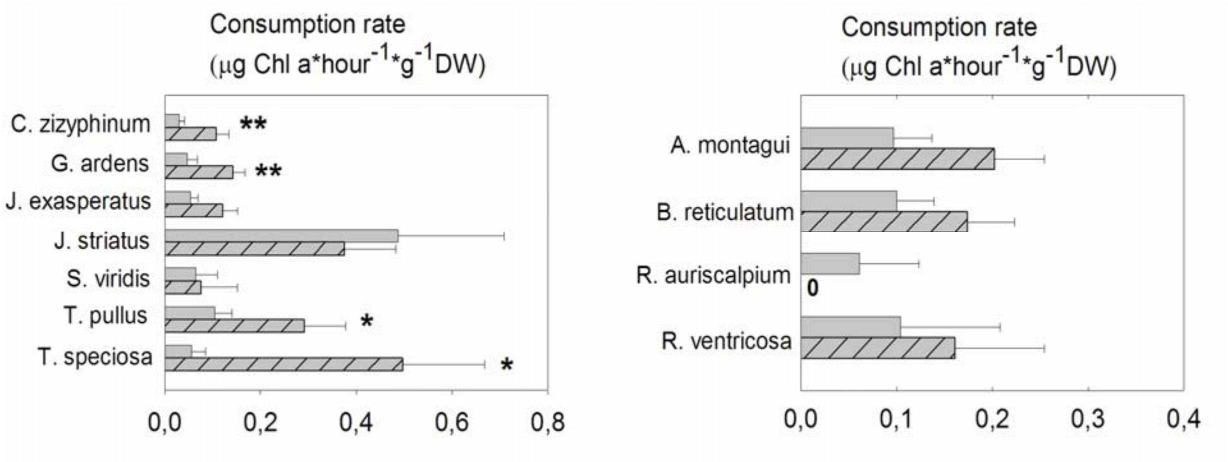


Figure 3. The consumption rates (µg Chl a * hour^{-1} * g^{-1}DW) of each species on the epiphyte biomass on the meshes colonized under ambient and nutrient- enriched conditions (mean ± SD). Significant differences found of T-test results are showed in the figure by: * = p<0.05, ** = p<0.01, *** = p<0.001

The increase of consumption rates on the meshes colonized with increased nutrients was not linked to the increase of epiphyte biomass on the meshes (Fig. 4).

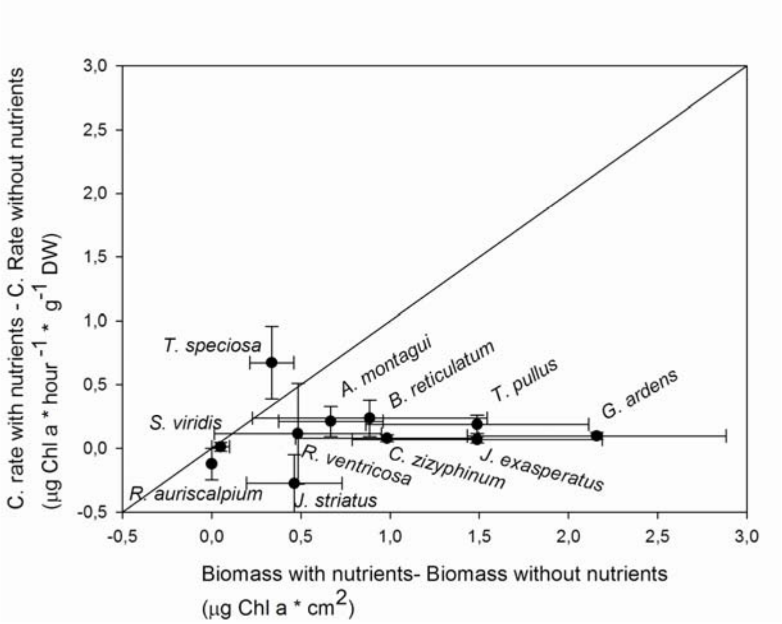


Figure 4. Biplot of mean and SE values for the difference of consumption rates and biomass on the meshes colonized under ambient and enriched nutrient conditions (µg Chl a * hour $^{-1}$ * g^{-1} DW).

Discussion

Our results show that epiphyte biomass was higher in the mesh colonized with increased nutrients and this type of epiphytic community promoted the rise of consumption rates of some species of gastropods. *C. zizyphinum, G. ardens, T. pullus and T. speciosa* are able of increasing their consumption rates in response to higher epiphytic biomass in nutrient-enriched conditions. The increase of consumption rates can be both a functional response to the quantitatively and to the qualitatively better food supply (Sommer 1999; Jaschinski and Sommer 2011) of epiphytes in meshes under nutrient-enriched conditions. Although the nutrient content of epiphyte biomass in the meshes was not determined it might be assumed to be higher in the nutrient-enriched conditions because nutrient content of *P. oceanica* epiphytes mirrors nutrient availability in the water column (Perez et al. 2008). Our results show that the increase of consumption rates of epiphytic biomass under nutrient-enriched conditions did not clearly respond to the increase of epiphyte biomass and may suggest that *C. zizyphinum, G. ardens, T. pullus and T. speciosa* consumption rates respond to an increased food quality (higher nutrient content) supply.

Gastropod grazing reduced epiphyte biomass on the meshes but the species-specific differences of consumption highlight the importance of the species composition of grazer community in the feeding pressure exerted on epiphytes. *G. ardens, J. exasperatus, J. striatus, T. pullus and T. speciosa* will be leading consumers in a scenario of an early succesional epiphytic community as microalgal grazers. The herbivore pressure on early successional epiphytic community is important because it may determine the community structure in the mature stages of succession (Kennelly 1983; Keats et al. 1994; Figueiredo et al. 1996). We did not found differences in the consumption rates between radular type although the most noticeable increases of consumption rates occurred in species with rhipidoglossan radula. Riphidoglossan radula facilitates microalgal consumption while the trophic niche of gastropods with taenioglossan radula will be the advanced stages of epiphytic succession dominated by coralline algae and macrophytes (Steneck and Watling 1982).

Although the gastropod *Smaragdia viridis* is strongly related with seagrasses its feeding preferences seem to be especially addressed to seagrass leaves (Rueda et al. 2008; Rueda and Salas 2007) because our results showed that this species did not consume epiphytic microalgae. All the gastropods collected for the experiment are common species in *P. oceanica* meadows and represented the main component of the total mollusc abundance in previous studies (Gambi et al 1991; Mazzella et al. 1989), thus they have a potential leading role in grazing pressure and epiphyte consumption in *P. oceanica* meadows. Further studies of mesograzers consumption in Mediterranean meadows are needed especially on mature stages of epiphytic community with presence of macroalgae.

Summary

Increased nutrient availability promoted epiphyte biomass in the meshes and gastropods actively consume on epiphytes and reduced epiphyte biomass in the control and enriched-nutrient meshes. Gastropod consumption rates increased in the meshes colonized in enriched-nutrient conditions but did not clearly respond to the increased of epiphyte biomass. The most noticeable increase of consumption rates occurred in species with rhipidoglossan radula and might be a response to higher food quality.

CHAPTER 6: Synthesis and discussion

This doctoral project has focussed on the interactions among nutrient availability and consumer pressure on epiphyte load of *Posidonia oceanica* leaves. The aim of this doctoral project was to assess the strength of bottom-up and top-down control in the regulation of epiphytic biomass in *Posidonia oceanica* leaves. Our specific objectives were:

1) To evaluate the spatial variability of epiphyte load and nutrient availability in *P. oceanica* meadows.
2) To assess the importance of grazing as a buffer of the effects of nutrient enrichment on epiphyte load.
3) To evaluate the effects of the fish communities in the control of epiphyte biomass.
4) To assess the feeding rates of frequent species of mesograzers in *P. oceanica* meadows.
5) To elucidate if the mesograzer community of *P. oceanica* meadows is affected by a nutrient-driven increase of epiphyte biomass.

In this chapter we focus on the general discussion of the partial findings corresponding to the objectives to gather a more general understanding and obtain new insights on the results of the research.

1. Spatial variability and relationship between variables in natural conditions

Assessment of the status of seagrass meadows and their epiphytic community is usually performed by extrapolating punctual data from samples obtained along a depth or disturbance gradient (Mazzella et al. 1989; Perez et al. 2008) or from samples collected at random sites and subsites (Alcoverro et al. 1995; Giovannetti et al. 2010). However the multi-scale spatial variability in ecosystem traits may lead to equivocal interpretations when punctual data are used to make general conclusions about the processes acting in a system (Wiens 1989; Levin 1992). In this thesis I evaluated the spatial distribution of epiphyte biomass, shoot size and nutrient availability at spatial scales from centimetres to hundreds of metres. The simultaneous evaluation of those variables allows us to evaluate whether their patterns of spatial variability were associated. We calculated correlations among epiphyte biomass, nutrient content in the leaves and in the epiphytes and shoot size to evaluate the model of bottom-up control of epiphyte and leaf biomass in natural conditions (e.g. Borum 1987; Tomasko and Lapointe 1991; Hauxwell et al. 2001). Nutrient content in biological tissues (epiphytes and leaves) is considered in this thesis as indicator of nutrient availability as suggested by the literature. The nutrient content of epiphytes has been suggested to be an indicator of nutrient availability in the water column (Lin et al. 1996; Perez et al 2008). The nutrient content of seagrass leaves as been traditionally measured as indicator of nutrient availability although it is in fact the result of the balance between nutrient availability and nutrient requirements for seagrass growth (Duarte 1990; Fourqurean et al. 1992; McClelland and Valiela 1998; Lepoint et al. 2008)

High spatial heterogeneity of epiphyte biomass, nutrient content in the epiphytes, nutrient content in the *Posidonia oceanica* leaves and shoot size was present between centimetres and hundred of metres (chapter 2). Epiphyte biomass was relatively homogeneous among shoots separated tens of metres in keeping with the results found by Moore and Fairweather (2006) in Australian multi-specific meadows. There is previous evidence of the heterogeneity of the composition of the *P. oceanica* epiphytic community at a wide range of spatial scales but of a relative homogeneity at intermediate spatial scales

(<100 metres) (Pardi et al. 2006; Balata et al. 2007). The size of *P. oceanica* shoots was extremely variable at spatial scales ranging from metres to hundreds of metres in keeping with the results of Balestri et al. (2003) and Gobert et al. (2003) during the summer season. Our study showed that the size of *P. oceanica* shoots and the nitrogen and phosphorus content of the leaves and epiphytes are highly variable spatially in November-December when shoot size is at its annual minimum. Most of the variability in epiphyte biomass, shoot size and nutrient content in the leaves and in the epiphytes happened among shoots separated some centimetres. This result advises to increase the number of shoots collected within 0.25 square metres to properly sample the variability in the distribution of those variables.

Epiphyte biomass did not correlate with nutrient content in the leaves or with nutrient content in the epiphytes in natural conditions. It could be expected that both variables correlated for two reasons. First, epiphyte biomass has been shown to respond to nutrient availability in the water column (Leoni et al. 2006; Prado et al. 2008a, 2010a; chapter 3 and chapter 4) and second, the nutrient content in the biological tissues (leaves and epiphytes) should be a reliable indicator of environmental nutrient availability (Perez et al. 2008). The lack of correlation of epiphyte nutrient content and epiphyte biomass in natural conditions (chapter 2) could be explained by the effect of other factors such as grazing pressure or hydrodynamics that may drive changes of epiphyte biomass buffering the effects of nutrient availability on epiphyte load. In this sense recent evidence indicates that epiphyte biomass is not an unbiased indicator of nutrient availability in carbonate low-nutrient environments (Terrados and Pons 2008; Fourqurean et al. 2010, Prado et al. 2010b).

We have performed two independent experiments of nutrient addition in the water column, in 2007 and 2008. Both have strongly promoted epiphyte biomass increase. However epiphyte nutrient content did not respond to the experimental nutrient additions carried out throughout this thesis in 2007 (chapter 3) nor in 2008 (chapter 4). The experimental nutrient addition in 2007 promoted the increase of nutrient content in the leaves, but not in the nutrient addition performed in 2008 (chapter 4). Leaf nutrient content should be used as indicator of nutrient availability together with the plant requirements and nutrient retranslocation assessment. The lack of response of the nutrient content in the leaves and in the epiphytes to the experimental nutrient additions (chapter 3 and chapter 4) dissuade from the use of epiphyte nutrient content as indicator of water column nutrient availability. Conversely, recent studies point out to epiphyte community composition as indicator of nutrient availability in the water column (Martinez-Crego et al. 2010; Giovanetti et al. 2010).

We evaluated the invertebrate community abundance and composition in different localities separated some kilometres (chapter 4). We found that differences of community composition of invertebrates were associated with differences of epiphyte biomass (chapter 4). This fact suggests a link between epiphyte biomass and invertebrate community; trophic dependence or shelter provision might be behind this response.

We found a consistent negative correlation between epiphyte biomass and shoot size in natural conditions (chapter 2). The experimental addition of nutrients (chapter 3 and chapter 4) increased epiphyte biomass and promoted smaller shoot sizes. The effects of epiphyte biomass shading on seagrasses reported in the literature includes shoot density reduction (e.g. Hauxwell et al. 2003) and above-ground net productivity reduction with a consequent reduction of shoot biomass (Hauxwell et al. 2001) among other negative effects on seagrass vitality. Our results also point out to shading by epiphyte biomass as most likely mechanism driving the reduction of shoot biomass (Ruiz and Romero 2003).

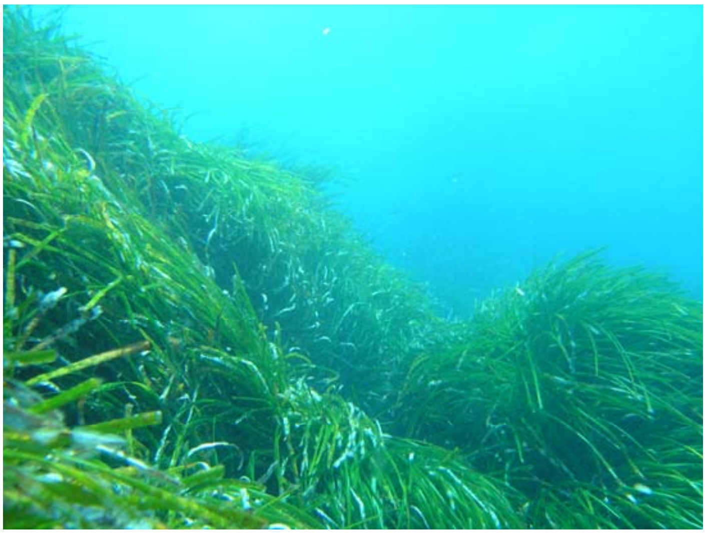


Figure 1. *Posidonia oceanica* meadow.
(Photo by Eduardo Infantes)

2. Bottom-up control in epiphyte biomass of *Posidonia oceanica* meadows

The experimental nutrient additions performed have consistently promoted an increase of epiphyte biomass. A five month manipulation of nutrient addition in the water column led to an 80% increase in epiphyte load in 2007 after two months of treatment (chapter 3). In 2008, epiphyte biomass increased about 50% after one month of increased-nutrient load in the water column (chapter 4). In chapter 5 the biomass of an early succesional epiphytic community increased by a thirty percent after three weeks of increased-nutrient treatment. The manipulation of nutrient availability in Mediterranean meadows during summer has previously led to an increase of epiphyte biomass on the leaves (Prado et al 2008a). The negative effect of epiphyte biomass increase on shoot size has also been a consistent finding. We found smaller shoots where nutrients were experimentally increased (chapter 3 and chapter 4) and we also found a negative correlation between shoot size and epiphyte biomass in natural conditions (chapter 2 and chapter 4). We found a gradient of decreasing epiphyte biomass along the coast line of the Bay of Palma (chapter 4) with higher epiphyte biomass in the inner part of the Bay. On the basis of the response of epiphyte biomass to nutrient availability in the water column (chapter 3, chapter 4 and chapter 5) this result suggests a gradient of nutrient availability in Palma Bay with higher values in the inner part of the Bay which is also the nearest area to human populations and wastewater outfalls.

Shoot size did not increase with nutrient addition in the water column. Indeed nutrient addition negatively affected *Posidonia oceanica* growth and led to a reduction of the size of the shoots (chapter 3 and chapter 4). Decline of seagrass productivity, density and vitality in response to intensive nutrient enrichment has been previously reported for *P. oceanica* and *Zostera marina* (Burkholder et al. 1992, 1994, 2007; Hauxwell et al. 2001, 2003; Leoni et al. 2006). The decline of seagrasses in response to increased nutrient availability may be related to physiological processes. Burkholder et al. (1992, 1994, 2007) suggest that seagrass species adapted to very oligotrophic waters do not have an inhibition mechanism to stop nitrate assimilation. Nitrate assimilation and reduction is energetically costly and continuous uptake of nitrate can promote substantial declines in plant growth (e.g. shoot density reduction, Burkholder et al. 1992, 1994; Touchette et al. 2003). The negative effects of nutrient addition on seagrass vitality are enhanced by increasing temperature, exposure time to enrichment and algal growth with the associated reduction of light (Leoni et al. 2008 and references herein). Our experimental nutrient additions were performed during the warmest season of the year and the addition of nutrients in the water column consistently promoted the increase of epiphytic algae on the *P. oceanica* leaves. In 2007 a reduction in shoot size was evident after two months of continuous nutrient enrichment at the same time that epiphyte biomass responded to nutrient addition (chapter 3). These facts would stimulate the negative response of *P. oceanica* to intensive nutrient additions.

Positive effects of nutrient addition on seagrass production have been also reported. Several studies (e.g. Alcoverro et al. 1997b; Terrados et al. 1999) have suggested that the increase in growth rate resulting from nutrient enrichment indicates that seagrasses are nutrient-limited and stimulated by enrichment. In summer *P. oceanica* shoot growth rates, shoot size and nutrient limitation are maximum (Alcoverro et al. 1997b) and nutrient addition in the water column could be expected to promote the increase of shoot size. However the magnitude of nutrient limitation of *P. oceanica* and thus also the response of the plant to nutrient addition can differ greatly among localities (Alcoverro et al. 1997b). In this sense Palma Bay has a dense human population and likely does not have one of the most oligotrophic waters around the island. The bay has 20 wastewater outfalls listed but not all are operational. In addition to the outfalls there are point source water discharges through torrential floods in heavy rain events that contribute to nitrate concentrations in the water column above 100 µm (Basterretxea 2011 et al.).

Experimental increase of nutrient availability in the water column promoted epiphyte biomass and cascaded-up to the increase in abundance of the mesograzer populations. However grazing pressure did not reverse the epiphyte biomass to a non-increased-nutrient situation despite the increase of the mesograzers abundance in increased nutrients treatments (chapter 4). The literature indicates that in oligotrophic meadows bottom up control seems to be more relevant in the control of algal growth that grazer control (Burkepile et al. 2006; Keuskamp 2004; Peterson et al. 2007). Epiphyte biomass should be considered resource controlled in *P. oceanica* meadows during summer and an important driver of the reduction of shoot size which probably suggests the decline of *P. oceanica* population in Palma Bay.

3. Top-down control of epiphyte biomass in *Posidonia oceanica* meadows

3.1 The role of the grazer community

Eleven common species of gastropods in *Posidonia oceanica* meadows have been found to actively graze on early succesional community of epiphytes in lab conditions (chapter 5). Some of them showed enhanced consumption rates in response to higher epiphyte biomass. We found *Jujubinus striatus* to be an efficient grazer both in natural and increased-nutrient conditions. We found that *Calliostoma zizyphinum, Gibbula ardens, Tricolia pullus and Tricolia speciosa* have the capacity for increasing their consumption rates in aquaria in response to higher epiphytic biomass developed in nutrient-enriched conditions (chapter 5). In a microcosm experiment maximum field densities of *J. striatus* and *Bittium reticulatum* were able to reduce by half the biomass of a natural community of epiphytes on *P. oceanica* leaves (Gacia et al. 2009). However our results suggest that grazing does not offset epiphyte response to increased nutrients in field conditions and that epiphyte load appears to be mostly controlled by bottom-up forces (chapter 3, chapter 4). In oligotrophic meadows grazer pressure appears to fail in the control of nutrient-driven increases of epiphyte biomass (Keuskamp 2004; Peterson et al. 2007; chapter 3 and chapter 4). In chapter 4 we sampled invertebrate community in two pairs of localities in Palma Bay with high versus low epiphyte load levels. The composition of the invertebrate community was similar among localities with comparable epiphytic biomass on the leaves. Furthermore, the invertebrate community in low epiphyte load localities becomes similar to that of high epiphyte load localities when nutrients were added, while the differences were kept among unfertilized plots in the same localities (chapter 4). The abundance of most invertebrate groups tended to increase when nutrients were added; suggesting that higher accumulation of epiphyte biomass in fertilized treatments stimulated the development of invertebrate populations, even for the non epiphyte-consumer groups (chapter 4). Since we did not include a grazer exclusion treatment in the field, the direct influence of grazers on epiphyte biomass was not evaluated in this thesis. Our results suggest that the abundance and composition of invertebrate community in *Posidonia oceanica* meadows is bottom-up controlled, driven by epiphyte biomass and, ultimately, nutrient additions.

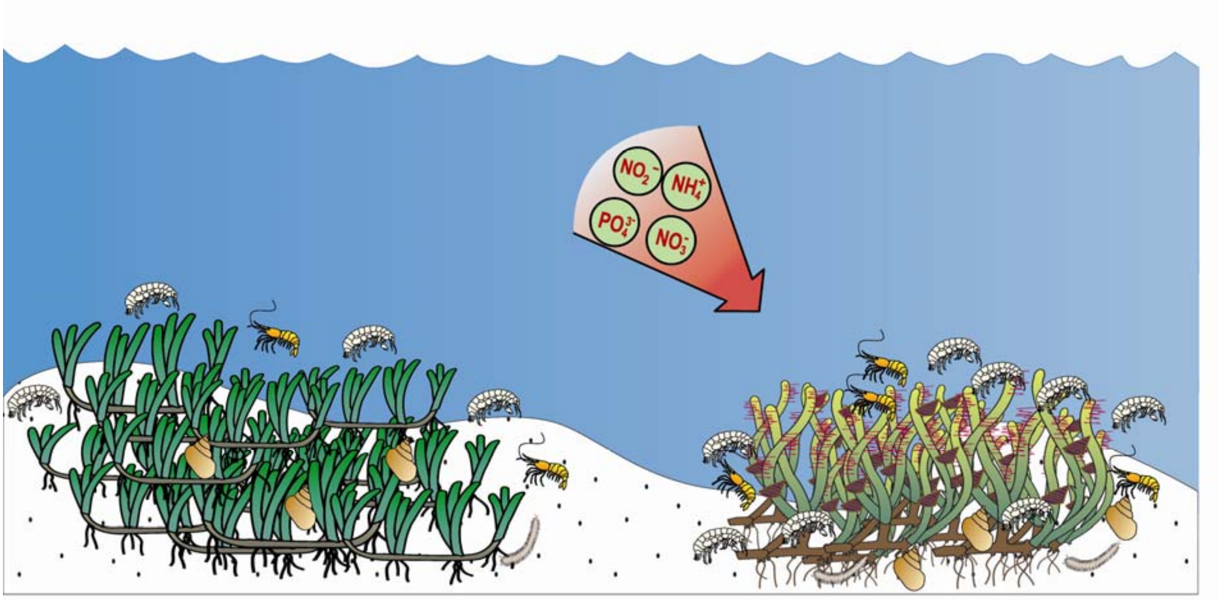


Figure 2. Conceptual framework of the epiphyte biomass and invertebrate populations abundance response to an scenario of increased nutrient availability in *Posidonia oceanica* meadows.

Local conditions and spatial variability are important in determining epifaunal communities and top-down control might be occurring in one of the four localities studied in chapter 4 (Cala Estancia), where the abundance of invertebrates was highest and nutrient addition promoted the lowest rise of epiphyte load. On the other hand, Estancia showed higher epiphyte load in the controls than the rest of localities, which could also suggest that the environmental nutrient supply already fulfils epiphyte requirements.

3.2 The role of fish community

A recent review highlights the importance of top predators modulating the abundance of autotrophs through a trophic cascade mechanism in marine shallow ecosystems. Kelp forest collapse occurs after the reduction of the abundance of sea otters that cascades with the increase of sea urchins populations and higher herbivore pressure. In coral reefs ecosystems, fishing activity removes large fish and alters the patterns of predation and herbivory, leading to shifted benthic dynamics, in an unfavourable competitive situation of reef-building corals and coralline algae. Similar examples are shown for rocky intertidal and algal bottoms ecosystems (Estes et al. 2011). It could be expected that the exclusion of the fish community would produce changes on epiphyte load through indirect effects in *Posidonia oceanica* trophic web. However the exclusion of the fish community that we carried out in 2007 (chapter 3) did not modify the epiphyte biomass or the response of epiphyte biomass to nutrient addition.

The results of the factorial manipulation of fish presence and nutrient availability showed that fish communities do not modify the response of the epiphyte biomass to nutrient availability by consuming directly epiphytes nor indirectly predating on the grazer population (chapter 3). Our results suggest that predation pressure by fish on invertebrate communities does not change epiphyte consumption by invertebrate grazers or at least not enough to modify the nutrient-driven epiphyte biomass increase (chapter 3). The composition of the benthic community of grazers results from fish community structure and abundance (Sieben et al. 2011) and will determine the grazing pressure exerted on epiphytes.

Leoni et al. (2006) found that the exclusion of the fish community slightly reduced epiphyte biomass on the leaves and it was suggested to be caused by light reduction caused by the fish exclusion netting (Dalla-Via et al. 1998). Epiphyte biomass was not light limited inside our fish exclusion cages as shown by the similar epiphyte biomass found on the shoots inside the fish exclusion-cages and in the controls. The traditional fishing activity in the Mediterranean Sea has reduced the trophic grad of fish capture between 1950 and 1994 (Pauly et al. 1998). Nowadays the scenario is a *P. oceanica* fish community dominated in number and biomass by labrids, sparids, serranids, gobids and scorpaenids and omnivory is the most frequent feeding behaviour (Deudero et al. 2008; Stergiou and Karpouzi 2001). The strength of trophic cascades in our system is probably buffered by the complex food web, with four trophic levels into the fish community, and the high abundance of omnivores (Coll et al. 2006).

Leoni et al. (2006) found that the exclusion of fishes in a *P. oceanica* meadow led to maximum leaf length by excluding herbivorous fishes (Leoni et al. 2006). In our five-month experimental fish exclusion the number of fish bites on the leaves was reduced until they disappear inside the exclusion-cages which prove the effectiveness of the treatment. The grazing role of herbivore fishes has been low throughout the thesis. The number of shoots with fish bites marks was very scarce (chapter 3 and chapter 4) which indicate low herbivore fish pressure in our study and explains the negligible role of direct fish herbivory in controlling epiphyte biomass (chapter 3). Previous studies have also found low influence of fish consumption on epiphyte biomass in *P. oceanica* shoots (Alcoverro et al. 1997a; Leoni et al. 2006; Tomas et al. 2005a). The consumption of epiphytes by fish did not increase with increased nutrients despite the higher nutrient content of the leaves and the higher epiphytic biomass found in nutrient enriched plots (chapter 3). This result contrasts with the literature (e.g. Prado et al. 2010b) and may be consequence of the very low herbivore fish pressure that is frequent in the broad extensions of continued *P. oceanica* cover (Prado et al. 2008b). Both direct and indirect effects of fish community were negligible in this work which suggests a scenario of depressed fish populations in Mediterranean meadows.

The work performed in this thesis could be completed in the future by using stable-isotope analysis to characterize the trophic linkages existing in the community of grazers inhabiting *Posidonia oceanica* meadows (but see Gacia et al. 2009). The assessment of species-specific feeding characterization of the main grazers in *P. oceanica* meadows and density-dependent effects on the consumption rates over epiphytes abundance should be also considered. I would propose also the evaluation of top-down control in areas with different natural abundances of fish using fish exclusion to test the role of fish community in both scenarios.

CHAPTER 7: Conclusions

- Resource availability is the main driver of change of epiphyte biomass in *Posidonia oceanica* shoots.

- Nutrient availability in the water column drives strong increases of epiphyte biomass and has negative effects on *P. oceanica* shoot size.

- Fish community has not an important role modifying the epiphyte biomass increase, driven by nutrient availability, neither by direct consumption nor by cascade changes of grazing pressure.

- The increase of epiphyte biomass cascades up to a global increase of the populations of invertebrates, even those that are not directly trophic related with epiphytes.

- The increase of invertebrate populations does not reverse epiphyte biomass to a non-nutrient-enriched situation.

- The increased epiphyte biomass results in the increase of consumption rates of some of the most frequent species of gastropod in *P. oceanica* meadows.

- Variability of shoot size, epiphyte biomass and nutrient content occurs at shoot scale and concentrate sampling efforts for these variables within 0,25 squared meters in Palma Bay is recommended.

- This thesis suggests a mainly bottom-up control scenario.

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