Diversidad y funcionamiento de la comunidad epífita eucariota de las hojas de *Posidonia oceanica*

Tesis doctoral

AUTOR: Francisco Javier Medina Pons
DIRECTORES: Dr. Jorge Miguel Terrados Muñoz / Dr. Ramón Rosselló-Móra
PONENTE: Dr. Gabriel Moyà Niell

Departament de Biologia
Universitat de les Illes Balears
12 de diciembre de 2011

Agradecimientos

En primer lugar me gustaría decir que esta tesis me ha ayudado a madurar, no tan solo desde un punto de vista científico sino, también, y especialmente, en el plano personal. Este trabajo no habría sido posible sin la ayuda y colaboración de un buen número de personas. Me gustaría disculparme de antemano si olvido a alguna de ellas en las próximas líneas.

Me gustaría dedicar esta tesis a mi mujer, Teresa, a mi madre, Carmina, a mis hermanas, Marga y Carmina, y al resto de mi familia y amigos. Desearía tener un recuerdo especial para mi 'padre', Javier, y para mi abuela, Margarita, los cuales nos dejaron hace unos años. Siempre he encontrado en ellos las palabras, el ánimo y el cariño necesarios para que este trabajo llegara a buen puerto.

Esta tesis no hubiera sido posible sin la contribución de mis directores de tesis, el Dr. Jorge Terrados Muñoz y el Dr. Ramón-Rosselló Móra, del Instituto Mediterráneo de Estudios Avanzados (IMEDEA). Me gustaría agradecer la ayuda y el ánimo que me han prestado a lo largo de todo este trabajo, especialmente durante las épocas en las cuales el trabajo no salía y había que repetir una y otra vez los mismos experimentos. Querría agradecer su colaboración en el trabajo de campo y laboratorio, así como sus consejos, sugerencias y ayuda en el diseño y realización de los experimentos, análisis de resultados y discusión de los mismos desde un punto de vista crítico. Asimismo, querría agradecer al Dr. Jorge Terrados el haberme permitido disfrutar de varias campañas en el Parque Nacional de Cabrera, de las cuales guardo un recuerdo especial por el interesante trabajo realizado en ellas, el intercambio de conocimientos y experiencias con otros científicos y la belleza del entorno.

A lo largo de todo el trabajo que he desarrollado durante estos años, siempre he creído que la ayuda y el buen ambiente que he encontrado entre todos mis compañeros del IMEDEA ha sido clave. Desearía agradecer especialmente la ayuda y colaboración de Inés (¡perdona por esos ratos de música indie con los que te hacía sufrir en el laboratorio!), Edu y Fiona del Grupo de Macrófitos Marinos, así como de Ana, Jocelyn (¡perdona por mis burdas imitaciones del locutor de Radio Infinita de Chile!), Arantxa, Maese Pablo y Mercedes, los cuales me hicieron sentir como uno más en la familia de Microbiología Marina del IMEDEA. Recuerdo con especial cariño las comidas en casa de María José, siempre atenta y amable con todos, además de ser una cocinera espectacular, y la competición de rimas que hacíamos al final de ellas cuando firmábamos en su libro de visitas.

Finalmente, desearía agradecer la ayuda imprescindible de la Conselleria de Economia, Hisenda i Innovació del Govern Balear para llevar a cabo esta tesis, al concederme una beca pre-doctoral en el año 2005, así como diversas bolsas de viaje que me permitieron disfrutar de estancias en Barcelona y Aarhus (Dinamarca) y participar activamente en el 8º Congreso Internacional de Angiospermas Marinas (ISBW8) en Bamfield (Canadá). Todo ello ha supuesto unas experiencias tremendamente enriquecedoras desde el punto de vista personal y científico. Desearía agradecer a la Dra. Amelia Gómez Garreta, de la Unidad de Botánica de la Facultad de Farmacia (Universidad de Barcelona (UB)), y a la Dra. Birgit Olesen, del Departamento de Biología Vegetal (Universidad de Aarhus, Dinamarca) haberme aceptado en sus grupos de investigación y haberme permitido usar sus instalaciones durante un período de tiempo de esta tesis.

Acknowledgements

First of all, I would like to say that this PhD thesis have helped me to mature, not just from a scientific point of view but, also, especially in a personal manner. This work would not have been possible without help and support from lots of people. I would previously like to apologize for forgetting someone in next lines.

I would like to dedicate this PhD thesis to my wife, Teresa, to my mother, Carmina, to my sisters, Marga and Carmina, and to the rest of my family and friends. I would wish to dedicate it in memory of my 'father', Javier, and my grandmother, Margarita, who died some years ago. I have always found on them words, motivation and affection that were necessary to successfully end this work.

This PhD thesis would have not been possible without the contribution of my two supervisors, Dr. Jorge Terrados Muñoz and Dr. Ramón Rosselló-Móra, from Mediterranean Institute of Advanced Studies (IMEDEA). I would like to be grateful to them for help and support throughout this PhD thesis, especially those moments in which work was not successful and experiments must be repeated again and again. I would like to than them for supporting me in field and lab work, as well as their advices and analyses and discussion of results from a critical point of view. I would also like to thank Dr. Jorge Terrados for allowing me to enjoy some scientific campaigns in Cabrera National Park. I have happy memories of those because of the interesting work developed, the knowledge and experiences exchanged with other scientists and beauty of the scenery.

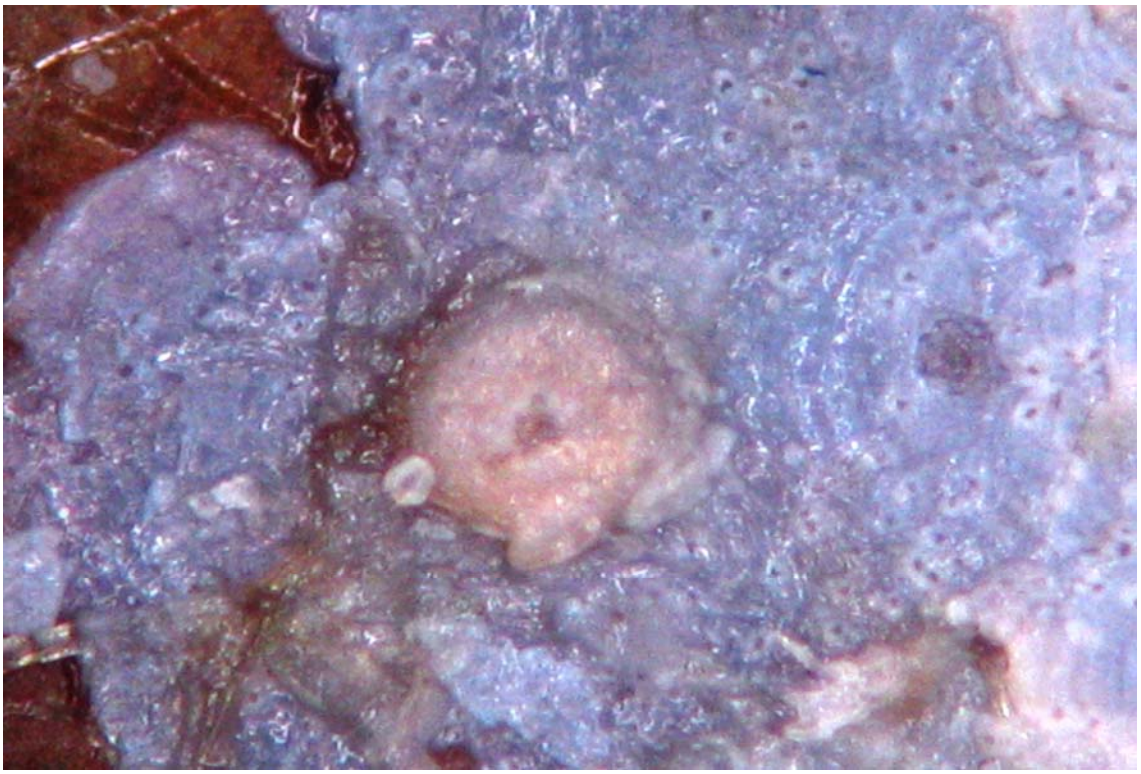
Throughout the work I have developed during these years, I have always believed that help and nice atmosphere that I have found among my colleagues from IMEDEA have played a key role. I would especially wish to thank help and support of

Inés (please, excuse for those moments with indie music you suffered in lab!), Edu and Fiona from Marine Macrophyte Group, as well as Ana, Jocelyn (please, excuse me for my bad imitations of the announcer of 'Radio Infinita' from Chile), Arantxa, Maese Pablo and Mercedes, who accepted me as another member of Marine Microbiology family from IMEDEA. I have special memories from lunchs in María José house, always nice and kind to everybody, apart from being an incredible cooker, and rhyme competition at the end of lunchs when we signed in her guests' book.

Finally, I would like to be grateful to 'Conselleria de Economia, Hisenda i Innovació' from Balearic Government for granting me a PhD fellowship, as well as some funds that allowed me enjoying short stays in Barcelona and Aarhus (Denmark) and actively participate in 8th International Seagrass Biology Workshop (ISBW8) in Bamfield (Canada). All these experiences have been incredibly important from either a scientific or personal point of view. I would like to thank Dr. Amelia Gómez Garreta, from Botany Department in Pharmacy Faculty (University of Barcelona (UB)), and Dr. Birgit Olesen, from Plant Biology Department (University of Aarhus, Denmark) for accepting me in their investigation groups during PhD stays and allowing me to use their facilities during a period of time of this PhD thesis.

INDEX

SECTION I - Summary



Detail of epiphyte encrusting Rhodophyta on *Posidonia oceanica* leaf

The macroeukaryotic leaf-epiphytic fraction of the seagrass *Posidonia oceanica* is a highly dynamic assemblage that significantly contributes to biomass, nutrient cycling, sediment carbonate and productivity of the ecosystem. The structure of this assemblage is characterized by showing variability in a relatively short period of time and by being influenced by abiotic and biotic factors and disturbances (natural and anthropogenic).

Spatio-temporal variability in the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves has been previously described in a wide variety of studies under optical microscope or dissecting microscope (classical microscopy approach). Our first objective was testing the sensitivity of two molecular techniques (Temperature gradient gel electrophoresis (TGGE) and small ribosomal subunit (SSU) clone libraries), as an alternative to classical microscopy method, to detect variability in the structure of that epiphytic assemblage and, therefore, to evaluate if it was feasible to use those molecular techniques to study this epiphytic community.

TGGE was optimized to study the structure of the macroeukaryotic leaf-epiphytic assemblage of *P. oceanica* (Chapter 1), allowing to observe variability in the structure of that assemblage between two stages (February vs. September) of the seasonal succession (Chapter 2), between the mature stage (summer) in two different locations (Chapter 4) and between different stages of the seasonal succession of this community (Chapter 5). The mature stage of the assemblage (September) was characterised by showing the highest richness (the highest Operational Taxonomic Units (OTUs)), according to previous studies. The composition of the assemblage differed between all stages, with the exception of spring stages (April and June), when the community has been described as highly homogeneous because of the relative abundance and biomass of Phaeophyta in relation to the whole assemblage.

SSU clone libraries were also able to detect variability in the structure of three different stages of the macroeukaryotic epiphytic assemblage (February, June and September) (Chapter 3). On the one hand, SSU clone libraries resulted to be more time-consuming than TGGE, but on the other hand they allowed the putative identification of epiphyte taxa.

Results suggested that molecular approaches were able to detect more differences than microscopy method. It could be explained for different reasons: (1) underestimation of the real diversity: leaf-macroeukaryotic epiphytic assemblage description based on microscopy could be biased by the taxonomical expertise of the researcher, (2) taxonomic impediment: some individuals (juvenile, broken,…) were difficult to determine under microscope and (3) molecular techniques could detect the presence of taxa even with low relative abundance in the assemblage.

We were also interested in studying the relationship between diversity/composition of the leaf-epiphytic macroalgae and Nitrate Reductase (NR) activity (as a measure of nitrogen assimilation capacity from water column, which is an ecosystem key process) (Chapter 6). Macroalgal epiphytes were selected because they are the most abundant and diverse component of the macroeukaryotic leaf-epiphytic fraction and they show variability in biomass, species composition and richness thorough seasonal succession. Results suggested that the diversity and composition of the epiphytic macroalgal component is a relevant factor to explain differences in NR activity between two stages of the assemblage (winter vs. summer).

Main conclusions of this PhD thesis can be summarized in:

- TGGE resulted to be an excellent parallel approach to monitor changes in the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves. This

molecular technique was able to detect variability in the structure of the epiphytic assemblage between two stages of the seasonal succession, between the mature stage in two different locations and between the different stages of the seasonal succession.

- SSU clone libraries also constituted an alternative method to study the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves. This technique showed variability in the structure of that assemblage between three stages of the seasonal succession.
- TGGE and SSU clone libraries were suitable to simultaneously analyse large amounts of samples. Both methods overcame some disadvantages of the classical microscopy approach: (1) underestimation of the real diversity: leaf-macroeukaryotic epiphytic assemblage description based on microscopy could be biased by the taxonomical expertise of the researcher, (2) taxonomic impediment: some individuals (juvenile, broken,…) were difficult to determine under microscope and (3) low abundant taxa: molecular techniques were able to detect the presence of taxa even with low relative abundance in the assemblage.
- Comparing the two molecular techniques, TGGE resulted to be less time-consuming than SSU clone libraries, but the later approach was more appropriate than the former one to putatively identify epiphytic taxa explaining variability in the structure of that assemblage. TGGE could be more recommended than SSU clone libraries to quickly assess the variability of the structure of the macroeukaryotic leaf-epiphytic assemblage.
- Diversity/composition of the macroalgae epiphytic component was linked to variability in NR activity of the assemblage, reinforcing the relationship between

diversity and ecosystem functioning found in other communities. The composition of the assemblage suggested playing a relevant role in determining nitrogen assimilation rates in different stages of the epiphytic assemblage.

SECTION II - Introduction

Detail of epiphyte Bryozoa on *P. oceanica* leaf

1. Seagrasses: *Posidonia oceanica*

Seagrasses are macrophytes distributed along coastal areas worldwide (with the exception of polar coasts). They mainly colonize unconsolidated substrata (sandy or muddy), although some species are able to establish on rocky bottoms (Procaccini et al. 2003; Spalding et al. 2003). Seagrasses are clonal plants[1] that share a common architecture based on a repetition of units (ramets). Each ramet is composed of a horizontally growing rhizome portion, a vertically growing rhizome portion in some species, a leafy shoot, a root system, and sometimes flowers or inflorescences and fruits. Seagrasses provide ecological services in coastal areas, which can be summarized in a high productivity, promotion of marine biodiversity, regulation of the quality of waters and protection of the shore line (Marbà et al. 2004; Terrados and Borum 2004).

Posidonia oceanica (L.) Delile is an endemic[2] Mediterranean seagrass that forms monospecific meadows with a shoot density ranging from 150 - 300 shoots m^{-2} (low density meadows) to more than 700 shoots m^{-2} (high density meadows) in shallow waters (< 10 m depth) (Spalding et al. 2003; Gobert et al. 2006). *P. oceanica* vegetative structure is composed of highly lignified horizontal (plagiotropic) and vertical rhizomes (ortotropic), leafy shoots and a root system (Fig. 1a). Plagiotropic rhizomes horizontally expand by means of terminal apexes, allowing substrata colonization and inter-connection between different ramets, maintaining plant integrity. Ortotropic rhizomes are attached to plagiotropic rhizomes and support leafy shoots that avoid sediment burial as a consequence of the vertical growth of rhizomes. Shoots are composed of 5 – 10 linear broad leaves (5 - 12 mm) with lengths, generally, from 20 to 40 cm, whereas roots are generally largely branched and are 3 to 4 mm thick and up to 40 cm long, respectively. Progressive accumulation of particulated organic matter from *P. oceanica*

and sediment inside the meadow, together with the very slow decay of the rhizomes and roots forms a typical structure called "matte" composed of rhizomes, roots and sediment (Fig. 1b). Flowering period goes from August to November, although *P. oceanica* shoots rarely flower (about 0.007 inflorescences shoot^{-1} year^{-1} in Western Mediterranean meadows) (Díaz-Almela et al. 2006). The flowers are large and produce a large fruit (10 mm). Young individuals originating from seeds (seedlings) are rarely found and spreading of this seagrass primarily occurs vegetatively by branching of the plagiotropic rhizomes (Borum and Greve 2004; Marbà et al. 2004). *P. oceanica* has a long life span and a very slow growth rate in comparison with other seagrass species (Table 1) (Marbà et al. 2004).

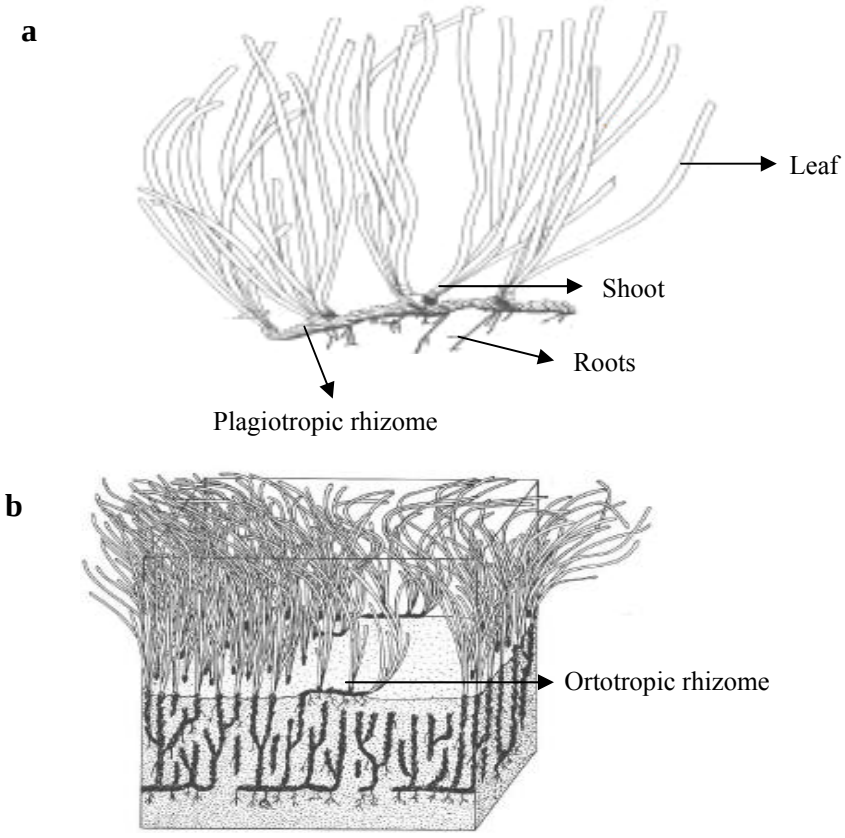


Fig. 1. General structure **(a)** and "matte" **(b)** of *P. oceanica*.
Adapted from Massutí Pascual et al. (2000).

Table 1. Average architectural features and growth rates of *P. oceanica* and the other three European seagrasses. Variables are in rows and seagrass species in columns. nd: no available data; NP: ortotropic rhizomes are not present in *Zostera marina* and *Zostera noltii*. Adapted from Marbà et al. (2004).

	Cymodocea nodosa	***P. oceanica***	***Zostera marina***	***Zostera noltii***
Shoot elongation rate (cm shoot^{-1} day^{-1})	1.3	0.8	3.2	0.7
Plagiotropic rhizomes elongation rate (cm apex^{-1} year^{-1})	40	2	26	68
Ortotropic rhizomes elongation rate (cm apex^{-1} year^{-1})	1.4	1	NP	NP
Leaf life span (days)	79	295	88	86
Shoot life span (days)	876	4,373	554.8	nd

P. oceanica requires stable environmental conditions, being mainly found over sandy carbonated sediments, although it can also colonize rocky substrata. This seagrass is stenohaline[3], not resistant to desiccation and tolerates a wide range of temperatures (9ºC – 29ºC). Its bathymetric distribution goes from a few meters to 40 meters depth in very clean waters, being light availability and wave exposure the main factors determining lower and upper limit, respectively (Greve and Binzer 2004; Gobert et al. 2006; Infantes et al. 2009). Light availability, substratum and wave exposure, together with nutrient availability (specially, nitrogen and phosphorus) are the most relevant abiotic factors that control *P. oceanica* growth and distribution (Greve and Binzer 2004; Gobert et al. 2006). This seagrass often appears in sediments with low nutrient content that might constrain its growth rate. However, there are several reasons that explain how *P. oceanica* can survive in those habitats: (1) nutrient uptake can be performed either from water column or pore water sediment, (2) nutrient requirements are lower than in macroalgae and phytoplankton, (3) nutrients can be stored in rhizome and (4) nutrient

retranslocation[4] is possible among different parts of the plant (Romero et al. 1994; Alcoverro et al. 2000; Invers et al. 2002; Lepoint et al. 2002a; Lepoint et al. 2002b). Experimental nutrient addition to water column has shown a positive relationship with filamentous and epiphyte[5] algae development in summer season that can negatively affect *P. oceanica* growth (Leoni et al. 2006; Prado et al. 2008). Grazing affecting *P. oceanica* distribution and growth, mainly caused by sea urchins (*Paracentrotus lividus*) and fishes (*Sarpa salpa*), has been traditionally considered as relatively low (Cebrián et al. 1996), although recent studies have concluded that it might have been underestimated (Tomas et al. 2005a; Prado et al. 2007a). Some authors have suggested an indirect and positive effect of fishes, sea urchins and mesograzers (Crustacea, Mollusca Gasteropoda,…) on *P. oceanica* because they would control epiphyte accumulation on their leaves (Alcoverro et al. 1997; Peirano et al. 2001; Tomas et al. 2005b; Prado et al. 2007b; Gacia et al. 2009).

P. oceanica meadows are highly relevant for the functioning of Mediterranean coastal areas because they provide some ecological services:

- Marine biodiversity promotion. *P. oceanica* meadows provide habitat for a large number of species belonging to a wide variety of taxonomic groups (Van der Ben 1971; Ballesteros 1987; Mazzella et al. 1989).
- High productivity (Romero 1989; Pergent et al. 1994; Cebrián et al. 1997).
- Protection of the shore line from erosion caused by waves (Granata et al 2001).
- Regulation of the quality of coastal waters through the trapping of suspended particles and the absorption of nutrients (Gacia and Duarte 2001; Gacia et al. 2002).

A high number of studies have used different parameters of *P. oceanica* (nitrogen and phosphorus rhizome content, shoot density, leaf area,…) as a multivariate indicator of the quality of coastal waters related to Water Framework Directive (2000/60/CE) (Romero et al. 2007; Fernández-Torquemada et al. 2008; Montefalcone 2009). The usefulness of this seagrass as a bioindicator of the status of Mediterranean coastal waters has been recognised because of four main reasons: (1) high sensitivity to perturbations (Ruiz et al. 2001; Cancemi et al. 2003; Pérez et al. 2008), (2) wide distribution along mediterranean coasts (Procaccini et al. 2003), (3) long life span allowing the identification of temporal changes in environmental conditions that affect growth rate (Marbà et al. 2004) and (4) high knowledge about biology and ecology of this species (Massutí Pascual et al. 2000; Gobert et al. 2006).

2. The macroeukaryotic epiphytic fraction of *P. oceanica* leaves

2.a) Temporal succession and factors determining the structure of the macroeukaryotic epiphytic fraction of P. oceanica leaves

Leaves of *P. oceanica* and other seagrasses provide excellent conditions for the establishment of micro- and macroepiphytes. These leaves are exposed to higher light availability, lower sedimentation rates and higher hydrodynamics[6] than the rhizomes (Trautman and Borowitzka 1999; Borowitzka et al. 2006) (Fig. 2).

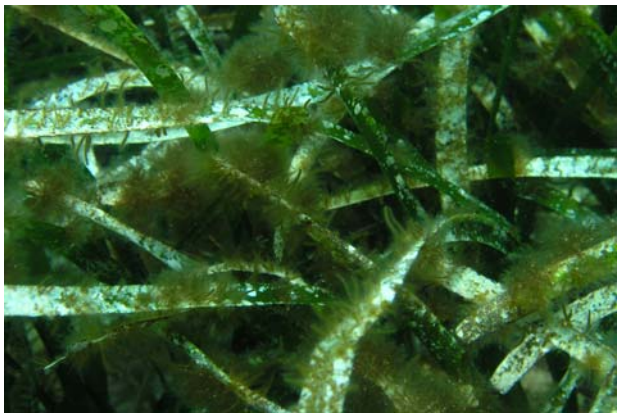


Fig. 2. Epiphytic community of *P. oceanica* leaves. Photography by E. Infantes.

Seasonality of growth and high renovation rates of *P. oceanica* leaves determine a seasonal succession of the macroeukaryotic epiphytic fraction that starts in autumn (related to the annual maximum of production of new leaves) and finishes in summer (related to the annual maximum of fallen leaves) (Ott 1980; Pergent and Pergent-Martini 1993). Significant changes in the structure of this fraction (biomass, species composition and richness) have been observed along this seasonal succession (Van der Ben 1971; Ballesteros 1987; Romero 1988; Mazzella et al. 1989). The first stage of the sucesssion is typically composed of an encrusting layer of Phaeophyta (mainly, *Myrionema magnusii*) and Rhodophyta (basically, *Hydrolithon* spp. and *Pneophyllum*

spp.). This encrusting layer, which is present through the entire succession, is generally covered by zooepiphytes (mainly, Bryozoa) in winter, by filamentous Phaeophyta (especially, *Giraudia sphacelarioides* and *Cladosiphon* spp.) in spring and by filamentous Rhodophyta in summer (basically, species belonging to Ceramiales Order) (Van der Ben 1971; Ballesteros 1987; Romero 1988). The highest biomass values along the succession are found in spring and summer because of the important abundance of filamentous Phaeophyta and Rhodophyta, respectively (Ballesteros 1987; Romero 1988). Epiphyte species richness generally increases along the succession, reaching a maximum at the end of summer, when the highest similarity in epiphytic species composition among different shoots is also found (Ballesteros 1987).

The structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves and other seagrasses is the result of the interaction of several abiotic (mainly, light, nutrient availability and water column hydrodynamics) and biotic factors (basically, grazing) that operate at different scales. Some authors have suggested that the interaction among phenomena that operate at large spatial scales (such as hydrodynamics or nutrient availability) and at local scale (such as light attenuation by self-shading among shoots) is relevant to explain the spatial variability in the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves. Results obtained in those studies indicate that the highest variability in the structure of this fraction is found at small (shoots separated 10s-100s cm from each other) and large spatial scales (shoots separated some km from each other) (Piazzi et al. 2004; Pardi et al. 2006; Balata et al. 2007; Piazzi et al. 2007).

The most studied abiotic factor related to the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves has been nutrient availability. Different studies

have concluded a positive relationship in summer between nutrient availability and epiphytic biomass over *P. oceanica* leaves and changes in specific composition mainly related to the abundance of filamentous Phaeophyta (Leoni et al. 2006; Balata et al. 2008; Prado et al. 2008). However, Terrados and Medina-Pons (2008) did not observe significant differences in epiphytic biomass when comparing locations with different loading rates of anthropogenically-derived nutrients which suggested that this positive relationship might occur when differences in nutrient availability exceed a specific threshold.

Light availability has been suggested as a key factor to explain the variability in the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves along depth gradients (Mazzella et al. 1989; Lepoint et al. 1999; Nesti et al. 2009). Lepoint et al. (1999) observed that the epiphytic fauna/flora ratio in *P. oceanica* leaves increased with depth because of the increase in relative abundance of Bryozoa and Hydrozoa against algae. Mazzella et al. (1989) and Nesti et al. (2009) showed a higher relative abundance in encrusting Rhodophyta against Phaeophyta in *P. oceanica* shoots located in depth, which could be explained by the fact that the former group exhibited a higher tolerance to variations in light availability (Figueiredo et al. 2000).

The influence of hydrodynamics in the structure of the macroeukaryotic epiphytic fraction of seagrasses has been basically studied in *Posidonia sinuosa* (Kendrick and Burt 1997; Lavery et al. 2007). Kendrick and Burt (1997) and Lavery et al. (2007) observed a negative relationship between hydrodynamics and epiphytic algae biomass in *P. sinuosa* leaves, because only encrusting algae were able to establish in locations exposed to a higher effect of waves and currents. However, Schanz et al. (2002) found an indirect and positive relationship between hydrodynamics and

epiphytic biomass in *Z. marina*, because higher hydrodynamics negatively affected the abundance of the invertebrate *Hydrobia ulvae*, which was a relevant epiphyte grazer in those seagrass meadows.

Among relevant biotic factors determining the structure of the macroeukaryotic epiphytic fraction of seagrass leaves, grazing has been much studied. Prado et al. (2007b) and Nesti et al. (2009) showed an indirect and negative relationship between epiphytes in *P. oceanica* leaves and the relative abundance of macrograzers (*S. salpa* and *P. lividus*) along a depth gradient. Those studies concluded that in shallow *P. oceanica* meadows (0 – 10 m) relative abundance of macrograzers *S. salpa* and *P. lividus* was higher, inducing a higher leaf consumption and a reduction in leaf area for epiphytic colonization (Alcoverro et al. 1997; Tomas et al. 2005a; Prado et al. 2007b). The number of studies about effects of mesograzers and the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves is significantly lower (Gacia et al. 2009), although mesograzers have shown that can significantly influence specific composition and biomass of this epiphytic fraction in other seagrasses (Duffy and Harvilicz 2001; Duffy et al. 2001).

2.b) Methodology to study the structure and diversity of the macroeukaryotic epiphytic fraction of P. oceanica leaves.

The study of the structure and diversity of the macroeukaryotic fraction of *P. oceanica* leaves has been traditionally performed by means of optical microscopy techniques and/or dissecting microscope and has been mainly focused in macroalgae that are the most abundant and diverse component of the epiphytic assemblage (Van der Ben 1971; Cinelli et al. 1984; Antolic 1985; Antolic 1986; Ballesteros 1987) (Fig. 3). A significant number of studies based on different zooepiphytic taxonomic groups are also found in

literature (Hayward 1975; Boero 1981; Pansini and Pronzato 1985). However, studies about the structure and diversity of the whole macroeukaryotic epiphytic fraction are significantly lower (Casola et al. 1987; Mazzella et al. 1989). This fact probably reflects the difficulty to identify the wide variety of taxonomic groups colonizing *P. oceanica* leaves.

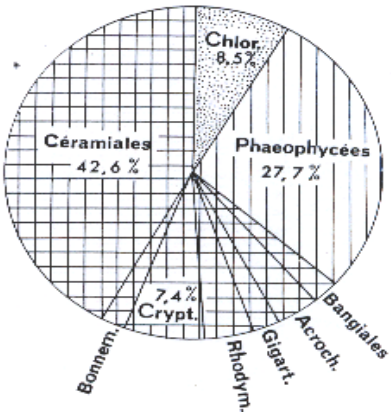


Fig. 3. Relative abundance of the different taxonomic groups of macroalgae over *P. oceanica* leaves. Chlorophyta (Chlor.), Phaeophyta (Phaeophycées), Rhodophyta is composed of Order Ceramiales (Céramiales) + Bonnemaisoniales (Bonnem.) + Cryptonemiales (Crypt.) + Rhodymeniales (Rhodym.) + Gigartinales (Gigart.) + Acrochaetiales (Acroch.) + Bangiales. Adapted from Van der Ben (1971).

The description of the structure, diversity and dynamics of microbial communities by means of molecular biology has progressively replaced the use of classical techniques from mid 80s (Giovannoni et al. 1990; DeLong 1992; Head et al. 1998). The evaluation of diversity from a molecular perspective has been based on the inference of phylogenetic relationships among organisms that compose a community. Most of these molecular approaches have been performed using codifying genes[7] for small ribosomal subunit (16S ARNr in prokaryotes / 18S ARNr in eukaryotes, hereafter abbreviated as SSU) (Head et al. 1998; Fuhrman 2008). The usefulness of these genes to infer phylogenetic relationships have been mainly explained by three reasons (Van de Peer et al. 1993; Head et al. 1998; Eickbush and Eickbush 2007):

1) They are homologous genes[8] that exist in all cellular organisms.

2) They share a high identity in nucleotide sequence among individuals belonging to the same species, and less with different taxa. Multiple copies of these genes occurring within the genome[9] of an individual (paralogous genes) also share a high identity in nucleotide sequence.
3) Primary structure of these genes is composed of a mosaic of highly conserved and highly variable regions. In addition, they have a high percentage of complementary sequences that are responsible of matchings inside the same nucleotide chain determining a functional secondary structure (Fig. 4).

Development of molecular techniques based on SSU has been primarily performed to study diversity and dynamics of prokaryotic communities. They have allowed overcoming limitations of classical culture techniques of microorganisms (Head et al. 1998; Fuhrman 2008), discovering an unknown diversity in different environments (Giovannoni et al. 1990; DeLong 1992; Bruemmer at al. 2004).

Recently, these molecular approaches have also shown to be useful to study the structure of microeukaryotic communities (Díez et al. 2001; López-García et al. 2001; Massana et al. 2002). These approaches have allowed obtaining diversity estimations independent from the knowledge of the taxonomy of groups composing a community. However, the application of molecular approaches in macroeukaryotic assemblages has been basically restricted to the description of the diversity of terrestrial nematodes (Floyd et al. 2002; Foucher and Wilson 2002), the diet of some species (Blankenship and Yayanos 2005; Passmore et al. 2006) and paleoecological[10] studies (Willerslev et al. 2003; Willerslev et al. 2007).

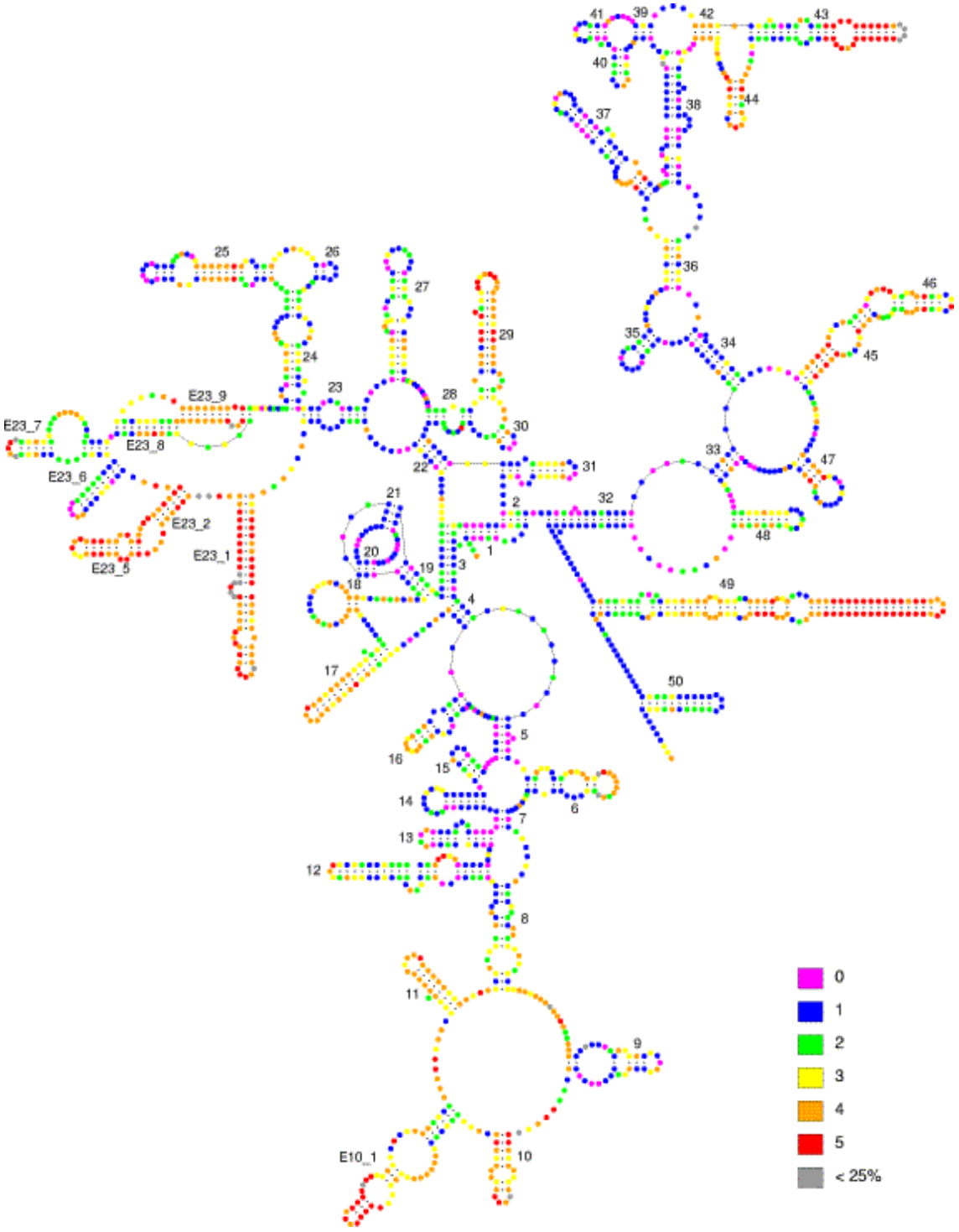


Fig. 4. Secondary model structure of SSU in *Saccharomyces cerevisiae* yeast. Different positions inside the molecule has been divided in 5 groups of variability (from totally conserved (pink) to highly variable (red)). Positions in grey colour either represent nucleotides present in *S. cerevisiae* but only present in less than 25% of other selected 500 eukaryotic SSU sequences, or not well-aligned regions. Variability of different positions is calculated by means of nucleotide substitution rate (Van de Peer et al. 1993) based on the alignment of 500 eukaryotic SSU sequences. Variability map adapted from Van de Peer et al. (1997).

2.c) Relationship between structure/diversity/identity of species of the macroalgal epiphytic component of P. oceanica leaves and ecosystem functioning.

The study of diversity/structure/identity of species composing a community has been traditionally descriptive. However, recent studies have focused in the relationship between diversity/structure/species identity and key processes of ecosystem functioning (Bengtsson 1998; Loreau et al. 2001). Most of these studies in aquatic environments have found a significant relationship between the community structure and productivity (McGrady-Steed et al. 1997; Downing and Leibold 2002), resource consumption (Cardinale et al. 2002), nitrogen uptake from water column (Lepoint et al. 2007), resistance to alien species colonization (Stachowicz et al. 1999) and the ecosystem stability against disturbances (Aoki and Mizushima 2001).

Although different studies have suggested a significant effect of the whole epiphytic community of different seagrasses in key processes of ecosystem functioning such as productivity (Morgan and Kitting 1984; Moncreiff et al. 1992), light availability (Sand-Jensen 1977; Sand-Jensen et al. 1985; Drake at al. 2003), oxygen exchange (Sand-Jensen et al. 1985) and nitrogen uptake from water column (Cornelisen and Thomas 2002; Lepoint et al. 2004; Cornelisen and Thomas 2006), the effect of diversity/specific composition of this epiphytic community in these processes has received less attention (Lepoint et al. 2007). Lepoint et al. (2007) concluded that encrusting epiphytic Rhodophyta exhibited significantly higher values of nitrogen uptake from water column than epiphytic Phaeophyta in *P. oceanica* leaves.

Nitrogen assimilation is a key process in coastal ecosystems dominated by seagrasses, where the most common nitrogen form is nitrate (NO_3^-) (Romero et al. 2006). The capacity of autotrophic organisms to assimilate nitrogen from water column

strongly depends on the enzyme Nitrate Reductase (NR), which is responsible of the initial reduction of NO_3^- to nitrite (NO_2^-). NO_2^- is subsequently reduced to ammonia (NH_4^+) and finally assimilated into aminoacids (Solomonson and Barber 1990; Romero et al. 2006) (Fig. 5). The activity of the NR enzyme, measured as NO_2^- production along the time, has been widely used as an estimation of nitrogen assimilation capacity in different species of phytoplankton (Berges and Harrison 1995), vascular plants (Doddema and Howari 1983; Scheible et al. 1997) and macroalgae (Davison and Stewart 1984; Lartigue and Sherman 2002), showing a significant relationship with abiotic (mainly, temperature, light and NO_3^- availability) and biotic factors (interspecific differences) (Gao et al. 2000; Lartigue and Sherman 2002; Young et al. 2007). However, the application of NR activity to estimate nitrogen assimilation capacity in communities is very scarce and has been limited to the macroalgal epiphytic component of *Amphibolis antarctica* and *P. sinuosa* seagrass species (Young et al. 2005).

Contrasting to seagrasses, such as *P. oceanica*, epiphytic algae are characterized by (1) high nutrient requirements, (2) nutrients are exclusively uptaken from water column and (3) a reduced capacity to store nutrients (Williams and Ruckelshaus 1993; Alcoverro et al. 1997; Terrados and Williams 1997; Romero et al. 2006; Lepoint et al. 2007). Because of that, epiflora productivity and growth is frequently more limited than in *P. oceanica* in oligotrophic[11] environments, such as Mediterranean Sea. Although a progressive increase in nutrient availability (eutrophication) has been recently observed in Mediterranean Sea (Turley 1999) and the effects of this environmental change have been mainly related to increases in abundance and biomass of epiphytic opportunistic algae (Cancemi et al. 2000; Prado et al. 2008), no relationship has been established in

literature between eutrophication and nitrogen uptake depending on community structure and diversity.

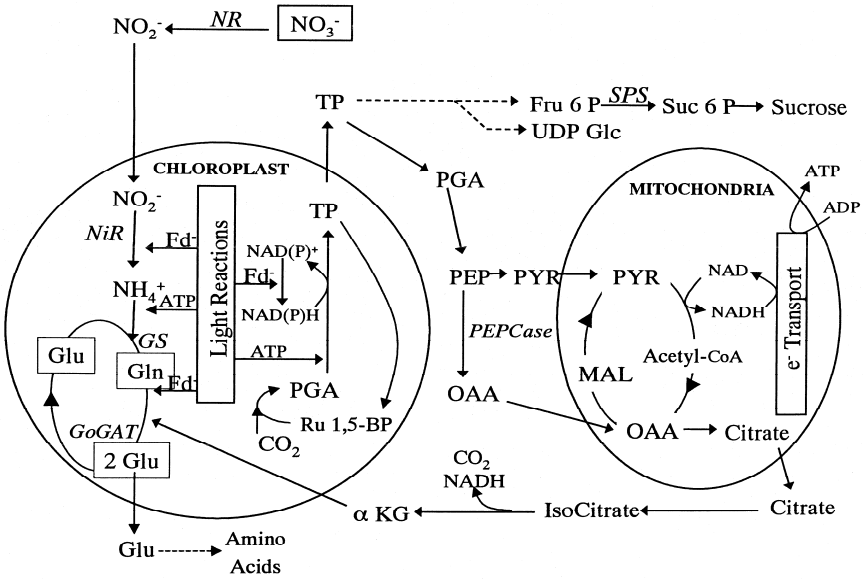


Fig. 5. Nitrogen uptake and assimilation in autotrophic organisms. NO_3^- uptake and NH_4^+ assimilation primarily occurs in cytosol and plasts (chloroplasts and mitochondria), respectively. NO_3^- can be stored in vacuoles inside cells or can be reduced in cytosol by means of NR enzyme. After that, NO_2^- produced from NO_3^- reduction enters in GS/GOGAT cycle, where glutamine synthetase (GS) catalyzes the transformation of glutamate in glutamine. The addition of carbon skeletons (a-ketoglutarate) produces two glutamate molecules, which is catalyzed by the enzyme glutamate synthetasa (GOGAT). One of these molecules can be recycled through GS/GOGAT cycle, whereas the other one can be used to form complex aminoacids. Adapted from Touchette and Burkholder (2000).

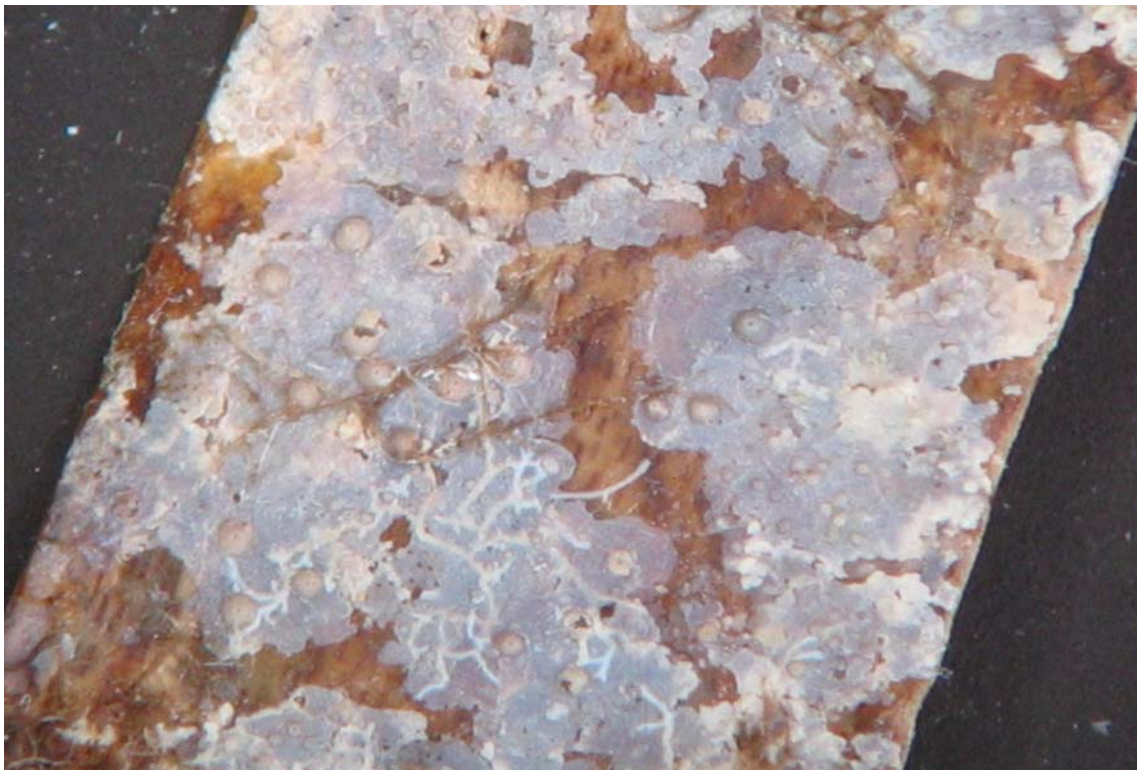
3. Main objectives of the PhD thesis

The present PhD thesis had two main goals:

1. Describe the diversity and structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves and its spatio-temporal variability applying molecular techniques as an alternative to classical microscopy/dissecting microscope techniques. Macroeukaryotic epiphytic fraction of *P. oceanica* leaves has been selected as a community model because of different reasons: (1) its significant contribution to biomass, nutrient cycling, sediment carbonate and productivity of the ecosystem (Ballesteros 1987; Romero 1988; Canals and Ballesteros 1997; Lepoint et al. 2007), (2) a relatively short period of time is enough to detect significant changes in its structure and (3) its structure can be influenced by abiotic and biotic factors and disturbances (natural or anthropogenic).
2. Evaluate the relationship between species identity/diversity/structure of macroalgal epiphytic component of *P. oceanica* leaves and NR activity (as a measure of nitrogen assimilation capacity from water column, which is an ecosystem key process). Macroalgal epiphytes were selected because they are the most abundant and diverse component of the macroeukaryotic epiphytic fraction and they show changes in biomass, species composition and richness thorough seasonal succession (Borowitzka et al. 2006).

Different techniques used to accomplish these objectives are explained in Section III.

SECTION III - Fundamentals of techniques used



Detail of epiphytes on old *P. oceanica* leaf

4. Molecular techniques used to describe the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves

4.a) Desoxirribonucleic acid (DNA) preparation of the macroeukaryotic epiphytic fraction of P. oceanica leaves

Molecular approaches used in the present PhD thesis involve the preparation of desoxirribonucleic acid (DNA) from the macroeukaryotic epiphytic fraction of *P. oceanica* leaves. DNA preparation consists in its isolation from the rest of cell components (polysaccharides, proteins,...) and the amplification or copy of the isolated DNA (DNA template) applying Polymerase chain reaction (PCR). PCR molecular technique allows obtaining multiple copies of the entire DNA or, more frequently, a desired DNA portion (target DNA) and is composed of the repetition of three basic stages:

I. Denaturation. Heating causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
II. Annealing. Two designed primers (short length DNA sequences) anneal to complementary sequences of each of the single-stranded DNA molecules. It allows, in the final stage, synthesizing new and complementary single-stranded DNA molecules which contains the target DNA.
III. Extension. The enzyme Taq DNA polymerase attaches to primers and synthesizes the new strands containing the target DNA.

Final number of copies of the target DNA is dependent on the number of repetitions of the three stages of the PCR (2^n, n = number of cycles) (Sambrook and Russell 2001a).

The present PhD thesis assessed the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves amplifying the whole or a portion of SSU by means of PCR. SSU was selected because of its characteristics and advantages that offer against other DNA regions (see Section II-*2.b*). Amplification of SSU produced multiple copies of the target DNA with the same length but different sequence, mirroring the complexity of the studied sample.

DNA isolated and amplified was visualized by means of agarose gel electrophoresis. Agarose gel electrophoresis is a technique that, in the presence of an electric field, allows separating DNA molecules depending on its size. Under these circumstances, DNA molecules migrate from the negative (anode) to the positive pole (cathode) because of the presence of fosfate (PO_4^{3-}) groups in its structure. Migration velocity of the different DNA molecules is inversely related to its size. Migration front was observed by adding a dye to DNA molecules and the results of the electrophoresis were visualized under an ultraviolet lamp (UV) with ethidium bromide (EtBr) staining. In order to determine that DNA fragment obtained by means of PCR was the desired, we used a DNA marker that consisted on a mix of DNAs with different size. One of these DNAs had a similar size to DNA fragment obtained by PCR and their migration velocity in agarose gel should be similar if PCR was correctly developed.

4.b) Barcoding or fingerprinting techniques

Barcoding or fingerprinting techniques are molecular approaches frequently applied to the study of the structure, dynamics and diversity of communities. They are based on the separation of DNA fragments, previously amplified with PCR, with the same size but different sequence in acrilamide/formamide gels either with a temperature gradient (Temperature gradient gel electrophoresis (TGGE)) or a denaturing chemicals gradient

(Denaturing gradient gel electrophoresis (DGGE)) (Muyzer and Smalla 1998; Muyzer 1999). In the present PhD thesis we amplified DNA fragments from SSU of about 600 base pairs (bp) with different sequence in TGGE gels. We chose TGGE against DGGE (analogous techniques) because the former approach allowed obtaining a clear separation of DNA fragments.

TGGE is based on the fact that DNA molecule structure and, therefore, its migration velocity in agarose gels with an electric field are affected by temperature. Double-stranded DNA molecules progressively denature (depending on the composition of nitrogenised bases on its sequence) and reduce its migration velocity as they migrate to the cathode because of the temperature gradient. DNA molecules with a sequence rich in nitrogenised bases guanine (G) and cytosine (C) require a higher temperature to denature and migrate quicker in the gel. The presence of a tail rich in G and C in designed primers for PCR avoids the total denaturation of the double-stranded DNA molecules. Each of those molecules is visualized in the gel as an unique band representing a partially denatured double-stranded DNA fragment (Muyzer 1999; Gadanho and Sampaio 2004) (Fig. 6). In the present PhD thesis, bands obtained in TGGE gels were revealed using silver staining because it has proven to be more sensitive and less toxic in comparison with other methods such as EtBr staining (Radojkovic and Kusic 2000).

Diversity measurements calculated from the application of TGGE (or DGGE) are based on the analysis of the band patterns obtained. These bands are commonly named molecular operational taxonomic units ((MOTUs or OTUs) (Rosselló-Móra and López-López 2008; Valentini et al. 2008), and the OTUs pattern complexity depends on the diversity of the studied community (Muyzer 1999).

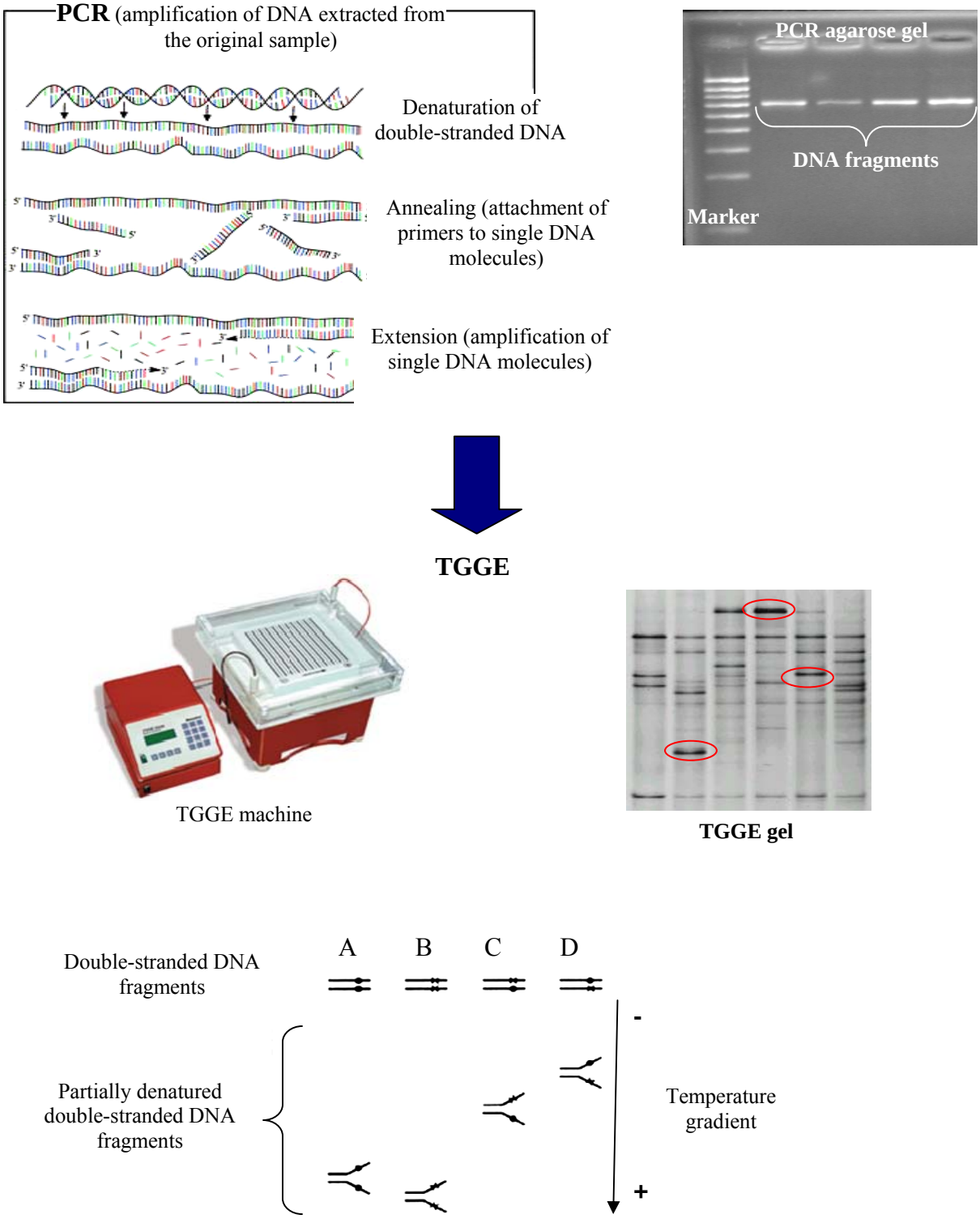


Fig. 6. TGGE molecular technique diagram. A, B, C and D represent different samples. Red ellipses are different OTUs detected in TGGE gel.

Because of the fact that SSU are present in all living organisms (homologous genes) and in multiples copies within the genome of an individual (paralogous genes), OTUs obtained with fingerprinting techniques could hypothetically reflect the diversity of paralogous and homologous genes of a community. However, it has been observed that SSU interspecific diversity is higher than SSU intraspecific diversity (Hebert et al. 2003), and, therefore, OTUs pattern mirror taxa richness from the original sample (Muyzer 1999).

Similarity among OTUs pattern from different samples in a TGGE (or DGGE) gel is calculated constructing a presence (1) – absence (0) matrix (columns = samples, rows = OTUs) and is graphically represented using a cluster or a non-metric multi-dimensional scaling (MDS) (Schäffer and Muyzer 2001) (Fig. 7).

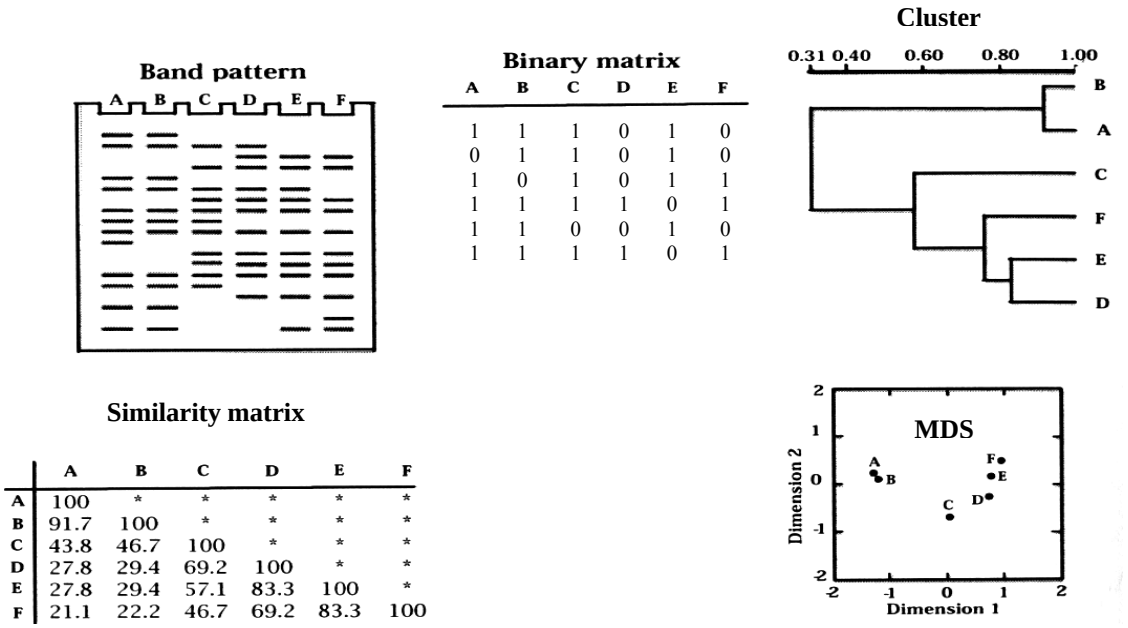


Fig. 7. OTUs pattern analyses. A, B, C, D, E and F represent different samples. Similarity matrix represent OTUs percentage (%) shared between two samples (from 0 (no OTUs shared) to 100 (the same OTUs pattern)). Cluster scale represents the similarity in OTUs pattern. MDS is a non-dimensional representation in which samples that are close share a high similarity of their OTUs pattern. Adapted from Schäffer and Muyzer (2001).

4.c) DNA libraries

DNA libraries are collections of DNA sequences that represent a portion or the whole genome from an organism or a group of organisms. DNA libraries construction, as in the case of barcoding techniques, involves DNA isolation and amplification by means of PCR. The presence of universally conserved regions in 5' and 3' ends of DNA molecules allows the amplification of the almost complete sequence of SSU (Head et al. 1998), which offers some advantages discussed above. For those reasons, the DNA fragment selected in this PhD thesis for the construction of DNA libraries was almost the entire SSU (about 1.800 bp long).

DNA libraries construction starts with the ligation phase, where DNA fragments from SSU are inserted in a vector (a DNA molecule, generally from a phage[12] or a plasmid[13]), forming recombinant vectors by means of an enzyme ligase. Because of the fact that the percentage of recombinant vectors is never 100%, some non-recombinant vectors (DNA fragments from SSU not inserted in vector) are obtained. After that, in cloning phase, vectors (either recombinant or non-recombinant) are introduced in host cells[14], where they are amplified producing a large number of copies. Each of the vectors introduced in a host cell produce a clone colony (group of clones with the same DNA sequence) and some of them are selected after a screening phase. Screening consists in selecting only clones with DNA from SSU, which allows describing the community structure. Screening is, generally, based on the fact that chosen vectors present in their structure some genes codifying for the resistance against different antibiotics or for the synthesis of different enzymes. In the present PhD tesis we used pGEM-T Easy Vector plasmid (Promega), which presented in its structure a gen codifying for the resistance against ampiciline antibiotic and the lacZ gen, codifying for

the synthesis of β-galactosidase enzyme. When inserted in vectors, DNA fragment from SSU, avoided the lacZ gen expression, inhibiting β-galactosidase synthesis. Because of that, clones obtained were screened by means of cultures (LB as culture medium) with ampiciline, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). IPTG is responsible of inducing the transcription of β-galactosidase enzyme and X-gal is a substratum hydrolyzed in the presence of that enzyme producing indoxil, which is oxidized by exposure to the air and converted in indigo (blue colour) (Sambrook and Russell 2001b). Three possibilities were possible in screening clones in this PhD thesis:

1) Clones from host cell without vector were sensitive to ampiciline and were not able to grow in LB cultures.
2) Clones from host cell with non-recombinant vector were able to grow in LB cultures because they were resistant to ampiciline. They formed blue clone colonies because of the production of indigo in the reaction catalyzed by β-galactosidase enzyme.
3) Clones from host cell with recombinant vector were able to grow in LB cultures because they were resistant to ampiciline. They formed white clone colonies because of β-galactosidase enzyme was not synthesized and indigo was not produced.

When screening is ended, sequencing and aligning of selected clones is developed. Sequence aligning is performed to compare sequences from the studied gene to detect similarities and establish evolutive or functional relationships. The construction and analyses of a phylogenetic tree with aligned sequences is the final

phase of DNA libraries. A phylogenetic tree is a graphic representation composed of branches and nodes in which evolutive relationships among sequences from the studied gene are shown, allowing obtaining a measure of the diversity in the sample studied. Branches are lines that connect two nodes, which can be external (representing the end of a branch where sequences are located) or internal (points representing a common ancestor of two or more nodes) (Fig. 8).

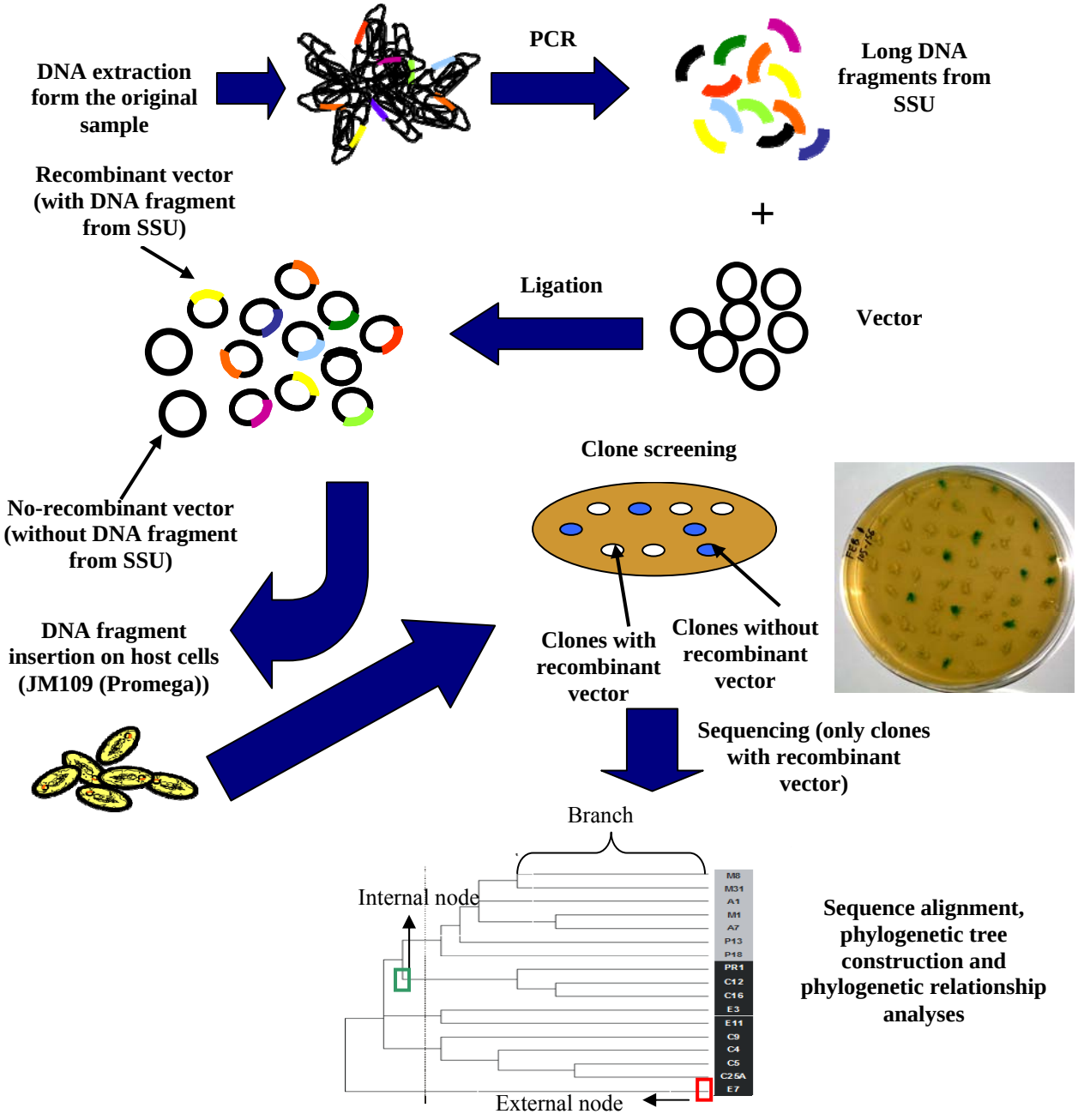


Fig. 8. DNA library construction and analyses based on SSU used in this PhD thesis.

Methods most frequently used for phylogenetic trees construction are:

- Distance methods, such as “Neighbor-Joining” (NJ).
- “Maximum Parsimony” (MP).
- “Maximum Likelihood” (ML).

NJ is the most used distance method. Distances calculated among sequences using NJ are commonly based on Jukes-Cantor evolutive model. Jukes-Cantor model assumes that (i) the frequencies of the different nucleotides in a sequence is the same and (ii) nucleotide substitution rate is the same and independent of the identity of the nucleotide. To obtain a distance value *d* (*d = -3/4 Ln [1-(4p/3)]*) between two sequences applying Jukes-Cantor correction, firstly it is necessary to calculate distance *p*, which is the combination of the number of differences between two sequences divided by total number of positions considered in the sequence. Distance matrix is used by NJ algorithm to generate the phylogenetic tree. However, MP and ML methods are more powerful and accepted in scientific community.

MP method takes all the sequences and generates multiple phylogenetic trees, which are evaluated following the criterion that the best explanation is the most simple. The final topology is the one that requires less number of evolutive changes (such as nucleotide substitutions or mutations) to explain differences observed among different sequences.

In the same way, ML method uses original nucleotide sequences data to generate and evaluate multiple trees. A likelihood value, which is inversely related to accumulated evolutive changes, is calculated for each topology. For a determined topology, the total probability is calculated as a product of the likelihood in each

alignment position (taking into account the frequency of each nucleotide and the probabilities of transition[15] / transversion[16] according to the chosen evolutive model). Contrasting to other methods, ML assumes that each sequence position can independently evolve and the evolution rate can vary from some lineages to others. The exhaustive evaluation of all possible topologies (for instance, $2.8x10^{74}$ trees with only 50 sequences) is generally impossible and ML represents, in most of the cases, the best approach to the optimal topology (Head et al. 1998; Sambrook and Russell 2001b; Hall 2004; Fuhrman 2008).

4.d) Advantages and limitations of molecular barcoding techniques and DNA libraries construction in the description of community structure.

Molecular barcoding techniques and DNA libraries construction present some advantages and limitations related to study the community structure. On the one hand, barcoding methods are more appropriated to study community succession because they simultaneously allow processing an elevated number of samples being less time-consuming and more economic. DNA fragments belonging to SSU for barcoding techniques must contain phylogenetically informative regions (regions with a nucleotide sequence variable among species) to detect the presence of different taxa in the original sample (Valentini et al. 2008). On the other hand, DNA library construction is a more powerful approach because it is phylogenetically more informative (as they generally works with longer and more quality DNA sequences) (Head et al. 1998; Muyzer 1999; Fuhrman 2008), being more appropriated to analyse deeply the community structure.

5. Relationship between structure/ diversity of macroalgal epiphytic component of *P. oceanica* and NR activity (as a measure of nitrogen assimilation capacity)

NR activity measurements in scientific literature have been performed following *"in vivo"* or *"in vitro"* methods. The main difference between them is that the former one is based on the tissue permeabilization to allow the contact of a buffer solution containing NO_3^- with cells, where NO_3^- is reduced to NO_2^- using endogenous nicotinamide adenine dinucleotide (NADH) in a reaction catalysed by NR enzyme. On the contrary, *"in vitro"* method consists on tissue homogenising allowing the release of the NR enzyme in a buffer solution, and the subsequently NR activity measurement with a colorimetric method in an assay solution containing NO_3^- and NADH. Extraction buffer is the responsible of this tissue homogenisation and NR release, whereas assay solution contains the necessary reagents (NO_3^- and NADH) to reduce NO_3^- to NO_2^-. After the incubation phase, reagents inhibiting NR enzyme activity are added and NO_2^- concentration is measured in a spectrophotometer with a colorimetric method, obtaining NR activity as a function of NO_2^- concentration, biomass and time (Fig. 9). The major limitation of *"in vivo"* against *"in vitro"* methods is the unknown NO_3^- diffusion rates from outside to inside cells and NO_2^- diffusion rates from inside to outside the cells (Lartigue and Sherman 2002). In the present PhD thesis, we adapted an *"in vitro"* method to measure NR activity (Scheible et al. 1997), because it allowed us controlling some factors, such as temperature, that can modify this enzymatic activity.

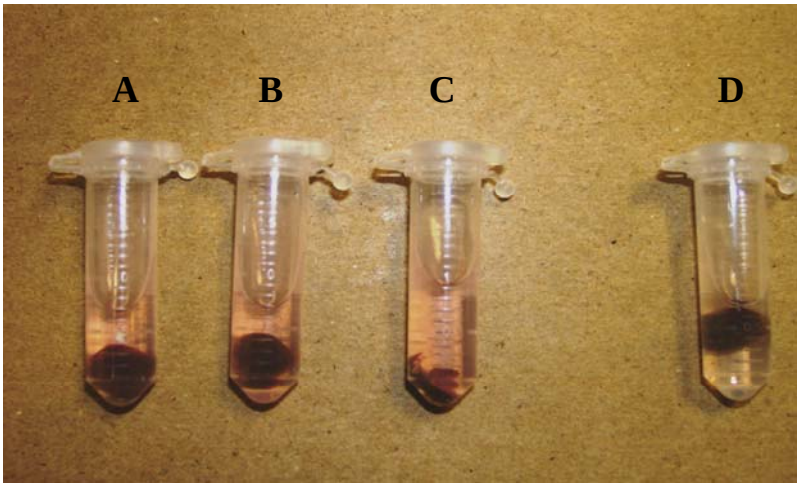


Fig. 9. Colorimetric method to measure NR using an *"in vitro"* technique. After incubating samples with a buffer solution containing NO_3^- and NADH, reagents are added to measure NO_2^- concentration in a spectrophotometer with a colorimetric method. A, B and C are three replicates from a sample and D is a control used to determine NO_2^- concentration present in tissues previously to perform the assay. NR activity is expressed as the average of NO_2^- concentration in A, B and C tubes (substracting that from control tube) per biomass and time.

SECTION IV - Chapter 1

Detail of old *P. oceanica* leaf

Optimisation of a molecular fingerprinting technique (TGGE) to the study of the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves

Optimisation of a molecular fingerprinting technique (TGGE) to the study of the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves

Background

The application of a molecular fingerprinting technique to the study of the structure of a community requires the previous extraction of DNA of enough quality and quantity. This has been recognised to be a major achievement in order to guarantee successful results (Head et al. 1998). There are many published procedures for extracting DNA from different communities, which use different physical (i.e. freezing, thawing, bead beating) and/or chemical treatments (lysis with detergents) to release DNA (Díez et al. 2001; Foucher and Wilson 2002; Gadanho and Sampaio 2004). The selection of an appropriate DNA extraction procedure from the macroeukaryotic epiphytes of *P. oceanica* leaves must take into account that macroalgae are the most abundant and diverse component of this fraction (Lepoint et al. 1999; Borowitzka et al. 2006). DNA extraction of enough quality and quantity from different macroalgae has previously proven to be difficult because of the co-extraction of polysaccharides and secondary metabolites that can inhibit the subsequent PCR reaction (Hong et al. 1997; Vidal et al. 2002; Varela-Álvarez et al. 2006). Furthermore, a procedure that allows obtaining successful results with one macroalgae group often fail with others, probably because of the differences in their cell wall composition, and that of storage and secondary compounds (Doyle 1990; Hong et al. 1997).

The optimisation of the subsequent PCR is another relevant matter to obtain successful results. It includes the selection of the adequate target regions within the SSU genes, designing of the primers targeting the selected regions and identification of the

PCR conditions that are appropriate to detect the diversity from the studied sample. The selection of the SSU gene regions to amplify must follow some criteria: (1) they must contain regions nearly identical among individuals of the same species, but different between species, and other regions highly conserved between species, (2) they must be phylogenetically informative and (3) they must have the correct size to apply the subsequent molecular approach that allows detecting the diversity from the studied sample (Valentini et al. 2008). The design of primers complementary to the selected target regions inside SSU is another key point to obtain an optimal amplification. Ideally, designed PCR primers should have between 40-60% GC content and both should be similar in size (18-25 bp), in melting temperatures (T_m)[17] and in nucleotide ratios. For fingerprinting techniques (DGGE and TGGE), one of both primers should contain a 3'-terminal GC clamp (at least one or two 3' G's and/or C's nucleotides). In addition, they should be free of repetitive motifs[18], palindromes[19], excessive degeneracy[20], and long stretches of polypurines[21] or polypyrimidines[22] (Roux 1995). Although slight deviations from these recommendations are allowed, several useful computer programs have been developed to design efficient PCR primers (Primer-BLAST, National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Other factors that could be necessary to optimize for amplification purposes are the DNA template quantity and magnesium (Mg^{2+}) concentration in the amplification buffer, pH and cycling conditions (i.e. changes in annealing temperature, which is dependant on primer T_m values and in number of cycles). On the one hand, the increase in DNA template may increase number of amplicons (or PCR products) obtained but it also may increase the presence of inhibitors of the PCR. In addition, a decrease in annealing temperature and an

increase in number of cycles may also increase in number of amplicons obtained but it also might produce a decrease in the specificity of the reaction (Roux 1995). Finally, the addition of different reagents, such as dimethyl sulfoxide (DMSO), glycerol, non-ionic detergents and formamide, have also showed to increase the specificity of the PCR (Roux 1995; Sambrook and Russell 2001a).

Some factors also need to be optimized in the selected fingerprinting technique (in this PhD thesis, TGGE). The most important are the temperature gradient, the quality and quantity of DNA fragments used, and the staining method of the gel. The optimisation of the temperature gradient is relevant to obtain a clear resolution of the amplicons in TGGE gel (i.e. a narrow gradient may produce a better segregation between bands than a wide one, but wide gradients may show more defined bands) (TGGE Maxi System Manual, Biometra, http://www.biometra.com). The staining method of the gel is also a relevant factor that should be optimized. The most used nucleic acids-staining procedures in literature have been silver-staining, EtBr, SYBR Green and SYBR Gold methods (Muyzer et al. 1993; Heuer et al. 1997; Muyzer et al. 1997; Harper et al. 2006). The silver-staining procedure has been found as the most sensitive method in the detection of DNA bands in fingerprinting gels (Felske et al. 1996; Radojkovic and Kusic 2000). In addition, this method has been shown to be more economic than EtBr staining procedure (Radojkovic and Kusic 2000). SYBR Green/Gold staining methods, which are economically more expensive, have been recognised as alternative procedures to silver-staining and EtBr methods because of the lack of background staining and the detection of DNA bands even at very low concentrations (Muyzer and Smalla 1998).

Our main goal is the optimization of the complete molecular fingerprinting protocol (DNA extraction, PCR, TGGE and staining method) to study the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves. The obtained molecular protocol could be used as an alternative approach to classical dissecting microscope/optical microscope methods to study this epiphytic fraction.

Materials and methods

1.- DNA extraction with DNeasy Plant Mini Kit and subsequent PCR.

P. oceanica shoots collected in the field were gently scraped with a clean razorblade and filtered with sterile seawater to isolate the macroeukaryotic epiphytic fraction. Before that, these shoots were distributed in three different methods of preservation after arrival to the lab: i.e. directly frozen at -20ºC, stored in 4% formaldehyde seawater and stored at 4ºC. Epiphytic DNA was extracted using a commercial kit for DNA extraction from terrestrial plants (DNeasy Plant Mini Kit (Qiagen)).

First assay. Epiphyte cells (about 0.1 g Fresh weight (FW)) were broken following two procedures: (1) using pipette tips followed by a cycle of freezing (15 min at -80ºC) and thawing (5 min at 65ºC); and (2) vortexing the cell material with 5 mm stainless steel beads for 3 min (Qiagen). Simultaneously, DNA extractions from fresh cypress leaves (*Cupressus sempervirens*) and from fresh different Rhodophyta (*Spiridea* spp., *Padina pavonica*, *Peyssonellia* spp.) were done as controls following the same assayed procedure. DNA extractions were run in 1% (wt/v) agarose gel, stained with EtBr at least during 10 min and visualized under an UV light.

Second assay. Fresh cypress leaves, fresh Rhodophyta and epiphytes were disrupted following a more aggressive procedure: two cycles of freezing (15 min at -80ºC) and thawing (5 min at 65ºC) followed by three ultrasonication cycles (5 min at 40 KHz), and

finally vortexing with 5 mm stainless steal beads for 3 min. DNA extractions were visualized as above mentioned.

For both assays, PCR was done using 1 µl DNA template in a final volume of 20 µl using Master Mix (Eppendorf) according to manufacturer's instructions. 5% DMSO was also added to the reaction. The two universal primers designed for eukaryotic SSU amplification, Euk1A/516r-GC and EukA/B, were tested (Díez et al. 2001). We also tested two different PCR programs. The first one included an initial denaturation at 94°C for 130 s, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 130 s, with a final extension step of 72°C for 10 min. The second PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min.

PCR products were visualized under an UV light in a 2-3% (wt/v) MS-8 agarose gel (Pronadisa) after staining with EtBr. Molecular weight DNA marker used was λ/HindII-Eco RI (Durviz).

2.- DNA extraction following procedures designed for samples with high concentrations of secondary compounds (aromatic plants and algae) and subsequent PCR

First assay. DNA extraction from epiphytes was performed following Wattier et al. (2000) procedure. Samples were broken by means of agitation (11.000 rpm during 6 min at room temperature) followed by vortexing with 5 mm stainless steel beads for 3 min. Two parallel tests were done, the first one strictly following the DNA extraction procedure from Wattier et al. (2000), and the second just changing centrifuging conditions (to 3900 rpm for 30 min) of the same procedure. DNA extractions were visualized as above mentioned.

Second assay. DNA extraction from epiphytes was performed following Khanuja et al. (1999) procedure. Samples were broken using liquid nitrogen followed by mixing with vortex and 5 mm stainless steel beads. The composition of cethyl trymethylammonium bromide (CTAB) buffer cited in Khanuja et al. (1999) was slightly modified (2% CTAB, 2% polyvinylpirrolidone (PVP), 1.4 M sodium chloride (NaCl), 20 mM ethylenediaminetetraacetic acid (EDTA) (pH = 8), 100 mM Tris-hydrochloric acid (Tris-HCl) (pH = 8), 2% 2-mercaptoethanol). DNA extractions from leaves of terrestrial plants (*Brassica oleracea*, *C. sempervirens* and *Rosmarinus officinalis*) were also done as controls. DNA extractions were visualized as above mentioned.

3.- DNA extraction following procedures designed for soils and subsequent PCR

First assay. DNA extraction from epiphytes and terrestrial plants (*B. oleracea*, *C. sempervirens* and *R. officinalis*) was performed following Girvan et al. (2003) procedure. A higher amount of sample was used (about 1 g FW) in comparison with assays explained above. Samples were broken using two different protocols: (1) two cycles of freezing (10 min at -80ºC) and thawing (10 min at 65ºC) followed by vortexing with 5 mm stainless steel beads for 3 min; and (2) freezing the sample with liquid nitrogen followed by vortexing with 5 mm stainless steel beads for 3 min. DNA extractions were visualized as above mentioned.

Second assay. DNA extraction from epiphytes was performed following Girvan et al. (2003) procedure. This procedure was modified using two alternatives: (1) addition of 0.1 g PVP g^{-1} epiphyte FW and (2) addition of a CTAB buffer (1 ml CTAB buffer g^{-1} epiphyte FW). CTAB buffer composition was 2% CTAB, 2% PVP, 1.4 M NaCl, 20 mM EDTA (pH = 8), 100 mM Tris-HCl (pH = 8), 2% 2-mercaptoethanol. Samples were broken using two cycles of freezing (10 min at -80ºC) and thawing (10 min at 65ºC)

followed by mixing with vortex and 5 mm stainless steel beads. DNA extraction was visualized as above mentioned.

Third assay. DNA extraction from epiphytes and different algae (*Udotea* spp. and *Corallina* spp.) was performed following Zhou et al. (1996) procedure. Samples were broken using liquid nitrogen followed by agitation with vortex and 5 mm stainless steel beads. DNA extraction was visualized as above mentioned.

A PCR was performed using DNA extracted using protocols optimised for soils. PCR was done using 1 µl DNA template in a final volume of 20 µl using Master Mix (Eppendorf) according to manufacturer's instructions. 5% DMSO was also added to the reaction. Sets of primers used were EukA/B and Euk1A/516r-GC (Díez et al. 2001) and PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min. PCR products were visualized as above mentioned.

Fourth assay. DNA extraction from epiphytes was performed following Zhou et al. (1996). Samples were broken using liquid nitrogen followed by agitation with vortex and 5 mm stainless steel beads. Finally, a step of purification using DNeasy Plant Mini Kit (Qiagen) was performed. DNA extraction was visualized as above mentioned.

A PCR was performed using DNA extracted from the fourth assay using a protocol optimised for soils. PCR was done using a Master Mix (Eppendorf) in a final volume of 20 µl according to manufacturer's instructions. A total of 5% DMSO was also added to the reaction and DNA template was increased (2 µl) in comparison with previous PCR. We used the set of primers EukA/B (Díez et al. 2001) and Euk1A/New 516r-GC. Euk/New 516r-GC resulted as a modification of primer Euk 516r-GC (Díez et

al. 2001), in which the nitrogenised base at the 3' position was removed. ARB-SILVA database (http://www.arbsilva.de, Pruesse et al. 2007) from ARB package (http://www.arb-home.de, Ludwig et al. 2004) allowed us to design that new primer, which was more appropriated to detect a wider spectrum of Rhodophyta, a relevant component of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves (Van der Ben 1971; Ballesteros 1987). PCR program included an initial denaturation at 94°C for 130 s, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 130 s, with a final extension step of 72°C for 10 min. PCR products were visualized as above explained.

A new PCR was tested in order to diminish the concentration of PCR inhibitors. PCR was done using a Master Mix (Eppendorf) in a final volume of 20 µl according to manufacturer's instructions. A total of 5% DMSO and polyvinylpolyipirrolidone (PVPP) (about. 0.12 µg μl^{-1}) (Zhou et al. 1996) were also added to the reaction. DNA template was 2 µl. Sets of primers used were EukA/B (Díez et al. 2001) and Euk1A/New 516r-GC. PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 44°C for 60 s, and extension at 72°C for 2 min, with a final extension step of 72°C for 10 min. PCR products were visualized as above mentioned.

We finally tested another modification of PCR, in where different dilutions (1:10, 1:50, 1:100) of DNA template were used in order to diminish the concentration of PCR inhibitors. Sets of primers, PCR conditions and PCR program was the same as above. PCR products were visualized as above mentioned.

We discarded set of primers Euk A/B as the combination Euk1A/New 516r-GC allowed an optimal DNA fragment (about 600 bp long) for the application of a

molecular fingerprinting technique (Valentini et al. 2008). PCR was done using a Master Mix (Eppendorf) in a final volume of 20 µl according to manufacturer's instructions. A total of 5% DMSO was also added to the reaction. PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 60 s, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min. PCR products were visualized as above mentioned.

4.- Optimisation of PCR conditions

Because DNA extraction assays following procedures designed for soils and subsequent PCR allowed us to obtain the best results, we tested some modifications to those PCR conditions in order to eliminate non-specific DNA products (DNA fragments different from the desired size). The presence of non-specific DNA products after PCR has been linked both to low T_m and/or prolonged melting time (Sambrook and Russell 2001a). These amplifications were done after a purification step of the DNA extracted by using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

First assay. We tested a PCR program increasing annealing temperature (from 48ºC to 52ºC). PCR was done as above explained. A total of 5% DMSO was also added to the reaction. Set of primers Euk1A/New 516r-GC was used. PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 60 s, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min. PCR products were visualized as above.

Second assay. We tested a 'Reconditioning' PCR and 'Touchdown' PCR, which has previously shown a positive effect in the reduction of the presence of non-specific products (Don et al. 1991; Thompson et al. 2002; Acinas et al. 2004). 'Reconditioning'

PCR is based on a dilution of amplification products into fresh reaction mixture followed by amplification for a low number of cycles (Thompson et al. 2002) and was performed using 1 µl of DNA template in a final 20 µl volume. PCR program, which was explained in the first assay, included 15 cycles instead of 30. After that, we took 2 µl of that DNA product in a final 20 µl volume and we performed 3 additional cycles. 'Touchdown' PCR is based on the decrease of the annealing temperature to a 'touchdown' to obtain more specific products (Don et al. 1991) and was performed decreasing 1ºC annealing temperature during 10 cycles. 'Touchdown' PCR program assayed included an initial denaturation at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 1 min and annealing at 58°C for 60 s (decreasing 1ºC each cycle). Finally, 29 cycles of denaturation at 94°C for 1 min, annealing at 48ºC for 60 s and extension at 72°C for 3 min were performed. PCR products were visualized as above explained.

Third assay. A final PCR was tested using a Master Mix (Eppendorf) in a final volume of 20 µl according to manufacturer's instructions. A total of 5% DMSO was also added to the reaction. Set of primers Euk1A/New 516r-GC was used. PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 60 s, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min. PCR products were visualized as above.

5.- Optimisation of TGGE conditions

The optimisation of TGGE conditions (temperature gradient, migration time, band staining) was necessary to determine which conditions allowed obtaining a clear segregation of DNA products after PCR. Those assays were performed using PCR

products obtained after DNA extraction from soils procedures, because they gave us the best results.

First assay. The temperature gradient tested was 45-60ºC, the migration time was 16 h and the voltage was 130 V. TGGE gel was EtBr stained (at least during 10 min) and DNA bands were revealed after visualization with an UV lamp.

Second assay. We tested a narrower temperature gradient (35-45ºC) and a higher migration time (17 h). The voltage remained invariable. TGGE gel was EtBr stained (at least during 10 min) and DNA bands were revealed as above in the first assay.

Third assay. We used the same temperature gradient, migration time and voltage conditions of the second assay. Finally, gel was silver-stained following a modification of a protocol of Heuer et al. 1997. In brief, the gel was fixed in 10% (v/v) ethanol (CH_3CH_2OH) plus 0.5% acetic acid (CH_3COOH) (10 min). After removing fixing solution, the gel was stained with 0.2% (wt/v) silver nitrate ($AgNO_3$) (30 min). After four thorough washes with bi-distilled water, a freshly prepared developing solution containing sodium borohydride ($NaBH_4$) (around 0.12 g l^{-1}), 0.15% formaldehyde, and 1.5% (wt/v) sodium hydroxide (NaOH) was added to the gel. The development of the gel was stopped by adding 0.75% (wt/v) sodium carbonate (Na_2CO_3) solution (10 min.). We tested silver staining because it has previously shown to be more sensitive to detect DNA bands than EtBr (Radojkovic and Kusic 2000).

Fourth assay. We tested a narrower temperature gradient (35-40ºC) and the other TGGE conditions remain invariable. TGGE gel was silver-stained as above in third assay.

6.- Excision of DNA bands obtained with TGGE

Our goal was obtain a procedure to sequence DNA bands from TGGE gel, link these DNA sequences to known and sequenced epiphyte taxa and reveal the identity of the epiphytes present in the sample studied.

First assay. We slightly modified a procedure optimized to sequence DNA bands from silver-stained gels (Gadanho and Sampaio 2004). In brief, bands were extracted from TGGE gel and eluted in 50 µl ultra-pure water at 4ºC overnight. After that, we performed a re-amplification using 2 µl of the elution following PCR program above mentioned. Finally, sequence of those bands was obtained.

Results

Both the DNA extractions following procedures designed for samples with high concentrations of secondary compounds (aromatic plants and algae) and using DNeasy Plant Mini Kit failed in the visualization of DNA bands in agarose gels for all the tests performed due to the low yields in the extraction. However, the DNA extractions with aromatic plants (such as *R. officinalis*) yielded good DNA concentrations. In addition, PCR tests using these DNA extracts also failed for epiphytic samples, but were positive for the DNA extracted from *C. sempervirens* leaves using both set of primers EukA/EukB and Euk 1A/Euk 516r-GC.

DNA extractions following procedures designed for soil samples allowed the visualization of DNA bands in an agarose gel when epiphytic cells were disrupted using liquid nitrogen and agitation with vortex and stainless steel beads. However, only DNA extractions that were purified in a final step with DNeasy Plant Mini Kit and diluted just before the PCR allowed to amplify DNA using the new set of primers (Euk 1A/EukNew516r-GC) (Fig. 10).

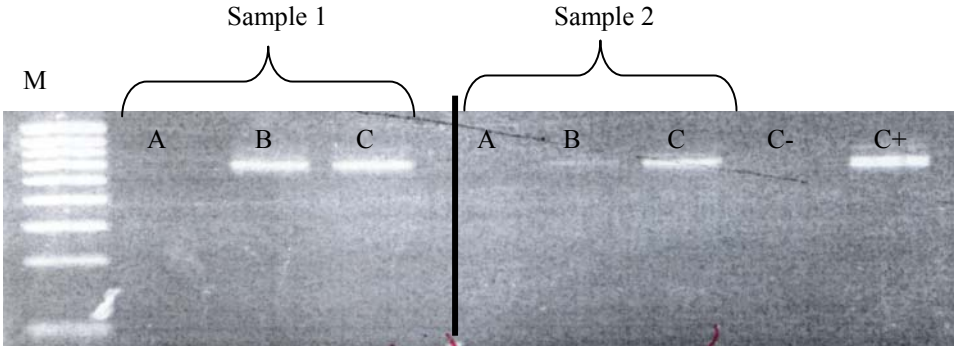


Fig. 10. PCR 2% (wt/v) agarose gel after purifying DNA extracts with DNeasy Plant Mini Kit (Qiagen) in two different samples. A = non-diluted extract, B = extract 1:10 diluted, C = extract 1:25 diluted. C-: negative control (ultra-pure water), C+: positive control. Molecular weight DNA marker (M) used was 'Real Escala 2'.

The best results in PCR tests assayed were obtained by maintaining a low annealing temperature (48ºC) and adding 5% DMSO to the reaction, which was able to significantly reduce non-specific DNA products.

TGGE was also optimised and best results were obtained using a narrow temperature gradient (35-40ºC) and a high migration time (17 h). These conditions allowed obtaining a clear segregation of DNA bands in TGGE gel. In addition, the silver-straining method was able to detect a higher number of bands than that using the EtBr staining method.

The sequencing of DNA from excised bands of the TGGE gel did not render clear results. The sequenced fragments were short-length (less than 300 bp in most cases) and the quality was very low in all cases.

DNA extractions, PCR and TGGE and staining procedures assayed and results obtained are summarized in Fig. 11, 12 and 13, respectively.

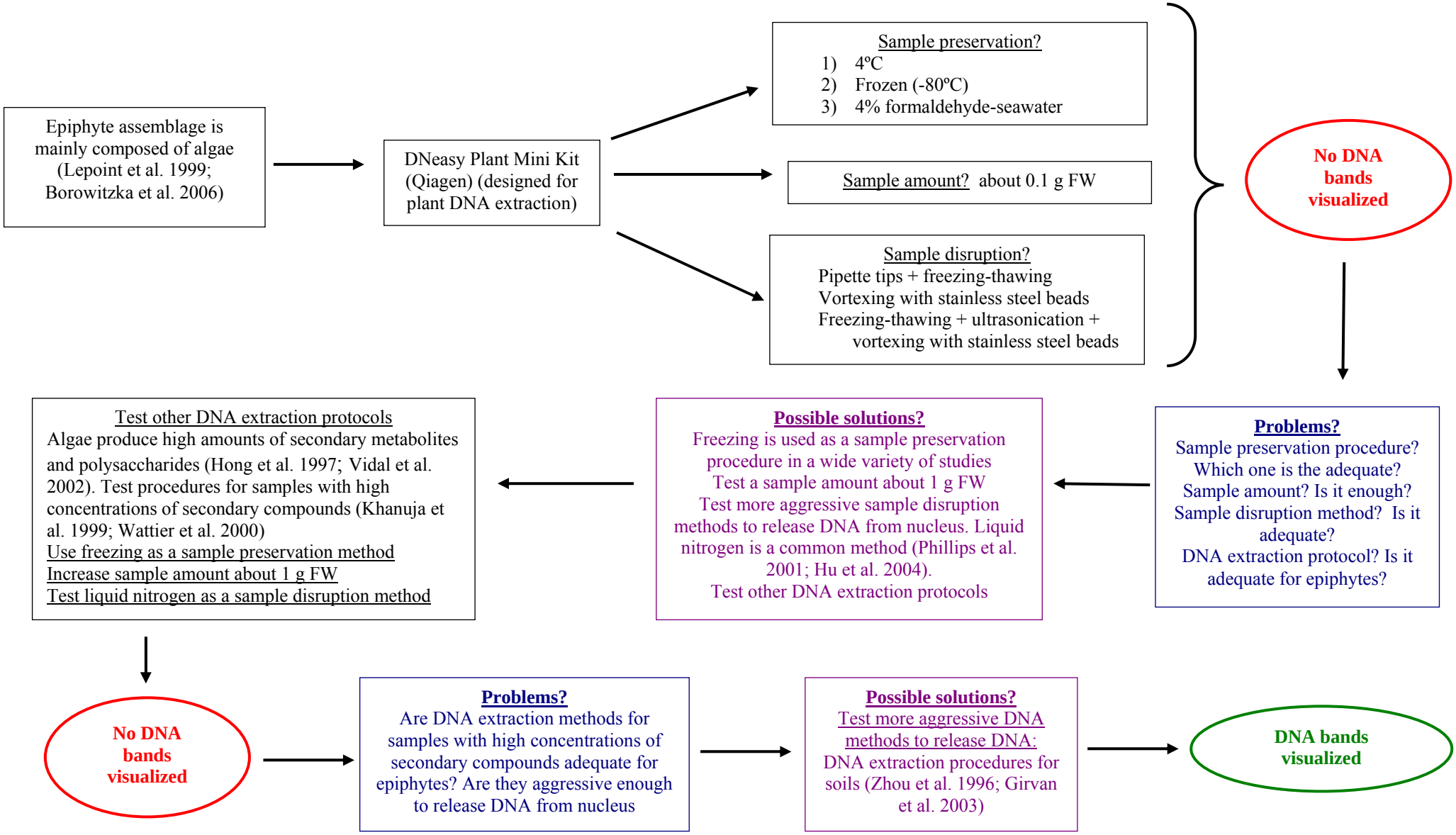


Fig. 11. Flux diagram of main DNA extraction procedures assayed and results obtained.

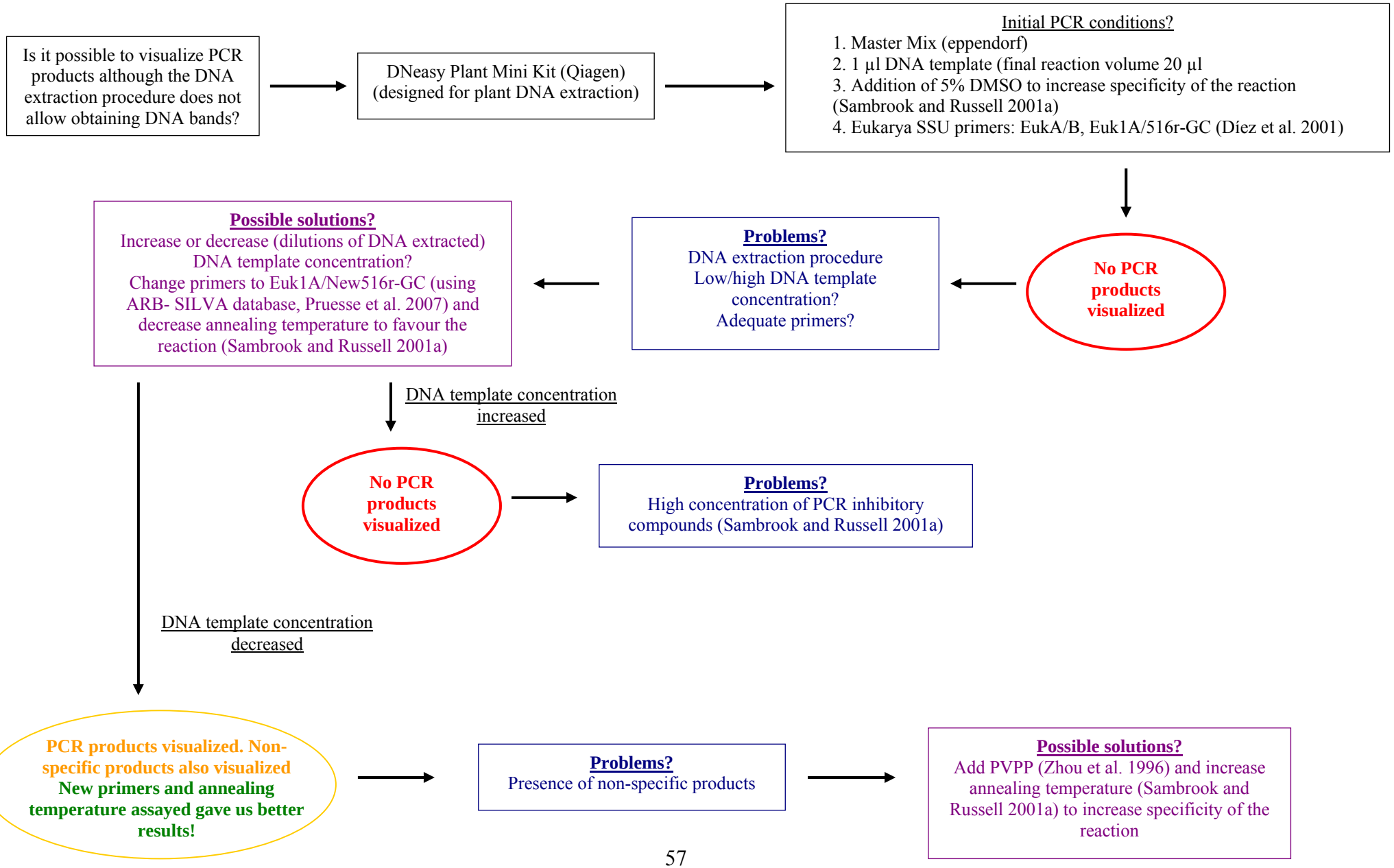

Is it possible to visualize PCR products although the DNA extraction procedure does not allow obtaining DNA bands?
DNeasy Plant Mini Kit (Qiagen) (designed for plant DNA extraction)
Initial PCR conditions?
1. Master Mix (eppendorf)
2. 1 µl DNA template (final reaction volume 20 µl
3. Addition of 5% DMSO to increase specificity of the reaction (Sambrook and Russell 2001a)
4. Eukarya SSU primers: EukA/B, Euk1A/516r-GC (Díez et al. 2001)
No PCR products visualized
Problems?
DNA extraction procedure
Low/high DNA template concentration?
Adequate primers?
Possible solutions?
Increase or decrease (dilutions of DNA extracted) DNA template concentration?
Change primers to Euk1A/New516r-GC (using ARB- SILVA database, Pruesse et al. 2007) and decrease annealing temperature to favour the reaction (Sambrook and Russell 2001a)
DNA template concentration increased
No PCR products visualized
Problems?
High concentration of PCR inhibitory compounds (Sambrook and Russell 2001a)
DNA template concentration decreased
PCR products visualized. Non-specific products also visualized
New primers and annealing temperature assayed gave us better results!
Problems?
Presence of non-specific products
Possible solutions?
Add PVPP (Zhou et al. 1996) and increase annealing temperature (Sambrook and Russell 2001a) to increase specificity of the reaction

Fig. 12. cont.)

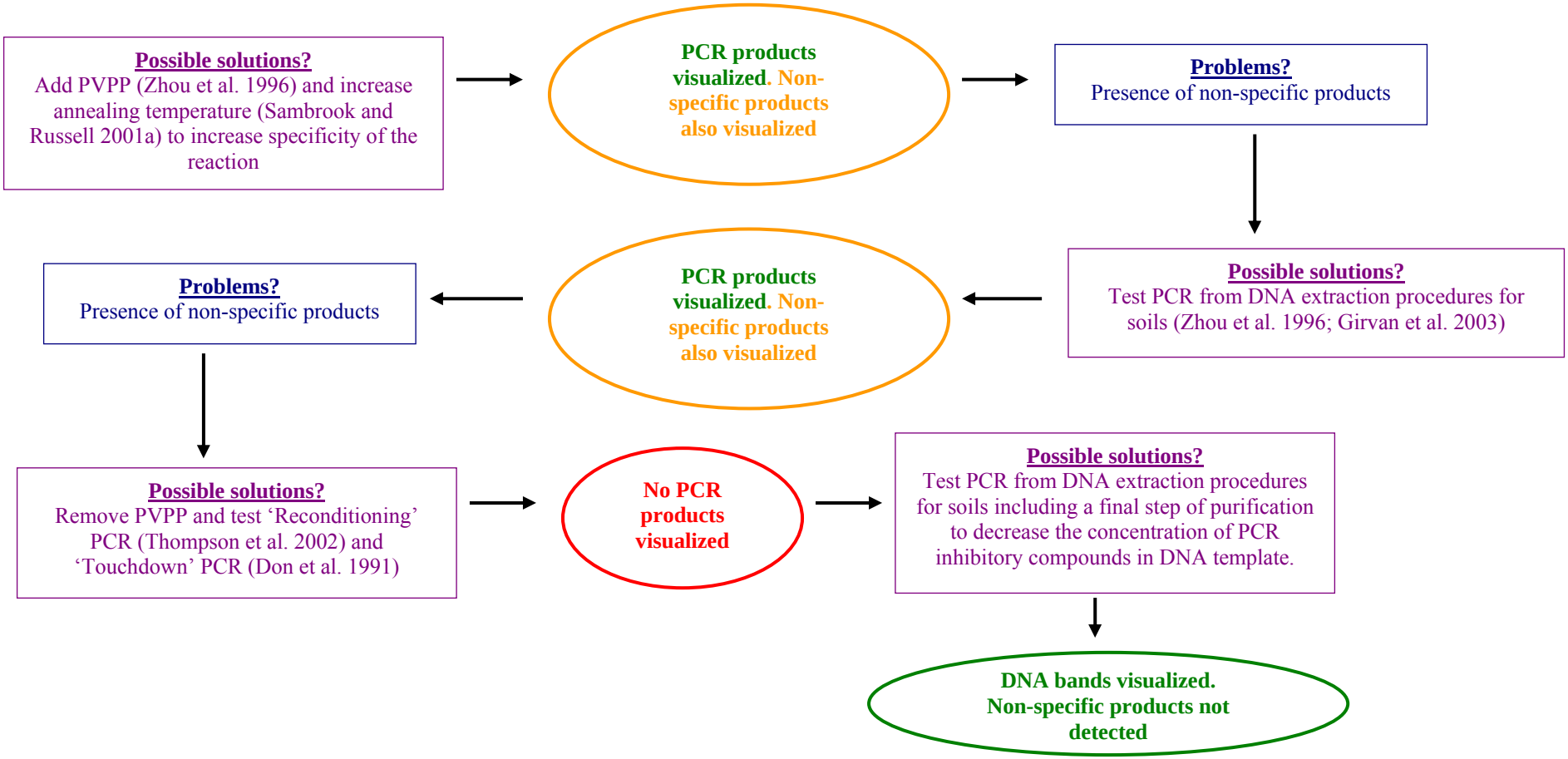


Fig. 12. Flux diagram of main PCR procedures assayed and results obtained.

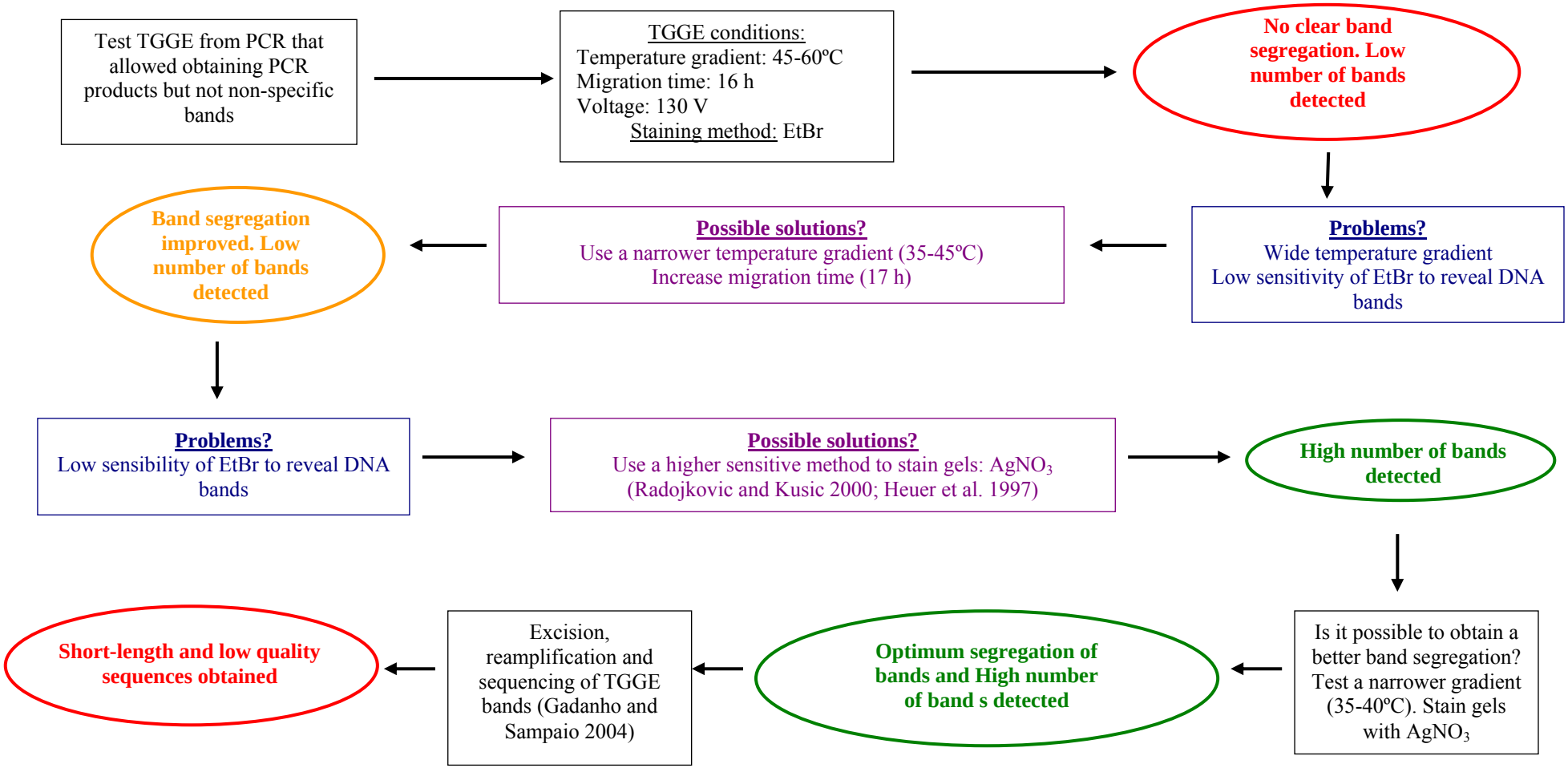


Fig. 13. Flux diagram of main TGGE and gel staining procedures assayed and results obtained.

Discussion

The application of a molecular fingerprinting technique to study the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves has showed that several steps must be previously optimised to obtain reliable results.

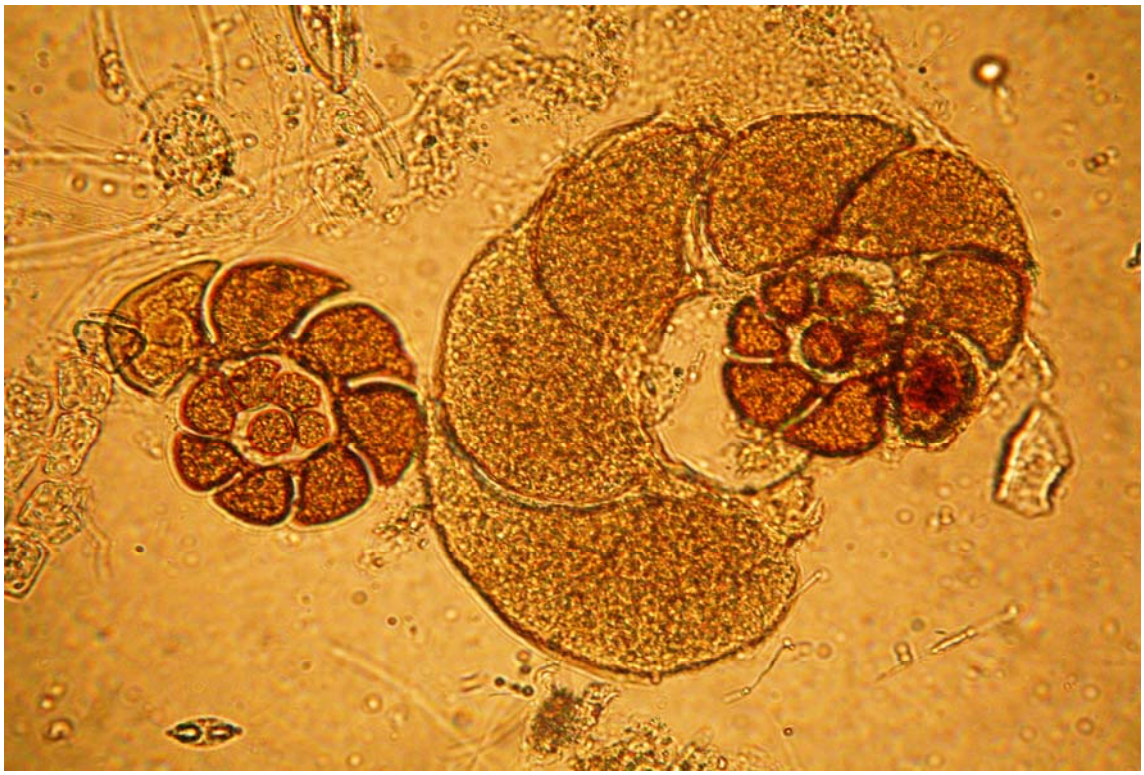
The first step to be optimised was the DNA extraction of the macroeukaryotic epiphytic fraction. Some assays were necessarily performed in order to visualize DNA bands in an agarose gel. Different methods of sample disruption were assayed, and the combination of liquid nitrogen and vortexing with stainless steel beads gave the best results. Liquid nitrogen has been previously used to disrupt samples allowing the DNA extraction of a wide variety of algae species (La Claire II and Herrin 1997; Phillips et al. 2001; Hu et al. 2004). DNA extraction was finally performed using a protocol for soil samples, because it allowed obtaining PCR products with the set of primers Euk1A/EukNew516r-GC. The use of less aggressive protocols (DNeasy Plant MiniKit) or adapted for samples with large concentrations of secondary compounds were positive for other samples but both failed in the amplification of epiphytic DNA. Those results suggested that secondary compounds might be co-isolated with epiphytic DNA in concentrations that were able to inhibit PCR. This was supported by the fact that the purification of the DNA extraction and the dilution of DNA from epiphytes just before PCR gave the best results. The addition of 5% DMSO showed to be effective in reducing the presence of non-specific products in PCR from epiphytic DNA, as it has been recognised previously (Sambrook and Russell 2001a).

TGGE fingerprinting technique was finally optimised using a narrow temperature gradient, which showed a clear resolution of DNA bands, and the silver-

staining was chosen because it was more sensitive in detecting bands than EtBR, as other studies had previously shown (Radojkovic and Kusic 2000).

In summary, the procedure that gave us the best results for epiphytic samples was the DNA extraction optimized for soil samples (Zhou et al. 1996) including a final step of purification with DNeasy Plant Mini Kit. Cell disruption was performed using liquid nitrogen and vortexing with stainless steel beads. DNA extracted after purification step needed necessarily to be diluted just before the amplification, and the addition of 5% DMSO was necessary to obtain DNA products without non-specific spurious bands. Set of primers selected was Euk1A/EukNew516r-GC because they produced an optimal length of fragment for fingerprinting purposes (Valentini et al. 2008) and were able to amplify DNA fragments of a wider spectrum of Rhodophyta, an abundant component of epiphytic macroalgae. TGGE optimal conditions were 35-40ºC (temperature gradient), 130 V (voltage), 17 h (migration time) and bands were revealed with a silver-staining protocol.

SECTION IV - Chapter 2



Detail of epiphytic Foraminifera on *P. oceanica* leaves

Application of TGGE to monitor changes in the structure of the eukaryotic leaf-epiphytic community of *P. oceanica*

In: Medina-Pons FJ, Terrados J, Rosselló-Móra R (2008). Mar Biol 155(4): 451-460

Application of TGGE to monitor changes in the structure of the eukaryotic leaf-epiphytic community of *P. oceanica*

Background

A molecular method (DNA extraction, PCR and TGGE) was optimised to study the structure of the macroeukaryotic leaf-epiphytic fraction of *P. oceanica* (Chapter 1). Our main objective was testing the sensitivity of that molecular approach to detect variability in the structure of two stages of the epiphytic assemblage. We were also interested in comparing results obtained with a molecular technique with those obtained with a classical microscopy method.

Materials and methods

Sample collection

The study was performed in February and September 2006 in a *P. oceanica* meadow located at a depth of 7 m in LaVictoria (39° 51' N, 3° 11' E, Alcudia Bay, Majorca, Spain). A total of 20 *P. oceanica* shoots were haphazardly collected at each sampling date by SCUBA diving and frozen at -80°C to keep nucleic acids intact. Five (in February) and ten (in September) additional shoots were harvested and preserved in 4% formalin seawater until their processing to identify the epiphytic taxa. Five to ten shoots are considered as an adequate number to assess the taxa composition of the leaf-epiphytic community using classical microscope methods (Panayotidis and Boudouresque 1981; Ballesteros 1987).

TGGE technique

DNA isolation and purification

We expected macroalgae to be a dominant fraction of the epiphytic biota (Lepoint et al. 1999). As DNA isolation of marine algae has proven to be very difficult due to the co-isolation of polysaccharides and secondary metabolites which can inhibit PCR (Hong et al. 1997; Vidal et al. 2002), we used a modification of an aggressive DNA isolation method for soils (Zhou et al. 1996). PCR inhibiting molecules were removed from the extracted DNA by using DNeasy Plant Mini Kit (Qiagen).

The epiphytic community of all leaves of a shoot (on average six leaves per shoot) was carefully scraped into sterile filtered seawater with a clean razor blade (we chose the whole epiphytic community of the shoot to obtain enough quantity of DNA to perform the analyses). Epiphytes were then collected by centrifugation at 2,000 g and room temperature for 15 min. Epiphyte mass (around 1 g of wet weight) was frozen with liquid nitrogen and ground to a fine powder using 5 mm stainless steel beads (Qiagen) and a vortex.

Disrupted cell material was suspended in 13.5 ml of extraction buffer [100 mM Tris- HCl (pH = 8), 20 mM EDTA (pH = 8), 1.4 M NaCl, 2% PVP and 2% CTAB (modified of Porebski et al. 1997)] and 100 μl of proteinase K (10 mg ml^{-1}). Tubes were incubated horizontally at 37°C for 1 h with shaking. Then, 1.5 ml of 25% sodium dodecyl sulfate (SDS) was added to each tube and samples were incubated for 2 h in a 65°C water bath. Tubes were mixed by inversion each 10–15 min. Extracts were then left at room temperature for at least 20 min. After incubation, 15 ml equilibrated phenol (pH = 8) (Amersham) was added to each sample and vigorously mixed and centrifuged for 15 min at 2,000 g at room temperature. Aqueous supernatant was collected and mixed with 15 ml of chloroform:isoamylic alcohol (24:1), vigorously shaken, and centrifuged at 2,000 g at room temperature for 10 min. This step was repeated at least

two times, or until no interface was seen. Finally, 1/9 volume of 3 M sodium acetate (AcNa) (pH = 7) and 0.6 volume of isopropanol were added to the supernatant to precipitate DNA. To increase recovery yields, samples were stored at 4°C overnight. DNA was collected by centrifugation at 2,000 g for 30 min at room temperature and the supernatant was discarded. Precipitated DNA was air dried at room temperature and finally dissolved in 200 µl MiliQ sterile water.

To remove PCR inhibitors, isolated DNA was further purified using DNeasy Plant Mini kit (Qiagen) following manufacturer's instructions. Quality and quantity of purified DNA was measured in 1% agarose gel with EtBr.

PCR conditions

Eukaryotic 18S ribosomal DNA gene fragments were amplified by PCR using (rDNA)-specific primers Euk1A (5'CTG GTT GAT CCT GCC AG3') and EukNew-516r-GC (5'CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC CAG ACT TGC CCT C3') (Díez et al. 2001). EukNew-516r-GC is a one nucleotide shorter version at the 3' end of the previously published oligonucleotide. Comparisons of 18S rRNA gene sequence alignments in the ARB-SILVA database (http://www.arbsilva.de, Pruesse et al. 2007), using ARB software package (http://www.arb-home.de, Ludwig et al. 2004) allowed us designing a wider spectra primer set for Eukaryotes. This new set allows distinguish Rhodophyta in contrary to the previously published (Díez et al. 2001).

A 600-bp fragment was generated with PCR using a Master Mix (Eppendorf) in a final volume of 20 µl according to manufacturer's instructions. A total of 5% DMSO was added to increase specificity of the reaction (Sambrook and Russell 2001a). The PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles

of denaturation at 94°C for 30 s, annealing at 48°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min. Amplicons were visualized in a 2–3% MS-8 Agarose gel (Pronadisa) with EtBr. "Real Escala No 2" was used as a quantification and size marker (Durviz).

TGGE conditions

PCR products were resolved by using the Biometra TGGE Maxi System with the procedure in accordance with the manufacturer's instructions. Acrylamide gel was prepared with 6% acrylamide/bisacrylamide (37.5:1), 8 M urea, 2% glycerol, 1x Tris–acetate–EDTA Buffer (TAE), and 20% deionized formamide. The gel was polymerized by adding 38 µl *N*,*N*,*N*,*N*-tetra-methylethylenediamine (TEMED) and 63 µl 10% ammonium persulfate (APS). The gel was let polymerizing at least for 3 h (modified of Van Dillewijn et al. 2002). About 70 ng of each PCR product were run at a constant voltage of 130 V for 17 h. For our purpose, the thermal gradient was optimal between 35 and 45°C. Finally, gels were silver-stained following a modification of a protocol of Heuer et al. 1997. The gel was fixed in 10% (v/v) ethanol plus 0.5% CH_3COOH (10 min). After removing fixing solution, the gel was stained with 0.2% (wt/v) $AgNO_3$ (30 min). After four thorough washes with bi-distilled water, a freshly prepared developing solution containing $NaBH_4$ (around 0.12 g l^{-1}), 0.15% formaldehyde, and 1.5% (wt/v) NaOH was added to the gel. The development of the gel was stopped by adding 0.75% (wt/v) Na_2CO_3 solution (10 min.). Gels were finally conserved with 25% ethanol and 10% glycerol and dried. Alternatively, gels were stained by using SYBR Gold Nucleic Acid Gel Stain according to manufacturarer's instructions (Molecular Probes).

Band reamplification, sequencing and phylogenetic analysis

To reveal the gene sequence, some bands were excised from the freshly stained gel with SYBR gold (Molecular Probes) soaked in 30 µl of sterile water, and let stand at overnight at 4°C. Acrylamide pieces were disaggregated by using a plastic stab. Between 1 and 3 µl of each supernatant were used for reamplification by using the same primers and PCR conditions as above. Sequencing was performed by using the Euk 1A primer by the sequencing company Secugen SL. Partial sequences were revised and corrected with Sequencher v 4.7 (Gene Codes Corp 2006). 18S rRNA gene alignments were produced with the use of the ARB software package (http://www.arb-home.de, Ludwig et al. 2004), introducing the new almost complete sequences into a preexisting alignment available of about 208,000 single sequences (http//www.arb-silva.de, Pruesse et al. 2007). Aligned partial sequences were inserted in a preexisting tree by the use of the ARB-Parsimony tool as implemented in the ARB package (Ludwig et al. 2004).

Optical microscope technique

Following standard methods to describe the epiphytic community of seagrasses, we studied the 10 cm apical portion of the oldest leaf (both outer and inner leaf sides) of each shoot for it has been shown to adequately represent the epiphyte community structure of the whole shoot (Vanderklift and Lavery 2000; Lepoint et al. 2007). Moreover, the species richness and biomass of epiphytes in the apical portion of seagrass leaves are usually higher that those of the basal portions (Reyes et al. 1998; Trautman and Borowitzka 1999; Lepoint et al. 2007). Random scrapings (between 3 and 5) of this apical segment were mounted on glass microscope slides for the identification of epiphytic taxa (Reyes and Sansón 1997) using an optical microscope (ZEISS AX10).

Data analysis

TGGE band profiles were manually translated into a binary (presence/absence) matrix for each sampling date. All bands migrating with an identical velocity in TGGE were identified as unique OTUs (Rosselló-Móra and López-López 2008). To obtain comparable results, amplicons of different sampling dates were run together in the same gel. An averaged rarefaction curve (cumulative number of different OTUs vs. number of shoots) was constructed by using 999 permutes of the band profiles of all shoots collected in each sampling date. The minimum number of shoots required to represent the band richness of each sample was reached when the relative increase of band richness after including an additional shoot was smaller than 5%.

MDS based on the Bray–Curtis similarity index, and an analysis of similarity (ANOSIM) were used to evaluate the differences between profiles.

Differences in number of OTUs per shoot between sampling dates were assessed by one-way analysis of variance (ANOVA). Data were previously tested for homogeneity of variances and normality using Levene's test ($P > 0.05$) and Kolmogorov–Smirnov test ($P > 0.05$), respectively. Variances were homogeneous and data followed normal distribution. Similar to the TGGE data analysis we calculated averaged rarefaction curves, and performed MDS, ANOSIM and one-way ANOVA analysis on the data obtained using optical microscope.

All analyses were performed using PRIMER 5 (Plymouth Routines in Multivariate Ecological Research) (Clarke and Gorley 2001), SIGMAPLOT 8.0 (SPSS Science 2002) and STATISTICA 7.1 software (StatSoft Inc 2005).

Results

A total of 43 different OTUs were identified in the complete set of samples of February and September shoots (Fig. 14). The February sample contained 30 of the total

identified OTUs, whereas the September sample contained 38 of them. The number of OTUs per shoot varied between 4 and 23 in February with a mean (±SD) of 11.9 ± 5.2 OTUs per shoot. In contrast, the number of OTUs per shoot in September varied between 16 OTUs and 30 OTUs with a mean of 22.3 ± 3.9 OTUs per shoot. One-way ANOVA revealed significant differences in the number of OTUs per shoot between the February and September samples ($F_{1,36} = 48.54$, $P < 0.05$).

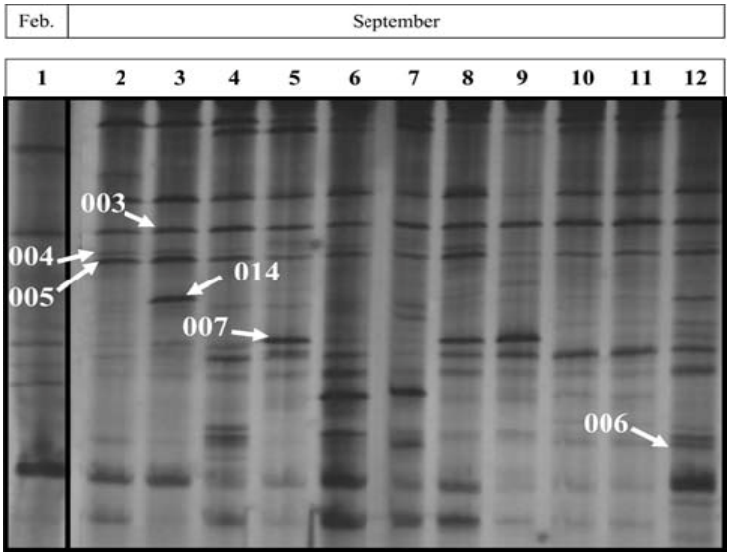


Fig. 14. Barcoding of the leaf-epiphytic community of some of the February and September shoots. *Lane 1* of the TGGE represents a February shoot and *lanes 2–12* represent September shoots. Sequenced OTUs and their codes are indicated and correspond to that listed in Table 3.

A total of 37 epiphytic taxa were identified by optical microscopy. The total number of epiphytic taxa identified was 10 in February shoots and 36 in September shoots. The mean number (±SD) of taxa per shoot was higher in September (14.9 ± 4.1) than in February (6 ± 2.1). One-way ANOVA revealed significant differences in taxa richness per shoot among February and September samples ($F_{1,13} = 20.32$, $P < 0.05$).

The analysis of the relative increase of OTUs richness compared to the number of shoots included in the sample indicated that four shoots in September and six in February were enough to represent TGGE band richness (Fig. 15a). Similarly, seven shoots in September and five shoots in February were necessary to represent the taxa richness using the optical microscope (Fig. 15b).

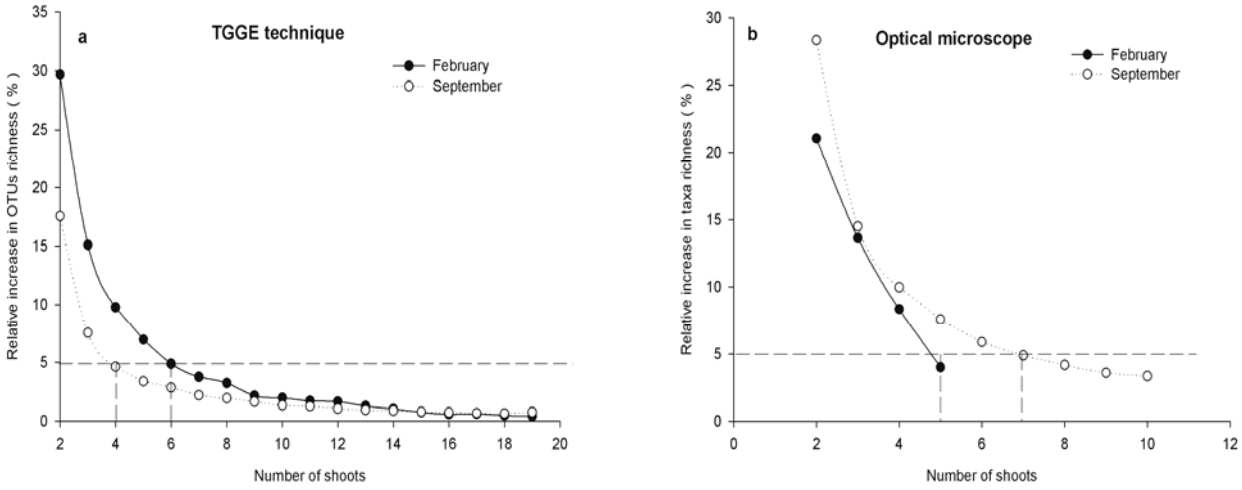


Fig. 15. Average rarefaction curves of the leaf-epiphytic community of *P. oceanica* in February and September. Relative increase in (**a**) OTUs richness and (**b**) taxa richness as a function of the number of shoots. The *horizontal discontinuous line* represents the 5% level considered to indicate minimum number of shoots needed to evaluate total OTU richness or taxa richness, respectively. One sample of each sampling date was unfortunately lost while processing in the lab.

MDS analysis produced a clear separation between the leaf epiphytic community of February and September shoots when based both on OTUs (Fig. 16a) and on taxa richness (Fig. 16b). ANOSIM tests confirmed the significance of the differences between the two sampling dates (TGGE: $R = 0.709$, $P < 0.01$; optical microscopy: $R = 0.877$, $P < 0.01$).

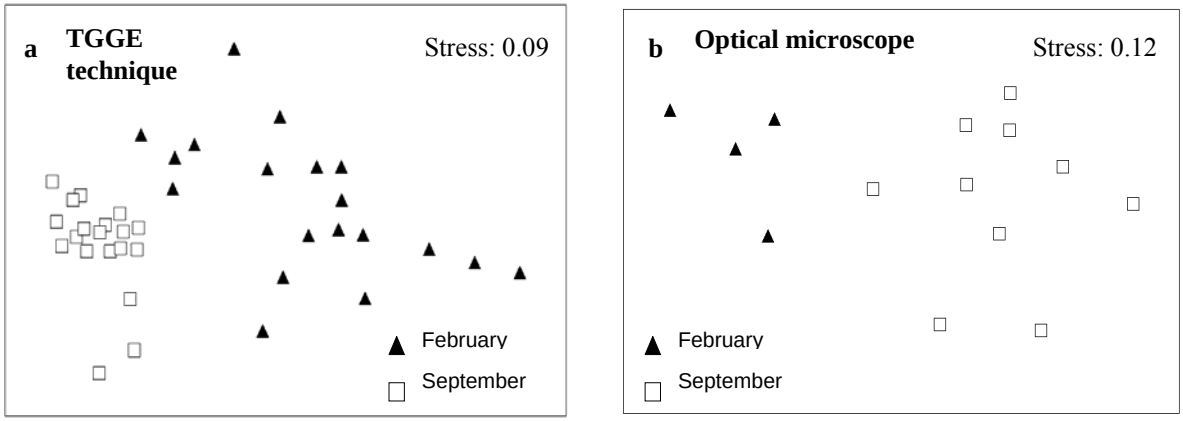


Fig. 16. MDS ordination of the leaf-epiphytic community of *Posidonia oceanica* shoots in February and September using (**a**) TGGE and (**b**) optical microscope

A list of the epiphytic taxa identified in the February and September shoots using optical microscopy is shown in Table 2 (according to the taxonomy of Furnari et al. 2003). In general, the February epiphytic community was basically composed of Rhodophyta and Phaeophyta, whereas in September, the community was much more complex and it included Rhodophyta, Phaeophyta, Chlorophyta, and zooepiphytes. Rhodophyta and Phaeophyta showed similar taxa richness in February shoots. However, Rhodophyta (Ceramiales mainly) dominated in September shoots. Some epiphytes were found in almost all samples (brown and red encrusting algae (*M. magnusii*, *Hydrolithon* sp. and *Pneophyllum* sp.) and filamentous red algae such as *Audouinella daviesii*, *Audouinella sp.*, *Erythrotrichia carnea* and *Spermothamnion* sp.). However, others were mainly present either in February (brown algae *G. sphacelarioides*) or in September (Ceramiales, Chlorophyta (*Phaeophila dendroides* and *Cladophora* sp.) and zooepiphytes (Bryozoa and Foraminifera). Zooepiphytes were less present than algae in both sampling dates.

Table 2. List of leaf-epiphytic taxa found in February and September shoots (taxonomy names according to Furnari et al. 2003)

Species	Shoots													
	February					September								
	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10
Bacillariophyta															
Diatoms		●	●						●			●	●	●	●
Chlorophyta															
Ulvales															
Ulvella or *Pringsheimiella* sp.								●				●			●
Cladophorales															
Chaetomorpha sp.									●						
Cladophora sp.						●		●	●	●	●	●	●	●	●
Phaeophilales															
Phaeophila dendroides							●	●		●	●	●	●	●	●
Phaeophyta															
Ectocarpales															
F. Ectocarpaceae					●										
Giraudia sphacelarioides	●	●	●	●	●							●	●	●	
Myrionema magnusii	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Rodophyta															
Acrochaetiales															
Audouinella daviesii	●	●	●	●	●	●	●			●	●	●	●	●	●
Audouinella sp.			●		●	●						●	●	●	●
Ceramiales															
Anotrichium tenue						●									
Callithamnion corymbosum								●							
Ceramium codii						●		●			●		●		
Chondria sp.											●	●	●	●	
Chondria or *Laurencia sp.*							●					●			
Dasya sp.											●		●		
Griffithsia sp.								●				●			
Herposiphonia secunda						●		●	●		●	●		●	●
Lophosiphonia sp.											●	●	●		
Polysiphonia spp.						●		●		●	●	●	●	●	●
F. Rodomelaceae															●
Spermothamnion spp.			●		●	●	●		●	●	●	●	●	●	●
Crytptonemiales															
Hydrolithon + *Pneophyllum* spp.	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Erythropeltidales															
Erythrotrichia carnea		●			●	●	●	●	●	●	●	●		●	●
Stylonematales															
Chroodactylon ornatum						●									
Stylonema alsidii											●				
Zooepiphytes															
Bryozoans									●	●	●	●	●	●	●
Hydrozoans					●							●			
Foraminifera							●	●	●	●	●	●	●	●	●

Table 2 continued

Species	Shoots													
	February					September								
	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10
Unknown epiphytes															
Sp. 1						●			●					●	●
Sp. 2									●						
Sp. 3								●					●		
Sp. 4										●					
Sp. 5											●	●	●	●	
Sp. 6														●	
Sp. 7															●
Sp. 8														●	

Six selected bands, representative of permanent and seasonal OTUs, were excised, reamplified and sequenced. The sequence identity (Table 3) indicated that they affiliated with Algae, Crustacea, Bryozoa, Annelida and Mollusca. Some of the bands (i.e. OTU 003 and 006) represented sequences of organisms that had been detected under the microscope.

Table 3. OTUs sequenced in this study. Similarity (%) is referred to the value of similarity between the OTU and the best match (according with ARB software).

Accession number	OTU	Sequence length	Best match (Accession Number)	Similarity (%)	Phylogenetic affiliation	Reference
EU888848	003	430	*Pneophyllum conicum* (DQ628985)	95	Rhodophyta Florideophyceae Corallinaceae	Unpublished
EU888849	004	527	*Pontoeciella abyssicola* (AY627031)	90	Crustacea Maxillopoda Copepoda	Huys et al. 2006
EU888850	005	497	*Parergodrilus heideri* (AJ310504)	81.5	Annelida Polychaeta	Rota et al. 2001
EU888851	006	550	*Bugula turrita* (AY210443)	91	Bryozoa Gymnolaemata Cheilostomata	Passamaneck and Halanych 2006
EU888852	007	541	*Brania sp.* (AY525633)	75	Annelida Polychaeta	Struck et al. 2005
EU888847	014	503	*Pomacea bridgesi* (DQ093436)	83.5	Mollusca Gastropoda	Giribet et al. 2006

Discussion

Our purpose was to evaluate the applicability of TGGE to the study of the epiphytic eukaryotic community of *P. oceanica* leaves comparing the results provided by this technique with those produced by taxa identification using optical microscopy. We obtained satisfactory amplification yields and clear TGGE banding profiles that could be easily compared between the two sampling dates. The TGGE technique was successful in detecting differences in the composition of the leaf epiphytic community of *P. oceanica* between the two sampling dates, and rendered comparable results to those produced by optical microscopy.

Each TGGE band represents a different SSU sequence. However, due to the highly conserved nature of the gene (Eckenrode et al. 1985), and the fact that paralogous genes may be nearly identical (Eickbush and Eickbush 2007; Ganley and Kobayashi 2007), different bands may be attributed to different taxa. In this regard, similar phylotype diversity evaluations have been previously made for microscopic eukaryotes (Rowan and Powers 1991a; Rowan and Powers 1991b). We considered, therefore, identically migrating bands as a unique OTU, and thus each OTU may be considered a different taxon. This hypothesis has been corroborated by the excision and sequencing of six representative bands on the gel. Despite the difficulties in reamplifying and sequencing partial amplicons, the sequences retrieved were relatively clean and could undoubtedly be affiliated to known sequences in the public databases.

TGGE and optical microscopy provided comparable results: (1) the number of both OTUs per shoot and epiphytic taxa per shoot were higher in September than in February; (2) the minimum number of shoots to represent OTUs richness and taxa richness were similar; (3) MDS and ANOSIM detected significant differences between

the epiphytic community in the two sampling dates and (4) TGGE detected a number of common bands between the September and February shoots, and optical microscopy identified some common taxa such as *Hydrolithon* sp., *Pneophyllum* sp. and *M.magnusii*. These results agree with the identification of common bands (as OTU 003 affiliating with *Pneophyllum conicum*) with sequences of organisms that appear permanent on shoots after classical optical inspection.

We found higher OTUs and taxa richness in September than in February shoots, a result that is consistent with previous microscopy studies (Antolic 1986; Ballesteros 1987). Those studies reported that taxa richness of the epiphytic community of *P. oceanica* was also higher in summer than in winter. The minimum number of shoots required to represent the richness of the epiphytic community (either 4 or 7 shoots using OTUs or identified taxa, respectively) was similar to that calculated in previous studies (from 7 to 8 shoots: Panayotidis and Boudouresque 1981; Ballesteros 1987). Furthermore, previous studies also found significant differences in the composition of the leaf epiphytic community (Antolic 1986; Ballesteros 1987) between summer and winter. Finally, the presence of a permanent group of taxa in the epiphytic community of *P. oceanica* is widely accepted (Van der Ben 1971; Romero 1988; Buia et al. 1989; Mazzella et al. 1989).

The use of optical microscopy to analyse the taxa composition of the epiphytic community produced similar results to those described previously: higher taxa richness in September shoots explained by the presence of numerous Rhodophyta (mainly filamentous Ceramiales) as Van der Ben (1971) and Antolic (1986); encrusting Rhodophyta and Phaeophyta characterized the community in February (Van der Ben

1971; Ballesteros 1987); Chlorophyta were totally absent in winter (Tsirika et al. 2007; but see Antolic 1986).

It is clear to us that both approaches are complementary and do not give identical information. In first instance, TGGE targets all eukaryotes in the sample, including microscopic taxa, whereas the microscopy study basically focuses on macroalgae. This may explain the higher number of OTUs that TGGE renders, and could be seen as an advantage of the molecular approach. While it is true that DNA-based methods are biased (Dahllöf 2002), we have optimized a DNA aggressive purification method and PCR primer set to better reflect the real diversity of the community. On the other hand, the optical identification may be biased towards macroepiphytes and by the taxonomical expertise of the researcher.

In summary, the TGGE provides a consistent barcoding of the composition of the eukaryotic epiphytic community, and allows the simultaneous handling of large amounts of samples. Furthermore, TGGE also allows the study of a broader set of eukaryotic organisms, thus giving a better idea of the whole community and not only on specific groups. A practical benefit of the technique is that it relaxes the level of taxonomical expertise necessary to describe the diversity of the community. This goal is generally hampered by the fact that taxonomists are relatively specialized, and seldom can identify organisms that are excluded of their taxonomic expertise. Hence, fingerprinting techniques like TGGE appear as an alternative and excellent approach to the study of the structure of the epiphytic assemblage of *P. oceanica* leaves. The possibility to excise, reamplify and sequence single bands on the gel enhances the resolution power of the approach. As we have shown by selecting representative bands, the analysis of the affiliation of partial sequences by the use of the parsimony tool of the

ARB program package allows the identification of putative taxa colonizing the *P. oceanica* shoots. In this regard, we could identify the sequences of some detected taxa by optical microscopy. However, as an additional benefit of the approach, we could as well detect the presence of taxa not listed in our inventory. This may help in overcoming the problems derived from the lack of expertise in morphologically recognize observable taxa.

SECTION IV - Chapter 3

Detail of epiphyte filamentous Rhodophyta on *P. oceanica* leaf

Evaluation of SSU clone library approach to study the diversity of the macroeukaryotic leaf-epiphytic community of the seagrass *P. oceanica* (L.) Delile

In: Medina-Pons FJ, Terrados J, López-López A, Yarza P, Rosselló-Móra R (2009). Mar Biol 156(9): 1963-1976

In: Medina-Pons FJ, Terrados J, López-López A, Yarza P, Rosselló-Móra R (2009). Mar Biol 156(9): 1963-1976.

Evaluation of SSU clone library approach to study the diversity of the macroeukaryotic leaf-epiphytic community of the seagrass *P. oceanica* (L.) Delile

Background

TGGE was able to monitor changes in the structure of the macroeukaryotic leaf-epiphytic fraction of *P. oceanica.* However, sequence data obtained from the excised OTUs in TGGE gel were of low quality due to the low information content of DNA fragments (short sequences), and the difficulties in obtaining clean sequences (Chapter 2). Our main objective was to overcome these limitations by obtaining high-quality (almost complete) 18S rRNA gene sequences and evaluating the usefulness of the SSU clone libraries to describe changes in the structure of the macroeukaryotic leaf-epiphytic assemblage of *P. oceanica.* To that end, we compared the results of these libraries with those provided by classical microscopic techniques.

Materials and methods

Sample collection

The samples were taken in February, June and September 2006 in a *P. oceanica* meadow located at a depth of 7 m in La Victoria (39° 51' N, 3° 11' E, Pollença Bay, Majorca, Spain). Seven shoots (each one containing about six leaves) were haphazardly collected by SCUBA diving in each sampling date and frozen at -80°C to keep nucleic acids intact for the SSU clone libraries approach. This number of shoots was previously considered adequate to represent the diversity of the epiphytic community in molecular studies (Chapter 2). Additionally, five to ten shoots were collected in each sampling event to assess the epiphytic taxa richness and the relative abundance of each taxon

using classical microscopy methods (Panayotidis and Boudouresque 1981; Ballesteros 1987).

DNA extraction and eukaryotic SSU amplification

DNA extraction was performed according to Chapter 2. The eukaryotic SSU gene (18S rRNA gene) was amplified using the specific primers Euk_A_Q and Euk_B_Q (Table 4). A fragment of a length of about 1,800 bp, covering the almost entire SSU, was obtained by PCR using the Master Mix (Eppendorf) in a final volume of 20 µl according to the manufacturer's instructions. About three independent PCR reactions per shoot were performed and mixed to diminish the bias of DNA polymerase. To increase the specificity of the reaction, 5% DMSO was added to the PCR mixture (Sambrook and Russell 2001a). PCR conditions included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min. Amplicons were visualised under UV light after ethidium bromide staining in 1% MS-8 agarose gels (Pronadisa). PCR products of each sampling date were mixed in equal concentrations (about 70 ng each amplicon) in eppendorf tubes and purified using Qiaquick PCR Purificación Kit (Qiagen). Finally, they were resuspended in 50 µl of sterile MiliQ water according to the manufacturer's recommendations.

Cloning and sequencing of SSU

SSU clone libraries were constructed for each sampling date. Purified amplification products were cloned into the pGEM-T Easy vector system (Promega). Ligation reactions were performed overnight at 4°C and transformed into JM 109 competent cells (Promega) following the manufacturer's instructions. Insert-containing clones were selected by ampicillin resistance in a medium containing LB broth (15 g l^{-1})

(Pronadisa), agar (16 g l^{-1}), X-gal (2 µl ml^{-1}), IPTG (0.5 µl ml^{-1}) and ampicillin (1 µl ml^{-1}). About 100 positive clones (containing eukaryotic SSU gene) from each SSU library were randomly selected for further analysis. Cloned fragments were partially sequenced (about 500–700 bp length) using M13F primer (Table 4) according to BigDye Terminator v3.1 Cycle Sequencing Kit procedures (Applied Biosystems). Sequencing was performed in "Servicios Científico-Técnicos" of the Balearic Islands University (Majorca, Spain) onto an ABI 310 Capillary DNA Sequencer (Applied Biosystems).

Screening for *P. oceanica* SSU-coding clones

Scraping off leaf-epiphytes from *P. oceanica* may introduce leaf material and therefore seagrass DNA may appear in the DNA extracts, and can be susceptible for amplification. For this reason, a set of *P. oceanica* SSU specific primers, Euk_A_Q and Pos_602_R (Table 4), was designed and used to screen for clones containing inserts from this seagrass species. All the positive clones were discarded from the study prior to the sequencing step. The PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min.

Screening for *Bugula* spp. SSU-coding clones

A preliminary analysis of the first clone sequences with BLAST tool (National Center for Biotechnology Information (NCBI), Maryland, USA, http://www.ncbi.nlm.nih.gov/BLAST/) showed the presence of a high number of sequences closely related to the Bryozoa *Bugula turrita*. We designed a set of *Bugula* spp. SSU specific primers, Bugula_F and Bugula_R (Table 4), to make a pre-screening of clone libraries and discard them for sequencing (although they were considered for further analysis). The PCR program included an initial denaturation at 94°C for 3 min,

followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min.

Sequence analyses

Partial SSU sequences were revised and corrected with Sequencher v 4.7 (Gene Codes Corp 2006). Those with similarity values higher than 98% among their sequences were grouped (this cut-off has been considered as a suitable value in other eukaryotic SSU libraries) (Romari and Vaulot 2004; Luo et al. 2005). One representative of each of these groups was chosen for the almost complete sequencing (about 1,800 bp length) using the primers M13R, Euk_570 and Euk_1200 (Table 4). All these sequences have been deposited in GenBank database (NCBI, http://www.ncbi.nlm.nih.gov/) under the accession numbers FJ485946-FJ486117 (partial sequences) and FJ153747-FJ53795 (almost complete sequences).

Table 4. Primers used in this study

Primer	Sequence (5'- 3')	Sense	Specifity	Source
Euk_A_Q	WACCTGGTTGATCCTGCCAGT	Forward	Universal for eukaryotic SSU gene	Present study
Euk_B_Q	TGATCCTTCYGCAGGTTCACCTAC	Reverse	Universal for eukaryotic SSU gene	Present study
Pos_602_R	ACACCATAGGCAGACC	Reverse	*Posidonia oceanica* SSU gene	Present study
Bugula_F	GAGACTGCGGACGGCTCA	Forward	*Bugula* spp. SSU gene	Present study
Bugula_R	CCCGCGTCTGACGCTTCA	Reverse	*Bugula* spp. SSU gene	Present study
M13F	GACGTTGTAAAACGACGGCCAG	Forward	pGEM-T Easy vector	Promega
M13R	CAGAGGAAACAGCTATGACCATG	Reverse	pGEM-T Easy vector	Promega
Euk_570	TGCCAGCAGCCGCGGKWA	Forward	Universal for eukaryotic SSU gene	Present study
Euk_1200	CTTAATTTGACTCAACAC	Forward	Universal for eukaryotic SSU gene	Present study

The phylogenetic inferences were carried out using the ARB software package (Ludwig et al. 2004) and the corresponding SILVA SSUref 96 database (Pruesse et al. 2007). All sequences were automatically aligned using the SINA aligner implemented

in the ARB program package (http://www.arb-silva.de/aligner/). The quality of the alignments was after manually improved taking into account helix pairing bases. These manual improvements were necessary due to the sparse sequence diversity of macroeukaryotic organisms in the database. Almost complete sequences generated in this study were inserted in a pre-existing tree using the ARB parsimony tool as previously reported (Chapter 2). Upon the recognition of the clone affiliations, a selection of 421 representative sequences, and our 49 almost complete sequences in this study were submitted to different treeing approaches encompassing NJ (using the Jukes-Cantor correction), ML (using RAxML version 7.0 with the GTRGAMMA model (Stamatakis 2006), and ARB_PARSIMONY as implemented in the ARB program package. Each of the algorithms was tested using the dataset treated with 30, 40 and 50% conservational filters, allowing 1,738, 1,671 and 1,544 positions of the entire alignment, respectively. A bootstrap analysis of 100 replicates was carried out for additional support of the final topology.

The 123 partial sequences reported in this study were then inserted into the pre-existing trees using ARB_PARSIMONY with local topology optimisation (data not shown). All these trees showed equivalent topologies and finally the ML tree with 40% conservational filter was selected. This tree was split into three subtrees corresponding to Metazoa (Fig. 17a), Macroalgae (Fig. 17b) and 'Other taxa' (Fig. 17c).

Optical microscope technique

Epiphytic species present in shoots from each sampling date were recorded following the procedure of Chapter 2. In brief, three to five random scraping of the 10 cm apical portion of the oldest leaf in each shoot were performed using a razor blade and mounted on glass microscope slides to identify the epiphytic taxa. In addition, 25 field views of 1

mm^2 were observed in each shoot under 400x magnification optical microscope (Zeiss AX10) to determine the relative abundance of each epiphytic taxon. The eyepiece of the microscope had grid points providing one hundred intersections per field view.

Data analysis and comparisons between microscopy and SSU clone libraries

Partial sequences from the three different SSU clone libraries were grouped in operational phylogenetic units (OPUs) (Rosselló-Móra and López-López 2008) after the evaluation of the affiliations in the final ML tree. OPUs are considered as those groups of clones forming independent clades in a phylogenetic tree that may reflect better a biological significant unit rather than just a specific gene sequence similarity cut-off. Rarefaction curves, representing number of OPUs as a function of the number of analysed clones in each SSU library, were constructed using Analytic Rarefaction software (Stratigraphy Lab, Department of Geology, University of Georgia, USA, http://www.uga.edu/strata/sofware/).

In order to simplify the comparisons between microscopy and SSU libraries data, OPUs (from SSU libraries analysis) and taxa (from microscopy data) were classified in groups. These groups were Metazoa Bryozoa, other Metazoa, encrusting Rhodophyta, filamentous Rhodophyta, Phaeophyta and 'other taxa'. The relative abundance of each group (%) was calculated from microscopy counts and SSU library data. Shannon Diversity Index (*H*') that considers both species richness and evenness was calculated using the relative abundances of each of these groups in the SSU libraries and the microscopy counts (Magurran 1989). Bray–Curtis similarity index (I_{BC}) was used to compare the epiphytic community between any two sampling dates in the SSU libraries and the microscopy counts (Brower and Zar 1984).

Results

Current status of eukaryotic SSU gene databases

The reliability of the results on the identification of new reported sequences from SSU clone libraries is linked to the amount and quality (length, number of ambiguities[23]) of sequences deposited in databases. In addition, representativeness, as referred to the number and quality of sequences of each group in databases, is another important parameter for this purpose. For this reason, we have done an evaluation of the public repositories. Up to September 2008, 1,443,326 total SSU sequences were submitted to EMBL (http://www. arb-silva.de/documentation/release-96/). Among them, only 41,331 sequences could be considered as eukaryotic SSU gene sequences with a minimum quality according to SILVA-SSU Ref threshold (at least 1,200 bp length, less than 2% ambiguities, less than 2% homopolymers[24] and less than 5% identity with known vector sequence in their sequences). The number of complete eukaryotic sequences (equal or over 1,800 bp length) with no ambiguities decreases until 9,700. In this work we deposited 49 fully sequenced clones corresponding to almost complete 18S rRNA genes with an adequate quality (on average 1,770 bp length and less than 0.5% of ambiguities in their sequences). The tree was calculated by selecting 470 representative sequences of the database in regard to the affiliation of our clones. The tree reconstruction with the almost complete sequences was biased due to the fact that the different taxonomic groups were differently represented by unequal amount of sequences (Table 5). In addition, just 111 were equal or over 1,800 bp length and without ambiguities. However, and despite of the sequence quality and amount biases, this is the best tree reconstruction based on the current public dataset.

Table 5. Current status of eukaryotic SSU gene databases, new reported sequences in this study and approximated numbers of described species of the relevant groups for this study. Data according to Hofrichter 2005, algaebase database (Guiry and Guiry 2009) and EMBL taxonomy (release 96)

Group	Described species	SSU sequences in SILVA96 [a]	Complete SSU sequences in SILVA96 [b]	New reported SSU sequences [c]
Metazoa Bryozoa	4,500	25	8	10
Other Metazoa [d]	>165,000	2,235	395	17
Phaeophyta	1,800	157	38	1
Encrusting Rhodophyta [e]	>600	129	10	9
Filamentous Rhodophyta [f]	>3000	374	8	6
Other taxa	>30,000	2,929	378	6
Total	>200,000	>5,500	>800	49

[a] SSU gene sequences with a minimum quality according to SILVA-SSU Ref threshold, [b] Considered as equal or over 1,800 bp-length, [c] Referred to almost complete SSU sequences (on average, 1,770 bp-length and less than 0.5% ambiguities in their sequences), [d] Phylum Platyhelminthes (Order Polycladida and Order Rhabdocoela), Phylum Nematoda, Phylum Mollusca (Class Gastropoda), Phylum Arthropoda (Subclass Copepoda and Subclass Podocopa), Phylum Echinodermata (Class Ophiuroidea), [e] Order Corallinales, [f] Order Acrochaetiales, Order Ceramiales and Order Rhodymeniales

Screening for *P. oceanica* SSU-coding clones

We screened for *P. oceanica* clones with a set of *P. oceanica* SSU specific primers, Euk_A_Q and Pos_602_R. The proportion of clones belonging to *P. oceanica* was low (< 10%) compared to those affiliated to epiphytic taxa. As mentioned, these clones were not taken into account for our analyses (see "Materials and methods").

OPUs analysis in SSU libraries

Three epiphytic SSU clone libraries were constructed, one for each sampling date. A total of 354 clones were screened with the *P. oceanica* (see above), and *Bugula* spp. (see below) specific primers (107 from February, 143 from June and 104 from September). From these clones, 172 were further partially sequenced (70 from February, 40 from June and 62 from September). From them, 37 clones (29 from June and 8 from

September) were not sequenced, but included for further analyses, as they were identified as *Bugula* spp. with the specific PCR amplification. A total of 30 different OPUs for the complete set of partial sequences of this study were detected. We obtained the almost complete SSU sequence of at least one representative clone of each OPU and their phylogeny were reconstructed using the RAxML algorithm. As it can be observed in Fig. 17, the vast majority of OPUs are affiliated with Metazoa (especially Bryozoa that represented one OPU with more than 50% of the partially sequenced clones). The second major group was Macroalgae (about 20% of the partially sequenced clones distributed in eleven different OPUs), whereas other groups such as Fungi, Protozoa, Oomycetes and Labyrinthulida were much less represented (altogether five OPUs and about 2% of the partially sequenced clones) (Table 5; Fig. 17).

Regarding the seasonal occurrence of clones, February SSU clone library contained the lowest number of OPUs (7), June SSU library contained an intermediate number of OPUs (14), whereas September exhibited the highest number (18). From the 30 different OPUs, only two were detected in all SSU clone libraries, and affiliated with the Bryozoa *B. turrita* and the encrusting red algae *Spongites yendoi*. The highest number of common OPUs (6) between two sampling dates occurred between June and September libraries, and affiliated with the Bryozoa *B. turrita*, the encrusting red algae *P. conicum* and *S. yendoi*, the filamentous red algae *Laurencia filiformis* and the Annelida Polychaeta *Grubeosyllis limbata* (Table 6; Fig. 17).

In: Medina-Pons FJ, Terrados J, López-López A, Yarza P, Rosselló-Móra R (2009). Mar Biol 156(9): 1963-1976.

Table 6. Number of partial SSU sequences generated in this study, closest relative in the SILVA 96 database, its phylogenetic affiliation in database and group assigned to each OPU

OPU code	Closest relative (accession number)	Closest relative similarity (minimum-maximum) (%)	Similarity between sequences in OPU (minimum–maximum (%)	Number of partial SSU sequences	Phylogenetic affiliation	Group
POE001 (*)	*Bugula turrita* (AY210443)	76.25–88.87	77.82–100	132	Metazoa Bryozoa Gymnolaemata	Metazoa Bryozoa
POE002 (S)	*Proxenetes flabellifer* (AY775764)	93.86–93.86	–	1	Platyhelminthes Turbellaria Rhabdocoela	Other Metazoa
POE003 (S)	*Trisaccopharynx westbladi* (AY775774)	83.50–83.50	–	1	Platyhelminthes Turbellaria Rhabdocoela	Other Metazoa
POE004 (S)	*Prostheceraeus vittatus* (AJ312272)	96.48–97.28	98.17–99.87	5	Platyhelminthes Turbellaria Polycladida	Other Metazoa
POE005 (S)	*Balcis eburnea* (AF120519)	97.96–98.97	99.11–99.87	5	Mollusca Gastropoda	Other Metazoa
POE006 (J, S)	*Grubeosyllis limbata* (AF474289)	98.29–99.54	98.03–99.74	7	Annelida Polychaeta	Other Metazoa
POE007 (J)	*Viscosia viscosa* (AY854198)	96.50–97.64	99.00–99.00	2	Nematoda Enoplea	Other Metazoa
POE008 (J)	Uncultured nematode (AY763131)	83.69–83.69	–	1	Nematoda (environmental sample)	Other Metazoa
POE009 (S)	*Chromadora nudicapitata* (AY854205)	96.71–97.99	97.17–97.17	2	Nematoda Chromadorea	Other Metazoa
POE010 (S)	*Praeacanthonchus* sp. (AF036612)	98.94.98.84	–	1	Nematoda Chromadorea	Other Metazoa
POE011 (F)	*Xestoleberis hanaii* (AB076633)	98.54–98.54	–	1	Crustacea Ostracoda Podocopa	Other Metazoa
POE012 (S)	*Xiphichilus* sp. (AB076624)	96.08–96.08	–	1	Crustacea Ostracoda Podocopa	Other Metazoa
POE013 (S)	*Bryocamptus pygmaeus* (AY627015)	96.91–96.91	–	1	Crustacea Maxillopoda Podocopa	Other Metazoa
POE014 (F)	*Amphipholis squamata* (X97156)	99.15–99.50	99.01–99.72	5	Echinodermata Eleutherozoa Ophiuroidea	Other Metazoa
POE015 (J)	*Malassezia globosa* (AAYY01000016)	99.88–99.88	–	1	Fungi Basidiomycota	Other taxa
POE016 (J)	*Scytosiphon lomentaria* (D16558)	97.41–98.18	97.76–99.55	4	Phaeophyta Ectocarpales	Phaeophyta
POE017 (S)	*Haliphthoros milfordensis* (AB284573)	99.04–99.04	–	1	Oomycetes Lagenidiales	Other taxa
POE018 (F)	Uncultured eukaryote (EF100415)	96.10–96.10	–	1	Eukaryote Environmental sample	Other taxa
POE019 (S)	Uncultured eukaryote (AB275005)	84.62–84.62	–	1	Eukaryote Environmental sample	Other taxa
POE020 (F,J)	*Aspidisca aculeata* (EF123704)	81.50–82.22	96.35–96.35	2	Alveolata Ciliophora Spirotrichea Euplotida	Other taxa
POE021 (S)	*Gloiosaccion brownii* (AF085259)	99.23–99.43	99.61–99.61	2	Rhodophyta Florideophyceae Rhodymeniales	Filamentous Rhodophyta
POE022 (J)	*Pterothamnion villosum* (DQ343658)	98.84–98.84	-	1	Rhodophyta Florideophyceae Ceramiales	Filamentous Rhodophyta
POE023 (S)	*Womersleyella setacea* (AF427537)	93.32–96.62	95.89–95.89	2	Rhodophyta Florideophyceae Ceramiales	Filamentous Rhodophyta
POE024 (J, S)	*Laurencia filiformis* (AF203894)	94.48–97.82	94.10–99.52	4	Rhodophyta Florideophyceae Ceramiales	Filamentous Rhodophyta
POE025 (F)	*Audouinella arcuata* (AF079786) and *Audouinella tenue* (AF079796)	99.34–99.71	99.21–99.61	2	Rhodophyta Florideophyceae Acrochaetiales	Filamentous Rhodophyta
POE026 (J, S)	*Pneophyllum conicum* (DQ628983, DQ628985)	97.59–98.63	97.59–100	10	Rhodophyta Florideophyceae Corallinales	Encrusting Rhodophyta
POE027 (J, S)	*Pneophyllum conicum* (DQ628983, DQ628985, DQ628986, DQ628987, DQ628989, DQ628994)	98.23–99.37	99.39–100	5	Rhodophyta Florideophyceae Corallinales	Encrusting Rhodophyta
POE028 (J)	*Hydrolithon gardineri* (DQ628992)	97.78–97.78	–	1	Rhodophyta Florideophyceae Corallinales	Encrusting Rhodophyta
POE029 (J)	*Metagoniolithon chara* (U60743)	98.37–98.37	–	1	Rhodophyta Florideophyceae Corallinales	Encrusting Rhodophyta
POE030 (*)	*Spongites yendoi* (U60948)	97.04–98.59	98.90–99.70	6	Rhodophyta Florideophyceae Corallinales	Encrusting Rhodophyta

OPUs were classified as permanent (asterisk) (if present in all SSU libraries) or seasonal (*F* February, *J* June, *S* September) (if not present in all SSU libraries) in OPU code column

In: Medina-Pons FJ, Terrados J, López-López A, Yarza P, Rosselló-Móra R (2009). Mar Biol 156(9): 1963-1976.

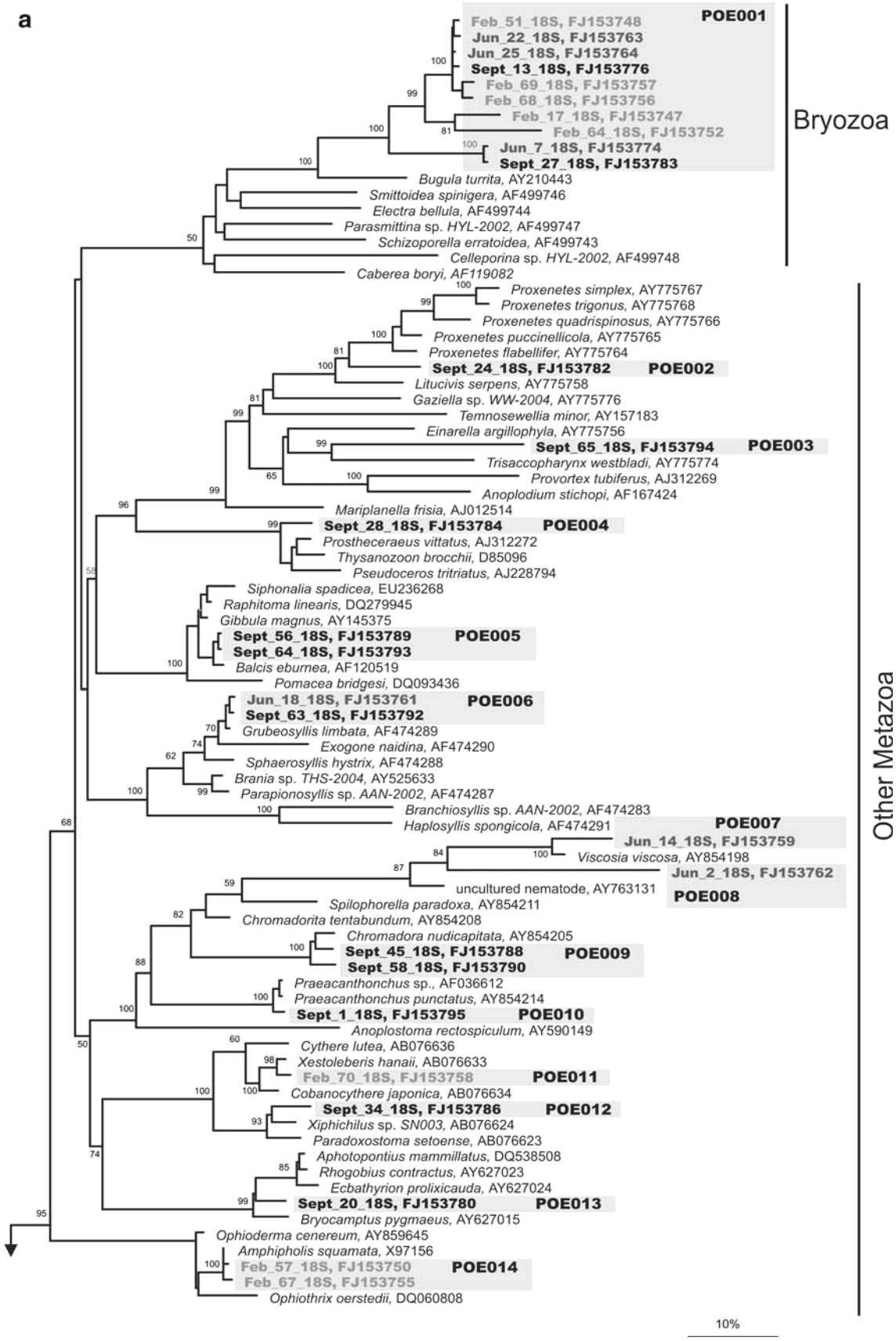


Fig. 17.

In: Medina-Pons FJ, Terrados J, López-López A, Yarza P, Rosselló-Móra R (2009). Mar Biol 156(9): 1963-1976.

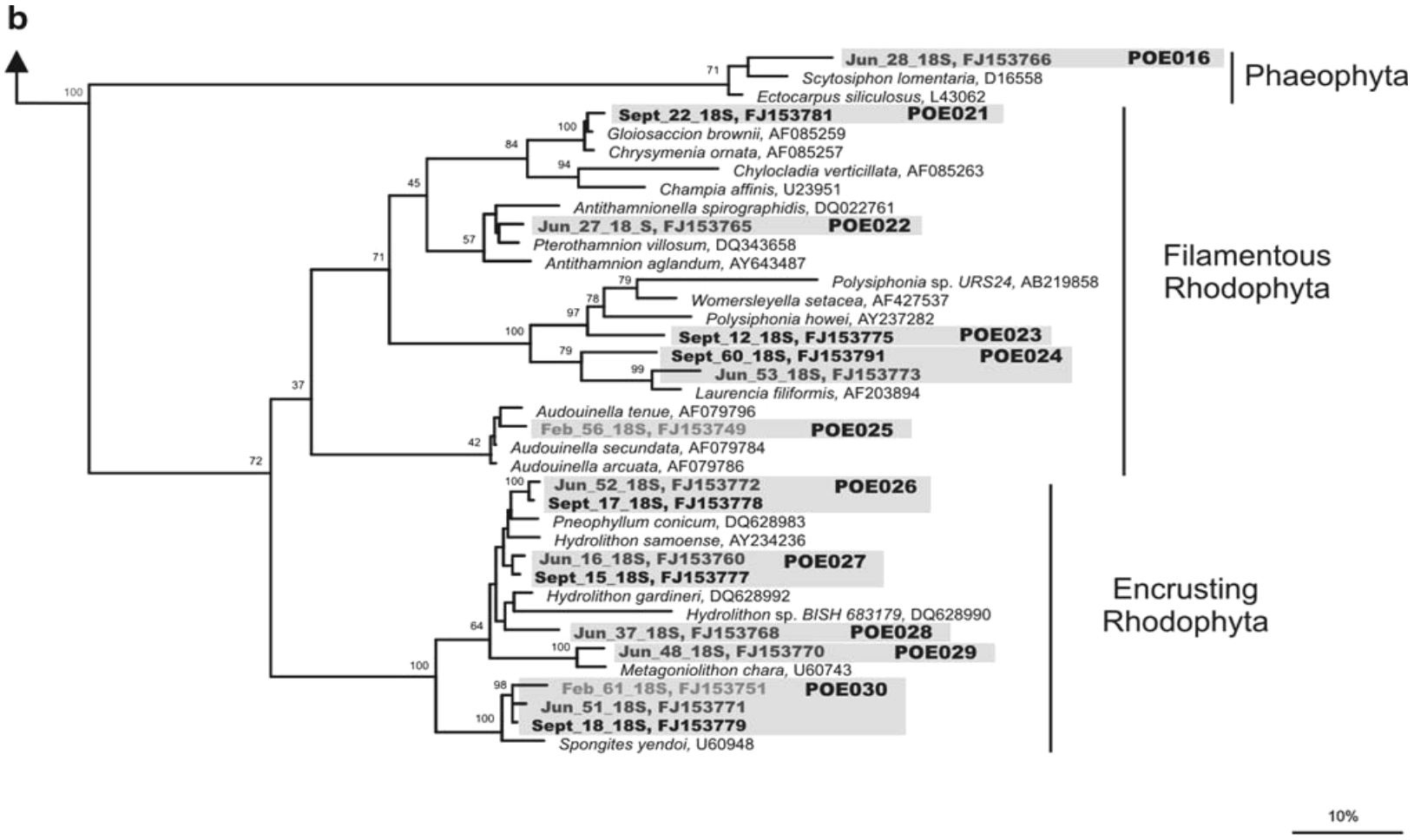


Fig. 17. cont.)

In: Medina-Pons FJ, Terrados J, López-López A, Yarza P, Rosselló-Móra R (2009). Mar Biol 156(9): 1963-1976.

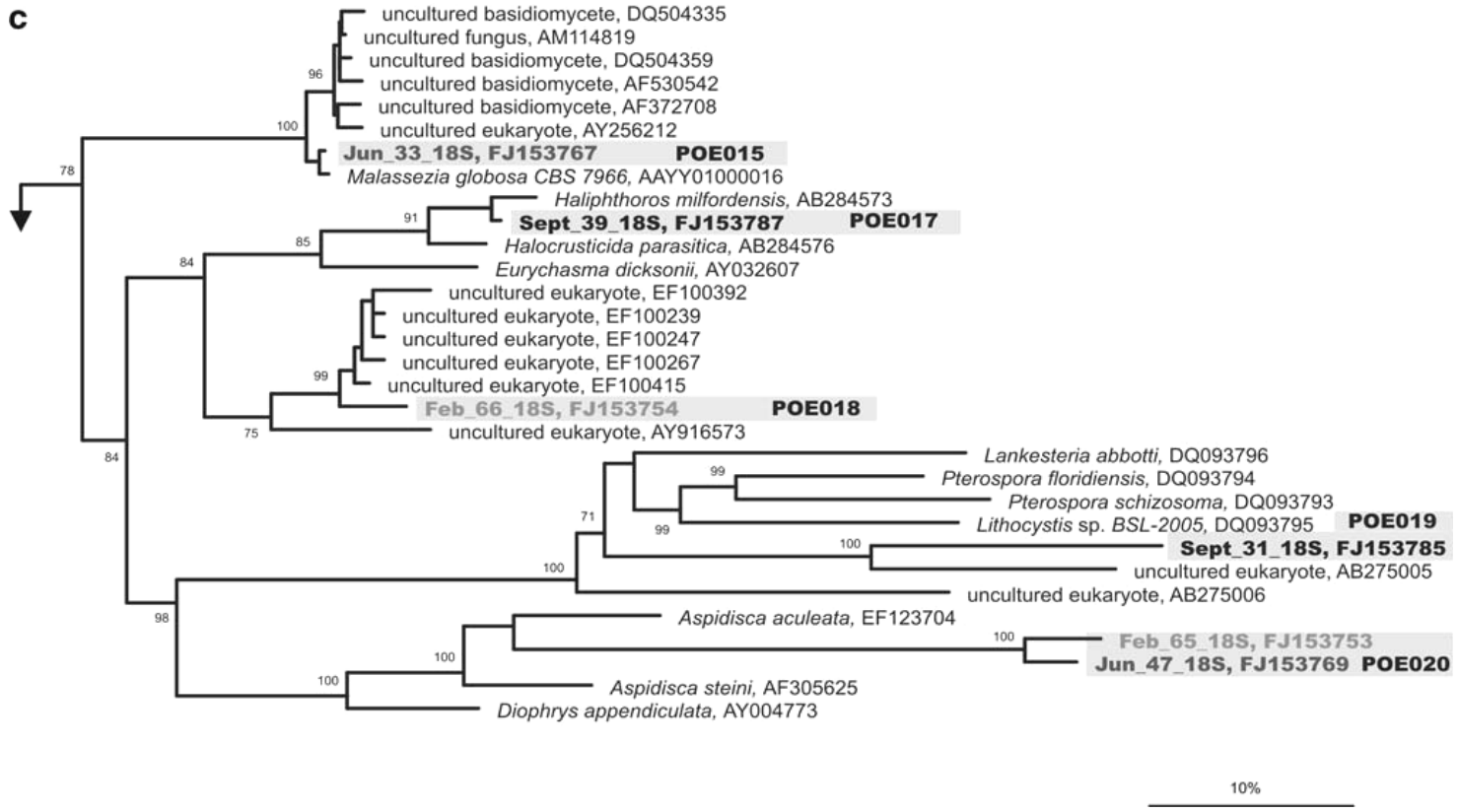


Fig. 17. ML tree split in Metazoa (**a**), Macroalgae (**b**), and 'Other taxa'(**c**) subtrees. New reported almost complete SSU sequences for the three sampling dates (*Feb* February, *Jun* June, *Sept* September) are distributed in operational phylogenetic units (OPUs, shown inside *shadowed areas*) and groups. *Arrows* indicate the rooting point for each subtree. *Numbers* at nodes represent the bootstrap percentages from 100 replicates. The *scale bar* corresponds to a distance of ten substitutions per 100 nucleotide positions.

Rarefaction curves, *H'* index and I_{BC} index

Rarefaction curves, representing number of OPUs as a function of the number of analysed clones, showed that earlier stages of the succession (February) tended to be saturated before than intermediate and later stages of the succession (June and September, respectively) (Fig. 18). *H*' values from the three sampling dates showed the same trend for microscopy and the SSU libraries approach indicating that the highest diversity occurred in September. In addition, this index showed that OPUs and epiphytic taxa were more evenly distributed among different groups in September than in February and June. I_{BC} values showed that the highest similarity between sampling dates was obtained when comparing February and June data for both approaches (Table 7).

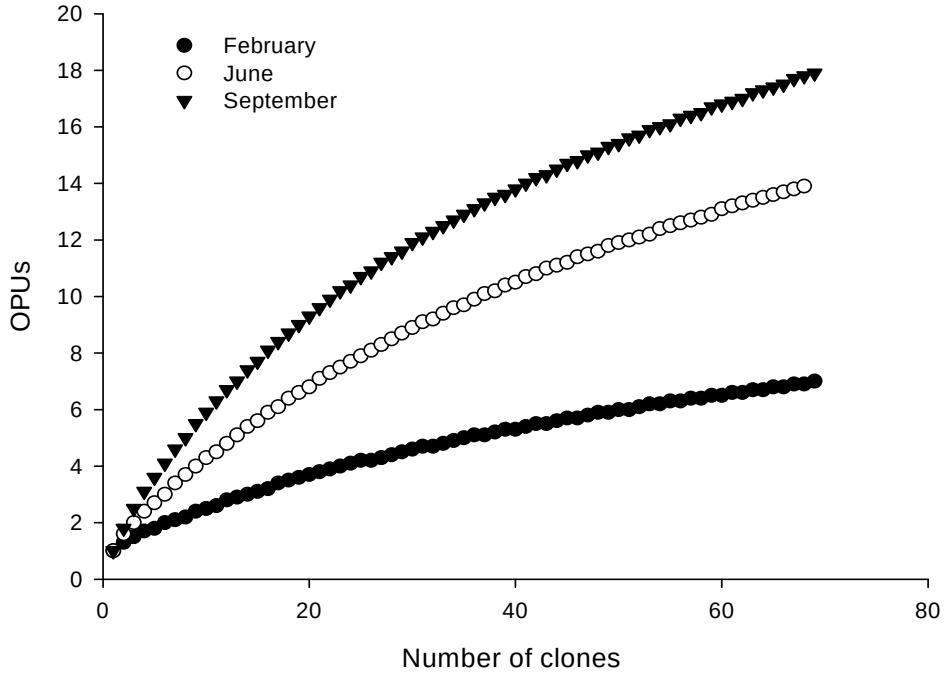


Fig. 18. Rarefaction curves for February, June and September SSU libraries

Table 7. Shannon Diversity index (*H'*) and Bray-Curtis similarity index (I_{BC}) (expressed as a percentage of similarity between any two sampling dates (%)) from SSU libraries data and microscopy counts in the three sampling dates

Sampling date	**SSU libraries**	**Microscopy counts**
	H'	
February	0.67	0.41
June	0.88	0.42
September	1.33	0.76
Comparison between sampling dates	I_{BC} (%)	
February-June	81	76
February-September	59	49
June-September	69	49

Relative abundance of each group in SSU clone libraries and microscopy approach

The relative abundance of each group in SSU libraries showed that Metazoa Bryozoa was the most abundant group in all sampling dates (Fig. 19). At earlier stages of the succession (February), the library was dominated by Metazoa (mainly Bryozoa; about 90% of total sequences) followed by a small fraction of encrusting and filamentous Rhodophyta (about 5%). Phaeophyta were only detected in June (about 5%), when the assemblage was mainly composed of Metazoa (specially Bryozoa; about 70%) and a higher fraction of encrusting red algae (about 15%) compared to February (less than 5%). The September SSU library was dominated by Metazoa (Bryozoa (about 40%), and other groups such as Mollusca, Crustacea and Annelida (about 30%)) and Rhodophyta (about 25%). An increase of the presence of filamentous Rhodophyta was detected in September (about 10%) when compared to February and June libraries (about 3% in both cases), whereas the percentage of encrusting Rhodophyta was similar to that in June (Fig. 19).

Microscopy data indicated that macroalgae were much more represented (according to relative abundance) than Metazoa (Bryozoa and other Metazoa) in the

three sampling dates. The February and June epiphytic assemblages were clearly dominated by Phaeophyta (about 75% of microscopy counts in both cases). In February, this group was basically represented by encrusting *M. magnusii*, whereas in June they were represented by *M. magnusii* (about 50%), and filamentous *Cladosiphon* spp., *Giraudia sphacelarioides* and *Sphacelaria* spp. (altogether about 25%). The relative abundance of encrusting Rhodophyta was higher in later stages of the succession (June and September (about 20 and 40%, respectively) than in February (about 1%)). Filamentous Rhodophyta were also more represented in September (about 30%) than in February (about 25%) and June (less than 1%) Although filamentous red algae represented a slightly higher percentage in September than in February, the former one was composed by a high diversity of taxa (such as *Polysiphonia* spp., *Lophosiphonia* spp., *Herposiphonia secunda*, *Ceramium codii* and *Chondria* spp.), whereas the later one was basically represented by *Spermothamnion* spp. (Fig. 19).

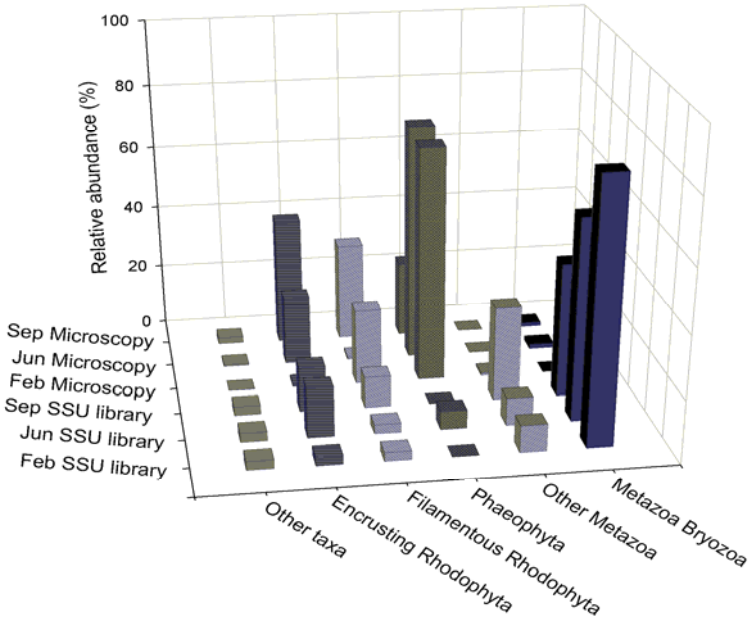


Fig. 19. Relative abundance of each group (%) in three sampling dates from SSU libraries data and microscopy counts (*Feb* February, *Jun* June, *Sep* September).

Discussion

Our main purpose was to evaluate the usefulness of SSU libraries to describe changes in taxa richness and composition of the macroeukaryotic leaf-epiphytic assemblage of *P. oceanica* in three different stages of the community. The SSU libraries were able to detect changes in the structure of epiphytic communities along the succession because we obtained a large number of seasonal OPUs (not present in all stages of the succession studied). In addition, the vast majority of the partial SSU sequences from this study were affiliated with macroeukaryotic organisms (more than 95% of the partially sequenced clones). These are important facts that highlight the usefulness of the SSU clone libraries approach to study diversity changes of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves. Indeed, these results were consistent with those provided by DNA barcoding techniques, where all obtained SSU gene sequences were assignable to macroepiphytes (Chapter 2).

The February SSU library represents an early stage of the succession and it was characterised by the lowest diversity (according to number of OPUs and *H*' value). For this reason, the rarefaction curve of the February SSU clone library tended to saturation at a faster rate than those of June and September libraries. These results were consistent with the information provided by our microscopy study. Similar results were also observed in previous works when late and early successional stages of the epiphytic community were compared using microscopy techniques (Van der Ben 1971; Ballesteros 1987) and a DNA barcoding technique (Chapter 2). I_{BC} values from SSU libraries data were generally consistent with those from microscopy data. However, similarity values obtained when comparing June and September data were significantly lower in the microscopy approach than in the SSU libraries. This may be explained by

the fact that Phaeophyta were a very significant group in June but not in September in the microscopy data.

Not all groups were equally represented with both approaches. Macroalgae were the most abundant epiphytes in all sampling dates when analysed by microscopy, whereas Metazoa (specially Bryozoa) were much more abundant in the SSU libraries. The dominance of zooepiphytes using molecular techniques has been previously recognised by our group (Medina-Pons et al. 2008). Previous microscopy studies show that Bryozoa are dominant in winter (Romero 1988) and reach their maximum development in spring (Ballesteros 1987). Our microscopy-based data revealed that Bryozoa were only detected in spring (June) probably because of their highest development (Ballesteros 1987). However, the SSU libraries showed that this group was present in all sampling dates being more abundant in winter (February) as it has been reported previously (Romero 1988). This result suggests that size or developmental stage of Bryozoa (and probably other zooepiphytes) are not affecting their detection by molecular approaches, which could be seen as an advantage of SSU libraries over microscopy. Both techniques revealed that encrusting Rhodophyta were present in all sampling dates, as many other studies have found (Van der Ben 1971; Romero 1988; Buia et al. 1989; Mazzella et al. 1989). The application of DNA barcoding techniques was also able to detect the presence of this group in winter and summer (Chapter 2). Filamentous Rhodophyta (mostly Order Ceramiales) were also detected in all sampling dates applying both techniques, being more represented in later stages of the succession in agreement with previous reports (Van der Ben 1971; Antolic 1986; Ballesteros 1987). On the other hand, Phaeophyta were just represented in the spring (June) SSU library, but detected in all sampling dates by microscopy. Actually, Phaeophyta were mostly

represented by encrusting *M. magnusii* in all sampling dates, whereas filamentous brown algae were especially relevant in spring, which agrees with previous reports (Ballesteros 1987; Romero 1988). The difference in *M. magnusii* presence between SSU libraries and microscopy data suggests that this species is underrepresented in SSU libraries. This bias could be explained either by the difficulties in extracting its DNA or by the unsuitability of the primers used for this taxon. The SSU libraries were also able to detect the colonisation of *P. oceanica* leaves by other much less represented taxa such as Protozoa, Fungi, Labyrinthulida and Oomycetes, not recorded by microscopy in our study. A wide taxa detection capacity is an advantage of the SSU clone libraries approach to describe the diversity of the epiphytic community.

Our results show that both approaches give complementary information. SSU libraries provided a wider spectrum of epiphytic taxa and a more accurate detection of Metazoa colonising *P. oceanica* leaves, whereas our microscopy expertise gave us a better description of macroalgal diversity. These differences might be explained by the fact that DNA extraction from marine macroalgae has proven to be difficult due to the presence of the cell wall and the co-isolation of polysaccharides and secondary metabolites that can inhibit PCR (Hong et al. 1997; Vidal et al. 2002). For this reason, DNA extraction and amplification from zooepiphytes could be favoured against macroalgae when SSU libraries are constructed. On the other hand, the microscopic identification of epiphytic taxa may be biased towards macroalgae, which were more easily recognised under the microscope.

Reliable affiliation of novel sequences with known taxa is linked to both quality and representativeness of taxa in the databases. The presence of highly underrepresented

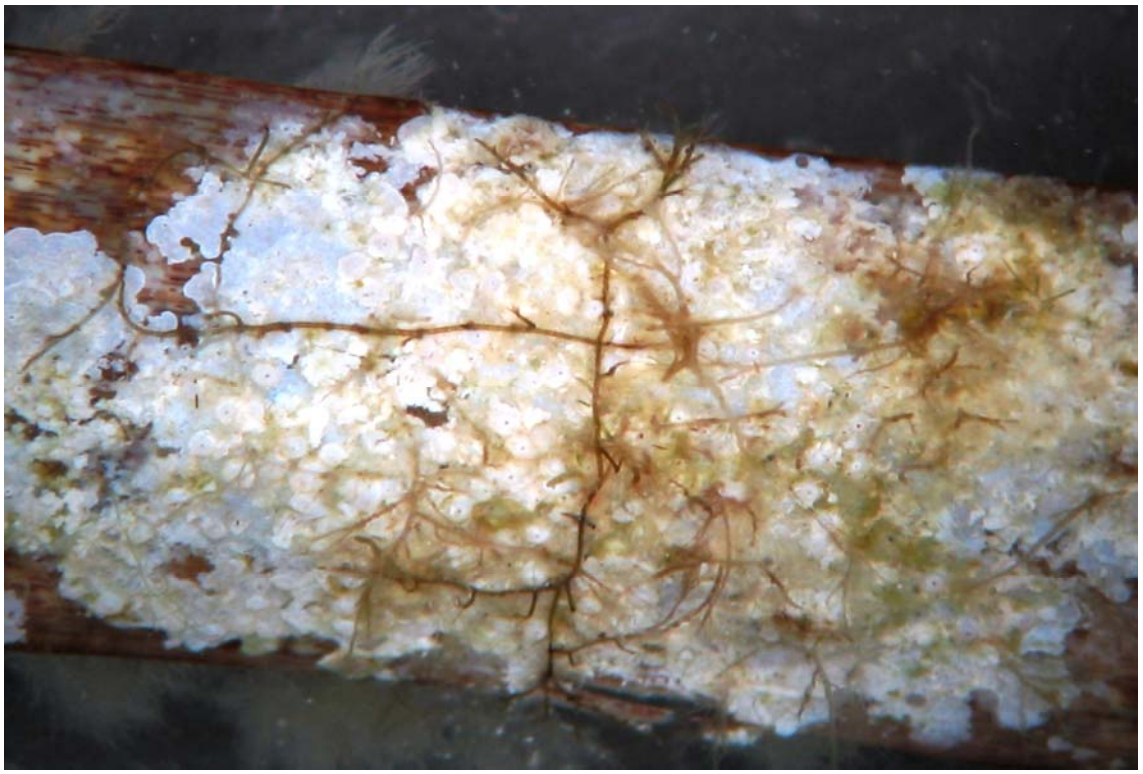
groups (as it is shown in Table 5) could be the explanation for the low sequence-similarity values found in some OPUs against their closest relative in the database. This fact, together with the generally low number of complete eukaryotic SSU sequences in databases reveals the difficulty of selecting reference subsets for reliable phylogenetic reconstructions. This fact highlights an additional value of our study due to our contribution with a significant number of almost complete SSU sequences of some underrepresented groups (specially Bryozoa and encrusting and filamentous Rhodophyta), that in some cases increases in about 100% the high-quality sequences in public repositories.

In summary, the clone library approach used here has demonstrated to be complementary to that based on microscopical observations. An added value of the approach for our goal is the fact that the microeukaryotic communities may be underrepresented due to their relative low contribution on the community biomass, or to DNA extraction difficulties. This macroeukaryotic-enriched DNA may better reflect the microscopy counts that generally underestimate single cell eukaryotes towards multicellular organisms. Clone libraries should be considered as complementary to microscopy descriptions because both combined produce a wider view of the community structure and dynamics. The former approach is less time-consuming, independent of the know-how of the taxonomy, and targets both zooepiphytic and phytoepiphytic biota. The later has the advantage of identifying organisms that due to their structure are difficult to lysate and thus may remain underrepresented in the DNA pools.

In addition, in the previous study (Chapter 2), we demonstrated similar complementarities among TGGE and microscopical counts as that reported here. The

choice of the molecular method for further studies may depend on the needs of the researcher and the amount of samples in study. In one way, TGGE allows the manipulation and comparison of large sets of samples, but the affiliation capabilities of the DNA fragments is restricted due to the short size of the amplicon (low information content), and the difficulties in obtaining clean sequences from reamplified excised bands. The TGGE method would be useful for comparing the structure of communities at different spatial or temporal scales. On the other side, clone libraries are not suitable for comparing large amounts of samples, but better reflect their diversity due to the screening capabilities and the larger information content of the cloned fragments (almost complete 18S rRNA gene sequences). The 18S rRNA gene clone libraries would be appropriate in studies that require a thorough description of the taxa composition and genealogy of the community.

SECTION IV - Chapter 4



Detail of epiphytes on *P. oceanica* leaf

Comparing the summer structure of the macroeukaryotic leaf-epiphytic community in two *P. oceanica* (L.) Delile meadows by means of TGGE molecular approach and classical microscopy techniques

Comparing the summer structure of the macroeukaryotic leaf-epiphytic community in two *P. oceanica* (L.) Delile meadows by means of TGGE molecular approach and classical microscopy techniques

Background

TGGE molecular technique demonstrated to be sensitive to detect variability in the structure of macroeukaryotic epiphytic fraction of *P. oceanica* when comparing two stages of the seasonal succession (Chapter 2). The main goal of this chapter is testing the usefulness of TGGE to observe variability in the mature stage of the epiphytic assemblage (summer) between two *P. oceanica* meadows. Summer stage of the epiphytic assemblage was selected because it is generally characterised by showing the highest diversity and biomass (together with spring stage) of the seasonal succession. We also compared results obtained applying TGGE with those provided by classical microscopy techniques.

Materials and Methods

Sample collection

The study was conducted in August 2006 and 2007 in two *P. oceanica* meadows separated by 6 km in Majorca, western Mediterranean Sea. The two locations were Victoria (39° 5' N, 3° 11' E, Pollença Bay, 7 m depth) and Torrent de Sant Jordi (39° 52' N, 3° 5' E, Pollença Bay, 5 m depth). Sampling was conducted in August because the mature stage of the epiphytic community is achieved in summer (Antolic 1986; Ballesteros 1987).

Three quadrates of 0.25 m^2 tens of metres apart were haphazardly selected in the two locations and sampling dates. In each quadrate, seven shoots were haphazardly

collected in each sampling event by SCUBA divers and frozen at -80 °C to keep nucleic acids intact until their processing by means of TGGE molecular method. This number of shoots was previously considered adequate to represent the diversity of the epiphytic community in molecular studies within an area of 0.25 m^2 (Chapter 2).

Two 10 m transects separated thirty meters were also laid in each location and sampling date. Five shoots were haphazardly harvested along each transect and immediately preserved in 4% formalin-seawater until their processing to determine eukaryotic leaf-epiphytic community structure (composition, taxa richness and relative abundance of the morphological epiphyte groups (%)) by means of microscopy method following the procedure of Chapter 2. This number of shoots has been recognised as a representative number of the epiphytic community in classical microscopy studies (Panayotidis and Boudouresque 1981; Ballesteros 1987). The distribution of epiphyte taxa in morphological groups has demonstrated to be useful to describe variability in community structure (Pardi et al. 2006; Piazzi et al. 2007).

Finally, ten additional shoots were haphazardly harvested along each transect in each location and sampling date to quantify main descriptors of *P. oceanica* meadows in the two locations.

TGGE molecular technique and optical microscope methods

DNA extraction, PCR, TGGE and optical microscope methods were performed according to Chapter 2 procedure.

Epiphyte extracted DNA from Torrent de Sant Jordi samples in August 2006 and August 2007 was additionally cleaned using Wizard DNA Clean-Up System (Promega) before PCR was performed. This was done because we observed a large presence of PCR inhibitors in those samples. PCR products from the extracted epiphyte DNA of

shoots belonging to the same quadrate were mixed in equal concentrations for the two locations and the two sampling dates. This was done to facilitate the comparisons between OTUs profiles of both locations and sampling dates in TGGE.

Main descriptors of *P. oceanica* meadows

P. oceanica description in the two locations included epiphyte load and epiphyte organic matter load on the shoots, vegetative features at shoot scale (number of leaves, nitrogen (N) and phosphorus (P) content, biomass) and at meadow scale (shoot density), and some variables associated to herbivory pressure on this seagrass and their epiphytes (sea urchin density, fish bite marks $shoot^{-1}$) (Romero et al. 2007; Fernández-Torquemada et al. 2008).

Shoots collected for description of *P. oceanica* were gently cleaned from epiphytes with a razor blade. Epiphytes collected in pre-weighed Whatman GF/C glass fibre filters and cleaned shoots were separately dried at 60ºC for at least 48 h to determine dry weight (DW) and estimate shoot biomass (g DW) and epiphyte load (g epiphyte DW g^{-1} shoot DW). Six of the dried epiphytic samples were burned in an oven furnace at 450ºC for 6 h to estimate epiphyte organic matter load (g epiphyte Ash-free Dry Weight (AFDW) g^{-1} shoot DW) (Kendrick and Lavery 2001). N and P shoot content (% DW) were estimated from at least four of these ten *P. oceanica* shoots for each sampling date and location. N shoot content was estimated using an elemental analyser CHN-O-RAPID (Heraeus). P content was estimated according to Fourqurean et al. 1992 procedure. BCR standard beech leaves (SIGMA) was used to calculate P recovery of the method and to make reliable comparisons among P content of different sets of samples. Seagrass nutrient content has been previously considered as a way to compare the nutrient availability for seagrass growth in coastal waters (Fourqurean et al.

2007). Between five to ten quadrates of 0.04 m^2 were haphazardly selected along two transects in the field and shoots were counted to estimate shoot density (shoots m^{-2}) in the two locations and sampling dates (Delgado et al. 1999).

Herbivory pressure has been revealed as a relevant phenomenon over *P. oceanica* shoots and their epiphytes. Sea urchins and *S. salpa* have demonstrated to be able to decrease up to 50% of shoot biomass and between 60% and 80% of the epiphyte load, respectively (Tomas et al. 2005a; Tomas et al. 2005b). While sea urchin density has been considered relatively constant over time in other *P. oceanica* meadows (Tomas et al. 2004), intense grazing on *P. oceanica* and epiphytes by fish *S. salpa* appears to be a very relatively common phenomenon in *P. oceanica* meadows during summer months (Tomas et al. 2005a). Sea urchin herbivory pressure over *P. oceanica* shoots and their epiphytes was investigated in the two locations and sampling dates counting number of sea urchins in quadrates of 0.25 m^2. Fish herbivory pressure caused by *S. salpa* was indirectly estimated counting the number of bite marks $shoot^{-1}$ in ten shoots collected in both locations (Tomas et al. 2005a; Tomas et al. 2005b).

Statistical analyses

Samples belonging to the same transect (microscopy approach) or to the same quadrat (TGGE) were grouped for statistical analyses constituting representative replication units for each location and sampling date.

MDS based on the Bray-Curtis similarity index was used to represent epiphyte taxa composition (presence/absence), relative abundance (%) of the morphological epiphyte groups and OTUs profile (presence/absence) for the two locations and sampling dates.

A two-way crossed Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson 2001) was used to evaluate significant differences in epiphyte taxa composition (presence/absence), relative abundance of the morphological epiphyte groups and OTUs profile (presence/absence) between the two locations and sampling dates.

Similarity percentages - species contributions (SIMPER) analysis (Clarke 1993) was performed to determine the morphological epiphyte groups (in terms of relative abundance) that contributed most to the significant differences between locations and sampling dates. We used the average contribution of each taxon/OTU to the overall dissimilarity between locations and/or sampling dates, δ_i, and the standard deviation of this contribution SD (δ_i). We used the ratio of mean to SD as a statistic δ_i / SD (δ_i) to evaluate the contribution of each epiphyte group/OTU to the dissimilarity between pairs of replication units. We used as an arbitrarily defined cut-off point a mean to SD ratio of 2, so that the mean contribution was higher than the variation.

Significant differences between the two locations and sampling dates in OTUs richness and epiphyte taxa richness were analysed using a two-way crossed ANOVA. An analogous statistical analyses was done to evaluate significant differences in the main descriptors of *P. oceanica* meadows (epiphyte load, epiphyte organic matter load, number of leaves shoot^{-1}, N and P shoot content, shoot biomass, shoot density, sea urchin density and fish bite marks shoot^{-1}) between the two locations and sampling dates.

Data used in ANOVA tests were previously tested for homogeneity of variances and normality test using Levene's test and Kolmogorov-Smirnov test, respectively. All analyses were performed using PRIMER 5 (Clarke and Gorley 2001), SIGMAPLOT 8.0

(SPSS Science 2002), STATISTICA 7.1 (StatSoft Inc 2005) and PERMANOVA software (Anderson 2001). Significance level in all statistical tests was 0.05.

Results

TGGE molecular technique and optical microscope methods

MDS showed differences in epiphyte taxa composition (presence/absence), relative abundance (%) of the morphological epiphyte groups and OTUs profiles between locations. It also showed differences between sampling dates in OTUs profiles for the two locations and between sampling dates in relative abundance of the morphological epiphyte groups for Torrent de St. Jordi location. PERMANOVA confirmed significant differences between locations in epiphyte taxa composition, relative abundance of the morphological epiphyte groups and OTUs profile. It also confirmed significant differences between sampling dates in OTUs profile and between sampling dates and the interaction of the two factors (location and sampling date) in the relative abundance of the morphological epiphyte groups (Table 8; Fig. 20).

A list of epiphyte taxa (presence/absence) for the two locations and sampling dates is provided (Table 9). Results (Mean $\pm$ SE) indicated that the most abundant (%) morphological epiphyte groups in Victoria for the two sampling dates were encrusting Rhodophyta (60.99 $\pm$ 4.56% and 38.92 $\pm$ 1.99% in August 2006 and 2007, respectively) and encrusting Phaeophyta (26.60 $\pm$ 1.84% and 36.93 $\pm$ 7.16% in August 2006 and 2007, respectively). The most abundant morphological epiphyte groups in Torrent de St. Jordi for August 2006 were Hydrozoa and encrusting Rhodophyta (43.25 $\pm$ 13.02% and 12.01 $\pm$ 0.84%, respectively) and for August 2007 were filamentous Phaeophyta and encrusting Rhodophyta (66.32 $\pm$ 2.65% and 19.33 $\pm$ 1.59%, respectively). Chlorophyta and Bryozoa generally exhibited lower relative abundances than Phaeophyta and

Rhodophyta groups for all the sampling events. The highest values for Chlorophyta and Bryozoa were detected in Torrent de St. Jordi for August 2006 (10.05 ± 6.92%) and in Victoria August 2006 (12.62 ± 5.12%), respectively. Foraminifera and Nematoda relative abundances generally exhibited the lowest relative abundances (less than 1%) in all sampling events (Fig. 21).

SIMPER analyses showed that dissimilarities between Victoria and Torrent de St. Jordi in August 2006 and in August 2007 were mainly explained by differences in relative abundances of encrusting Rhodophyta and filamentous Phaeophyta, respectively. It also showed that dissimilarity in Torrent de St. Jordi between August 2006 and 2007 was mainly explained by differences in relative abundances of filamentous Phaeophyta and Hydrozoa. SIMPER analysis between Victoria August 2006 and August 2007 was not performed because MDS clearly showed no differences in relative abundances of the morphological epiphyte groups (Fig. 20; Table 10).

Table 8. Results of two-way crossed PERMANOVA to evaluate significant differences in epiphyte taxa composition (presence/absence), relative abundance (%) of the morphological epiphyte groups and OTUs profile (presence/absence) between locations (Victoria vs. Torrent de St. Jordi) and sampling dates (August 2006 vs. August 2007). PERMANOVA was conducted on Euclidean distance matrix. Significant values in **bold**. ***p < 0.05; ** p < 0.01; *** p < 0.001.**

	Source	df	MS	Pseudo-F	P
Epiphyte taxa composition (presence/absence)	Location	1	10.87	1.85	**0.0217***
	Sampling date	1	6.37	1.08	0.3931
	Location x Sampling date	1	4.87	0.83	0.7219
	Residual	8	5.87		
	Total	11			
Relative abundance (%) of the morphological epiphyte groups	Location	1	131.41	25.43	**0.0040****
	Sampling date	1	25.58	4.95	**0.0385***
	Location x Sampling date	1	36.46	7.06	**0.0263***
	Residual	8	5.17		
	Total	11			
OTUs profile (presence/absence)	Location	1	15.92	6.16	**0.0002*****
	Sampling date	1	9.58	3.71	**0.0119***
	Location x Sampling date	1	6.25	2.42	0.0599
	Residual	8	2.58		
	Total	11			

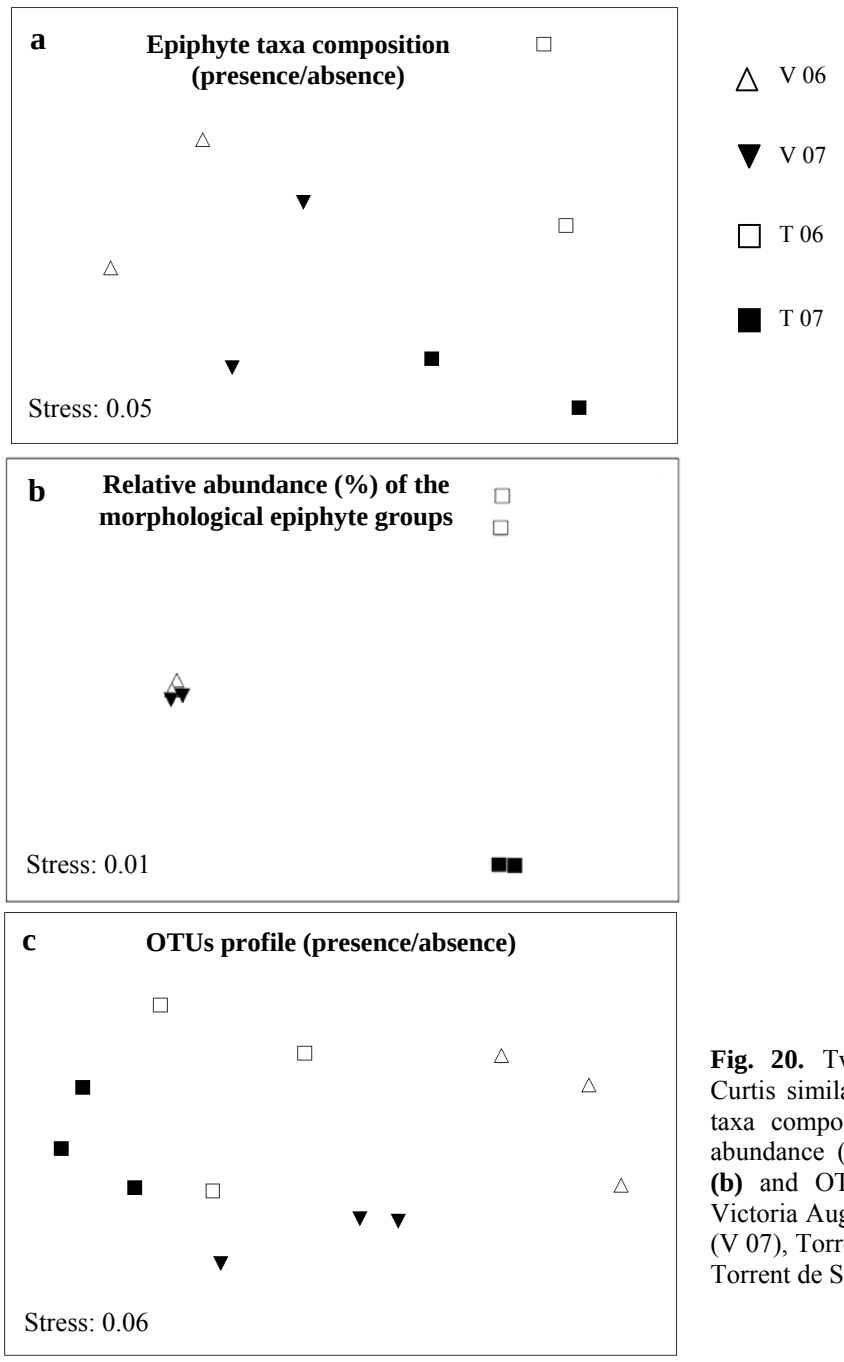


Fig. 20. Two-factor MDS obtained from Bray-Curtis similarities showing differences in epiphyte taxa composition (presence/absence) **(a)**, relative abundance (%) of morphological epiphyte groups **(b)** and OTUs profile (presence/absence) **(c)** in Victoria August 2006 (V 06), Victoria August 2007 (V 07), Torrent de St. Jordi August 2006 (T 06) and Torrent de St. Jordi August 2007 (T 07).

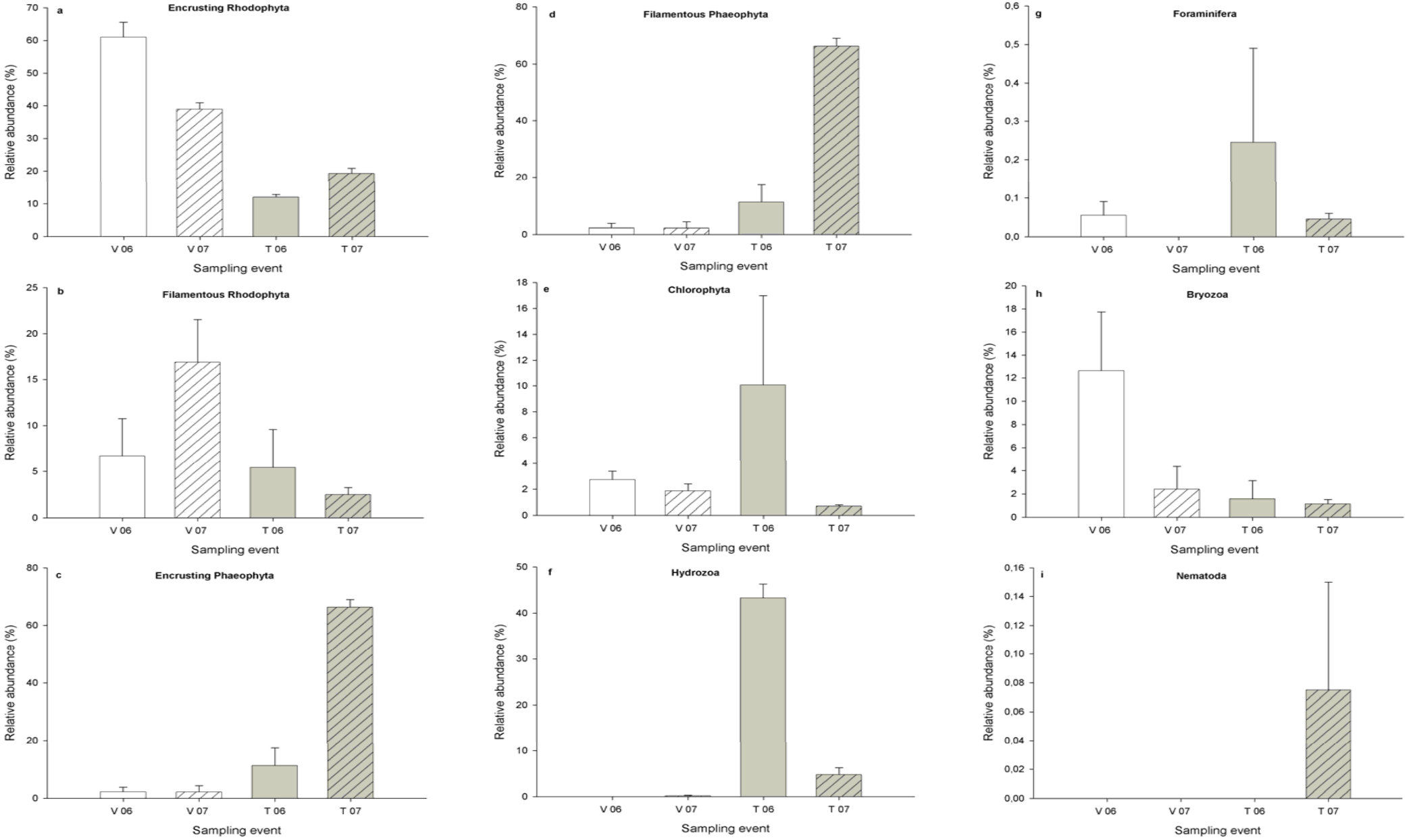


Fig. 21. Relative abundance (%) of encrusting Rhodophyta **(a)**, filamentous Rhodophyta **(b)**, encrusting Phaeophyta **(c)**, filamentous Phaeophyta **(d)**, Chlorophyta **(e)**, Hydrozoa **(f)**, Foraminifera **(g)**, Bryozoa **(h)** and Nematoda **(i)** in Victoria August 2006 (V 06), Victoria August 2007 (V 07), Torrent de St. Jordi August 2006 (T 06) and Torrent de St. Jordi August 2007 (T 07).

Table 9. List of epiphyte taxa for each replication unit. The presence of epiphyte taxa is symbolized with (●). Victoria (V), Torrent de Sant Jordi (T), August 2006 (06), August 2007 (07). (1) and (2) indicate number of replication unit. Bryozoa group was mainly composed by *Electra posidoniae*. Foraminifera group was mainly composed by *Lobatula lobatula*, *Spirillina vivipara* and *Rosalina* spp. Polychaeta group was mainly composed by Family Syllidae.

Morphological epiphyte group	Epiphyte taxa	V 06 (1)	V 06 (2)	V 07 (1)	V 07 (2)	T 06 (1)	T 06 (2)	T 07 (1)	T 07 (2)
Chlorophyta	*Chaetomorpha* spp.			●					●
	Cladophora spp.		●	●		●	●	●	●
	Phaeophila dendroides	●	●	●	●	●	●	●	●
	Pringsheimiella + *Ulvella* spp.		●				●		
	Pseudochlorodesmis furcellata		●	●		●	●	●	●
Diatoms		●	●	●	●	●	●	●	●
Encrusting Phaeophyta	*Myrionema magnusii*	●	●	●	●	●	●	●	●
	Myrionema strangulans		●						
Encrusting Rhodophyta	*Hydrolithon* + *Pneophyllum* spp.	●	●	●	●	●	●	●	●
Filamentous Phaeophyta	*Dictyota* spp.	●	●	●		●	●	●	●
	Giraudia sphacelarioides	●	●	●	●	●		●	●
	Sphacelaria cirrosa	●				●		●	●
	Sphacelaria fusca			●		●			●
	Sphacelaria spp.	●		●	●	●	●	●	●
Filamentous Rhodophyta	*Antithamnion plumula*		●						
	Audouinella daviesii	●	●	●	●	●	●	●	●
	Audouinella spp.	●			●				
	Callithamnion spp.						●		
	Ceramium spp.	●		●	●	●	●	●	●
	Chondria + *Laurencia* spp.	●	●	●	●	●	●	●	●
	Chroodactylon ornatum		●	●	●	●			●
	Dasya spp.			●					
	Discosporangium mesarthrocarpum					●			

Table 9. cont.)

Morphological epiphyte group	Epiphyte taxa	V 06 (1)	V 06 (2)	V 07 (1)	V 07 (2)	T 06 (1)	T 06 (2)	T 07 (1)	T 07 (2)
Filamentous Rhodophyta									
	Erythrotrichia carnea	●	●	●	●	●	●	●	●
	Falkenbergia spp.						●	●	●
	Family Ceramiaceae		●	●			●		
	Family Rodomelaceae	●	●	●	●	●	●	●	●
	Haliptilon virgatum				●				
	Herposiphonia secunda		●	●	●				
	Jania spp.	●		●					●
	Lomentaria chylocladiella							●	
	Lophosiphonia spp.	●		●		●			
	Polysiphonia spp.	●	●	●	●	●	●		●
	Spermothamnion spp.	●	●	●	●		●		●
	Stylonema alsidii	●	●	●	●	●	●	●	●
Bryozoa		●	●	●	●	●	●	●	●
Crustacea	Class Acarida					●	●	●	
	Class Copepoda		●	●	●	●	●	●	●
	Unknown crustacea					●	●		
Foraminifera		●	●	●	●	●	●	●	●
Hydrozoa	*Obelia geniculata*				●	●	●	●	●
	Unknown Hydrozoa				●			●	●
Nematoda		●	●	●	●	●	●	●	●
Polychaeta						●	●		
Porifera	Class Calciospongiae							●	●
Unknown epiphytes		●	●	●	●	●	●	●	●

Table 10. Results of SIMPER analysis on relative abundance of morphological epiphyte groups for the two locations and sampling dates. δ_i is the contribution of each taxon to the average Bray–Curtis dissimilarity between Victoria August 2006 (V 06), Victoria August 2007 (V 07), Torrent de St. Jordi August 2006 (T 06) and Torrent de St. Jordi August 2007 (T 07). Each morphological epiphyte group was considered important for dissimilarities observed between groups if δ_i / SD was equal or higher than 2.

	Average Relative abundance (%)			
Morphological epiphyte group	Group V 06	Group V 07	δ_i	δ_i / SD
Encrusting Rhodophyta	61.00	38.93	10.24	5.04
Morphological epiphyte group	Group V 06	Group T 06	δ_i	δ_i / SD
Encrusting Rhodophyta	61.00	12.02	23.12	26.82
Hydrozoa	0.00	43.25	20.55	8.87
Encrusting Phaeophyta	26.60	6.93	9.40	3.66
Morphological epiphyte group	Group V 07	Group T 07	δ_i	δ_i / SD
Filamentous Phaeophyta	2.16	66.32	32.09	16.22
Encrusting Phaeophyta	36.93	2.51	17.22	4.09
Encrusting Rhodophyta	38.93	19.33	9.80	6.63
Filamentous Rhodophyta	16.93	2.49	7.22	2.68
Morphological epiphyte group	Group T 06	Group T 07	δ_i	δ_i / SD
Filamentous Phaeophyta	11.31	66.32	27.79	7.38
Hydrozoa	43.25	4.79	19.44	9.48
Encrusting Rhodophyta	12.02	19.33	3.70	3.55

Two-way crossed ANOVA showed significant differences in OTUs richness between locations. Maximum OTUs richness (Mean $\pm$ SE) was recorded in Victoria August 2006 (16.33 $\pm$ 2.03%) and minimum in Torrent de St. Jordi August 2007 (3.67 $\pm$ 0.33%). No significant differences were observed for epiphyte taxa richness both for locations and sampling dates and data (Mean $\pm$ SE) ranged from 13.50 $\pm$ 1.50% (Victoria August 2006) to 17.70 $\pm$ 2.10% (Torrent de St. Jordi August 2007) (Fig. 22; Table 11).

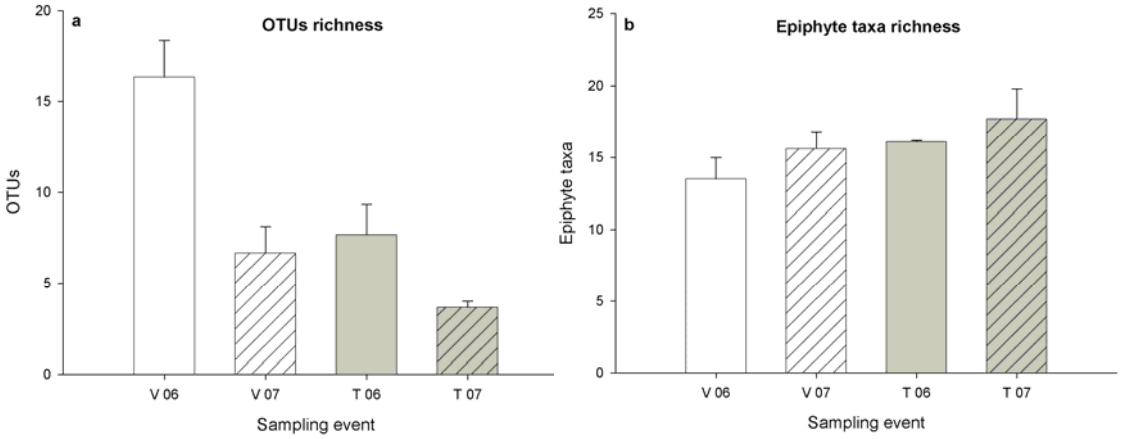


Fig. 22. OTUs richness **(a)** and epiphyte taxa richness **(b))** in Victoria August 2006 (V 06), Victoria August 2007 (V 07), Torrent de St. Jordi August 2006 (T 06) and Torrent de St. Jordi August 2007 (T 07).

Table 11. Summary of values and results of the two-way factorial ANOVA to assess significant differences in OTUs richness and epiphyte taxa richness between locations and sampling dates. Significant values in **bold. *p < 0.05, **p < 0.01, ***p < 0.001**

	Source	df	MS	F	p
OTUs richness	Location	1	102.08	14.94	**0.0048****
	Sampling date	1	140.08	20.50	**0.0019****
	Location x Sampling date	1	24.08	3.52	0.097
	Error	8	6.83		
	Total	11			
Epiphyte taxa richness	Location	1	11.05	2.72	0.1742
	Sampling date	1	6.84	1.69	0.2637
	Location x Sampling date	1	0.12	0.03	0.8691
	Error	4	4.05		
	Total	7			

Relevant descriptors of *P. oceanica* meadows

Significant differences in epiphyte load (g epiphyte DW g^{-1} shoot DW) and epiphyte organic matter load (g epiphyte AFDW g^{-1} shoot DW) between locations were detected. Epiphyte load (Mean $\pm$ SE) was clearly higher in Torrent de St. Jordi (0.54 $\pm$ 0.05 g epiphyte DW g^{-1} shoot DW and 0.87 $\pm$ 0.10 g epiphyte DW g^{-1} shoot DW in August 2006

and 2007, respectively) than in Victoria (0.22 ± 0.03 g epiphyte DW g^{-1} shoot DW and 0.23 ± 0.05 g epiphyte DW g^{-1} shoot DW in August 2006 and 2007, respectively). Epiphyte organic matter load (Mean ± SE) was also higher in Torrent de St. Jordi (0.14 ± 0.02 g epiphyte AFDW g^{-1} shoot DW and 0.22 ± 0.04 g epiphyte AFDW g^{-1} shoot DW in August 2006 and 2007, respectively) than in Victoria (0.05 ± 0.01 g epiphyte AFDW g^{-1} shoot DW and 0.08 ± 0.02 g epiphyte AFDW g^{-1} shoot DW in August 2006 and 2007, respectively). Number of leaves $shoot^{-1}$ (Mean ± SE) differed between locations and sampling dates, and the highest value was recorded in Torrent de St. Jordi in August 2006 (7.50 ± 0.50). N and P shoot content (% DW) did not show any significant differences between locations and between sampling dates, but the highest values (Mean ± SE) were achieved in Torrent de St. Jordi in August 2007 (1.22 ± 0.14% DW and 0.138 ± 0.014% DW, respectively). Shoot biomass (g DW) was significantly higher (Mean ± SE) in Victoria (0.96 ± 0.11 g DW and 1.06 ± 0.08 g DW in August 2006 and 2007, respectively) than in Torrent de St. Jordi (0.75 ± 0.10 g DW and 0.66 ± 0.05 g DW in August 2006 and 2007, respectively). Significant differences in shoot density (shoots m^{-2}) between locations and between sampling dates were observed. Higher values (Mean ± SE) were recorded in Victoria (25.90 ± 3.98 shoots m^{-2} and 33 ± 0.41 shoots m^{-2} in August 2006 and 2007, respectively) than in Torrent de St. Jordi (14.40 ± 1.53 shoots m^{-2} and 18.50 ± 1.57 shoots m^{-2} in August 2006 and 2007, respectively). No significant differences in fish bite marks $shoot^{-1}$ (Mean ± SE) between locations or between sampling dates were detected. The highest value was achieved in Victoria August 2006 (1.10 ± 0.67 fish bite marks $shoot^{-1}$) and the lowest in Torrent de St. Jordi in August 2006 (0.20 ± 0.13 fish bite marks $shoot^{-1}$). No sea urchins were detected in any location and sampling date and ANOVA was not performed (Fig. 23; Table 12).

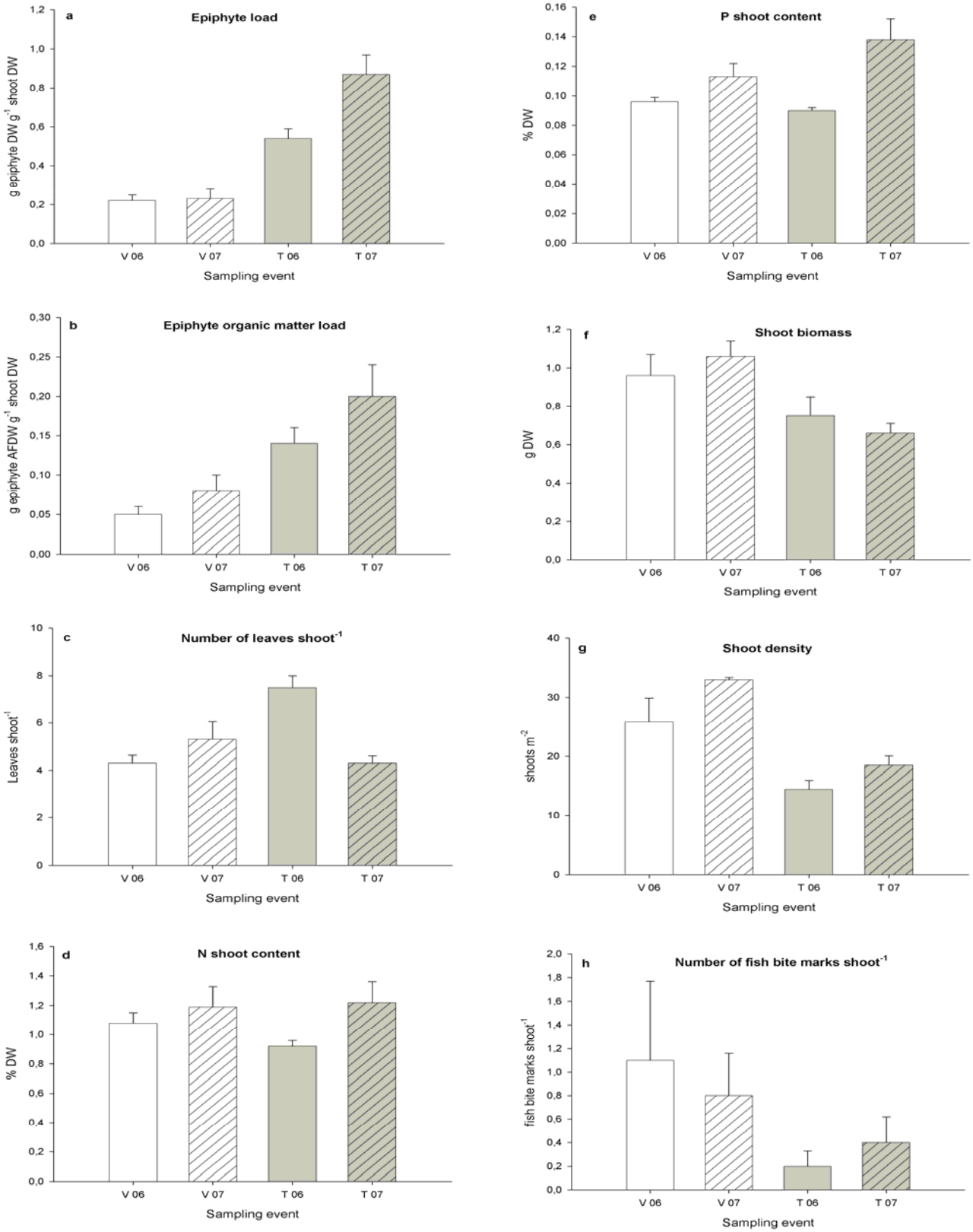


Fig. 23. Main descriptors of *P. oceanica* meadows: epiphyte load **(a)**, epiphyte organic matter load **(b)**, number of leaves shoot^{-1} **(c)**, N shoot content **(d)**, P shoot content **(e)**, shoot biomass **(f)**, shoot density **(g)** and number of fish bite marks shoot^{-1} **(h)** in Victoria August 2006 (V 06), Victoria August 2007 (V 07), Torrent de St. Jordi August 2006 (T 06) and Torrent de St. Jordi August 2007 (T 07).

Table 12. Summary of values and results of the two-way factorial ANOVA to assess significant differences in epiphyte load (g epiphyte DW g^{-1} shoot DW), epiphyte organic matter load (g epiphyte AFDW g^{-1} shoot DW), number of leaves $shoot^{-1}$, N shoot content (% DW), P shoot content (% DW), shoot biomass (g DW), shoot density (shoots m^{-2}), fish bite marks $shoot^{-1}$) between two locations and sampling dates. Significant values in **bold. *p < 0.05, **p < 0.01, ***p < 0.001.**

	Source	df	MS	F	p
Epiphyte load (g epiphyte DW g^{-1} shoot DW)	Location	1	0.46	20.62	**0.0105***
	Sampling date	1	0.06	2.69	0.1761
	Location x Sampling date	1	0.05	2.40	0.1962
	Error	4	0.02		
	Total	7			
Epiphyte organic matter load (g epiphyte AFDW g^{-1} shoot DW)	Location	1	0.02	8.35	**0.0029****
	Sampling date	1	< 0.01	1.18	0.3384
	Location x Sampling date	1	< 0.01	0.17	0.6993
	Error	4	< 0.01		
	Total	7			
Number of leaves $shoot^{-1}$	Location	1	3.38	3.07	**0.0011****
	Sampling date	1	1.62	1.47	**0.0125***
	Location x Sampling date	1	7.22	6.56	0.1112
	Error	4	1.10		
	Total	7			
N shoot content (% DW)	Location	1	0.01	0.13	0.7381
	Sampling date	1	0.08	1.45	0.2953
	Location x Sampling date	1	0.02	0.31	0.6085
	Error	4	0.06		
	Total	7			
P shoot content (% DW)	Location	1	< 0.01	0.90	0.3973
	Sampling date	1	< 0.01	3.39	0.1396
	Location x Sampling date	1	< 0.01	1.71	0.2608
	Error	4	< 0.01		
	Total	7			
Shoot biomass (g DW)	Location	1	0.19	12.37	**0.0245***
	Sampling date	1	< 0.01	0.01	0.9326
	Location x Sampling date	1	0.02	1.16	0.3417
	Error	4	0.01		
	Total	7			
Shoot density (shoots m^{-2})	Location	1	463.60	69.39	**0.0011****
	Sampling date	1	124.03	18.56	**0.0125***
	Location x Sampling date	1	27.75	4.15	0.1112
	Error	4	6.68		
	Total	7			
Fish bite marks $shoot^{-1}$	Location	1	0.84	1.16	0.3411
	Sampling date	1	< 0.01	0.01	0.9378
	Location x Sampling date	1	0.12	0.17	0.6993
	Error	4	0.72		
	Total	7			

Discussion

Both TGGE and microscope techniques were able to detect significant differences between locations and/or sampling dates in the structure of the mature stage of the leaf-epiphytic community of *P. oceanica*. TGGE showed significant differences in OTUs richness and OTUs profile (composition) between locations and sampling dates, whereas microscopy only detected significant differences in epiphyte taxa composition between locations. Those results could be explained for different reasons: (1) underestimation of the real diversity: epiphytic assemblage description based on microscopy could be biased by the taxonomical expertise of the researcher, (2) taxonomic impediment: some individuals (juvenile, broken,…) are difficult to determine under microscope and (3) TGGE technique could detect the presence of taxa even with low relative abundance in the assemblage (Valentini et al. 2008). Although microscopy approach was able to detect a higher number of OTUs than TGGE, which was not in accordance with results obtained in Chapter 2, it must be taken into account that PCR products belonging to the same quadrat were joined before applying TGGE to simplify comparisons between locations. For this reason, OTUs from the highest abundant taxa were probably favoured against those from the lowest abundant taxa in TGGE gel.

Microscopy approach showed that the relative abundance of the morphological epiphyte groups was more sensitive than epiphyte taxa composition to detect differences in the composition of the assemblage either between locations or sampling dates. Our results showed that the mature stage of the epiphytic community in Victoria was mainly composed by encrusting and filamentous Rhodophyta, whereas in Torrent de St. Jordi the assemblage differed between sampling dates, being mainly composed by Hydrozoa

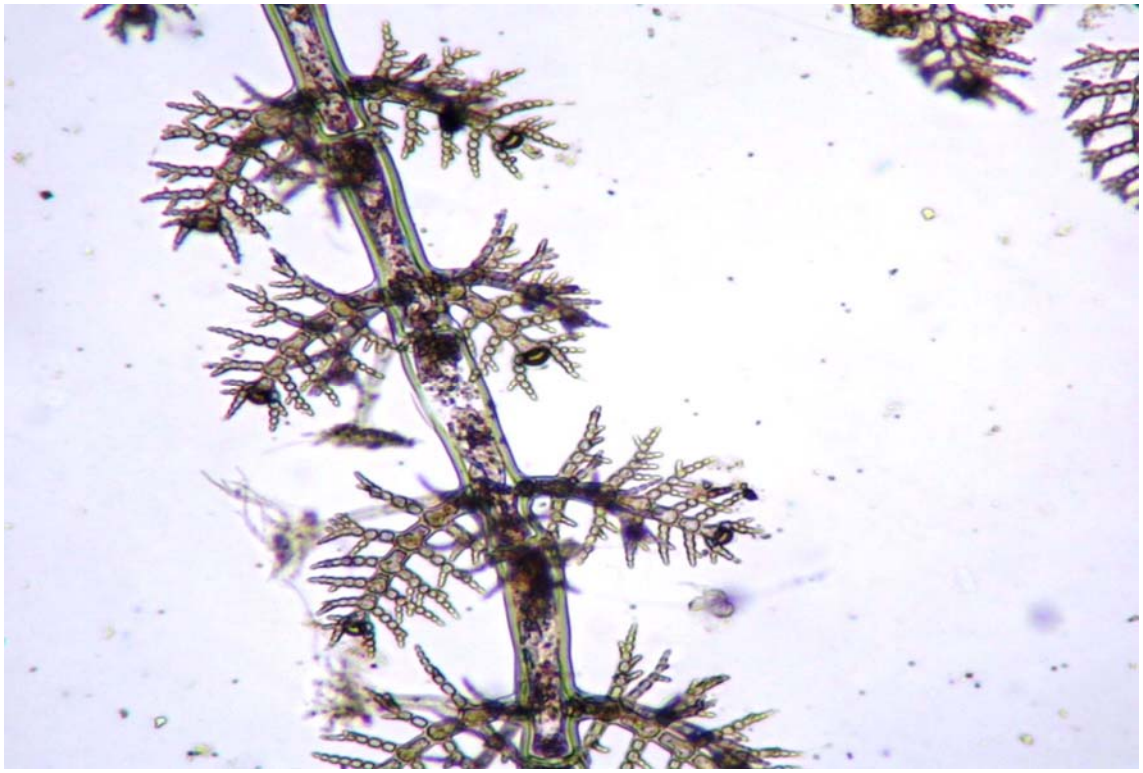
and by encrusting and filamentous Phaeophyta in August 2006 and 2007, respectively. Significant differences in the relative abundance of the morphological (or functional) epiphyte groups in the mature stage of the assemblage have been previously observed when comparing different locations (Piazzi et al. 2004; Pardi et al. 2006). According to previous studies, the composition of the mature stage of the epiphytic assemblage followed the general trend (Van der Ben 1971; Ballesteros 1987) in Victoria, but not in Torrent de St. Jordi. Other studies have documented an increase in the relative abundance of filamentous Phaeophyta related to water column nutrient additions in *P. oceanica* meadows (Prado et al. 2008).

Balata et al. 2007 concluded that differences in epiphytic assemblages between meadows appeared to be related to local differences in environmental factors that were more evident in habitats influenced by human disturbance. Here, we analysed patterns of relevant descriptors of *P. oceanica* meadows, which have been previously linked to environmental conditions (Romero et al. 2007). Significant differences in epiphyte load, epiphyte organic matter load, number of leaves shoot^{-1}, shoot biomass and shoot density were mainly found between locations. Higher values of epiphyte load and epiphyte organic matter load in Torrent de St. Jordi than in Victoria could be explained because of the higher relative abundance of filamentous Phaeophyta in the former location. While filamentous Phaeophyta has been recognised as a relevant contributor to epiphyte biomass in other studies, other epiphyte groups (i.e. filamentous Rhodophyta) have been considered that represent a limited biomass in the assemblage (Romero 1988; Lepoint et al. 2007). Shoot biomass and shoot density also exhibited differences between locations reaching higher values in Victoria than in Torrent de St. Jordi. It could be explained by the fact that a biomass increase in an epiphytic assemblage, as it was observed in

Torrent de St. Jordi, might reduce the amount of light reaching *P. oceanica* shoots, causing seagrass decline (Drake et al. 2003). Although differences in the epiphyte load and epiphyte assemblage composition have been linked in previous studies to differences in nutrient inputs (Prado et al. 2008) and herbivory magnitude of macroherbivores (sea urchins and the fish *S. salpa*) on *P. oceanica* (Tomas et al. 2005b; Prado et al. 2007b), we did not detect differences in N and P shoot content, sea urchin density and fish bite marks $shoot^{-1}$ between locations.

In summary, both methods used here (TGGE and microscopy) were able to detect spatial variability in epiphyte community, although molecular method allowed to overcome some problems related to microscopy approach, such as taxonomic impediment. The use of the relative abundance of morphological epiphyte groups revealed to be more sensitive than epiphyte taxa composition to determine that spatial variability in microscopy approach. Differences found in epiphyte assemblages and relevant descriptors of *P. oceanica* meadows between locations could not be explained by differences in environmental conditions (nutrient availability and herbivory pressure), suggesting that other factors are operating to explain that variability.

SECTION IV - Chapter 5



Detail of alien epiphyte filamentous Rhodophyta

Seasonal variability of the structure of the macro-eukaryotic leaf-epiphytic assemblage of *P. oceanica* (L.) Delile applying TGGE and classical microscopy approach

Seasonal variability of the structure of the macro-eukaryotic leaf-epiphytic assemblage of *P. oceanica* (L.) Delile applying TGGE and classical microscopy approach

Background

TGGE was able to detect variability in the structure of two stages (February and September) of the macroeukaryotic leaf-epiphyte assemblage (Chapter 2) and in the structure of the mature stage (summer) of that assemblage in two *P. oceanica* meadows (Chapter 4). We were interested in applying TGGE to study seasonal variability in the structure of the epiphyte community along a two-year cycle. Results obtained applying this molecular technique was compared with those from classical microscopy approach.

Materials and Methods

Sample collection

The study was conducted in a *P. oceanica* meadow located in Victoria (39°51 N, 3°11 E, Pollença Bay, Majorca western Mediterranean Sea, Spain) in 2006 and 2007. Samples were collected at intervals of about 2-3 months.

Three quadrates of 0.25 m^2 tens of metres apart were haphazardly selected in each sampling date. In each quadrate, seven shoots were haphazardly collected in each sampling event by SCUBA divers and frozen at -80 °C to keep nucleic acids intact until their processing by means of TGGE molecular method. This number of shoots was previously considered adequate to represent the diversity of the epiphytic community in molecular studies within an area of 0.25 m^2 (Chapter 2).

Two 10 m transects separated thirty meters were also laid in each sampling date. Five shoots were haphazardly harvested along each transect and immediately preserved

in 4% formalin-seawater until their processing to determine eukaryotic leaf-epiphytic community structure (composition, taxa richness and relative abundance of the morphological epiphyte groups) by means of microscopy method following the procedure of Chapter 2. This number of shoots has been recognised as a representative number of the epiphytic community in classical microscopy studies (Panayotidis and Boudouresque 1981; Ballesteros 1987). The distribution of epiphyte taxa in morphological groups has demonstrated to be useful to describe variability in community structure (Pardi et al. 2006; Piazzi et al. 2007).

Finally, ten additional shoots were haphazardly harvested along each transect in each sampling date to quantify main descriptors of *P. oceanica* meadow in Victoria.

TGGE molecular technique and optical microscope methods

DNA extraction, PCR, TGGE and optical microscope methods were performed according to Chapter 2 procedure. Samples belonging to the same quadrat (TGGE) or transect (microscopy approach) were grouped to constitute a representative unit of replication.

Some relevant OTUs along the sampling dates were picked from TGGE gels, re-amplified and sequenced, and a putative identification was assigned to each of them (see Chapter 2 for further details).

Main descriptors of *P. oceanica* meadow

P. oceanica description in Victoria included epiphyte load and epiphyte organic matter load on the shoots, and vegetative features at shoot scale (number of leaves, N and P content, biomass) and at meadow scale (shoot density).

Shoots collected for description of *P. oceanica* were gently cleaned from epiphytes with a razor blade. Epiphytes collected in pre-weighed Whatman GF/C glass

fibre filters and cleaned shoots were separately dried at 60ºC for at least 48 h to estimate shoot biomass (g DW) and epiphyte load (g epiphyte DW g^{-1} shoot DW). Six of the dried epiphytic samples were burned in an oven furnace at 450ºC for 6 h to estimate epiphyte organic matter load (g epiphyte AFDW g^{-1} shoot DW) (Kendrick and Lavery 2001). N and P shoot content (% DW) were estimated from at least four of these ten *P. oceanica* shoots for each sampling date. N shoot content was estimated using an elemental analyser CHN-O-RAPID (Heraeus). P content was estimated according to Fourqurean et al. 1992 procedure. BCR standard beech leaves (SIGMA) was used to calculate P recovery of the method and to make reliable comparisons among P content of different sets of samples. Seagrass nutrient content has been previously considered as a way to compare the nutrient availability for seagrass growth in coastal waters (Fourqurean et al. 2007). Between five to ten quadrates of 0.04 m^2 were haphazardly selected along two transects in the field and shoots were counted to estimate shoot density (shoots m^{-2}) in each sampling date (Delgado et al. 1999).

Statistical analyses

Samples belonging to the same transect (microscopy approach) or to the same quadrat (TGGE) were grouped for statistical analyses constituting representative replication units for each location and sampling date.

MDS based on the Bray-Curtis similarity index was used to represent epiphyte taxa composition (presence/absence), relative abundance (%) of the morphological epiphyte groups and OTUs profile (presence/absence) for each sampling date.

A one-way PERMANOVA (Anderson 2001) was used to evaluate significant differences in epiphyte taxa composition (presence/absence), relative abundance of the

morphological epiphyte groups and OTUs profile (presence/absence) between sampling dates.

Significant differences between sampling dates in OTUs richness and epiphyte taxa richness were analysed using a one-way ANOVA. An analogous statistical analyses was done to evaluate significant differences in the main descriptors of *P. oceanica* meadows (epiphyte load, epiphyte organic matter load, number of leaves shoot^{-1}, N and P shoot content, shoot biomass and shoot density) between sampling dates.

The Spearman rank correlation (r_s) analysis was used to evaluate if there was any association between epiphyte taxa richness, relative abundance of the morphological epiphyte groups, OTUs richness and *P. oceanica* meadow descriptors in Victoria.

Data used in ANOVA tests were previously tested for homogeneity of variances and normality test using Levene's test and Kolmogorov-Smirnov test, respectively. All analyses were performed using PRIMER 5 (Clarke and Gorley 2001), SIGMAPLOT 8.0 (SPSS Science 2002), STATISTICA 7.1 (StatSoft Inc 2005) and PERMANOVA software (Anderson 2001). Significance level in all statistical tests was 0.05.

Results

TGGE molecular technique and optical microscope methods

MDS showed differences in OTUs profile and epiphyte taxa composition between sampling dates (Fig. 24). PERMANOVA confirmed those differences in OTUs profile ($F = 6.6498$, $p = 0.0001$) and epiphyte taxa composition ($F = 2.6040$, $p = 0.0001$) between sampling dates. Post-hoc Tukey HSD test showed significant differences in OTUs profile between all the sampling dates ($p < 0.05$), with the exception of June 2006

vs. April 2007 (p = 0.1510), April 2007 vs. June 2007 (p = 0.1150) and October 2007 vs. November 2007 (p = 0.2360). Epiphyte taxa composition differed between February 2006 and April 2006 (p = 0.0256), February 2006 and June 2006 (p = 0.0343) and February 2006 and November 2007 (p = 0.0443). A list of epiphytic taxa in each sampling date is provided (Table 13). Some epiphytic taxa were present in all sampling dates (*Hydrolithon* + *Pneophyllum* spp. (encrusting Rhodophyta) and *M. magnusii* (encrusting Phaeophyta)) while others were characteristic of a season (*Cladosiphon* spp. (spring)).

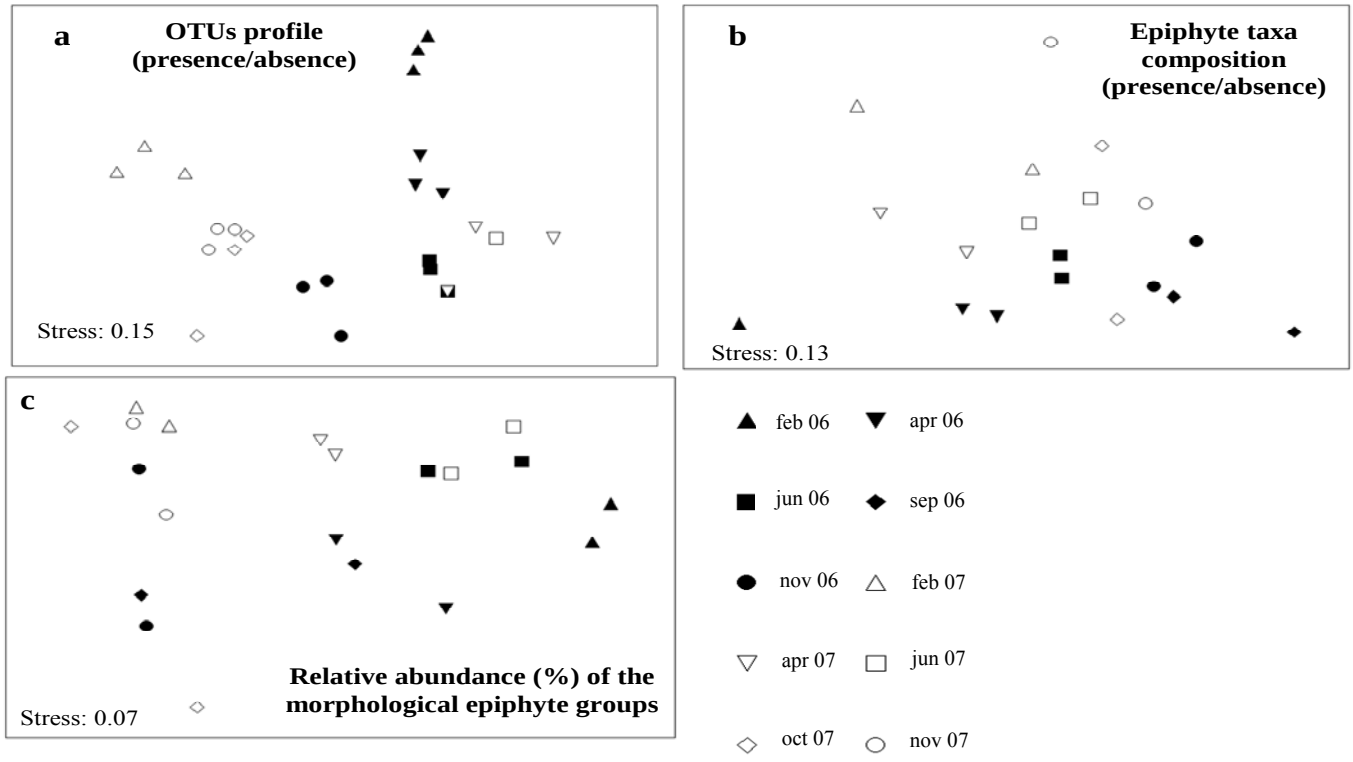


Fig. 24. MDS ordination of OTUs profile **(a)**, epiphyte taxa composition **(b)** and relative abundance (%) of morphological epiphyte groups **(c)** for different sampling dates. Each sampling date is represented for two replication units. February (feb), April (apr), June (jun), September (sep), October (oct), November (nov), 2006 (06), 2007 (07).

Table 13. List of epiphyte taxa for each replication unit and sampling date. The presence of epiphyte taxa is symbolized with (●). February (F), April (A), June (J), September (S), October (O), November (N), August 2006 (06), August 2007 (07). (1) and (2) indicate number of replication unit. Bryozoa group was mainly composed by *Electra posidoniae*. Foraminifera group was mainly composed by *Lobatula lobatula, Spirillina vivipara* and *Rosalina* spp. Polychaeta group was mainly composed by Family Syllidae.

Morphological epiphyte group	Epiphyte taxa	F06 (1)	F06 (2)	A06 (1)	A06 (2)	J06 (1)	J06 (2)	S06 (1)	S06 (2)	N06 (1)	N06 (2)	F07 (1)	F07 (2)	A07 (1)	A07 (2)	J07 (1)	J07 (2)	O07 (1)	O07 (2)	N07 (1)	N07 (2)
Chlorophyta	*Chaetomorpha* spp.				●											●					●
	Cladophora spp.							●	●	●	●						●	●	●	●	
	Entocladia viridis												●							●	●
	Phaeophila dendroides			●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
	Pringsheimiella + *Ulvella* spp.			●	●	●		●	●												
	Pseudochlorodesmis furcellata					●	●			●	●						●				
Diatoms		●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●	●	●	●
Encrusting Phaeophyta	*Myrionema magnusii*	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Encrusting Rhodophyta	*Hydrolithon* + *Pneophyllum* spp.	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Filamentous Phaeophyta	*Cladosiphon* spp.			●	●	●	●							●	●	●	●				
	Dictyota spp.									●	●					●	●		●		
	Ectocarpus spp.			●	●											●					
	Family Ectocarpaceae	●	●	●	●																
	Giraudia sphacelarioides	●	●	●	●	●	●		●		●	●	●	●	●	●	●	●		●	
	Sphacelaria cirrosa															●					
	Sphacelaria spp.					●	●			●	●		●			●	●			●	
	Acrothamnion preissii					●					●										

Table 13.
cont.)

Morphological epiphyte group	Epiphyte taxa	F06 (1)	F06 (2)	A06 (1)	A06 (2)	J06 (1)	J06 (2)	S06 (1)	S06 (2)	N06 (1)	N06 (2)	F07 (1)	F07 (2)	A07 (1)	A07 (2)	J07 (1)	J07 (2)	O07 (1)	O07 (2)	N07 (1)	N07 (2)
Filamentous Rhodophyta	*Aglaothamnion* spp.																			●	
	Anotrichium tenue							●													
	Audouinella daviesii	●	●	●	●	●	●	●	●	●	●			●	●	●	●	●	●	●	●
	Audouinella spp.	●	●	●	●	●	●	●	●	●	●								●	●	
	Callithamnion spp.							●													
	Ceramium spp.			●			●	●	●	●	●	●		●		●		●	●	●	
	Champia spp.																				●
	Chondria + *Laurencia* spp.					●		●	●		●								●		
	Chroodactylon ornatum							●			●								●		
	Ctenosiphonia spp.									●											
	Dasya spp.								●												
	Erythrotrichia carnea			●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●
	Family *Ceramiaceae*					●	●	●	●	●	●	●				●	●	●		●	●
	Family *Rodomelaceae*					●	●		●	●	●	●				●	●	●	●	●	●
	Griffithsia spp.							●	●											●	
	Herposiphonia secunda							●	●												
	Jania spp.										●										
	Lophosiphonia spp.								●	●	●										
	Polysiphonia spp.							●	●	●	●						●			●	
	Spermothamnion spp.	●	●	●	●	●	●	●	●	●	●	●	●	●		●	●		●	●	
	Stylonema alsidii			●	●		●		●	●	●								●	●	
Bryozoa				●	●	●	●	●	●	●	●	●		●		●	●	●	●	●	●
Crustacea	Class Copepoda			●	●	●	●				●			●	●	●			●		
	Unknown crustacea			●	●	●	●					●		●	●	●	●	●			

Table 13.
cont.)

Morphological epiphyte group	Epiphyte taxa	F06 (1)	F06 (2)	A06 (1)	A06 (2)	J06 (1)	J06 (2)	S06 (1)	S06 (2)	N06 (1)	N06 (2)	F07 (1)	F07 (2)	A07 (1)	A07 (2)	J07 (1)	J07 (2)	O07 (1)	O07 (2)	N07 (1)	N07 (2)
Hydrozoa	*Obelia geniculata*												•	•							
	Plumularia obliqua																			•	
	Sertularia perpusilla																	•			
	Unknown Hydrozoa	•	•	•	•				•		•	•		•	•				•	•	•
Nematoda					•	•	•	•	•	•	•	•		•					•		
Polychaeta								•													
Unknown epiphytes				•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•

OTUs picked from TGGE gels, re-amplified and sequenced were putatively identified to encrusting Rhodophyta, Crustacea Copepoda, Annelida Polychaeta (2 OTUs), Mollusca Gastropoda and Bryozoa taxa (see Chapter 2 for further details). OTU corresponding to encrusting Rhodophyta taxon was present in all sampling dates, which mirrored with results obtained from classical microscopy approach.

Significant differences in OTUs richness between sampling dates were found, but they were not mirrored with differences in epiphyte taxa richness. The highest OTUs richness (± SE) was observed in September 2006 (21.16 ± 1.01) and the lowest in June 2007 (8.28 ± 0.74) (Fig.25; Table 14).

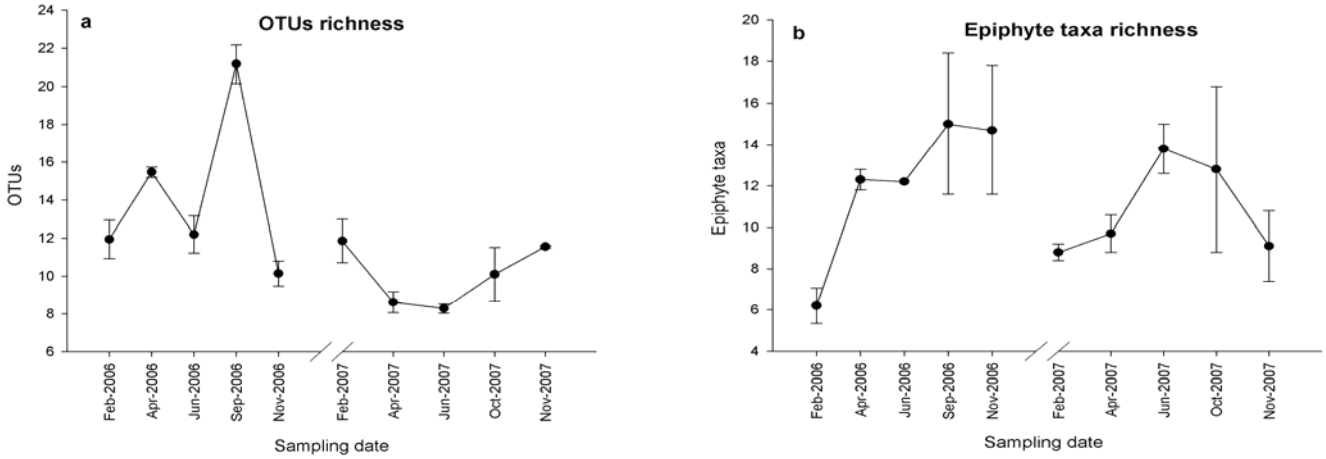


Fig. 25. Mean (± SE) OTUs richness **(a)** and epiphyte taxa richness **(b)** in February 2006 (Feb-2006), April 2006 (Apr-2006), June 2006 (Jun-2006), September 2006 (Sep-2006), November 2006 (Nov-2006), February 2007 (Feb-2007), April 2007 (Apr-2007), June 2007 (Jun-2007), October 2007 (Oct-2007), November 2007 (Nov-2007).

Table 14. Results of one-way ANOVA to evaluate significant differences in OTUs richness and epiphyte taxa richness between sampling dates. Significant values in **bold**. ***p < 0.05; ** p < 0.01; *** p < 0.001.**

	Source	df	MS	F	P
OTUs richness	Sampling date	9	42.78	19.87	**0.0000*** **
	Residual	20	2.15		
	Total	29			
Epiphyte taxa richness	Sampling date	9	16.66	1.95	0.1564
	Residual	10	8.54		
	Total	19			

One-way ANOVA showed significant differences in relative abundance (%) of encrusting Rhodophyta, encrusting Phaeophyta, filamentous Phaeophyta and Chlorophyta morphological epiphyte groups (Table 15). Encrusting Phaeophyta and encrusting Rhodophyta were present in all sampling dates. Post-Hoc Tukey HSD test detected significant differences between sampling dates in relative abundance of encrusting Phaeophyta, but no clear temporal trend was observed, whereas relative abundance of encrusting Rhodophyta remained very constant along two-year cycle. Maximum relative abundance of encrusting Rhodophyta and encrusting Phaeophyta was in November 2007 (about 70%) and in February 2006 (about 74%) and minimum was in February 2006 (about 3%) and October 2007 (about 5%), respectively. Relative abundance of filamentous Phaeophyta was significantly higher in spring, especially in 2006 (about 23%, either in April or June), than in other sampling dates (Post-hoc Tukey HSD test, $p < 0.05$). Chlorophyta relative abundance was lower than Phaeophyta and Rhodophyta (encrusting and filamentous) and significantly higher in November 2006 (about 4%) than in other sampling dates (Post-Hoc Tukey HSD test, $p < 0.05$) (Fig. 26). Zooepiphytes (Bryozoa, Foraminifera and Hydrozoa) were far less abundant than

macroalgae in the assemblage for all the sampling dates. Those differences were mirrored in MDS representation (Fig. 24c).

Table 15. Results of one-way ANOVA to evaluate significant differences in relative abundance (%) of morphological epiphyte groups between sampling dates (a). Significant values in **bold**. ***p < 0.05; ** p < 0.01; *** p < 0.001.**

	Source	df	MS	F	P
Encrusting Rhodophyta	Sampling date	9	1188.84	4.34	**0.0158***
	Residual	10	274.08		
	Total	19			
Filamentous Rhodophyta	Sampling date	9	296.19	1.20	0.3891
	Residual	10	247.41		
	Total	19			
Encrusting Phaeophyta	Sampling date	9	1086.73	9.93	**0.0006*****
	Residual	10	109.48		
	Total	19			
Filamentous Phaeophyta	Sampling date	9	183.04	7.36	**0.0022****
	Residual	10	24.88		
	Total	19			
Chlorophyta	Sampling date	9	3.24	5.83	**0.0055****
	Residual	10	0.55		
	Total	19			
Hydrozoa	Sampling date	9	1.29	0.76	0.6541
	Residual	10	1.70		
	Total	19			
Foraminifera	Sampling date	9	0.04	2.24	0.1122
	Residual	10	0.02		
	Total	19			
Bryozoa	Sampling date	9	10.14	1.22	0.3778
	Residual	10	8.30		
	Total	19			

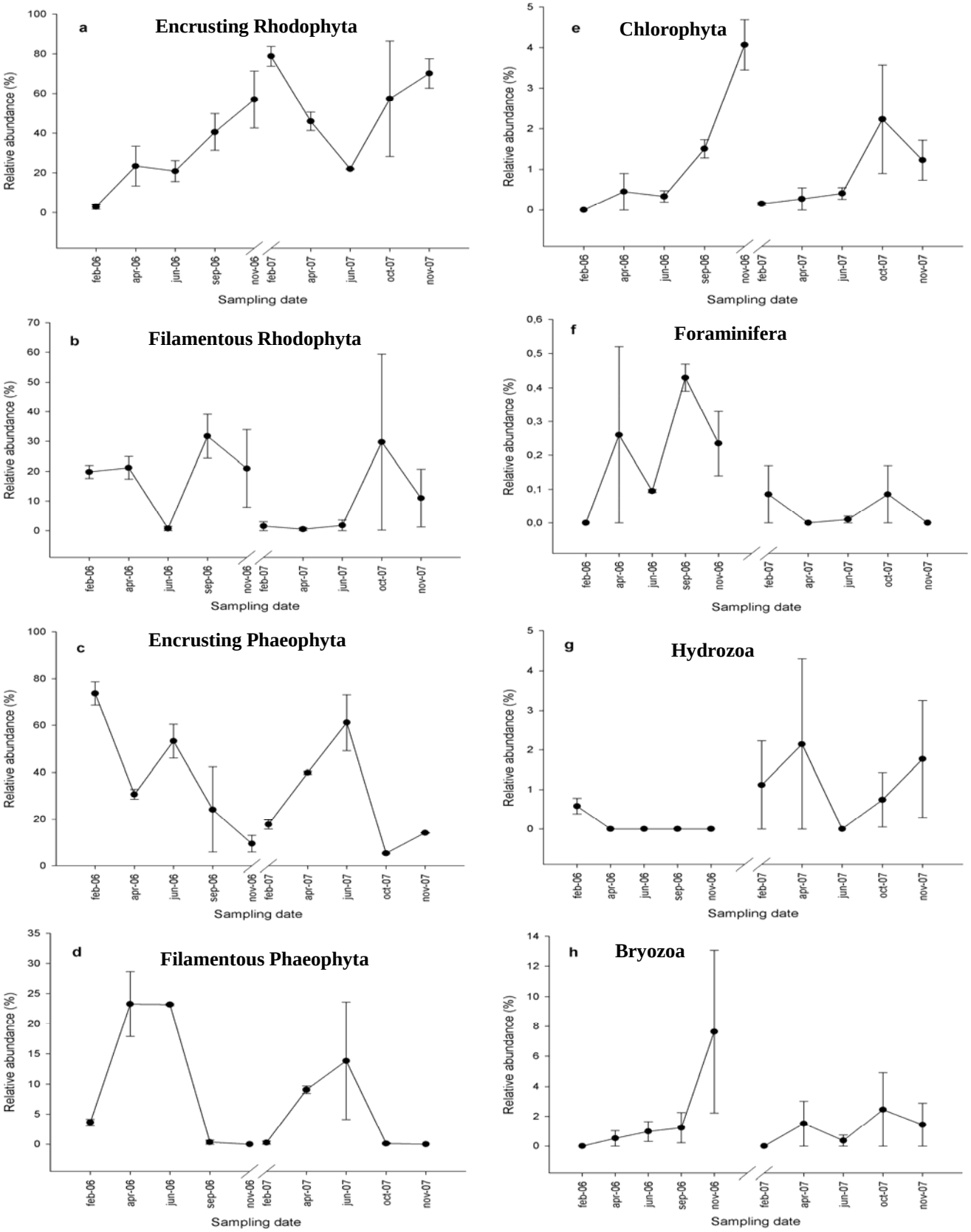


Fig. 26. Relative abundance (%) of encrusting Rhodophyta **(a)**, filamentous Rhodophyta **(b)**, encrusting Phaeophyta **(c)**, filamentous Phaeophyta **(d)**, Chlorophyta **(e)**, Foraminifera **(f)**, Hydrozoa **(g)** and Bryozoa **(h)** morphological epiphyte groups for different sampling dates (February (feb), April (apr), June (jun), September (sep), October (oct), November (nov), 2006 (06), 2007 (07)).

Relevant descriptors of the *P. oceanica* meadow

Significant differences were also found in epiphyte load, epiphyte organic matter load number of leaves shoot^{-1} and shoot biomass between sampling dates (Table 16). Epiphyte load and epiphyte organic matter load also exhibited the highest values in spring (April and June) (about 0.2 g epiphyte DW g^{-1} shoot DW and about 0.07 g epiphyte AFDW g^{-1} shoot DW) and the lowest in autumn (November) (about 0.1 g epiphyte DW g^{-1} shoot DW and about 0.03 g epiphyte AFDW g^{-1} shoot DW). Post-Hoc Tukey HSD test ($p < 0.05$) confirmed that epiphyte load and epiphyte organic matter load significantly differed between those two seasons. Number of leaves shoot^{-1} differed between June 2007 (about 5 leaves shoot^{-1}) and autumn 2007 (October and November) (about 6.5 leaves shoot^{-1}) (Post-Hoc Tukey HSD test, $p < 0.05$). The highest values of N shoot content (% DW) were recorded in autumn and winter (about 1.8% DW) and the lowest in late summer and spring (about 1.4% DW). Differences between those seasons in N shoot content were confirmed by Post-Hoc Tukey HSD test ($p < 0.05$). P shoot content (% DW) not exhibited a clear trend along two-year cycle. Shoot biomass showed a maximum in June 2006 and June 2007 (about 1.4 g DW and 0.9 g DW, respectively) and post-hoc Tukey HSD test revealed significant differences between June 2006 and all other sampling dates ($p < 0.001$). No significant differences in shoot density between sampling dates were found, but slightly higher values in spring (April) (about 700 shoots m^{-2}) than in autumn (November) (about 650 shoots m^{-2}) were observed (Fig. 27).

Spearman rank correlation (r_s) analysis showed significant correlations ($p < 0.05$) between some of the variables studied. Strong positive correlation was found between epiphyte load and epiphyte organic matter load ($r_s = 0.891$). Those two variables were also positively correlated with shoot biomass ($r_s = 0.673$ and $r_s = 0.648$,

respectively), whereas epiphyte load showed a negative correlation with number of leaves shoot^{-1} (r_s = -0.719) and epiphyte organic matter load with N shoot content (r_s = -0.745). N shoot content was positively correlated with P shoot content (r_s = 0.636). The relative abundances of encrusting and filamentous Phaeophyta exhibited positive correlations with epiphyte load (r_s = 0.782 and r_s = 0.948, respectively) and epiphyte organic matter load (r_s = 0.636 and r_s = 0.936, respectively). Shoot biomass was also positively correlated with relative abundance of filamentous Phaeophyta (r_s = 0.644) (Fig. 28).

Table 16. Results of one-way ANOVA to evaluate significant differences in epiphyte load, epiphyte organic matter load, number of leaves shoot^{-1}, N and P shoot content, shoot biomass and shoot density between sampling dates. Significant values in **bold**. ***p < 0.05; ** p < 0.01; *** p < 0.001.**

	Source	df	MS	F	P
Epiphyte load (g epiphyte DW g^{-1} shoot DW)	Sampling date	9	0.004	6.609	**0.0034****
	Residual	10	0.001		
	Total	19			
Epiphyte organic matter load (g epiphyte AFDW g^{-1} shoot DW)	Sampling date	9	0.001	5.410	**0.0072****
	Residual	10	< 0.001		
	Total	19			
Number of leaves shoot^{-1}	Sampling date	9	0.862	4.491	**0.01410***
	Residual	10	0.192		
	Total	19			
N shoot content (% DW)	Sampling date	9	0.167	6.882	**0.0029****
	Residual	10	0.024		
	Total	19			
P shoot content (% DW)	Sampling date	9	0.002	5.727	**0.0058****
	Residual	10	< 0.001		
	Total	19			
Shoot biomass (g DW)	Sampling date	9	0.124	16.257	**< 0.0001*****
	Residual	10	0.008		
	Total	19			
Shoot density (shoots m^{-2})	Sampling date	9	8.063	0.436	0.7392
	Residual	10	18.477		
	Total	19			

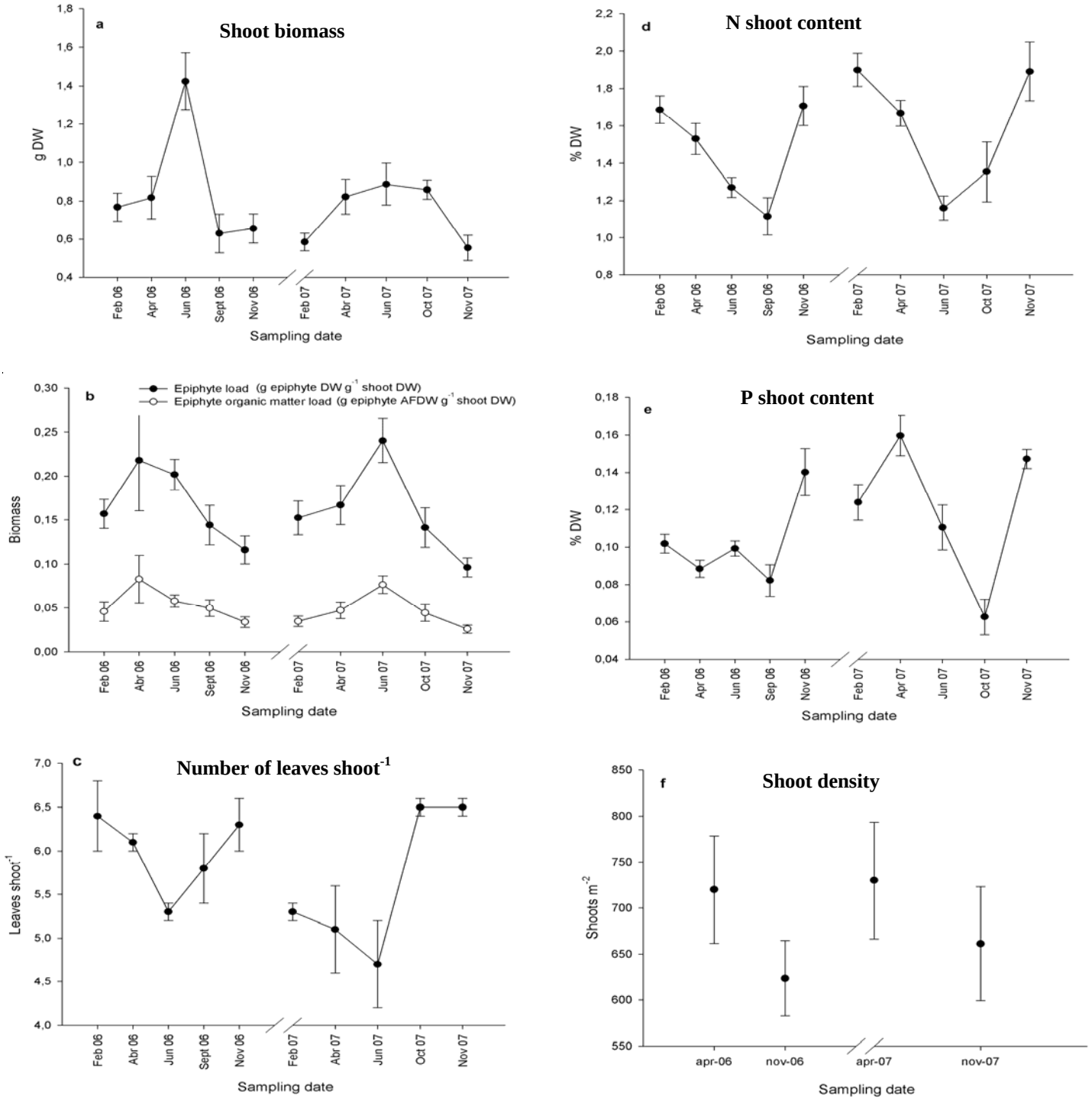


Fig. 27. Relevant descriptors of *P. oceanica* meadows (Mean ± SE): shoot biomass **(a)**, epiphyte load and epiphyte organic matter load **(b)**, number of leaves shoot^{-1} **(c)**, N shoot content **(d)**, P shoot content **(e)** and shoot density **(f)** for different sampling dates. February (feb), April (apr), June (jun), September (sep), October (oct), November (nov), 2006 (06), 2007 (07).

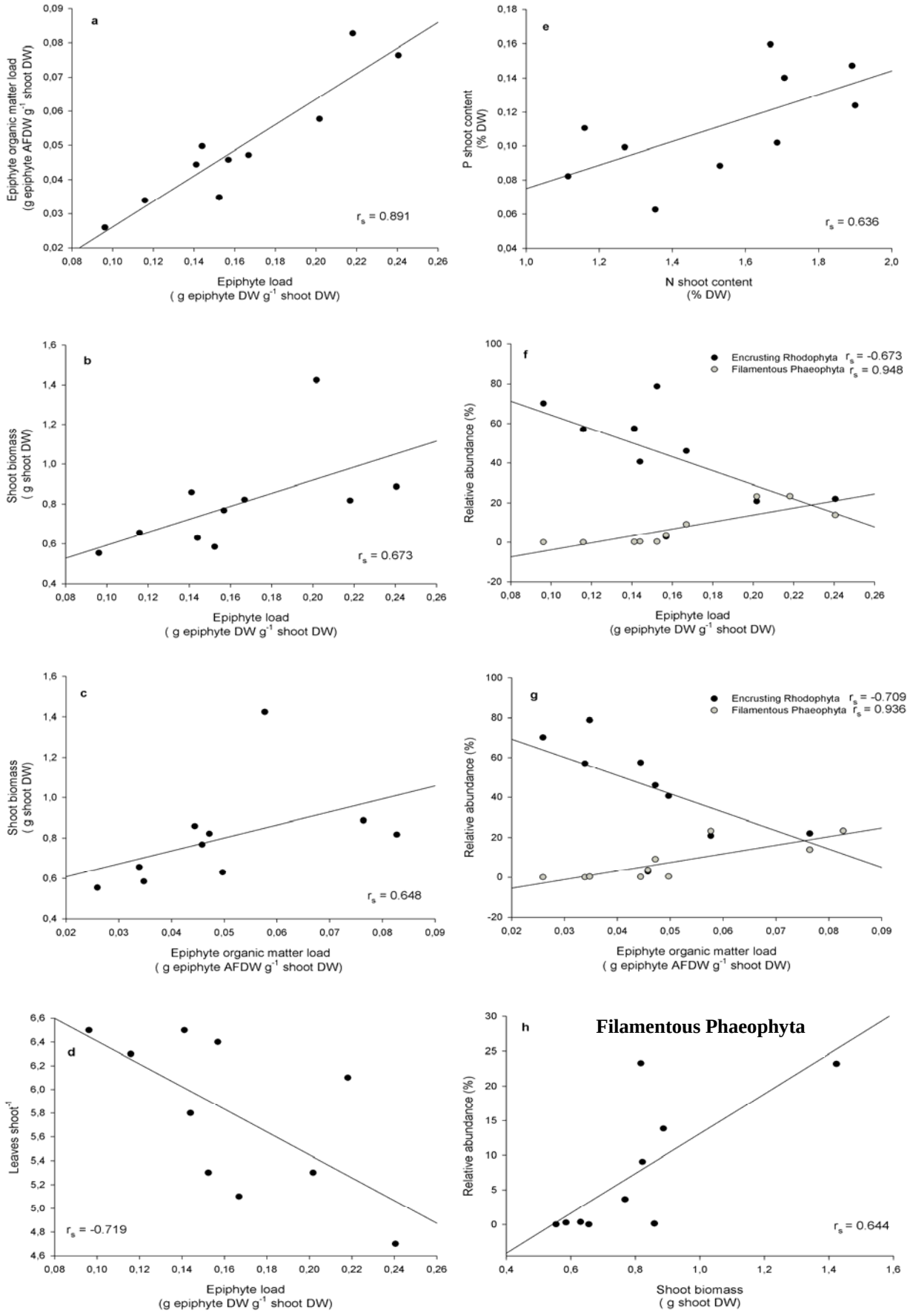


Fig. 28. Spearman rank correlations **(r_s)** between variables studied. Only main significant correlations are shown.

Discussion

Our study showed significant differences between sampling dates for almost all the variables studied. The application of TGGE fingerprinting technique and classical microscopy techniques were able to detect significant differences in composition of the macroeukaryotic leaf-epiphytic fraction of *P. oceanica*, when analysing OTUs profile and epiphyte taxa composition, respectively. Hence our results confirm those of previous evaluations of the sensitivity of the molecular technique to analyze the structure of this community (Chapters 2 and 4). On the one hand, OTUs profile differed between almost all pairs of sampling dates, but it was noticeable that composition not differed between June 2006 and April 2007 and between April 2007 and June 2007. Spring assemblage composition has been previously considered as highly homogeneous between *P. oceanica* shoots (Ballesteros 1987). In accordance with other studies (Van der Ben 1971; Ballesteros 1987; Romero 1988), our microscopy approach also detected a high relative abundance of encrusting and filamentous Phaeophyta (between 50% and 80%) and the presence of epiphytic taxa (*Cladosiphon* spp.), restricted to spring months (April and June). On the other hand, although microscopy approach found significant differences in epiphyte taxa composition between sampling dates, those differences were only attributed to the comparison of February 2006 with April 2006, February 2006 with June 2006 and February 2006 with November 2007. Results obtained suggested that TGGE molecular approach was able to detect more differences than microscopy method. It could be explained for different reasons: (1) underestimation of the real diversity: leaf-macroeukaryotic epiphytic assemblage description based on microscopy could be biased by the taxonomical expertise of the researcher, (2) taxonomic impediment: some individuals (juvenile, broken,…) are difficult to

determine under microscope and (3) TGGE technique could detect the presence of taxa even with low relative abundance in the assemblage (Valentini et al. 2008). In addition, OTUs richness and epiphyte taxa richness did not show the same trend (Fig. 25). Those reasons could also explain the detection of significant differences between sampling dates in OTUs richness, but not in epiphyte taxa richness. The highest OTUs richness was detected in late summer (September 2006), which corresponds to the mature stage of the assemblage and was consistent with other studies (Ballesteros 1987; Mazzella et al. 1989) and the lowest in late spring (June 2007), when the biomass and relative abundance of Phaeophyta is significantly higher than other epiphyte morphological groups (Ballesteros 1987; Romero 1988).

All relevant descriptors of *P. oceanica* meadows, with the exception of shoot density, showed significant differences between sampling dates. Mean shoot density ($\pm$ SE) in April was 725 $\pm$ 5.0 and 642.5 $\pm$ 18.7 in November, but no statistical differences were observed between those sampling dates. That result was consistent with previous studies (Guidetti et al. 2002). Shoot biomass reached a maximum in late spring (June), which was consistent with other studies that found a peak in May-June (Mazzella and Ott 1984; Buia et al. 1992; Guidetti et al. 2002). Number of leaves shoot^{-1} also differed between sampling dates although temporal variations were lowest when comparing with other descriptors of *P. oceanica*. The highest values were generally recorded in autumn (October and November) and the lowest in spring (April and June). Guidetti et al. 2002 also found an increase in number of juvenile and adult leaves shoot^{-1} in autumn. Maximum epiphyte load and epiphyte organic matter load were basically detected in spring and were related with the significant relative abundance of filamentous Phaeophyta (Ballesteros 1987; Romero 1988). High values of epiphyte organic matter

load were also detected in late summer (September 2006), related to significant relative abundance of filamentous Rhodophyta (Ballesteros 1987; Mazzella et al. 1989). Although N shoot content exhibited higher values in autumn and winter than in spring and late summer, as it was previously recorded (Fourqurean et al. 2007), P shoot content not showed this clear trend.

The relative abundance of filamentous Phaeophyta was positively correlated with epiphyte load and epiphyte organic matter load, whereas the relative abundance of encrusting Rhodophyta was negatively correlated with those descriptors of *P. oceanica* meadows. While Phaeophyta has been previously recognised as the most relevant contributor to epiphyte biomass assemblage, the contribution of Rhodophyta to biomass is limited (Romero 1988; Lepoint et al. 2007). On the contrary, epiphyte taxa richness and OTUs richness did not exhibit significant correlations with any relevant descriptor of *P. oceanica* meadows.

In summary, TGGE approach has demonstrated to be an excellent method to study the structure of the macroeukaryotic lef-epiphytic fraction because it was more sensitive than microscopy approach to detect changes along the temporal succession of the assemblage. Furthermore, TGGE fringerprinting technique allowed simultaneously processing a large number of samples and resulted useful to overcome taxonomy limitations contrasting microscopy approach. It was relevant that relative abundance of the main morphological epiphyte groups was more correlated with relevant descriptors of *P. oceanica* meadows than OTUs richness and epiphyte taxa richness. That result suggested that finding a method to measure the relative contribution of different OTUs in different stages of the assemblage could be useful in the description of the structure of the community.

SECTION V - Chapter 6

Detail of *P. oceanica* shoots in a refrigerated incubator

NR activity in seagrass (*P. oceanica*) leaf-epiphytic macroalgae

NR activity in seagrass (*P. oceanica*) leaf-epiphytic macroalgae

Background

Relationships between diversity and ecosystem functioning have been deeply explored in literature in different assemblages. Nitrogen acquisition from water column, which strongly depends on the activity of the enzyme NR in autotrophic organisms, is a key process in ecosystems dominated by seagrasses. Leaf-epiphytic macroalgae of the Mediterranean seagrass *P. oceanica* has been recognised as a highly diverse and dynamic assemblage that significantly contributes to nitrogen acquisition from water column. The present study evaluated NR activity, using an "*in vitro*" assay, in two stages of the leaf-epiphytic macroalgae with different taxa composition (summer vs. winter). Our main hypothesis is that leaf-epiphytic macroalgae composition is a relevant factor to explain NR activity of the leaf-epiphytic macroalgae fraction.

Materials and methods

Study site and sampling design

The study was performed in a shallow (depth < 12 m) *P. oceanica* meadow located in western Mediterranean Sea (Sant Elm, Majorca, Spain) (39º 34' N, 2º 20' E).

Leaf-epiphytic macroalgae NR activity and community structure (taxa richness and composition) were assessed in *P. oceanica* shoots collected inside quadrates of 0.25 m^2 both in late winter (February 2009) and in late summer (September 2009). Three quadrates were haphazardly placed in each of three sites (haphazardly selected) that were located hundreds of meters apart from each other. Within each site, the three quadrates were separated tens of meters from each other. Fourteen *P. oceanica* shoots were haphazardly collected in each quadrate at each sampling date. Half of the shoots

were used to measure leaf-epiphytic macroalgae NR activity and the remaining seven shoots were used to describe the assemblage structure. This number of shoots is representative of the *P. oceanica* leaf-epiphytic community in an area of 0.25 m^2 (Chapter 2).

Fourteen additional shoots were collected in each of the quadrates at one of the sites to assess the response of leaf-epiphytic macroalgae NR activity to a short-term NO_3^- pulse both in winter and in summer.

All shoots harvested in the field were kept in ice until arrival to the laboratory.

P. oceanica leaf-epiphytic macroalgae assemblage structure

We pooled the leaf-epiphytic community of seven shoots to constitute a single sample and thus we had three replicate samples from each site and sampling date (one from each quadrate). We only considered the epiphytes present on 10 cm leaf sections closest to the tip of the oldest and entire leaf (both outer and inner leaf sides) of each shoot, as it represents the mature stage of the community (Lepoint et al. 2007). Leaf-epiphytic macroalgae taxa richness (number of taxa until the lowest taxonomic level possible) and composition, measured as relative abundance (%) of the main macroalgae groups in *P. oceanica* leaves (encrusting Phaeophyta, encrusting Rhodophyta, filamentous Phaeophyta, filamentous Rhodophyta and other algae) were recorded for each sample following the procedure of Chapter 2. Other algae groups were referred to Chlorophyta and unknown epiphytic macroalgae. Macroalgae groups have been extensively used in other studies to study the spatio-temporal variability of the composition of macroalgae epiphytic assemblages (Piazzi et al. 2004; Pardi et al. 2006; Balata et al. 2008).

NR "*in vitro*" assay

Shoots harvested to measure leaf-epiphytic macroalgae NR activity were frozen in liquid nitrogen and subsequently stored at -80ºC until their processing. This sample storage procedure preserved NR activity allowing us to obtain an estimation of *"in situ"* enzymatic activity (Lartigue and Sherman 2002). NR "*in vitro*" assay was performed adapting the procedure described in Scheible et al. (1997) for *P. oceanica* leaf-epiphytic macroalgae. All glass material used to measure NR activity was cleaned with 4.5% HCl and rinsed with distilled water before performing the analyses.

NR activity measurements have been performed in the literature following either an "*in vivo*" or "*in vitro*" method. The "*in vivo*" approach requires membrane permeabilization to allow the buffered solution with NO_3^- to contact with the enzyme inside otherwise intact cells, where it is reduced to NO_2^- by NR using endogenous NADH. In the "*in vitro*" method the tissue is homogenised to obtain a suspension of the enzyme in a buffer solution that is supplied with NO_3^- and NADH to estimate NR activity. Interpretation of the *"in vivo"* assay is constrained by the difficulty to know substrate concentrations and the diffusion rates of NO_3^- into the cells and NO_2^- out of the cells (Hurd et al. 1995; Lartigue and Sherman 2002). The *"in vitro"* approach provides an estimation of the potential maximum NR capacity because enzyme activity is not limited by NO_3^- diffusion or transport into the cells. *"In vitro"* measurements of NR activity have been used in several studies to estimate the maximum capacity of nitrogen uptake (Berges 1997).

Similar to the community structure samples, we pooled the leaf-epiphytic community of seven shoots to constitute a single sample and thus we had three replicate samples from each site and sampling date (one from each quadrate). The epiphytic community of the shoots was gently scrapped off the leaves, pooled and a portion of

each sample was weighed (FW) and ground to a fine powder in a mortar with liquid nitrogen for determination of NR activity.

The homogenised sample was mixed and extracted with 2.5 ml ice-cold extraction buffer (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid – potassium hydroxide (HEPES-KOH) (pH = 7.5), 5 mM magnesium acetate ($Mg(CH_3COO)_2$), 1 mM EDTA, 1% (w/v) bovine serum albumin (BSA), 5 mM (w/v) dithiothreitol (DTT), 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 0.5 mM (w/v) phenylmethylsulfonyl fluoride (PMSF) (diluted in 99% (w/v) ethanol), 20 µM flavin adenine dinucleotide (FAD), 25 µM leupeptin, 5 µM sodium molybdate (Na_2MoO_4) and 1% (w/v) PVPP). BSA is included in the buffer because it could stabilize NR by binding inhibitory compounds and provide protein in higher concentration that the proteases can degrade in preference to NR (Berges and Harrison 1995; Lartigue and Sherman 2002). Leupeptin has been previously observed as an effective compound against NR-specific proteases in other species (Ingemarsson 1987). The addition of EDTA, DTT and the detergent Triton X-100 into the extraction buffer has been also related to maintaining NR activity (Hurd et al. 1995). Similarly, the addition of FAD to the extraction buffer was based on enhancement of NR activity in the presence of FAD in some species of microalgae (Vennesland and Solomonson 1972; Berges and Harrison 1995).

500 µl from an assay mix solution containing 100 mM HEPES-KOH (pH = 7.5), 5 mM potassium nitrate (KNO_3), 5 mM EDTA and 0.25 mM NADH were immediately mixed with 100 µl of sample extract obtained in the previous step and incubated at ambient seawater temperature (15ºC for winter and 26ºC for summer) and at the constant temperature of 17.5ºC (annual mean seawater temperature in the location

studied). Incubations were performed at a constant temperature to separate variability in NR activity associated to seasonal changes of leaf-epiphytic macroalgae assemblage structure to that linked to seasonality of water temperature.

Samples were incubated in a water bath with slight shaking (Lauda E100) for 60 min. The linearity between NO_2^- produced (from NO_3^- reduction) and incubation time was previously checked at reaction times of 2, 5, 10, 30 and 60 min and we found that the optimal incubation time to detect leaf-epiphytic macroalgae NR activity was 60 min (data not shown). After the incubation time, the reaction was stopped by adding to each tube 25 µl 0.6 M zinc acetate ($Zn(O_2CCH_3)_2(H_2O)_2$) and 75 µl 0.15 mM phenazine methosulphate (PMS). The NO_2^- formed by NR was determined by adding to each tube 300 µl 1% (w/v) sulphanilamide ($C_6H_8N_2O_2S$) in 3 M HCl and 300 µl 0.02% (w/v) N-(1-naphthyl)-ethylenediamine (NED) and measuring the absorbance (OD) at 540 nm in a Hitachi U-2900 spectrophotometer after 20 min. Three replicate tubes and a time zero control tube (containing 25 µl 0.6 M $Zn(O_2CCH_3)_2(H_2O)_2$ and 75 µl 0.15 mM PMS prior to adding the assay mix solution) were run for each sample and temperature. To correct for any internal NO_2^- present in algal tissue when performing the analyses, NO_2^- found in time zero control tube was subtracted from NO_2^- determined in the three replicate tubes.

After measuring NO_2^-, we dried the samples at 60ºC during at least 48 h and determined the leaf-epiphytic DW to establish a relationship between FW and DW (see below).

NR activity recovery

The possible loss of NR activity during extract preparation was assessed by adding a commercial NR enzyme (NR from *Arabidopsis thaliana,* SIGMA) into the extracts and

calculating the recovery of its activity (Munzarova et al. 2006). NR activity was measured in three different extract preparations: 100 µl extraction buffer with leaf-epiphytes (a), 1 µl commercial NR + 100 µl extraction buffer with leaf-epiphytes (b) and 1 µl commercial NR + 100 µl extraction buffer without leaf-epiphytes (c). The recovery of NR activity for each sample was calculated from values of enzymatic activity measured in the three different extracts according to the following formula: NR activity recovery (%) = (b – a / c) *100.

A slight modification of this procedure was tested by adding 5 mM nickel chloride ($NiCl_2$) to our extraction buffer (Munzarova et al. 2006). $NiCl_2$ has been previously recognised as an agent preventing negative effect of cyanogenic glycosides on NR activity (Munzarova et al. 2006), but it failed in increasing the recovery of commercial NR enzyme (data not shown).

Relationship between leaf-epiphytic FW, DW and AFDW

Leaf-epiphytic FW and DW determinations were used to calculate a conversion factor between them (DW = 0.1757 FW + 0.0227, $r^2 = 0.85$). It is important to notice that more than 80% of leaf-epiphytic DW are minerals (carbonates of encrusting epiphytes, sediment particles) (Terrados and Medina-Pons 2008), which do not contain NR enzyme. For this reason, we considered that NR activity based on leaf-epiphytic DW might be underestimated and expressed that variable as nmol $NO_3^- g^{-1}$ AFDW min^{-1} using an AFDW = 0.2 DW conversion factor (unpublished data).

Relationship between NO_3^- availability and leaf-epiphytic macroalgae NR activity

The response of leaf-epiphytic macroalgae NR activity to a short-term NO_3^- pulse was investigated by incubating seven shoots with the attached leaf-epiphytic community in filtered seawater (Whatman GF/C glass fibre filters) supplied with NO_3^- to achieve an

external concentration 5 μM above ambient concentrations. Water column NO_3^- concentrations in NW Mediterranean Sea generally varies from 0.1 μM to 0.5 μM (Lepoint et al. 2004), reaching values of 5 μM in highly eutrophic areas (i.e. NW Adriatic Sea) (Unesco (1990)). For this reason, we considered that short term NO_3^- pulses of that magnitude could be ecologically relevant in that area. The remaining seven shoots were incubated in filtered seawater without supplying NO_3^- (control). All incubations were performed in a refrigerated incubator (Raypa, IRE-160) for two days. This duration of the exposure to high NO_3^- availability has previously demonstrated to be long enough to increase NR activity in algae and vascular plants species (Cedergreen and Madsen 2003; Chow and de Oliveira 2008). Winter and summer shoots were incubated in filtered seawater collected at each sampling date and exposed to ambient photoperiod and temperature conditions (winter: 13:11 h light–dark (LD) cycle and 15ºC, summer: 13:11 h LD cycle and 26ºC). Both types of shoots were irradiated with 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (LI-COR Biosciences, LI-1400) and supplied with continuous air bubbling (Resum, AC9906). After incubations, shoots were frozen in liquid nitrogen, subsequently stored at -80ºC and processed following the NR "*in vitro*" assay (see above).

Data analysis

Nested ANOVA was performed to evaluate if leaf-epiphytic macroalgae taxa richness and relative abundance (%) of the main groups in *P. oceanica* leaves (encrusting Phaeophyta, encrusting Rhodophyta, filamentous Phaeophyta, filamentous Rhodophyta and other algae) were significantly different between sampling dates (winter vs. summer) and sites (1 to 6). Site was nested in sampling date (winter sites: 1 to 3, summer sites: 4 to 6).

Significant differences in leaf-epiphytic macroalgae NR activity (nmol NO_3^- g^{-1} AFDW min^{-1}) between sampling dates (winter vs. summer), temperatures (ambient vs. mean) and sites (1 to 6) were also assessed by means of a nested ANOVA. Site was nested in the interaction between temperature and sampling date.

A two-way factorial ANOVA was used to determine if leaf-epiphytic macroalgae NR activity (nmol NO_3^- g^{-1} AFDW min^{-1}) was significantly different between treatments (short-term 5 μM NO_3^- pulse vs. control) and temperatures (ambient vs. mean) for each sampling date (winter and summer).

Significance level in all statistical analyses was 0.05. Homogeneity of variances and normality of data were tested before performing ANOVA analyses using Cochran's C test and Kolmogorov–Smirnov test, respectively.

All analyses were performed using SIGMAPLOT 8.0 (SPSS Science 2002) and STATISTICA 7.1 software (Statsoft Inc. 2005).

Results

P. oceanica leaf-epiphytic macroalgae assemblage structure

P. oceanica leaf-epiphytic macroalgae assemblage showed significant differences in relative abundance (%) of the main algal groups between sampling dates. Winter assemblage was mainly composed by an encrusting layer of Phaeophyta (basically, *M. magnusii*) and Rhodophyta (*Hydrolithon* + *Pneophyllum* spp.). These two groups constituted more than 80% of the total leaf-epiphytic macroalgae abundance in winter samples. A small fraction (about 10% of total abundance) of filamentous Phaeophyta (mainly, *G. sphacelarioides* and *Cladosiphon* spp.) was also recorded in winter samples. Summer leaf-epiphytic macroalgae assemblage was basically composed of a mix of encrusting Rhodophyta (*Hydrolithon* + *Pneophyllum* spp.) and filamentous Rhodophyta

(generally, species belonging to Acrochaetiales and Ceramiales). These two groups could reach up to 84% and 22%, respectively, of the total abundance in the summer leaf-epiphytic macroalgae assemblage (Fig. 29a; Table 17). Leaf-epiphytic Chlorophyta (basically, *Cladophora* spp. and *Phaeophila dendroides*) were less abundant than other macroalgae groups in both sampling dates. Zooepiphytic groups (Bryozoa, Foraminifera, Hydrozoa and Nematoda) were more frequent in summer than in winter, although they represented a very low fraction compared tot epiphytic macroalgae on *P. oceanica* leaves in the two sampling dates. The significant differences in dominance patterns of the main groups of leaf-epiphytic macroalgae between sampling dates were not mirrored in taxa richness differences (Table 17).

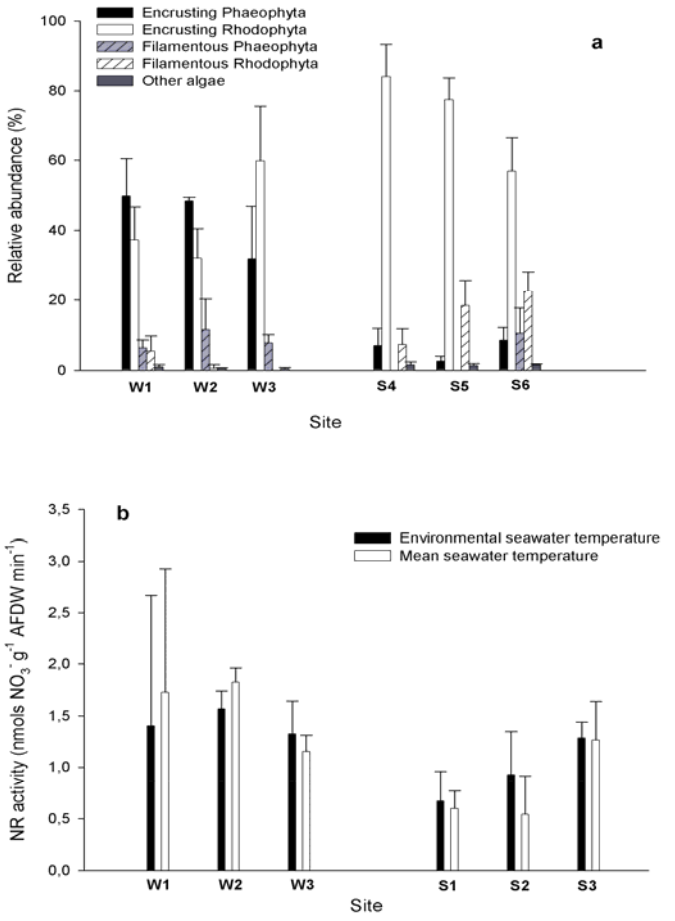


Fig. 29. Relative abundance of the main leaf-epiphytic macroalgae groups in winter (W) and summer (S) **(a)** and leaf-epiphytic macroalgae NR activity in W and S sites at ambient (15ºC (winter), 26ºC (summer)) and mean annual (17.5ºC) seawater temperature **(b)** in Sant Elm (Majorca, Western Mediterranean Sea).

Table 17. Results of nested ANOVA to evaluate if leaf-epiflora taxa richness and relative abundance (%) of the main leaf-epiflora groups of *P. oceanica* were different between sampling dates (winter vs. summer) and sites (1-6). Site was nested in sampling date (winter sites: 1-3, summer sites: 4-6). Significant values in **bold**. ***p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.** [#] Data were square root-transformed.

	Source of variation	df	MS	F	p
Encrusting Rhodophyta [#] (%)	Sampling date	1	21.76	13.71	**0.003 ****
	Site (Sampling date)	4	3.24	2.04	0.152
	Error	12	1.59		
	Total	17			
Encrusting Phaeophyta [#] (%)	Sampling date	1	85.01	39.23	**< 0.001 *****
	Site (Sampling date)	4	2.03	0.94	0.475
	Error	12	2.17		
	Total	17			
Filamentous Rhodophyta [#] (%)	Sampling date	1	31.54	21.37	**< 0.001 *****
	Site (Sampling date)	4	4.61	3.12	0.056
	Error	12	1.47		
	Total	17			
Filamentous Phaeophyta [#] (%)	Sampling date	1	19.09	8.80	**0.012 ***
	Site (Sampling date)	4	5.47	2.52	0.096
	Error	12	2.17		
	Total	17			
Other algae [#] (%)	Sampling date	1	0.72	3.30	0.094
	Site (Sampling date)	4	0.06	0.30	0.872
	Error	12	0.22		
	Total	17			
Leaf-epiflora taxa richness	Sampling date	1	10.89	2.36	0.150
	Site (Sampling date)	4	14.44	3.13	0.056
	Error	12	4.61		
	Total	17			

NR activity

Leaf-epiphytic macroalgae NR activity (nmol NO_3^- g^{-1} AFDW min^{-1}) showed significant differences ($p = 0.01$) between sampling dates. The winter assemblage exhibited higher NR activity than the summer assemblage. Leaf-epiphytic macroalgae NR activity measured at ambient temperature did not differ from that measured at mean annual

seawater temperature at Sant Elm in both seasons. The comparison between sites (nested in the interaction temperature and sampling date) showed no differences (Fig. 29b; Table 18).

Table 18. Results of nested ANOVA to evaluate if leaf-epiphytic macroalgae NR activity (nmol NO_2^- g^{-1} AFDW min^{-1}) was different between sampling dates (winter vs. summer), temperatures (ambient vs. mean) and sites (1-6). Ambient seawater temperature in St. Elm was 15 ºC and 26 ºC in winter and summer, respectively. Mean annual seawater temperature in St. Elm was 17.5 ºC. Site (random factor) was nested in the interaction between temperature and sampling date (fixed factors) (winter sites: 1-3, summer sites: 4-6). Significant values in **bold**. ***p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001**.

	df	NR activity MS	NR activity F	NR activity p
Temperature	1	0.0710	0.5378	0.471
Sampling date	1	1.0316	7.8094	**0.010 ****
Site(Temperature x Sampling date)	9	0.0857	0.6485	0.745
Error	23	0.0011		
Total	35			

Relationship between NO_3^- availability and leaf-epiphytic macroalgae NR activity

No significant differences were observed between NR activity of leaf-epiphytic macroalgae exposed to short-term 5 µM NO_3^- pulse and ambient seawater (control) either in winter or in summer. The interaction between treatment and temperature (ambient seawater temperature vs. mean seawater temperature) was also not significant (Fig. 30; Table 19).

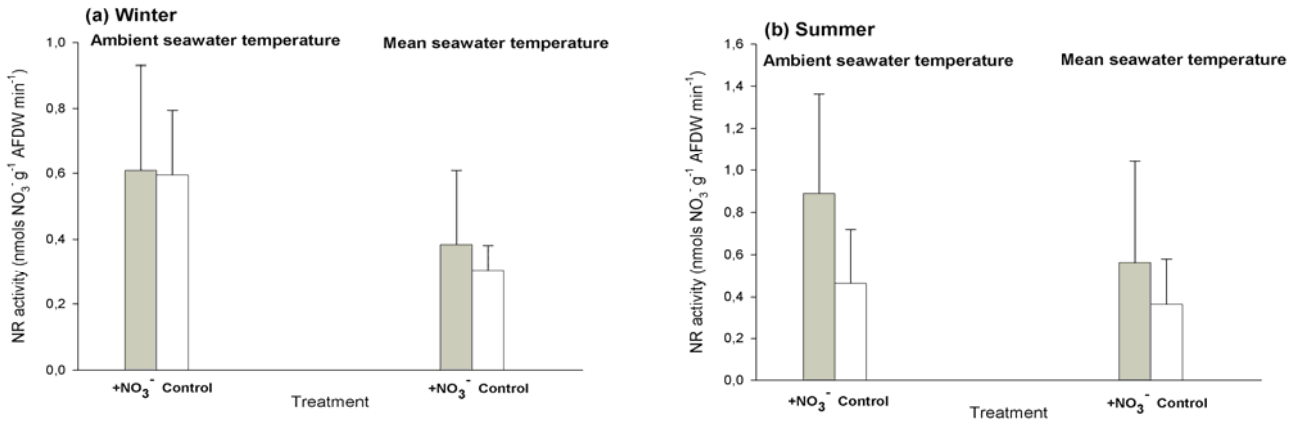


Fig. 30. Leaf-epiphytic macroalgae NR activity in shoots exposed to short-term 5 µM NO_3^- pulse and control treatment (not exposed to a 5 µM NO_3^- pulse) in winter (a) and summer (b) at ambient (15ºC (winter), 26ºC (summer)) and mean annual (17.5ºC) seawater temperature in Sant Elm (Majorca, Western Mediterranean Sea).

Table 19. Results of two-way factorial ANOVA to evaluate if leaf-epiphytic NR activity (nmol NO_3^- g^{-1} AFDW min^{-1}) was different between treatments (short-term 5 µM NO_3^- pulse vs. control) and temperatures (ambient vs. mean). Winter and summer analyses were individually performed. Ambient seawater temperature in St. Elm was 15 ºC and 26 ºC in winter and summer, respectively. Mean annual seawater temperature in St. Elm was 17.5 ºC. Significant values in **bold**.
*** p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.**

	Source of variation	df	MS	F	p
Winter	Treatment	1	0.006514	0.0433	0.840
	Temperature	1	0.198134	1.3166	0.284
	Treatment x Temperature	1	0.003161	0.0210	0.888
	Error	8	0.150487		
	Total	11			
Summer	Treatment	1	0.28914	0.6799	0.433
	Temperature	1	0.136228	0.3203	0.587
	Treatment x Temperature	1	0.038831	0.0913	0.770
	Error	8	0.425259		
	Total	11			

Discussion

Leaf-epiphytic macroalgae assemblage structure on *P. oceanica* varied significantly between sampling dates. The relative abundance of the main leaf-epiphytic macroalgae groups changed from dominance of an encrusting layer of Rhodophyta and Phaeophyta in winter to a dominance of encrusting and filamentous Rhodophyta in the summer assemblages. Similar seasonal changes have been previously described for the leaf-epiphytic macroalgae assemblage of *P. oceanica* (Van der Ben 1971; Ballesteros 1987; Mazzella et al. 1989). However, in those studies leaf-epiphytic macroalgae taxa richness increased from winter to summer, whereas taxa richness in our study remained unaffected. *P. oceanica* leaf-epiphytic macroalgae composition and taxa richness did not vary significantly among the sampling sites separated by hundreds of meters, suggesting similar growth conditions and availability of epiflora propagules. This is consistent with other studies where variability of *P. oceanica* leaf-epiphytic macroalgae

assemblage is lower at hundreds of m scale than at cm or km scale (Piazzi et al. 2004; Piazzi et al. 2007).

The winter and summer leaf-epiphytic macroalgae communities also differed in NR activity, with higher rates in the winter than in the summer assemblage. Pedersen and Borum (1997) suggested that slow-growing (e.g. encrusting) algae should display lower NO_3^- uptake rates than fast-growing (e.g. filamentous) algae. However, Lepoint et al. (2007) observed higher NO_3^- uptake rates in encrusting Rhodophyta than in encrusting and filamentous Phaeophyta, while growth rates of filamentous Phaeophyta were higher than those of encrusting Rhodophyta. NO_3^- uptake, NR activity, and algal growth are not always positively correlated (Thompson and Valiela 1999; Teichberg et al. 2007). NO_3^- uptake and NR activity depend on the internal NO_3^- pools, Nitrogen concentration in tissues, and the Nitrogen requirements established by growth rates (Thompson and Valiela 1999; Lartigue and Sherman 2005; Young et al. 2007). Phycobiliproteins are major pigments and nitrogen storage of Rhodophyta (Ryther et al. 1981; Lapointe and Duke 1984; Rico and Fernández 1996; Smit et al. 1997) which implies high Nitrogen requirements. Rhodophyta dominance of community composition, as we found in summer, and the high NO_3^- uptake rates of encrusting Rhodophyta measured by Lepoint et al. (2007) in *P. oceanica* leaf epiphytes suggest, that NR activity might be higher in summer than in winter. We found the opposite though because NR activity was higher in the winter assemblage when Phaeophyta accounted up to 60% of the epiphytic macroalgae abundance, most of it as encrusting forms. High NO_3^- availability promotes NR activity (Thompson and Valiela 1999; Cedergreen and Madsen 2003; Lartigue and Sherman 2005; Teichberg et al. 2007) which suggests that winter NR activity might reflect a higher availability of NO_3^- during

this season compared to summer. However we did not detect an increase of the NR activity of the leaf-epiphytic macroalgae assemblage when exposed to short-term 5 μM NO_3^- pulse neither in summer nor in winter which suggests that the differences in NR activity between sampling dates might not be related to differences of NO_3^- availability.

Short-term NO_3^- pulses (equal to or less than two days) have been observed to increase NR activity in different aquatic macrophytes (Cedergreen and Madsen 2003; Chow and de Oliveira 2008). Nevertheless, the stimulating effect of NO_3^- pulses on NR activity was achieved in those studies at much higher NO_3^- availability (about 500 μM) than in our study. Short-term NO_3^- pulses similar to those assayed here induced an increased NR activity for 12 h immediately after 6 and 30 μM NO_3^- additions in *Enteromorpha* sp. (Lartigue and Sherman 2005). Other studies have also found that NR activity remained high when measured after an exposure period of 24 h - 48 h with 500 μM NO_3^- (Touchette and Burkholder 2001; Cedergreen and Madsen 2003). All these data suggest that the duration of the exposure to the NO_3^- used in our study could be adequate to detect induction of leaf-epiphytic macroalgae NR activity but at pulses of a higher concentration than ours. It is important to notice that a moderate NO_3^- increase, similar in magnitude to the pulse assayed here, has shown significant effects on *P. oceanica* leaf-epiphytic macroalgae composition and biomass at least in summer assemblages. However, these effects were observed when the community was exposed to increased NO_3^- availability during longer periods of time than assayed here (Balata et al. 2008; Prado et al. 2008). All these evidences would indicate that a NO_3^- pulse much higher than assayed in our study might increase leaf-epiphytic macroalgae NR activity but it would not be ecologically relevant.

NR activity recovery assay with commercial NR enzyme showed that a significant portion of activity was lost during extract preparation. In order to improve NR stability in the epiphytic macroalgae extracts we added $NiCl_2$ (following the procedure of Munzarova et al. (2006)) but no improvement of the recovery of enzyme activity was obtained (data not shown), probably because vascular plants and algae species may differ in the content of chemical compounds inhibiting NR activity. Munzarova et al. (2006) is the only study that has evaluated the loss of NR activity during extraction, showing that enzyme activity recovery from *Glyceria maxima* leaf extracts was less than 20%. In that study, the addition of NiCl improved NR activity recovery in *G. maxima* leaf extracts but no significant effect was observed in other extracts from the same species. Previous estimations of NR activity in literature should be considered with caution because the recovery of NR activity is almost never done and we are aware that we might have underestimated leaf-epiphytic macroalgae NR activity. Hence, comparison between our data and results obtained in other studies is difficult because of two reasons: (1) information about loss of NR activity during sample preparation is not available in most of the studies and (2) NR activity has mainly been studied in individual species and not in assemblages with a high fraction of minerals such as seagrass epiphytic communities.

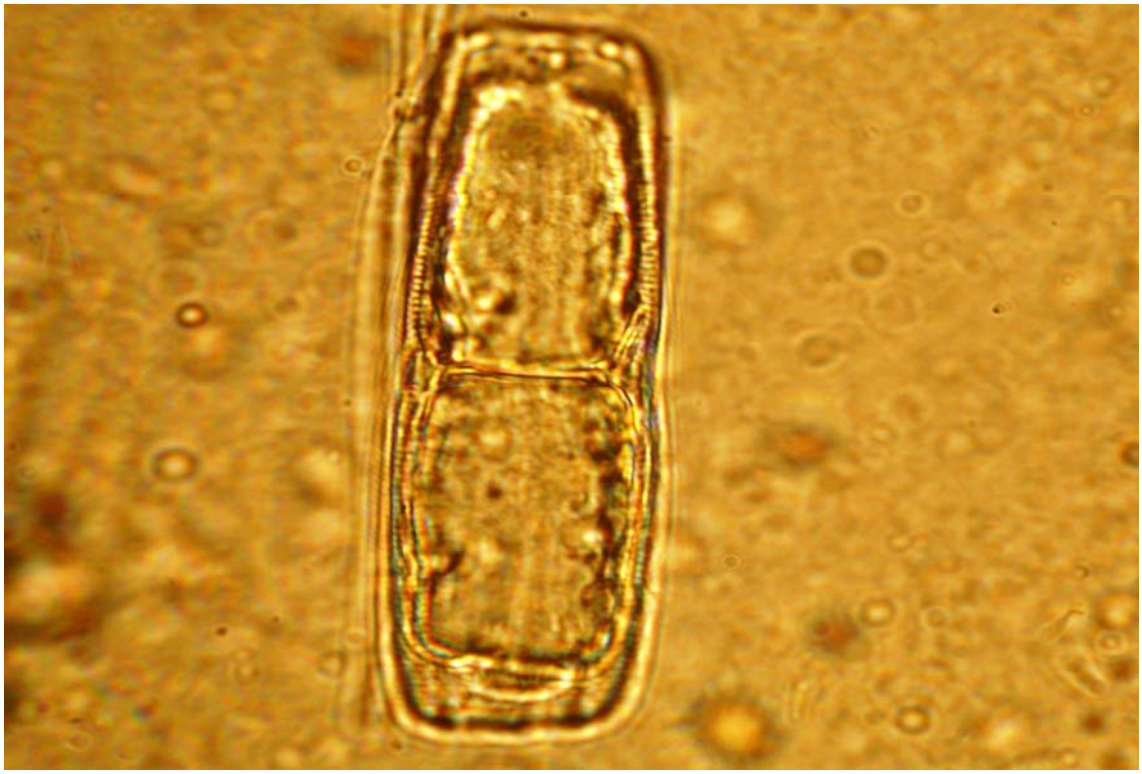
The NR activity of leaf-epiphytic macroalgae of the tropical seagrass *T. testudinum* varied between 4 and 12 nmol NO_3^- g^{-1} frozen weight min^{-1} while those of the temperate seagrass *A. antarctica* varied between 3 and 7 nmol NO_3^- g^{-1} frozen weight min^{-1} (Young et al. 2005). These values are higher than the NR rates we measured in the epiphytic algae on *P. oceanica* leaves but those studies does not provide information about the mineral content of the epiphytic communities assayed or

about its species composition, which makes it difficult to conclude anything about the differences in the NR activity of epiphytic communities of those seagrass species and that of *P. oceanica.*.

Assuming that NR activity indicates the potential NO_3^- uptake rate, we compared the *P. oceanica* leaf-epiflora NR activity measured in the present study with *P. oceanica* epiflora NO_3^- uptake rates measured by Lepoint et al. (2007). They estimated that the annual mean NO_3^- uptake rate was about 2 nmol NO_3^- g^{-1} AFDW min^{-1} in encrusting Rhodophyta and 0.6 nmol NO_3^- g^{-1} AFDW min^{-1} in encrusting and filamentous Phaeophyta. These NO_3^- uptake rates are similar to the NR rates measured here suggesting that the NR activity in the leaf-epiphytic macroalgae assemblage on *P. oceanica* was high enough to account for a major part of the expected NO_3^- uptake from the water column.

In summary, the results obtained in the present study suggest an association between NR activity and the composition leaf-epiphytic macroalgae assemblage on *P. oceanica*. Although temperature and NO_3^- availability have been previously reported as major factors determining NR activity (Gao et al. 2000; Touchette and Burkholder 2001), they were not able to explain the detected differences in NR activity between winter and summer leaf-epiphytic macroalgae assemblages. Moreover, both leaf-epiphytic macroalgae composition and NR activity were similar among sites hundreds of meters apart, emphasising the importance of species composition for enzymatic activity. Hence, the seasonal succession of the *P. oceanica* leaf-epiphytic macroalgae assemblage is likely to play a significant role in nitrogen dynamics in this ecosystem. Further investigations are needed to evaluate how changes in leaf-epiphytic macroalgae assemblage composition driven by eutrophication will affect NR activity.

SECTION VI - General discussion



Detail of epiphyte diatom on *P. oceanica* leaf

Studies about the structure of the macroeukaryotic epiphytic fracion of *P. oceanica* leaves, a highly dynamic and diverse assemblage, have been traditionally performed using classical microscopy methods. Most of those studies have focused in a specific group of that assemblage (Van der Ben 1971; Antolic 1986; Ballesteros 1987; Hayward 1975; Boero 1981). Number of studies focusing on whole macroeukaryotic epiphytic fraction is significantly lower (Casola et al. 1987; Mazzella et al. 1989). This fact is probably related to limitations in the knowledge of taxonomy of such a wide spectrum of epiphytic groups.

Alternative molecular techniques based on SSU genes have progressively replaced classical techniques to study the structure and dynamics of microbial and microeukaryotic communities since mid-80's. They have solved some limitations of classical culture techniques of microorganisms (Head et al. 1998; Fuhrman 2008), discovering an unknown diversity in different environments (Giovannoni et al. 1990; DeLong 1992; Bruemmer at al. 2004) and allowing obtaining diversity estimations independently from the taxonomical expertise of the researcher. Based on those advantages, we proposed to apply two commonly used molecular techniques, TGGE and clone libraries, to study the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves as an alternative to classical microscopy approach. Main conclusions obtained could be summarized in:

1. TGGE and SSU clone libraries resulted to be excellent approaches to monitor changes in the structure of the macroeukaryotic epiphytic fraction of *P. oceanica* leaves when comparing different stages of the seasonal succession (Chapters 2, 4 and 5) and the mature stage in two different locations (Chapter 3).

2. Results obtained applying the two molecular techniques were similar to those from microscopy approach and were in accordance with that from previous studies, although molecular techniques were generally able to detect higher diversity than microscopy approach. Some reasons that could explain that fact, in addition to the bias caused by taxonomical expertise of the researcher, were the taxonomic impediment (the determination of some individuals (juvenile, broken,…) was difficult under microscope) and molecular methods allowed the detection of low abundant taxa in the assemblage (Valentini et al. 2008).

3. On the one hand, TGGE and SSU clone libraries were suitable to simultaneously analyse large amounts of samples and, therefore, involving less time-consuming than classical microscopy approach. The application of molecular approaches was also independent from the knowledge of the taxonomy of groups composing the epiphytic assemblage, contrasting to microscopy methods.

4. On the other hand, the application of molecular methods involved a higher economic cost (mainly due to the use of reagents, including phenol, acrilamide, $AgNO_3$,…) than microscopy approach.

5. Comparing the two molecular techniques, TGGE resulted to be less time-consuming than SSU clone libraries, but the later approach was more appropriate than the former one to putatively identify epiphytic taxa explaining variability in the structure of that assemblage. Contrasting to SSU clone libraries, TGGE would be more recommended to rapidly assess the structure of different stages of the assemblage. SSU clone libraries would be more recommended to deeply assess the structure of different stages of the

assemblage, allowing the identification of epiphytic taxa composing the community.

The second main objective of this PhD thesis was studying the relationship between diversity/composition of the epiphytic assemblage and ecosystem functioning (Chapter 6). Traditional studies have been focused in the description of the structure and the diversity of a wide spectrum of communities. However, recently, relationships between diversity/composition of assemblages and ecosystem functioning in aquatic and terrestrial environments have been found (McGrady-Steed et al. 1997; Stachowicz et al. 1999; Aoki and Mizushima 2001; Cardinale et al. 2002; Downing and Leibold 2002; Lepoint et al. 2007). We focused our attention in studying the relationship between the diversity/composition of the macroalgae epiphytic component of *P.oceanica* leaves and nitrogen assimilation capacity from water column, which is a key process in coastal ecosystems dominated by seagrasses (Romero et al. 2006). We measured NR activity, which is responsible of the initial reduction of NO_3^- to NO_2^-) (Solomonson and Barber 1990; Romero et al. 2006), in two stages of the macroalgae epiphytic component (winter vs. summer). We selected those two different stages because they presented a different composition in macroalgae epiphytic component, as we detected using both classical microscopy and molecular methods. Main conclusion obtained could be summarized in:

1. Diversity/composition of the macroalgae epiphytic component was linked to variability in NR activity of the assemblage, reinforcing the relationship between diversity and ecosystem functioning found in other communities. The composition of the assemblage suggested playing a relevant role in determining

nitrogen assimilation capacity from water column in different stages of the epiphytic assemblage.

SECTION VII - References

Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL et al. (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature 430(6999): 551-554

Alcoverro T, Duarte CM, Romero J (1997) The influence of herbivores on *Posidonia oceanica* epiphytes. Aquat Bot 56: 93-104

Alcoverro T, Manzanera M, Romero J (2000) Nutrient mass balance of the seagrass *Posidonia oceanica:* the importance of nutrient retranslocation. Mar Ecol Prog Ser 194: 13-21

Anderson MJ (2001) A new method for a non-parametric multivariate analysis of variance. Austral Ecology 26: 32-46

Antolic B (1985) Distribution of epipphytic flora on *Posidonia oceanica* (L.) Delile leaves (National Park "Kornati" - central Adriatic). Acta Adriat 26(2): 135-143

Antolic B (1986) Epiphytic flora on leaves of *Posidonia oceanica* (L.) Delile from the area of Dubrovnik (South Adriatic). Acta Adriat 27(1-2): 37-49

Aoki I, Mizushima T (2001) Biomass diversity and stability of food webs in aquatic ecosystems. Ecol Res 16: 65-71

Balata D, Bertocci I, Piazzi L, Nesti U (2008) Comparison between epiphyte assemblages of leaves and rhizomes of the seagrass *Posidonia oceanica* subjected to different levels of anthropogenic eutrophication. Estuar Coast Shelf Sci 79: 533-540

Balata D, Nesti U, Piazzi L, Cinelli F (2007) Patterns of spatial variability of seagrass epiphytes in the north-west Mediterranean Sea. Mar Biol 15: 2025-2035

Ballesteros E (1987) Estructura i dinàmica del poblament algal de les fulles de *Posidonia oceanica* (L.) Delile als herbeis de Tossa de Mar (Girona). Bullt Inst Cat Hist Nat 54: 13-30

Bengtsson J (1998) Which species? What kind of diversity? Which ecosystem function? Some problems in studies of relations between biodiversity and ecosystem function. Appl Soil Ecol 10: 191-199

Berges JA (1997) Miniview: Algal nitrate reductases. Eur J Phycol 32(1): 3-8

Berges JA, Harrison PJ (1995) Nitrate reductase activity quantitatively predicts the rate of nitrate incorporation under steady state light limitation: a revised assay and characterization of the enzyme in three species of marine phytoplankton. Limnol Oceanogr 40(1): 82-93

Blankenship LE, Yayanos AA (2005). Universal primers and PCR of gut contents to study marine invertebrate diets. Mol Ecol 14: 891-899

Boero F (1981) Systematics and ecology of the Hydroid population on two *Posidonia oceanica* meadows. PSZNI Mar Ecol 2(3): 181-197

Borowitzka MA, Lavery PS, Van Keulen M (2006) Epiphytes of seagrasses. In: Larkum AWD, Orth RJ, Duarte CM (eds) Seagrasses: biology, ecology and conservation. Springer, Dordrecht, The Netherlands. pp 441–461

Borum J, Greve TM (2004) The four European seagrass species. In: Borum J, Duarte CM, Krause-Jensen D, Greve TM (eds). European seagrasses: an introduction to monitoring and management. The M&Ms project. pp 1-7

Brower JE, Zar JH (1984) Field and laboratory methods for general ecology. 2nd edition. W.C. Brown Publishers, Dubuque, USA, 226 pp

Bruemmer IHM, Felske ADM, Wagner-Doebler I (2004) Diversity and Seasonal Changes of Uncultured Planctomycetales in River Biofilms. Appl Environ Microbiol 70: 5094-5101

Buia MC, Cormaci M, Furnari G, Mazzella L (1989) *Posidonia oceanica* off Capo Passero (Sicily, Italy): leaf phenology and leaf algal epiphytic community. In: Boudouresque CF, Meinesz A, Fresi E, Gravez V (eds) 2nd Int Workshop on *Posidonia oceanica* Beds. GIS Posidonie publ, Marseille, France, pp 127-143

Buia MC, Zupo V, Mazzella L (1992) Primary production and growth dynamics in *Posidonia oceanica*. PSZNI Mar Ecol 13(1): 2-16

Canals M, Ballesteros E (1997) Production of carbonate particles by phytobenthic communities on the Mallorca-Menorca shelf, northwestern Mediterranean Sea. Deep-Sea Res Pt II 44(3-4): 611-629

Cancemi G, De Falco G, Pergent G (2000) Impact of a fish farming facility on a *Posidonia oceanica* meadow. Biol Mar Medit 7(2): 341-344

Cancemi G, De Falco G, Pergent G (2003) Effects of organic matter input from a fish farming facility on a *Posidonia* oceanica meadow. Estuar Coast Shelf Sci 56: 961-968

Cardinale BJ, Palmer MA, Collins SC (2002) Species diversity enhances ecosystem functioning through interspecific facilitation. Nature 415: 426-429

Casola E, Scardi M, Mazzella L, Fresi E (1987) Structure of the epiphytic community of *Posidonia oceanica* leaves in a shallow meadow. PSZNI Mar Ecol 8(4): 285-296

Cebrián J, Duarte CM, Marbà N, Enríquez S (1997) Magnitude and fate of the production of four co-occurring Western Mediterranean seagrass species. Mar Ecol Prog Ser 155: 29-44

Cebrián J, Duarte CM, Marbà N, Enríquez S, Gallegos M, Olesen B (1996) Herbivory on *Posidonia oceanica:* magnitude and variability in the Spanish Mediterranean. Mar Ecol Prog Ser 130: 147-155

Cedergreen N, Madsen TV (2003) Nitrate reductase activity in roots and shoots of aquatic macrophytes. Aquat Bot 76(3): 203-212

Chow F, de Oliveira MC (2008) Rapid and slow modulation of nitrate reductase activity in the red macroalga *Gracilaria chilensis* (Gracilariales, Rhodophyta): influence of different nitrogen sources. J Appl Ecol 20(5): 775-782

Cinelli F, Cormaci M, Furnari G, Mazzella L (1984) Epiphytic macro-flora of *Posidonia oceanica* (L.) Delile leaves around the island of Ischia (Gulf of Naples). In: Boudouresque CF, Jeudy de Grissac A, Olivier J (eds) 1st Int Workshop on *Posidonia oceanica* Beds. GIS Posidonie publ, Marseille, France, pp 91–99

Clarke KR (1993) Non-parametric multivariate analyses of change in community structure. Aust J Ecol 18: 117-143

Clarke KR, Gorley RN (2001) Primer (Plymouth Routines in Multivariate Ecological Research) V5: User Manual/Tutorial. Primer-E Ltd, Plymouth. UK

Cornelisen CD, Thomas FIM (2002) Ammonium uptake by seagrass epiphytes: isolation of the effects of water velocity using an isotope label. Limnol Oceanogr 47: 1223-1229

Cornelisen CD, Thomas FIM (2006) Water flow enhances ammonium and nitrate uptake in a seagrass community. Mar Ecol Prog Ser 312: 1-13

Dahllöf I (2002) Molecular community analysis of microbial diversity. Curr Opin Biotechnol 13: 213-217

Davison IR, Stewart WDP (1984) Studies on nitrate reductase activity in *Laminaria digitata* (Huds.) Lamour. I. Longitudinal and transverse profiles of nitrate reductase activity within the thallus. J Exp Mar Biol Ecol 74: 201-210

Delgado O, Ruiz JM, Pérez M, Romero J, Ballesteros E (1999) Effects of fish farming on seagrass (Posidoniaoceanica) in a Mediterranean bay: seagrass decline after organic loading cessation. Oceanol Acta 22:109–117

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89: 5685-5689

Díaz-Almela E, Marbà N, Álvarez E, Balestri E, Ruiz-Fernández JM, Duarte CM (2006) Patterns of seagrass (*Posidonia oceanica*) flowering in the Western Mediterranean. Mar Biol 148(4): 723-742

Díez B, Pedrós-Alió C, Marsh TL, Massana R (2001) Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl Environ Microbiol 67(7): 2942-2951

Doddema H, Howari M (1983) In vivo nitrate reductase activity in the seagrass *Halophila stipulacea* from the Gulf of Aqaba (Jordan). Bot Mar 26: 307-312

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19(14): 4008

Downing AL, Leibold MA (2002) Ecosystem consequences of species richness and composition in pond food webs. Nature 416: 837-841

Doyle JJ (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13-15

Drake LA, Dobbs FG, Zimmerman RG (2003) Effects of epiphyte load on optical properties and photosynthetic potential of the seagrasses *Thalassia testudinum* Banks ex Kδnig and *Zostera marina* L. Limnol Oceanogr 48: 456-63

Duffy JE, Harvilicz AM (2001) Species-specific impacts of grazing amphipods in an eelgrass-bed community. Mar Ecol Prog Ser 223: 201-211

Duffy JE, MacDonald KS, Rhode JM, Parker JD (2001) Grazer diversity, functional redundancy, and productivity in seagrass beds: an experimental test. Ecology 82(9): 2417-2434

Eckenrode VK, Arnold J, Meagher RB (1985) Comparison of the nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs. J Mol Evol 21(3): 259-269

Eickbush TH, Eickbush DG (2007) Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics 175: 477-485

Felske A, Engelen B, Nübel U, Backhaus H (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. Appl Environ Microbiol 62(11): 4162-4167

Fernández-Torquemada Y, Díaz-Valdés M, Colilla F, Luna B, Sánchez-Lizaso JL, Ramos-Esplá A (2008). Descriptors from *Posidonia oceanica* (L.) Delile meadows in coastal waters of Valencia, Spain, in the context of the EU Water Framework Directive. ICES J Mar Sci 65: 1492-1497

Figueiredo MA de O, Kain (Jones) JM, Norton TA (2000) Responses of crustose corallines to epiphyte and canopy cover. J Phycol 36: 17-24

Floyd R, Abebe E, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. Mol Ecol 11: 839-850

Foucher A, Wilson M (2002) Development of a polymerase chain reaction-based denaturing gradient gel electrophoresis technique to study nematode species biodiversity using the 18s rDNA gene. Mol Ecol Notes 2(1): 45-48

Fourqurean JW, Marbà N, Duarte CM, Díaz Almela E, Ruiz-Halpern S (2007) Spatial and temporal variation in the elemental and stable isotopic content of the seagrasses *Posidonia oceanica* and *Cymodocea nodosa* from the Illes Balears, Spain. Mar Biol 151: 219-232

Fourqurean JW, Zieman JC, Powell GVN (1992) Phosphorus limitation of primary production in Florida Bay: evidence from the C:N:P ratios of the dominant seagrass *Thalassia testudinum*. Limnol Oceanogr 37: 162-171

Fuhrman J (2008) Measuring Diversity In: Zengler K (ed) Accessing uncultivated microorganisms. From the environment to organisms and genomes and back. ASM press, Washington, USA, pp 131-152

Furnari G, Giaccone G, Cormaci M, Alongi G, Serio D (2003) Biodiversità marina delle coste italiane: catalogo del macrofitobenthos. Biol Mar Medit 10: 1-482

Gacia E, Costalago D, Prado P, Piorno D, Tomas F (2009) Mesograzers in *Posidonia oceanica* meadows: an update of data on gastropod-epiphyte-seagrass interactions. Bot Mar 52: 439-447

Gacia E, Duarte CM (2001) Sediment retention by a Mediterranean *Posidonia oceanica* meadow: the balance between deposition and resuspensión. Estuar Coast Shelf Sci 52: 505-514

Gacia E, Duarte CM, Middelburg JJ (2002) Carbon and nutrient deposition in a Mediterranean seagrass (*Posidonia oceanica*) meadow. Limnol Oceanogr 47: 23-32

Gadanho M, Sampaio JP (2004) Application of temperature gradient gel electrophoresis to the study of yeast diversity in the estuary of the Tagus river, Portugal. FEMS Yeast Res 5(3): 253-261

Ganley ARD, Kobayashi T (2007) Highly efficient concerted evolution in the ribosomal DNA repeats: total rDNA repeat variation revealed by whole-genome shotgun sequence data. Genome Res 17: 184-191

Gao Y, Smith GJ, Alberte RS (2000) Temperature dependence of nitrate reductase activity in marine phytoplankton: biochemical analysis and ecological implications. J Phycol 36: 304-313

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345: 60-63

Giribet G, Okusu A, Lindgren AR, Huff SW, Schrödl M, Nishiguchi MK (2006) Evidence for a clade composed of molluscs with serially repeated structures: monoplacophorans are related to chitons. Proc Natl Acad Sci USA 103(20): 7723-7728

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003) Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl Environ Microbiol 69(3): 1800-1809

Gobert S, Cambridge ML, Velimirov B, Pergent G, Lepoint G, Bouquegneau J-M, Dauby P, Pergent-Martini C, Walker DI (2006) Biology of *Posidonia*. In: Larkum AWD, Orth RJ, Duarte CM (eds) Seagrasses: Biology, ecology and conservation. Springer, Dordrecht, the Netherlands, pp 387-408

Granata TC, Serra T, Colomer J, Casamitjana X, Duarte CM, Gacia E (2001) Flow and particle distributions in a nearshore seagrass meadow before and after a storm. Mar Ecol Prog Ser 218:95-106

Greve TM, Binzer T (2004) Which factors regulate seagrass growth and distribution?. In: Borum J, Duarte CM, Krause-Jensen D, Greve TM (eds) European seagrasses: an introduction to monitoring and management. The M&Ms project, pp 19-23

Guidetti P, Lorenti M, Buia MC, Mazzella L (2002) Temporal Dynamics and Biomass Partitioning in Three Adriatic Seagrass Species: *Posidonia oceanica, Cymodocea nodosa, Zostera marina.* PSZNI Mar Ecol 23(1): 51-67

Guiry MD, Guiry GM (2009) AlgaeBase. World-wide electronic publication, National University of Ireland, Galway, Ireland. http://www.algaebase.org

Hall BG (2004) Phylogenetic trees made easy: a how-to manual. 2nd Edition. Sinauer Associates, Inc. Sunderland, UK, 221 pp

Harper GL, Sheppard SK, Harwood JD, Read DS, Glen DM, Bruford MW, Symondson WOC (2006) Evaluation of temperature gradient gel electrophoresis for the analysis of prey DNA within the guts of invertebrate predators. B Entomol Res 96:295-304

Hayward PJ (1975) Observations on the bryozoan epiphytes of *Posidonia oceanica* from the island of Chios (Aegean Sea). In: Pouyet S (ed) Bryozoa 1974. Université Claude Bernard, Lyon, France, pp 347-356

Head IM, Saunders JR, Pickup RW (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb Ecol 35(1):1-21

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci 270: 313-321

Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63(8): 3233-3241

Hofrichter R (2005) El Mar Mediterráneo. Fauna-Flora-Ecología. Vol II/1 Guía sistemática y de identificación. Ediciones Omega. Barcelona, España, 849 pp

Hong YK, Sohn CH, Lee KW, Kim HG (1997) Nucleic acid extraction from seaweed tissues for polymerase chain reaction. J Mar Biotechnol 5(2–3): 95-99

Hu Z, Zeng X, Wang A, Shi C, Duan D (2004) An efficient method for DNA isolation from red algae. J Appl Ecol 16: 161-166

Hurd CL, Berges JA, Osborne J, Harrison PJ (1995) An in vitro nitrate reductase assay for marine macroalgae: optimization and characterization of the enzyme for *Fucus gardneri* (Phaeophyta). J Phycol 31: 835-843

Huys R, Llewelyn-Hughes J, Olson PD, Nagasawa K (2006) Small subunit rDNA and Bayesian inference reveal *Pectenophilus ornatus* (Copepoda *incertae sedis*) as highly transformed Mytilicolidae, and support assignment of Chondracanthidae and Xarifiidae to Lichomolgoidea (Cyclopoida). Biol J Linn Soc Lond 87: 403-425

Infantes E, Terrados J, Orfila A, Cañellas B, Álvarez A (2009) Wave Energy and the Upper Depth Limit Distribution of *Posidonia oceanica*. Bot Mar 52: 419-427

Ingemarsson B (1987) Nitrogen utilization in *Lemna*. I. Relations between net nitrate flux, nitrate reduction, and *in vitro* activity and stability of nitrate reductase. Plant Physiol 85: 856-859

Invers O, Pérez M, Romero J (2002) Seasonal nitrogen speciation in temperate seagrass *Posidonia oceanica* (L.) Delile. J Exp Mar Biol Ecol 273: 219-240

Kendrick GA, Burt GA (1997) Seasonal changes in epiphytic macroalgal assemblages between offshore exposed and inshore protected *Posidonia sinuosa* Cambridge et Kuo seagrass meadows, Western Australia. Bot Mar 40:77-85

Kendrick GA, Lavery PS (2001) Assessing biomass, assemblage structure and productivity of algal epiphytes on seagrasses. In: Short FT, Coles RG, Short CA (eds) Global Seagrass Research Methods. Elsevier, Amsterdam, The Netherlands, pp 199–222

Khanuja SPS, Shasany AK, Darokar MP, Kumar S (1999) Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol Biol Rep 17: 1-7

La Claire II JW, Herrin DL (1997) Co-isolation of high-quality DNA and RNA from coenocytic green algae. Plant Mol Biol Rep 15: 263-272

Lapointe BE, Duke CS (1984) Biochemical strategies for growth of *Gracilaria tikvahiae* (Rhodophyta) in relation to light intensity and nitrogen availability. J Phycol 20: 488-495

Lartigue J, Sherman TD (2002) Field assays for measuring nitrate reductase activity in *Enteromorpha* sp. (Chlorophyceae), *Ulva* sp. (Chlorophyceae) and *Gelidium* sp. (Rhodophyceae). J Phycol 38(5): 971-982

Lartigue J, Sherman TD (2005) Response of *Enteromorpha* sp. (Chlorophyceae) to a nitrate pulse: nitrate uptake, inorganic nitrogen storage and nitrate reductase activity. Mar Ecol Prog Ser 292: 147-157

Lavery PS, Reid T, Hyndes GA, Van Elven BR (2007) Effect of leaf movement on epiphytic algal biomass of seagrass leaves. Mar Ecol Prog Ser 338: 97-106

Leoni V, Pasqualini V, Pergent-Martini C, Vela A, Pergent G (2006) Morphological responses of *Posidonia oceanica* to experimental nutrient enrichment of the canopy water. J Exp Mar Biol Ecol 339: 1-14

Lepoint G, Defawe O, Gobert S, Dauby P, Bouquegneau JM (2002a) Experimental evidence for N recycling in the leaves of the seagrass *Posidonia oceanica*. J Sea Res 48: 173-179

Lepoint G, Gobert S, Dauby P, Bouquegneau JM (2004) Contributions of benthic and planktonic primary producers to nitrate and ammonium uptake fluxes in a nutrient-poor shallow coastal area (Corsica, NW Mediterranean). J Exp Mar Biol Ecol 302: 107-122

Lepoint G, Havelange S, Gobert S, Bouquegneau JM (1999) Fauna vs flora contribution to the leaf epiphytes biomass in a *Posidonia oceanica* seagrass bed (Revellata Bay, Corsica). Hydrobiologia 394: 63-67

Lepoint G, Jacquemart J, Bouquegneau JM, Demoulin V, Gobert S (2007) Field measurements of inorganic nitrogen uptake by epiflora components of the seagrass *Posidonia oceanica* (Monocotyledons, Posidoniaceae). J Phycol 43: 208-218

Lepoint G, Millet S, Dauby P, Gobert S, Bouquegneau JM (2002b) Annual nitrogen budget of the seagrass *Posidonia oceanica* as determined by *in situ* uptake experiments. Mar Ecol Prog Ser 237: 87-96

López-García P, Rodriguez-Valera F, Pedrós-Alió C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409: 603-607

Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime JP, Hector A, Hooper DU, Huston MA, Raffaelli D, Schmid B, Tilman D, Wardle DA (2001) Biodiversity and Ecosystem Functioning; Current Knowledge and Future Challenges. Science 294: 804-808

Ludwig W, Strunk O, Westram R, Richter L, Meier H Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonho VB, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucl Acids Res 32: 1363-1371

Luo Q, Krumholz LR, Najar FZ, Peacock AD, Roe BA, White DC, Elshahed MS (2005) Diversity of the microeukaryotic community in Sulfide-Rich Zodletone Spring (Oklahoma). Appl Environ Microbiol 71(10): 6175-6184

Magurran AE (1989) Diversidad ecológica y su medición. Ediciones Vedrá, Barcelona, España, 200 pp

Marbà N, Duarte CM, Alexandra A, Cabaço S (2004). How do seagrasses grow and spread?. In: Borum J, Duarte CM, Krause-Jensen D, Greve TM (eds) European

seagrasses: an introduction to monitoring and management. The M&Ms project, pp 11-18

Massana R, Guillou L, Diez B, Pedros-Alio C (2002) Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl Environ Microbiol 68(9):4554-4558

Massutí Pascual E, Grau Jofre AM, Duarte CM, Terrados J, Marbà N (2000). La Posidònia: l'alga que no ho és. Quaderns de Pesca 5. Conselleria d'Agricultura i Pesca, Direcció General de Pesca, "Sa Nostra" Obra social i cultural. Palma de Mallorca, España, 59 pp

Mazzella L, Ott JA (1984) Seasonal changes in some features of *Posidonia oceanica* (L.) Delile leaves and epiphytes at different depths. In: Boudouresque C-F, Jeudy de Grisssac A, Olivier J (eds) Int Workshop on *Posidonia oceanica* Beds. GIS Posidonie publ, Marseille, France, pp 119-127

Mazzella L, Scipione MB, Buia MC (1989) Spatio-temporal distribution of algal and animal communities in a *Posidonia oceanica* meadow. Mar Ecol Evol Perspect 10:107-129

McGrady-Steed J, Harris PM, Morin PJ (1997) Biodiversity regulates ecosystem predictability. Nature 390:162-165

Medina-Pons FJ, Terrados J, López-López A, Yarza P, Rosselló-Móra R (2009) Evaluation of the 18S rRNA clone library approach to study the diversity of the macroeukaryotic leaf-epiphytic community of the seagrass *Posidonia oceanica* (L.) Delile. Marine Biology 156(9): 1963-1976

Medina-Pons FJ, Terrados J, Rosselló-Móra R (2008) Application of temperature gradient gel electrophoresis technique to monitor changes in the structure of the

eukaryotic leaf-epiphytic community of *Posidonia oceanica*. Mar Biol 155(4): 451-460

Moncreiff CA, Sullivan MJ, Daehnick AE (1992) Primary production dynamics in seagrass beds of Mississippi Sound: the contributions of seagrass, epiphytic algae, sand microflora and phytoplankton. Mar Ecol Prog Ser 87: 161-171

Montefalcone M (2009) Ecosystem health assessment using the Mediterranean seagrass *Posidonia oceanica*: A review. Ecol Indic 9(4): 595-604

Morgan MD, Kitting CL (1984) Productivity and utilization of the seagrass *Halodule wnghtii* and its attached epiphytes. Limnol Oceanogr 29: 1066-1076

Munzarova E, Lorenzen B, Brix H, Vojtiskova L, Votrubova O (2006) Effect of NH_4^+/NO_3^- availability on nitrate reductase activity and nitrogen accumulation in wetland helophytes *Phragmites australis* and *Glyceria maxima*. Environ Exp Bot 55(1-2): 49-60

Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol 2(3): 317-322

Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Wawer C (1997) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (eds) Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 1-27

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. Appl Environ Microbiol 59: 695-700

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73(1): 127-141

Nesti U, Piazzi P, Balata D (2009) Variability in the structure of epiphytic assemblages of the Mediterranean seagrass *Posidonia oceanica* in relation to depth. Mar Ecol 1-12

Ott JA (1980) Growth and production in *Posidonia oceanica* (L.) Delile. PSZNI Mar Ecol 1: 47-64

Panayotidis P, Boudouresque CF (1981) Vegetation marine de l'Ile de Port-Cros (Parc National) XXI. Aire minimale et patchiness de la flore epiphyte des feuilles de *Posidonia oceanica*. Trav Sci Parc Nation Port-Cros 7: 71-84

Pansini M, Pronzato R (1985) Distribution and ecology of epiphytic Porifera in two *Posidonia oceanica* (L.) Delile meadows of the Ligurian and Tyrrhenian Sea. PSZNMar Ecol 6: 1-11

Pardi G, Piazzi L, Balata D, Papi I, Cinelli F, Benedetti-Cecchi L (2006) Spatial variability of *Posidonia oceanica* (L.) Delile epiphytes around the mainland and the islands of Sicily (Mediterranean Sea). Mar Ecol 27: 397-403

Passamaneck Y, Halanych KM (2006) Lophotrochozoan phylogeny assessed with LSU and SSU data: evidence of lophophorate polyphyly. Mol Phylogenet Evol 40: 20-28

Passmore AJ, Jarman SN, Swadling KM, Kawaguchi S, McMinn A, Nicol S (2006) DNA as a Dietary Biomarker in Antarctic Krill, *Euphausia superba*. Mar Biotechnol 8: 686-696

Pedersen MF, Borum J (1997) Nutrient control of estuarine macroalgae: growth strategy and the balance between nitrogen requirements and uptake. Mar Ecol Prog Ser 161: 155-163

Peirano A, Niccolai I, Mauro R, Bianchi CN (2001) Seasonal grazing and food preference of herbivores in a *Posidonia oceanica* meadow. Sci Mar 65(4): 367-374

Pérez M, García T, Invers O, Ruiz JM (2008) Physiological responses of the seagrass *Posidonia oceanica* as indicators of fish farm impact. Mar Pollut Bull 56: 869-879

Pergent G, Pergent-Martini C (1993) Leaf renewal cycle of *Posidonia oceanica* in the bay of Lacco Ameno (Ischia, Italy) using lepidochronological method. Posidonia Newsl 4(2): 11-20

Pergent G, Romero J, Pergent-Martini C, Mateo MA, Boudouresque CF (1994) Primary production, stocks and fluxes in the Mediterranean seagrass *Posidonia oceanica*. Mar Ecol Prog Ser 106: 139-146

Phillips N, Smith CM, Morden CW (2001) An effective DNA extraction protocol for brown algae. Phycol Res 49: 97-102

Piazzi L, Balata D, Cinelli F, Lisandro BC (2004) Patterns of spatial variability in epiphytes of *Posidonia oceanica*. Differences between a disturbed and two reference locations. Aquat Bot 79: 345-356

Piazzi L, De Biasi AM, Balata D, Pardi G, Boddi S, Acunto S, Pertusati M, Papi I, Cinelli F, Sartoni GF (2007) Species composition and patterns of spatial variability of morphological forms of macroalgal epiphytic assemblages of the seagrass *Posidonia oceanica*. Vie Et Milieu 57: 1-9

Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep 15(1): 8-15

Prado P, Alcoverro T, Martínez-Crego B, Vergés A, Pérez M, Romero J (2007b) Macrograzers strongly influence patterns of epiphytic assemblages in seagrass meadows. J Exp Mar Biol Ecol 350: 130-143

Prado P, Alcoverro T, Romero J (2008) Seasonal response of *Posidonia oceanica* epiphyte assemblages to nutrient increase. Mar Ecol Prog Ser 359: 89-98

Prado P, Tomas F, Alcoverro T, Romero J (2007a) Extensive direct measurements of *Posidonia oceanica* defoliation confirms the importance of herbivory in temperate seagrass meadows. Mar Ecol Prog Ser 340: 63-71

Procaccini G, Buia MC, Gambi MC, Pérez M, Pergent G, Pergent-Martini C et al (2003) The seagrasses of the Western Mediterranean. In: Green EP, Short FT (eds) World Atlas of Seagrasses. UNEP World Conservation Monitoring Centre. University of California Press, Berkeley, USA, pp 48–58

Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35(21): 7188-7196

Radojkovic D, Kusic J (2000) Silver staining of denaturing gradient gel electrophoresis gels. Clin Chem 46(6): 883-884

Reyes J, Sansón M (1997) Temporal distribution and reproductive phenology of the epiphytes on *Cymodocea nodosa* leaves in the Canary Islands. Bot Mar 40(3): 193-201

Reyes J, Sansón M, Afonso-Carrillo J (1998) Distribution of the epiphytes along the leaves of *Cymodocea nodosa* in the Canary Islands. Bot Mar 41(6): 543-551

Rico JAM, Fernández CA (1996) Seasonal nitrogen metabolism in an intertidal population of *Gelidium latifolium* (Gelidiaceae, Rhodophyta). Eur J Phycol 31(2): 149-155

Romari K, Vaulot D (2004) Composition and temporal variability of picoeukaryote communities at a coastal site of the English Channel from 18S rDNA sequences. Limnol Oceanogr 49(3): 784-798

Romero J (1988) Epífitos de las hojas de *Posidonia oceanica*: variaciones estacionales y batimétricas de biomasa en la pradera de las islas Medes (Girona). Oecol Aquat 9: 19-25

Romero J (1989) Primary production of *Posidonia oceanica* beds in the Medas Islands (Girona, NE Spain). In Boudouresque CF, Meinesz A, Fresi E, Gravez V (eds) 2nd Int Workshop on *Posidonia oceanica* Beds. GIS Posidonie publ, Marseille, France, pp 85-91

Romero J, Kun-Seop Lee K-S, Pérez M, Mateo MA, Alcoverro T (2006) Epiphytes of seagrasses. In: Larkum AWD, Orth RJ, Duarte CM (eds) Seagrasses: biology, ecology and conservation. Springer, Dordrecht, The Netherlands, pp 227-254

Romero J, Martínez-Crego B, Alcoverro T, Pérez M (2007) A multivariate index based on the seagrass *Posidonia oceanica* (POMI) to assess ecological status of coastal waters under the Water Framework Directive (WFD). Mar Pollut Bull 55: 196-204

Romero J, Pérez M, Mateo MA, Sala E (1994) The belowground organs of the Mediterranean seagrass *Posidonia oceanica* as a biogeochemical sink. Aquat Bot 47: 13-19

Rosselló-Móra R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25(1): 39-67

Rosselló-Móra R, López-López A (2008) The least common denominator—species or OTU's. In: Zengler K (ed) Accessing uncultivated microorganisms. From the environment to organisms and genomes and back. ASM press, Washington, USA, pp 117-130

Rota E, Martin P, Erséus C (2001) Soil-dwelling polychaetes: enigmatic as ever? Some hints on their phylogenetic relationships as suggested by a maximum parsimony analysis of 18S rRNA gene sequences. Contrib Zool 70(3): 127-138

Roux KH (1995) Optimization and troubleshooting in PCR. Genome Res 4: S185–S194

Rowan R, Powers DA (1991a) A molecular genetic identification of zooxanthellae and the evolution of animal-algal symbioses. Science 251: 1348-1351

Rowan R, Powers DA (1991b) Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae). Mar Ecol Prog Ser 71: 65-73

Ruiz JM, Pérez M, Romero J (2001) Effects of Fish Farm Loadings on Seagrass (*Posidonia oceanica*) Distribution, Growth and Photosynthesis. Mar Pollut Bull 42(9):749-760

Ryther JH, Corwin N, DeBusk TA, Williams LD (1981) Nitrogen uptake and storage by the red alga *Gracilaria tikvahiae* (McLachlan, 1979). Aquaculture 26: 107-115

Sambrook J, Russell DW (2001a) In vitro amplification of DNA by PCR. Molecular cloning: a laboratory manual. 3rd edition. Vol II. Chapter 8. Sect 8.1. Cold Spring Harbor Laboratory Press, New York, USA

Sambrook J, Russell DW (2001b) Preparation of cDNA Libraries and Gene Identification. Molecular cloning: a laboratory manual. 3rd edition. Vol II. Chapter 11. Sect 11.1. Cold Spring Harbor Laboratory Press, New York, USA

Sand-Jensen K (1977) Effect of epiphytes on eelgrass photosynthesis. Aquat Bot 3: 55-63

Sand-Jensen K, Revsbech NP, Jorgensen BB (1985) Microprofiles of oxygen in epiphyte communities on submerged macrophytes. Mar Biol 89: 55-62

Schäffer H, Muyzer G (2001) Denaturing Gradient Gel Electrophoresis in Marine Microbial Ecology. In: Paul J (ed) Methods in Microbiology, 30. Academic Press, London, UK, pp 425-468

Schanz A, Polte P, Asmus H (2002) Cascading effects of hydrodynamics on an epiphyte–grazer system in intertidal seagrass beds of the Wadden Sea. Mar Biol 141:287-297

Scheible W-R, Lauerer M, Schulze E-D, Caboche M, Stitt M (1997) Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. Plant J 11(4):671-691

Sequencher v4.7 (2006) Gene Codes Corporation, Ann Arbor, Michigan, USA. http://www.sequencher.com

Solomonson LP, Barber MJ (1990) Assimilatory nitrate reductase: functional properties and regulation Annu Rev Plant Physiol. Plant Mol Biol 41:225-253

Spalding M, Taylor M, Ravilious C, Short FT, Green EP (2003) Global overview: the distribution and status of seagrasses. In: Green EP, Short FT (eds) World Atlas of Seagrasses: Present Status and Future Conservation. University of California Press, Berkeley, USA, pp 5–26

SPSS Science (2002) SigmaPlot 8.0 User's Guide, USA. http://www.sigmaplot.com

Stachowicz JJ, Whitlatch RB, Osman RW (1999) Species diversity and invasion resistance in a marine ecosystem. Science 286:1577-1579

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688-2690

StatSoft Inc (2005) STATISTICA (data analysis software system), version 7.1. http://www.statsoft.com

Struck TH, Purschke G, Halanych KM (2005) A scaleless scale worm: Molecular evidence for the phylogenetic placement of *Pisione remota* (Pisionidae, Annelida). Mar Biol Res 1(4): 243-253

Teichberg M, Heffner LR, Fox S, Valiela I (2007) Nitrate reductase and glutamine synthetase activity, internal N pools, and growth of *Ulva lactuca*: responses to long and short-term N supply. Mar Biol 151: 1249-1259

Terrados J, Borum J (2004) Why are seagrasses important? – Goods ans services provided by seagrass meadows. In: Borum J, Duarte CM, Krause-Jensen D, Greve TM (eds) European seagrasses: an introduction to monitoring and management. The M&Ms project, pp 8-10

Terrados J, Medina-Pons FJ (2008) Epiphyte load on the seagrass *Posidonia oceanica* (L.) Delile does not indicate anthropogenic nutrient loading in Cabrera

Archipelago National Park (Balearic Islands, Western Mediterranean). Sci Mar 72(3): 503-510

Terrados J, Williams SL (1997) Leaf versus root nitrogen uptake by the surfgrass *Phyllospadix torreyi*. Mar Ecol Prog Ser 149: 267-277

Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucl Acids Res 30(9): 2083-2088

Thompson SM, Valiela I (1999) Effect of nitrogen loading on enzyme activity of macroalgae in estuaries in Waquoit Bay. Bot Mar 42: 519-529

Tomas F, Romero J, Turon X (2004) Settlement and recruitment of the sea urchin *Paracentrotus lividus* in two contrasting habitats in the Mediterranean. Mar Ecol Prog Ser 282: 173-184

Tomas F, Turon X, Romero J (2005a). Seasonal and small-scale variability of herbivory pressure on the temperate seagrass *Posidonia oceanica* (L.). Mar Ecol Prog Ser 301: 95-107

Tomas F, Turon X, Romero J (2005b) Effects of herbivores on a *Posidonia oceanica* seagrass meadow: importance of epiphytes. Mar Ecol Prog Ser 287: 115-125

Touchette BW, Burkholder JM (2000) Review of nitrogen and phosphorus metabolism in seagrasses. J Exp Mar Biol Ecol 250: 133-167

Touchette BW, Burkholder JM (2001) Nitrate reductase activity in a submersed marine angiosperm: Controlling influences of environmental and physiological factors. Plant Physiol Biochem 39(7-8): 583-593

Trautman DA, Borowitzka MA (1999) Distribution of the epiphytic organisms on *Posidonia australis* and *P.sinuosa*, two seagrasses with differing leaf morphology. Mar Ecol Prog Ser 179: 215-229

Tsirika A, Skoufas G, Haritonidis S (2007) Seasonal and bathymetric variations in epiphytic macroflora on *Posidonia oceanica* (L.) Delile leaves in the National Marine Park of Zakynthos (Greece). Mar Ecol (Berl) 28(Suppl 1): 146-153

Turley CM (1999) The changing Mediterranean Sea-a sensitive ecosystem?. Prog Oceanogr 44: 387-400

UNESCO (1990) Review of potentially harmful substances. Nutrients. Reports and Studies GESAMP 34, 40 pp

Valentini A, Pompanon F, Taberlet P (2008) DNA barcoding for ecologists. Trends Ecol Evol 24(2): 110-117

Van de Peer Y, Jansen J, De Rijk P, De Wachter R (1997) Database on the structure of small ribosomal subunit RNA Nucleic Acids Res 25: 111-116

Van de Peer Y, Neefs JM, De Rijk P, De Wachter R (1993) Reconstructing evolution from eukaryotic small-ribosomal-subunit RNA sequences: calibration of the molecular clock. J Mol Evol 37: 221–232.

Van der Ben D (1971) Les épiphytes des feuilles de *Posidonia oceanica* Delile sur les côtes françaises de la Méditerranée. Mem Inst R Sci Nat Belg 168: 1-101.

Van Dillewijn P, Villadas PJ, Toro N (2002) Effect of a *Sinorhizobium meliloti* strain with a modified putA gene on the rhizosphere microbial community of alfalfa. Appl Environ Microbiol 68(9): 4201-4208

Vanderklift MA, Lavery PS (2000) Patchiness in assemblages of epiphytic macroalgae on *Posidonia coriacea* at a hierarchy of spatial scales. Mar Ecol Prog Ser 192: 127-135

Varela-Álvarez E, Andreakis N, Lago-Lestón A, Pearson GA, Serrao EA, Procaccini G, Duarte CM, Marbà N (2006) Genomic DNA isolation from green and brown algae (Caulerpales and Fucales) for microsatellite library construction. J Phycol 42: 741-745

Vennesland B, Solomonson L P (1972) The nitrate reductase of *Chlorella*: species or strain differences. Plant Physiol 49: 1029-1031

Vidal R, Meneses I, Smith M (2002) Enhanced DNA extraction and PCR amplification of SSU ribosomal genes from crustose coralline algae. J Appl Phycol 14(3): 223-227

Wattier RA, Prodöhl PA, Maggs CA (2000) DNA isolation protocol for red seaweed (Rhodophyta). Plant Mol Biol Rep 18: 275-281

Willerslev E, Cappellini E, Boomsma W, Nielsen R, Hebsgaard MB, Brand TB, Hofreiter M, Bunce M, Poinar HN, Dahl-Jensen D, Johnsen S, Steffensen JP, Bennike O, Schwenninger J-L, Nathan R, Armitage S, de Hoog C-J, Alfimov V, Christl M, Beer J, Muscheler R, Barker J, Sharp M, Penkman KEH, Haile J, Taberlet P, Gilbert MTP, Casoli A, Campani E, Collins MJ (2007) Ancient biomolecules from deep ice cores reveal a forested southern Greenland. Science 317(5834): 111-114

Willerslev E, Hansen AJ, Binladen J, Brand TB, Gilbert MTP, Shapiro B, Bunce M, wiuf C, Gilichinsky DA, Cooper A (2003) Diverse plant and animal genetic records from Holocene and Pleistocene sediments. Science 300(5620): 791-795

Williams SL, Ruckelshaus MH (1993) Effects of nitrogen availability and herbivory on eelgrass (*Zostera marina*) and epiphytes. Ecology 74: 904-918

Young EB, Dring MJ, Savidge G, Birkett DA, Berges JA (2007) Seasonal variations in nitrate reductase activity and internal N pools in intertidal brown algae are correlated with ambient nitrate concentrations. Plant Cell Environ 30: 764-774

Young EB, Lavery PS, van Elven B, Dring MJ, Berges JA (2005) Nitrate reductase activity in macroalgae and its vertical distribution in macroalgal epiphytes of seagrasses. Mar Ecol Prog Ser 288: 103-114

Zhou JZ, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microbiol 62(2): 316-322

ANNEX I - Glossary

[1]**clonal plants**: a group of genetically identical plants that have grown in a given location, all originating vegetatively (not sexually) from a single ancestor.

[2]**endemic**: an organism exclusively native to a place or biota.

[3]**stenohaline**: an organism that can not handle a wide fluctuation in salinity of water.

[4]**nutrient retranslocation**: nutrient removal from plant tissue into the perennial part of the plant prior to senescence. The percentage of nutrients retranslocated is generally greater on nutrient-poor sites reflecting the role of retranslocation as a nutrient conservation mechanism.

[5]**epiphyte**: an organism growing on plants.

[6]**hydrodynamics**: the branch of science that deals with the dynamics of fluids, especially incompressible fluids, in motion.

[7]**genes**: the fundamental physical and functional units of heredity, which carries information from one generation to the next. Segments of DNA, composed of a transcribed region and regulatory sequences that make possible transcription.

[8]**homologous genes**: two or more genes that are derived from the same ancestral gene.

[9]**genome**: the total genetic content contained in a haploid set of chromosomes in eukaryotes, in a single chromosome in bacteria, or in the DNA or RNA in viruses.

[10]**paleoecological**: the study of fossil animals and plants in order to deduce their ecology and the environmental conditions in which they lived.

[11]**oligotrophic**: lacking in plant nutrients and having a large amount of dissolved oxygen throughout.

[12]**phage**: an organism composed by an outer protein capsid enclosing genetic material.

[13]**plasmid**: a DNA molecule that is separate from, and can replicate independently of, the chromosomal DNA. They are double-stranded and, in many cases, circular. Plasmids usually occur naturally in bacteria, but are sometimes found in eukaryotic organisms.

[14]**host cells**: living cells in which a virus reproduces. Host cells used for DNA libraries are generally rapid growth.

[15]**transition**: DNA substitution mutations consisting in interchanges of two-ring purines (A↔G) or of one-ring pyrimidines (C↔T), which involve bases of similar shape.

[16]**transversion**: DNA substitution mutations consisting in interchanges of purine for pyrimidine bases, which involve exchange of one-ring and two-ring structures.

[17]**melting Temperature (T_m)**: Temperature at which half of a DNA molecule will dissociate and become single strand DNA. Depends on both the length of the molecule and the specific nucleotide sequence composition of that molecule. It is generally calculated from the following formula: $T_m = 4\ (G+C) + 2\ (A+T)$.

[18]**motifs**: short, recurring patterns in DNA that are pressumed to have a biological function. They often indicate sequence-specific binding sites for proteins such as nucleases and transcription factors. Others are involved in important processes at the RNA level, including ribosome binding, mRNA processing and transcription termination.

[19]**palindromes**: nucleic acid sequences that are the same whether read 5' to 3' on one strand or 5' to 3' on the complementary strand. Primers that contain those sequences should be avoided in PCR because they can form secondary structures.

[20]**degeneracy**: number of unique sequence combinations in a PCR primer sequence. Degenerate primers may be used to amplify DNA in situations where only the protein sequence of a gene is known, or where the aim is to isolate similar genes from a variety of species.

[21]**polyprurines**: a continuous sequence of purines. Purines are nitrogen-containing bases consisting of a six-membered and a five-membered nitrogen-containing ring, fused together.

[22]**polypyrimidines**: a continuous sequence of pyrimidines. Pyrimidines are nitrogen-containing bases consisting of a six-membered nitrogen-containing ring.

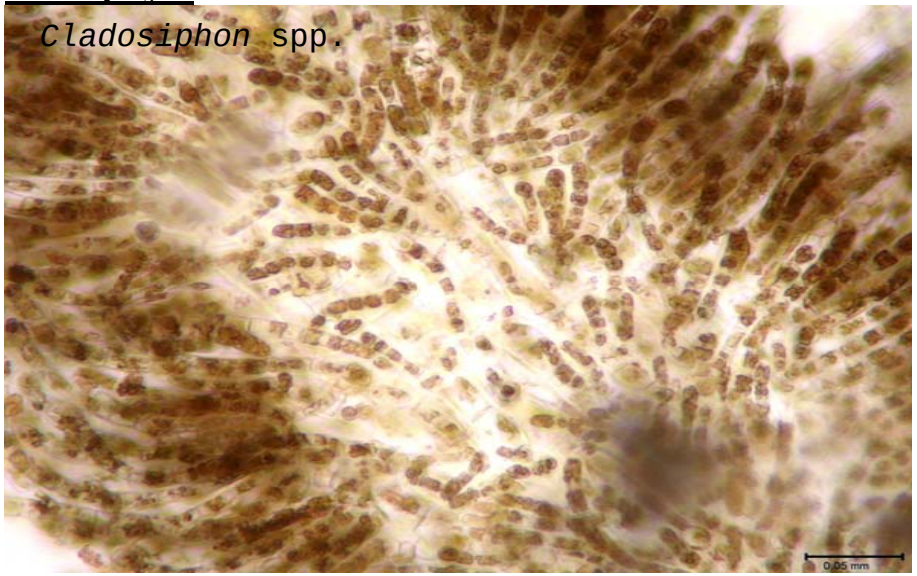
[23]**ambiguities**: positions in nucleic acid sequences that can be occupied for more than one kind of nucleotide. For example, R symbolizes A or G, according to the rules of the International Union of Pure and Applied Chemistry (IUPAC).

[24]**homopolymers**: a sequence of identical bases, such as AAAA or TTTT.

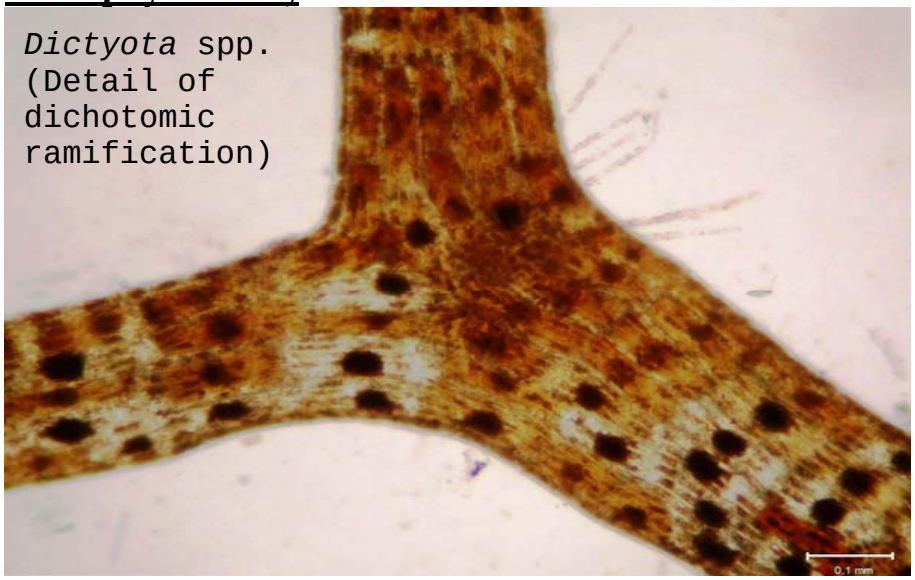
ANNEX II - Images of selected taxa of the macroeukaryotic leaf-epiphytic assemblage of P. oceanica

Chlorophyta

Phaeophyta



Phaeophyta. cont.)



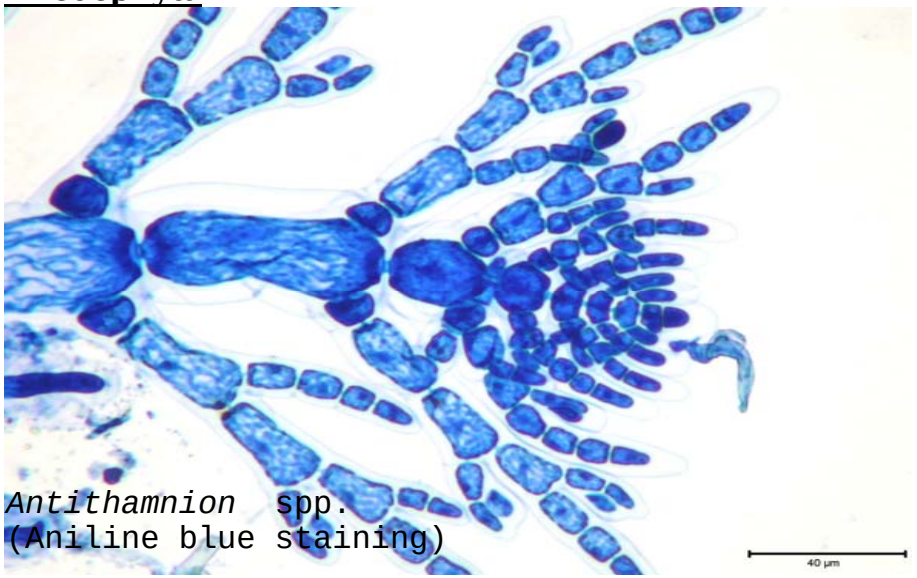
Dictyota spp.
(Detail of dichotomic ramification)

Dictyota spp.
(Detail of apicall cell)

Sphacelaria spp.

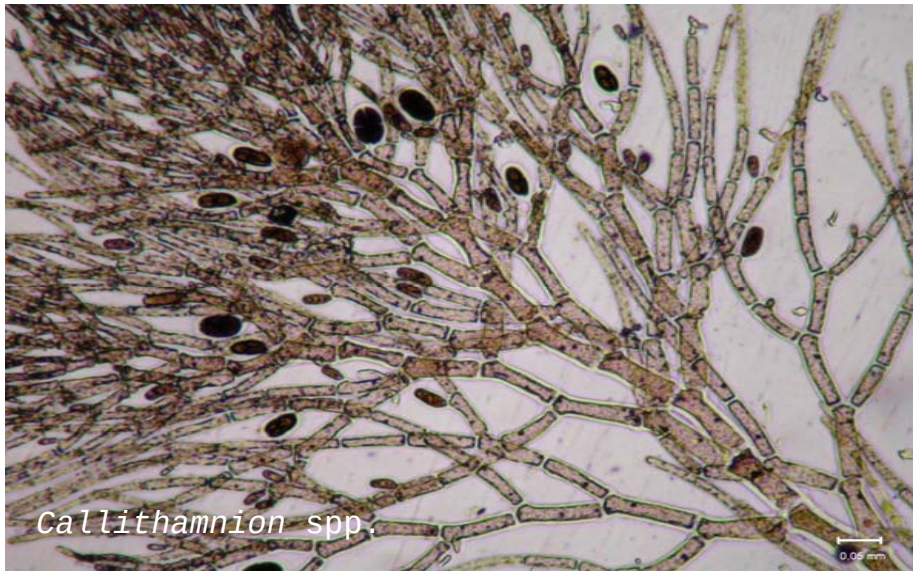
Sphacelaria cirrosa
(Detail of a propagule)

Rhodophyta

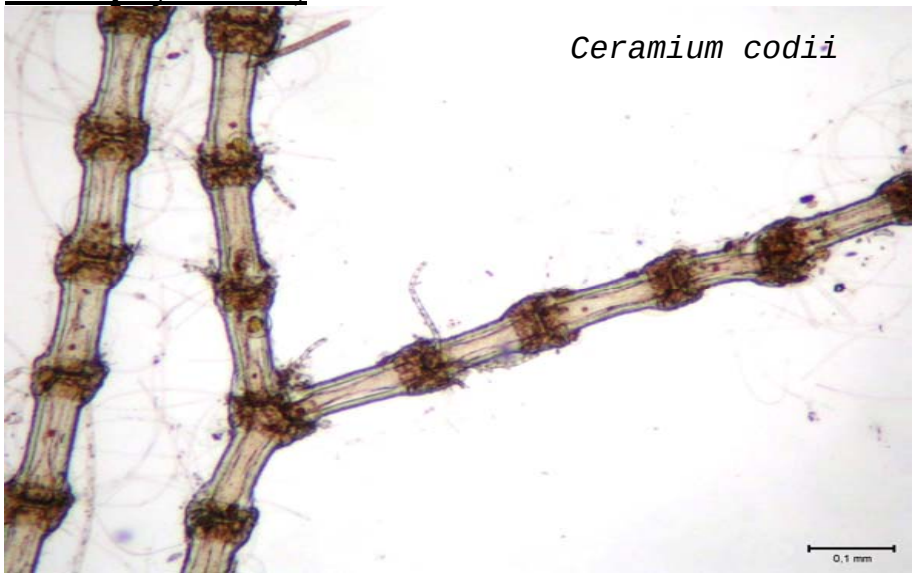

Antithamnion spp.
(Aniline blue staining)
40 µm

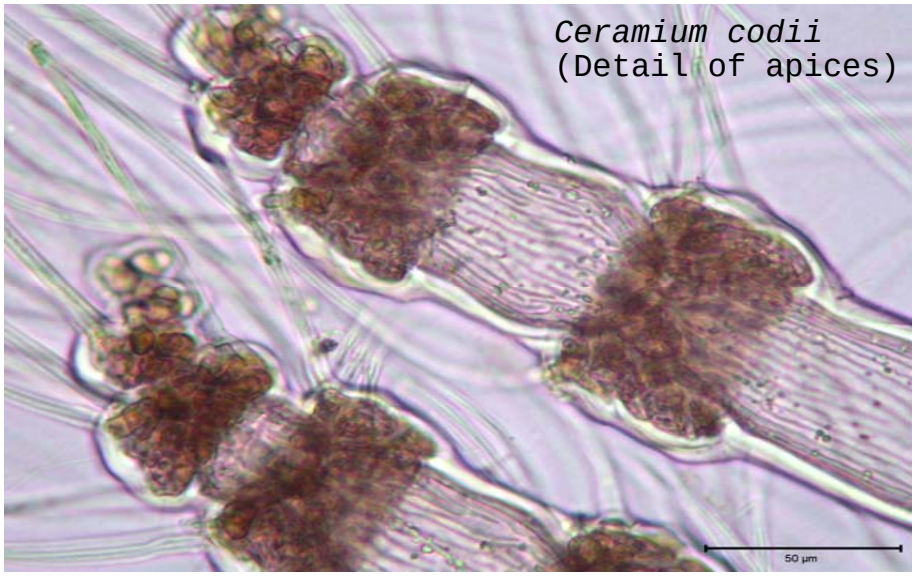

Antithamnion spp.
(Detail of gland cell;
Aniline blue staining)
10 µm


Audouinella daviesii
(Detail of monosporangia)
10 µm


Callithamnion spp.

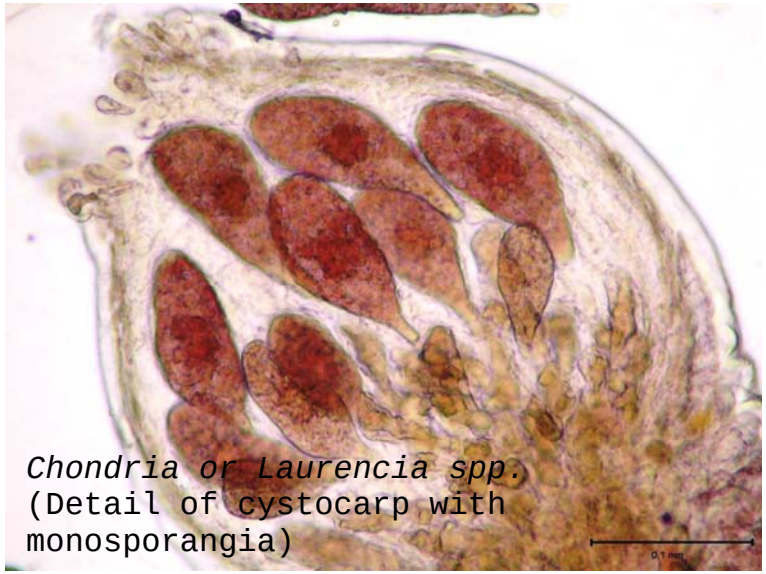
Rhodophyta. cont.)





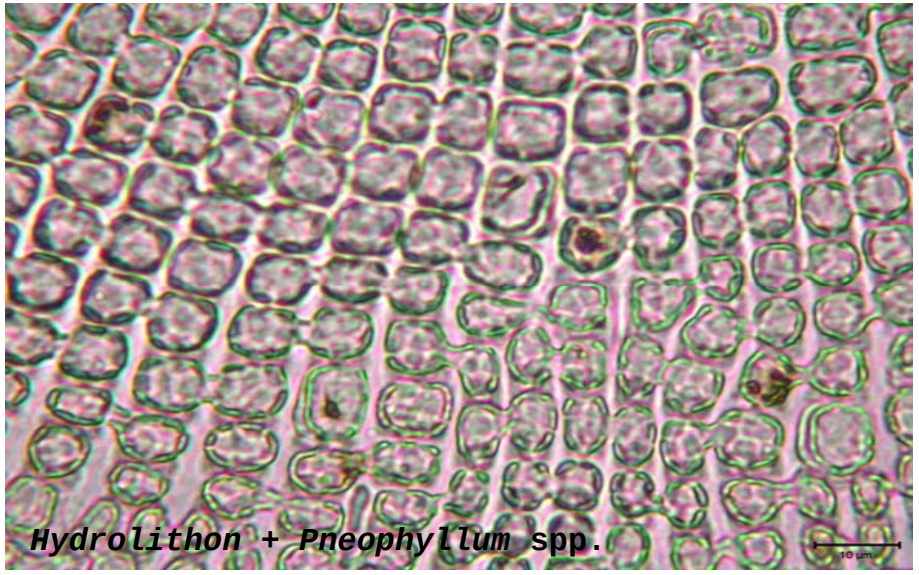
Rhodophyta. cont.)

Chondria or Laurencia spp.
(Detail of apex)



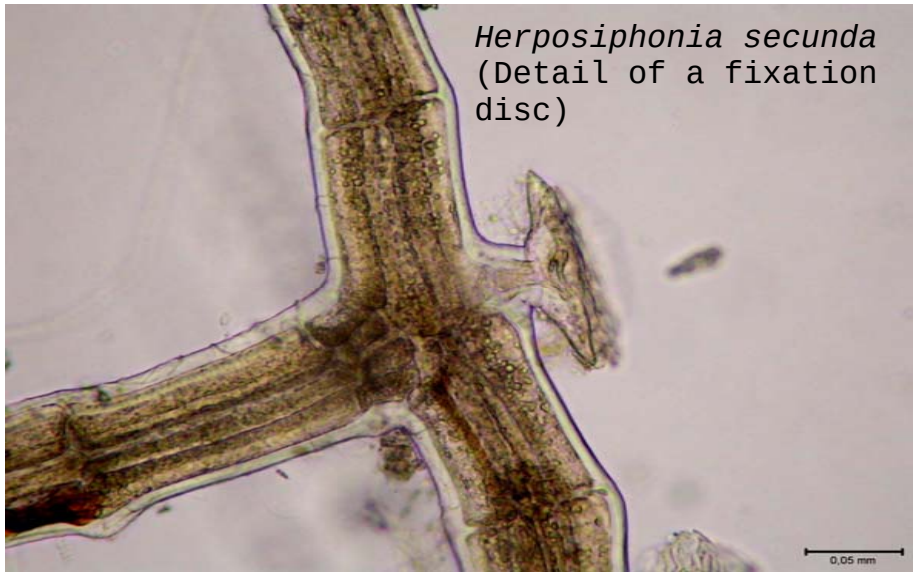
Chondria or Laurencia spp.
(Detail of cystocarp with monosporangia)

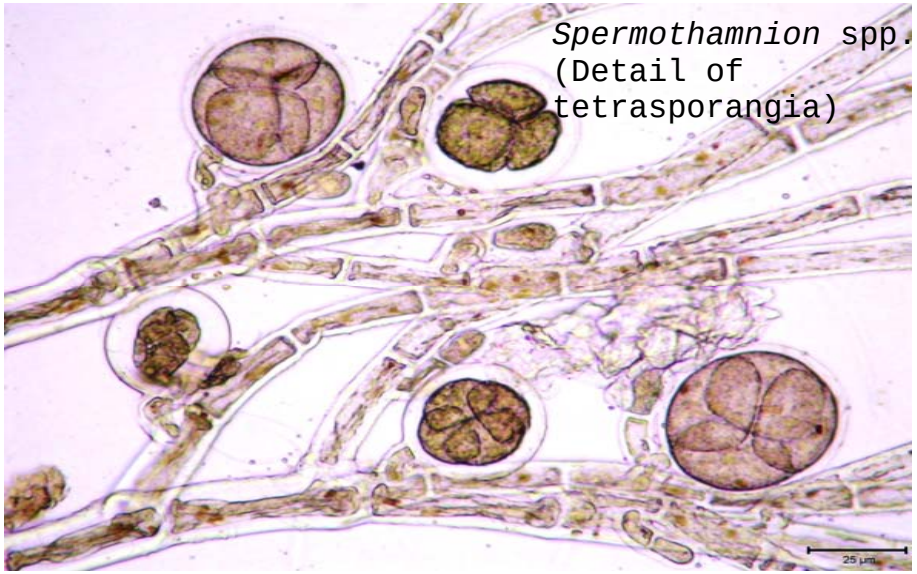
Erythrotrichia carnea



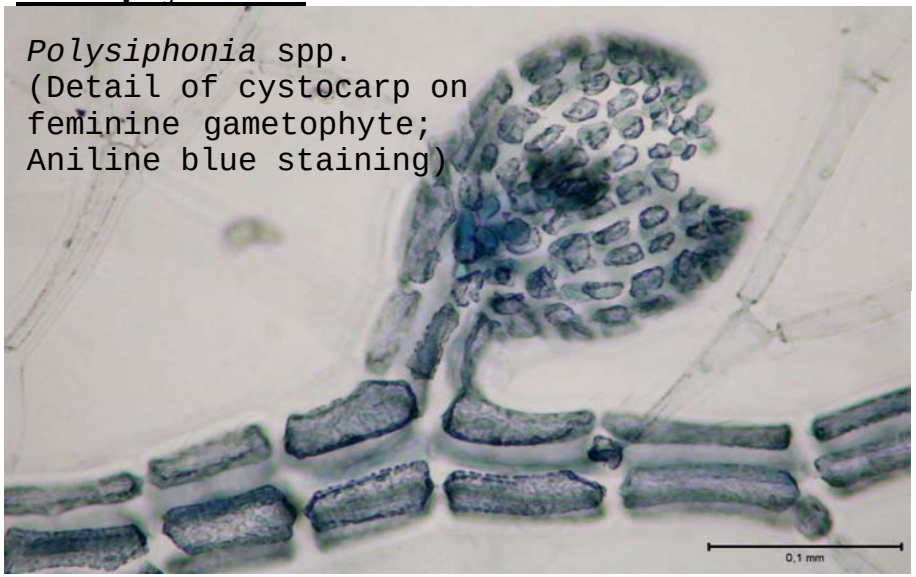
***Hydrolithon* + *Pneophyllum* spp.**

Rhodophyta. cont.)

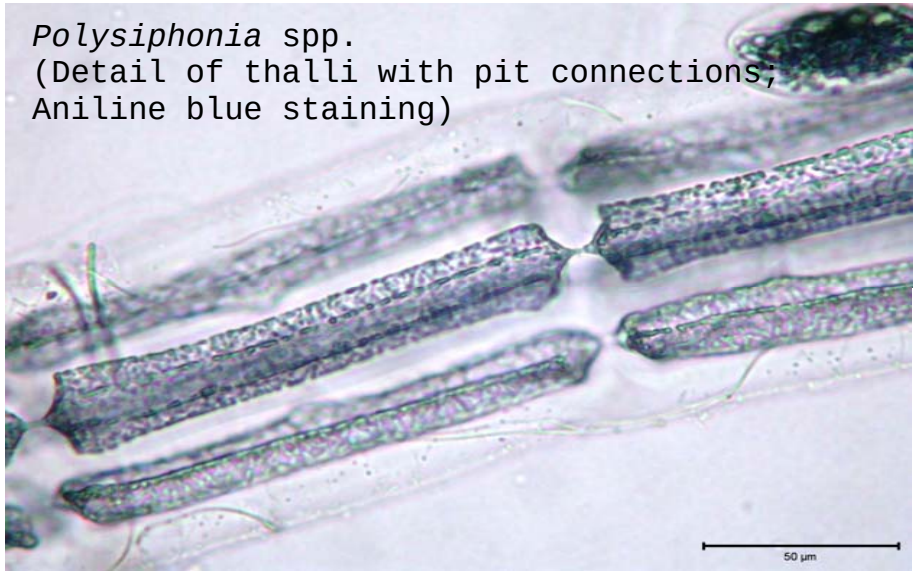




Rhodophyta. cont.



Polysiphonia spp.
(Detail of cystocarp on feminine gametophyte;
Aniline blue staining)

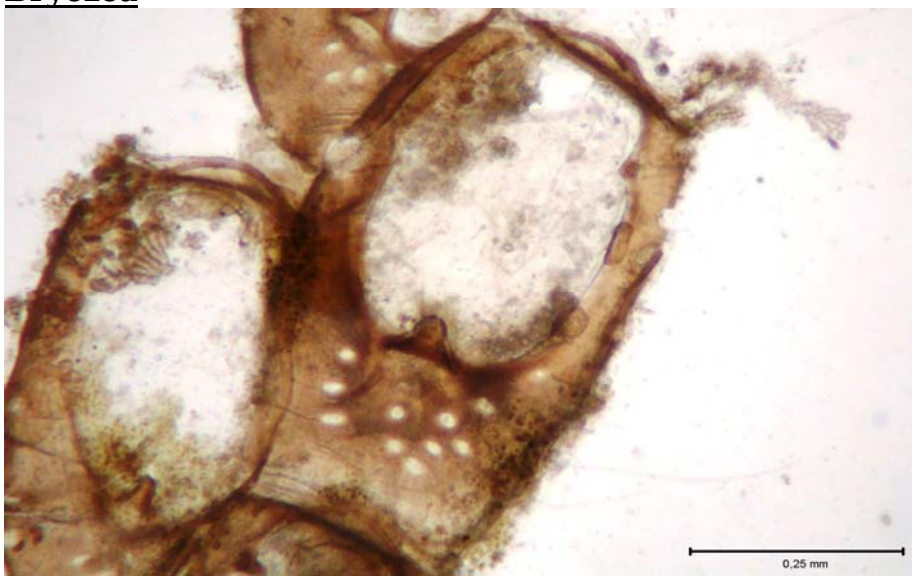


Polysiphonia spp.
(Detail of thalli with pit connections;
Aniline blue staining)

Polysiphonia spp.
(Detail of spermatangia;
Masculine gametophyte;
Aniline blue staining)

Polysiphonia spp.
(Detail of apical region
with trichoblasts)

Bryozoa



Hydrozoa