Universitat de les Illes Balears

DIVERSITY AND FUNCTIONS OF MICROBIAL COMMUNITIES IN SEAGRASSES

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Departamento de Biología de la Universitat de les Illes Balears

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Key words: seagrass, *Posidonia oceanica*, microbial community, pathogen, *Labyrinthula* sp., bacterial community, endophytes, diazotrophism, nitrogen-fixation, sulfate-reducers, sulfide pools, meristematic activity.

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Abstract

Because of the importance of microbial communities in the maintenance of the seagrass meadows, we investigated the diversity of microbes in Mediterranean seagrasses. We investigated the presence, distribution and pathogenicity of *Labyrinthula* sp. in *Posidonia oceanica* meadows in Balearic Islands in order to assess if this pathogen could threat seagrass meadows. The results confirmed that *Labyrinthula* sp. could be detrimental agent involved in *P. oceanica* decline. Moreover, we have confirmed the possibility of cross infection of *P. oceanica* by *Labyrinthula* strains isolated from other seagrass species located in other geographic regions. On the contrary, the absence of viral particles in the *P. oceanica* tissues examined suggests that, at least, these meadows are not threatened by diseases caused by viruses.

Similarly, the bacterial endophytic community has been characterized in leaves, rhizomes and roots of *P. oceanica*, identifying some bacteria that presumably can play key roles in the fitness of this seagrass species. In fact, we demonstrated the presence of endophytic nitrogen-fixing bacteria in *P. oceanica* roots, by detection of a functional gene, *nifH*, involved in nitrogen fixation process. The endophytic diazotrophic community found in *P. oceanica* roots could be seagrass-specific and exhibited low diversity. Only two species of diazotrophic bacteria were detected in *P. oceanica* roots.

Additionally, we compiled all published information regarding identification of bacteria in seagrass tissues and rhizospheres to provide an overview of the seagrass bacterial community composition, and to identify the main bacterial taxonomic groups present in seagrass meadows worldwide. This compilation provided a global perspective of the research conducted to present in this topic and highlighted that bacteria diversity

remains unexplored in meadows of many seagrass species and along vast coastal regions.

The most frequent bacterial family identified in seagrass rhizosphere was *Desulfobacteraceae*, composed by sulfate reducer members that reduce sulfate into sulfides in the oxidation of organic compounds. The sulfides produced are often accumulated in Mediterranean sediments, due to the absence of available iron to precipitate it as iron sulfides. We developed a new methodology based on the quantification of the meristematic activity and we confirmed the detrimental effect of sediment sulfide pools in the meristems of *P. oceanica* growing in sediments with high sulfide concentrations, drastically declining the percentage of diving cells.

Resumen

Debido a la importancia de las comunidades microbianas en el mantenimiento de las praderas de angiospermas marinas, en este estudio se ha investigado la diversidad microbiana asociada a plantas marinas mediterráneas. Se ha investigado la presencia, distribución y patogenicidad del protista *Labyrinthiula* sp. en las praderas de *Posidonia oceanica* en las Islas Baleares con la finalidad de determinar qué papel juega este patógeno en el declive que padecen estas angiospermas marinas. Los resultados confirman que *Labyrinthula* sp. podría ser un factor perjudicial relacionado con el deterioro de las praderas de *P. oceanica*. Además, se ha confirmado la capacidad de infección cruzada de hojas de *P. oceanica* por cepas de *Labyrinthula* sp. aisladas a partir de tejidos de otras angiospermas marinas creciendo en otras regiones separadas. Sin embargo, la ausencia de partículas víricas en tejidos de *P. oceanica* examinados sugiere que al menos las praderas investigadas no están amenazadas por enfermedades víricas.

Del mismo modo, en este estudio se ha caracterizado la comunidad bacteriana endófita en hojas, rizomas y raíces de *P. oceanica*, identificando algunas bacterias que podrían tener un papel importante en el mantenimiento de las praderas. De hecho, se demuestra la presencia de bacterias endófitas fijadoras de Nitrógeno en raíces de *P. oceanica*, mediante la detección del gen funcional *nifH* implicado en el proceso de fijación de Nitrógeno. La comunidad diazotrófica descrita en raíces de *P. oceanica* parece ser específica y encontrarse en baja diversidad ya que sólo se han detectado 2 especies de diazotrofos en raíces de *P. oceanica*.

Adicionalmente, se recopiló toda la literatura publicada referente a la identificación taxonómica de bacterias relacionadas con tejidos de plantas marinas y su rizosfera, obteniendo una visión general de la composición de la comunidad bacteriana e identificando los principales grupos taxonómicos relacionados con las angiospermas marinas de todo el mundo. Con esta recopilación pudimos obtener una perspectiva global de la investigación realizada hasta la fecha en este campo y detectar algunos puntos donde la información resulta más escasa, como muchos géneros de angiospermas marinas inexploradas en vastas regiones costeras. *Desulfobacteraceae* fue la familia más frecuentemente identificada en las rizosferas de las angiospermas marinas. Esta familia se compone de miembros con capacidad sulfato reductora que reducen sulfatos a ácido sulfhídrico en la oxidación de compuestos orgánicos. El ácido sulfhídrico generado por estas bacterias se acumula a menudo en los sedimentos mediterráneos, debido a la ausencia de hierro disponible para precipitarlo en forma de pirita. En este estudio, se desarrolló una metodología nueva basada en el estudio de la actividad meristemática, con el que evidenciamos el efecto negativo de los acúmulos de ácido sulfhídrico en la actividad meristemática de *P. oceanica* creciendo en sedimentos con elevadas concentraciones de sulfhídrico, con una reducción drástica del porcentaje de células en división.

Resum

A causa de la importància de les comunitats microbianes en el manteniment de les praderies d'angiospermes marines, en aquest estudi s'ha investigat la diversitat microbiana associada a plantes marines mediterrànies. S'ha investigat la presència, distribució i patogenicitat del protist *Labyrinthiula* sp. en les praderies de *Posidonia oceanica* a les Illes Balears amb la finalitat de determinar quin paper juga aquest patogen en el deteriorament que pateixen aquestes angiospermes marines. Els resultats confirmen que *Labyrinthula* sp. podria ser un factor perjudicial relacionat amb el deterioramnet de les praderies de *P. oceanica*. A més, s'ha confirmat la capacitat d'infecció creuada de fulles de *P. oceanica* per per soques de *Labyrinthula* sp. aillades a partir de teixits d'altres angiospermes marines que creixen a altres regiones separades. Però, l'absència de partículas víriques als teixits de *P. oceanica* examinats suggereix que almanco les praderies examinades no estan amenaçades per malaties víriques.

De la mateixa manera, en aquest estudi s'ha caracteritzat la comunitat bacteriana endòfita a fulles, rizomes i arrels de *P. oceanica*, i s'han identificat alguns bacteris que podrien tenir un paper important en el manteniment de les praderies. De fet, es demostra la presència de bacteris endòfits fixadors de nitrogen a arrels de *P. oceanica*, mitjançant la detecció del gen funcional *nifH*, implicat en el procés de fixació de nitrogen. La comunitat diazotròfica descrita a arrels de *P. oceanica* pareix ser específica i trobar-se en baixa diversitat ja que només s'han detectat dues espècies de diazotrofs a les arrels de *P. oceanica*.

Addicionalment, es va recopilar tota la literatura publicada referent a la identificació taxonòmica de bacteris relacionats amb teixits de plantes marines i la seva rizosfera, i es va obtenir una visió general de la composició de la comunitat bacteriana al mateix temps que s'identificanven els principals grups taxonòmics realcionats amb les angiospermes marines de tot el món. Amb aquesta recopilació vàrem poder obtenir una perspectiva global de la investigació ftea fins avui en aquest camp, amb molts de gèneres d'angiospermes marines inexplorats en vastes regions costaneres. *Desulfobacteraceae* va ser la família més freqüentment identificada a les rizosferes de les angiospermes marines. Aquesta família es compon de membres amb capacitat sulfat-reductora que redueixen els sulfats a àcid sulfhídric durant l'oxidació de compostos orgànics. L'àcid sulfhídric generat per aquests bacteris s'acumula sovint als sediments mediterranis, a causa de l'absència de ferro disponible per precipitar-lo en froma de pirita. En aquest estudi, es desenvolupà una nova metodologia basada en l'estudi de l'activitat meristemàtica, metodologia amb el qual vàrem evidenciar l'efecte negatiu dels acumulaments d'àcid sulfhídric en l'activitat meristemàtica de *P. oceanica* creixent sobre sediments amb elevades concentracions de sulfhídric, amb una dràstica reducción del percentatge de cèl·lules en divisió.

General Introduction

Seagrasses are clonal angiosperms that colonized the marine environment in the Cretaceous (e.g. Den Hartog and Kuo 2006). Despite they developed in an early stage of the evolution of the angiosperms, seagrass flora only encompass 6 families, 12 genera and about 60 species (Den Hartog and Kuo 2006). The low diversity of seagrass flora is to some extend attributed to the environmental constrains of marine environment for reproduction (e.g. hydrophilic pollination, Duarte et al. 1994a). Sexual reproduction, by flowering events, is scant in seagrasses, but frequencies vary among species (Gallegos et al. 1992). Similarly, life in the sea constrains seagrass form, as reflects the rather similar plant architecture across seagrass flora despite the large differences in species size (Duarte 1991). Seagrass growth pattern relies on the repetition of the structural unit, named ramet, which contains a fragment of rhizome, roots and a shoot with leaves. Seagrasses expand into new areas vegetatively by adding ramets produced by the meristems located at the rhizome apices (Tomlinson 1974). Meristematic activity, therefore, regulates vegetative growth, which is the main mechanism of seagrasses to occupy the space, and thus the dynamics and productivity of seagrass metapopulations. The rates and patterns of clonal growth vary among seagrass species, ranging from few centimeters to few meters per year (Sintes et al. 2005) and this variability scales to plant size (Marbà and Duarte 1998). The slow clonal growth of large species is compensated by long life spans of ramets, clones and meadows. For instance, the ramets of *P. oceanica*, one of the largest and slowest growing seagrasses, which is endemic of the Mediterranean Sea, live up to 50 years old and its clones, which can spread for several kilometers (Díaz-Almela et al. 2007), can be several millennia old (Arnaud-Haond et al. in review). Similarly, there is evidence that meadows of *P. oceanica*

live for millenia (Mateo et al. 1997). The long life span of some seagrasses suggests that they might be important reservoirs of microbiota.

Despite the low diversity of flora, seagrasses successfully colonize the coastal regions of all continents except Antarctica from 0 m to 40 m water depth (Hemminga and Duarte 2001), often in nutrient-poor environments (Perez et al. 1990). Global seagrass area is estimated at about 300 000 to 600 000 km^2 (Charpy-Roubaud and Sournia 1990, Duarte et al. 2005). In these areas, seagrasses extend their leaf surfaces in dense canopies from few centimeters to more than 1 m height, depending on the species (Duarte 1991), into the water column. Similarly, seagrass belowground modules (i.e. rhizomes and roots) can form dense rhizospheres (e.g. >100g DW of roots m^{-2}, >200g DW of rhizomes m^{-2}; Duarte et al. 1998) up to more than 1 m thick (Duarte et al. 1998). The above and belowground structures of seagrasses provide habitat to marine species of different phyla, which live in the canopy and rhizosphere, on seagrass surfaces as epiphytes or inside seagrass tissues. Seagrasses, therefore, enhance coastal biodiversity (Hemminga and Duarte 2001). Whereas the role of seagrass meadows enhancing coastal fauna and flora diversity is well documented (e.g. Hemminga and Duarte 2001), the diversity of microbiota, particularly that of endophytic and epiphytic organisms, in seagrass meadows is largely unexplored.

Seagrass meadows play other important ecological functions both at coastal and global scale despite they only occupy less than 1 % of the global ocean surface (Duarte et al. 2005). Seagrasses rank among the most productive marine ecosystems and among the largest carbon sinks on Earth, since it is estimated that they bury 15 % of the total organic carbon buried in the global ocean (Duarte and Chiscano 1999). Seagrasses, therefore, play an important role in climate regulation (Nellemann et al. 2009). Seagrass meadows also

prevent coastline erosion, by trapping sediment particles, preventing sediment re-suspension, protecting beaches from storms and contributing as a source of beach carbonate materials. The valuable functions of seagrass meadows are compromised by worldwide coastal deterioration. The global overpopulation, mainly in coastal zones, during the last decades has led to high coastal pollution, enhanced coastal siltation due to land deforestation, modification of the coastal line, increased organic matter and nutrient inputs, largely because the use of fertilizers by agriculture and wastewater discharges, to the coastal seawater, mechanical coastal destruction, increased maritime traffic and global climate change (Waycott et al. 2009). All these disturbing changes are causing a worldwide decline of seagrass ecosystems at a global rate of 7 % yr^{-1} (Waycott et al. 2009), revealing that seagrasses rank among the most threatened ecosystems on Earth (Duarte et al. 2008).

During the last four decades, the scientific community has dedicated big efforts to elucidate the decline rates of seagrasses (Waycott et al. 2009). Firstly quantifying losses in meadow extent and shoot density, and later using shoot population demographic approaches, initially applying retrospective techniques (Duarte et al. 1994b) and more recently with direct census of seagrass vertical shoots in permanent plots annually monitored (Marbà et al. 2005). Because the rapid loss rate of seagrass meadows, it urges to develop methodologies able to detect meadow decline before losses are evident at areal and/or shoot density scale in order to be able to apply managerial measures towards reverting the disturbing conditions triggering stress and, therefore, plant death.

Seagrass decline has mostly been attributed to abiotic disturbances, while the role of the microbiota in the survival and growth of seagrass meadows has been less studied. However, there are some studies confirming the presence of bacteria and protists in the

seagrass tissues. There is evidence of pathogen protists that caused massive mortality events in seagrasses, reducing vast extensions of seagrass meadows. These die off events were called wasting disease and the causative agent was identified as the unicellular protist *Labyrinthula* sp. The first mass mortality event related to *Labyrinthula* infections was recorded in 1930s when *Zostera marina* meadows declined in the Atlantic Coast (Renn 1936, Young 1943, Muehlstein et al. 1991). Later in 1960s, *Labyrinthula* sp. also caused a mass mortality event in New Zealand, in this case, infecting *Z. capricornii* (Armiger 1964) and in 1980s Robblee et al. (1991) associated *Labyrinthula* sp. with a mass mortality observed in *Thalassia testudinum* in Florida Bay. After those seagrass mass mortality events, the interest in understanding the infection mechanisms, transmission, and distribution of *Labyrinthula* spp. increased. *Labyrinthula* spp. has been reported to be present in many seagrass species, although its presence not always has been linked to lesion appearance, pointing out the possibility of some strains being more virulent than others.

The process of *Labyrinthula*-induced lesion formation on seagrass leaves is presumed to begin with enzymatic degradation of the cell wall (Muehlstein 1992), allowing *Labyrinthula* sp. to enter the tissue, followed by condensation and destruction of chloroplasts and cytoplasmic contents as well as vesiculation of cell membranes. Ultimately, *Labyrinthula* sp. becomes widespread within the leaf and can even be found inside vascular tissues of the seagrass (Muehlstein 1992). The resulting necrotic tissue experiences a strong decrease in its photosynthetic activity, which extends to areas adjacent to damaged tissue with apparently healthy status, especially in the acropetal part of the leaves (Ralph and Short 2002), implying that lesions produced by *Labyrinthula* sp. compromise much more tissue than the visibly damaged area. In fact, *Labyrinthula* sp.

infections in *Thalassia testudinum* reduce net photosynthetic rate to below zero, even when only 25% of the leaf is damaged tissue (Durako and Kuss 1994). In the slow growing seagrass *Posidonia oceanica,* this could have severe implications. The determination of the presence, distribution and pathogenicity of *Labyrinthula* sp. in *P. oceanica* meadows would be desirable in order to gain any insight in the role this pathogen can play in Mediterranean seagrass meadows.

Viruses might also compromise seagrass functioning. There are many studies where the role of viruses in marine ecosystems has been evaluated, although they are mostly restricted to plancktonic communities (Culley et al. 2003). There are some evidences of the presence of viruses in the filamentous brown algae *Ectocarpus siliculosus* and *Feldmannia* (Van Etten and Meints 1999), in the green macroalga *Bryopsis cinicola* (Koga et al. 2003) and in the seagrass *Zostera marina* (Fukuhara et al. 2006). However, to our knowledge, the presence of viruses in other seagrasses has never been explored.

The study of bacterial communities related to marine angiosperms is scarce, and most of the studies have focused on describing the abundance, biomass or diversity of bacteria mainly in seagrass sediments (Cifuentes et al. 2000, Bagwell et al. 2002, García-Martínez et al. 2009). Less information is available in terms of bacteria closely related to seagrass tissues, in contrast with the wide knowledge of endophytes in terrestrial plants. There are some studies evidencing colonization of seagrass surfaces, pointing out the existence of a specific epiphytic bacterial community (Weidner et al. 2000, Jensen et al. 2007, Uku et al. 2007, Crump and Koch 2008). Moreover, there are evidences of endophytic bacteria present in seagrasses. Kuo et al. (1993) and Barnabas (1993) demonstrated the presence of bacteria inside seagrass tissues by microscopy techniques. Some endophytic bacteria have

been isolated from surface-sterilized seagrass tissues, demonstrating the existence of an endophytic bacterial community (Nielsen et al. 1999).

In most seagrass species, such is the case for *Posidonia oceanica*, the presence of endophytic bacteria remains largely unexplored. However, recent investigations suggest that *P. oceanica* may host endophytes, since 7 new bacteria species belonging to the genus *Marinomonas* have been isolated from *P. oceanica* tissues using culturing methods (Espinosa et al. 2010 and Lucas-Elío et al. in press). The characterization of the total endophytic bacterial community will provide new insight to help understanding the functioning of seagrass meadows. Characterization of endophytic bacterial community from surface-sterilized tissues is the first step for identification of endophytic bacterial species specific of each tissue and therefore for further studies to elucidate the role these bacteria may play in the physiology of the plant, allowing the discovery of symbiotic and/or pathogenic relations never described before.

The marine bacterial communities participate in many elemental cycles, as they are involved in many changes in compounds making them available for the rest of the marine organisms. Specifically, some steps can only be catalyzed by bacteria and other microorganisms, as is the case of atmospheric N_2 fixation (Dresler-Nurmi et al. 2009). In other cases the reduction state of some compounds is modified by bacteria, since they use them as electron donors or acceptors in some respiration processes where O_2 does not participate (Hines 2006). By changing the redox status of some compounds, bacteria are changing their availability, and subsequently shaping the environmental nutrient availability. This is particularly important in those ecosystems where a nutrient limitation

exists and therefore, the primary production can be constrained by the underlying bacterial activity.

The ubiquity of bacteria in marine environments has been well documented although the total diversity is unknown (Pedrós-Alió 2006). The development of molecular methods, and its application in microbiology, has lead to an increase of the knowledge in terms of bacterial diversity because only about the 1% of the bacteria present in an environmental sample can be detected by culturing methods (Amann et al. 1995). Despite the increase in non-cultured bacteria identification by 16s rDNA sequencing, their activity and their role is often difficult to assess. Bacteria inhabiting marine sediments have been studied with high detail, identifying bacteria participating in many elemental cycles. Sediment bacterial communities play an important ecological and biogeochemical roles in marine ecosystems, regulating and shaping the cycles of biogenic elements such as C, N, P, Fe, O and S (Nealson 1997). The new techniques allowing the study of functional genes provide the opportunity to investigate further in this direction.

Bacteria are also known to inhabit inside other marine organisms, in all phyla of Eukarya domain. Therefore, bacteria are present, for instance, in the intestinal conducts of many animals, in association with corals (e.g. Rohwer et al. 2002). The relationship between the host and bacteria present in tissues can be very divergent. We can distinguish a type of relation among organisms in which the host organism not only don't receive any advantage but also develops a disease. In this case, bacteria are acting as pathogens. In other cases, we can identify a beneficial relation for both bacteria and the host organism, called symbiosis. In this case, both organisms have a new condition more advantageous when they are together. In some cases, coevolution can occur between host organisms and

bacterial symbioses, as is the case of the marine red alga *Prionitis* and bacteria belonging to the genus *Roseobacter* (Ashen and Goff, 2000). The symbiosis can be obligated or not, depending on the capability of the host organisms for living free of symbionts. The long life span of *P. oceanica* clones might have allowed coevolution of symbiotic bacteria.

The low nitrogen (and phosphorous) availability of coastal waters where often seagrasses form lush and productive meadows could be solved or minimized by the possible presence of nitrogen fixers in symbiosis with seagrasses. Globally, the biological nitrogen fixation accounts for 90% of the global amount of nitrogen fixed that has been estimated as 250 million tons of N_2 per year. The 10% remaining is due to lighting processes (Dresler-Nurmi et al. 2009). Therefore, biological nitrogen fixation (BNF) is the main process by which inorganic nitrogen is converted to nitrates and nitrites and incorporated into the food web. The main enzyme involved in this process is nitrogenase, and the organisms capable of such fixation are known as diazotrophic bacteria. These organisms are spread along many taxonomic groups, suggesting horizontal genetic transfer in many cases, as species of specific groups may or may not have nitrogen-fixing capabilities. Diazotrophism is divided in three groups: a) non-symbiotic diazotrophs that are free-living organisms; b) associative symbiotic organisms that are in symbiosis with plants but they don't form nodules; and c) symbiotic diazotrophs that are responsible of nodule formation. The only two groups capable of inducing nodule formation are *Frankia* and rhizobia (before *Rhizobium*). It has been widely documented that bacteria present in vegetated sediments and rhizospheres of seagrasses are responsible for a big amount of the nitrogen uptake by the plants (Welsh 2000). High rates of nitrogen fixation in the rhizosphere of *Zostera capricorni*, *Thalassia testudinum*, and *Zostera marina* have been

quantified (O'Donohue et al. 1991, Patriquin 1972, Shieh et al. 1989), and incorporation of nitrogen fixed by diazotrophs by seagrass tissues, inferred from the analysis of the nitrogen isotopic composition of seagrass tissues in *Posidonia oceanica,* has been reported (Papadimitriou et al. 2005). However, there is, to date, no evidence of nodule formation in seagrasses (Nielsen et al. 1999), but the presence of other endophytic diazotrophs in seagrass tissues has not been investigated.

The existence of sulfate reducing bacteria in marine sediments is very common, as sulfate is very abundant in the seawater, being the third most concentrated ion in seawater, after Cl^- and Na^+ (Millero et al. 2008). Desulfobacteraceae, which members are sulfate reducers, is the family more frequently described in seagrass sediments (Chapter 5). The sulfate-reducing bacteria obtain their energy by oxidizing organic carbon compounds and reducing sulfate to sulfide, in anaerobic conditions. The sulfate reducing bacteria are well documented in marine environments and are responsible, with other organisms, of the degradation of organic matter in marine environments and most of all in coastal zone, where the deposition of organic matter in the sediment can reach big amounts, for example in river discharge, after phytoplankton blooms, in mangroves, or in seagrass meadows where decaying material is abundant. There are evidences of increased sediments sulfide concentration in seagrass rhizospheres after organic matter additions (Terrados et al. 1999, Perez et al. 2007, Ruiz-Halpern et al. 2008). The sulfate reducing bacteria are able to drastically change sediment conditions, affecting other organisms inhabiting the same sediment, as for example seagrasses (Holmer et al. 2003). The sulfide can be oxidized again in presence of oxygen or can be sequestered in pyrite form when conjugates with iron. In the Balearic sediments, which are biogenic carbonate rich, and because the lack of river

discharges, iron is deficient (Holmer et al. 2003), and, thus, the sulfide remains in the sediment. The low iron availability in the Balearic sediments, therefore, makes seagrass meadows particularly vulnerable to organic inputs (Marbà et al. 2008). The sulfide is toxic for seagrasses (Terrados et al. 1999), inhibiting the cytochrome oxidase activity (Raven and Scrimgeour 1997). There are evidences of sulfide intrusion in meristems of *P. oceanica* (Frederiksen et al. 2007) and the rate of seagrass decline increases with increasing concentration of sulfide in sediment pore water (Calleja et al. 2007). Seagrasses can minimize sulfide intrusion by generating an oxic layer around roots and rhizome by pumping directly oxygen from the water column and from photosynthesis. However, this mechanism does not always suffice to avoid the intrusion of sulfide in plant tissues, enhancing seagrass stress. This stress condition caused by sediment bacterial activity can be early detected using cytometer techniques to assess the shoot meristematic activity (Garcias-Bonet et al. 2008, Chapter 7).

The meristematic activity can be assessed by calculating the mitotic division rate of meristematic cells. A reduction in the percentage of dividing cells would reflect a decrease in shoot growth rates that might constrain plant survival. This method of determination of the percentage of nuclei in G2 phase of the cell cycle provides useful information for further studies focused on detection of thresholds of stress in marine angiosperms due to the activity of bacterial communities inhabiting sediments colonized by seagrasses. The development of new techniques based on cell cycle measurements allows the understanding of the mechanisms taking place in the interaction of bacterial communities in sediments and seagrasses inhabiting them.

General goal and objectives

The general goal of this thesis is to study the diversity of microbial communities associated to seagrass meadows and identify the effect of some microbes on seagrass growth and survival. This goal will be achieved through these specific objectives grouped in the following two sections:

1. **Section 1**. Characterization of endophytic microbiota in *Posidonia oceanica*.

   The objective of this section is to characterize the endophytic microbial community in *Posidonia oceanica*. This objective will be addressed by these 3 specific objectives:

   1.1. To explore the presence of unicellular protists and viruses in *Posidonia oceanica* meadows. This objective will be addressed in Chapters 1 and 2:

Chapter 1.

Neus Garcias-Bonet, Timothy D. Sherman, Carlos M. Duarte and Núria Marbà. 2011. Distribution and pathogenicity of the protist *Labyrinthula* sp. in the western Mediterranean seagrass meadows. *Estuaries and Coasts*, 34: 1161-1168.

Chapter 2.

Neus Garcias-Bonet, José Guerri, Pedro Moreno, Mariano Cambra, Núria Marbà, Carlos M. Duarte and Luis Navarro. Screening for viruses in seagrass (*Posidonia oceanica*) tissues. *Manuscript*.

1.2. To identify the endophytic bacterial community in *P. oceanica* meadows by molecular methods, and to assess the presence of nitrogen-fixing bacteria in seagrass tissues. This objective is addressed in Chapters 3 and 4:

Chapter 3.

Neus Garcias-Bonet, Jesus M. Arrieta, Carlos M. Duarte and Núria Marbà. Endophytic bacterial community of a mediterranean marine angiosperm (*Posidonia oceanica*). Submitted to *Applied and Environmental Microbiology*.

Chapter 4.

Neus Garcias-Bonet, Jesus M. Arrieta, Carlos M. Duarte and Núria Marbà. Endophytic nitrogen-fixing bacteria in surface-sterilized roots of *Posidonia oceanica* seagrass. *Manuscript*.

1.3. To provide an overview of the diversity of epiphytic, endophytic and rhizosphere bacterial communities of seagrass meadows worldwide. This objective is addressed in Chapter 5:

Chapter 5.

Neus Garcias-Bonet, Núria Marbà, Ester Marco-Noales and Carlos M. Duarte. Bacterial diversity in seagrass meadows *Manuscript*.

2. **Section 2.** Seagrass meristematic activity: technique development and its evaluation as an early warning indicator of seagrass sulfide stress.

This second section aims at detecting the effects of the activity of sediment sulfate reducing bacterial communities on seagrass health. This objective is addressed (1) by developing a new methodology based on cell cycle analysis of seagrass meristematic cells and flow cytometry techniques in order to quantify meristematic activity and test if it can be used as an early warning indicator of seagrass stress and (2) to use meristematic activity to detect seagrass sediment sulfide stress. This objective is addressed in chapters 6 and 7:

Chapter 6.

Neus Garcias-Bonet, Carlos M. Duarte and Núria Marbà. Meristematic activity of Mediterranean seagrass (*Posidonia oceanica*) shoots. Submitted to *Aquatic Botany*.

Chapter 7.

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Section I

Characterization of endophytic microbiota in seagrasses

Chapter 1

Distribution and pathogenicity of the protist *Labyrinthula* sp. in western mediterranean seagrass meadows

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Abstract

The presence of the pathogenic protist *Labyrinthula* sp., the causative agent of seagrass wasting disease and mass mortality events, was assessed in 18 seagrass meadows in the Balearic region (western Mediterranean). This protist was found in 70 % of seagrass meadows investigated, and in all seagrass species present in the region (i.e. *Posidonia oceanica*, *Cymodocea nodosa,* and *Zostera noltii*). *Labyrinthula* spp. cultures isolated from seven *P. oceanica* and one *Thalassia testudinum* meadows were used as inocula to perform cross-infection experiments in order to test seagrass vulnerability to *Labyrinthula* spp. infection. These isolates produced lesions on *P. oceanica* and other seagrass species (*Zostera marina*, *Z. noltii*, and *C. nodosa*). *Posidonia oceanica* and *Z. noltii*, both species autochthonous to the Mediterranean Sea, were the seagrasses most vulnerable to infection by the tested isolates. One of the *P. oceanica* isolates of *Labyrinthula* sp. also infected the Atlantic seagrass *Z. marina* and all of the Mediterranean seagrasses were infected by *Labyrinthula* sp. isolated from the *Thalassia testudinum*, native to the Caribbean and Gulf of Mexico. This work confirms that *Labyrinthula* sp. is commonly found on seagrasses of the Mediterranean Sea and demonstrates that *Labyrinthula* sp. can infect seagrasses in different genera, in contrast to previous studies where *Labyrinthula* sp. was considered to be genus-specific. This finding points out the broadly pathogenic nature of some *Labyrinthula* sp. isolates. Finally, this work identifies *Labyrinthula* sp. as a possible detrimental agent for *P. oceanica*.

Introduction

Seagrasses rank among the most productive ecosystems on earth (Duarte and Chiscano 1999) and play important functions in the marine environment, such as increasing biodiversity, protecting coastline, and sequestering carbon and other nutrients (Orth et al. 2006). *Posidonia oceanica* is the dominant seagrass in the Mediterranean Sea and, despite its very slow clonal growth (rhizome extension rates ranging from 1 to 6 cm y^{-1} $apex^{-1}$, Marbà and Duarte 1998), develops meadows that exist for millennia (Mateo et al. 1997). *Posidonia oceanica* meadows are experiencing a general decline across the Mediterranean Sea (Marbà et al. 2005), as reported for seagrass meadows elsewhere (Waycott et al. 2009). This decline has been associated with disturbances such as altered sediment dynamics, climate change, and nutrient and organic matter inputs to coastal waters (Duarte 2002). However, damage to *P. oceanica* shoots and, subsequent meadow decline, also could be caused by pathogenic diseases, a possibility that is relatively unexplored.

Historically, there has been evidence of mass mortality of seagrass due to pathogenic disorders. The widespread decline of *Zostera marina* meadows along the Atlantic coasts during the 1930s was associated with infection by the marine protist *Labyrinthula zosterae* which caused the so-called "wasting disease" (Renn 1936, Young 1943, Muehlstein et al. 1991). Similarly, *Labyrinthula* sp. was apparently associated with mass mortality of *Zostera capricorni* in New Zealand in the early 1960s (Armiger 1964) and *Thalassia testudinum* in Florida Bay during the late 1980s (Robblee et al. 1991). Interestingly, *Labyrinthula* spp. have been found ubiquitously in seagrasses (Muehlstein et al. 1988, Short et al. 1993, Vergeer and den Hartog 1994, den Hartog et al. 1996), yet outbreaks of disease are uncommon. Some authors have suggested that *Labyrinthula* spp. infections

only contribute to seagrass mass mortality when plant survival is already compromised by other detrimental factors, such as hypoxia or light limitation (e.g. Robblee et al. 1991, Vergeer et al. 1995). While this may be true, factors that affect the potential pathogenicity of *Labyrinthula* spp. (e.g. salinity and temperature) are also likely to play a role (e.g. Tutin 1938, McKone and Tanner 2009). Moreover, it has been suggested that there are inherent differences in pathogenicity among *Labyrinthula* sp. isolates (Muehlstein et al. 1988, Boettcher and Martin, personal communication).

Labyrinthula spp. produce lesions on seagrass leaves through enzymatic degradation of the cell wall, condensation and destruction of chloroplasts and cytoplasm, and vesiculation of cell membranes, thereby spreading inside the leaf and even reaching the vascular tissues (Muehlstein 1992). Infections by *Labyrinthula* spp. strongly decrease photosynthesis in the lesions and in adjacent, apparently healthy leaf areas (Ralph and Short 2002).

Labyrinthula spp. have been reported to be associated with most marine seagrasses, including: *Zostera marina, Z. mucronata, Z. noltii, Z. japonica, Heterozostera tasmanica, Posidonia oceanica, Halodule universis, H. wrightii, Cymodocea nodosa, Phyllospadix scouleri, Syringodium isoetifolium, Thalassodendron ciliatum, Ruppia cirrhosa, R. maritima, Thalassia testudinum*, and *Halophila ovalis* (Vergeer and den Hartog 1991, Vergeer and den Hartog 1994, Martin and Boettcher personal communication). In the Mediterranean Sea, *Labyrinthula* spp. have been isolated from *P. oceanica, C. nodosa* and *Z. marina* meadows along the southern and eastern Italian coasts (Vergeer and den Hartog 1994, den Hartog et al. 1996), but *Labyrinthula* sp. remains unreported for the western Mediterranean.

Although *Labyrinthula* spp. appear to be widespread, previous infection experiments with these protists suggested that the occurrence of infection is genus-specific with regard to *Labyrinthula* spp. isolates (Muehlstein et al. 1988, Vergeer and den Hartog 1991, Short et al. 1993, Vergeer and den Hartog 1994). However, more recent work showed that an isolate from *T. testudinum* cross-infected *Z. marina* (Caldwell 2006). Given the possibility of cross-infection among seagrass genera, determination of the vulnerability of Mediterranean seagrasses to *Labyrinthula* spp. is particularly important because of the increased potential hazard associated with globalization and global transport (Ruiz et al. 2000) of invasive species introduction and spread of pathogens in aquatic ecosystems.

Here we examine the presence of *Labyrinthula* spp. in seagrass meadows along the Balearic Island coasts (western Mediterranean) to evaluate its occurrence on seagrass species in this region. In addition, we use cross-infection experiments to assess the vulnerability of seagrasses from the Mediterranean (*Posidonia oceanica*, *Cymodocea nodosa*, *Zostera noltii*), Gulf of Mexico (*Thalassia testudinum*), and Atlantic (*Zostera marina*) to infection by *Labyrinthula* sp. isolated from *P. oceanica*.

Materials and Methods

Study sites

The Balearic Islands comprise four main islands (Mallorca, Menorca, Ibiza, and Formentera) and the Cabrera Archipelago, with small islands and inlets. *Posidonia oceanica* meadows are the dominant subtidal ecosystems along the Balearic Island coasts, covering ~ 1200 km^2. These seagrass stands are monospecific and occur at depths of 0−45

m. *Cymodocea nodosa* meadows are restricted to shallow bays and below the depth limit of *P. oceanica*, and *Zostera noltii* is relatively rare and occurs in shallow bays.

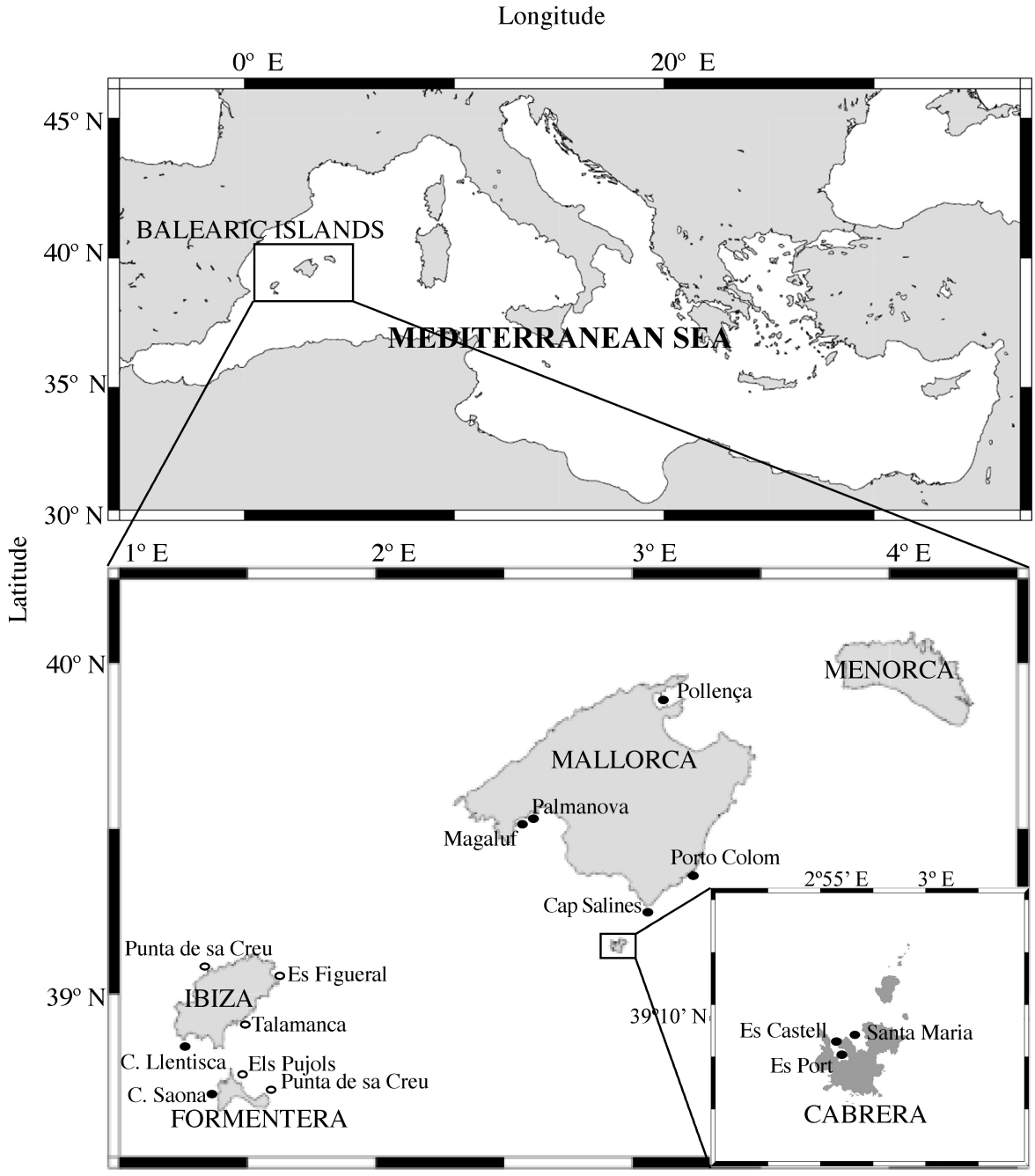

Figure 1. Location of 18 seagrass meadows of *Posidonia oceanica* (14), *Zostera noltii* (1), and *Cymodocea nodosa* (3) sampled for presence of *Labyrinthula* spp. on the Balearic Islands (Mallorca, Ibiza, Formentera, and Cabrera). Filled circles represent meadows where *Labyrinthula* sp. was isolated. Open circles represent absence of *Labyrinthula* sp. in seagrass tissues sampled.

In August-September 2006, when seawater temperature was 26.3 ± 0.02 ºC (Marbà, personal communication) and salinity was 37.4 ± 0.1 (Lasternas et al. 2010), we sampled 18 seagrass meadows distributed along the 950 km of the Balearic Islands coast (Fig. 1). These included five meadows of *P. oceanica* and two meadows of *C. nodosa* along the coast of Mallorca Island, seven meadows of *P. oceanica* along the coast of Formentera and Ibiza Islands, and two *P. oceanica* meadows, one of *C. nodosa* and one of *Z. noltii* in Cabrera Island (Table 1). The *P. oceanica* meadows were growing at 3-13 m, and those of *C. nodosa* and *Z. noltii* at 2-3 m water depth. The study sites were distributed from relatively pristine areas (e.g. Es Castell and Santa Maria in the National Park of Cabrera Archipelago; Cap Salines) with low coastal human pressure to areas with intense touristic development (e.g. Magalluf, Palmanova, Pollença, Porto Colom, Talamanca, and Es Figueral). The Balearic Islands receive around ten million tourists annually (data from the Spanish Institute of Statistics), which are mainly concentrated in coastal zones, reaching 6082 vessels around Mallorca Island on the busiest day in high summer (Balaguer et al. 2010).

At each sampling site, SCUBA divers collected three shoots of each seagrass present. The plant materials were placed in plastic bags underwater to avoid cross-contamination of the samples during transport to the laboratory.

Isolation of *Labyrinthula* sp.

Labyrinthula spp. were isolated from seagrass leaves as previously described (e.g. Porter 1990). Briefly, pieces of seagrass leaves were cut into 1−2 cm length fragments immediately after return to the laboratory. The fragments were dipped in 0.5 % sodium hypochlorite solution for 2 min, rinsed in sterile distilled water for 2 min, and then in

Table 1. List of 18 sampled seagrass meadows at 15 sites in the Balearic Islands. Source indicates seagrass species from which *Labyrinthula* spp. were isolated, indicating location of the meadow (Island and Location). (+) indicates presence and (-) indicates absence of *Labyrinthula* spp. on seagrass leaves. Water depth of sampled meadows is indicated.

Source (seagrass)	Island	Location	*Labyrinthula* sp. presence	Water depth (m)
Zostera noltii	Cabrera	Es Port	+	3
Cymodocea nodosa	Cabrera	Es Port	+	2
Cymodocea nodosa	Mallorca	Palmanova	+	2
Cymodocea nodosa	Mallorca	Pollença	+	2
Posidonia oceanica	Cabrera	Es Castell	+	12
Posidonia oceanica	Cabrera	Sta. Maria	+	13
Posidonia oceanica	Mallorca	Magaluf	+	6
Posidonia oceanica	Mallorca	Palmanova	+	5
Posidonia oceanica	Mallorca	Porto Colom	+	6.5
Posidonia oceanica	Mallorca	Pollença	+	4
Posidonia oceanica	Mallorca	Cap Salines	+	5
Posidonia oceanica	Ibiza	Cap Llentisca	+	6
Posidonia oceanica	Ibiza	Es Figueral	-	6.5
Posidonia oceanica	Ibiza	Talamanca	-	3
Posidonia oceanica	Ibiza	Pta Sa Creu	-	4.5
Posidonia oceanica	Formentera	Cala sahona	+	7
Posidonia oceanica	Formentera	Pta Sa Creu	-	5
Posidonia oceanica	Formentera	Els Pujols	-	5.5

sterile seawater for 2 min. These surface-sterilized leaf pieces were placed on Serum-Seawater Agar (SSA) medium (1.2 % agar in filtered (0.4 μm) and autoclaved seawater, 3 mg L^{-1} germanium dioxide (GeO_2), 25 ml L^{-1} penicillin/streptomycin [10,000 units

penicillin G/ mL and 10 mg mL^{-1} streptomycin], 1% (v/v) horse serum) in plastic Petri dishes and maintained at 25ºC. When the growth of *Labyrinthula* sp. was visible, a portion of the agar containing the culture was transferred to a new plate in order to isolate the protists from marine fungi, which sometimes grow on the plates. The cultures were maintained by sub-culturing on to new plates every 2 weeks.

Identification of *Labyrinthula* sp.

The *Labyrinthula* spp. were identified by light microscopy of the cultures using phase contrast optics (Zeiss). Representative isolates of *Labyrinthula* sp. were recognized based on gross cell morphology and growth patterns on SSA medium (Muehlstein et al. 1988), cell ultrastructure using transmission electron microscopy (Porter 1969, Muehlstein et al. 1991), and the presence of an ectoplasmic network (Fig. 2). Additionally, in 2009, sequence analysis of a 240-bp diagnostic region of the 18S rDNA (Martin et al. 2006) was used to identify the *P. oceanica* isolate from Pollença, Mallorca, Spain (Martin unpublished data). The sequences in the18S rRNA gene were defined by the primers 960bF (a eukaryote-specific primer without the GC clamp) and 1200R (a universal 18S primer) described by Gast et al. (2004). Blastn analysis yielded best matches with *Labyrinthula* spp. accession numbers AB290459.1 and AB290458.1 (score=304, E=2e-79, Identity=89%, gaps=9). Of the top 20 hits, all were for *Labyrinthula* sp. or *Aplanochytrium* sp. Although both groups are closely related, colony growth morphologies of the two are quite different and easily discernible. Unfortunately, at the time of this sequencing, the *P. oceanica* isolate from Pollença, (Mallorca, Spain) was the only isolate available, because all other Mediterranean isolates ceased to grow in culture. Based on our experience with numerous isolates taken

globally over the past 5 years, cessation of growth in culture is common with this protist (Sherman and Martin, unpublished data).

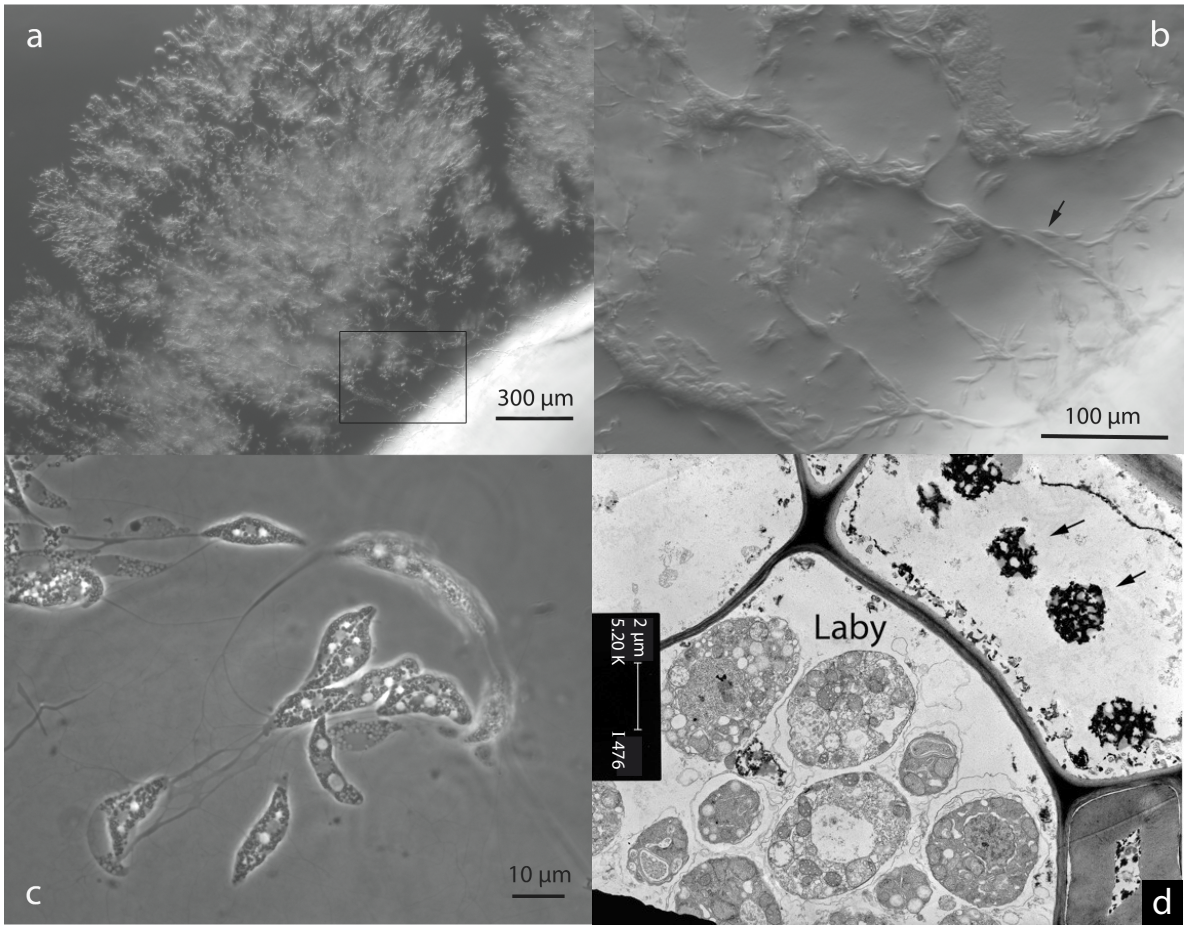

Figure 2. Representative images of *Labyrinthula* sp. used in this work. All images are of *Thalassia testudinum* isolate. Insets A-C represent light microscope images of cultures growing on Serum Seawater Agar (SSA) medium. A) Cells display typical growth morphology of *Labyrinthula* sp. in culture, with cells on the surface and growing into the agar medium. B) An enlargement of boxed area of inset A. Plane of focus images cells on surface of agar, where cells can be seen following the ectoplasmic network (arrow). C) Higher magnification shows typical features of *Labyrinthula* sp. cell structure and ectoplasmic network. D) TEM of *Z. marina* tissue in area of leaf associated with a lesion caused by *Labyrinthula* sp. isolate from *T. testudinum*. Note numerous *Labyrinthula* sp. cells within the plant cell at bottom of figure and extensively degraded cytoplasmic structures in the surrounding plant cells (arrows denote remnants of degraded chloroplasts).

Cross-infection experiments

To evaluate the pathogenicity (i.e. capacity to produce lesions on healthy seagrass shoots) of *Labyrinthula* spp. isolated from seven *Posidonia oceanica* meadows in the Balearic Islands, we conducted five cross-infection experiments using the following target seagrass species: *Thalassia testudinum* collected from the coast of Florida, USA (30° 18.186' N, 87° 24.264' W), *Zostera marina* collected from the temperate Western Atlantic (37° 15.50' N, 76° 01.30' W), and *Posidonia oceanica*, *Z. noltii* and *Cymodocea nodosa* from three meadows around Mallorca and Cabrera Islands (*P. oceanica* from Es Cargol 39º 16.62' N, 3º 2.449'E; *Z. noltii* from Es Port-Cabrera 39º 9.18' N , 2º 55.86' E; and *C. nodosa* from Cala Estancia 39º 32.13' N, 2º 42.65' E). From each meadow containing the target seagrass species, we collected ~100 healthy shoots. These were maintained in aerated seawater to allow them to acclimate to aquarium conditions for 3-4 d before the onset of the experiments.

We conducted the infection experiments according to the procedures of Muehlstein et al. (1988). The infection vectors used were autoclaved pieces of seagrass leaves that were placed on *Labyrinthula* sp. cultures in Petri dishes for 7 d at 25ºC. The leaf sections carrying the *Labyrinthula* sp. were clamped onto healthy leaves of seagrass shoots with a piece of clear, flexible PVC tubing. Negative controls were sterile leaf pieces that had no contact with *Labyrinthula* sp. cultures. These were fixed to healthy leaves with PVC tubing. In addition, we used a *Labyrinthula* sp. isolated from *Thalassia testudinum* (Steele et al. 2005) as a positive control as it was previously reported to infect not only *Thalassia testudinum* (≈ 100% efficiency), but also *Zostera marina* (≈ 70% efficiency) (Caldwell 2006). Only one leaf per seagrass plant was inoculated with the *Labyrinthula* sp. vector

clamp. We distributed the 6−10 replicated seagrass shoots treatment^{-1} (Table 2) between two aquaria (2 L) filled with filtered (0.4 μm), aerated seawater (salinity 38). There was no water flow among aquaria to avoid contamination. We placed the aquaria in an incubator at 25ºC for 2 wk with a 12 h light: 12 h dark photoperiod, receiving light intensity of 150 μmol m^{-2} s^{-1}.

We monitored the plants daily for lesions and, at the end of the experiment, we assessed the virulence of the *Labyrinthula* spp. by calculating the percentage of shoots with lesions out of the total number of shoots inoculated per treatment. All lesions observed in the experiments were restricted to an area in or around the *Labyrinthula* spp. inoculation point. *Labyrinthula* spp. were re-isolated from randomly selected lesions that appeared on the seagrass leaves at the end of the infection experiments, following the methodology described above, in order to satisfy Koch's postulates. These isolates were examined for colony growth morphology as well as protist size and shape to ascertain the presence of *Labyrinthula* sp. in the lesions.

Electron microscopy

During infection experiments on *Zostera marina* (conducted at the University of South Alabama), *Labyrinthula* sp. isolated from *T. testudinum* readily produced lesions and these lesions were processed for transmission electron microscopy to confirm the presence of the pathogen in the tissue. Samples of both uninfected and infected tissue at the margins of lesions were cut into thin sections while immersed in 3% (v/v) glutaraldehyde in seawater and allowed to stand on ice for 2 h. Samples then were washed twice in 100 mM cacodylate (pH 7.2) for 15 min each, post-fixed in 2% osmium in cacodylate (pH 7.2) for 2

h, and then given two water rinses before incubation in 2% uranyl acetate overnight at 4˚C. Samples were washed in water, dehydrated in a graded acetone series, and transferred to propylene oxide. A 1:1 (v/v) mixture of Spurr's low-viscosity resin and Polybed 812 (Polysciences, Warrington, Pennsylvania) was added in increasing increments to 75% plastic, with the remaining 25% of propylene oxide allowed to evaporate through small holes in aluminum foil covers over the sample vials. The samples then were oriented in Teflon flat molds (Ted Pella Inc., Redding, California) and Epon/Spurr's was used to completely fill the cavity. A coverslip was applied and the samples polymerized at 60˚C for 16 h. Thin sections (gray-silver reflectance) were cut with a Delaware Diamond Knife on a LKB 8800 Ultratome III, post-stained with uranyl acetate and lead citrate, and observed with a Phillips CM 100 electron microscope.

Statistical analysis

Data from the cross-infection experiments were evaluated via 3-way log-linear analysis (G-test of independence) (Sokal and Rohlf 1995, Quinn and Keough 2002) to look for differences in rates of infection between seagrass species and differences in infection due to different *Labyrinthula* spp. isolates.

Results

Geographic distribution of *Labyrinthula* spp.

Labyrinthula spp. organisms were found in 13 of the 18 seagrass meadows examined. Specifically, *Labyrinthula* spp. were isolated from seagrass leaves from all four meadows at Cabrera Island and in all seven of those around Mallorca Island. Conversely, *Labyrinthula* spp. were isolated in only two of the seven meadows examined at Ibiza and

Formentera Islands (Fig. 1). *Labyrinthula* spp. were cultured from 9 of the 14 *Posidonia oceanica* meadows, from all three *Cymodocea nodosa* meadows, and from the single *Zostera noltii* meadow investigated (Table 1). As determined by light microscopy, cell shape of the various isolates from the Mediterranean was in keeping with the morphology ascribed to *Labyrinthula* sp., with spindle-shaped cells within a filamentous network (as shown in Fig. 2).

Cross-infection experiments

No lesions were observed on the negative control treatments for any of the infections conducted (Table 2). Conversely, the *Labyrinthula* sp. used as a positive control (isolate from *T. testudinum*) produced lesions on the leaves of all replicated shoots of all seagrass species tested. This isolate was the most pathogenic of all isolates tested. *Labyrinthula* sp. was observed within the infected tissue of *Z. marina* with evidence of extensive cellular damage in the surrounding seagrass cells (Fig. 2).

The most virulent *Labyrinthula* spp. isolates from *P. oceanica* were all from meadows around Mallorca Island (Palmanova, Pollença, and Cap Salines). *Labyrinthula* spp. from Pollença and Cap Salines were capable of producing lesions on all three Mediterranean seagrass species tested. *Labyrinthula* sp. isolated from the Palmanova location produced lesions on *Zostera marina* from the temperate Western Atlantic, as well as the Mediterranean seagrasses *P. oceanica* and *Z. noltii*. The least virulent *Labyrinthula* sp. isolate was that from Santa Maria Bay (Cabrera Island), which did not produce lesions in any of the examined seagrass species.

Table 2. Results of cross-infection experiments using *Labyrinthula* spp. isolated from *Posidonia oceanica* with different targeted seagrass species (*Thalassia testudinum, Zostera marina, Posidonia oceanica, Zostera noltii,* and *Cymodocea nodosa*). Occurrence of infection (% of shoots presenting lesions out of total shoots inoculated) on each treatment and target seagrass species are indicated. Number of replicates is shown in parenthesis under % of shoots infected. Additionally, the total number of target seagrass species infected by each *Labyrinthula* sp. isolate and total number of isolates that infected a particular target seagrass species are indicated. (nd: no data.) Frequencies of infection differed significantly among target species and sources of *Labyrinthula* sp. (3-way log-linear analysis, G=70.35, df=9, p<0.0001).

Treatment	**Seagrass source**	**Seagrass collection site**	**Percentage of shoots infected**					**Target spp. infected**
			Thalassia testudinum	***Zostera marina***	***Posidonia oceanica***	***Zostera noltii***	***Cymodocea nodosa***	
Positive Control	*Thalassia testudinum*	Perdido Bay (Florida)	100 (7)	100 (7)	90 (10)	100 (8)	100 (6)	**5**
Isolate 1	*Posidonia oceanica*	St. Maria (Cabrera)	0 (7)	0 (7)	0 (7)	0 (8)	0 (7)	**0**
Isolate 2	*Posidonia oceanica*	Es Castell (Cabrera)	0 (7)	0 (7)	100 (7)	83 (6)	0 (7)	**2**
Isolate 3	*Posidonia oceanica*	C. Sahona (Formentera)	0 (7)	0 (7)	100 (7)	0 (7)	nd	**1**
Isolate 4	*Posidonia oceanica*	Palmanova (Mallorca)	0 (7)	100 (7)	67 (6)	100 (7)	0 (9)	**3**
Isolate 5	*Posidonia oceanica*	Magaluf (Mallorca)	0 (7)	0 (7)	0 (7)	100 (8)	0 (6)	**1**
Isolate 6	*Posidonia oceanica*	Pollença (Mallorca)	0 (7)	0 (7)	50 (6)	100 (7)	100 (6)	**3**
Isolate 7	*Posidonia oceanica*	Cap Salines (Mallorca)	nd	nd	63 (8)	50 (6)	100 (6)	**3**
Negative Control			0 (7)	0 (7)	0 (7)	0 (7)	0 (7)	**0**
***Posidonia oceanica* isolates producing lesions**			**0**	**1**	**5**	**5**	**2**	

The seagrass species most vulnerable to infection by *Posidonia*-derived *Labyrinthula* spp. isolates were *P. oceanica* and *Z. noltii*, with five out of the seven *Labyrinthula* spp. isolates producing lesions in the cross-infection experiments. Only two of the seven isolates from *P. oceanica* produced lesions on *C. nodosa* and only one of them produced lesions on *Z. marina*. It is also of note that *T. testudinum* was the seagrass most resistant to infection, since none of the seven *Labyrinthula* spp. isolated from *P. oceanica* produced lesions on its leaves (Table 2).

Analysis of these data confirms that the various seagrass species tested had significantly different susceptibilities to the pathogens and that there were differences in pathogenicity of the various *Labyrinthula* spp. isolates (3-way log linear analysis, $G=70.35$, $df=9$, $p<0.00010$).

Discussion

This is the first report of *Labyrinthula* spp. in the western Mediterranean Sea, and it was found on leaves of all three seagrass species in the Balearic Islands: *Posidonia oceanica*, *Cymodocea nodosa,* and *Zostera noltii*. The presence of *Labyrinthula* spp. on *P. oceanica* was previously reported in the Ionian Sea (Gallipoli, Italy), on *C. nodosa* in Taranto, Italy (Vergeer and den Hartog 1994) and in the Lagoon of Venice, Adriatic Sea (den Hartog et al. 1996); and in association with *Z. noltii* in the Netherlands and France (Vergeer and den Hartog 1991). In this study, we found *Labyrinthula* spp. in most (70%) of the sampled meadows, indicating that *Labyrinthula* spp. are widespread in the seagrass meadows of the Balearic Islands, and very common in the Mediterranean Sea, with some of them producing lesions like those described previously during wasting disease events (Muehlstein 1992).

Our cross-infection experiments revealed that *Labyrinthula* sp. infectivity was not specific to the seagrass species from which it was isolated. *Labyrinthula* sp. isolates from *Thalassia testudinum* produced lesions on 90-100% of all leaves in experiments with *T. testudinum, Posidonia oceanica*, *Zostera marina*, *Z. noltii*, and *Cymodocea nodosa*. Those from *P. oceanica* produced lesions on 50-100% of all treated leaves of *P. oceanica, Z. marina, Z. noltii, and C. nodosa*. These results contrast with those in previous studies (Muehlstein et al. 1988, Short et al. 1993, Vergeer and den Hartog 1994), where the capacity for infection by *Labyrinthula* sp. was restricted to the seagrass species from which it was isolated, or at most to a congeneric species. However, our results parallel those of Caldwell (2006), where *Labyrinthula* sp. isolates from *T. testudinum* cross-infected *Z. marina*, but not vice versa.

The target host range for the pathogen could be much broader than was thought previously. The *Labyrinthula* sp. isolated from *T. testudinum* in Perdido Bay, Florida infected the shoots of all seagrass species tested here, including representatives of the western Mediterranean Sea, the temperate western Atlantic, and Florida coast. The *Labyrinthula* spp. isolates from *P. oceanica* were, to varying degrees, somewhat less virulent, but still produced infections on species from other genera. Additionally, *Labyrinthula* spp. used in the present study had different capacities to produce lesions, indicating a range of virulence among isolates from a given seagrass species, consistent with the findings of Muehlstein et al. (1988). Our results also reveal that vulnerability to *Labyrinthula* spp. infections depends on the seagrass species as evidenced by the fact that the two seagrass species not autochthonous to the Mediterranean Sea (*T. testudinum* and *Z. marina*) were the most resistant to infection by *Labyrinthula* sp. isolated from *P. oceanica*.

Collectively, these results highlight the fact that virulence of some *Labyrinthula* spp. isolates may not be defined by phylogenetic or geographical boundaries.

In summary, this work demonstrates that *Labyrinthula* spp. are prevalent in seagrass meadows of the western Mediterranean Sea, and can damage seagrass leaves. *Posidonia oceanica* may be particularly vulnerable to *Labyrinthula* spp. infections, as it is very slow growing and supports a large belowground biomass, so that any loss of photosynthetic capacity may compromise its carbon budget and, therefore, plant survival. As some *Labyrinthula* spp. isolates can infect seagrasses from outside their geographical region, Mediterranean seagrass meadows may be threatened by *Labyrinthula* spp. infections. Increasing worldwide maritime traffic may contribute to enhanced vulnerability to *Labyrinthula* spp. infections of these valuable seagrass meadows. Implications for avoiding propagation of this disease are then apparent and further examination of the conditions favoring disease transmission and development are needed.

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Chapter 2

Screening for viruses in seagrass (*Posidonia oceanica*) tissues

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Manuscript

Abstract

The presence of viruses was tested in *Posidonia oceanica* tissues (leaves, rhizomes and roots) from four meadows located in the Balearic Islands (Western Mediterranean Sea), experiencing different rates of population decline. The presence of plant viruses in *P. oceanica* tissues was examined by i) transmission electron microscopy (TEM), ii) indirect tissue print-ELISA using polyclonal and specific monoclonal antibodies, iii) analysis of double-stranded RNA (dsRNA), and iv) reverse transcription (RT) and PCR amplification using primers based on sequences conserved in several viral families or genera: genus *Luteovirus*, family *Closteroviridae* and family *Potyviridae* Viruses were not detected in the studied *P. oceanica* meadows by applying such diverse techniques of detection, however we cannot conclude the absence of viruses in *P. oceanica* meadows of Western Mediterranean Sea.

Introduction

Viruses are present in marine environments at very large abundance, including about 10^5 -10^8 particles ml^{-1} in water column (Fuhrman 1999, Azam & Worden 2004) and about 10^9 viruses g^{-1} (dry weight) of marine sediments (Danovaro et al. 2001). Their depth distribution in water is coupled to abundances of phytoplankton and bacterioplankton communities (Marie et al. 1999), where viruses cause infections on these communities, regulating their abundances (Culley et al. 2003), and therefore, their population dynamics. However, there is a paucity of information regarding the presence of viruses in marine macrophytes and seagrasses, since most studies focused on viruses infecting crop plant species. Despite this fact, there are evidences of large double-stranded DNA (dsDNA) viruses in the filamentous brown algae *Ectocarpus siliculosus* and *Feldmannia* (Van Etten J.L. & Meints R.H. 1999) and dsRNA viruses in chloroplasts and mitochondria of the green macroalga *Bryopsis cinicola* (Koga et al. 2003). Moreover, there are evidences of presence of dsRNA viruses in symptomless *Zostera marina* seagrass tissues collected in Tokyo Bay. These viruses found in seagrass tissues were recognized as members of the genus *Endornavirus*, non-enveloped viral capsid viruses holding a linear non-segmented single molecule of double-stranded RNA classified in large-dsRNA virus, greater than 10Kbp (Fukuhara et al. 2006).

In the Mediterranean Sea, the seagrass *Posidonia oceanica* forms the most productive coastal ecosystem in sandy areas. *P. oceanica* is a clonal plant with a very slow growth, ranging from 1 to 6 cm yr^{-1} $apex^{-1}$ (Marbà et al. 1996), and with clones living for millennia (Mateo et al. 1997). *P. oceanica* meadows are experiencing a general decline across the Mediterranean Sea (Marbà et al. 2005). This decline has been associated with disturbances

such as sediment dynamics, climate change, and nutrient and organic matter inputs to coastal waters (Duarte et al. 2004). However, the mortality of *P. oceanica* shoots and the subsequent loss of meadows could be also influenced by diseases produced by viruses, a possibility that has not yet been explored. The clonal nature of seagrasses would favor the spreading of viral particles (Oparka et al. 1997). Currently, there are no reports on the role of viruses on seagrass health.

In this work, we screened *P. oceanica* tissues (leaves, rhizomes and roots) from four meadows in the Balearic Islands (Mediterranean Sea, Spain) experiencing different rates of population decline for the presence of viruses by applying a broad range of detection techniques.

Materials and Methods

Sample collection and site description

Posidonia oceanica shoots, rhizomes and roots were collected by scuba diving during March 2004 in four meadows in the Balearic Islands (Mediterranean Sea, Spain) experiencing different rates of shoot mortality, recruitment and net population growth rates. The meadow at Pollença (Mallorca Island) was exhibiting the highest shoot mortality rate and the fastest rate of decline, whereas that at Porto Colom (Mallorca Island) was expanding (Table 1). The collected plant material was transported in plastic bags to the laboratory and maintained in four aquaria (one per site) with aerated seawater until processed.

Table 1. Water depth, latitude, longitude, and shoot mortality, recruitment and net population growth rates of *Posidonia oceanica* growing at the sampling sites. Source of demographic data: Marbà (unpublished). Positive net population growth rate indicates the rate of meadow expansion (mortality < recruitment); and, negative net population growth rate indicates the rate of meadow decline.

Sampling site	Depth (m)	Latitude (ºN)	Longitude (ºE)	Mortality rate (yr^{-1})	Recruitment rate (yr^{-1})	Net population growth rate (yr^{-1})	Meadow status
Porto Colom	6.4	39º25.05'	3º16.18'	0.1	0.12	0.02	expanding
Pollença	4	39º53.7'	3º05.52'	0.25	0.1	- 0.15	fast decline
Magalluf	6	39º30.25'	2º32.59	0.12	0.04	- 0.08	decline
Illetes	9	39º32.10'	2º35.56'	0.11	0.03	- 0.07	decline

Detection of viruses

The presence of viruses in *P. oceanica* tissues was examined by i) transmission electron microscopy (TEM), ii) indirect tissue print-ELISA using polyclonal and specific monoclonal antibodies, iii) analysis of double-stranded RNA (dsRNA), and iv) reverse transcription (RT) and PCR amplification using primers based on sequences conserved in several viral families or genera.

i) TEM observations were performed according to procedures described by Milne (1993). Extracts were prepared by homogenizing about 6 mm^2 leaf tissue in 50 µl of 0.1M phosphate buffer, pH 7, containing 2% (w/vol) PVP-44000 (BioRad). Drops of each sample extract were placed on two carbon-Formvar coated copper grids per sample and kept for 10 min. Then, the grids were rinsed with distilled water, stained with 1% uranyl acetate, dried and examined in a Jeol 100S microscope at 80 KV. Observations were performed at 21,000X instrument magnification using a 10X binocular.

ii) Rhizome and leaf sections were imprinted following the methodology described in Cambra et al. (2000) on nitrocellulose membranes (Plantprint Diagnostics) and processed by a conventional tissue print-ELISA protocol (OEPP, 2004) indirect version using polyclonal and monoclonal antibodies to several plant viruses (IVIA collection) and then goat anti-rabbit or anti-mouse immunoglobulins conjugated with alkaline phosphatase. Polyclonal antibodies were against *Tobacco mosaic virus* (TMV), *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Barley yellow dwarf virus* (BYDV) and *Cucumber mosaic virus* (CMV), and monoclonal antibodies were specific for the potyvirus-group (Richter et al. 1995), *Potato virus Y* (PVY) 1E10 and 10E3 epitopes (Sanz et al. 1990) and *Potato virus X* (PVX) conserved epitope.

iii) Preparations enriched in dsRNA were obtained from total RNA extracts of *P. oceanica* leaves and rhizomes by non-ionic cellulose column chromatography in the presence of 16.5% ethanol (Moreno et al. 1990), and then analyzed by polyacrylamide gel (6% acrylamide) electrophoresis at 100 V for 3 h. Gels were stained with silver nitrate (Beidler et al. 1982).

iv) Detection of viruses was also attempted using RT-PCR amplification using total RNA and dsRNA-rich extracts as templates and primers PV21/T7 and PV1/SP6 conserved in members of the family *Potyviridae* (Gibbs & Mackenzie 1997), Luteo 1f and Luteo 1r conserved in luteoviruses (Robertson et al. 1991) and HSP-p-1/HSP-P-2 conserved in *Closteroviridae* (Tian et al. 1996). The reaction mix and thermocycling conditions were as described by Gibbs & Mackenzie (1997).

Results

TEM observation of extracts from five different *Posidonia* samples did not reveal the presence of particles that could be potential virions, whereas similar grids prepared with extracts from citrus plants infected with *Citrus tristeza virus* (CTV) showed characteristic CTV filamentous particles. Negative results were also obtained by indirect tissue print-ELISA with five broad-spectrum polyclonal antibodies against tested viruses and four monoclonal antibodies representing conserved epitopes in potyviruses and PVX. Electrophoretic analysis of dsRNA-rich preparations from CTV-infected plants showed a typical dsRNA pattern (Moreno et al. 1990), whereas no dsRNA bands could be observed in equivalent extracts from *P. oceanica* samples. Finally, no DNA could be RT-PCR amplified from the same samples with either of the four primer sets assayed.

Discussion

Failure to detect viral particles by TEM or viral coat protein by tissue print-ELISA indicated either that the *P. oceanica* tissues analyzed were virus free or that the putative virus infecting them would be at low titer and/or unevenly distributed in the plant. On the other hand, plants infected with RNA viruses usually contain large dsRNA molecules (corresponding to replicative intermediates of the genomic and subgenomic RNAs) that are not present in virus free plants. The absence of detectable dsRNAs in *P. oceanica* samples is consistent with the absence of viral particles in the TEM preparations, although it does not exclude the possibility of infection with DNA viruses or RNA viruses with very low accumulation of dsRNA. Finally, lack of RT-PCR amplification with primers specific for plant viruses of known members of the genus *Luteovirus*, and families *Closteroviridae* and *Potyviridae* further supports previous data and strongly suggests that viruses of these taxa

are not present, at least at detectable levels, in sampled *P. oceanica*. The viruses of the family *Potyviridae*, however, have been found associated with the bacterioplankton in the marine environment (Culley et al. 2003).

Consequently, it can be concluded that viruses belonging to these groups tested do not seem to be present in the studied *P. oceanica* meadows and that they are unlikely to compromise *P. oceanica* health.

Acknowledgements

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Chapter 3

Endophytic bacterial community of a mediterranean marine angiosperm (*Posidonia oceanica*)

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Abstract

We described the endophytic bacterial community of surface-sterilized tissues (roots, rhizomes and leaves) of the long living Mediterranean marine angiosperm Posidonia oceanica by applying Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA amplified from total DNA extracts of seagrass tissues. We sampled 26 Posidonia oceanica meadows around the Balearic Islands and compared the band patterns obtained for each meadow and for the three sampled tissues. A total of 34 OTUs (Operational Taxonomic Units) were detected. We found that the band patterns of roots significantly (ANOSIM test: $R=0.201$ and $R=0.126$; Bonferroni Post-hoc test: $P<0.005$) differed from those of rhizomes and leaves. Moreover, we sequenced the main OTUs detectable by DGGE analysis in order to identify those bacteria present endophytically in P. oceanica tissues. The 33% of sequences belonged to Bacteroidetes class and the rest belonged to Proteobacteria: 41.7% were affiliated to the α-subclass, 16.7% to the γ-subclass and 8.3% to the δ-subclass. The major families or groups represented in endophytic bacterial community were Desulfovibrionaceae (15.38%), Flammeovirgaceae (15.38%), Rhodobacteracerae (15.38%), Sphingobacteriaceae (15.38%), Non identified Coral Black Band Disease (BBD) isolates (15.38%), Oceanimonaceae (7.69%), Rhizobiaceae (7.69%) and Non identified Sulfur-Oxidizing-Symbionts (7.69%). The results of this study provide a pioneer step towards the characterization of the endophytic bacterial community associated with tissues of a marine angiosperm and reveal the presence of some bacteria that may play key roles for maintenance of this marine angiosperm in its ecosystem.

Introduction

Bacteria are commonly found living endophytically within plant tissues (e.g. Hallmann and Berg 2006). Endophytic bacteria, typically defined as those living inside plant tissues not harming the host plant (Schulz and Boyle 2006), often promote plant growth by, for instance, providing nutrients or controlling plant pathogens through mutualistic bacteria-plant interactions (e.g. Hallmann and Berg 2006, Ikeda et al. 2010, Li et al. 2011). However, plant tissues can also host pathogenic bacteria that, when present at high abundances, cause plant disease outbreaks. Whereas information on composition and ecological roles of endophytic (including pathogenic) bacterial communities of terrestrial, and to some extent freshwater plants abounds, particularly for crop species (e. g. Ueda et al. 1995), the presence and relevance of endophytic bacterial communities of marine plants remain unexplored.

Seagrasses are marine clonal angiosperms that evolved from freshwater angiosperm ancestors that colonized the marine environment in the Cretaceous (den Hartog 1970). Despite the fact that seagrass flora is restricted to about 50-60 species, they develop lush and highly productive meadows, particularly in oligotrophic waters, along the coasts of all continents except Antarctica (Hemminga and Duarte 2000, Short et al. 2007). Seagrass meadows are important global carbon sinks, enhance coastal biodiversity and prevent coastal erosion (Hemminga and Duarte 2000, Orth et al. 2006). Bacterial communities play important roles in seagrass meadows, particularly in the recycling of materials (Hemminga and Duarte 2000). However, information about bacterial communities associated with seagrasses is scant with most studies focusing on bacterial communities in seagrass sediments (Cifuentes et al. 2000, Bagwell et al. 2002, García-Martínez et al. 2009) or

associated with plant surfaces (i.e., epiphytic bacterial community) above (Weidner et al. 2000, Jensen et al. 2007, Uku et al. 2007, Crump and Koch 2008) or belowground (García-Martínez et al. 2005). However, endophytic bacteria in seagrass (*Thalassia hemprichii*, *Cymodocea serrulata*, *Halodule uninervis*, *Syringodium isoetofolium*) tissues have been reported using optical microscopy (Kuo 1993). *Clostridium glycolicum* has been isolated from the rhizoplane and deep cortex cells of *Halodule wrightii* (Küsel et al. 1999), a new species of the genus *Sulfitobacter* has been isolated from a homogenate of *Zostera marina* (Ivanova et al. 2004) and *Desulfovibrio zosterae* from the surface-sterilized roots of *Z. marina* (Nielsen et al. 1999), pointing out that endophytic bacteria may be prevalent in seagrass tissues.

Posidonia oceanica is the dominant seagrass species in the Mediterranean Sea. Although, *P. oceanica* ranks among the slowest growing seagrasses (rhizome extension rates ranging from 1 to 6 cm yr^{-1} $apex^{-1}$, Marbà and Duarte 1998), it develops meadows living for millennia (Mateo et al. 2007) and occupies an estimated 50,000 km^2 in the Mediterranean Sea. The unique environments found in and around *P. oceanica* tissues constitute niches well differentiated from those in surrounding waters and sediments. Moreover, the millenary life span of *P. oceanica* clones suggest that endophytic bacteria can remain isolated within the *P. oceanica* tissues over extended periods of time, relevant for microbial evolutionary processes. Thus, it is likely that *P. oceanica* meadows harbor distinct microbial communities, previously undescribed microbial species or even microbial symbionts. Indeed, recent studies using culturing methods have described seven new bacterial species belonging to the genus *Marinomonas* isolated from *P. oceanica* (Espinosa

et al. 2010, Lucas-Elío et al. in press), supporting the idea of the existence of a distinct bacterial community associated with *P. oceanica*.

However, culturing techniques do not provide a thorough description of the bacterial community, often biasing the results towards bacterial species able to grow in rich media while other bacteria that have specific culture requirements or are adapted to grow in more oligotrophic conditions, remain undetectable to culturing approaches (Hugenholtz et al. 1998, Pedrós-Alió, 2006). The introduction of molecular techniques in microbial ecology has enabled the identification of abundant and relevant members of natural bacterial communities in a cultivation-independent manner. Molecular techniques have been successfully applied in the characterization of bacterial communities in marine sediments (Gray and Herwig 1996), the total bacterial community associated with corals (Rohwer et al. 2002) or the microbiota associated with seagrass sediments (James et al. 2006), to cite a few examples.

The interest in exploring the endophytic bacterial community of *P. oceanica* extends beyond that of exploring a potential biodiversity niche. The characterization of the microbes found inside the tissues of *P. oceanica* can offer significant clues about the health and ecology of *P. oceanica* meadows since endophytic bacteria can be pathogens but also symbionts. The number of disease outbreaks in the marine environment appears to be rising (Harvell et al. 1999). This trend is possibly facilitated by anthropogenic pressures (e.g. global movement of ballast waters by ships, Ruiz et al. 2000) and global warming (Harvell et al. 1999, 2002), as they may facilitate the occurrence of pathogens in areas with previously unexposed host populations. Symbiotic microorganisms can also play a key role in determining seagrass meadows, as they can facilitate the uptake of elements like

nitrogen, which can be limited in marine environments. The role of bacteria in sulfur cycling can also determine the health of marine angiosperms in marine sediments receiving high organic matter inputs. H_2S produced from decomposition of organic matter under anoxic conditions can intrude seagrass tissues (Pedersen et al. 2004) with negative consequences for seagrass meristematic activity (Garcias-Bonet et al. 2008). Bacteria can, therefore, play a major role in the survival and growth of seagrass meadows. The characterization of the microbiota closely associated with Posidonia oceanica, such as endophytic bacteria, is a first step that may provide further insights into the complex interactions between bacteria and seagrass.

Here we describe the bacterial communities associated with surface-sterilized tissues (roots, rhizomes, leaves) collected in summer in 26 meadows of *Posidonia oceanica* around the 950 km of coast of the Balearic Islands (Western Mediterranean). We used DGGE (Denaturing Gradient Gel Electrophoresis) analyses to screen for the diversity of the endophytic community through the band profiles of endophytic bacteria in plant tissues. The profiles derived were compared across locations and dominant bands were sequenced to provide a first snapshot of the diversity and identity of the bacterial endophytes of Balearic *P. oceanica*.

Materials and Methods

Sampling strategies

Posidonia oceanica shoots were collected at 26 locations across the Balearic Islands (Fig. 1), by SCUBA diving during the summers of 2005 and 2006. The plants were transported to the laboratory in seawater from the same location and processed

immediately. The leaves, rhizomes and roots from 3 shoots per meadow were separated and subsequently subjected to a surface-sterilization protocol adapted from Coombs and Franco (2003). Briefly, the protocol consisted in immersing each sample in ethanol 99% for 1 minute, then in NaOCl 3.125% for 6 minutes, then in ethanol 99% for 30 seconds and finally washing gently with autoclaved seawater, These surface-sterilized samples were frozen in liquid nitrogen until nucleic acid extraction was performed.

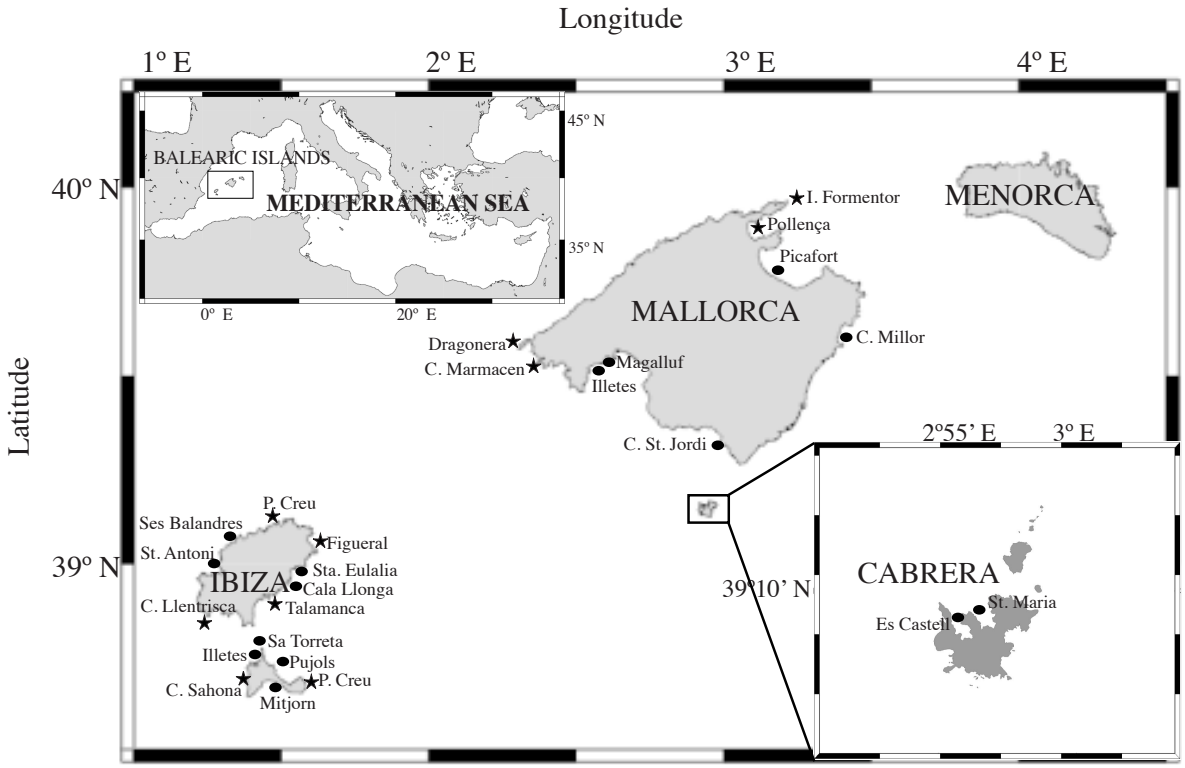

Figure 1. Location of Posidonia oceanica meadows sampled during two consecutives sampling events in summer 2005 (black circles) and summer 2006 (black stars).

Nucleic Acid extraction

Surface-sterilized plant material (100 mg of fresh tissue) was grinded with the help of a sterilized tool inside an eppendorf tube prior to nucleic acid extraction. The total nucleic

acid extraction was performed with the help of a commercial kit specific for plant tissues (Partec®). Nucleic acid extracts were stored at -20ºC.

PCR amplification

The DNA extract, containing plant and endophyte DNA when present, was amplified by PCR with primers 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') and 341F-GC containing a 40bp GC clamp at the 5' end (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C/CC TAC GGG AGG GAG CAG-3') specific for the bacteria domain (Muyzer et al. 1998). Each 50 µl PCR reaction containing template DNA plus (final concentrations) 2mM of dNTPs mixture, 20 µM of each primer and 0.5 units of *Taq* Polimerase (Takara) suspended in the buffer provided by the manufacturer of the polymerase. Additional negative (no DNA) and positive (*E. coli* DNA) control reactions were run with each batch of PCR reactions to check for possible contamination. The PCR consisted of an initial denaturing step at 94ºC for 5 min, followed by 10 touchdown cycles (94ºC for 1 min, 65ºC for 1 min (decreased by 1ºC cycle-1) and 72ºC for 1 min); and 30 cycles of standard amplification (94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min) with a final elongation step of 72ºC for 7 min. The PCR products were checked by electrophoresis on 1% agarose gels. For each sample, the products of several replicate reactions (minimum of 2) were pooled prior to DGGE.

Denaturing Gradient Gel Electrophoresis (DGGE)

The amplification products of the fragment of the 16S ribosomal DNA gene were separated by DGGE in a 6% polyacrylamide gel containing a gradient of denaturants ranging from 40% to 70% (where 100% is 7 M urea and 40% formamide). Gels were run

for 18 h at 150V in 1X TAE (Tris-Acetate-EDTA) buffer at 60º C in a CBS Scientific Co. DGGE system. Following electrophoresis, the gels were stained with SyberGold for 30 min in the dark and photographed using a G:BOX imaging system (Syngene). All the detectable bands were excised and stored frozen at -20ºC for further processing.

Analysis of DGGE profiles

The digital images of DGGE gels were analyzed by measuring the relative migration of each band, normalized to the migration of the 16S rDNA band corresponding to *Posidonia oceanica* chloroplasts, which were detectable on every sample. The bands with the same normalized migration distance were identified as the same OTU. This was also confirmed by sequencing of some of the bands identified as the same OTU.

Species accumulation curves (i.e., accumulated increase of detected OUTs vs. number of samples) were constructed in R (http://www.R-project.org/, ref R) using Packaged vegan (Oksanen et al. 2011) in order to check accuracy and representativeness of the sampling strategy and, therefore, of our results. Estimates of species richness (Chao, Jacknife and Bootstrap) were obtained from accumulation curves using the function *specaccum* in package vegan.

A binary matrix (presence/absence) was constructed for all the identified OTUs, in order to determine the similarity between samples. The binary matrix was used to generate a distance matrix based on Jaccard's coefficient as the basis for a non-metric multidimensional scaling (NMDS) diagram using package vegan in R. We performed an Analysis of Similarity (ANOSIM) using the vegan package (10,000 permutations), to test for the existence of differences in band patterns among groups defined as: tissue, island

(each of the four islands sampled) and location (within each island). The R value generated by ANOSIM test, indicates the magnitude of difference among groups, where an R>0 indicates differences between groups and R<0 indicates no difference between groups, because differences between groups are lower than differences within a group. The significance of ANOSIM results was tested using the Bonferroni correction as post-hoc test.

Finally, we performed an indicator species test (Dufrene and Legendre 1997) using package labdsv (Roberts 2010) in R software, in order to identify those OTUs that are characteristics of each tissue and island. The indicator species are defined as the most characteristic species of each group, found mostly in a single group and present in the majority of the sites or samples belonging to that group.

Sequentiation of the OTUs detected in DGGE

The reamplification of the excided bands (OTUs) was conducted using the same pair of primers used before (907R and 341F-GC), several PCR were done using, as template, the DNA of the band excided. The amplification products were pooled together, precipitated and concentrated. The concentrated amplification products were cleaned and purified from primers and dNTPs by an enzymatic reaction with a mixture of Exonuclease I (1U/ reaction) and Alkaline Phosphatase (1U/ reaction) at 37ºC during 60 min, followed by an enzyme denaturing step at 72ºC for 15 min and by a precipitation and resuspension protocol. Finally, we quantified the concentration of each amplified OTU and 150 ng of the amplified product was used for the sequencing reaction with the reverse primer 907R. The sequencing was performed by Secugen, using the chemistry BigDye ® Terminator v3.1.

The sequences of about 500bp were checked for existence of chimeras using the Bellerophon tool available at http://greengenes.lbl.gov and compared to the public DNA database of NCBI by using BLAST (Basic Local Alignment Search Tool) service at the National Center of Biotechnology Information (NCBI) web page (www.ncbi.nlm.nhi.gov). Further validation of the phylogenic identity of the sequences, was performed by aligning the sequences to those in the greengenes database (http://greengenes.lbl.gov) using ARB (Ludwig et al. 2004).

The sequences obtained in this study have been deposited in Genbank under the accession numbers JF292432 to JF292446.

Results

A total of 34 different OTUs were identified in DGGE profiles from total number of plant tissue samples (n=186). Rhizome samples (n=57) and root samples (n=67) hosted 28 different OTUs while leaf samples (n=62) showed 24 different OTUs detectable by DGGE analyses. Thirteen rhizome samples, five root samples, and eight leaf samples did not show any band, except the band corresponding to chloroplast sequence. In the meadows tested, 18.6% of rhizomes, 6.9% of roots and 11.4% of leaf samples were free of endophytes.

The species accumulation curves (Fig. 2) confirmed that the sampling effort was adequate to characterize the bacterial community richness associated with *P. oceanica* tissues, as curves showed saturation (i.e., approached a plateau), suggesting that more intensive sampling was likely to yield only minor improvements in coverage. The Chao, Jacknife and Bootstrapping estimates of species richness (Table 1) indicated that the percentages of OTUs detected in our DGGE gels accounted for 97.03% to 99.63% of the

total community richness for all tissues sampled. Large coverage was estimated for all tissue classes, the percentages of detected richness varied between 95.64% to 99.59% (leaves), 93.44% to 98.99% (rhizomes) and from 69.14% to 93.08% (roots) depending on the particular estimate used. Despite the total number of OTUs found in *P. oceanica* tissues was large, individual root, rhizome and leaf samples only presented on average (± standard error) 3.56±0.3, 2.46± 0.29 and 2.7±0.3, respectively, different OTUs.

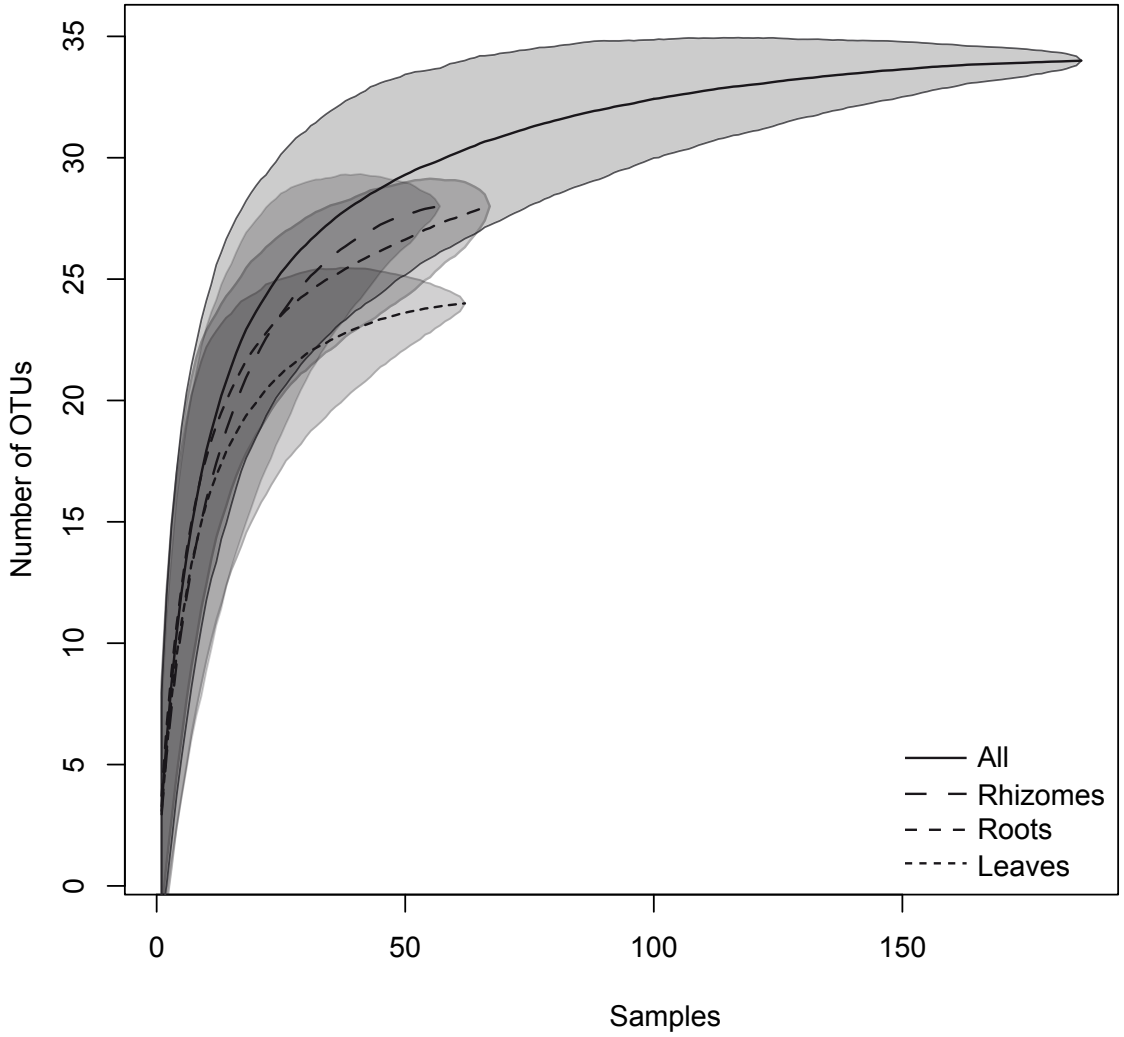

Figure 2. Species accumulation curves of the endophytic bacterial community found in P. oceanica tissues. The averaged (10,000 random sampling events) accumulated increase of detected OTUs and the standard deviation (shadowed area) vs. number of samples, for all samples pooled together, and separately by tissues.

Table 1. Number of total OTUs detected in each tissue, and pooling all samples together; number of expected number of OTUs by different approaches: Chao, Jacknife and Bootstrapping; and the percentage of identified OTUs from the expected number. N= number of samples.

			Number of OTUs expected by			**Percentage of sampled OTUs from the expected number by**		
Groups	**N**	**Number of OTUs detected**	**Chao Approach**	**Jacknife Approach**	**Bootstrap-ping Approach**	**Chao Approach**	**Jacknife Approach**	**Bootstrap-ping Approach**
All	186	34	34.12 ± (0.44)	34.99 ± (0.99)	35.04 ± (0.98)	99.65	97.16	97.03
Leaves	62	24	24.1 ± (0.38)	24.98 ± (0.98)	25.09 ± (1.28)	99.59	96.06	95.64
Rhizomes	57	28	28.29 ± (0.68)	29.96 ± (1.38)	29.89 ± (1.67)	98.97	93.44	93.67
Roots	67	28	40.5 ± (17.14)	32.93 ± (2.61)	30.08 ± (1.34)	69.14	85.04	93.08

The NMDS analysis showed no clear separation between groups: roots, rhizomes and leaves, in terms of band patterns (Fig. 3). However, ANOSIM test confirmed statistically significant differences in band patterns among tissues, although these differences were small, suggesting other variables playing a role in the endophytic bacterial composition of *P. oceanica* tissues. The band patterns obtained in DGGE analysis for root tissues were different from those obtained for rhizome and leaf tissues ($R= 0.201$, $P<0.005$ and $R=0.126$, $P<0.005$, respectively) and greater than the differences obtained between band patterns in leaf and rhizome tissues ($R=0.046$, $P<0.05$). Conversely, ANOSIM test for location groups did not show differences in band patterns among plants sampled in different islands.

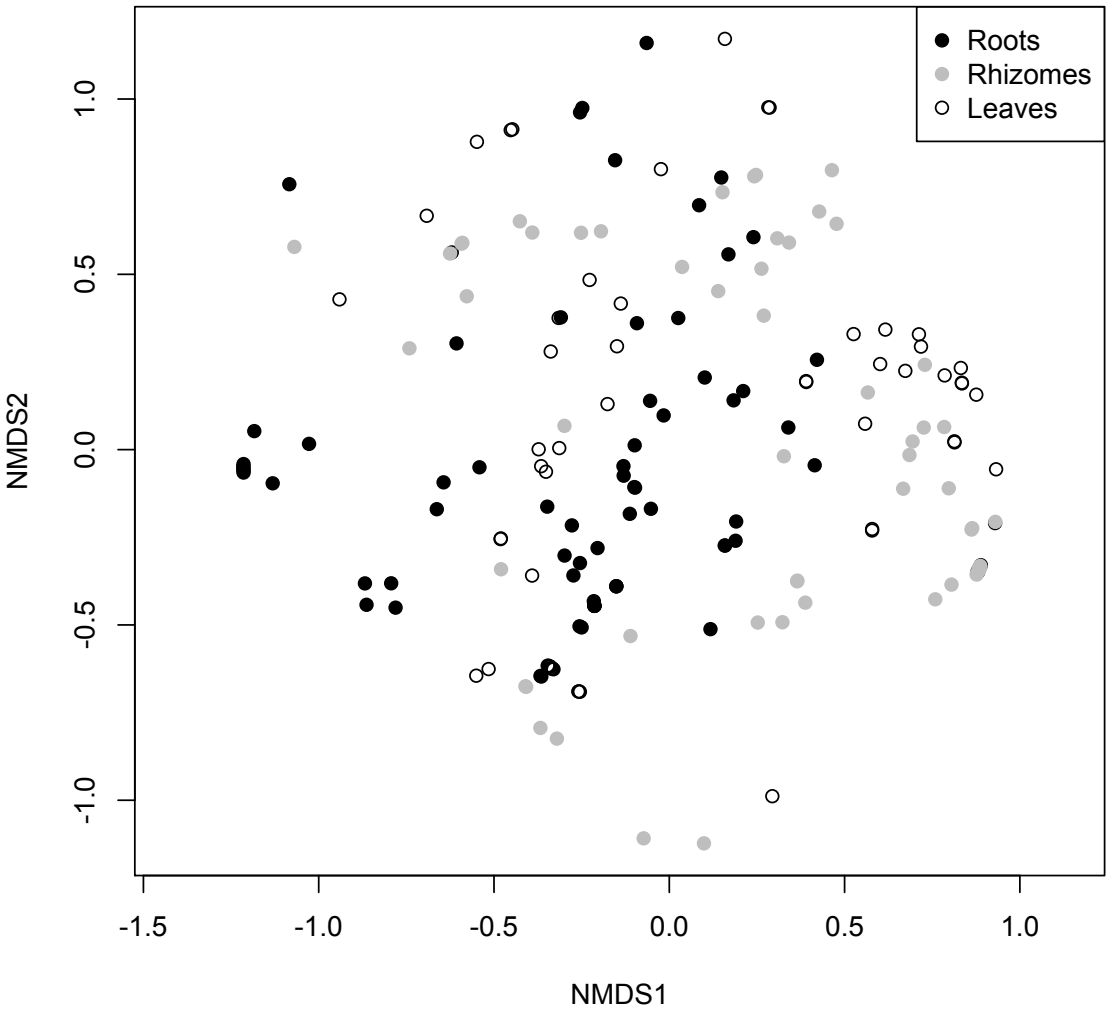

Figure 3. Non-metric Multidimensional Scaling analysis of endophytic bacterial community of *Posidonia oceanica* tissues.

The indicator species analysis identified some OTUs characteristic of each tissue (Table 2), although the indicator values were low. We found two OTUs associated with leaves, five OTUs associated with rhizomes and six OTUs associated with roots, but these OTUs were not present in all samples within each tissues, neither exclusively associated with each tissue.

Table 2. Indicator species test. OTUS' times of appearance in each tissue group, and in all samples. Cluster indicates the group for which each OUT is considered indicator, with the indicator value and the probability. The bold ones are statistically significant.

	Times of appearance in						
OTUs	**Roots**	**Rhizomes**	**Leaves**	**All**	**Cluster**	**Indicator value**	**Probability**
OTU_15	**1**	**4**	**12**	**17**	**Leaves**	**0.1344**	**0.001**
OTU_14	**7**	**5**	**17**	**29**	**Leaves**	**0.1612**	**0.004**
OTU_36	5	9	13	27	Leaves	0.0994	0.186
OTU_1	0	0	2	2	Leaves	0.0323	0.209
OTU_5	0	0	2	2	Leaves	0.0323	0.216
OTU_7	6	1	7	14	Leaves	0.0579	0.232
OTU_16	5	5	8	18	Leaves	0.0571	0.482
OTU_6	12	7	12	31	Leaves	0.0756	0.63
OTU_11	3	5	6	14	Leaves	0.0408	0.698
OTU_37	**0**	**8**	**0**	**8**	**Rhizomes**	**0.1404**	**0.001**
OTU_4	**15**	**21**	**6**	**42**	**Rhizomes**	**0.197**	**0.005**
OTU_19	**0**	**4**	**0**	**4**	**Rhizomes**	**0.0702**	**0.011**
OTU_10	**5**	**13**	**6**	**24**	**Rhizomes**	**0.1302**	**0.019**
OTU_12	**10**	**26**	**25**	**61**	**Rhizomes**	**0.2063**	**0.019**
OTU_34	0	2	0	2	Rhizomes	0.0351	0.074
OTU_23	1	3	0	4	Rhizomes	0.041	0.079
OTU_17	0	2	0	2	Rhizomes	0.0351	0.095
OTU_21	1	2	0	3	Rhizomes	0.0246	0.199
OTU_25	2	3	2	7	Rhizomes	0.0241	0.675
OTU_9	1	2	2	5	Rhizomes	0.015	0.875
OTU_26	**39**	**13**	**21**	**73**	**Roots**	**0.2949**	**0.001**
OTU_32	**24**	**2**	**0**	**26**	**Roots**	**0.3263**	**0.001**
OTU_33	**13**	**2**	**0**	**15**	**Roots**	**0.1643**	**0.001**
OTU_30	**14**	**0**	**8**	**22**	**Roots**	**0.1292**	**0.004**
OTU_2	**14**	**1**	**5**	**20**	**Roots**	**0.1422**	**0.005**
OTU_24	**17**	**8**	**1**	**26**	**Roots**	**0.1569**	**0.006**
OTU_8	7	2	2	11	Roots	0.0635	0.104
OTU_35	3	0	0	3	Roots	0.0448	0.136
OTU_18	11	3	7	21	Roots	0.0818	0.157
OTU_20	11	3	9	23	Roots	0.0745	0.288
OTU_22	5	0	4	9	Roots	0.04	0.289
OTU_13	18	12	11	41	Roots	0.1099	0.405
OTU_31	5	4	3	12	Roots	0.0288	0.921
OTU_3	1	0	0	1	Roots	0.0149	1

Table 3. Closest relatives of the sequenced OUTs indicating the tissue and the location from which the sequence was obtained. The percentage of similarity of the closest relative is given as well as a description of the isolation source and the reference. The family or group in which is affiliated is indicated.

Sequence ID (Accession Number)	Tissue/ Location	Length (bp)	Similarity (%)	Closest relative in BLAST (Accession Number)	Description	Author	Family/Group in Greengens
P.o_rhiz_3b (JF292432)	Rhizome / C.Sahona	494	99	Uncultured bacteria (EU181012)	Isolated from seagull fecal sample	Lu et al. 2008	Non-Identified Coral BBD isolates / α-proteobacteria
			99	Uncultured bacterium clone (GU118071)	Isolated from the coral *Acropora palmata*	Sunagawa et al. 2010	
			99	Uncultured bacterium clone (FJ202069)	Isolated from the coral *Montastraea faveolata* displaying signs of White Plague Disease type II	Sunagawa et al. 2009	
			99	Uncultured bacterium clone (EF123439)	Isolated from Black Band Diseased tissues of the coral *Siderastrea siderea*	Sekar et al. 2008	
			99	*Cohaesibacter* sp. (GQ200200)	Rhizobiales; Cohaesibacteraceae. Isolated from sediment of a seawater pond	Qu et al. 2011	
P.o_rhiz_6b (JF292433)	Rhizome / Figueral	433	97	Uncultured bacteria (EU181012)	Isolated from seagull fecal sample	Lu et al. 2008	Non-Identified Coral BBD isolates / α-proteobacteria
			97	Uncultured bacterium clone (GU118071)	Isolated from the coral *Acropora palmata*	Sunagawa et al. 2010	
			97	Uncultured bacterium clone (FJ202069)	Isolated from the coral *Montastraea faveolata* displaying signs of White Plague Disease type II	Sunagawa et al. 2009	
			97	Uncultured bacterium clone (EF123439)	Isolated from Black Band Diseased tissues of the coral *Siderastrea siderea*	Sekar et al. 2008	
			97	*Cohaesibacter* sp. (GQ200200)	Rhizobiales; Cohaesibacteraceae. Isolated from sediment of a seawater pond	Qu et al. 2011	

Sequence ID (Accession Number)	Tissue/ Location	Length (bp)	Similarity (%)	Closest relative in BLAST (Accession Number)	Description	Author	Family/Group in Greengens
P.o_rhiz_23b (JF292434)	Rhizome / Talamanca	518	92	Uncultured δ-proteobacteria (AY133092)	Isolated from TCE-contaminated site	Carrol and Zinder, Unpublished	*Desulfovibrionaceae*/ δ-proteobacteria
			92	Uncultured bacterium clone (GU118736)	Isolated from the coral *Montastraea franksi*	Sunagawa et al. 2010	
			91	Bacterium enrichment culture clone (HQ622261)	polluted estuarine sediment	Abed et al. Unpublished	
			91	*Desulfarculus baarsii* (CP002085)	Desulfarculales; Desulfarculaceae; Desulfarculus.	Lucas et al. Unpublished	
P.o_root_15c (JF292435)	Root / C. Marmacen	471	96	*Roseovarius sp.* (HQ871860 + HQ871851)	Rhodobacterales; Rhodobacteraceae; Roseovarius.	Jeanthon et al. Unpublished	*Rhodobacteraceae* / α-proteobacteria
			96	*Pelagibaca sp.* (EU440959)	Rhodobacterales; Rhodobacteraceae; Pelagibaca.	Yuan et al. Unpublished	
			96	Rhodobacterales bacterium (HQ537377 + HQ537273)	Isolated from 75 m depth on C-MORE BLOOMER cruise, Hawaii Ocean Time Series (HOT) station ALOHA	Sher et al. Unpublished	
			96	Uncultured Rhodobacterales bacterium (GU474886)	Isolated from Hawaii Oceanographic Time-series study site ALOHA	Rich et al. 2011	
			96	*Marinovum algicola* (FJ752526)	Rhodobacterales; Rhodobacteraceae; Marinovum.	Pradella et al. 2010	

Sequence ID (Accession Number)	Tissue/ Location	Length (bp)	Similarity (%)	Closest relative in BLAST (Accession Number)	Description	Author	Family/Group in Greengens
P.o_root_26c (JF292436)	Root / Magalluf	520	94	*Celerinatantimonas diazotrophica* (DQ913889)	N-fixing bacteria isolated from stuarine grasses *Spartina alterniflora* and *Juncus roemerianus*	Cramer in press	*Oceanimonaceae* / γ-proteobacteria
			93	*Celeribacter arcticus* gen. nov., sp. nov. (FJ039852)	Alteromonadales. Halophilic denitrifying bacteria isolated from water brine in Siberian permafrost	Shcherbakovaet al. Unpublished	
			92	Uncultured *Agarivorans sp.* (DQ647161)	Alteromonadales; Alteromonadaceae; Agarivorans;	Dahle et al. 2008	
			92	*Agarivorans* sp. (GQ200591)	Alteromonadales; Alteromonadaceae; Agarivorans. Isolated from surface of seaweeds	Du et al. 2011	
P.o_rhiz_0BDSB (JF292437)	Rhizome / C Torreta	391	91	Uncultured bacterium clone (GU946163)	Isolated from agricultural soil	Ros et al. Unpublished	*Sphingobacterriaceae* / Bacteroidetes
			90	*Pedobacter sp.* (AM988948)	Isolated from lake water.	Berg et al. 2009	
			90	*Pedobacter koreensis* (DQ092871)	Isolated from fresh water.	Baik et al. 2007	
P.o_rhiz_0BDSD (JF292438)	Rhizome / C.Torreta	412	96	Uncultured bacterium clone (HM125351)	Isolated from soils	Bissett Unpublished	*Sphingobacteriaceae* / Bacteroidetes
			95	Uncultured bacterium (AM158409)	Isolated from *Typha* rhizosphere in constructed wetlands	Saenz de Miera et al. Unpublished	
			95	Bacterium (FJ654260)	Isolated from soil	Kim et al. Unpublished	
			95	Uncultured bacterium clone (GU946163)	Isolated from agricultural soil	Ros et al. Unpublished	
			95	*Pedobacter sp.* (HM204919)	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Pedobacter	Im,W.-T., Unpublished	

Sequence ID (Accession Number)	Tissue/ Location	Length (bp)	Similarity (%)	Closest relative in BLAST (Accession Number)	Description	Author	Family/Group in Greengens
P.o_rhiz_0BDSG (JF292439)	Rhizome / C.Torreta	368	92	Unidentified bacterium clone (EF606109)	Isolated from rhizosphere soil from former arable field sown with low seeds diversity	Kielak et al. 2008	*Rhizobiaceae* / α-proteobacteria
			92	Uncultured bacterium clone (HM066499)	Isolated from environmental sample	Gray and Engel Unpublished	
			92	Uncultured bacterium clone (AB583099)	Isolated from soybean leaf	Ikeda et al. 2011	
			92	*Agrobacetrium tumefaciens* (HQ003411)	Rhizobiales; Rhizobiaceae; Rhizobium/Agrobacterium group; Agrobacterium. Isolated from a lake in India	Sahay et al. Unpublished	
			92	*Agrobacetrium tumefaciens* (FJ999942)	Rhizobiales; Rhizobiaceae; Rhizobium/Agrobacterium group; Agrobacterium. Isolated from a plant, endophytic microbiota.	Zheng and Feng Unpublished	
P.o_rhiz_0BDSJ (JF292440)	Rhizome / P. Colom	404	97	*Nautella italica* (HQ908722)	Rhodobacterales; Rhodobacteraceae; Nautella. Bacteria associated with sponges	Feby and Nair Unpublished	*Rhodobacteraceae* / α-proteobacteria
			97	*Nautella* sp. (HQ188608)	Rhodobacterales; Rhodobacteraceae; Nautella. Isolated from surface seawater	Cho and Hwang 2011	
			95	Uncultured bacterium clone (GU472165)	Isolated from BBD affected corals	Arotsker et al. Unpublished	
			97	*Ruegeria* sp. (GU176618)	Rhodobacterales; Rhodobacteraceae; Ruegeria. Isolated from surface of the red macroalgae, *Delisea pulchra*	Case and Kjelleberg Unpublished	
			97	Rhodobacteraceae bacterium (FJ937900)	Rhodobacterales; Rhodobacteraceae. Isolated from *Gelliodes carnosa* (marine sponge)	Li et al. Unpublished	
			97	Uncultured bacterium clone (FJ202604)	Isolated from *Montastraea faveolata* kept in aquarium for 23 days	Sunagawa et al. 2009	

Sequence ID (Accession Number)	Tissue/ Location	Length (bp)	Similarity (%)	Closest relative in BLAST (Accession Number)	Description	Author	Family/Group in Greengens
P.o_leaf_0BDVV (JF292441)	Leaf / Pujols	436	80	Uncultured γ-proteobacterium clone (DQ269096)	Isolated from surface of marine macro-alga *Delisea pulchra*	Longford et al. Unpublished	Sulfur-Oxidizing-Symbionts / γ-proteobacteria
			80	Uncultured γ-proteobacterium clone (FJ205337)	Isolated from deep marine sediments	Dong and Shao Unpublished	
			80	Uncultured bacterium clone (EU491600 + EU491489 + EU491463)	Isolated from seafloor lavas from the East Pacific Rise	Santelli et al. 2008	
			80	Uncultured γ-proteobacterium (AB611274)	Isolated from abdominal setae of galatheid crab (*Shinkaia crosnieri*) at the Hatoma Knoll in the Okinawa Trough	Yoshida-Takashima et al. Unpublished	
			80	Uncultured γ-proteobacterium clone (AY534017)	Isolated from oxic surface sediments of eastern Mediterranean Sea	Polymenakou et al. 2005	
P.o_leaf_0BDVW (JF292442)	Leaf / Pujols	467	95	Sphingobacteriales bacterium (FJ952766)	Isolated from healthy tissue of coral *Montastrea annularis*	Rypien et al. 2010	*Flammeovirgaceae* / Bacteroidetes
			95	*Flammeovirga aprica* (FJ917551)	Isolated from *Enteromorpha* (Green algae)	Zhang et al. Unpublished	
			95	*Flammeovirga* sp. (EF587968)	Isolated from natural subtidal biofilm	Huang et al. Unpublished	
			95	Uncultured bacterium clone (EF433127)	Isolated from *Favia* sp. mucus layer adjacent to Black Band mat	Barneah et al. 2007	
			95	*Flammeovirga* sp. (FN377813)	Bacteroidetes; Cytophagia; Cytophagales; Flammeovirgaceae; Flammeovirga. Isolated from a marine gastropod mollusk *Haliotis diversicolor*	Lu Unpublished	

Sequence ID (Accession Number)	Tissue/ Location	Length (bp)	Similarity (%)	Closest relative in BLAST (Accession Number)	Description	Author	Family/Group in Greengens
P.o_leaf_0BDVX (JF292443)	Leaf / Pujols	447	87	*Flammeovirga aprica* (FJ917551)	Isolated from *Enteromorpha* (Green algae)	Zhang et al. Unpublished	*Flammeovirgaceae* / Bacteroidetes
			86	Sphingobacteriales bacterium (FJ952766)	Isolated from healthy tissue of coral *Montastrea annularis*	Rypien et al. 2010	
			86	*Flammeovirga sp.* (EF587968)	Isolated from natural subtidal biofilm	Huang et al. Unpublished	
			86	Uncultured bacterium clone (EF433134)	Isolated from *Favia* sp. mucus layer adjacent to Black Band mat	Barneah et al. 2007	
			86	*Flammeovirga aprica* (FN554611)	Isolated from a marine gastropod mollusk *Haliotis diversicolor*	Zhao Unpublished	

We sequenced about 200 bands detected by DGGE analysis, trying to cover all identified OTUs. However, we only could sequence properly 12 bands corresponding to Bacteria. The 33.3% of the sequences analyzed belonged to class Bacteroidetes, while the rest (66.7%) belonged to the class Proteobacteria: 41.7% were affiliated to the α-subclass, 16.7% to the γ-subclass and 8.3% to the δ-subclass. More specifically 15.38% of the sequences belonged to the *Desulfovibrionaceae*, 15.38% to the *Flammeovirgaceae*, 15.38% to the *Rhodobacteracerae*, 15.38% were *Sphingobacteriaceae*, 15.38% non-identified Coral Black Band Disease isolates, 7.69% *Oceanimonaceae*, 7.69% *Rhizobiaceae* and 7.69% were non-identified Sulfur-Oxidizing-Symbionts. We identified 3 endophytic bacteria from leaf tissues, 7 from rhizome tissues, and 2 from roots tissues. The closest relative sequences to our OTUs are listed in Table 3 and the phylogenetic assignment is illustrated in Figure 4.

Discussion

The research reported here provides a pioneer step towards the characterization of the endophytic bacterial community associated with tissues of a marine angiosperm, both quantitatively (comparing DGGE band patterns) and qualitatively (sequencing the main OTUs found). Our results show that endophytic bacteria are prevalent in tissues of *P. oceanica* in the Balearic Islands, as only a modest percentage of samples (6.9% of roots, 18.6% of rhizomes and 11.4% of leaves) seemed to be free of endophytic bacteria.

Whereas our study appeared to yield a thorough inventory of OTUs in tissues of *P. oceanica,* the number of different OTUs identified in *P. oceanica* tissues, by DGGE analysis, appears relatively small, with 34 OTUs detected in *P. oceanica* tissues, which suggest that the endophytic microbiota must be highly specialized. Yet, this endophytic bacterial diversity is similar to that found in other plants, such as rice (*Oryza sativa*), where

52 different endophytic OTUs were identified by clone library. Moreover 60% of endophytic OTUs detected on rice were proteobacteria (Sun et al. 2008), similar to the percentage obtained for *P. oceanica* tissues. However, the endophytic bacterial community characterized by DGGE analysis in potato (*Solanum tuberosum*) and maize (*Zea mays*) plants was less diverse with 11 OTUs for potato plants (Garbeva et al. 2001) and 6 different bacterial species, all identified as proteobacteria, for maize roots (Seghers et al. 2004). When comparing with other marine organisms, the bacterial diversity described in *P. oceanica* is similar to that found in the marine sponge *Rhopaloides odorabile*, where 34 different bacterial sequences were obtained from clone library analysis of 3 samples (Webster et al. 2001). However, the bacterial diversity estimated for corals (*Montastraea franksi*, *Diploria strigosa*, and *Porites astreoides*), where 6000 different bacterial ribotypes were derived from statistical analysis, is much larger than that in *P. oceanica* tissues, as only 14 coral samples yielded 430 distinct bacterial ribotypes by clone library techniques (Rohwer et al. 2002). Yet this studied epiphytic bacterial and the fraction of the OTUs that were endophytic was not reported.

The comparison of patterns in endophytic bacterial communities between tissues suggested that bacteria associated with roots differs from that of rhizomes and leaves, confirmed by the R-values obtained in ANOSIM test, similar to differences in DGGE band patterns found among rice tissues (García de Salamone et al. 2010). Moreover, some OTUs were identified as indicator species in roots, rhizomes and leaves of *P. oceanica*. Differences in endophytic bacterial community patterns in *P. oceanica* due to the type of tissue were small, pointing out the importance of the meadow from which the tissue was collected, and therefore, the importance of environmental factors affecting each location.

However, the bacterial community composition seemed to be unrelated to the geographical location of the sampled meadows, derived from the results obtained by ANOSIM test when comparing the band profiles among islands ($R<0$; factor = ISLAND). Yet, the meadows sampled in each island, ranged from very pristine areas to very disturbed areas in all the islands. Thus, the degree of disturbance, pollution, organic matter inputs and human pressure differs largely among the different meadows sampled in each island, and therefore is likely that the main drivers affecting the composition of the endophytic bacterial community are other environmental parameters rather than just geographical location. Likewise, the patterns of bacterial communities associated with different tissues, are probably affected by the different environmental conditions. For example in an oligotrophic meadow, the bacterial community associated with roots may be different of that in eutrophic environments, as the requirements are not the same.

The sequenciation of the main OTUs detected by DGGE analysis, allowed us to draw the first characterization of the endophytic bacterial community in *Posidonia oceanica* tissues. The main group represented is the Proteobacteria class with a 66% of representative, being the α-subclass the majority group, as it is characteristic for marine environments. The other main group is represented by Bacteroidetes class, with many representatives characteristics of marine environments. From these OTUs identified as belonging to bacteria domain, most of them belonged to these groups or families: *Desulfovibrionaceae, Flammeovirgaceae, Rhodobacteracerae, Sphingobacteriaceae* and Non-identified Coral Black Band Disease isolates. Whereas, the families or groups less represented were: *Oceanimonaceae, Rhizobiaceae* and Non-identified Sulfur-Oxidizing-Symbionts.

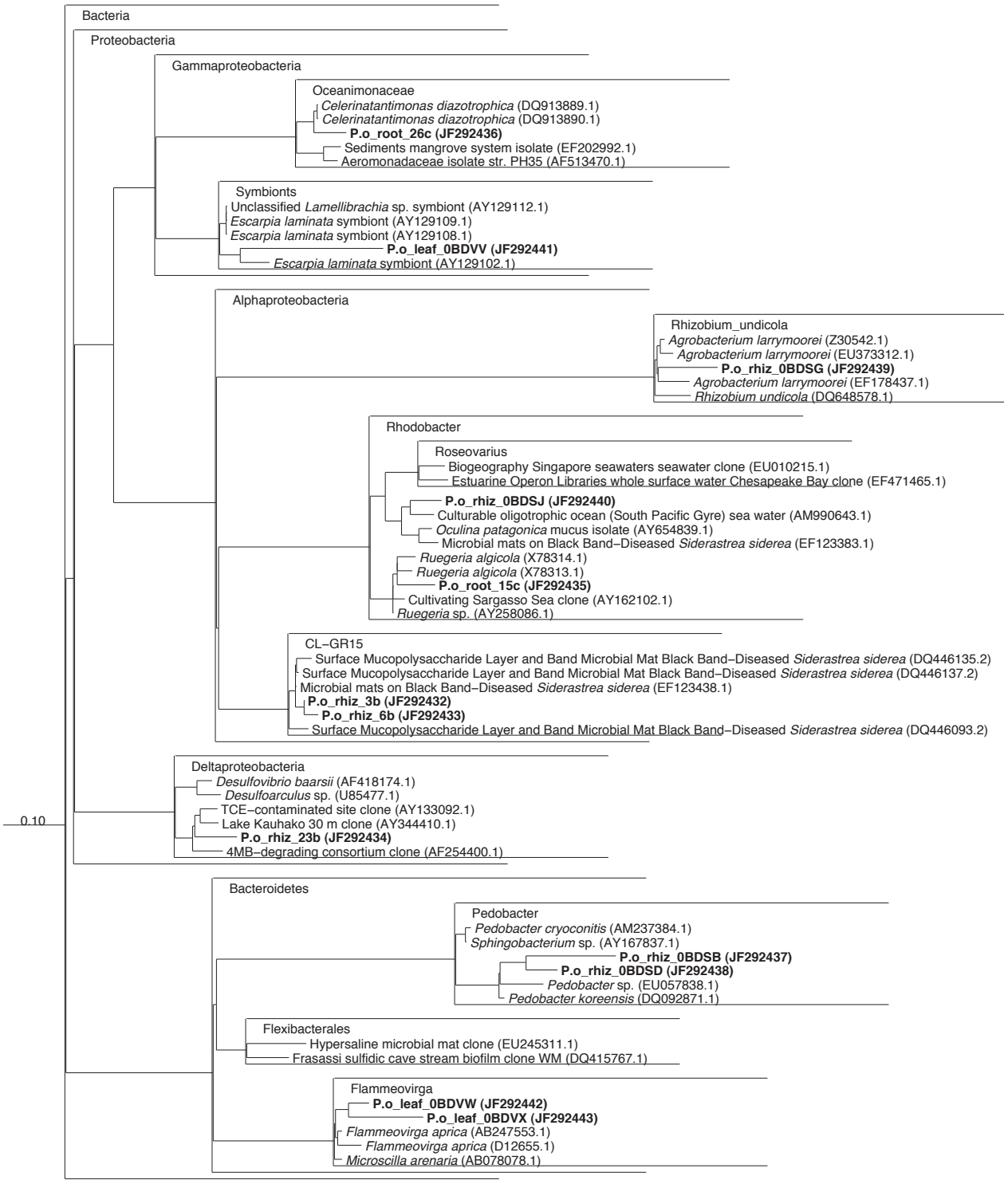

Figure 4. Phylogenetic position of the sequences obtained from endophytic bacterial community of *P. oceanica* in ARB.

The sequences with the accession numbers JF292432 and JF292433, both found in rhizome samples, were very similar in sequence (99% and 97%, respectively) to sequences of bacteria found in coral tissues, mainly related to diseased coral tissue (Table 3). The phylogenetic assignment in ARB, positioned these sequences in the cluster corresponding to α-proteobateria, specifically in the cluster identified as Non-Identified Coral BBD isolates (Fig. 4). The identification of bacteria very similar in sequence to those found in diseased coral tissues opens a new and exciting venue, as there is no evidence, to our knowledge, of specific bacterial pathogens of seagrasses. However, further research is needed in order to clearly elucidate the role these endophytic bacteria can play in seagrass tissues, because some of them were identified in association with macroalgae without relation to diseases (Table 3).

The sequence with the accession number JF292440, isolated from a rhizome sample was very similar (97-95%) to sequences of uncultured bacteria found in corals, and BBD corals. However, this sequence was also similar (97%) to sequences of *Nautella italica* (HQ908722) and *Rhodobacteraceae* bacterium (FJ937900) found in association with marine sponges and very similar (97%) to sequences of *Ruegeria* sp. (GU176618) isolated from surface of the red macroalgae, *Delisea pulchra* (Table 3). The phylogenetic analysis in ARB, assigned this sequence to the *Rhodobacteraceae* within the α-proteobateria cluster (Fig. 4).

The sequences with the accession number JF292442 and JF292443, isolated from leaves samples were similar (95% and 86%, respectively) to sequences of *Sphingobacteriales* bacterium (FJ952766) isolated from healthy tissue of coral *Montastrea annularis* and to sequences of an uncultured bacterium clone (EF433127) isolated from

coral *Favia* sp. mucus layer adjacent to Black Band mat. These sequences were also similar (95% and 86-87%, respectivelly) to sequences of *Flammeovirga* sp. (FN377813) isolated from a marine gastropod mollusk *Haliotis diversicolor* and to *Flammeovirga aprica* (FJ917551) isolated from the green algae *Enteromorpha* (Table 3). Phylogenetic analysis in ARB, assigned these sequences to the *Flammeovirgaceae* (Bacteroidetes) (Fig.4).

The closest Genbank match (94%) to sequence JF292436, found in a root sample, was the nitrogen-fixing bacterium *Celerinatantimonas diazotrophica* (DQ913889) isolated from estuarine grasses *Spartina alterniflora* and *Juncus roemerianus* (Table 3). More precise assignment in ARB, assigned this sequence in the cluster corresponding to *Oceanimonaceae* in γ-proteobacteria (Fig.4).

The closest Genbank match (92%) to sequence JF292439, corresponding to *P. oceanica* rhizome sample was *Agrobacetrium tumefaciens* (HQ003411 and FJ999942) isolated from a lake and from a plant, being endophytic microbiota. This sequence was also similar (92%) to sequences of an uncultured bacterium clone (AB583099) isolated from soybean leaf. Moreover, this sequence was also similar (92%) to sequences of uncultured bacteria (EF606109 and HM066499) isolated from sediments and rhizosphere (Table 3). Despite the relatively low similarity values to the closest Genbank relatives, phylogenetic analysis in ARB positioned this sequence in the cluster corresponding to *Rhizobiaceae* in the α-proteobacteria (Fig. 4). Many species of *Rhizobiaceae* are diazotrophs able to fix nitrogen in symbiosis with plants.

The identification of bacteria related to well known nitrogen fixers is especially interesting because the Mediterranean sediments are known to be oligotrophic and the

existence of bacteria with capabilities of shaping the nutrient conditions may have a beneficial role in the establishment, growth and survival of *P. oceanica* in this environment. This is particularly the case for bacteria belonging to the *Rhizobiaceae,* associated to marine plants, as *Agrobacterium* species are aerobic bacteria that can live free as well as some strains are responsible of tumor formation in terrestrial plants. In fact, there is a marine subdivision of *Agrobacterium* species (Uchino et al. 1997), although their role is still not clear. Specific research will be needed in order to elucidate the possibility and the role of endophytic nitrogen-fixing bacteria in *P. oceanica* roots.

The sequence with the accession number JF292434, corresponding to *P. oceanica* rhizome sample was similar, although with low percentage of similarity (92%) to sequences of uncultured bacterium clone (GU118736) isolated from the coral *Montastraea franksi*; to sequences of uncultured bacteria related to contaminated sediments and also similar (91%) to *Desulfarculus baarsii* (CP002085) (Table 3). The ARB analysis positioned this sequence in the cluster of *Desulfovibrionaceae* within the δ-proteobacteria (Fig. 4).

The sequence with the accession number JF292441, from leaf sample was similar, although with low percentage of similarity (80%) to sequences of uncultured γ-proteobacteria, isolated from the surface of marine macroalgae *Delisea pulchra* (DQ269096), deep marine sediments (FJ205337), seafloor lavas from the East Pacific Rise (EU491600, EU491489 and EU491463), abdominal setae of galatheid crab *Shinkaia crosnieri* (AB611274) and oxic surface sediments of eastern Mediterranean Sea (Table3). The phylogenetic analysis in ARB, assigned this sequence to the cluster corresponding to Sulfur-Oxidizing-Symbionts in γ-proteobacteria (Fig. 4).

The presence of Sulfur-Oxidizing bacteria capable of oxidizing the sulfide to elemental sulfur, would have an important role in detoxifying sediments because hydrogen sulfide produced as a consequence of organic matter decomposition is toxic for plants (Garcias-Bonet et al. 2008). This would be particularly beneficial for *P. oceanica* survival in carbonate and iron poor sediments, characteristic of the Balearic coast and many other Mediterranean areas, where low iron available in these sediments prevents formation of iron sulfur compounds, and thus even small inputs of organic matter are able to enhance porewater hydrogen sulfide concentration (Holmer et al. 2003, Marbà et al. 2007).

The sequences with the accession numbers JF292437 and JF292438, corresponding to rhizome samples were similar (91-90% and 96-95%, respectively) to sequences of uncultured bacteria isolated from agricultural soils and were similar also to *Pedobacter* sp. (AM988948, DQ092871 and HM204919) (Table 3). The phylogenetic analysis in ARB, positioned these two sequences in the cluster corresponding to *Sphingobacterriaceae* within the Bacteroidetes class (Fig. 4).

The sequence with the accession number JF292435, isolated from rhizome sample was similar (96%) to uncultured Rhodobacterales bacterium clones (HQ537377, HQ537273 and GU474886) and different bacterial genus: *Roseovarius* sp. (HQ871860 and HQ871851), *Pelagibaca* sp. (EU440959) and *Marinovum algicola* (FJ752526) (Table 3). The phylogenetic analysis in ARB, assigned this sequence to the *Rhodobacteraceae* within the α-proteobacteria (Fig. 4).

In summary, this work is the first characterization of endophytic bacterial community in *Posidonia oceanica* tissues, suggesting an specialization of bacterial species found in

roots. However further research is needed to explain the different patterns observed across tissues and meadows. Moreover, this work represents the first identification of endophytic bacterial present in *P. oceanica* tissues, with very suggestive results. Some of the sequences were closely related to major groups of bacteria able to fix nitrogen, some others related to the sulfur cycle and finally a group of sequences had their closest known relatives among those found in corals affected by black band disease. It is not possible to infer whether or not the functional genes and capacities associated to the closest matching relatives will be present in our samples, due to the low similarity of some sequences to known cultured bacteria or even to environmental sequences. However, the fact that the closest matches are related to these three categories suggests that endophytic bacteria may play an important role in the health of *P. oceanica*, by providing nitrogen or protecting the plants against the invasion of toxic sulfides, whereas some others may be pathogenic. Moreover, the low sequence similarity to previously reported sequences in Genbank indicates that many of these sequences correspond to unknown bacteria some of which could be specific of *P. oceanica* tissues. Subsequent research should include a search for functional genes involved in nitrogen fixation and the Sulfur cycle and also a more detailed study on healthy vs. damaged tissues of *P. oceanica*, which could lead to the discover of new pathogens of marine angiosperms.

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Chapter 4

Endophytic nitrogen-fixing bacteria in surface-sterilized roots of *Posidonia oceanica* seagrass

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Manuscript

Abstract

Seagrass *Posidonia oceanica* roots were analyzed for the presence of endophytic diazotrophs in 26 meadows located at Balearic Islands (Western Mediterranean). The presence of endophytic nitrogen-fixing bacteria in surface-sterilized roots of seagrass was detected by amplification of a fragment of the nitrogenase gene (*nifH*), and analyzed by Denaturing Gradient Gel Electrophoresis (DGGE). The detected bands were sequenced and compared with a *nifH* gene database, containing 16,989 sequences from known and unknown diazotrophs. We detected *nifH* gene amplification in 13 out of the 78 roots sampled, corresponding to 9 locations. Our results confirmed the presence of endophytic nitrogen-fixing bacteria in *P. oceanica* roots. Only two different bands were detected in DGGE gels. Sequence analysis allowed classifying the endophytic nitrogen-fixing bacteria inhabiting *P. oceanica* roots as a proteobacterium closely related to sequences isolated from the rhizosphere of salt marsh cord grass and a putative anaerobe. The finding of only two different *nifH* sequences suggests a species-specific relation between endophytic diazotrophs and *P. oceanica* seagrass, revealing a possible symbiotic interaction that could play a major role in nitrogen acquisition by seagrasses in oligotrophic environments where they form lush meadows.

Introduction

Seagrasses are clonal marine angiosperms that form lush and highly productive meadows in often nutrient-poor coastal areas around the world (Hemminga and Duarte 2000), where nutrients often limit their growth (e.g. Short et al. 1985, Powell et al. 1989, Perez et al. 1990, Lee et al. 2007). Seagrasses acquire inorganic (e.g. Touchette and Burkholder 2000, Lee et al. 2007) and organic (Vonk et al. 2008) nutrients, supplied from land (e.g. run off, riverine, agriculture, groundwater and sewage discharges) and sediment mineralization, through leaves and roots. Moreover, atmospheric nitrogen has been identified as an important additional source of nitrogen to fulfill seagrass nutrient requirements (Patriquin 1972, O'Donohue et al. 1991, Welsh 2000). To date, nitrogen fixation in seagrass meadows has been described in the sediments, the rhizosphere and leaf epiphytes (Patriquin 1972, Bagwell et al. 2002, Lyimo and Hamisi 2008). Contrary to what occurs in their terrestrial relatives, the presence of endophytic bacteria able to acquire atmospheric nitrogen has not been reported in seagrasses.

Biological nitrogen fixation is the main process by which atmospheric nitrogen is converted to nitrates and nitrites and incorporated into the food web. The main enzymatic complex involved in this process is nitrogenase, and the organisms capable of such nitrogen fixation are diazotrophic bacteria. Diazotrophs can live as free-living organisms or as symbionts of plants, forming nodules or not (Dresler-Nurmi et al. 2009). In terrestrial plants, nitrogen-fixing bacteria a re able to enter into roots from the rhizosphere, particularly at the base of emerging lateral roots, between epidermal cells and trough roots hairs, colonizing endophytically plant tissues (Cocking 2003). There is no evidence of nodule formation in seagrasses, despite many years of targeted searching (Nielsen et al.

1999). On the contrary, the scarce information available on seagrass endophytic microbiota reveals that endophytic bacteria is often present in seagrasses tissues (Nielsen et al. 1999, Küsel et al. 1999, Finster et al. 2001, Chapter 3) and recently a bacterial sequence highly similar to a diazotroph has been found in the dominant, endemic and long living Mediterranean seagrass *Posidonia oceanica* (Chapter 3). These recent findings point out that seagrass tissues may contain diazotrophic endophytes, which could help explaining the successful seagrass colonization of oligotrophic environments.

The nitrogenase enzyme is a multisubunit protein highly regulated by transcriptional and post-transcriptional controls. The nitrogenase enzyme consists of 2 proteins: dinitrogenase containing Fe and Mo formed by α-subunit (encoded by *nifD* gene) and β-subunit (encoded by *nifK* gene); and dinitrogenase reductase, a dimer of two identical iron-containing subunits (encoded by *nifH* gene) (Madigan et al. 2003). The dinitrogenase genes (*nifHDK*) are highly conserved and show a high degree of similarity among organisms. The *nifH* gene can be found in a variable number of copies in a single organism, yet its sequence variability shows good correspondence with the taxonomic affiliation of diazotrophic bacteria (Zehr and Capone 1996). Thus, sequences of an amplified fragment of *nifH* gene can be used to determine the taxonomic affiliation of uncultured and unknown environmental nitrogen-fixing bacteria (Zehr et al. 1995, Ueda et al. 1995). The detection of the nitrogenase gene has been widely used in biological samples as an indicator of nitrogen-fixing capabilities and also to estimate the diversity of diazotrophic communities in many environments (e.g. salt marsh rhizospheres, Cyanobacterial mats, seagrass rhizospheres; Piceno et al. 1999, Zerh et al. 1995, Bagwell et al. 2002). Different sets of primers have been designed in order to detect nitrogen-fixing bacteria in natural samples.

The development of degenerated sets of primers for the *nifH* gene allowed the detection of all groups of known diazotrophs (Zehr and McReynolds 1989). These amplified sequences can subsequently be separated by DGGE allowing the characterization of the diversity of diazotrophic communities in nature (Lovell et al. 2008). Sequence analysis of *nifH* genes results in 5 major clusters (Gaby and Buckley 2011). Cluster I groups *nifH* genes from most Proteobacteria, all Cyanobacteria, some Firmicutes and Actinobacteria while sequences from alternative FeV and FeFe nitrogenases and sequences from some methanogenic *Archaea* fall in Cluster II and the *nifH* sequences from anaerobic Bacteria and Archaea fall in Cluster III. Finally, paralogous sequences not involved in nitrogen fixation fall into Clusters IV and V. Thus, it is possible to assign environmental sequences to one of these large, loosely-defined taxonomic groups even when no close relative is available in public sequence databases.

In this study, we screened surface-sterilized roots collected from 26 meadows of *Posidonia oceanica* in Balearic Coasts (Western Mediterranean) for the presence of endophytic nitrogen-fixing bacteria by amplification of *nifH* genes. When a positive result was found, we separated and characterized the diversity of amplified *nifH* genes by DGGE. The resulting bands were sequenced and the sequences obtained were used to identify the source organisms by comparison to an aligned database of *nifH* genes (Gaby and Buckley 2011). These analyses allowed us to identify the nitrogen-fixing bacterial endophytes found in *P. oceanica* roots and their diversity.

Materials and Methods

Sampling strategy. Triplicate samples of *Posidonia oceanica* containing leaf shoots, rhizome and roots were randomly collected at 26 locations spread along the Balearic Islands (Fig. 1), by scuba diving during the summers of 2005 and 2006.

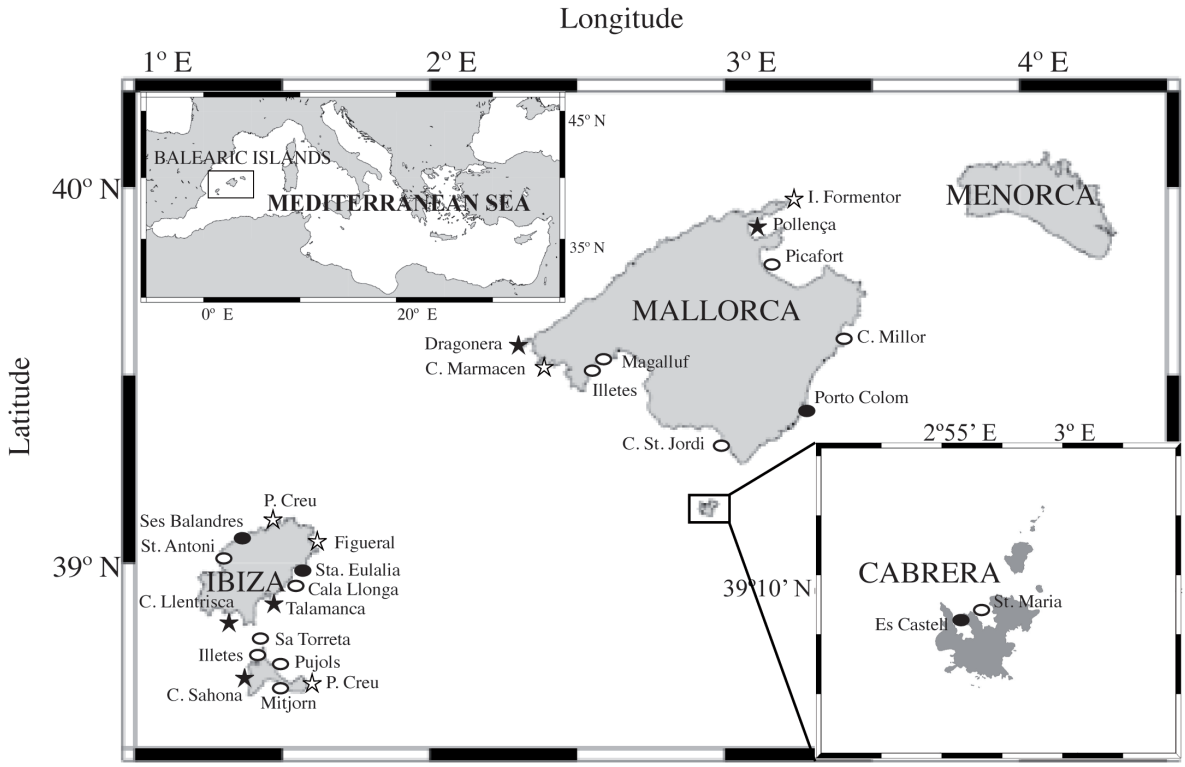

Figure 1. Location of *Posidonia oceanica* meadows sampled during two consecutive sampling seasons in summer 2005 (circles) and summer 2006 (stars). Filled circles and stars indicate those meadows where endophytic N-fixers were detected by amplification of *nifH* gene in surface-sterilized roots.

The plants were transported to the laboratory in seawater from the same location and processed immediately. The plant material was separated into roots, rhizomes and leaves and subsequently subjected to a surface-sterilization protocol adapted from Coombs and Franco (2003). Briefly, the protocol consisted in immersing each sample in ethanol 99% for 1 minute, then in NaOCl 3.125% for 6 minutes, then in ethanol 99% for 30 seconds and

finally washing gently with autoclaved seawater. The surface-sterilized roots (2 roots per replicate) were frozen in liquid nitrogen until nucleic acid extraction was performed.

In addition, the youngest leaf (free of epiphytes) of three *P. oceanica* shoots and three young rhizome fragments were collected in each meadow to analyze nitrogen isotopic composition.

Isotopic composition of nitrogen content in seagrass tissues. Isotopic analyses were conducted as described by Fourqurean et al. (2007) for three replicate samples of seagrass tissues. The samples were dried at 60ºC for 48 h and ground to a fine powder using a motorized agate mortar and pestle. All isotopic analyses were measured using standard elemental analyzer isotope ratio mass spectrometer (EA-IRMS) procedures. The EA was used to combust the organic material and to reduce the formed gas into N2, which was measured on a Finnigan MAT Delta C IRMS in a continuous flow mode. Isotopic ratios (R) are reported in the standard delta notation (δ, ‰),

$$\delta_{sample} = 1000\ [(R_{sample}/R_{standard}) - 1],$$

where $R = {}^{15}N/{}^{14}N$. These results are presented with respect to the International standard of atmospheric nitrogen (AIR, N2). Analytical reproducibility of the reported d values, based on sample replicates, was better than ±0.2‰.

Nucleic Acid extraction. Surface-sterilized roots (100 mg of fresh tissue from one or two roots per sample in triplicate) were ground with the help of a sterilized tool inside an eppendorf tube prior to nucleic acid extraction. The total nucleic acid extraction was performed with the help of a commercial kit specific for plant tissues (Partec®). Nucleic acid extracts were stored at -20ºC.

PCR. The DNA extract, containing plant and endophyte DNA when present, was amplified by PCR with degenerated primers for *nifH* gene sequences containing a GC-clamp for DGGE analysis described previously by Piceno et al. (1999). Each 20 µl PCR reaction containing template DNA plus (final concentrations) 2mM of dNTPs mixture, 20 µM of each primer and 0.5 units of *Taq* Polimerase (Takara) suspended in the buffer provided by the manufacturer of the polymerase. Additional negative (no DNA) and two positive control reactions were run with each batch of PCR reactions. Two known diazotrophic bacteria were used as positive controls: *Mesorhizobium ciceri* (DSM 1978) and *Vibrio diazotrophicus* (DSM 2605) DNA provided by DSMZ (Germany). The PCR protocol, slightly modified from Piceno et al. (1999), consisted of an initial denaturing step at 94ºC for 5 min, followed by 20 touchdown cycles (94ºC for 1 min, 58ºC for 1 min (decreased by 0.5ºC $cycle^{-1}$) and 72ºC for 1 min); and 10 cycles of standard amplification (94ºC for 1 min, 48ºC for 1 min and 72ºC for 1 min) with a final elongation step of 72ºC for 7 min.(The PCR products were checked for the presence or absence of a product of adequate size by electrophoresis on 1.5% agarose gels. For each positive sample, the products of several replicate reactions (minimum of 2) were pooled prior to DGGE in order to load approximately 1 µg of PCR product per lane on the DGGE gel.

Denaturing Gradient Gel Electrophoresis (DGGE). The amplification products of the fragment of the *nifH* gene were separated by DGGE in a 6.5% polyacrylamide gel containing a gradient of denaturants ranging from 72.5% to 95% (where 100% is 7 M urea and 40% formamide). Gels were run for 9 h at 200 V in 1X TAE (Tris-Acetate-EDTA) buffer at 48º C in a CBS Scientific Co. DGGE system. Following electrophoresis, the gels were stained with SyberGold for 30 min in the dark and photographed using a G:BOX

imaging system (Syngene). All the detectable bands were excised and stored frozen in 20 μL of autoclaved Mili-Q water at -20ºC for further processing.

Sequencing of bands detected in DGGE. The reamplification of the excised bands (OTUs) was conducted using the same pair of primers used before. Several PCRs were done using, as template, the DNA of the band excised. The amplification products were pooled together, precipitated and concentrated. The concentrated amplification products were cleaned and purified from primers and dNTPs using a commercial kit (MSB Spin PCRapace) Finally, we quantified the purified product and 150 ng of the amplified product was used for the sequencing reaction with the forward primer (not containing the GC-clamp). The sequencing was performed on an ABI 3730 sequencer (Applied Biosystems), using the chemistry BigDye ® Terminator v3.1. The sequences obtained in this study have been deposited in GenBank under the accession numbers JN987872 and JN987873.

Sequence analysis. The sequences (~400 bp) were checked for existence of chimeras using the Bellerophon tool available at http://greengenes.lbl.gov and the primer sequences were removed. The sequences were imported into an ARB (Ludwig et al. 2004) database including all *nifH* sequences available in GenBank until April 2009 (16,989 sequences), compiled and constructed by Gaby and Buckley (2011). The sequences were aligned with the guide of a PT-server constructed from all the *nifH* sequences already aligned in the database. The resulting alignment was checked manually and the edges were manually re-aligned when necessary. The aligned sequences were added by maximum parsimony into the existent tree constructed with the *nifH* sequences on the database, in order to assess their phylogenetic identity.

Table 1. Nitrogen stable isotopic composition of *Posidonia oceanica* leaves and rhizomes in the 26 sampled meadows around Balearic Islands, as average (AVG) of $\delta^{15}N$ and the standard error (SE) of 3 replicates. Those meadows where *nifH* gene was detectable are indicated with a plus sign (+).

Sampling Site	Island	*nifH* gene detetction	δ15N of *P. oceanica* leaves		δ15N of *P. oceanica* rhizomes	
			AVG	SE	AVG	SE
C. Sahona	Formentera	+	2.28	0.16	3.18	0.51
C. Llentrisca	Ibiza	+	3.65	0.05	4.66	0.17
Talamanca	Ibiza	+	4.31	0.10	4.60	0.63
Sta. Eulàlia	Ibiza	+	7.41	0.34	6.09	0.67
Ses Balandres	Ibiza	+	3.13	0.11	3.31	0.09
Pollença	Mallorca	+	3.33	0.12	3.13	0.28
Porto Colom	Mallorca	+	5.39	0.24	5.83	0.18
Es Castell	Cabrera	+	2.58	0.30	2.43	0.30
Dragonera	Mallorca	+	3.77	0.33	2.94	0.11
Figueral	Ibiza	-	2.65	0.19	3.40	0.31
Cala Llonga	Ibiza	-	4.50	0.19	4.28	0.40
P. Creu	Ibiza	-	4.29	0.09	3.69	1.23
P. Creu	Formentera	-	n.d	n.d	3.24	1.17
Illetes	Mallorca	-	5.66	0.29	5.62	0.16
Magalluf	Mallorca	-	4.08	0.11	3.96	0.35
C. Marmacen	Mallorca	-	3.78	0.16	3.34	0.13
Els Pujols	Formentera	-	3.28	n.d.	2.61	0.21
Mitjorn	Formentera	-	2.50	0.28	2.95	0.37
Illetes	Formentera	-	2.60	0.70	0.69	n.d
Sa Torreta	Formentera	-	2.80	0.30	2.58	0.16
St. Antoni	Ibiza	-	3.53	0.26	3.45	0.36
I. Formentor	Mallorca	-	3.55	0.20	3.48	0.39
Picafort	Mallorca	-	7.57	0.13	7.62	0.28
C. Millor	Mallorca	-	5.50	0.22	5.47	0.30
St. Maria	Cabrera	-	3.40	0.33	2.56	0.24
C. St. Jordi	Mallorca	-	6.27	0.17	6.17	0.17
TOTAL AVERAGE			4.07	0.29	3.90	0.30

Results

The *nifH* gene could be amplified in 13 samples of *Posidonia oceanica* roots (out of the total 78 samples), corresponding to 9 locations (out of 26 meadows, Fig. 1). In Mallorca Island, *nifH* sequences were detected in 3 out of the 10 sampled meadows (30%); in Ibiza and Cabrera Islands *nifH* sequences were detected in 50% of the sampled locations (4 out

of the 8 sampled meadows, and 1 out of the 2 sampled meadows, respectively); and in Formentera Island only 1 out of the 6 sampled locations (17%). However, the *nifH* gene could not be amplified and detected in all replicates of the same location. The finding of nitrogen-fixing bacteria by detection of *nifH* gene was in agreement with the average low isotopic nitrogen content ($\delta^{15}N$) of *P. oceanica* tissues (4.07‰ ± 0.29 and 3.9‰ ± 0.3 in leaves and rhizomes, respectively, Table 1).

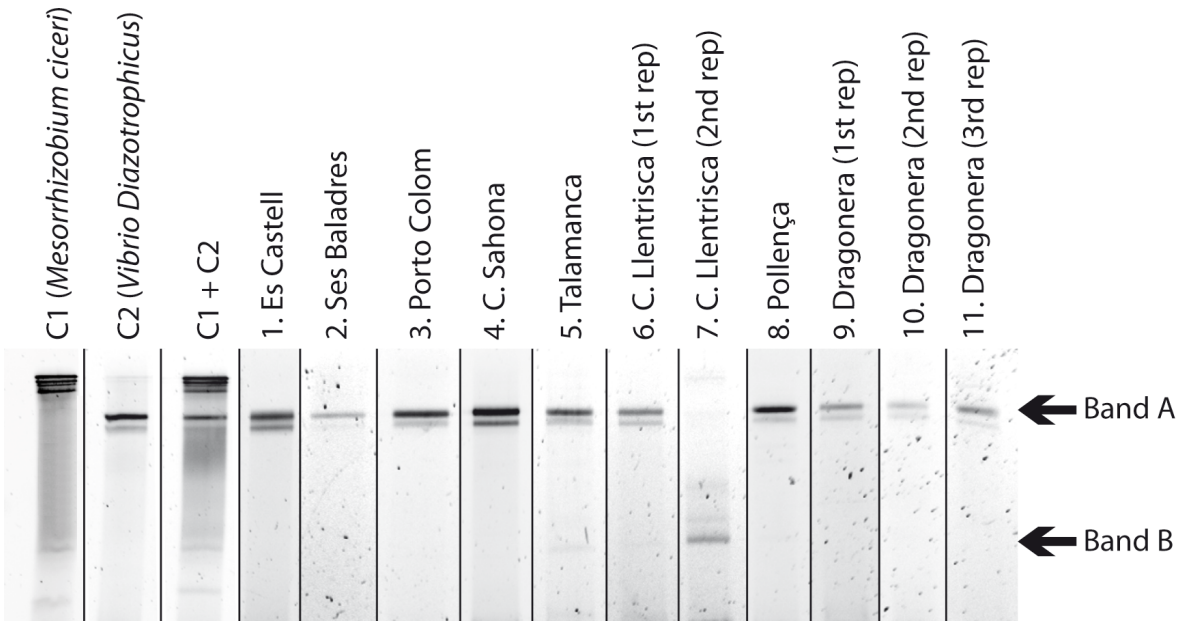

Figure 2. DGGE image showing the band patterns generated by the amplification products of the *nifH* gene from surface-sterilized roots of *P. oceanica*. The lane C1 and C2 belong to the amplified positive control *nifH* gene from *Mesorrhizobium ciceri* and *Vibrio diazotrophicus,* respectively. The lane C1+C2 is a mixture of the both amplified *nifH* genes from positive controls. The lanes 1 to 11 belong to *nifH* gene amplimers from surface-sterilized roots of *P. oceanica* collected in different locations along Balearic Islands (Mediterranean Sea). Arrows indicate the positions of Band A (P.o_root_A_nifH) and Band B (P.o_root_B_nifH).

The DGGE analysis of the amplified *nifH* gene could be resolved for 11 of the 13 samples where *nifH* was amplified and detected, yielding a clear and homogenous band profile among all samples (Fig. 2), revealing a low diversity of nitrogen-fixing endophytes.

Only two different bands were detected (Band A-P.o_root_A_nifH- and Band B-P.o_root_B_nifH), which were never found simultaneously in the same root sample. Sequencing all the detected DGGE bands yielded only 2 different nifH sequences. One sequence corresponded to Band A (P.o_root_A_nifH, JN987872) and the other corresponded to Band B (P.o_root_B_nifH, JN987873, Fig. 2). The *nifH* sequences obtained from Band A in all lanes were identical, and they were different from the sequence obtained from Band B, only detected in one DGGE lane (Lane 7) of one root sample collected in one location at Ibiza Island (C. Llentrisca, Fig. 2).

According to our ARB analysis, the different *nifH* sequences obtained from *P. oceanica* roots were affiliated to two different clusters. The sequence obtained from Band A (P.o_root_A_nifH, JN987872) fell into cluster I (Fig. 3), which contains *nifH* genes from most Proteobacteria, all Cyanobacteria and some Firmicutes and Actinobacteria (Gaby and Buckley, 2011). The closest relative of the sequence obtained from Band A (P.o_root_A_nifH, JN987872) with 87.24% of similarity, was a *nifH* sequence (DQ402931) amplified from a salt marsh cord grass (*Spartina alterniflora*) rhizosphere (Lovell et al. 2008) that was very similar to the N-fixing *Vibrio diazotrophicus* (AF111110). The other closest relatives were unknown diazotrophs isolated from diverse environments, as seawater, soil and mangrove sediments (Fig. 4A), although with lower similarity (from 79.5% to 87%). The sequence obtained from Band B (P.o_root_B_nifH, JN987873) fell into cluster III (Fig. 3), which contains *nifH* genes from anaerobic diazotrophs (Gaby and Buckley 2011). The closest relatives of Band B sequence (P.o_root_B_nifH, JN987873) were two *nifH* sequences (AY091864 and DQ402844, with 83.16% and 82.62% of similarity, respectively) that belong to uncultured bacterium clones from the rhizosphere of

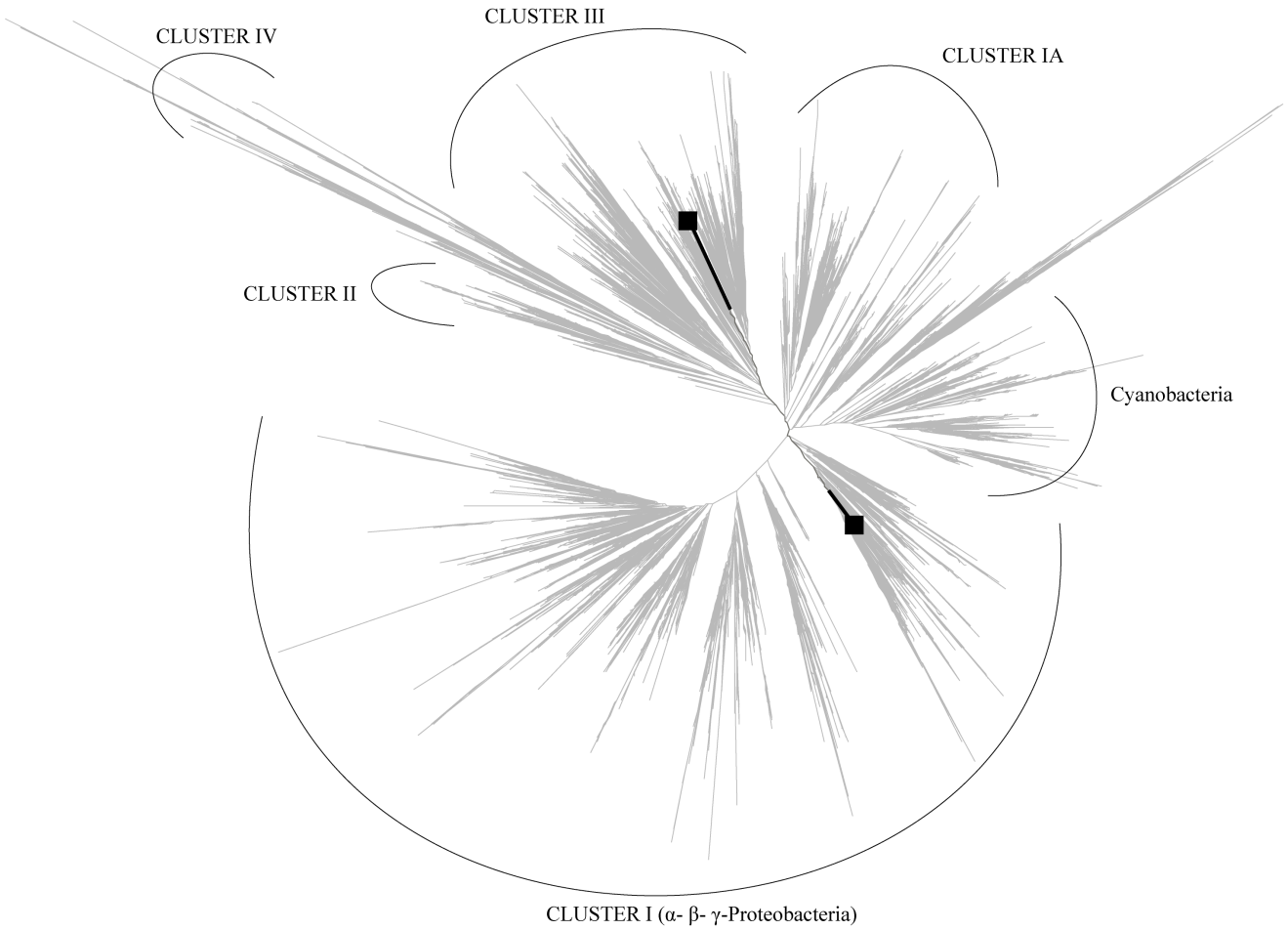

Figure 3. Phylogenetic affiliation of *nifH* sequences obtained from *Posidonia oceanica* surface-sterilized roots. The *nifH* sequences obtained in this study are in black and marked with a black square at the edge. Cluster labeling according to Gaby and Buckley (2011).

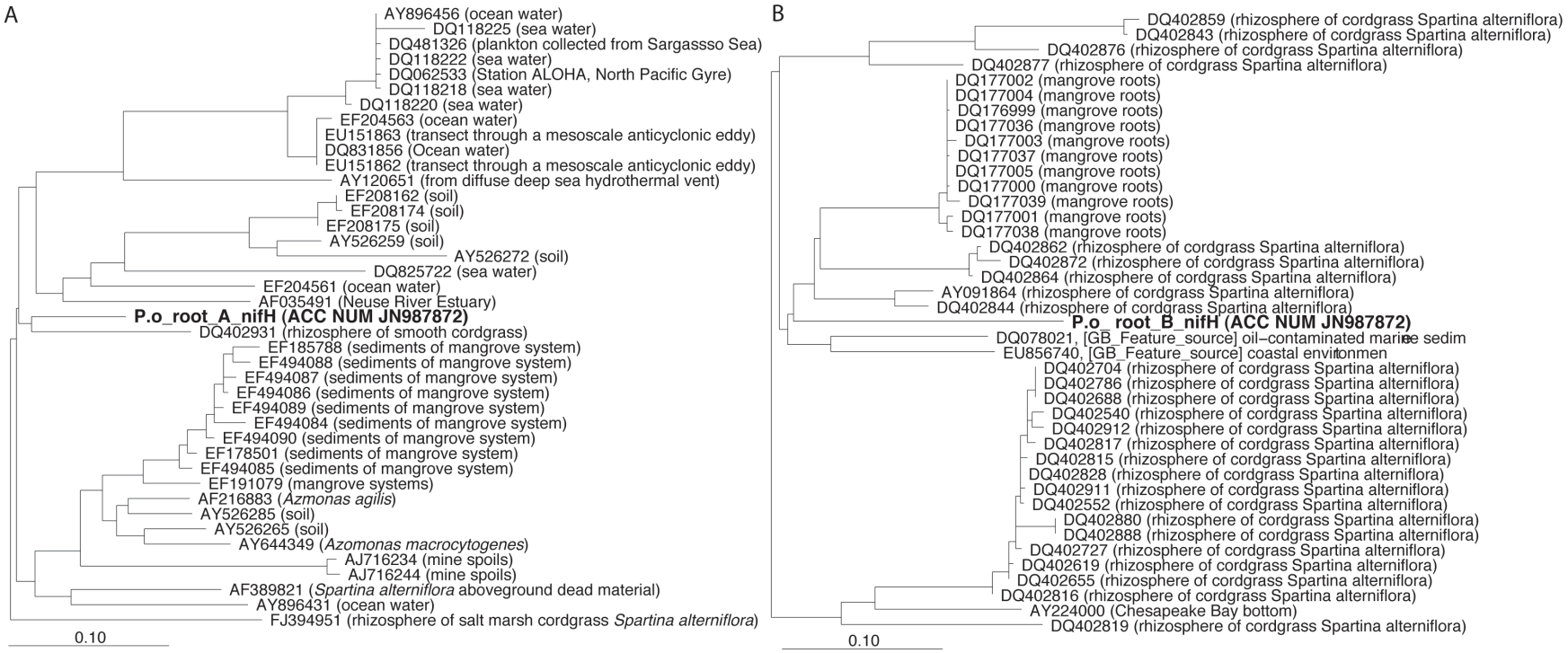

Figure 4. Phylogenetic analysis in ARB of *nifH* sequences obtained from *Posidonia oceanica* surface-sterilized roots, showing the 40 closest relatives for both of the 2 different *nifH* sequences obtained from endophytic N-fixers. Panel A corresponds to P.o_root_A_nifH, JN987872. Panel B corresponds to P.o_root_B_nifH, JN987873.

salt marsh cord grass (*Spartina alterniflora*, Lovell et al. 2008), without any cultured close relative, preventing its taxonomic identification. The other closest relatives (with 82% of similarity) were also unknown diazotrophs isolated from *S. Alterniflora* rhizosphere and non-sterilized mangrove roots (Flores-Mireles et al. 2007, Fig. 4B).

Discussion

These results demonstrate for the first time the presence of endophytic nitrogen-fixing bacteria in seagrass (*P. oceanica*) roots, despite the possibility of the presence of seagrass endophytic bacteria very similar to bacteria with nitrogen-fixing capabilities in *P. oceanica* has been recently suggested (Chapter 3). The use of primers specific for the *nifH* gene, has allowed us to demonstrate the genetic machinery needed for nitrogen fixation is present inside seagrass surface-sterilized roots. Diazotrophic endophytes have been described also in roots of salt marsh grasses, as *Spartina alterniflora* and *Leptochloa flusca* (McClung and Patriquin 1980, Hurek et al. 1994), and identified as *Campylobacter* spp. and *Azoarcus* spp., respectively. Similarly, the existence of nitrogen-fixing endophytes has been widely described for many terrestrial plants. For instance, crop species as rice contain diazotrophic endophytes (Ebeltagy et al. 2001, Govinderajan et al. 2008) as components of a highly diverse bacterial community (Ueda et al. 1995). The finding of diazotrophs in seagrass tissues indicates the presence of symbiotic bacteria that might play an important role in seagrass nitrogen acquisition, particularly in nutrient poor environments where they form lush and extensive meadows.

The fact that endophytic diazotrophs were detected in 13 samples out of the total 78 samples screened and in 9 locations out of the total 26 sampled locations, indicates that

endophytic diazotrophs may not be present in all roots, but it reveals that endophytic diazotrophs are a common feature of the seagrass *P. oceanica*. Moreover, the absence of diazotrophs in some samples from the same meadow, suggests that not all the plants in the same location host endophytic diazotrophs or that these endophytes are not homogenously distributed along the roots, and may escape detection in small sections of root tissue. However, in terrestrial plants (e.g. rice) the N-fixing endophytes are located within root apoplast (i. e. intercellular spaces and/or the xylem vessels, James et al. 2002), suggesting a non-patchy distribution along roots. Anyway, the clonal architecture of seagrasses allows mobilization and sharing of nutrients acquired at different regions of the clone with their neighbors (Marbà et al. 2002). Specifically, for *P. oceanica* meadows, a translocation experiment using labeled nitrogen isotopes (Marbà et al. 2002), demonstrated that acquired nitrogen can travel within the clone at spatial scales of decimeters (Marbà et al. 2002). This clonal connectivity suggests that although endophytic nitrogen-fixing bacteria were only present in some roots, the whole clone might benefit from the nitrogen acquired by them.

The nitrogen isotopic content of *P. oceanica* tissues evidenced incorporation of atmospheric nitrogen into plant tissues, as its average $\delta^{15}N$ indicates a ^{15}N depletion when compared with values reported for P. oceanica growing in other Mediterranean regions as the Iberian Peninsula (Papadimitriou et al. 2005). However, those meadows where nitrogenase gene was detected did not show significant differences in the nitrogen isotopic content comparing with those meadows where nitrogenase gene was not detected. With this technique we cannot distinguish the nitrogen fixation produced by endophytic bacteria from that of bacteria inhabiting the rhizosphere and the epiphytic community in leaves. Thus, a large fraction of the nitrogen fixation detected could have been caused by the presence of

diazotrophic community in sediments as well as diazotrophic epiphytes that contribute to the global pool of atmospheric fixed nitrogen available.

The identification of only two different *nifH* sequences in all the 11 *P. oceanica* surface-sterilized root samples analyzed by DGGE suggests a very species-specific diazotroph-seagrass relationship. This contrasts with rhizosphere diazotrophic communities, which have been reported to be highly diverse. For instance, in the rhizosphere of the salt marsh cord grass *Spartina alterniflora* the analysis of diazotrophic community by DGGE of the amplified *nifH* gene yielded 58 different *nifH* sequences (Lovell et al. 2008). Similarly, in the sediments colonized by mixed seagrasses meadows (*Thalassia testudinum* and *Syringodium filiforme*) 67 different *nifH* sequences were recovered by DGGE analysis, as well (Bagwell et al. 2002).

The most frequent *nifH* sequence found in this study, corresponding to Band A (P.o_root_A_nifH, JN987872), fell into cluster I, pointing out this sequence corresponds to a proteobacterium (Gaby and Buckley, 2011). Moreover, its closest known relative as determined by BLAST scores and ARB alignment (87.25% similarity) was a *nifH* sequence clustering with *nifH* sequence of *Vibrio diazotrophicus* (Lovell et al. 2008), a marine nitrogen-fixing Gamma-proteobacterium. The other *nifH* sequence obtained in this study, corresponding to Band B (P.o_root_B_nifH, JN987873), was only present in one sample and fell into cluster III, suggesting it can be a putative anaerobe (Gaby and Buckley 2011). However, its phylogenetic affiliation cannot be more precisely elucidated, as the closest relatives derived from ARB analysis, were *nifH* sequences from unknown diazotrophs. Moreover, the phylogenetic division in clusters, based on *nifH* gene, pooled together in cluster III all *nifH* sequences from anaerobic members of Bacteria and Archaea belonging

to very different taxonomic groups, including spirochetes, methanogens, acetogens, sulfate-reducing bacteria, green sulfur bacteria and clostridia (Zehr et al. 2003, Gaby and Buckley 2011). This polyphyletic clustering can be explained by lateral transfer but also by environmental selection (Zehr et al. 1995). The finding of a not yet taxonomically described diazotroph bacteria in *P. oceanica* roots also supports the hypothesis of strong endophytic diazotroph bacteria-seagrass symbiosis relationship, at least for this seagrass species.

In summary, this is the first report of presence of endophytic nitrogen-fixing bacteria in marine angiosperms tissues. Moreover, the diazotrophic endophytes seem to be species-specific as derived from the 2 unique different sequences obtained in the 11 *P. oceanica* root samples analyzed by DGGE, suggesting the presence of a Gamma-proteobacterium similar to *Vibrio diazotrophicus* and an anaerobic nitrogen-fixing bacterium. The presence of diazotrophic endophytic bacteria in seagrass tissues might explain the success of these plants in nutrient-poor environments, encouraging further efforts to quantify the contribution of endophytic bacteria to the nitrogen cycle of seagrasses.

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Chapter 5

Bacterial diversity in seagrass meadows

Neus Garcias-Bonet, Núria Marbà, Ester Marco-Noales and Carlos M. Duarte

Manuscript

Abstract

The bacterial diversity described in association with seagrasses was compiled in order to obtain a general view of the composition of bacterial communities related to seagrass ecosystems around the world. The existence of bacterial communities in association with seagrass meadows is known, although relative scarce information is available. The majority of the literature found and compiled in this work referred to the characterization of the bacterial communities in sediments colonized by seagrasses, while the endophytic or epiphytic communities remained less studied. In this compilation we analyzed the use of different techniques and approaches to perform a characterization, finding a prevalence of culture over molecular techniques. Moreover, we realized that the sampling effort done in this research topic was biased to some geographic zones, with underrepresentation of many areas in the globe. Similarly, information regarding the diversity of the bacterial communities in seagrass ecosystems was only available in 30% of the seagrass species. Despite these biases, this compilation of the available literature allowed the analysis of the composition of the bacterial communities described in seagrasses. The composition of bacterial communities in sediments was different from that of those related to tissues, as it was expected. We discussed the possible implication of some of the bacterial identifications; however, the role of these bacteria cannot be inferred from this compilation, and further and specific studies will be needed in order to achieve this goal.

Introduction

Bacteria are present in the ocean at very large abundance, with densities around 10^6 cells ml^{-1} (Azam & Worden 2004). Most of the research on marine microorganisms has been focused on assessing their abundance (Azam & Worden 2004), diversity (e.g. Sogin et al. 2006, Hugenholtz et al. 1998), and their role in biogeochemistry (La Ferla et al. 2005), mainly in the water column, and to a less extent in the sediment (e.g. Danovaro et al. 1994, Nealson 1997). The characterization of the microbiota associated with seagrasses is scarce, although seagrasses constitute an important ecosystem. Moreover, the assessment of the bacterial diversity found in these ecosystems could result in new bacterial taxa descriptions, as seagrasses can be a large diversity reservoir, due to its particular features.

Seagrasses are marine clonal plants represented by 60 species (Short et al. 2001) restricted to nearshore environments, where they can meet their growth requeriments (i.e. substract and light), from shallow areas to up to 58m in depth, such as *Halophila* sp. found in the great Barrier Reef in very clear water (Lee Long et al. 1996). Seagrasses are distributed along most of the continents with an estimated extension of 300,000 to 600,000 km^2 (Charpy-Roubaud and Sournia 1990, Duarte et al. 2005). Seagrasses are mainly found in oligotrophic ambients, driving to nutrient limited situations. There is a general decline of seagrass ecosystems worldwide (Waycott et al. 2009) and the main drivers have been identified as anthropogenic. The characterization of the microbiota associated to these ecosystems can be useful to clarify the role bacteria can play in such decline, both by accelerating the detrimental conditions or improving seagrass fitness, depending on the bacteria under consideration.

There is little information about characterization of bacterial diversity associated with marine angiosperms and most of these studies describe the bacterial diversity in seagrass sediments (e. g. Cifuentes et al. 2000, Bagwell et al. 2002, García-Martínez et al. 2009). Some other studies focused on bacterial communities associated to surfaces (i.e., epiphytic bacterial communities) of seagrass tissues (e. g. Weidner et al. 2000, Jensen et al. 2007, Uku et al. 2007, Crump and Koch 2008). However, Barbanas (1992) demonstrated the presence of bacteria with marked cellulolytic activity on and within the leaf blade epidermal cells of *Thalassodendron ciliatum* by transmission electron microscopy. Endophytic bacteria in tropical seagrasses (*Thalassia hemprichii*, *Cymodocea serrulata*, *Halodule uninervis*, *Syringodium isoetofolium*) tissues had also been evidenced by optical microscopy (Kuo, 1993). Moreover, bacteria were isolated from the rhizoplane and deep cortex cells of *Halodule wrightii* (Küsel et al. 1999) and from the surface-sterilized roots of *Zostera marina* (Nielsen et al. 1999). Specifically, a new species of the genus *Sulfitobacter* was isolated from a homogenate of *Z. marina* (Ivanova et al. 2004), and *Clostridium glycolicum* from *H. wrightii* (Küsel et al. 2001), evidencing endophytic bacteria in seagrasses. The presene of bacteria was also shown in ineer tissues of *Posidonia oceanica* (Marco-Noales et al. 2006).

Some previous works focused in the bioactive potential of bacterial communities present in marine angiosperms, some of these bacteria showing activity against human bacterial pathogens (Ravikumar et al. 2010). Yet, most of the studies were based on culturing methods, which are known to be less representative than molecular methods because those bacteria unable to grow on culture media cannot be detected. The recent

application of molecular methods in this field has allowed a more accurate description of the microbiota in environmental samples (Hugenholtz et al. 1998).

The role of bacterial communities associated with seagrasses remains uncertain, evidencing the needing of further studies to elucidate the nature of the relationship bacterium-seagrass. A compilation of studies with a description of the bacterial diversity both in sediments colonized by seagrasses and close related to seagrass tissues could be useful in order to achieve this goal and may cast some light on the possible causes of the decline that seagrasses are experiencing worldwide. The characterization of these complex communities is the first step in order to further explore the possible role or effects on seagrass fitness.

In this work, we reviewed and compiled the published literature available up to date for bacterial diversity found in seagrass ecosystems all over the world in order to characterize this diversity and try to infer the possible role bacteria can play in seagrass fitness and/or seagrass decline.

Materials and Methods

We searched for published papers containing the words: bact* AND seagrass in topic field using the ISI web of Knowledge web site. From the resulting list of 275 published papers, 50 contained information regarding bacterial diversity in seagrass meadows. These 50 studies, in which the bacterial taxonomic groups were identified, were compiled and taken in account, both characterizating the sediment diversity as well as the bacterial diversity closed related to seagrass tissues (endo- and epiphytically). Studies without bacterial taxonomic identifications were discarded. We analyzed the main differences

between the diversity described for sediments and the diversity found related to plant tissues.

We analyzed the representation of the studies published up to date, showing the different effort done depending on the geographic zone and depending on the seagrass species studied. The geographic distribution of the compiled studies was addressed by extracting the geographic coordenates of each meadow sampled in each published work. The decimal coordenates were used to perform a map using GIS methodology, where the number of different meadows per site was indicated as well as the number of works describing the bacterial diversity in each location. Information regarding geographic sampling distribution was compared with globlal seagrass distribution reproduced from UNEP-WCMC (http://data.unep-wcmc.org/).

Moreover, we reported the methodology used in each case, comparing the use of culturing methods versus molecular methods in the characterization of the bacterial diversity in seagrass meadows. We classified as molecular methods those in which the characterization of the bacterial community was done without culturing and then avoiding the biases related to culturing methods.

Results

Out of the 50 papers found in literature, where a description and taxonomic identification of the bacterial diversity was assessed, 20 referred to sediments, 12 referred to the epiphytic community, and only 7 to bacteria found endophytically in seagrass tissues. Ten out of the remaining 11 did not specify the treatment of the plant material or did not sterilize the surfaces, impossibiliting to discern the origin of the isolate between endophytic

or epiphytic nature, and one referred to decaying tissue without specification of the seagrass species. The 30% of the compiled works related to sediment bacterial diversity performed a general description of the bacterial community, another 30% described new bacterial species or looked for specific bacterial species. The remaining 40% of the compiled works focused on functional bacterial groups, where more than the half were related to nitrogen cycle and the rest were related to sulfate-reducing bacteria. In the case of the compiled works referring to bacterial diversity on seagrass tissues, the 34% focused on a general community description, 38% on new bacterial species and the remaining 28% on functional bacterial groups, mainly on nitrogen cycle and in less extend on sulfate-reducing bacteria, as well.

More frequently methodologies used in published literature

Most of the studies used in this compilation, 66%, were performed using culturing techniques, whereas only the remaining 34% used molecular techniques. The molecular methods used in the compiled works were varied, ranging from probe hybridations detectable by microscopy to techniques where amplification steps were involved. Some of the works were based on characterization by DGGE (Muyzer et al. 1993) and later sequencing of the detected OTUs. Others were based on the performance of clone libraries from the amplified products obtained directly from the sample. In less proportion, the bacterial diversity was assessed by ARDRA and T-RFLP. Some of the works using molecular techniques combined some of them in a single study. The performance of clone libraries was the most extended methodology in the studies based on molecular techniques, followed by DGGE, probe hybridation and TEM.

Geographical sampling distribution

The sampling effort seemed to be biased to some geographical zones, with some areas poorly represented or even never studied (Fig. 1). Moreover, some locations were more intensively sampled than others, in terms of sampling sites and number of studies. For example, in the Western Mediterranean Sea, the sampling effort was the biggest; with 10 meadows of *Posidonia oceanica* sampled along the Balearic Islands coasts in 5 published works. Similarly, in the Atlantic coast of France, the Arcachon Bay location was widely studied with 8 published works, although these studies only characterized the bacterial community in seagrass sediments, mainly in *Zostera noltii* meadows, but also in *Ruppia cirrhosa*, although with less representation (2 works out of the 8).

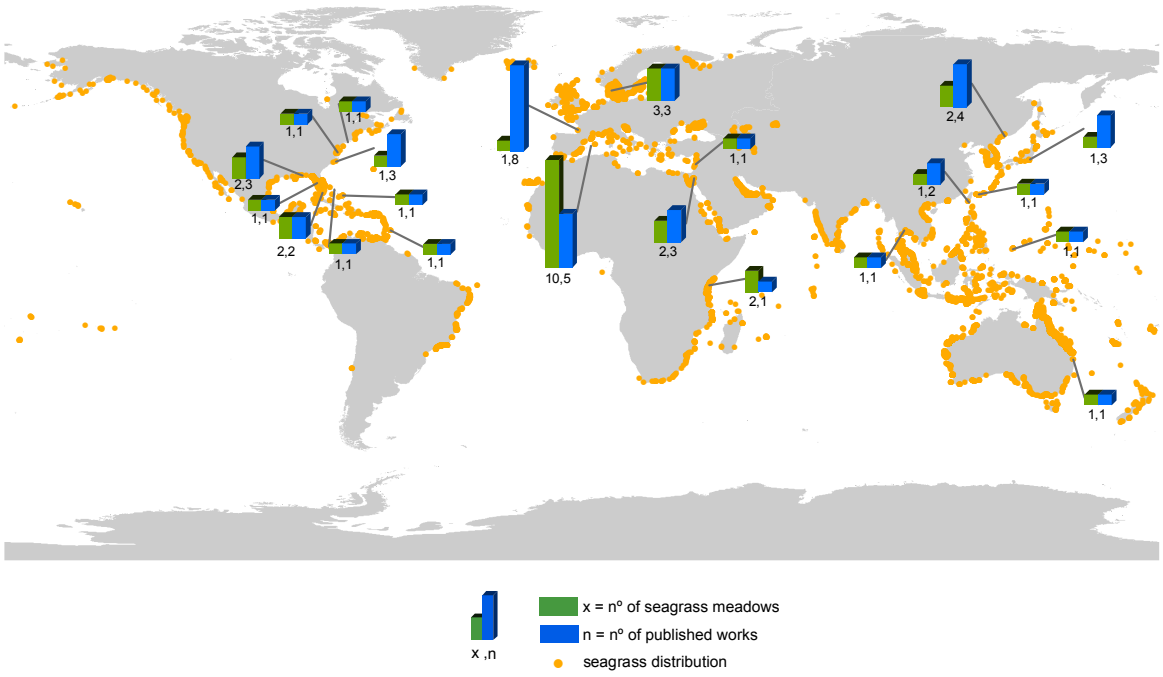

Figure 1. Geographic distribution of the published literature on bacterial identification in seagrass ecosystems, indicating the number of sites sampled in a geographic zone (green bars) and the number of papers describing the bacterial diversity (blue bars). The global seagrass distribution (in orange) is reproduced from UNEP-WCMC (http://data.unep-wcmc.org/).

Seagrass species representation

Bacterial diversity information was only available for 18 seagrass species out of the total 60 species of seagrasses taxonomically classified (Short et al. 2001). The most studied seagrass species was *Zostera marina*, with 15 works describing its bacterial communities. The ranking of the most studied seagrass species followed with *Halodule wrightii* and *Thalassia testudinum* with 7 works for each one, *Posidonia oceanica* and *Zostera noltii* with 6 each one (Fig. 2). Studies on bacterial diversity lacked in many seagrass species found in other zones, even with some genus unrepresented.

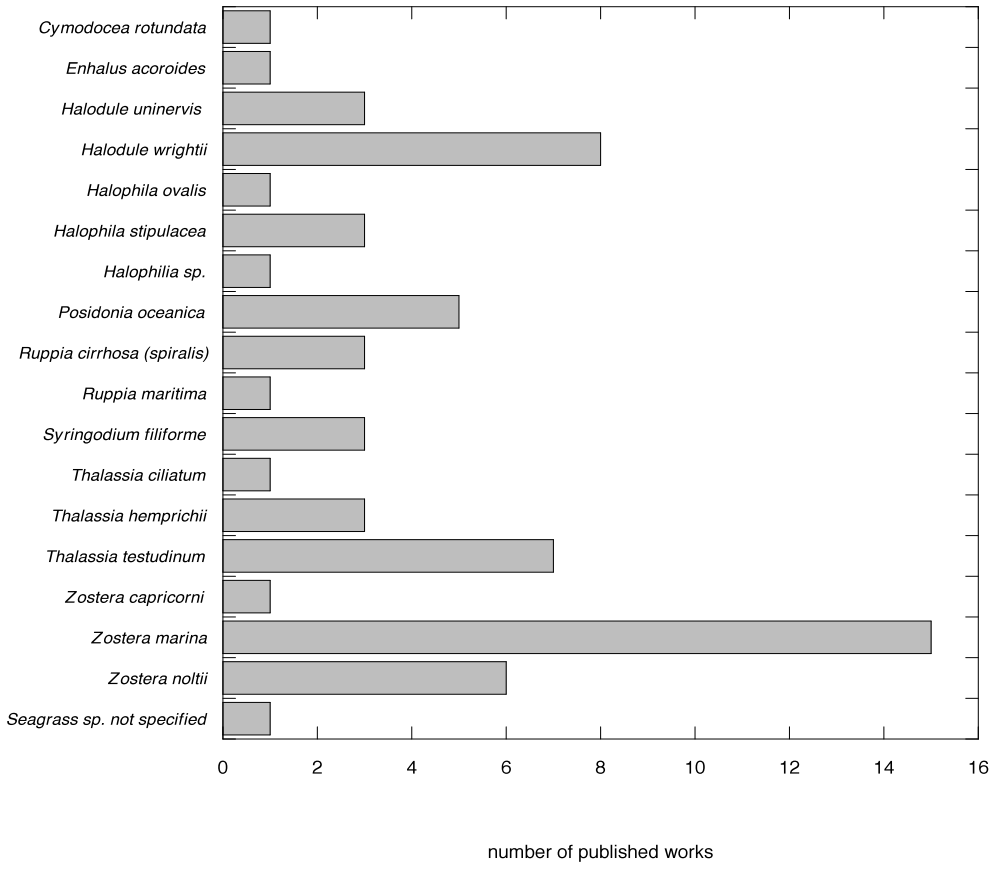

Figure 2. Seagrass species from which bacterial diversity is available in published literature and the number of studies compiled.

Table 1. List of bacterial species reported on published literature related to seagrass meadows. Bacteria identified associated to seagrass sediments as well as bacteria related to seagrass tissues (endo- and epiphytically) are compiled and listed. The seagrass sp., the location of the meadows and the technique used, are indicated as well.

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Enhalus acoroides	*Pelagicoccus croceus*	Puniceicoccaceae	Opitutae	Verrucomicrobia	Okinawa (Japan)	Leaves. Endo- and epiphytes	Culturing	Yoon et al. 2007
Cymodocea rotundata	*Calothrix* sp.	Rivulariacea	Cyanobacteria	Cyanobacteria	Nyali and Vipingo (Kenya)	Leaves. Epiphytes	Clone libary and PCR-DGEE-Sequncing 16s rDNA	Uku et al. 207
	Anabaena sp.	Nostocaceae	Cyanobacteria	Cyanobacteria				
	Lyngbya sp.	Oscillatoriaceae	Cyanobacteria	Cyanobacteria				
	Leptolyngbya sp.	Pseudanabaenaceae	Cyanobacteria	Cyanobacteria				
	Spirulina sp.	Oscillatoriaceae	Cyanobacteria	Cyanobacteria				
	Chroococcus sp.	Chroococcaceae	Cyanobacteria	Cyanobacteria				
	Cyanosarcina sp.	Chroococcaceae	Cyanobacteria	Cyanobacteria				
	Mantelum sp.	Merismopediaceae	Cyanobacteria	Cyanobacteria				
	Chroococidiopsis sp.	Xenococcaceae	Cyanobacteria	Cyanobacteria				
	Xenotholus sp.	Xenococcaceae	Cyanobacteria	Cyanobacteria				
	Enterobacter sp.	Enterobacteriaceae	γ-proteobacteria	Proteobacteria				
	Microbacterium sp.	Microbacteriaceae	Actinobacteria	Actinobacteria				
Cymodocea rotundata, Thalassia ciliatum and *T. hemprichii*	*Comamonas aquatica*	Comamonadaceae	β-proteobacteria	Proteobcteria				
	Agrobacterium sp.	Rhizobiaceae	α-proteobacteria	Proteobacteria				
Thalassia ciliatum	*Ochrobactrum anthropi*	Brucellaceae	α-proteobacteria	Proteobacteria				
Thalassia hemprichii	*Halomonas* sp.	Halomonadaceae	γ-proteobacteria	Proteobacteria				
	Mesorhizobium sp.	Phyllobacteriaceae	α-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Halodule wrightii	*Klebsiella* sp.	Enterobacteriaceae	γ-proteobacteria	Proteobacteria	Middle Marsh, North Carolina (USA)	Surface-sterilized roots. Endophytes	Culturing	Smith and Hayasaka 1982
Halodule wrightii	*Clostridium* sp.	Clostridiaceae	Clostridia	Firmicutes	Middle Marsh, North Carolina (USA)	Rhizosphere. Sediment	nifH-PCR and Clone lybrary	Kirshtein et al. 1991
Halodule wrightii	*Acetobacterium* sp.	Eubacteriaceae	Clostridia	Firmicutes	Big Sabine Point, Santa Rosa, Florida (USA)	Root. Endophytes	rRNA probe hybridation	Küsel et al. 1999
	Desulfovibrio sp.	Desulfovibrionaceae	δ-proteobacteria	Proteobacteria				
Halodule wrightii	*Clostridium glycolicum*	Clostridiaceae	Clostridia	Firmicutes	Big Sabine Point, Santa Rosa, Florida (USA)	Root. Endo- and epiphytes	Culturing	Küsel et al. 2001
Halodule wrightii, *Syringodium filiforme*, *Thalassia testudinum* and *Zostera marina*	*Clostridium* sp.	Clostridiaceae	Clostridia	Firmicutes	St. Lawrence (Barbados)	Rhizosphere. Sediment	Culturing	Patriquin and Knowles 1972
	Azotobacter sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Desulfovibrio sp.	Desulfovibrionaceae	δ-proteobacteria	Proteobacteria				
Halophilia sp.	*Microbulbifer* sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria	Ngermeaus, Palau Islands, Tropical Pacific Islands	Not specified. Epiphytes	Culturing	Nishijima et al. 2009

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Halophila ovalis	*Arthrobacter* sp.	Micrococcaceae	Actinobacteria	Actinobacteria	Jordan	Leves, Rhizomes and Roots. Epiphytes	Culturing	Whabeh and Mahasneh 1984
	Actinomyces sp.	Actinomycetaceae	Actinobacteria	Actinobacteria				
	Acinetobacter sp.	Moraxellaceae	γ-proteobacteria	Proteobacteria		Rhizomes and Roots. Epiphytes		
	Bacillus sp.	Bacillaceae	Bacilli	Firmicutes		Leves, Rhizomes and Roots. Epiphytes		
	Pseudomonas sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Staphylococcus sp.	Staphylococcaceae	Bacilli	Firmicutes		Rhizomes and Roots. Epiphytes		
	Streptococcus sp.	Streptococcaceae	Bacilli	Firmicutes				
Halophila stipulacea	*Arthrobacter* sp.	Micrococcaceae	Actinobacteria	Actinobacteria		Rhizomes and Roots. Epiphytes		
	Actinomyces sp.	Actinomycetaceae	Actinobacteria	Actinobacteria		Leves, Rhizomes and Roots. Epiphytes		
	Bacillus sp.	Bacillaceae	Bacilli	Firmicutes				
	Pseudomonas sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria		Rhizomes and Roots. Epiphytes		
Halodule uninervis	*Arthrobacter* sp.	Micrococcaceae	Actinobacteria	Actinobacteria		Leves, Rhizomes and Roots. Epiphytes		
	Actinomyces sp.	Actinomycetaceae	Actinobacteria	Actinobacteria				
	Bacillus sp.	Bacillaceae	Bacilli	Firmicutes				
	Micrococcus sp.	Micrococcaceae	Actinobacteria	Actinobacteria		Rhizomes and Roots. Epiphytes		
	Vibrio sp.	Vibrionaceae	γ-proteobacteria	Proteobacteria		Leves, Rhizomes and Roots. Epiphytes		
Halophila stipulacea	*Hyphomonas* sp.	Hyphomonadaceae	α-proteobacteria	Proteobacteria	Gulf of Elat (Israel)	Leaves. Epiphytes	ARDRA (Amplified rDNA restriction analysis)	Weidner et al. 1996

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Halophila stipulacea	*Vibrio splendidus*	Vibrionaceae	γ-proteobacteria	Proteobacteria	Gulf of Elat (Israel)	Leaves. Epiphytes	Clone library	Weidner et al. 2000
	Vibrio proteolyticus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Alteromonas sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Pseudoalteromonas sp.	Pseudoalteromonadaceae	γ-proteobacteria	Proteobacteria				
	Marinomonas sp.	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Pseudomonas sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Methylophaga sp.	Piscirickettsiaceae	γ-proteobacteria	Proteobacteria				
	Hyphomonas sp.	Hyphomonadaceae	α-proteobacteria	Proteobacteria				
	Janthinobacterium lividum	Oxalobacteraceae	β-proteobacteria	Proteobacteria				
	Pirellula sp.	Planctomycetaceae	Planctomycea	Planctomycetes or Planctobacteria				
	Oceanospirillum	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Roseobacter sp.	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Ruegeria sp.	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Rhizobiaceae group	Rhizobiaceae	α-proteobacteria	Proteobacteria				
Posidonia oceanica	*Pseudoalteromonas carrageenovora*	Pseudoalteromonadaceae	γ-proteobacteria	Proteobacteria	Illetes, Pollença, Magalluf and Portocolom, Mallorca, Balearic Islands (Spain)	Leaves, roots and rhizomes. Endo- and epiphytes	Culturing	Marco-Noales et al. 2006
	Pseudoalteromonas agarivorans	Pseudoalteromonadaceae	γ-proteobacteria	Proteobacteria				
	Pseudoalteromonas aurantia	Pseudoalteromonadaceae	γ-proteobacteria	Proteobacteria				
	Pseudoalteromonas porphyrae	Pseudoalteromonadaceae	γ-proteobacteria	Proteobacteria				
	Alteromonas atlantica	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Alteromonas carrageenovora	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Alteromonas stellaepolaris	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Alteromonas macleodii	Alteromonadaceae	γ-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Posidonia oceanica	*Glaciecola mesophila*	Alteromonadaceae	γ-proteobacteria	Proteobacteria	Illetes, Pollença, Magalluf and Portocolom, Mallorca, Balearic Islands (Spain)	Leaves, roots and rhizomes. Endo- and epiphytes	Culturing	Marco-Noales et al. 2006
	Agarivorans albus	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Shewanella pacifica	Shewanellaceae	γ-proteobacteria	Proteobacteria				
	Shewanella putrefaciens	Shewanellaceae	γ-proteobacteria	Proteobacteria				
	Shewanella frigidimarina	Shewanellaceae	γ-proteobacteria	Proteobacteria				
	Marinomonas mediterranea	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Marinomonas communis	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Marinomonas vaga	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Vibrio splendidus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio pomeyori	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio aesturianus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio nigripulchritudo	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio gazogenes	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio tasmaniensis	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio pelagicus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio natriegens	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio parahaemolyticus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio alginolyticus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Vibrio cyclitrophicus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Neptunomonas sp.	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Sulfitobacter pontiacus	Rhodobacteraceae	α-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Posidonia oceanica	*Desulfosarcina* sp.	Desulfobacteraceae	δ-proteobacteria	Proteobacteria	Es Port Bay, Cabrera, Balearic Islands (Spain)	Sediment	Clone library and FISH	García-Martínez et al. 2009
	Holophaga sp.	Holophagaceae	Holophagae	Acidobacteria				
	Desulfonema sp.	Desulfobacteraceae	δ-proteobacteria	Proteobacteria				
Posidonia oceanica	*Marinomonas balearica*	Oceanospirillaceae	γ-proteobacteria	Proteobacteria	P.Colom and Pollença, Balearic Islands (Spain)	Leaves, roots and rhizomes. Endo- and epiphytes	Culturing	Espinosa et al. 2010
	Marinomonas pollencensis	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
Posidonia oceanica	*Marinomonas foliarum*	Oceanospirillaceae	γ-proteobacteria	Proteobacteria	Balearic Islands (Spain)	Leaves. Endo- and epiphytes	Culturing	Lucas-Elío et al. In press
	Marinomonas rhizomae	Oceanospirillaceae	γ-proteobacteria	Proteobacteria		Rhizomes. Endo- and epiphytes		
	Marinomonas alcarazii	Oceanospirillaceae	γ-proteobacteria	Proteobacteria		Leaves, roots and rhizomes. Endo- and epiphytes		
	Marinomonas posidonica	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Marinomonas aquiplantarum	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
Posidonia oceanica	*Celerinatantimonas diazotrophica*	Celerinatantimonadaceae	γ-proteobacteria	Proteobacteria	Cala Sahona, Talamanca, Cala Marmacen, Magalluf, Cala Torreta, Portocolom, Pujols, Balearic Islands (Spain)	Roots. Endophytes	PCR-DGEE-Sequncing of 16s rDNA gene	Garcias-Bonet et al. Submitted
	No identification at genus level	*Rhodobacteraceae*	α-proteobacteria	Proteobacteria				
	Flammeovirga sp.	*Flammeovirgaceae*	Sphingobacteria	Bacteroidetes		Leaves. Endophytes		
	Sulfur-Oxidizing-Symbionts		γ-proteobacteria	Proteobacteria				
	Nautella italica	Rhodobacteraceae	α-proteobacteria	Proteobacteria		Rhizomes. Endophytes		
	No identification at genus level	*Desulfovibrionaceae*	δ-proteobacteria	Proteobacteria				
	No identification at genus level	*Sphingobacteriaceae*	Sphingobacteria	Bacteroidetes				
	No identification at genus level	*Rhizobiaceae*	α-proteobacteria	Proteobacteria				
	Non-Identified Coral BBD isolates		α-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Ruppia cirrhosa	*Thiorhodococcus minus*	Chromatiaceae	γ-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Culturing	Guyoneaud et al. 1997
Ruppia cirrhosa	*Roseospira marina*	Rhodospirillaceae	α-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Culturing	Guyoneaud et al. 2002
Ruppia maritima	*Klebsiella* sp.	Enterobacteriaceae	γ-proteobacteria	Proteobacteria	Not specified	Rhizosphere. Sediment	Culturing	Currin et al. 1990
	Pseudomonas sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
Ruppia spiralis	*Aeromonas hydrophila*	*Aeromonadaceae*	γ-proteobacteria	Proteobacteria	Bardawil (Hypersaline) Lagoon (Egypt)	Rhizosphere. Sediment	Culturing	Bahgat et al 2010
	Agrobacterium radiobacter	Rhizobiaceae	α-proteobacteria	Proteobacteria				
	Bacillus spp.	Bacillaceae	Bacilli	Firmicutes				
	Clostridium spp.	Clostridiaceae	Clostridia	Firmicutes				
	Corynebacterium spp.	Corynebacteriaceae	Actinobacteria	Actinobacteria				
	Erysipelothrix rhusiopathiae	Erysipelotrichaceae	Erysipelotrichia	Firmicutes				
	Flavobacterium indologenes	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Flavobacterium meningosept	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Micrococcus sp.	Micrococcaceae	Actinobacteria	Actinobacteria				
	Pseudomonas diminuta	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Pseudomonas florescens	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Pseudomonas versicularis	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Staphylococcus spp.	Staphylococcaceae	Bacilli	Firmicutes				
	Streptococcus spp.	Streptococcaceae	Bacilli	Firmicutes				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Thalassia hemprichii and *Halodule uninervis*	*Pseudomonas* sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria	Nanwan Bay of Kenting National Park (Taiwan)	Rhizosphere. Sediment	Culturing	Shieh and Yang 1997
	Alcaligenes sp.	Alcaligenaceae	β-proteobacteria	Proteobacteria				
	Cytophaga sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes				
	Moraxella sp.	Moraxellaceae	γ-proteobacteria	Proteobacteria				
	Vibrio sp.	Vibrionaceae	γ-proteobacteria	Proteobacteria				
Thalassia hemprichii and *Halodule uninervis*	*Vibrio aerogenes*	Vibrionaceae	γ-proteobacteria	Proteobacteria	Nanwan Bay of Kenting National Park (Taiwan)	Sediment	Culturing	Shieh et al. 2000
Thalassia testudinum	*Cytophaga* sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes	Great Abaco (Bahama Islands)	Decaying leaves. Endophytes	TEM	Porter et al. 1989
Thalassia testudinum and Halodule wrightii mixed meadow	*Desulfosarcina* sp.	Desulfobacteraceae	δ-proteobacteria	Proteobacteria	Pensacola Bay, Florida (USA)	Sediment	Clone library *drsA* and *B* (Dissimilator and bisulfite reductase) genes	Smith et al. 2004
	Desulfosporosinus sp.	Peptococcaceae	Clostridia	Firmicutes				
	sulfate-reducing bacteria							
Thalassia testudinum	*Vibrio* sp.	Vibrionaceae	γ-proteobacteria	Proteobacteria	Tampa Bay (USA)	Leaves. Epiphytes	Clone library *nasA* (Assimilatory nitrate reductase) gene	Adhitya et al. 2007
	Alteromonas sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Marinomonas sp.	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Thalassia testudinum	*Clostridium tetani*	Clostridiaceae	Clostridia	Firmicutes	Tarpon Bay, Florida (USA)	Sediment	T-RFLP	Milbrandt et al. 2008
	Desulfobacterium cetonicum	Desulfobacteraceae	δ-proteobacteria	Proteobacteria				
	Desulfomicrobium baculatum	Desulfomicrobiaceae	δ-proteobacteria	Proteobacteria				
	An unknown Sponge Endosymbiont							
	Geobacter Metallireducens	Geobacteraceae	δ-proteobacteria	Proteobacteria				
	Syntrophobacter pfenniii	Syntrophobacteraceae	δ-proteobacteria	Proteobacteria				
Thalassia testudinum, Syringodium filiforme and *Halodule wrightii*	*Azospirillum sp.*	Rhodospirillaceae	α-proteobacteria	Proteobacteria	Bahama Islands	Sediment, roots and rizhomes. Endo- and epiphytes	Clone library and PCR-DGEE-Sequncing of *nifH* gene	Bagwell et al. 2002
	Rhizobium sp.	Rhizobiaceae	α-proteobacteria	Proteobacteria				
	Vibrio diazotrophicus	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Desulfonema sp.	Desulfobacteraceae	δ-proteobacteria	Proteobacteria				
Thalassia testudinum, Syringodium filiforme and *Halodule wrightii*	phototrophic purple-sulfur bacteria and denitrifiers such as *Pseudomonas* sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria	Florida Bay (USA)	Sediment	PCR-DGEE-Sequencing 16s rDNA	Ikenaga M et al. 2010
	Sulfate-reducing bacteria and *Desulfosarcina* sp. and *Desulfocapsa* sp.	Desulfobacteraceae /Desulfobulbaceae	δ-proteobacteria	Proteobacteria				
	No identification			Cyanobacteria				
	No identification			Bacteroidetes				
	No identification		Spirochaetes	Spirochaetes				
	No identification			Planctomycetes or Planctobacteria				
Zostera capricorni	*Alteromonas* sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria	Moreton Bay, Australia	Root and rhizome. Endophytes	Inmuno-fluorescent microscopy	Glazebrook et al. 1996

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Zostera marina	*Spirillum* sp.	Spirillaceae	β-proteobacteria	Proteobacteria	Bellport Bay, Long Island, New York (USA)	Roots. Epiphytes	Culturing	Budin 1981
Zostera marina	*Bacteroides* sp. *Clostridium* sp.	Bacteroidaceae Clostridiaceae	Bacteroidia Clostridia	Bacteroidetes Firmicutes	Beaufort, North Carolina (USA)	Roots and rhizosphere. Sediment	Culturing	Roth and Hayasaka 1984
Zostera marina	*Vibrio* sp.	Vibrionaceae	γ-proteobacteria	Proteobacteria	Aburatsubo Inlet, Kanagawa (Japan)	Root. Endo- and epiphytes	Culturing	Shieh et al. 1987
Zostera marina	No identification at genus level	Vibrionaceae	γ-proteobacteria	Proteobacteria	Aburatsubo Inlet, Kanagawa (Japan)	Root. Endo- and epiphytes	Culturing	Shieh et al. 1988
Zostera marina	*Vibrio* sp. *Photobacterium* sp.	Vibrionaceae Vibrionaceae	γ-proteobacteria γ-proteobacteria	Proteobacteria Proteobacteria	Aburatsubo Inlet, Kanagawa (Japan)	Root. Endo- and epiphytes	Culturing	Shieh et al. 1989
Zostera marina	*Desulfovibrio* sp.	Desulfovibrionaceae	δ-proteobacteria	Proteobacteria	Logstor bredning, Limfjorden (Denmark)	Rhizomes and Roots. Epiphytes	Culturing	Blaabjerg and Finster 1998
Zostera marina	*Desulfovibrio zosterae*	Desulfovibrionaceae	δ-proteobacteria	Proteobacteria	Denmark	Surface-sterilized roots. Endophytes	Culturing	Nielsen et al. 1999
Zostera marina	*Desulfomusa hansenii*	Desulfobacteraceae	δ-proteobacteria	Proteobacteria	not specified	Surface-sterilized roots. Endophytes	Culturing	Finster et al. 2001

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Zostera marina	*Micrococcus* sp.	Micrococcaceae	Actinobacteria	Actinobacteria	Trinity Bay of Peter the Great Bay, Sea of Japan	Leves, Rhizomes and Roots. Epiphytes	Culturing	kurilenko et al. 2001
	Bacillus sp.	Bacillaceae	Bacilli	Firmicutes				
	Cytophaga sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes				
	Flavobacterium sp.	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Vibrio sp.	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Shewanella sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Alteromonas sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Pseudoalteromonas sp.	Pseudoalteromonadaceae	γ-proteobacteria	Proteobacteria				
	Marinobacter sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Marinobacterium sp.	Alteromonadaceae	γ-proteobacteria	Proteobacteria				
	Halomonas sp.	Halomonadaceae	γ-proteobacteria	Proteobacteria				
	Fundibacter sp.	Alcanivoracaceae	γ-proteobacteria	Proteobacteria				
	Pseudomonas sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
Zostera marina	*Sulfitobacter dubius*	Rhodobacteraceae	α-proteobacteria	Proteobacteria	Troitza Bay, Gulf of Peter the Great, Sea of Japan	Leves, Rhizomes and Roots. Endo- and epiphytes	Culturing	Ivanova et al. 2004
Zostera marina	*Algibacter lectus*	Flavobacteriaceae	Flavobacteria	Bacteroidetes	Roskilde Fjord and Rungsted harbour (Denmark)	Roots. Endo- and epiphytes	Clone library	Jensen et al. 2007
	Anaerophaga sp.	Marinilabiaceae	Bacteroidia	Bacteroidetes				
	Arcobacter nitrofigilis	Campylobacteraceae	ε-proteobacteria	Proteobacteria				
	Cellulomonas fermentans	Cellulomonadaceae	Actinobacteria	Actinobacteria				
	Clostridium hathewayi	Clostridiaceae	Clostridia	Firmicutes				
	Cycloclasticus spirillensus	Piscirickettsiaceae	γ-proteobacteria	Proteobacteria				
	Cytophaga diffluens	Cytophagaceae	Sphingobacteria	Bacteroidetes				
	Cytophaga latercula	Cytophagaceae	Sphingobacteria	Bacteroidetes				
	Cytophaga sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes				
	Desulfobacterium catecholicum	Desulfobacteraceae	δ-proteobacteria	Proteobacteria				
	Desulfotalea psychrophila	Desulfobulbaceae	δ-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Zostera marina	*Donghaeana dokdonensis*	Flavobacteriaceae	Flavobacteria	Bacteroidetes	Roskilde Fjord and Rungsted harbour (Denmark)	Roots. Endo- and epiphytes	Clone library	Jensen et al. 2007
	Eubacterium oxidoreducens	Eubacteriaceae	Clostridia	Firmicutes				
	Flavobacterium aquatile	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Gaetbulibacter saemankumensis	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Jannaschia rubra	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Leadbetterella byssophila	Cytophagaceae	Sphingobacteria	Bacteroidetes				
	Lewinella cohaerens	Saprospiraceae	Sphingobacteria	Bacteroidetes				
	Oceanospirillum beijerinckii	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	O. linum	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	O. multiglobuliferum	Oceanospirillaceae	γ-proteobacteria	Proteobacteria				
	Ochrobactrum anthropi	Brucellaceae	α-proteobacteria	Proteobacteria				
	Pelagibaca bermudensis	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Phycococcus omphalius	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Psychroserpens burtonensis	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Roseobacter gallaeciensis	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Ruminofilibacter xylanolyticum	Rikenellaceae	Bacteroidia	Bacteroidetes				
	Stenothermobacter spongiae	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Sulfurimonas autotrophica	Helicobacteraceae	ε-proteobacteria	Proteobacteria				
	S. paralvinella	Helicobacteraceae	ε-proteobacteria	Proteobacteria				
	Tannerella forsythensis	Porphyromonadaceae	Bacteroidia	Bacteroidetes				
	Tenacibaculum amylolyticum	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Candidatus *Thiobios zoothamnicoli*		γ-proteobacteria	Proteobacteria				
	Vibrio metschnikovii	Vibrionaceae	γ-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Zostera marina	*Cytophaga* sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes	Troitsa Bay of the Peter the gerat bay, Sea of Japan	Leaves. Epiphytes	Culturing	Kurilenko et al. 2007
	Pseudoalteromonas citrea	Pseudoalteromonadaceae	γ-proteobacteria	Proteobacteria				
Zostera marina	*Cytophaga* sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes	Chesapeake Bay (USA)	Leaves. Epiphytes	DGGE band sequencing and clone library	Crump and Koch 2008
	Lewinella agarilytica	Saprospiraceae	Sphingobacteria	Bacteroidetes				
	Methylotenera mobila	Methylophilaceae	β-proteobacteria	Proteobacteria				
	Rhodobacteraceae sp.	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Thalassobacter sp.	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Roseobacter sp.	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Arcobacter sp.	Campylobacteraceae	ε-proteobacteria	Proteobacteria		Roots. Epiphytes		
	Oceanospirillaceae	Oceanospirillaceae	γ-proteobacteria	Proteobacteria		Roots. Epiphytes		
Zostera marina	*Granulosicoccus coccoides*	Granulosicoccaceae	γ-proteobacteria	Proteobacteria	Troitsa Bay of the Peter the gerat bay, Sea of Japan	Leaves. Epiphytes	Culturing	Kurilenko et al. 2010
Zostera noltii	*Desulfovibrio* sp.	Desulfovibrionaceae	δ-proteobacteria	Proteobacteria	Arcachon Bay (France)	Rhizosphere. Sediment	Culturing	Bourguès 1996
Zostera noltii	*Sulfurospirillum arcachonense*	Campylobacteraceae	ε-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Culturing	Finster et al 1997a
Zostera noltii	*Desulfospira joergensenii*	Desulfobacteraceae	δ-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Culturing	Finster et al 1997b
Zostera noltii	*Desulfocapsa sulfoexigens*	Desulfobulbaceae	δ-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Culturing	Finster et al 1998
Zostera noltii	*Desulfosarcina* sp.	Desulfobacteraceae	δ-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Clone library	Cifuentes et al. 2000
	Desulfonema sp.	Desulfobacteraceae	δ-proteobacteria	Proteobacteria				
	Desulfobulbus sp.	Desulfobulbaceae	δ-proteobacteria	Proteobacteria				
	Syntrophobacter sp.	Syntrophobacteraceae	δ-proteobacteria	Proteobacteria				
	Geobacter sp.	Geobacteraceae	δ-proteobacteria	Proteobacteria				
	Desulfuromonas sp.	Desulfuromonadaceae	δ-proteobacteria	Proteobacteria				
	Nitrospina sp.	Nitrospinaceae	δ-proteobacteria	Proteobacteria				
	Desulforhabdus sp.	Syntrophobacteraceae	δ-proteobacteria	Proteobacteria				

Seagrass sp.	Bacteria sp.	Family	Class	Phylum	Location	Type of sample	Technique	Reference
Zostera noltii	*Desulfonatronovibrio* sp.	Desulfohalobiaceae	δ-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Clone library	Cifuentes et al. 2000
	Methylobacter sp.	Methylococcaceae	γ-proteobacteria	Proteobacteria				
	Pseudomonas sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Aeromonas sp.	*Aeromonadaceae*	γ-proteobacteria	Proteobacteria				
	Beggiatoa sp.	Thiotrichaceae	γ-proteobacteria	Proteobacteria				
	Azotobacter sp.	Pseudomonadaceae	γ-proteobacteria	Proteobacteria				
	Methylophaga sp.	Piscirickettsiaceae	γ-proteobacteria	Proteobacteria				
	Photobacterium sp.	Vibrionaceae	γ-proteobacteria	Proteobacteria				
	Dichelobacter sp.	Cardiobacteriaceae	γ-proteobacteria	Proteobacteria				
	Alcanivorax sp.	Alcanivoracaceae	γ-proteobacteria	Proteobacteria				
	Bacteroides sp.	Bacteroidaceae	Bacteroidia	Bacteroidetes				
	Capnocytophaga sp.	Flavobacteriaceae	Flavobacteria	Bacteroidetes				
	Microscilla sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes				
	Sphingomonas sp.	Sphingomonadaceae	α-proteobacteria	Proteobacteria				
	Sulfitobacter sp.	Rhodobacteraceae	α-proteobacteria	Proteobacteria				
	Rhodobium sp.	Rhodobiaceae	α-proteobacteria	Proteobacteria				
	Rhizobium sp.	Rhizobiaceae	α-proteobacteria	Proteobacteria				
	Rhodoplanes sp.	Hyphomicrobiaceae	α-proteobacteria	Proteobacteria				
	Arcobacter sp.	Campylobacteraceae	ε-proteobacteria	Proteobacteria				
	Spirochaeta sp.	Spirochaetaceae	Spirochaetes	Spirochaetes				
	Methylophilus sp.	Methylophilaceae	β-proteobacteria	Proteobacteria				
	Streptomyces sp.	Streptomycetaceae	Actinobacteria	Actinobacteria				
Zostera noltii	*Desulfobacula totuolica*	Desulfobacteraceae	δ-proteobacteria	Proteobacteria	Arcachon Bay (France)	Sediment	Culturing	Cifuentes et al. 2003
	Desulfospira joergensenii	Desulfobacteraceae	δ-proteobacteria	Proteobacteria				
	Desulfovibrio zosterae	Desulfovibrionaceae	δ-proteobacteria	Proteobacteria				
	Cytophaga sp.	Cytophagaceae	Sphingobacteria	Bacteroidetes				
Not specified	*Alkalibacterium pelagium*	Carnobacteriaceae	Bacilli	Firmicutes	Thailand	Decaying seagrass	Culturing	Ishikawa et al. 2009

Bacterial diversity in seagrass sediments

Analyzing the bacterial diversity described in sediments inhabited by seagrasses, we found that the majority of bacterial species identified belonged to Proteobacteria Phylum (70%), followed by Firmicutes (11%) and Bacteroidetes (10%). The most represented Classes were Delta-proteobacteria (29%), Gamma-proteobacteria (28%) and Alpha-proteobacteria (10.5%); and the most represented families were *Desulfobacteraceae* (13%), *Pseudomonadaceae* (10%), *Clostridiaceae* (6%) and *Vibrionaceae* (5%). At genus level, 56 different genus were found, the most frequently described were *Pseudomonas* sp. (7%), *Clostridium* sp. (6%), *Desulfonema* sp. (3.5%), *Desulfosarcina* sp. (3.5%), *Desulfovibrio* sp. (3.5%), *Rhizobium* sp. (3.5%) and *Vibrio* sp. (3.5%) (Table 1).

Bacterial diversity in seagrass tissues

The bacterial diversity described in association with seagrass tissues in the compiled literature was analyzed separately between endophytic and epiphytic studies. Some of the studies did not specify exactly the procedure to obtain the isolates or the genetic material. In this case, the studies were not taken in account in this section. Sixteen bacterial species were identified endophytically: three of them (*Desulfovibrio zosterae*, *Desulfomusa hansenii* and *Klebsiella* sp.) were detected by culture techniques, while other 13 bacterial species were determined by molecular methods. The majority of bacterial species found endophytically in seagrass tissues belonged to Proteobacteria Phylum (75%), followed by Bacteroidetes (19%) and, finally, Firmicutes (1%). Alpha-proteobacteria, Delta-proteobacteria and Gamma-proteobacteria were the most and equally (25%) represented Classes. The remaining 25% of the identifications were divided between Sphingobacteria

(18.75%) and Clostridia (6.25%). The most represented families were *Desulfovibronaceae* (21.4%) and *Rhodobacteraceae* (14.3%); the rest of bacteria identified belonged equally (7%) to *Alteromonadaceae*, *Celerinatantimonadaceae*, *Cytophagaceae*, *Desulfobacteraceae*, *Enterobacteriaceae*, *Eubacteriaceae*, *Flammeovirgaceae*, *Rhizobiaceae* and *Sphingobacteriaceae* families. At genus level, only 9 different genera were identified although 6 sequences were not identified at genus level; the most frequently described genus was *Desulfovibrio* sp. (12.5%). The species identified endophytically in seagrass tissues were *Acetobacterium* sp., *Alteromonas* sp., *Celerinatantimonas diazotrophica*, *Cytophaga* sp., *Desulfomusa hansenii*, *Desulfovibrio zosterae*, *Flammeovirga* sp., *Klebsiella* sp. and *Nautella italica* (Table 1).

In the analysis of the bacteria described epiphytically in seagrass tissues, we found 78 isolates identified in the 12 related publications, and in this case, the methodology used was 45% culture-dependent and 55% culture-independent (by applying molecular techniques). Half of the studies focused on the identification of bacteria in the surface of the leaves with a 56% of the identifications, whereas only the 4% of the bacteria described were isolated from root surfaces. The rest of the bacteria identified (38%) corresponded to leaves, rhizome and root without specification. The majority of bacterial species found epiphytically in seagrass tissues belonged to Proteobacteria Phylum (60%), followed by Cyanobacteria (13%), Actinobacetria (11.5%), Firmicutes (7.7%), Bacteroidetes (6.4%) and, finally, Planctomycetes or Planctobacetria (1,3%). The most represented Classes were Gamma-proteobacteria (38.5%), Alpha-proteobacteria (14%), Cyanobacteria (12.8%), Actinobacteria (11.5%), Bacilli (7.7%), Beta-proteobacteria (5%) and Sphingobacteria (5%). The rest of bacteria identified epiphytically belonged equally (1.3% each) to Delta-

proteobacteria, Epsilon-proteobacetria, Flavobacteria and Planctomycea. The most represented families were *Alteromonadaceae* (9%) and *Oceanospirillaceae* (7.7%), the rest of the bacteria belonged to many other families. At genus level, 56 different genus were identified and the most frequently described genus were *Vibrio* (6.4%), *Bacillus* (5%) and *Pseudomonas* (5%) (Table 1).

Comparison of the bacterial diversity between seagrass sediments and seagrass tissues.

The bacterial communities described in sediments colonized by seagrasses differed from those described for seagrass tissues. Thus, we compared the diversity found in sediments with that described in association with seagrass tissues. In this comparison, we pooled together the bacteria identified endo- and epiphytically, as well as those isolates whose origin was not clearly specified, disabling to discern between endophytic and epiphytic isolates or sequences. In both types of samples (i.e. sediment samples and seagrass tissues samples), the bacterial species identified belonged mainly, and in the same percentage, to Proteobacetria phylum (69% and 68%, respectively). However, in sediment samples, bacteria belonging to Firmicutes Phylum were more abundant (11%) than in seagrass tissues samples (6%). Conversely, the Bacteroidetes and Cyanobacteria Phylums were more abundant in tissue samples (13.5% and 5.3%) than in sediment samples (10% and 1%). Acidobacteria and Spirochaetes Phylums seemed to be represented only in sediment samples, whereas Actinobacteria and Verrucomicrobia were only present in tissue samples (Fig. 3).

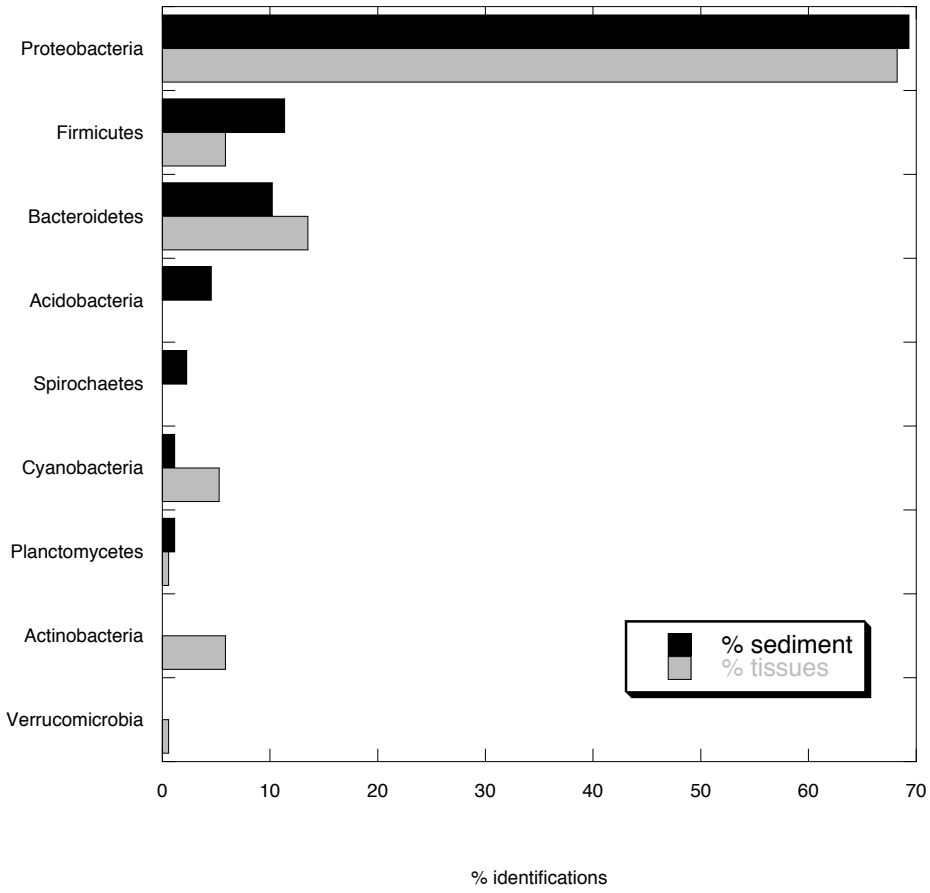

Figure 3. Percentage of bacteria identified belonging to each Phylum in sediment (black) and tissues samples (grey). N (sediment)= 88; N (tissues)= 170.

When the comparison was done at class level, we found differences in the dominant class. In sediment samples the most represented classes were Delta-proteobacteria and Gamma-proteobacteria (29% and 26.7%), while in tissue samples the dominant class was Gamma-proteobacteria (46.5%), (Fig. 4). The most represented families in sediment samples were *Desulfobacteraceae* and *Pseudomonadaceae* (13% and 10.5%), while in tissues samples were *Vibrionanceae* and *Oceanospirillaceae* (12.5% and 12%), (Fig. 5).

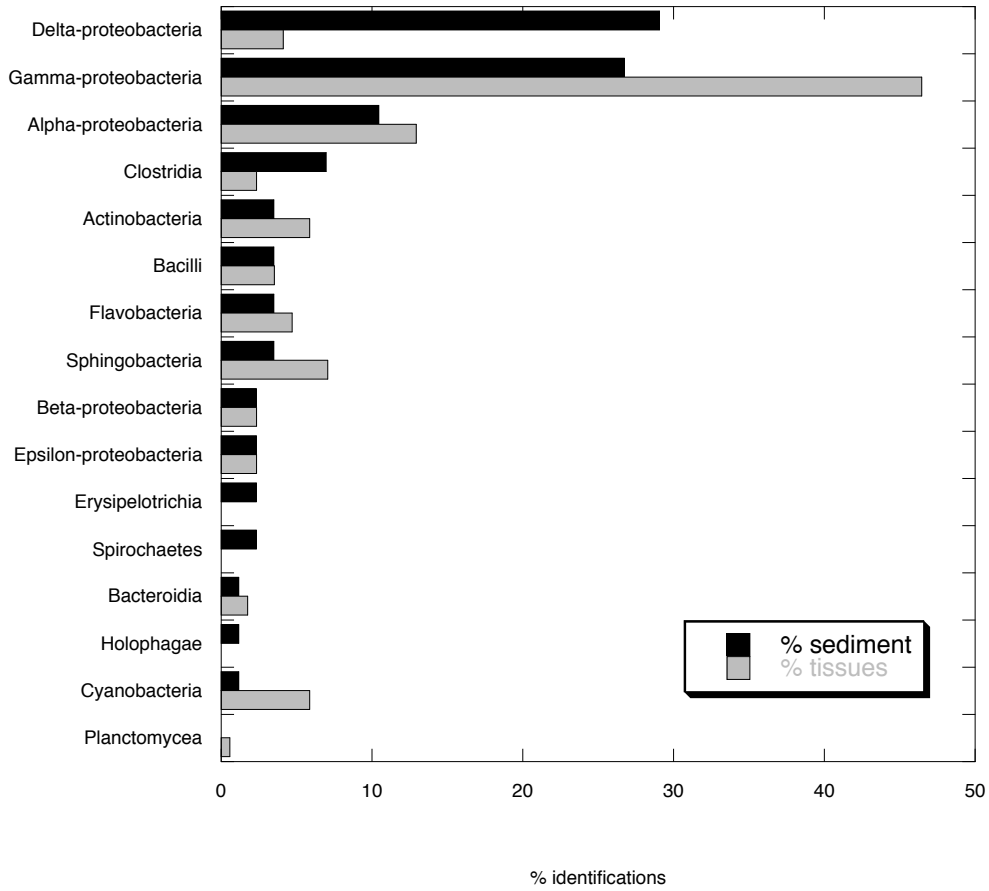

Figure 4. Percentage of bacteria identified belonging to each Class in sediment (black) and tissues samples (grey). N (sediment)= 86; N (tissues)= 170.

In general, the bacterial families more frequently found in seagrass meadows were: *Pseudomonadaceae*, found in association with 11 different seagrass species; *Rhizobiaceae*, in 10 seagrass species; *Vibrionaceae*, in 9 seagrass species; *Desulfobacteraceae* and *Desulfovibrionaceae*, in 6 seagrass species.

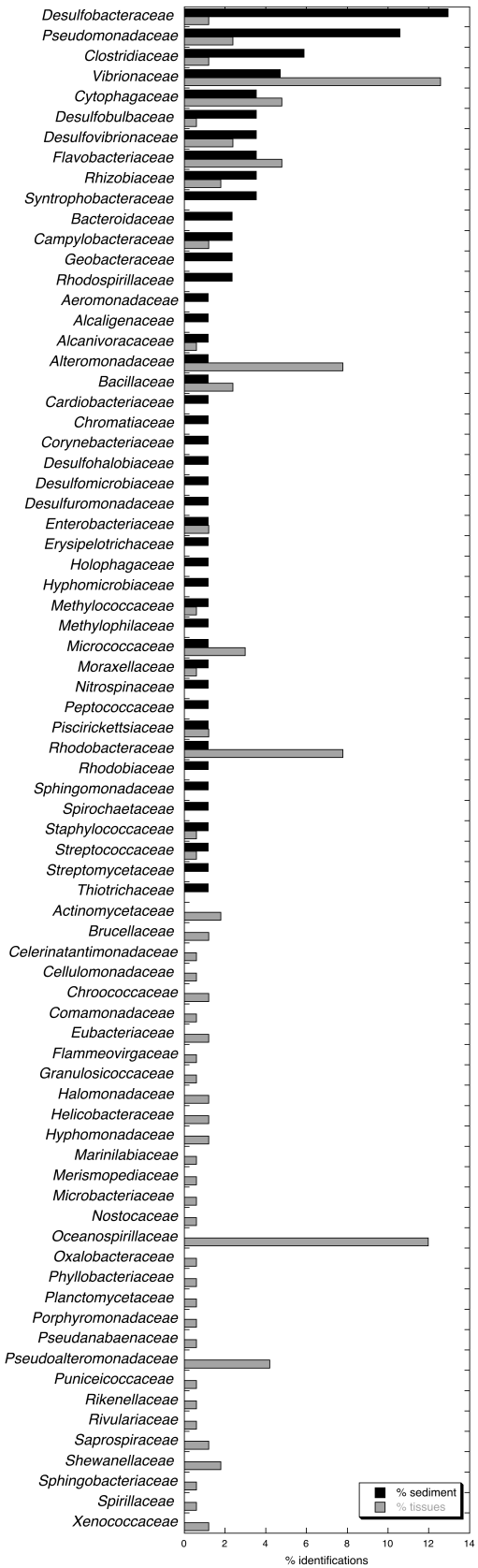

Figure 5. Percentage of bacteria identified belonging to each Family in sediment (black) and tissues samples (grey).
N (sediment)= 85; N (tissues)= 167.

In sediments and seagrass tissues, the 50% of the diversity reported in this study is covered with only one phylum in both types of samples, although the 90% of the total diversity is reached with 3 and 4 phylums, for sediment and tissues, respectively. Similarly, at class level, the sediment samples seemed to be more diverse than tissue samples, as the 90% of the total diversity belonged to 10 different classes, while for tissues samples, the 90% of the total diversity belonged only to 8 different classes. At family level, sediment also seemed to be more diverse than tissue samples, as the 50% of the diversity observed belonged to 9 families, and in tissues samples only 7 families; however, the 90% of the total diversity belonged to 36 families in both cases (Table 2).

Table 2. Number of bacteria identified at each taxonomic level. Number of different phylums, classes and families needed to cover a 50 % and a 90% of the diversity described in the published studies used in this compilation.

	Number of bacteria described		**Number of taxons for a coverage of the 50% of diversity**		**Number of taxons for a coverage of the 90% of diversity**	
	Sediment	**Tissues**	**Sediment**	**Tissues**	**Sediment**	**Tissues**
Phylum level	88	170	1	1	3	4
Class level	86	170	2	2	10	8
Family level	85	167	9	7	36	36

Discussion

This work provides the first compilation of studies where the diversity of the bacterial communities in seagrass ecosystems was assessed; giving a general view of the composition of the bacterial communities found in seagrass beds and close related to seagrass tissues. Information about bacterial diversity in seagrasses can be useful in order to characterize the complexity of the bacterial community in these ecosystems. Assessing the

bacterial diversity associated with seagrasses would allow to discern the variety of putative functions including new metabolic capabilities and also could be a reservoir of useful genes for medicine and biotechnology (Pedrós-Alió 2006).

Information on taxonomic composition of microbial communities associated with seagrasses is still scarce, as it is derived from this compilation, where diversity of bacterial communities was only available for the 30% of the seagrass species found around the world, suggesting the possibility of finding new bacterial species or new relations.

Although it has been demonstrated that the characterization of bacterial communities by culturing methods biases the real composition, as those bacteria able to grow in the selected media are favored, the majority of the works found in this compilation based their research on cultures of the samples. Specifically, the culturing methods represented the 67% of the works. The picture obtained for bacterial diversity found in seagrass ecosystems extracted from this compilation, thus, could be biased favoring those taxonomic groups that better grow in the routinely employed culture media. However, the use of molecular methods, although recognized to be more accurate, has others biases that need to be taken in account. The amplification process favors those sequences that are more abundant with detriment of the sequences found in low titer or those sequences in which the annealing site is less available to primer sets. These little differences after many cycles of amplification can lead to underrepresentation of some taxonomic groups, as well. The large discrepancy between prokaryotic biodiversity obtained applying culture-dependent and culture-independent techniques has been described elsewhere (de Wit 2008), pointing out the convenience of using both techniques in parallel in order to have complete information of the bacterial biodiversity that seagrass meadows hold.

In fact, the bacterial species identified and reported as epiphytes of *Halophila*

stipulacea tissues, differed substantially depending on the technique used. Wahbeh and Mahasneh (1984) identified 4 bacterial species by culture: 2 of them were Actinobacteria, 1 Firmicutes and 1 Proteobacteria; while Weidner et al. (2000) identified 12 bacterial species: 10 were Proteobacteria and only one was identified as Planctomycetes by applying molecular methods (Table 1- Supplementary material). Similarly, in *Posidonia oceanica*, the identification of bacterial species related to its tissues differed depending on the technique used. The species identified applying culture techniques by Marco-Noales et al. (2006) differed from those identified applying molecular techniques by Garcias-Bonet et al. (submitted, Chapter 3). Although molecular methods are supposed to yield a more realistic description, the use of culture techniques allowed the description of 7 new species belonging to the genus *Marinomonas* in *P. oceanica* tissues (Espinosa et al. 2010, Lucas-Elío et al. In press), confirming the convenience of combining both methodologies.

The comparison of the bacterial diversity for seagrasses reported in this study with bacterial diversity in others ecosystems is difficult, as we compiled only those studies where taxonomic information was given and those studies regarding bacterial diversity estimates without taxonomical identification were discarded. Even with this limitation, the bacterial diversity found in seagrass rhizospheres, with 56 different bacterial genus identified, is much more diverse than that calculated for rhizospheres of other terrestrial plants, such as strawberry (*Fragaria ananassa*), potato (*Solanum tuberosum*) and oil seed rape (*Brassica napus*), in which rhizospheres approximately only 9 OTUs were detected by DGGE (Smalla et al. 2001). This diversity found in seagrass rhizospheres is in agreement with the results derived from a review done about bacterial diversity comparing many environments, where marine sediments were one of the most diverse, although this category

included many types of marine sediments (Lazupone and Knight 2007). However, the diversity calculated for 1g of desert soil, prairie and rainforest was extremely higher, with more than 300 different OTUs for each sediment (Fierer et al. 2007). Similarly, other studies predicted a very high bacterial diversity in terrestrial soils, with 2,000 and 5,000 bacterial species in 0.5g of soil (Schloss and Handelsman 2006). Moreover, in unvegetated marine sediments, the bacterial diversity was reported to be different from seagrass colonized sediments, derived from the band patterns obtained by DGGE, although the number of total detected OTUs was not significantly different (James et al. 2006).

The diversity reported in this study related to seagrass tissues (with 83 different bacterial genus identified when pooling together the endophytes, epiphytes and those whose origin was not clearly specified, disabling to discern between endophytic and epiphytic isolates or sequences) was higher than the diversity identified for seagrass sediments. However, the identified endophytic bacterial diversity was lower, with only 9 bacterial genus identified, although the endophytic community in seagrasses seemed to be more diverse, as derived from the results reported for *Posidonia oceanica* where 34 different OTUs were detected in surface-sterilized tissues (Chapter 3). Unfortunately, there is very little information regarding endophytic diversity in seagrasses. However, the endophytic bacterial communities in rice were reported to be highly diverse as well, even analyzing only the diazotrophic community (Ueda et al. 1995).

The bacterial community composition described for both types of samples, seagrass sediments and related to seagrass tissues, differed, as it was expected. The most represented family in seagrass sediments was *Desulfobacteraceae*, which members are known to be sulfate reducers, although identification at genus level was not available for many of these isolates. The sulfate is one of the most abundant components in marine environments, and

these bacteria use it in the oxidation of organic carbon compounds, reducing the sulfate into sulfides under anaerobic conditions, typical of sediments. Thus, the sulfate reducing bacteria change sediment conditions affecting seagrasses that inhabit them. The sulfide produced is toxic for plants (Terrados et al. 1999), and when the sulfide pools exceed concentrations of 10μM in sediment porewater, a decline in seagrasses is derived (Calleja et al. 2007), driving to a decrease in meristematic activity (Garcias-Bonet et al. 2008, Chapet 7). Therein, the role of *Desulfobacteraceae* members may be very important for the health status of seagrasses. This finding is in contrast with bacterial communities in terrestrial soils, where *Bacillus* sp. was found the dominant population in rhizospheres of plants as barley (Normander and Prosser 2000) and terrestrial grass (Felske et al. 1998).

The bacterial species identified as endophytes differed from that identified as epiphytes, suggesting a very different communities. The epiphytic communities were more diverse and with more bacterial Phylums represented. While the endophytic identifications belonged to 3 bacterial Phylums (Proteobacetria 75%, Bacteroidetes 19% and Firmicutes 1%), the epiphytic community belonged to 5 bacetrial Phylums (Porteobacetria 60%, Cyanobacteria 13%, Actinobacteria 11.5%, Bacteroidetes 6.4% and Planctomycetes 1.3%). The most frequently identified bacteria in endophytic community was *Desulfovibrio* sp., while in epiphytic community was *Vibrio* sp.

The species found in seagrass tissues could play a role on nutrient acquisition, particularly in oligotrophic environments, as it is the case of the Balearic Islands in the Western Mediterranean Sea. Nitrogen fixation was previously suggested as an important process contributing to nitrogen pool in seagrass sediments along the Balearic Islands (Papadimitriou *et al*. 2005).

The bacterial species reported in relation to seagrass tissues may play a wide repertoire of functions in the ecosystem. The detection of *Pseudoalteromonas* spp. and *Vibrio* spp. in seagrass tissues is particularly interesting, as it is known that these genera include pathogenic species for marine invertebrates (Costa-Ramos and Rowley 2004), vertebrates (Austin and Zhang 2006) and algae, like that causing red spot disease in *Laminaria japonica* (Sawabe et al. 1998). *Pseudoalteromonas* spp. has also been shown to produce a variety of extracellular compounds with biological activity (Hölstrom & Kjelleberg 1999, Lee et al. 2000). Moreover, some *Vibrio* spp. produce lethal proteases in extracellular products (Farto et al. 2006), as *Vibrio splendidus*, species isolated in *Posidonia oceanica* and *Halophila stipulacea* (Table 1), suggesting the possibility of some bacteria acting as pathogens for seagrasses, although the existence of diseases in seagrasses produced by bacteria has never been described up to date.

Therefore, some of the bacterial strains reported in association with seagrass ecosystems could be detrimental, whereas others could be beneficial for seagrass meadows. However, the discernment of the bacterial roles in ecosystem functioning requires further experimental research. This compilation would help in the better understanding of the involvement of bacteria in seagrass ecosystems.

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Section II

Posidonia oceanica meristematic activity: technique development and its evaluation as an early warning indicator of stress

Chapter 6

Meristematic activity of Mediterranean seagrass (Posidonia oceanica) shoots

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Aquatic Botany, under review

Abstract

Shoot meristematic activity of Mediterranean seagrass *Posidonia oceanica* has been assessed in eleven different meadows located around Balearic Islands. Moreover, in six of them, the meristematic activity has been determined hourly (or each two or three hours) during at least 24hours, with the aim of detecting a possible circadian rhythm in cell divisions. Meristematic activity is inferred by applying flow cytometry techniques combined with DNA labeling to determine the percentage of nuclei in each phase of the cell cycle (i.e. G0+G1, S, G2). The percentage of nuclei in G2 phase of the cell cycle reflects the percentage of nuclei that are dividing in a specific moment. In the shoot meristems of *P. oceanica* the percentage of nuclei in G2 phase was on average 6.99% ± 0.11, although it ranged from 1.83 % to 12.16 % across the meadows studied. The average percentage of nuclei in G2 phase in *P. oceanica* meristems is lower than those reported in literature for other plants. No circadian rhythms were detected in meristems of *P. oceanica*. Meristematic activity could be used as indicator of seagrass stress, and, thus to assess impacts to meristems before population declines could be observed.

Introduction

Meristems are multicellular plant tissues formed by undifferentiated cells in continuous division that produce new cells that will develop and differentiate into new tissues and organs. Therefore, meristems are the major sites of cell divisions responsible for plant growth. Meristems are also responsible for forming primordia that will develop into new leaves and flowers, and in the case of clonal plants they are also responsible for extending rhizomes or stolons and, hence, the vegetative spread of the clone (Tomlinson 1974). In a meristem, different cell layers and zones can be distinguished with cells being different in size and shape. As well, there is heterogeneity of growth rates within the apex and heterogeneity in cell cycle duration can be detected in the cells of a single meristem (Lyndon 1998). Meristematic activity, assessed as temporal changes in the number of cells per apex, the rate of cell division (Lyndon 1998) or/and the cell cycle duration, has been widely studied in terrestrial angiosperms. On the contrary, the dynamics of meristems of seagrasses, i.e. marine angiosperms is largely unknown, despite its central role in clonal seagrass growth (Tomlinson 1974).

Posidonia oceanica is a Mediterranean endemic seagrass and the dominant species in this sea, with a total extension of 50 000 Km^2 (Béthoux and Copin-Montégut 1986). Like all seagrasses, *P. oceanica* is a clonal plant that occupies the space largely by growing vegetatively repeating the structural unit called ramet. In *P. oceanica*, shoots, which have basal meristems, emerge at the apexes of the rhizomes. Hence, in this species, shoot meristems are responsible of both shoot growth and rhizome elongation (Tomlinson 1974). *P. oceanica* ranks among the largest and the slowest-growing seagrass species (Duarte 1991). Despite the slow growth rate of *P. oceanica* rhizomes (1-6 cm yr^{-1}, Marbà and

Duarte 1998), the resulting clones may spread across several kilometers (Díaz-Almela et al. 2007, Arnaud-Haond et al. submitted) and, thus, achieve millenary ages, while the ramets may live for several decades (e.g. Marbà et al. 1996). *P. oceanica* plays an important role in ecological processes, such as shoreline protection, sediment retention and carbon burial, and enhancement of coastal biodiversity (Hemminga and Duarte 2001). *P. oceanica* is very sensitive to coastal deterioration and is suffering a widespread decline in the Mediterranean (Marbà et al. 2005). Detection of *P. oceanica* decline from thinning of the meadows identifies the problem at a time that maybe difficult to revert, and because the dynamics of *P. oceanica* shoot populations is largely the outcome of the dynamics of its meristems, *P. oceanica* meristematic activity may allow a rapid assessment of the health status of the plants. Indeed, there is evidence of a marked decrease in the percentage of dividing cells in shoot meristems of *P. oceanica* when sediments conditions become adverse to plant survival, such as accumulation of sulfides (Garcias-Bonet et al. 2008).

Eukaryotic cell division cycles comprise the Interphase and Mitosis phase. Interphase consists of a G1 phase (post-mitotic phase), where the cell grows and synthesizes proteins and RNA; an S phase (DNA synthetic phase), where the DNA is replicated; and a G2 phase, where the cell has doubled the DNA content and the nuclear proteins and is preparing to enter the mitotic phase (M). There are many regulation points across the cell division cycle. In this regulation, protein-kinases are involved regulating the transition from the G1 phase to the S phase and from the G2 phase to the Mitosis (Huntley and Murray 1999, Dewitte and Murray 2003). The percentage of nuclei in G2 phase provides information on the percentage of meristematic cells that are dividing. A reduction

in the percentage of dividing cells would be expected to result in decreased shoot growth rates, which may propagate to the population level to affect net population growth.

Cell division parameters have been estimated based on (Fiorani and Beemster 2006): 1) the observation of individual cells and determination of the time between two successive division events; 2) using cell cycle blocking methods, arresting the progression of the cycle at any point of the cell cycle and determining the rate at which this fraction of cells increases; and 3) using cell suspension cultures and determining the rate at which the cell number increases over time, in most of the cases using synchronized cell cultures. These methods combine labeling techniques with microscopy or flow cytometry. Another metric informing of the cell division parameters is the mitotic index, which is the percentage of the total number of cells in a sample that are in mitosis (Baskin 2000).

The mitotic activity of plant meristems can also be assessed by measuring the percentage of cells that are dividing, as demonstrated for intact tissues of *Nicotiana tabacum* (Galbraith et al. 1983). Studies on macroalgae *Ulva pseudocurvata* (Chlorophyceae) and *Porphyra yezoensis* (Rhodophyceae) have been able to detect a circadian rhythm in cell division rates (Oohusa 1980, Titlyanov et al. 1996). Flow cytometry has been extensively used in research with animal cells and bacteria but not so much with plant cells, because of the difficulties they present. However, flow cytometry techniques have been applied in plants to analyze the DNA content of cells or isolated nuclei suspensions (Galbraith et al. 1983, Le Gall et al. 1993, Koce et al. 2003), to elucidate the duration of the cell cycle in cell cultures of *Solanum aviculare* (Yanpaisan et al. 1998), to determine the rate of cell division in phytoplankton (Mann and Chisholm 2000, Sosik et al. 2003, Agawin and Agustí 2005) and to examine the response of cell division of *P.*

oceanica meristems to sediment deterioration due to macroalgae invasions (Garcias-Bonet et al. 2008).

The goal of this study is to determine the variability of *P. oceanica* meristematic activity by quantifying the percentage of nuclei in each phase of the cell cycle in meristems of shoots collected in 11 different meadows along the Balearic Islands. Moreover, we examine the diel fluctuations of the percentage of nuclei in each phase of the cell cycle in shoot meristems from 6 meadows to elucidate if the cellular divisions are synchronized and/or adjusted to a circadian rhythm, which would allow estimation of cell cycle duration.

Materials and Methods

Sampling strategies

We collected *Posidonia oceanica* shoots from 11 meadows located along the Balearic Sea (8 in the coasts of Mallorca Island and 3 in those of Cabrera Island, Fig. 1) growing at water depths between 4 and 17 m (Table 1). In one of these meadows, Es Port (Cabrera Island), we collected shoots to examine the meristematic activity in plots with sediments that were fertilized with iron for two years (station Es Port Fe, Marbà et al. 2007) and unfertilised plots (station Es Port C). The sediments of all the meadows studied, as those along most of the Balearic Sea, are biogenic, carbonate rich and iron deficient (Holmer et al. 2003). The biogeochemical condition of Balearic sediments renders seagrass meadows highly vulnerable to organic inputs and iron limited (Marbà et al. 2008). Cabrera Island is the largest island of Cabrera Archipelago, declared a Terrestrial-Maritime National Park since year 1991. In Cabrera Island, the access to the sites Santa Maria and Es Castell is restricted to visitors whereas Es Port is open to visitors, with 50 permanent moorings. In

Mallorca Island, the meadow at Ses Salines is free of anthropogenic pressures, while the meadows at Magalluf, Illetes, Portals Vells, Pollença, Cala Millor, Porto Colom, Sa Colònia de Sant Jordi (referred as Sa Colonia) are adjacent to touristic destinations.

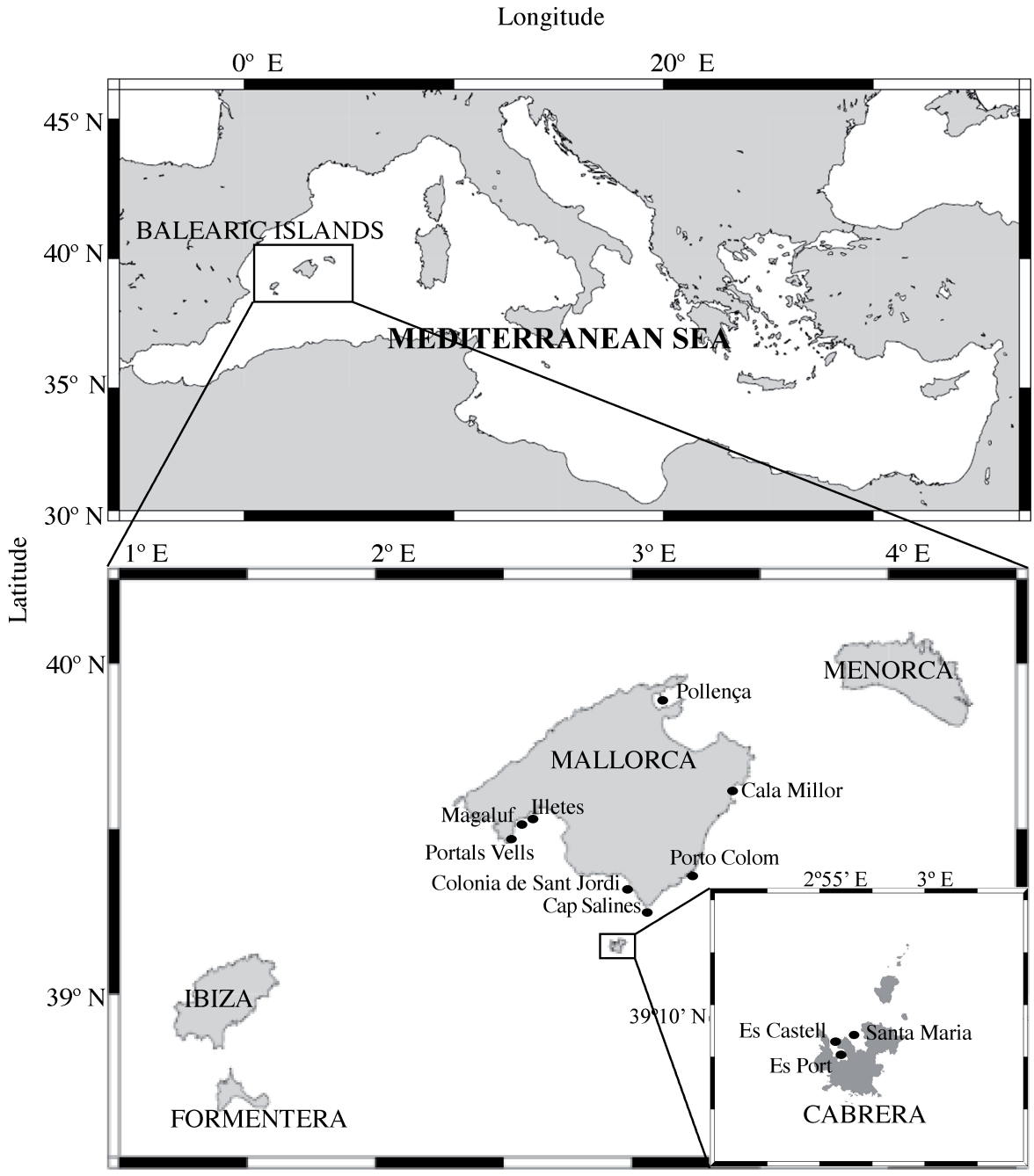

Figure 1. Map showing the location of the *Posidonia oceanica* meadows sampled.

The meadows were sampled during the growing season of *P. oceanica* (Barrón and Duarte, 2009; Table 1). The samples were immediately transported to the laboratory following collection of 100 healthy shoots randomly by SCUBA divers in each station and maintained in aerated aquaria with seawater from the same location. The meristematic activity was measured one day after sample collection.

Table 1. Summary of *Posidonia oceanica* meadows sampled in this survey. The length and the frequency of sampling are shown for each station.

Station	Depth (m)	Sampling Date	Sampling lenght	Sampling frequency
Cabrera Island				
Es Port C	17	July 2004	30 h	1-2h
Es Port Fe	17	July 2004	30 h	1-2h
Es Castell	5	July 2004	1 event	-
St Maria	7	July 2004	1 event	-
Mallorca Island				
Magalluf	6	July 2004	24 h	1h
Illetes	9	July 2004	24 h	1h
Portals Vells	6	June 2004	48 h	3h
Pollença	4	October 2005	22 h	1h
Cala Millor	7	August 2004	1 event	-
Porto Colom	6,5	June 2004	1 event	-
Sa Colonia	4	July 2004	1 event	-
Cap Salines	6	May 2006	1 event	-

Meristematic activity

In six of these eleven sampled *Posidonia oceanica* meadows, the activity of shoot meristems was estimated hourly (or every 2-3 hours), in three replicated shoots, during at least 24 hours. The duration of the experiments ranged from 22 to 48 h and the sampling frequency varied from 1 to 3 h depending on the meadows (Table 1). In the other six *P. oceanica* meadows, the activity of rhizome meristems was estimated only once, always between 11:00 and 12:00 am (Table 2).

Table 2. Average and Standard Error (SE) of the percentages of nuclei in each phase of the cell cycle (i.e. G1, S, G2) for all the *Posidonia oceanica* meadows tested in this study. The time of the day when the analysis was performed is detailed. n: number of shoot meristems analyzed.

Station	Time of day	n	Average % of nuclei in G1 Phase	SE % of nuclei in G1 Phase	Average % of nuclei in S Phase	SE % of nuclei in S Phase	Average % of nuclei in G2 Phase	SE % of nuclei in G2 Phase
Cabrera Island								
Es port C	12:00	3	85,96	1,06	5,55	0,70	8,50	1,18
Es Port Fe	12:00	3	87,09	1,06	5,72	1,47	7,19	0,46
Es Castell	11:00	5	78,87	1,99	14,81	2,12	6,32	0,74
St Maria	11:00	5	83,39	1,36	9,35	1,80	7,25	0,73
Mallorca Island								
Magalluf	11:00	3	77,68	2,75	16,85	3,24	5,46	0,52
Illetes	11:00	3	76,61	2,95	17,47	4,65	5,92	1,84
Portals Vells	11:00	5	82,53	3,51	11,81	4,68	5,75	1,20
Pollença	11:00	6	86,57	1,10	5,66	0,90	7,78	0,51
Cala Millor	11:00	4	89,73	1,82	4,86	0,93	5,41	0,90
Porto Colom	11:00	4	91,16	1,12	4,82	0,64	4,03	0,71
Sa Colonia	11:00	5	88,25	1,15	6,78	1,87	4,97	1,10
Cap Salines	12:00	6	73,67	2,69	21,03	3,16	5,30	1,52

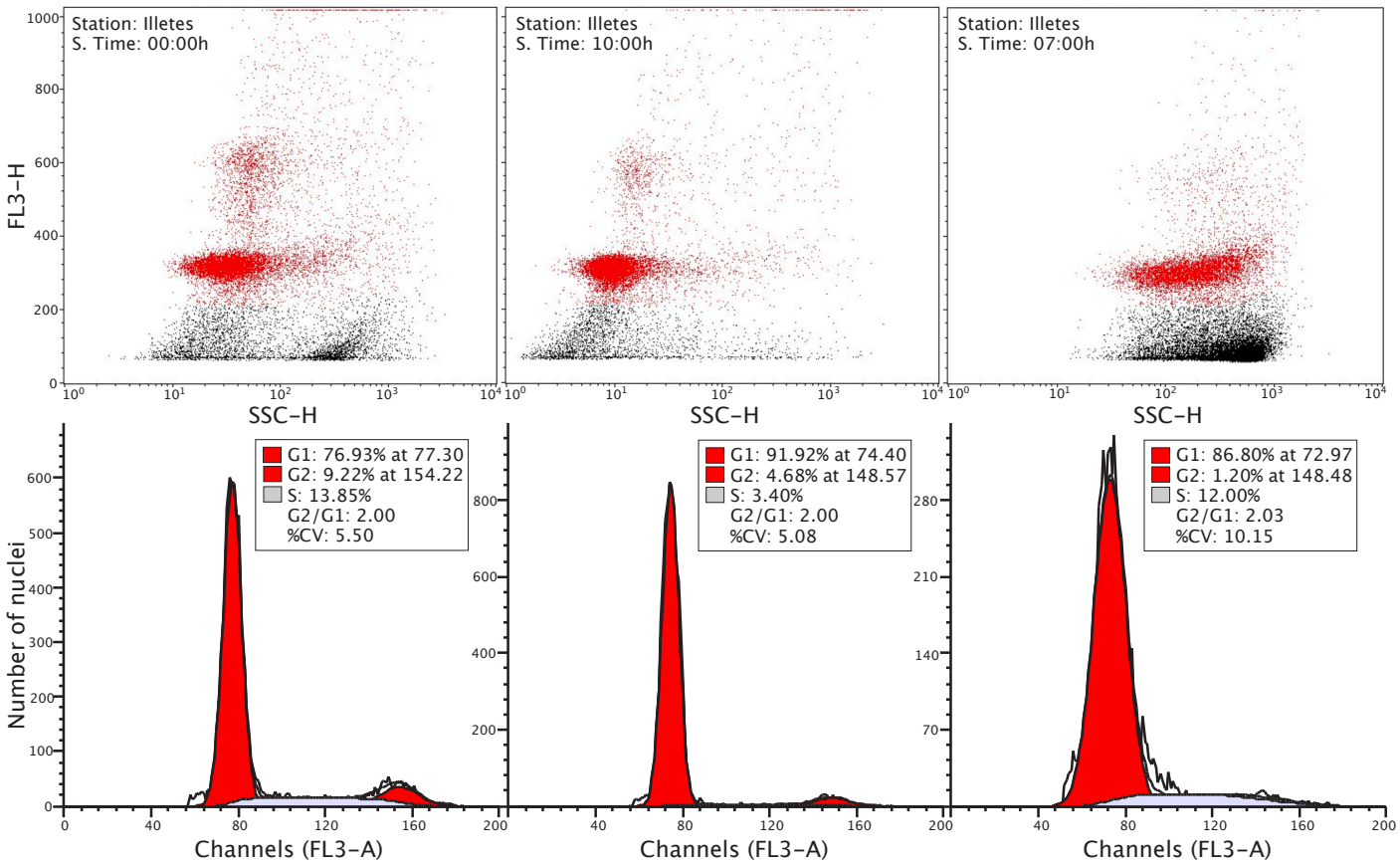

Figure 2. Flow cytometry plots: Red Fluorescence (FL3-H) vs Side Scatter (SSC-H) showing the nuclei detected in each sample (in red), and the corresponding histogram of nuclei fluorescence (FL3-A) with the cell cycle phases analysis (percentage of nuclei in each phase of the cell cycle: G1, S, G2) by ModFit software for three different sampling hours at Illetes station. Three different situations are shown: high (Illetes 00:00h), intermediate (Illetes 10:00h) and low (Illetes 07:00h) percentages of nuclei in G2 phase of the cell cycle.

The meristematic activity was calculated by quantifying the percentage of nuclei in each phase of the cell cycle (i.e. G0+G1, S, G2). Samples of 0.5 cm x 1 cm from the rhizome meristematic zone were dissected and the nuclei were isolated using a Partec® extraction kit and stained with propidium iodide (PI) for 1 hour in darkness at 4º C. The number of nuclei in each phase of the cell cycle (i.e. G0+G1, S, G2) was quantified using a Beckton-Dickinson flow cytometer equipped with an argon-ion laser measuring the red fluorescence emitted by the PI. For each meristem ten thousand nuclei were analyzed (Fig. 2). The histograms of nuclei fluorescence obtained from the flow cytometer were analyzed using cell cycle analysis software (ModFit), which provided the percentage of nuclei in G1, S, G2 phases of each meristem sampled (Fig. 2).

Statistical analysis

We assessed the statistical significance of the variability of percentages of nuclei in each phase of the cell cycle among stations and among sampling times using ANOVA. The significant differences were detected by comparing means pairs using Tukey's post-hoc test.

Table 3. Average (AVG), Standard Error (SE), Coefficient of Variation (CV), Maximum and Minimum values and its time of the day (MAX, MIN, Sampling hour) of the percentages of nuclei in each phase of the cell cycle (i.e. G1, S, G2) for the *Posidonia oceanica* meadows that have been analyzed during 24h or 48h long. Levels of Tukey post-hoc test are shown. A, B, C, D indicate significantly different percentage of nuclei in each phase of the cell cycle across stations. Stations not connected by the same letter are significantly different.

	Cabrera Island		**Mallorca Island**			
	Es port C n= 72	**Es Port Fe** n= 72	**Magalluf** n= 71	**Illetes** n= 72	**Portals Vells** n= 84	**Pollença** n= 126
% of nuclei in G1 Phase						
AVG	86,09^{A}	85,8^{A}	81,02^{B}	80,51^{B,C}	78,67^{C}	81,75^{B}
SE	0,36	0,27	0,59	0,63	0,8	0,43
CV	2826,35	3945,05	1636,44	1526,95	1083,08	1696,68
MAX	88,29	87,96	86,05	85,91	83,38	86,56
Sampling hour	14:00	08:00	03:00	07:00	23:00	11:00
MIN	78,09	80,16	71,18	71,19	70,39	74,91
Sampling hour	15:00	15:00	12:00	16:00	08:00	03:00
% of nuclei in S Phase						
AVG	7,02^{B}	6,9^{B}	13,78^{A}	13,45^{A}	15,56^{A}	8,50^{B}
SE	0,39	0,29	0,71	0,74	0,97	0,42
CV	212,39	287,99	232,86	216,44	176,42	179,09
MAX	16,43	13,26	22,65	24,91	27,77	14,66
Sampling hour	15:00	15:00	12:00	16:00	08:00	01:00
MIN	4,93	3,19	7,86	8,12	9,85	4,45
Sampling hour	02:00	08:00	00:00	01:00	14:00	15:00
% of nuclei in G2 Phase						
AVG	6,89^{B,C}	7,29^{B}	5,2^{D}	6,04^{C,D}	5,67^{D}	9,76^{A}
SE	0,17	0,15	0,25	0,22	0,31	0,2
CV	490,13	567,26	250,79	325,41	199,65	425,15
MAX	8,49	8,85	6,84	7,71	8,44	12,16
Sampling hour	12:00	08:00	10:00	21:00	14:00	04:00
MIN	5,47	5,59	2,37	3,89	1,83	7,71
Sampling hour	18:00	16:00	07:00	16:00	08:00	19:00

Results

Most of the nuclei were in G1 phase whereas those in G2 phase accounted for the smallest fraction of the nuclei populations (Table 3, Fig. 3-5). However, the percentage of nuclei of *Posidonia oceanica* meristems in the different phases of the cell cycle varied over time. In the *Posidonia oceanica* meadows where meristematic activity was measured over more than 24h, the percentage of nuclei in G2 phase of the cell cycle varied between 1.5 fold (Es Port C) and 4.6 fold (Portals Vells) over time (Table 3), the percentage of nuclei in G1 phase between 1.1 fold (Es Port Fe) and 1.2 fold (Magalluf) and that of nuclei in S phase between 2.8 fold (Portals Vells) and 4.2 fold (Es Port Fe), (Table 3). The largest diel fluctuations in G1, S and G2 phases were observed in the shoot meristems from Magalluf, Es Port Fe and Portals Vells meadows, respectively, (Table 3), whereas the amplitude of the fluctuations in G1, S and G2 phases was the smallest at Es Port Fe, Portals Vells and Es Port C respectively (Table 3). Despite the temporal fluctuations observed in the percentage of nuclei of *P. oceanica* meristems in the different phases of the cell cycles, they were statistically significant only in the shoots growing at Pollença for percentages of nuclei in G1 and G2 phase (ANOVA test, $p<0.0001$; Tukey post-hoc test, Table 3), the temporal changes in the percentages of nuclei in S phase were statistically significant in the shoots growing at Es Port Fe and Pollença (ANOVA test, $p<0.0001$; Tukey post-hoc test, Table 3). The temporal changes in meristematic activity observed in the shoots of *P. oceanica* did not exhibit a circadian rhythm (Fig. 3-5). Moreover, temporal trends in cell division of *P. oceanica* meristems differed across meadows (Table 3, Fig. 3-5).

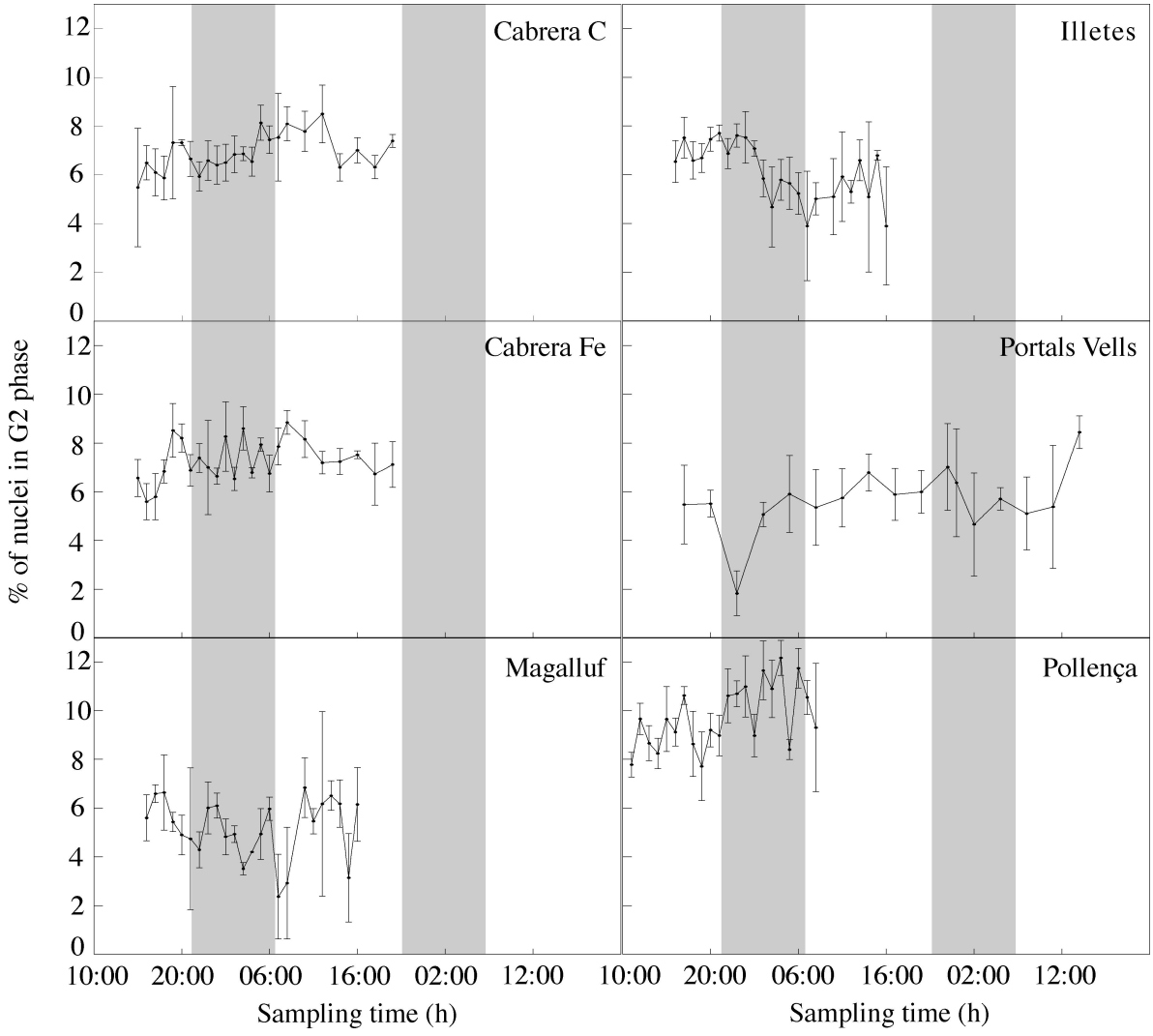

Figure 3. Percentage of nuclei in G2 phase of the cell cycle in meristems of *Posidonia oceanica* measured at different frequencies for at least 24h. Shaded areas correspond to night times.

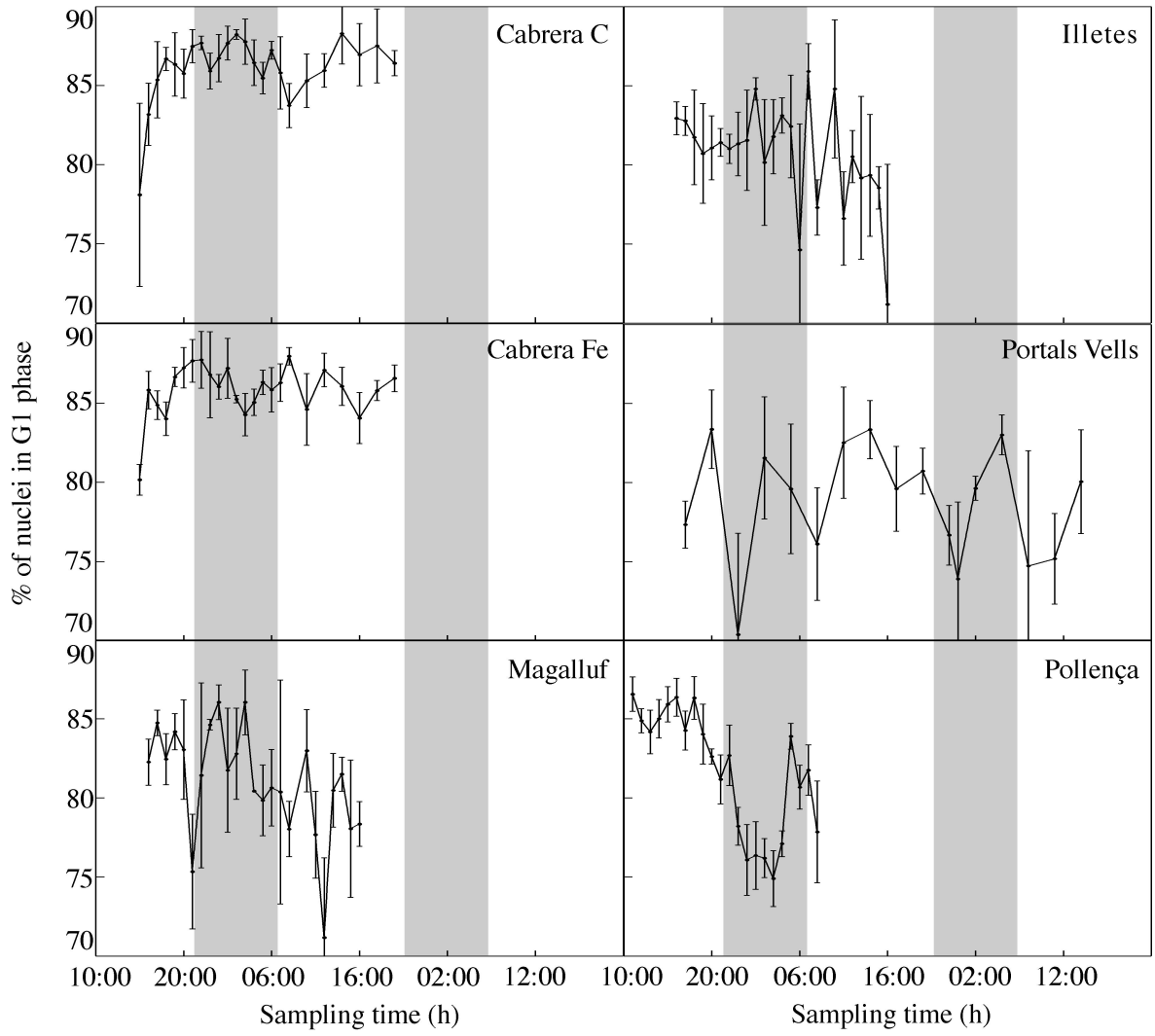

Figure 4. Percentage of nuclei in G1 phase of the cell cycle in meristems of *Posidonia oceanica* measured at different frequencies for at least 24h. Shaded areas correspond to night times.

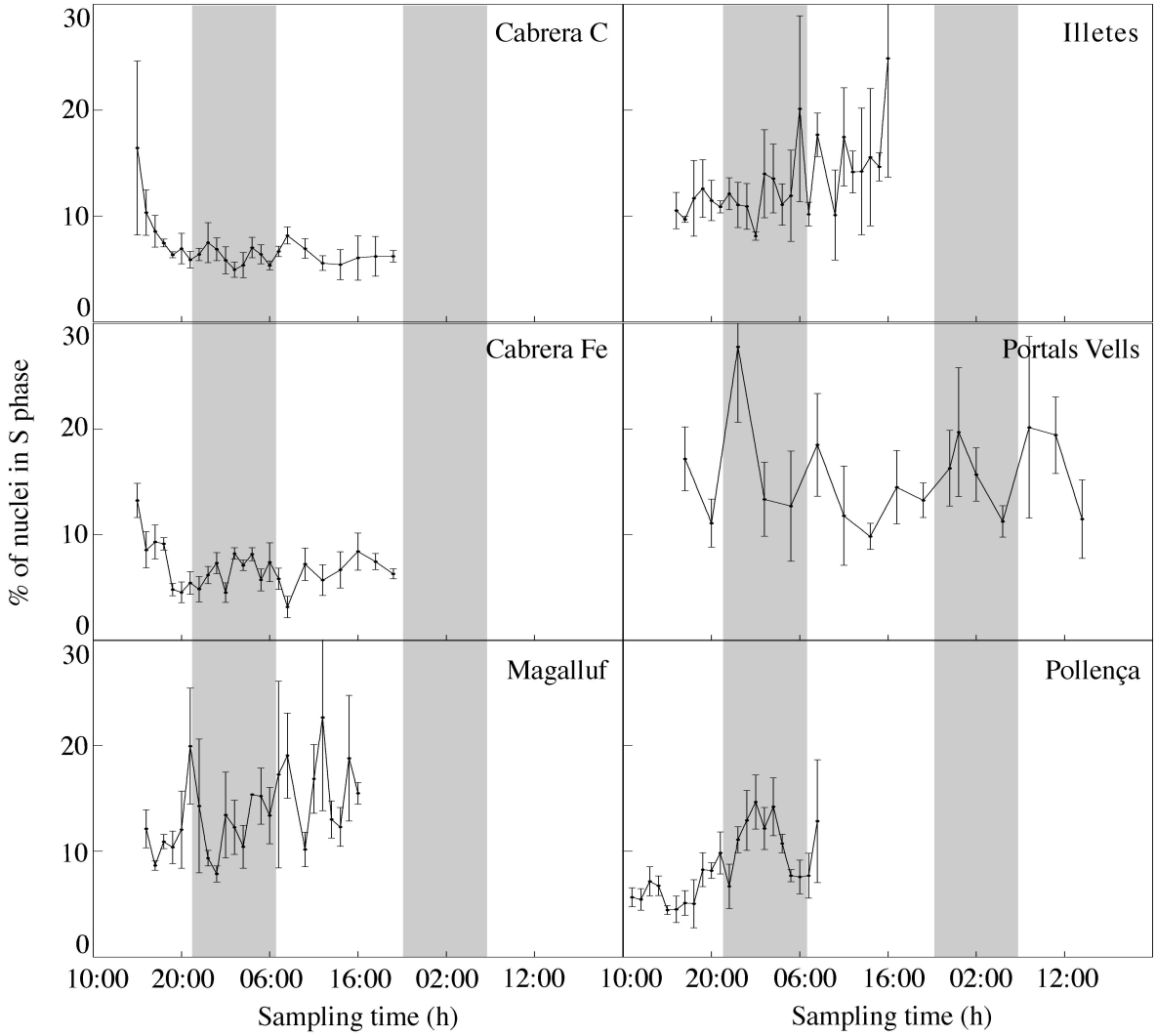

Figure 5. Percentage of nuclei in S phase of the cell cycle in meristems of *Posidonia oceanica* measured at different frequencies for at least 24h. Shaded areas correspond to night times.

The meristematic activity in *Posidonia oceani*ca shoots (i.e. percentage of nuclei in each phase of the cell cycle) at noon varied substantially across the 11 meadows studied (Table 2). At this time of the day, most meristematic nuclei in the shoots of all stations were in phase G1 of the cell cycle accounting, on average, for 73.67% to 91.16% of the total nuclei population (Table 2). The percentage of nuclei in phase S ranged between 4.82% and 21.03%, and between 4.03% and 8.50% of nuclei were in phase G2 of the cell cycle (Table 2).

Discussion

The percentage of dividing cells in *Posidonia oceanica* meristems, quantified as the percentage of cells in the G2 Phase of the cell cycle, is 6.99 ± 0.11 % on average for all the meadows studied here. This value reveals that cell division rates in *P. oceanica* are low when compared with those reported for other plant species, encompassing terrestrial and aquatic plants and macroalgae. In most studies assessing cell division of vegetation tissues, the rate of cell division is reported as mitotic index (sum of cells in prophase, metaphase, anaphase and telophase as a percentage of the total), and only few studies provide data on the percentage of cells in each phase of the cell cycle. The mitotic indexes reported in the literature indicate that they vary between 1 and 30% in other plant tissues (Table 4). Less data on the percentage of cells in each phase of the cell cycle, specifically for the G2 phase, are available, and range from 22.3 to 55.9 % (Table 4).

The low percentage of dividing cells obtained for *Posidonia oceanica* meristems is consistent with the very slow growth of this species, which ranks among the slowest-growing plants among the seagrass flora (Duarte 1991). The low fraction of dividing cells observed in this study could also reflect, to some extend, an underestimation of the amount

of dividing cells due to the methodology used. During nuclei extraction process we are loosing all the nuclei in Mitosis phase, because the nuclear membrane disappears to allow the separation of the chromosomes, which makes it impossible to recover those nuclei for flow cytometry analysis. However, this underestimation should be small since during the temporal experiments no clear peaks in G2 phase, i.e. nuclear phase prior to the Mitosis phase, were detected (Fig. 3).

Table 4. Values of dividing cells in plant tissues by applying different approaches found in literature.

Plant species	Tissue analyzed	Approach	Value (%)	Reference
Lemna minor	Root tips	Mitotic Index	7.1-7.7	Samardakiewicz and Wozny, 2005
Arabidopsis sp.	Seedling cotiledons	Mitotic Index	20-30	Stoynova-Bakalova et al., 2004
Dactylis glomerata	Shoot meristems	Mitotic Index	7.3-7.8	Kinsman et al., 1997
Dactylis glomerata	Roots	Mitotic Index	5.2-6.9	Kinsman et al., 1997
Arabidopsis thaliana	Shoot apical meristem	Mitotic Index	1	JacQMard et al., 2003
Vicia faba	Root tips	Mitotic Index	9.1	Dolezel et al., 1992
Stenocereus gummosus	Root meristem	Mitotic Index	4.8	Dubrovsky et al., 1998
Ferocactus peninsulae	Root meristem	Mitotic Index	8.8	Dubrovsky et al., 1998
Pachycereus pringlei	Root meristem	Mitotic Index	6.9	Dubrovsky et al., 1998
Nicotiana tabacum	Leaf primordia	Metaphase Index	5	Cockcroft et al., 2000
Nicotiana tabacum	Leaf	Nuclei in G2 phase by flow cytometry	22.3	Galbraith et al., 1983
Nicotiana tabacum	Root	Nuclei in G2 phase by flow cytometry	55.9	Galbraith et al., 1983
Nicotiana tabacum	Leaf	Nuclei in G2 phase by flow cytometry	27	Chen et al., 2001
Solanum aviculare	Cell suspension	Cells in G2 phase by flow cytometry	40	Yanpaisan, 1998
Vicia faba	Root tips	Cells in G2 phase by flow cytometry	26.5	Dolezel et al., 1992
Posidonia oceanica	Shoot meristems	Nuclei in G2 phase by flow cytometry	6.99	This study

The variability of the percentage of nuclei in G2 phase observed across *P. oceanica* meadows may reflect different local ambient conditions for seagrass growth and the extent of synchronization of cell cycles among populations. The largest percentages of nuclei in G2 phase were observed in *P. oceanica* shoot meristems growing at the relatively pristine meadows from Cabrera Island (Table 2). The percentage of nuclei in G2 phase observed in seagrasses from Cabrera rank within the range reported for *P. oceanica* growing in

sediments with low concentration of sulfides (Garcias-Bonet et al. 2008), a toxic compound for plant cells (Raven and Scrimgeour 1997). *P. oceanica* has been demonstrated to be highly sensitive to sediment sulfide concentrations as reflected by the increase of the rate decline of *P. oceanica* shoot population when sediment sulfide concentration exceeds 10 μM (Calleja et al. 2007). At high sediment sulfide concentrations there is evidence that sulfides intrude into seagrass tissues (e.g. Frederiksen et al. 2007) and may damage plant meristems (Greve et al. 2003). Indeed, a sharp decrease in meristematic activity of *P. oceanica* shoots when sediment sulfide concentration increase, due to enhanced sediment sulfate reduction rates of invasive *Caulerpa* species, has been reported (Garcias-Bonet et al. 2008), and suggests that meristematic activity below 5 % of nuclei in G2 phase could reflect sulfide intrusion. According to Garcias-Bonet et al. (2008), the percentage of nuclei in G2 phase observed in our study in *P. oceanica* shoots growing at Porto Colom and Sa Colonia (Table 2) could reflect sulfide stress and, hence, sediment conditions compromising seagrass growth and survival. These are indeed ecosystems that receive substantial inputs from dense tourist developments in the shore.

Cell division in the meristems of *P. oceanica* shoots does not exhibit a circadian rhythm nor does it display consistent temporal fluctuations across meadows. The absence of consistent rhythms in the percentage of meristematic cell division was expected due to the fact that meristems of vascular plants present many areas of division that have different rates and different lengths in the cell cycle (Lyndon 1998), which prevents detection of synchronization of cell division. However, on the marine green algae *Ulva pseudocurvata*, Titlyanov et al. (1996) reported the existence of a circadian rhythm in cell divisions, detecting a maximum value of 15% of cells in Mitosis phase. Oohusa (1980) also detected a

circadian rhythm in cell division in *Porphyra yezoensis* (*Rhodophyceae*) although no percentage of cell division was reported for this red alga.

The lack of temporal rhythms in cell division allows quantification of the average of the percentage of cells that are dividing, assessed as the % of nuclei in G2 phase, without requiring following cell divisions across time. Further studies are needed to understand how seagrass meristems are regulated and how these percentages of dividing cells are changing across the year, as leaf growth and rhizome elongation rates in temperate seagrass, as *Posidonia oceanica* and others marine angiosperms, exhibit marked seasonality.

In summary, this work reports, for the first time, the percentages of dividing cells (both by single measurements and time-series measurements) in the Mediterranean seagrass *Posidonia oceanica*, on the basis of 11 meadows located along the Balearic Islands and receiving different levels of anthropogenic pressures. These values of meristematic activity have potential to be used as an early warning indicator of *P. oceanica* meadow decline. Deterioration of sediment conditions (Calleja et al. 2007) and warming (Marbà and Duarte 2010) are accelerating the decline of *P. oceanica* in the Mediterranean Sea. The results provided here help conform a base line that can be used to assess impacts to meristems before population declines be observed, which may be useful to conserve this key species in the Mediterranean ecosystem.

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Chapter 7

Effects of sediments sulfides on seagrass (Posidonia oceanica) meristematic activity

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Feature article

Abstract

Meristematic activity response of *Posidonia oceanica* shoots has been assessed along a gradient of sediment sulfate reduction rates (SRR) and sediment sulfide pools (SSP) to test if meristematic activity could be used as an early indicator of seagrass health. The percentage of nuclei in the G2 phase of the cell cycle was used as a proxy of the cell division rate and therefore of the meristematic activity. The variability observed in the percentage of dividing cells (i.e. containing nuclei in G2 phase) in *P. oceanica* meristems was closely (>80% of total variance) coupled to variability in SSP and SRR. The percentage of nuclei in G2 phase exponentially declined with increasing SSP and SRR, reaching the lowest values (< 5 %) when plants were growing on sediments with SSP greater than 0.001 mol AVS (Acid Volatile Sulfides) m^{-2}. These results demonstrate that the meristematic activity can be used as an early warning indicator of seagrass stress.

Introduction

Seagrass meadows rank amongst the most productive ecosystems on Earth, growing along tropical and temperate coasts where they have important structural and biogeochemical functions (Hemminga and Duarte 2000). Seagrasses are clonal plants, expanding into new areas vegetatively by adding modules produced by the meristems located at the rhizome apices, rendering seagrass demographics and productivity closely dependent on rhizome meristematic activity (Tomlinson 1974).

Currently, seagrass meadows are undergoing worldwide decline as reflected by losses in cover and density during the 20th century (e.g. Orth et al. 2006). Excess inputs of nutrients and organic matter have been identified as the main drivers of the observed decline (e.g. Orth et al. 2006). Organic carbon and nutrient inputs to the sediment stimulate bacterial activity, increasing sediment oxygen demand and the production of bacterial metabolites, such as sulfides, toxic for seagrasses (Terrados et al. 1999). Several die-off events in subtropical (*Thalassia testudinum,* Borum et al. 2005) and temperate (*Zostera marina*, Goodman et al. 1995) seagrass meadows have been linked to episodes of high sediment sulfide concentrations. Recently, it has been suggested that *Caulerpa* spp invasions could also accelerate the decline of *Posidonia oceanica* meadows, since these macroalgae species increase sediment sulfate reduction rate and sulfide pools by 3 fold, when compared with non-invaded meadows (Holmer et al. submitted). The rate of decline of *P. oceanica* meadows is closely coupled to sediment sulfide pools, increasing when sulfide porewater concentration exceeds 10 μM H_2S (Calleja et al. 2007). There is evidence of sulfide intrusion in *P. oceanica* tissues when growing in sediments with high sulfate reduction rate (Frederiksen et al. 2007). The intrusion of sulfide into the meristematic zone

is considered detrimental for the plants, as it inhibits the cytochrome oxidase even at low concentrations (1-10 μM, Raven & Scrimgeour 1997). Hence, seagrass health is compromised by anoxia and sulfide concentration in the rhizosphere (Terrados et al. 1999, Duarte et al. 2005), since the activity of seagrass meristems growing in sediments with high sulfide concentrations may be affected.

The activity of plant meristems can be analysed by measuring the percentage of dividing cells at a specific time point, as demonstrated for intact tissues of *Nicotiana tabacum* (Galbraith et al. 1983), and for cell suspensions of *Solanum aviculare* (Yanpaisan et al. 1998). Flow cytometry has been extensively used to quantify the DNA content of cells or isolated nuclei suspensions (e.g. Galbraith et al. 1983, Le Gall et al. 1993). Eukaryotic cells have a cell division cycle that is divided into Interphase and Mitosis phase. The Interphase consists of a G1 phase (post-mitotic phase), when the cell grows and synthesizes proteins and RNA; an S phase (DNA synthetic phase), when the DNA is replicated; and a G2 phase, when the cell has doubled the DNA content and the nuclear proteins and is preparing to enter the mitotic phase. The percentage of nuclei in G2 phase provides information on the percentage of meristematic cells that are dividing. Hence, the percentage of nuclei in G2 phase could be used as an indicator of meristematic activity and, thus, at declining percentages as an indicator of seagrass stress.

In this study, we quantify the percentage of nuclei in each phase of the Interphase of the cell cycle in rhizome meristems of the dominant Mediterranean seagrass, *Posidonia oceanica*, growing alone and mixed with the native *Caulerpa prolifera*, and invasive *C. taxifolia* or *C. racemosa* in three meadows from the Balearic Islands (Spain). The *P. oceanica* examined grew on sediments encompassing the broad range of sediment sulfide

concentrations and sulfate reduction rates reported for the Balearic Islands region (Holmer et al. 2003, Calleja et al 2007). The percentage of nuclei in Interphase phases is quantified by using flow cytometer techniques and cell cycle analysis. We compare the variability found for the percentage of dividing cells, or nuclei in G2 phase, with sediment sulfide pools and sulfate reduction rates to evaluate the use of meristematic activity as an early warning indicator of seagrass stress.

Materials and Methods

Study site

This study was conducted at three *Posidonia oceanica* meadows, growing in Mallorca (Balearic Islands, Spain), that were partially colonized by the autochthonous macroalgae *Caulerpa prolifera* in Cala Llonga (39º 22.03'N, 3º 13.73'E), and the invasive *C. taxifolia* and *C. racemosa* in Cala d'Or (39º 22.164'N, 3º 13.88'E) and in Cala Estancia (39º 32.13'N, 2º 42.65'E), respectively. The *P. oceanica* meadow at Cala Llonga, located in a sheltered bay, at the entrance of a marina, is declining, as evidenced by large areas with dead rhizomes. It grows at 3 m depth and receives high sedimentary inputs (10 gDW m^{-2} d^{-1}, Holmer et al. submitted). The *P. oceanica* meadow at Cala d'Or is in steady-state growth condition (Marbà, unpublished data), it grows at 6 m depth and receives low sedimentary inputs (2 gDW m^{-2} d^{-1}, Holmer et al. submitted), and has been invaded by patches of *C. taxifolia* since 1992 (T. Grau, personal communication). The *P. oceanica* meadow at Cala Estancia grows at 3 m depth in front of a highly developed coastal area, receiving about 5 gDW m^{-2} d^{-1} sedimentary inputs (Holmer et al. submitted), and the presence of *C. racemosa* has been recorded since 1998 (T. Grau, personal communication). In all studied meadows *Caulerpa spp*. grow as monospecific patches on sandy areas, on dead *P. oceanica*

rhizomes, as well as mixed with P. oceanica. The study was conducted during July 2005, the time of the year when the biomass of all three *Caulerpa spp.* (Terrados and Ros 1991, Thibaut et al. 2004, Ruitton et al. 2005), and bacterial activity are the highest (Hobbie and Colle 1984).

Sampling

At each meadow we selected two sampling sites (hereafter called stations), one where *Posidonia oceanica* was growing monospecifically, and one where *P. oceanica* and *Caulerpa* spp. were growing together. At each station, we collected three 4.5 cm diameter, 10 cm long, sediment cores to estimate sediment density and porosity, and three 2.6 cm diameter, 20 cm long, sediment cores to quantify sediment sulfide pools and sulfate reduction rates. At each station, we harvested 12 *P. oceanica* vertical shoots that were transported to the laboratory in seawater, where their meristematic activity was analyzed immediately.

Sulfate Reduction Rates (SRR) and Sediment Sulfide Pools (SSP)

SRR and SSP were quantified as described in Holmer et al. (*submitted*) according to the two-step procedure (acid volatile sulfides, AVS, and chromium reducible sulfur, CRS) (Fossing & Jørgensen 1989). Sulfide concentrations were measured by the spectrophotometric method (Cline 1969), and sulfate reduction rates were calculated according to Jørgensen (1978). Only rates and pools from the AVS fraction are used here (SSR_{AVS} and SSP_{AVS}).

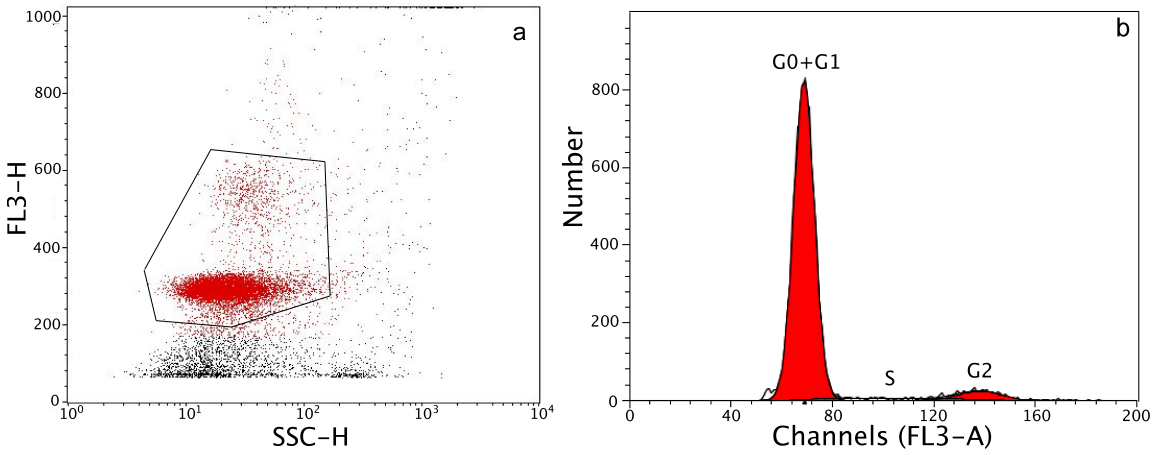

Figure 1. *Posidonia oceanica*. Red fluorescence (FL-3H) vs Side Scatter (SSC-H) of the nuclei extracted from the *P.oceanica* meristematic zone, measured with the flow cytometer; the polygon drawn in plot encloses the nuclei in the sample (a). Histogram of nuclei fluorescence used to estimate the percentage of nuclei in cell cycle phases (G0+G1, G2, S) with ModFit software (b).

Meristematic activity

The activity of *Posidonia oceanica* rhizome meristems was estimated by quantifying the percentage of nuclei in each phase of the cell cycle (i.e. G0+G1, S, G2). Samples of 0.5 cm x 1 cm from the rhizome meristematic zone were cut. The nuclei were isolated using a Partec® extraction kit. Each piece of basal shoot meristem was placed in a Petri dish, a few drops of the extraction buffer of Partec® extraction kit were added, and the tissue was chopped with a razor blade for about 5 minutes. Then, 0.5 ml of Partec® extraction buffer were added to the chopped sample and the sample was filtered through a 20 µm filter, and stained with propidium iodide (PI). The sample was stained for 1 hour in darkness at 4º C. The number of nuclei in each phase of the cell cycle (i.e. G0+G1, S, G2) was quantified using a Beckton-Dickinson flow cytometer equipped with an argon-ion laser measuring the red fluorescence emitted by the PI. For each meristem ten thousand nuclei were analysed (Fig. 1a). The histograms of nuclei fluorescence obtained from the flow cytometer were

analyzed using cell cycle analysis software (ModFit), which provided the percentage of nuclei in G0+G1, S, G2 phases of each meristem sampled (Fig. 1b).

Statistics

We assessed the statistical significance of the variability observed in the percentage of *Posidonia oceanica* nuclei in each cell cycle phase, and sediment AVS pools and SRR, among stations (i.e. presence/absence of *Caulerpa* sp.) and meadows using a 2-way ANOVA. The significance of differences between stations and meadows was assessed using Tukey's post-hoc test. We tested the relationships between *P. oceanica* meristematic activity and sediment sulfide pools and sulfate reduction rate using least square regression analysis on log-transformed variables to meet the requirements of the analysis.

Table 1. Summary of the factorial two-way ANOVA to assess significant differences in meristematic variables of *P. oceanica* (G1, S, G2) and sediment variables (SSP_{AVS} and SRR_{AVS}) between stations (presence/absence of *Caulerpa*) and meadows (Cala LLonga, Cala d'Or and Cala Estancia). *p*- Values correspond to those provided by a univariate F-test; df, degrees of freedom; n.s., not significant.

Variable	Efect	df	*F*	*p*- Value
SSP_{AVS}	Station	1	28.61	<0.0005
	Meadow	2	0.20	n.s
	Station*Meadow	2	9.59	<0.005
SRR_{AVS}	Station	1	15.95	<0.005
	Meadow	2	0.11	n.s
	Station*Meadow	2	4.33	<0.05
G1	Station	1	0.17	n.s
	Meadow	2	2.38	n.s
	Station*Meadow	2	1.51	n.s
G2	Station	1	0.56	n.s
	Meadow	2	11.75	<0.0001
	Station*Meadow	2	0.52	n.s
S	Station	1	0.46	n.s
	Meadow	2	2.67	n.s
	Station*Meadow	2	0.84	n.s

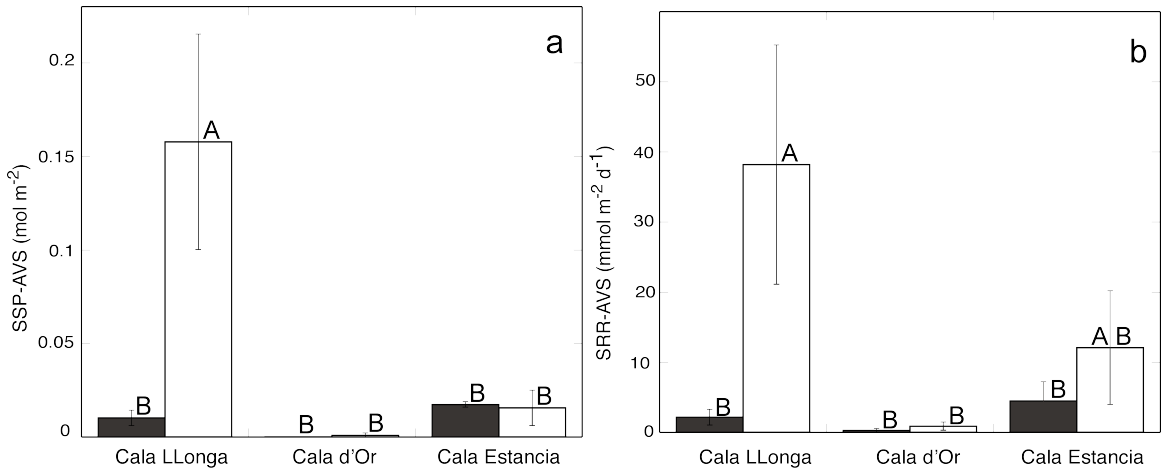

Fig. 2. Average SSP_{AVS} (a) and SRR_{AVS} (b) in top 10 cm sediment where *P. oceanica* was growing monospecifically (black bars) and mixed with *Caulerpa* species (white bars). The standard errors of the mean values are shown (n=3). A, B indicate significantly different SSP_{AVS} and SRR_{AVS} across meadows and stations (Tukey post-hoc test).

Results

Sediment sulfide pools (SSP_{AVS}) and sulfate reduction rates (SRR_{AVS}) ranged between 0.29 10^{-4} mol SSP_{AVS} m^{-2} and 0.226 mol SSP_{AVS} m^{-2} and 0.086 mmol SRR_{AVS} m^{-2} d^{-1} and 64.37 mmol SRR_{AVS} m^{-2} d^{-1}, respectively, across stations and meadows SSP_{AVS} were significantly higher in the sediments colonised by *Caulerpa* at Cala Llonga when compared with those from the rest of stations and meadows (Table 1, Fig. 2). The differences in SSP_{AVS} observed across meadows and stations reflected differences in SRR_{AVS}, as the highest rates were observed at Cala Llonga when sediments were colonised by *Caulerpa*, and the lowest ones in the both sediment types at Cala d'Or (Fig. 2). SRR_{AVS}, in *Caulerpa* sediments at Cala Llonga and Cala Estancia were significantly higher than those at the rest of stations and meadows (Fig. 2, Table 1). The sediments colonised by *Caulerpa* species mixed with *Posidonia oceanica* exhibited SSP_{AVS} up to 15.2 fold higher

than those colonised only by *P. oceanica*. SRR_{AVS} in sediments colonised by *Caulerpa* species mixed with *P. oceanica* were between 2.7 to 17.5 fold higher than those in adjacent sediments colonised by *P. oceanica* alone.

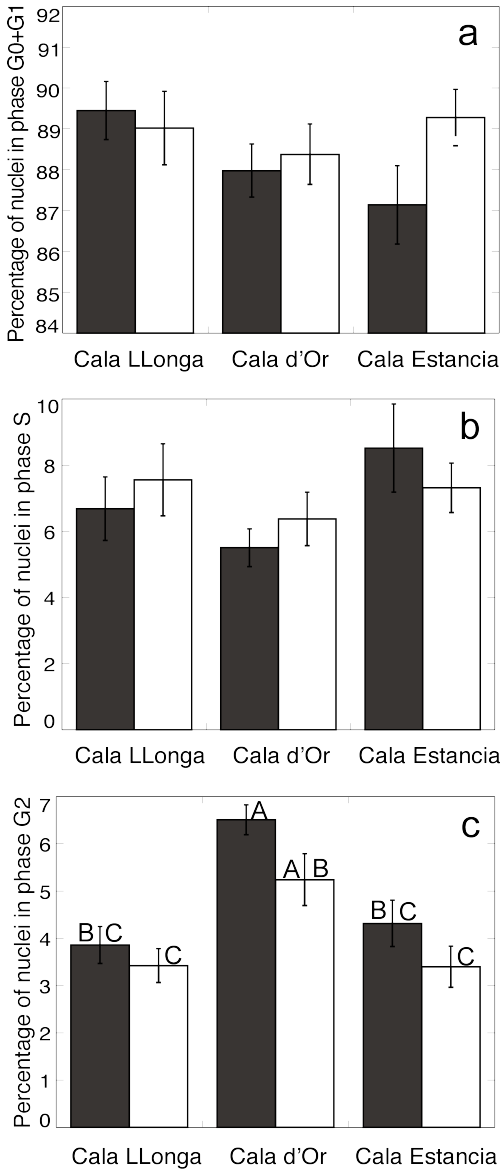

Figure 3. Average percentage of nuclei of *P. oceanica* meristems in G1+G0 phase (a), S phase (b) and G2 phase (c) growing monospecifically (black bars) and mixed with *Caulerpa* species (white bars) at the study sites. The standard errors of the mean values are shown (n= 12). A, B, C indicate significantly different percentage of nuclei in G2 phase across meadows and stations (Tukey post-hoc test); variability in G0+G1 and S phases across meadows and stations was not statistically different.

The meristems of *Posidonia oceanica* from the studied meadows presented between 86.02% and 90.90% of nuclei in phase G0+G1, 2.01% and 6.97% in phase S, and 4.67% and 10.21% in phase G2 of the cell cycle. There were no significant differences (Table 1) in the percentage of nuclei in phases G0+G1 and S across *P. oceanica* meadows. Similarly, the presence of *Caulerpa* species did not affect significantly the percentage of nuclei in G0+G1 and S phases (Fig. 3, Table 1). Conversely, the percentage of nuclei in phase G2 in the meristems of *P. oceanica* growing at Cala d'Or was significantly 1.5 fold higher than those colonizing Cala Llonga and Cala Estància (Fig.3, Table 1). The percentage of nuclei in phase G2 in the meristems of *P. oceanica* tended to decrease when growing mixed with *Caulerpa* species (Fig. 3), although this trend was not statistically significant (Table 1).

The variability observed in the percentage of dividing cells (i.e. containing nuclei in phase G2) in *Posidonia oceanica* meristems was closely (>80% of total variance) coupled to variability in SSP_{AVS} and SRR_{AVS} (Fig. 4). The percentage of nuclei in G2 phase declined with increasing SSP_{AVS} and SRR_{AVS}, reaching the lowest values (< 5 %) when plants were growing on sediments with SSP_{AVS} greater than 0.001 mol AVS m^{-2} (Fig. 4). No relationship was observed between the variability in the percentage of nuclei in G0+G1 and S phases and sediment sulfur dynamics (Regression Analysis, $p > 0.05$).

Discussion

These results demonstrate that the percentage of nuclei in G2 in *Posidonia oceanica* meristems is low, particularly when compared with available estimates for other plants. For instance, the percentage of dividing cells in leaves of *Nicotiana tabacum* has been reported to 22 % - 27 % (Galbraigth et al. 1983, Chen et al. 2001). The low fraction of dividing cells

in *P. oceanica* rhizome meristems is consistent with the slow growth of this species (rhizome growth rate: 4.2 cm yr^{-1}; leaf growth: 1.3 mm leaf $^{-1}$ d^{-1}, Duarte 1991), which ranks amongst the slowest-growing angiosperms on Earth (Nielsen et al. 1996).

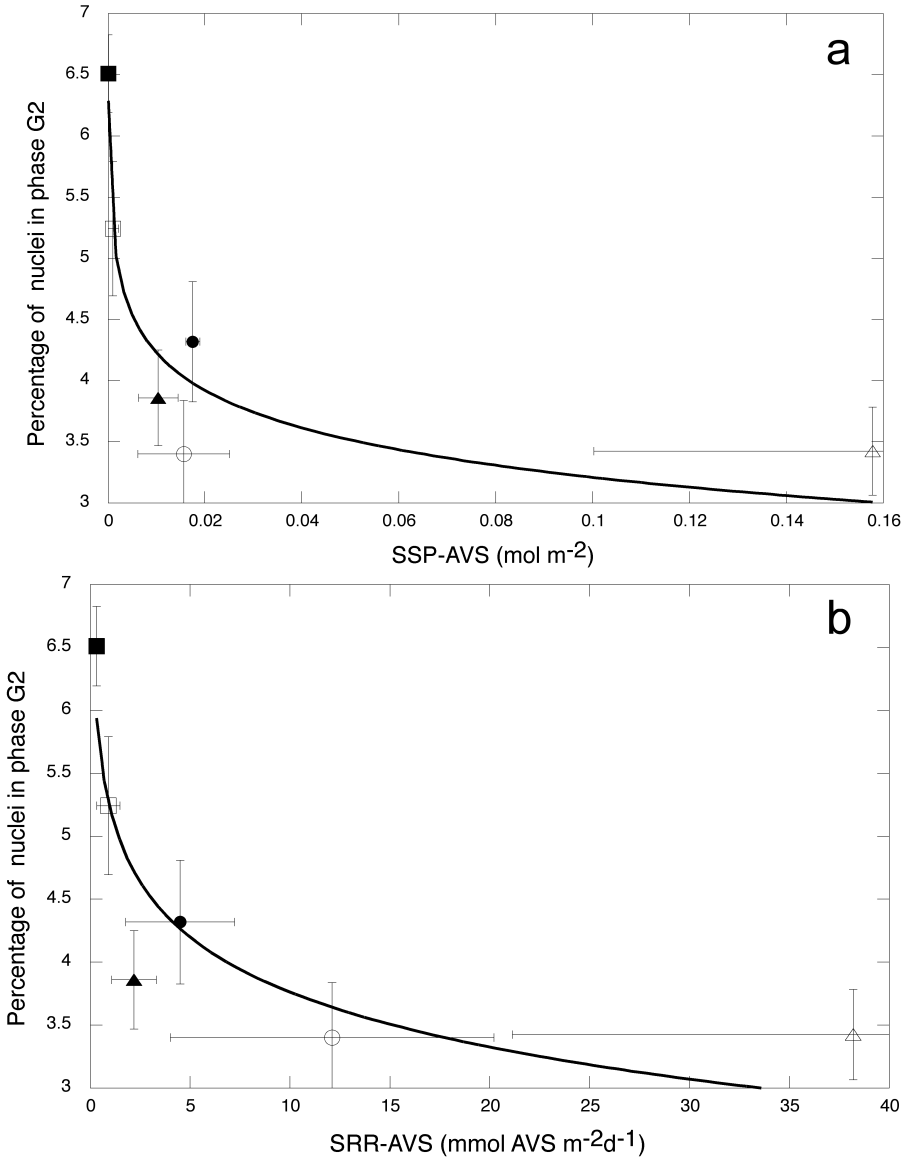

Figure 4. Relationship between the percentage of nuclei of *P. oceanica* meristems in G2 phase and SSP_{AVS} (a) and SRR_{AVS} (b) in monospecific (black symbols) and mixed (open symbols) meadows at Cala Llonga (triangles), Cala d'Or (squares) and Cala Estancia (circles). The standard errors of the mean values are shown (n_{G2}=12; n_{AVS}=3; n_{SRR}=3). The equations of the fitted logarithmic functions are %G2=2.19(± 0.45) –1.02(± 0.18) log SSP_{AVS} (R^2=0.88 n=6 p=0.005) (a); and %G2=5.21(± 0.3) –1.45(± 0.35) log SRR_{AVS} (R^2= 0.81 n=6 p=0.014) (b).

Despite the low fraction of dividing cells in *Posidonia oceanica* meristems, the fraction of cells in G2 phase varied by up to 50 % across the stations and meadows examined (Fig. 3). The variability observed in the percentage of dividing cells in *P. oceanica* meristems mainly reflected differences between sites (Table 1), the meristems in plants at Cala d'Or exhibiting a larger fraction of nuclei in G2 phase than those at Cala Llonga and Cala Estància. Despite the presence of *Caulerpa* spp. did not have statistically significant effect on the percentage of nuclei in G2 phase in *P. oceanica* meristems (Table 1), the activity of seagrass meristems tended to be lower when growing mixed with *Caulerpa* spp. than in monospecific stations at all three studied sites (Fig. 3). Recently, Holmer et al. (submitted) demonstrated that *Caulerpa* spp enhance sulfate reduction rate and anoxia in the sediments they colonise, and a strong coupling between *Caulerpa* biomass and sulfate reduction rate and sediment anoxia was found. The finding that meristems of *P. oceanica* tended to present less dividing cells when growing mixed with *Caulerpa* spp. than those of plants growing at monospecific sites (Fig. 3), suggests that *P. oceanica* health might be compromised by *Caulerpa* invasions due to the increase in sediment sulfides, and it supports the hypothesis that *Caulerpa* spp. act as ecological engineers (Holmer et al. submitted), which, by deteriorating sediment conditions, excludes *P. oceanica*.

The close and negative relationship between the percentage of dividing cells in *Posidonia oceanica* meristems and sediment sulfide concentrations and production reflects the high sensitivity of seagrasses to sulfides. Sulfide intrusion in seagrass tissues has been observed in *Zostera marina* (Pedersen et al. 2004), *Thalassia testudinum* (Borum et al. 2005) and, recently, *P. oceanica* (Frederiksen et al. 2007, Marbà et al. 2007). Indeed, mass

balance computations based on $\delta^{34}S$ signals in *P. oceanica* tissues and the potencial sulfur sources (sediment porewater sulfides and seawater sulfate) at the studied stations showed sulfide intrusion to occur, accounting for up to 40 % of total plant sulfur at Cala Llonga (Holmer et al. *submitted*), the location that supported the plants with meristems with the least percentage of dividing cells (Fig. 3).

The percentage of dividing cells in *Posidonia oceanica* meristems decreased sharply when SSP_{AVS} and SRR_{AVS} increased (Figs. 4a,b). The stability of *P. oceanica* meadows has been demonstrated to be closely related with sediment sulfide pools, accelerating meadow decline rates when sediment sulfide concentration exceeds 10 μM (Calleja et al. 2007). Considering that 10 μM of H_2S equals to 0.001 mol AVS m^{-2}, if all SSP_{AVS} within the top 10 cm sediment layer was released as H_2S in the porewater, our results (Fig. 4) indicate that *P. oceanica* meristems with less than about 5 % nuclei in G2 phase are under sulfide stress. Hence, there is a potential for seagrass meristematic activity to be used as an early warning indicator of plant health and meadow decline.

In summary, our results show that *Posidonia oceanica* meristems divide slowly, as the percentage of cells in G2 phase was lower than 7 %, below that observed for meristems of any other studied plant. *P. oceanica* meristematic activity, assessed as the percentage of nuclei in G2 phase, was closely coupled to sediment sulfur dynamics, the percentage of nuclei decreasing when SSP_{AVS} exceeded 0.001 m^{-2}. These findings confirm that seagrass meristematic activity is highly sensitive to increased sulfide production and points at seagrass meristematic activity as a potentially useful indicator of plant stress and seagrass health, as well as an early warning indicator of seagrass decline.

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General Discussion

The results of this PhD thesis contribute to the general knowledge of the poorly studied, although with a recent increased interest, microbial communities in seagrass meadows. We studied the endophytic microbial community associated with Mediterranean seagrasses: from pathogenic protists and viral particles to bacteria. Moreover, we conducted a literature compilation with the published information regarding bacterial diversity in seagrass ecosystems including endophytes, epiphytes and bacteria inhabiting seagrass rhizospheres, in order to characterize the bacterial species or groups mainly found in association with seagrasses. Finally, we studied how the bacterial activity in rhizosphere affects seagrass health and, thus, its maintenance, by modifying the sediment geochemical conditions.

The general goal of this PhD thesis has been achieved by combining a broad range of techniques encompassing molecular techniques, detection of functional genes and bacterial sequences analysis, culturing techniques of protists and seagrass infection experiments, microscopy and flow cytometry in combination with meristematic cell cycle analysis. The use of this variety of techniques has proved successful to expand the scientific knowledge about the diversity of microbial communities in seagrass meadows and seagrass-microbial interactions, as well as to identify key microorganisms that could constrain seagrass health in the Mediterranean Sea.

The results reported in this dissertation confirm the presence of endophytic microbial community (i.e. microscopic protist and bacteria) in seagrass tissues. The results obtained in Chapter 1 demonstrate that the pathogenic protist *Labyrinthula* sp., identified as the causative agent of mass mortalities events in seagrass meadows since the 1930s (Renn

1936, Young 1943, Arminger 1964, Muehlstein et al. 1991, Robblee et al. 1991), is ubiquitous in seagrass species growing in the Balearic region (Western Mediterranean). This protist was found in 70 % of the seagrass meadows investigated (18 in total) and in all seagrass species (i.e. *Cymodocea nodosa*, *Zostera noltii* and *Posidonia oceanica*) growing the region. Performing infection experiments, we identified some *Labyrinthula* sp. isolates as virulent strains, producing up to 100% of lesions in the infected shoots, while other strains seemed to be not pathogenic for *P. oceanica* (Chapter 1, Table 2). Moreover, the results obtained with cross infection experiments demonstrate that isolates of *Labyrinthula* spp. can infect seagrasses in different genera, even from different biogeographical distribution ranges. This finding contrasts with previous studies where *Labyrinthula* spp. were supposed to be genus-specific (Muehlstein et al. 1988, Vergeer and den Hartog 1991, Short et al. 1993, Vergeer and den Hartog 1994). The presence and pathogenicity of some *Labyrinthula* sp. isolates in seagrass meadows might be an additional threat to these vulnerable Mediterranean ecosystems, which so far had not been considered. The lesions caused by *Labyrinthula* sp. on seagrasses are characterized by a necrosed tissue in leaves, which drastically reduces the photosynthetic plant activity (Ralph and Short 2002). These pathogenic infections could have particularly negative effects for the slow-growing *P. oceanica*, due to its high belowground non-photosynthetic biomass and the need to maintain a balanced photosynthesis-respiration rate. Since, environmental conditions have been identified as triggers of seagrass *Labyrinthula* infections (McKone and Tanner 2009), it is highly relevant to investigate the role that environmental conditions such as temperature, salinity and pH could play in the development of infections, in order to prognosticate the possible impact of *Labyrinthula* spp. on Mediterranean seagrasses during the 21st Century under the IPCC scenarios of Global Change.

The lack of viral particles detection in *Posidonia oceanica* tissues from the 4 meadows tested in the Balearic region suggests that these meadows were not threatened by diseases caused by viruses (Chapter 2). However, further studies are required in order to state categorically the non-involvement of viruses in the decline of seagrasses, as viruses have been identified in other marine photosynthetic organisms, including other seagrass species as *Zostera marina* (Fukuhara et al. 2006). With the current new development of massive sequencing techniques, as pyrosequencing, it would be desirable to screen massively seagrass tissues from a wider region, in order to confirm the absence of viral particles observed in this thesis.

In parallel, we evidenced the frequent presence of endophytic bacteria in *P. oceanica* seagrass along the Balearic Islands by applying molecular techniques (Chapter 3). From the results obtained, we concluded that endophytic bacteria were widely present in almost all seagrass tissues analyzed, yielding a very low percentage of samples free of endophytic bacteria. Approximately, only the 7% of roots, the 19% of rhizomes and 12% of leaves analyzed appeared to be free of bacteria, pointing out that endophytic bacterial colonization of *P. oceanica* tissues is a common feature of this seagrass. Moreover, we described and analyzed the bacterial community composition found in its tissues (leaves, rhizomes and roots) by DGGE. We compared the band patterns obtained for each sample among all the sampled meadows and we identified a specific bacterial community for each tissue sampled, indicating the existence of a specific microbiota in roots that differed from the bacterial community found in rhizomes and leaves. However, the bacterial community composition was not related with the location of the samples, suggesting a bacterial community specific for each tissue, but not conditioned by the geographic location. The lack of geographic patterns of bacterial communities could be also attributed to the small

sample size of seagrass tissues examined in each meadow. Additionally, we analyzed the bacterial diversity by sequencing some of the main OTUs detected by DGGE, as a first attempt to identify taxonomically the endophytic bacterial community. The obtained 16s rDNA sequences from endophytic bacteria suggested the presence of specific bacteria that could play a role in the maintenance of the seagrass functions, although the attribution of bacterial functions and roles would need additional research. The most frequently found OTUs belonged to *Desulfovibrionaceae*, *Flammeovirgaceae*, *Rhodobacteraceae*, *Sphingobacteriaceae* families and to the group of Non-identified Coral Black Band Disease isolates. The finding of sequences with high similarity (up to 99%, Chapter 3, Table 3) with bacteria isolated from diseased coral tissues, suggested the presence of bacteria that could have degradative functions and, thus, could be pathogenic, although there is no previous evidence of lesions in seagrasses produced by bacteria.

Other bacterial sequences found in *P. oceanica* tissues were similar to bacteria related to sulfur and nitrogen cycles, which could have important implications for the maintenance of seagrass ecosystems, especially in the oligotrophic Mediterranean Sea (Dugdale and Wilkerson 1988). The Sulfur-oxidizing bacteria could have a positive effect on Mediterranean seagrass meadows by removing the toxic sulfide produced in organic matter decomposition process, that typically accumulates in sediment pore water due to the low content in available iron (Holmer et al. 2003). Similarly, the presence of bacteria similar to nitrogen fixers would be beneficial for seagrass development in oligotrophic waters, where seagrass growth is often nutrient limited. The finding of bacteria in surface-sterilized seagrass roots with very similar sequence to nitrogen fixers, pushed us to seek further research in this topic, as it was the first time it was described.

In order to confirm the nitrogen fixation capability of those identifications, we

performed an experiment based on the detection of functional genes related with nitrogen fixation. Consequently, we evidenced for the first time the presence of endophytic nitrogen-fixing bacteria in *P. oceanica* roots (Chapter 4) by detection of the functional gene *nifH*, coding for nitrogenase reductase. The detection of functional genes is a useful tool to identify the putative functional roles bacteria are playing (Zehr et al. 2003). The detection of this gene confirmed the existence of nitrogen-fixing capabilities in *P. oceanica* roots, in agreement with the results obtained in Chapter 3. The presence of endophytic nitrogen-fixing bacteria was widely detected (in 17% of root samples analyzed and in 9 locations out of the 26 meadows sampled) although when detected in a meadow they were not always observed in all samples from that location (Chapter 3). The clonal architecture of seagrasses allows mobilization and sharing of nutrients acquired at different regions of the clone with their neighbors (Marbà et al. 2002). This clonal connectivity suggests that although endophytic nitrogen-fixing bacteria were only present in some roots of the clone, the whole clone still might benefit from the nitrogen acquired by nitrogen fixers. The absence of endophytic nitrogen-fixing bacteria in some root samples could also reflect their heterogeneous distribution within the roots. The diversity of nitrogen-fixing community analyzed by DGGE reveled a unique OTU for each root sample, and the sequences obtained from the detected OTUs revealed the presence of only two nitrogen-fixing bacterial species. The very low diversity of nitrogen-fixing bacteria found in *P. oceanica* contrasted with studies conducted in crop plants, where the diazotrophic community was highly diverse (Ueda et al. 1995). The differences found in terms of diversity could be explained based on the very ancient origin of *P. oceanica* and its very long life-span, providing the perfect niche for coevolution. These results suggested the possibility of a symbiosis relation between nitrogen fixers and *P. oceanica*. Further studies will be required

in order to ascertain the exact position of these bacteria, the amount of nitrogen fixed and the contribution to seagrass growth and fitness.

The compilation of all the published literature, concerning bacterial diversity in seagrass meadows (in Chapter 5), casted light on the bacterial community composition, completing the information obtained in previous chapters (Chapter 3 and 4). This compilation allowed identification of the main bacterial groups inhabiting seagrass sediments and tissues of meadows around the world, and provide the first overview of bacterial taxonomic diversity in seagrass meadows. Moreover, seagrasses seemed to be a microbial diversity reservoir as it became evident from the many new bacterial species described from seagrass samples. This study also revealed a low and heterogeneous allocation of efforts on seagrass bacteria diversity research, and bacteria diversity remains unexplored in meadows of many seagrass species and along vast coastal regions.

The bacterial community composition differed greatly among the rhizosphere and seagrass tissues, as it was expected, and differed, as well, among endophytic and epiphytic bacterial communities (Chapter 5; Fig. 3, 4 and 5). The epiphytic communities were more diverse and encompassed a larger number of bacterial Phyla. While the identified endophytic bacteria belonged to 3 bacterial Phyla (Proteobacetria 75%, Bacteroidetes 19% and Firmicutes 1%), the epiphytic bacterial community belonged to 5 bacterial Phyla (Porteobacetria 60%, Cyanobacteria 13%, Actinobacteria 11.5%, Bacteroidetes 6.4% and Planctomycetes 1.3%). The bacterial genus most frequently identified in endophytic community was *Desulfovibrio* sp., whereas *Vibrio* sp. was the most common bacteria in epiphytic community.

The existence of a bacterial community in the rhizoplane has been long studied, and a close coupling between seagrass and sediment bacteria interactions has been largely

documented (e.g. Duarte et al 2005). In fact, some bacteria detected in the rhizoplane of seagrasses presented a positive chemotaxis through the root exudates composed mainly of amino acids (Wood and Hayasaka 1981) used as organic carbon source, confirming seagrass colonization of sediments modifies the bacterial community inhabiting the sediment (James et al. 2006). Similarly, bacteria present in the sediment can shape sediment conditions due to their metabolic activities.

The overview of bacteria diversity in seagrass meadows revealed that bacteria belonging to *Desulfobacteraceae* were the family most frequently identified in seagrass rhizospheres (Chapter 5). This family consists of sulfate-reducer members, which oxidize organic matter by reducing sulfate to sulfide. Sulfide is a toxic compound for eukaryotic cells and, thus, when it intrudes in seagrass tissues it can produce deleterious effects (Frederiksen et al. 2007) resulting in enhanced shoot seagrass decline (Calleja et al. 2007). In order to detect seagrass damage prior observations of net losses of shoot population we have developed a new methodology based in meristematic cell division analysis. Firstly, we examined the variability of percentage of meristematic nuclei in the different phases of the cell cycle along the day, which indicated the absence of daily fluctuations (Chapter 6). The average meristematic activity, calculated as the % of cells in the G2 Phase of the cell cycle, was 6.99 ± 0.11 % for the 6 meadows analyzed. The close relationship between meristematic activity and sediment sulfide pools and the sulfate reduction rate observed in the *P. oceanica* sediments colonized by macroalgae species (Chapter 7) suggests that this new methodology can be used as a early warning indicator of seagrass stress.

Under projected scenarios of marine warming, due to the excessive inputs of greenhouse gas emissions (IPCC 2007), it is expected that the frequency of events of detrimental sediment conditions for seagrass survival and growth might increase during the

current Century. An increase in seawater temperature combined with high sulfide concentrations in sediments increases significantly seagrass mortality rates (Koch and Erskine 2001). The increased risk of damage of seagrass meristems enhances the risk to accelerate the decline of these vulnerable ecosystems.

In summary, the seagrass associated microbiota (Chapter 1, 3, 4 and 5) contains microbial groups that could be able to modify seagrass fitness. Some of them might promote plant growth and survival by, for instance, providing nutrients or detoxifying excessive sulfides. On the contrary, other microbial groups may have a detrimental effect on seagrass health (Chapter 7) and thus compromise the persistence of these valuable ecosystems.

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Conclusions

1. The pathogenic protist *Labyrinthula* sp. is ubiquitous in Mediterranean seagrasses, being found in *Posidonia oceanica*, *Cymodocea nodosa* and *Zostera noltii* and in most locations investigated.
2. Some strains of *Labyrinthula* sp. isolated from *Posidonia oceanica* are virulent, causing necrotic lesions on leaves of *P. oceanica* in the infection experiments conducted.
3. Mediterranean seagrasses are vulnerable to infection by *Labyrinthula* sp. isolated from seagrasses from another bio-geographic region.
4. Viruses could not be detected on *P. oceanica* tissues samples, suggesting viral diseases are not driving current seagrass decline.
5. Endophytic bacterial community has been found in surface-sterilized tissues of *P. oceanica*, suggesting a specific community for roots that differed from rhizomes and leaves.
6. Endophytic bacteria were widely detected in almost all samples analyzed and identification of their sequences reveals that they mainly belong to *Desulfovibrionaceae*, *Flammeovirgaceae*, *Rhodobacteraceae*, *Sphingobacteriaceae* families and to the group of Non-identified Coral Black Band Disease isolates.
7. Diazotrophic endophytes were detected in *P. oceanica* roots by amplification of the nitrogenase reductase gene (*nifH*) in a very low diverse community, with only two *nifH* sequences detected.
8. The diazotrophs were identified as *Vibrio diazotrophicus* and as a putative anaerobe by phylogenetic analysis of their *nifH* sequences.

9. The taxonomic composition of bacterial communities inhabiting seagrass rhizospheres differed from that of bacterial communities found in seagrass tissues. Similarly, bacterial epiphytic and endophytic communities also differed.

10. Seagrasses are identified as a possible reservoir of bacterial diversity, evidenced by the high number of new bacterial species identified from their tissues.

11. Bacterial communities of seagrass tissues and the rhizosphere can play a major role on the sustainability of seagrass meadows.

12. Enhanced activity of sulfate-reducing bacteria inhabiting seagrass sediments may injure seagrasses. The detrimental effects of sulfide accumulation in sediment pore water are demonstrated by the reduction on dividing cells in the meristems.

13. Meristematic activity can be used as an early warning indicator of seagrass stress.

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