Vol. 551: 53–62, 2016
doi: 10.3354/meps11755

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published June 9

Significant nitrogen fixation activity associated with the phyllosphere of Mediterranean seagrass *Posidonia oceanica*: first report

Nona S. R. Agawin[1,*], Pere Ferriol[1], Callum Cryer[2], Elena Alcon[1], Antonio Busquets[1], Eva Sintes[3], Constanza Vidal[1], Gabriel Moyà[1]

[1]Interdisciplinary Ecology Group, Biology Department, Universidad de las Islas Baleares, 07122 Palma de Mallorca, Spain
[2]School of Biological Sciences, University of Portsmouth, Portsmouth, Hampshire PO1 2DY, UK
[3]Department of Marine Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

ABSTRACT: The present study provides first estimates of dinitrogen (N_2) fixation activities associated with the phyllosphere of *Posidonia oceanica* in the Mediterranean Sea. N_2 fixation rates in the phyllosphere reached values up to 2.53 µg N gDW h^{-1} or 15.2 mg N m^{-2} d^{-1} (integrated to daily rates and by areal basis), which is higher than most rates reported in the phyllosphere of tropical seagrasses. These rates can potentially supply the total N demand of *P. oceanica* and may contribute significantly to the N budget in the Mediterranean Sea. Our results showed differences in N_2 fixation rates among leaf segments of different age with higher activities in older segments that were heavily epiphytized and higher activities during summer when epiphyte biomass accumulation was higher than in spring. N_2 fixation activities occurred during day and night, suggesting that the community of N_2 fixers in the phyllosphere of *Posidonia oceanica* is complex and is not attributable to a single group of diazotrophs. Molecular analysis of *nifH* genes (coding for the nitrogenase enzyme) of epiphytic samples in the phyllosphere of *P. oceanica* revealed bacterial *nifH* sequences, closely related to as-yet uncultured and unidentified bacteria. To support the ecological importance of N_2 fixation in *P. oceanica* beds, more measurements with wider spatial and temporal scope are needed.

KEY WORDS: Nitrogen fixation · *Posidonia oceanica* · Mediterranean Sea

INTRODUCTION

Posidonia oceanica is an endemic angiosperm in the Mediterranean Sea, occupying 50 000 km^2 (representing 50% of the Mediterranean Sea floor between 0 and 35 m depth and 2% of the total Mediterranean Sea floor; Béthoux & Copin-Montégut 1986). In the Balearic Islands, it occupies approximately 1200 km^2 of coastal area, representing 2–3% of the *P. oceanica* along the entire Mediterranean coastline (Marbà 2009). Recent studies on the nutrient balance of *P. oceanica* meadows, based on fluxes of inorganic and organic nitrogen (N), revealed that N burial exceeded N uptake by the system in this region (Barrón & Duarte 2009). This suggests that there must be additional sources of N inputs, such as particulate matter derived from sestonic particles (Gacia et al. 2002) and N_2 fixation, which to date have not been measured in this area. Given the generally low rates of planktonic dinitrogen (N_2) fixation measured in the Mediterranean Sea in basin-wide studies (Ibello et al. 2010, Agawin et al. 2011, Bonnet et al. 2011), N_2 fixation associated with *P. oceanica* meadows may be an important source of N in the Mediterranean sea, as suggested by Béthoux & Copin-Montégut (1986). Biological N_2 fixation may help explain the N imbal-

*Corresponding author: nona.agawin@uib.es

ance reported for the Mediterranean Sea where the Atlantic surface inflow of N is deficient compared to the deep Mediterranean outflow of N through the Gibraltar strait (Béthoux & Copin-Montégut 1986, Pantoja et al. 2002).

Biological fixation is a characteristic feature of many marine benthic photosynthetic communities (Capone 1983, Stal et al. 1984). In seagrass benthic communities, N_2 fixation occurs in the phyllosphere (leaves), in the upper, oxic, millimeters of the sediment, and in the deeper, largely anoxic layers of the rhizosphere. N_2 fixation by seagrasses has mostly been studied in the upper sediment, and varies between 0.1–7.3 mg N m^{-2} d^{-1} and 7.5–47 mg N m^{-2} d^{-1} in temperate and tropical meadows, respectively (Hemminga & Duarte 2000). Studies on the phyllosphere of seagrass beds reported widely varying N_2 fixation rates (0.3–746 mg N m^{-2} d^{-1}; Goering & Parker 1972, McRoy et al. 1973), and more research in this area is needed.

In the phyllosphere, classical microscopic techniques and recent molecular techniques have revealed several N_2-fixing species of heterocystous, non-heterocystous and unicellular cyanobacteria in tropical seagrasses (i.e. *Thalassodendron*, *Thalassia*, *Cymodocea* and *Halodule*) (Hamisi et al. 2004, Uku et al. 2007). Since the diversity of the N_2 fixers can be species-specific (Uku et al. 2007) and may depend on the different colonization patterns of the epiphytes or the capacity of various seagrasses to host diazotrophs, it remains to be discovered what species of N_2-fixing organisms are epiphytic to *Posidonia oceanica* leaves.

This study reports, for the first time, N_2 fixation rates and identification of N_2 fixers associated with the phyllosphere of *P. oceanica* in the Mediterranean Sea.

MATERIALS AND METHODS

Study site and sampling

The study was conducted during 2 experimental periods: (1) summer (July 19 to 22) of 2010 and (2) spring (May 14 to 22) of 2012. The study was conducted in a 2 m depth seagrass meadow dominated by *Posidonia oceanica*, with co-occurring patches of *Cymodocea nodosa*, in Alcanada (39° 50′ 12″ N, 3° 10′ 15″ E; Alcudia Bay, Mallorca, Spain). During each experimental period, *P. oceanica* shoot density, aboveground (leaf) biomass and morphometry were determined using 6 randomly placed (0.4 × 0.4 m) quadrats. In 6 to 8 randomly selected shoots from the quadrats, the number of leaves per shoot and length and width of each leaf were measured, and each of the leaves was cut into 5 cm segments. Using a clean scalpel, epiphytes were gently scraped from both sides of each segment, and collected in previously dried and weighed (105°C, 24 h) Eppendorf tubes for epiphyte dry weight determination (80°C, 24 h; Kendrick & Lavery 2001). The corresponding epiphyte-free leaf segment was also processed for dry weight determination (60°C, 24 h; Short & Duarte 2001).

Quantification of N_2 fixation rates

N_2 fixation rates were measured in the phyllosphere of *P. oceanica* as well as in the water column using the acetylene reduction assay (ARA) (Stal 1988, Capone 1993, Agawin et al. 2014). Two types of independent N_2 fixation experiments were carried out: (1) on leaf segments during summer 2010 and spring 2012 and (2) on whole leaf shoots during spring 2012. For experiment type 1, 6 to 12 shoots of *P. oceanica* were collected randomly from the shallow site (2 m). For each incubation, the oldest leaf of 3 independent shoots was selected and cut in 5 cm segments: from the top (oldest, heavily epiphytized leaf segment), middle (moderately epiphytized) and bottom (scarcely epiphytized youngest segment). Each of the leaf segments was transferred to 16 ml Hungate tubes and humidified with ~3 ml Whatman GF/F filtered seawater (FSW). A volume of 3.2 ml of acetylene gas (final concentration 20% v/v) was injected into each of the tubes using gas-tight Hamilton syringes. The tubes containing the leaf segments were incubated for 3 h underwater in the *P. oceanica* meadow. Diurnal variability of N_2 fixation rates was checked by carrying out day and night incubations (conducted twice during summer), and/or multiple sampling over a day (every ~3.1–7.6 h in a 24 h cycle during spring). For each independent incubation experiment, a fresh set of leaf materials were used. Water column N_2 fixation rates were simultaneously measured during summer and spring, using three 2 l samples collected from the surface waters of the seagrass meadow with a 5 l Niskin bottle. The water samples were further processed using ARA as described in Agawin et al. (2014). After all the incubations, 10 ml of headspace were taken using a gas-tight Hamilton syringe from the incubation vials and transferred to and stored in Hungate tubes, and sealed with hot melt adhesive glue (SALKI, ref. 0430308) to minimize gas losses (Agawin et al. 2014). Ethylene and acety-

lene determinations were done as described in Agawin et al. (2014) using a gas chromatograph (Agilent Technologies, model HP-5890) equipped with a flame ionization detector. Ethylene produced was calculated using the equations in Stal (1988). The acetylene reduction rates were converted to N_2 fixation using a factor of 4:1 (C_2H_4:N_2 reduced; Jensen & Cox 1983), and reported per dry weight of plant biomass incubated. The mean (±SD) detection limit for C_2H_4 production based on our method was 0.005 (±0.003) nmol C_2H_4.

Experiment type 2 was additionally conducted during spring but instead of excised leaf segments, we enclosed ~6 to 8 shoots of the phyllosphere of *Posidonia oceanica* in methyl acrylic chambers containing ambient seawater. The chambers had a diameter of 14.4 cm, a height of 49 cm, enclosed a volume of 7.98 l and had a port for injection of acetylene-saturated seawater and for water sample extraction. At the beginning of the experiment, ~1.6 l acetylene-saturated FSW, were injected into the chambers through the port to achieve ~20% (v/v) final concentration of acetylene in the enclosed seawater, and were gently stirred with a stirrer handle, made of metallic wire, for 2 min to equilibrate the gas concentration in the jar. The treatments were 4 replicates of chambers enclosing *P. oceanica* shoots with acetylene added, and duplicates of controls enclosing only a water sample with acetylene added to check for any N_2 fixation not due to the intact leaves enclosed in the chambers. Additionally, duplicates enclosing only *P. oceanica* shoots and water without acetylene were included as controls to check for contaminating ethylene that may have been present. Samples were taken from the chambers every 2.6 to 4.2 h over a day. During each sampling period, the enclosed water was mechanically stirred for 2 min before removing 20 ml samples using 50 ml plastic syringes through the port. Moreover, the chambers were not fixed on the sediment, and with the natural wave movement, they were able to gently roll over back and forth. This mechanism provided constant additional stirring inside the chamber throughout the incubation, permitting gas diffusion. The enclosures unlikely limited gas exchanges within the chambers as evidenced by high water column O_2 concentrations measured during the day and night which were well above the critical values found for other seagrass species (e.g. 2.96 mg l^{-1} for *Zostera marina*; Pedersen et al. 2004). On land, 10 ml of each sample from each chamber was transferred to Hungate tubes containing 1.25 ml 20% trichloroacetic acid (TCA) solution to stop bacterial activity and immediately sealed with hot melt adhesive glue (SALKI, ref. 0430308). The tubes were incubated for 24 h at 35°C, in order to equilibrate the gas concentration in the 2 phases inside the tube (gas and liquid). Gas samples (2.5 ml) were taken using gas-tight syringes from the headspace of the experimental tubes for ethylene and acetylene determinations as described above. The amount of ethylene that passes from the aqueous solution into the vapor phase was calculated using a variation of Henry's Law (Flett et al. 1976), corrected for TCA addition. The acetylene reduction rate due to the phyllosphere of *P. oceanica* was the difference between treatment chambers with *P. oceanica* (+ acetylene) minus the controls enclosing seawater only (+ acetylene).

Identification of N_2 fixers in the *P. oceanica* phyllosphere

After the incubations during summer, the leaf segments were stored immediately with dry ice (–80°C) in the field for transport and further processing in the laboratory for molecular identification of N_2 fixers. The epiphytic community on each leaf from the incubation tubes which exhibited the highest rates of nitrogen activities (2 samples each from top, middle, bottom portions of the leaves during the day and night incubations, a total of 12 samples) were gently scraped into sterile FSW with a clean razor blade for molecular identification of N_2 fixers. The epiphytes from the same leaf segment were pooled together, centrifuged at 2000 × *g* for 15 min, and frozen in liquid nitrogen (–80°C) until further processing for nucleic acid extraction (Wilson 1987). DNA purification after nucleic acid extraction was done with Promega kit (Wizard® DNA Clean-Up System, A7280). *nifH* genes (coding for the nitrogenase enzyme) were subsequently amplified using a nested PCR described in Agawin et al. (2014) with modifications on the second round of PCR using 4 primer sets instead of 2. The first set is a mixture of primers amplifying *nifH* fragments for Groups A, B and C of unicellular N_2-fixing cyanobacteria (Agawin et al. 2014). The second primer set amplifies specific *nifH* genes for some of the non-heterocystous filamentous cyanobacterial genera (i.e. *Trichodesmium* group, *Katagnymene, Oscillatoria, Symploca*) (Agawin et al. 2014). The third was designed to preferentially amplify general cyanobacterial *nifH* fragments, while the fourth was intended to be specific for aerobic and microaerophilic diazotrophs (Olson et al. 1998). Amplicons of the predicted 370 bp size (cyanobacterial diazotrophs) and 389 bp size (aerobic and

microaerophilic diazotrophs) (Olson et al. 1998) as revealed by gel electrophoresis (using the third and fourth primer sets) were cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Randomly picked clones were then sequenced in the forward direction using the M13 primer with a BigDye 3.1. The phylogenetic analyses of the 20 *nifH* sequences obtained were analyzed using mega-BLASTn (www.ncbi.nlm.nih.gov). Closest related known and unknown published *nifH* sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov) and aligned with sequences obtained during the study using MEGA6. Alignments based on a 234 bp DNA sequence were used to construct a neighbour-joining phylogenetic tree rooted to the *nifH* gene of *Pelosinus fermentans* JBW45 (CP010978) and relationships were bootstrapped 1000 times using the Jukes-Cantor model in MEGA6.

Data and statistical analyses

Data on N_2 fixation rates are reported as the mean (±SD) of triplicate measurements. A 2-way ANOVA was used to test for significant differences between N_2 fixation rates with age of the leaf segments and with incubation time in experiment type 1. Normal distribution of rates N_2 fixation data was checked with significance of skewness and kurtosis and if necessary, data were logarithmically transformed prior to analyses. The statistical analyses were performed using the Plainstat (version 0.1.1) and R (version 3.2.2).

RESULTS

Study site and quantification of N_2 fixation rates

The ambient temperature of the seawater in the study site was ~10°C higher (28.1°C) in summer than in spring (18.5°C). Density and leaf areal biomass of *Posidonia oceanica* during summer were lower (455 ± 141 shoots m^{-2} and 441 ± 290 gDW m^{-2}, respectively) than in spring (942 ± 380 shoots m^{-2} and 1328 ± 1138 gDW m^{-2}, respectively). During summer, biomass of the total epiphyte community ranged from 50 to 635 $mgDW_{epiphyte}\ gDW_{leaf}^{-1}$ with a clear tendency of higher epiphyte biomass accumulation in older leaves than the younger leaves during summer. During spring, total epiphyte biomass ranged from 106 to 220 $mgDW_{epiphyte}\ gDW_{leaf}^{-1}$ with no clear differences in accumulation of epiphytes among different leaf ages.

The planktonic nitrogen fixation rates were low ($<4.76 \times 10^{-1}$ µg N m^{-3} h^{-1}) and ranged from 1.96 (± 3.36) $\times 10^{-3}$ to 3.56 ($\pm$ 3.53) $\times 10^{-2}$ µg N m^{-3} h^{-1} during summer, and from non-detectable to 2.82 ($\pm$ 1.87) $\times 10^{-1}$ µg N m^{-3} h^{-1} during spring. Table 1 presents the nitrogenase activities in the leaf segments of *P. oceanica* on a leaf dry weight basis. During the summer experiments, nitrogenase activity varied significantly (ANOVA, $p < 0.05$) among the leaf segments but not between light and dark incubations. Higher nitrogenase activities were found in the heavily epiphytized older segments (Table 1). During the spring experiments, the N_2 fixation rates were lower than during summer (Table 1) and the rates did not vary significantly with leaf parts nor with incubation times. For the whole phyllosphere *in situ* chamber incubation during spring using the ARA technique, the ethylene that accumulated through time in treatment chambers with acetylene and with *P. oceanica* was up to ~5 times higher than the controls (seawater without *P. oceanica* leaves). In control chambers without acetylene added (with and without *P. oceanica*), no detectable amounts of ethylene evolved (data not shown), indicating that the ethylene evolved from the chambers with acetylene (with and without *P. oceanica*) resulted from nitrogenase activities. The nitrogenase activities associated with the phyllosphere of *P. oceanica* using the chamber experiments were higher than the leaf segments (Table 1). The N_2 fixation rates in the chambers on leaf dry weight basis values in Table 1 correspond to 15.2 mg N m^{-2} d^{-1} (or 1.09 mmol N m^{-2} d^{-1}) calculated to daily rates by integrating the nitrogen fixation measurements in a span of 24 h and taking into account the leaf areal biomass of *P. oceanica* in the study site.

Identification of N_2 fixers in the *Posidonia oceanica* phyllosphere

During the summer sampling, results of gel electrophoresis after the second round of PCR gave positive bands of amplicons using the first set of a mixture of probes amplifying *nifH* fragments for Groups A, B and C unicellular N_2-fixing cyanobacteria, and using PCR primers specific for cyanobacteria and aerobic and microaerophilic diazotrophs. No visible bands were obtained using the primers specific for some of the non-heterocystous filamentous cyanobacterial genera (i.e. *Trichodesmium* group, *Katagnymene, Oscillatoria, Symploca*). Using the PCR primers specific for cyanobacteria and aerobic and microaerophilic diazotrophs, phylogenetic analysis of the

Table 1. Mean (±SD) nitrogenase activities (based on dry weight [DW] of seagrass leaf biomass) in the phyllosphere of *Posidonia oceanica* and of other seagrass meadows. nd: not detectable

Seagrass species	Nitrogenase activity (µg N gDW^{-1} h^{-1}) Min.	Max.	Incubation conditions	Source
Alcudia Bay, Mediterranean Sea				
Posidonia oceanica	$2.8 (\pm 1.1) \times 10^{-3}$	$1.29 (\pm 1.04) \times 10^{-1}$	Daytime incubations of 5 cm leaf segments	Present study
	$2.8 (\pm 2.8) \times 10^{-3}$	$1.18 (\pm 1.32) \times 10^{-1}$	Nighttime incubations of 5 cm leaf segments (qualitative epiphyte cover: bottom–top or few–many). Maximum values correspond to top older heavily epiphytized segments. Summer	
	nd	$7.03 (\pm 3.61) \times 10^{-2}$	Incubations every 3–7.6 h for ~24 h of 5 cm leaf segments (qualitative epiphyte cover: bottom–top or few–many). Spring	
	$2.6 (\pm 3.3) \times 10^{-1}$	$2.53 (\pm 0.74)$	Whole shoot phyllosphere *in situ* chamber incubations (multiple sampling in a span of ~24 h). Spring	
Dar es Salaam, Tanzanian coast, Western Indian Ocean				
Halodule uninervis	1.4×10^{-1}	2.69	Light	Hamisi et al. (2009)
Cymodocea rotundata	9.8×10^{-2}	1.12	Light	
Thalassia hemprichii	1.4×10^{-1}	1.05	Light	
Thalassodendron ciliatum	2×10^{-3}	3.05×10^{-2}	Light	
Mixed community of	5.6×10^{-2}	1.84	Day	
H. uninervis & *C. rotundata*	1.26×10^{-2}	9.74×10^{-1}	Night	
Florida keys				
Syringodium fiiforme	Trace		Light	McRoy et al. (1973)
	0		Dark	
Thalassia testudinum	Trace		Light	
	0		Dark	
	0	2.4×10^{-2}	Light	
	0	5.6×10^{-2}	Dark	
Bihimi harbor, Bahamas				
Thalassia testudinum	5.01×10^{-1}	1.37	Light	Capone et al. (1979)
	5.82×10^{-2}	1.87×10^{-1}	Dark	
Redfish Bay, Texas				
Thalassia testudinum	4.4×10^{1}	1.85×10^{2}	Light (qualitative epiphyte cover: few–many)	Goering & Parker (1972)
	3.66×10^{1}	7.69×10^{1}	Dark (qualitative epiphyte cover: few–many)	
Cymodocea manatorum	1.78×10^{2}	2.78×10^{2}	Light (qualitative epiphyte cover: many)	
	1.25×10^{2}	1.37×10^{2}	Dark (qualitative epiphyte cover: many)	
Ruppia maritima	5.31×10^{1}	7.51×10^{1}	Light (qualitative epiphyte cover: few)	
	1.28×10^{1}	1.46×10^{1}	Dark (qualitative epiphyte cover: few)	
Diplanthera wrightii	2.75×10^{1}	3.02×10^{2}	Light (qualitative epiphyte cover: few–many)	
	1.65×10^{1}	8.43×10^{1}	Dark (qualitative epiphyte cover: few–many)	

20 *nifH* clones obtained here corresponded to 7 distinct bacterial sequences (assigned GenBank accession numbers from HG739058 to HG739064) which were most related to uncultured bacterial clones. Sequences HG739061, HG739062 and HG739063 are closely related to uncultured bacterial clones MTT1.02 (HM999387), PJD3-2 (KF854531) and PJ1-26 (KF854513) with ~90% sequence similarities. The closest cultured relatives (sequence similarity index >75%) of these clones were from *Prosthecochloris, Chlorobium* and *Chlorobaculum* genera with 78% to 84% sequence similarities (Fig. 1). Sequence HG739058 is closely related to uncultured bacterial clone 119 (JN655425) and clone bal25mar34 (KC140438) with sequence similarity of 99% and the closer cultured relative (75% similarity) is an Opitutaceae bacterium strain TAV5 (CP007053) (Fig. 1). Sequence HG739059 is identical to an uncultured bacterium clone P2_H8 (GU946064) (Fig. 1). The sequence HG739060 is highly related to an uncultured bacterium PJD2-7 (KF854544) isolated from *Porites lutea* coral and nf1 (KP685551) isolated from seagrass sediments from China, both with ~99% sequence similarities (Fig. 1).

DISCUSSION

The present study provides the first known report to date on the nitrogenase activities in the phyllosphere of *Posidonia oceanica* in the Mediterranean. The planktonic N_2 fixation rates were low ($<4.76 \times 10^{-1}$ µg N m^{-3} h^{-1}, or by areal basis, $<9.52 \times 10^{-1}$ µg N m^{-2} h^{-1}) compared to those reported in the adjacent Atlantic ocean ($>4.08 \times 10^{1}$ µg N m^{-2} h^{-1}; Capone et al. 2005 and data therein) but corroborates with data previously reported in the Mediterranean region (Ibello et al. 2010, Agawin et al. 2011). These planktonic rates were 3 to 4 orders of magnitude lower than the N_2 fixation rates in the phyllosphere of *P. oceanica* from *in situ* chamber incubations, which in spring were as high as 3.36 (± 0.98) $\times 10^3$ µg N m^{-2} h^{-1}. The N_2 fixation rates obtained from the chamber experiments (experiment type 2) where the leaves were fully submerged in 20% v/v acetylene by addition of acetylene-saturated seawater were higher than the values obtained from leaf segments exposed to air containing 20% v/v acetylene by directly adding acetylene gas in the humidified tubes (experiment type 1). Theoretically, the exposure of the leaf segments to air containing acetylene avoids solubility and diffusion problems of acetylene that may arise when the leaves are completely submerged. However, when the leaves are directly exposed to air, the N_2-fixing cells may be negatively affected by O_2 levels that are higher than when leaves are fully submerged in seawater. As the nitrogenase enzyme is irreversibly inactivated by O_2, exposure to air can reduce the N_2 fixation rates. The experiment type 1 can be useful for investigation of N_2 fixation patterns by excluding possible problems with dissolution and diffusion of the gasses but for environmentally relevant rates, incubation should be as close as possible to environmental conditions as in experiment type 2 where the leaves are fully submerged in chambers with seawater. The estimated N_2 fixation rates in the phyllosphere of *P. oceanica* based on the chamber experiments were generally higher than those found in tropical seagrasses (Table 1). However, our estimates and those from the rest of the available literature on seagrass phyllosphere N_2 fixation are 2 to 3 orders magnitude lower than the exceptionally high rates of N_2 fixation reported by Goering & Parker (1972) (Table 1).

What is the possible ecological significance of the estimated high rates of phyllosphere N_2 fixation (15.2 mg N m^{-2} d^{-1} or 396 mmol N m^{-2} yr^{-1}) in *Posidonia oceanica* reported here? N_2 fixation can contribute significantly to seagrass N demand. N_2 fixation can provide from <5 to 100% of the plant N demand (Capone 1988, Howarth & Marino 1988). Although ammonia (NH_3) is the direct product of the N_2 fixation process ($N_2 \rightarrow 2NH_3 + H_2$), it is quickly ionized to ammonium (NH_4^+) that can be directly absorbed by the seagrass by leaf uptake, or the NH_4^+ can be converted to NO_3 by nitrifying bacteria and then absorbed readily by the seagrasses (Cornelisen & Thomas 2004). McRoy & Goering (1974) reported that N_2 fixed by the epiphytes can be transferred to and absorbed by the seagrass host. Using the C:N ratio of *P. oceanica* leaves (~24; Barrón & Duarte 2009), it can be estimated that the amount of N needed to support the net community production of 6086 ± 1832 mmol C m^{-2} yr^{-1} in *P. oceanica* ecosystems (Barrón & Duarte 2009) should be 262 mmol N m^{-2} yr^{-1}. Our estimated N_2 fixation in the phyllosphere alone could therefore supply the total N demand of *P. oceanica*. In comparison with the few estimates attempting to quantify the contribution of N_2 fixation to the N demand of seagrasses (i.e. 6–12% in *Zostera noltii*: Welsh et al. 1996; <5% in *Zostera marina*: McGlathery et al. 1998), our initial rough estimate for *P. oceanica* based on the phyllosphere alone is higher. There is a potential for even a higher contribution when studies are done taking into account N_2 fixation associated with the other

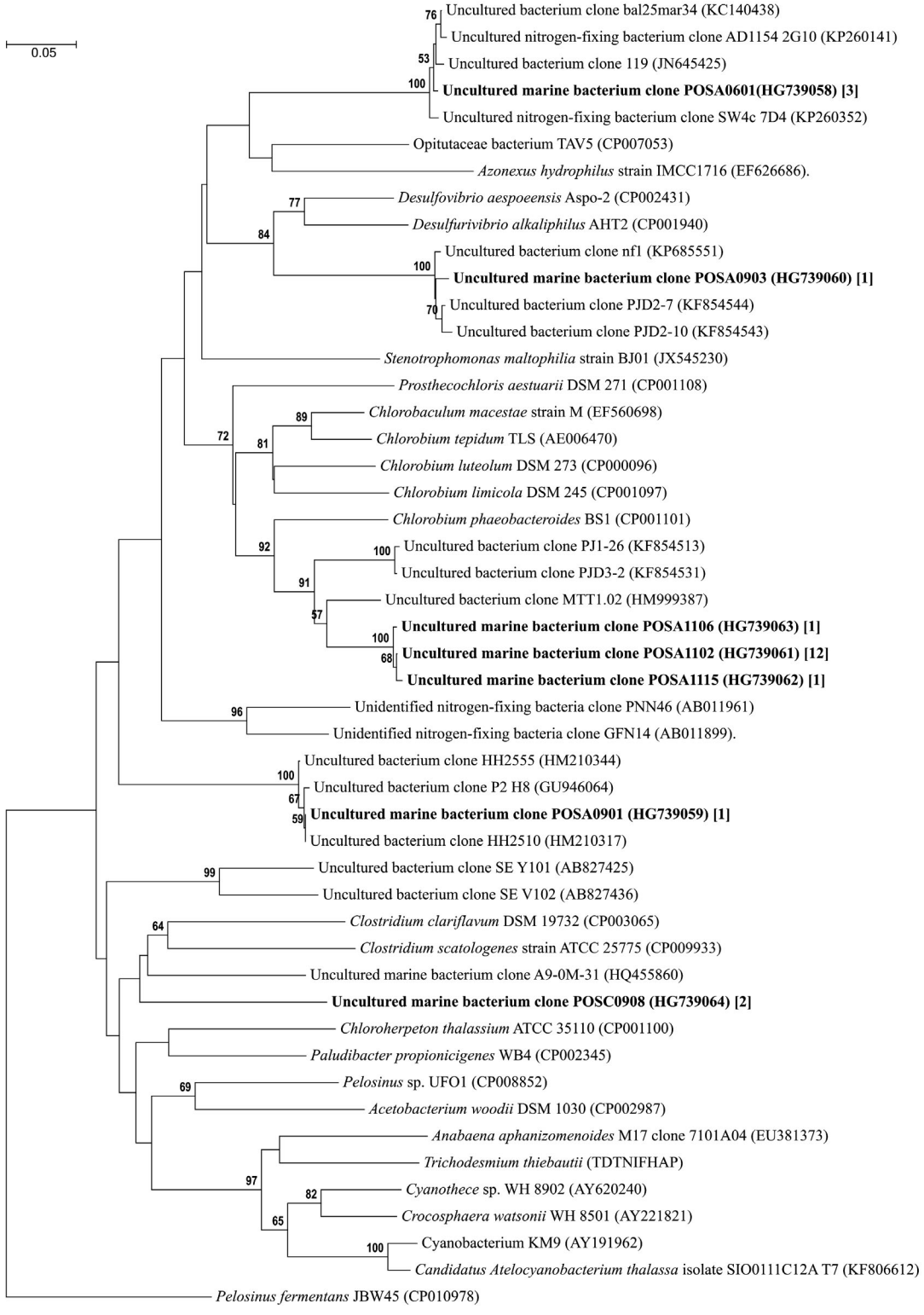

Fig. 1. Rooted neighbor-joining phylogenetic tree of nitrogen-fixing bacteria associated with the epiphytes on the seagrass *Posidonia oceanica* taken during summer 2010, based on a 234 bp DNA sequence alignment with sequences of known phylogeny obtained from GenBank (www.ncbi.nlm.nih.gov). Assigned GenBank sequence ID indicated. Sequences obtained in this study are marked in **bold** and the numbers of identical clones of each sequence are in square brackets. Relationships were bootstrapped 1000 times, and bootstrap values greater than 50% are indicated at respective nodes. Tree is rooted to *nifH* gene of *Pelosinus fermentans* JBW45 (CP010978)

compartments or niches of *P. oceanica* (i.e. in the upper, oxic, millimeters of the sediment, and in the deeper, largely anoxic layers of the rhizosphere). However, our measurement of N_2 fixation was based only on values during spring, and thus seasonal variations were not taken into account.

The high *Posidonia oceanica* N_2 fixation rates reported here may also be of significant ecological importance because of its potential contribution to the Mediterranean N budget. Béthoux & Copin-Montégut (1986) suggested that N_2 fixation in *P. oceanica* can significantly contribute to the N budget and theoretically calculated this to be 4.1×10^{10} mol N yr^{-1} for the entire Mediterranean basin. Our estimate of 15.2 mg N m^{-2} d^{-1} or 396 mmol N m^{-2} yr^{-1} of *P. oceanica* phyllosphere N_2 fixation based on experimental measurement translates roughly to 1.98×10^{10} mol N yr^{-1}, taking into account that *P. oceanica* occupies 50 000 km^2 of Mediterranean basin (Béthoux & Copin-Montégut 1986). This estimate, although lacking spatial and temporal scope of measurements, is the first experimental calculation and corroborates in the magnitude of what has been previously theoretically calculated in Béthoux & Copin-Montégut (1986). Of the estimates of N deficit in the Mediterranean Sea (14.4×10^{10} to 25×10^{10} mol N yr^{-1}; Béthoux and Copin-Montégut 1986) *P. oceanica* phyllosphere N_2 fixation alone could possibly contribute 8 to 14 % of the N in the Mediterranean Sea.

P. oceanica is one of the longest living seagrass species (Duarte 1991). It is slow-growing, producing on average 8 leaves $shoot^{-1}$ yr^{-1} or 1 leaf every 47 d, and leaves can reach up to 50 cm length along the Spanish Mediterranean coasts (Marbà et al. 1996). The high surface area and the long life span of *P. oceanica* leaves (Romero 1988) offer great potential for heavy epiphyte colonization, including the epiphytic N_2-fixers, and thus high N_2 fixation rates particularly for old segments. There was significant variability in N_2 fixation rates among the different leaf segments (bottom, middle and top segments) or leaf ages of *Posidonia oceanica,* particularly during summer when epiphyte accumulation on leaves was highest and there were clear differences in the epiphyte accumulation among the leaf segments. The higher N_2 fixation rates in older, more heavily epiphytized leaves, is consistent with the pattern observed in tropical seagrasses (Goering & Parker 1972; our Table 1). This is because of increased colonization and abundance of more actively N_2-fixing organisms in older leaves and/or that conditions are more favorable for N_2 fixation in older leaves (Hamisi et al. 2009), or differences in diazotroph colonization patterns with leaf age (Hamisi et al. 2004). However, the mechanisms of these possibilities remain to be experimentally investigated. N_2 fixation rates, particularly on the top (older) segments during summer, were higher than during spring and may be due to the greater epiphyte cover (and corresponding colonization and abundance of actively N_2-fixing organisms) and/or indicate that conditions are more favorable for N_2 fixation in summer than spring (e.g. warmer temperatures; Stal 2009).

The non-significant differences between day and night measurements of N_2-fixing activities suggest that the community of N_2-fixers in the phyllosphere of *Posidonia oceanica* is complex, and is not attributable to a single group of diazotrophs which can only fix N_2 during the day or night. The ability to fix N_2 during the day depends on the organism's strategic mechanisms to cope with the O_2 evolution resulting from daytime photosynthesis by the plant that can irreversibly deactivate the N_2-fixing enzyme, nitrogenase. Among the mechanisms employed by cyanobacterial N_2-fixers that fix N_2 during the day is the formation of heterocysts (thick-walled cells containing the nitrogenase) that act as physical barrier to prevent O_2 from diffusing to the enzyme (Fay 1992). However, using the primers specific for cyanobacterial diazotroph *nifH* genes (Olson et al. 1998), sequencing of the clones did not show any relations with heterocystous cyanobacteria nor with the cyanobacterial *nifH* gene cluster in general. This may be due to non-specificity of the general cyanobacterial primer used, and errors in the resolution of analysis of phylogeny when the cyanobacteria gene cluster intermingles with those of other bacterial phyla (e.g. Firmicutes; Hirsch et al. 1995). Some anaerobic heterotrophic N_2-fixing bacteria (members of Cluster III) can reside in anoxic microzones, attached to particles and can fix N_2 in oxygenated coastal waters (Man-Aharonovich et al. 2007). In this study, we encountered *nifH* gene sequences related with *Chlorobium* of the green sulphur group, Cluster III (Zehr et al. 2003) that may adopt this strategy. However, the bacterial *nifH* sequences from the phyllosphere of *P. oceanica* are more closely related to uncultured bacteria than known cultured bacterial clones: to be able to investigate comprehensively their N_2-fixing strategies in a challenging oxygenated habitat, future studies must try to culture these organisms. Comparing the 7 distinct N_2-fixing bacterial sequences obtained here from updated GenBank data, only one (HG739060) has been reported associated with seagrass (sediments from Xincun Bay, China; J. Ling, J. Dong & Y. Jiang unpubl.). This suggests that *P.*

oceanica harbors a distinct community of N_2 fixers that has never been reported before.

In summary, for the first time, we have provided experimental evidences of significant rates of N_2 fixation associated with the phyllosphere of *Posidonia oceanica*. Differences in these rates may be due to the age of the leaves, seasonal fluctuations and diversity of the N_2-fixers present. Future studies must include more comprehensive investigations of N_2 fixation activities with wider spatial and temporal scope and measurements in the other compartments (i.e. the sediments and rhizosphere of *P. oceanica*) to comprehensively assess the role of N_2 fixation as a source contributing to the N demand of a *P. oceanica* meadow and to the N budget of the Mediterranean Sea basin.

Acknowledgements. This work was supported by the projects CTM2007-66408-C02-02, CTM2008-00915-E and ECT017/09, CTM2011-23538 and a Ramón y Cajal grant to NSRA. We thank C. Barrón, A. Beckett, A. Deininger and A. Garcia-Faria for help with fieldwork, B. Nogales and M. Gomila for valuable advice on the molecular analyses, and L. Stal and R. Vaquer-Sunyer for valuable advice on the manuscript. SKUALO Alcudia dive shop and Can Picafort Club Nautico collaborated in this work.

LITERATURE CITED

Agawin NSR, Tovar-Sanchez A, Stal LJ, Alvarez M, Agustí S, Duarte CM (2011) Low water column nitrogen fixation in the Mediterranean Sea (Basin-wide experimental evidence). Aquat Microb Ecol 64:135–147

Agawin NSR, Benavides M, Busquets A, Ferriol P, Stal LJ, Aristeguí J (2014) Dominance of unicellular cyanobacteria in the diazotrophic community in the Atlantic Ocean. Limnol Oceanogr 59:623–637

Barrón C, Duarte CM (2009) Dissolved organic matter release in a *Posidonia oceanica* meadow. Mar Ecol Prog Ser 374:75–84

Béthoux JP, Copin-Montégut G (1986) Biological fixation of atmospheric nitrogen in the Mediterranean Sea. Limnol Oceanogr 31:1353–1358

Bonnet S, Grosso O, Moutin T (2011) Planktonic dinitrogen fixation in the Mediterranean Sea: a major biogeochemical process during the stratified period? Biogeosciences 8:1197–1225

Capone DG (1983) N_2 fixation in seagrass communities. Mar Technol Soc J 17:32–37

Capone DG (1988) Benthic nitrogen fixation. In: Blackburn H, Sorensen J (eds) Nitrogen cycling in coastal marine environments. Springer, New York, NY, p 85–123

Capone DG (1993) Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. CRC Press, Boca Raton, FL, p 621–631

Capone DG, Penhale P, Oremland R, Taylor B (1979) Relationship between productivity and N_2 (C_2H_2) fixation in *Thalassia testudinum* community. Limnol Oceanogr 24: 117–125

Capone DG, Burns J, Montoya J, Subramanian A and others (2005) Nitrogen fixation by *Trichodesmium* spp.: An important source of new nitrogen to the tropical and subtropical North Atlantic Ocean. Global Biogeochem Cycles 19:GB2024, doi:10.1029/2004GB002331

Cornelisen CD, Thomas FIM (2004) Ammonium and nitrate uptake by leaves of the seagrass *Thalassia testudinum*: effects of hydrodynamic regime and epiphyte cover on uptake rates. J Mar Syst 49:177–194

Duarte CM (1991) Allometric scaling of seagrass form and productivity. Mar Ecol Prog Ser 77:289–300

Fay P (1992) Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56:340–373

Flett RJ, Hamilton RD, Campbell NER (1976) Aquatic acetylene-reduction techniques: solutions to several problems. Can J Microbiol 22:43–51

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

Goering JJ, Parker PL (1972) Nitrogen fixation by epiphytes on seagrasses. Limnol Oceanogr 17:320–323

Hamisi MI, Lyimo TJ, Muruke MHS (2004) Cyanobacterial occurrence and diversity in seagrass meadows in coastal Tanzania. Western Indian Ocean. J Mar Sci 3:113–122

Hamisi MI, Lyimo TJ, Muruke MHS, Bergman B (2009) Nitrogen fixation by epiphytic and epibenthic diazotrophs associated with seagrass meadows along the Tanzanian coast, Western Indian Ocean. Aquat Microb Ecol 57:33–42

Hemminga MA, Duarte CM (2000) Seagrass ecology. Cambridge University, Cambridge

Hirsch AM, McKhann HI, Reddy A, Liao J, Fang Y, Marshall CR (1995) Assessing horizontal transfer of *nifDK* genes in eubacteria: nucleotide sequence of *nifK* from *Frankia* Strain HFPCcl3. Mol Biol Evol 12:16–27

Howarth RW, Marino R (1988) Nitrogen fixation in freshwater, estuarine, and marine ecosystems. 2. Biogeochemical controls. Limnol Oceanogr 33:688–701

Ibello V, Cantoni C, Cozzi S, Civitarese G (2010) First basin-wide experimental results on N_2 fixation in the open Mediterranean Sea. Geophys Res Lett 37:L03608, doi: 10.1029/2009GL041635

Jensen BB, Cox RP (1983) Direct measurements of steady-state kinetics of cyanobacterial N_2 uptake by membrane-leak mass spectrometry and comparisons between nitrogen fixation and acetylene reduction. Appl Environ Microbiol 45:1331–1337

Kendrick GA, Lavery PS (2001) Assessing biomass, assemblage structure and productivity of algal epiphytes on seagrasses. In: Short FT, Short CA, Coles RG (eds) Global seagrass reseach methods. Elsevier, Amsterdam, p 199–222

Man-Aharonovich D, Crees N, Zeev EB, Berman-Frank I, Beja O (2007) Molecular ecology of *nifH* genes and transcripts in the eastern Mediterranean Sea. Environ Microbiol 9:2354–2363

Marbà N (2009) Estat ecologic de les masses d'aigua a les Illes Balears, Palma de Mallorca. I Jornada científcotécnica Directiva Marc de l'Aigua

Marbà N, Duarte CM, Cebrián J, Gallegos ME, Olesen B, Sand-Jensen K (1996) Growth and population dynamics of *Posidonia oceanica* on the Spanish Mediterranean coast: elucidating seagrass decline. Mar Ecol Prog Ser 137:203–213

McGlathery JK, Risgaard-Petersen N, Christensen PB (1998) Temporal and spatial variation in nitrogen fixation activity in the eelgrass *Zostera marina* rhizosphere. Mar Ecol Prog Ser 168:245–258

McRoy CP, Goering JJ (1974) Nutrient transfer between the seagrass *Zostera marina* and its epiphytes. Nature 248: 173–174

McRoy CP, Goering JJ, Chaney B (1973) Nitrogen fixation associated with seagrasses. Limnol Oceanogr 18:998–1002

Olson JB, Steppe TF, Litaker RW, Paerl HW (1998) N_2-fixing microbial consortia associated with the ice cover of Lake Bonney, Antarctica. Microb Ecol 36:231–238

Pantoja S, Repeta DJ, Sachs JP, Sigman DM (2002) Stable isotope constraints on the nitrogen cycle of the Mediterranean Sea water column. Deep-Sea Res I 49: 1609–1621

Pedersen O, Binzer T, Borum J (2004) Sulphide intrusion in eelgrass (*Zostera marina* L.). Plant Cell Environ 27: 595–602

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

Short FT, Duarte CM (2001) Methods for the measurement of seagrass growth and production. In: Short FT, Short CA, Coles RG (eds) Global seagrass reseach methods. Elsevier, Amsterdam, p 155–182

Stal LJ (1988) Nitrogen fixation in cyanobacterial mats. Methods Enzymol 167:474–481

Stal LJ (2009) Is the distribution of nitrogen-fixing cyanobacteria in the oceans related to temperature? Environ Microbiol 11:1632–1645

Stal LJ, Grossberger S, Krumbein WE (1984) Nitrogen fixation associated with the cyanobacterial mat of a marine laminated microbial ecosystem. Mar Biol 82:217–224

Uku J, Björk M, Bergman B, Díez B (2007) Characterization and comparison of prokaryotic epiphytes associated with three East African seagrasses. J Phycol 43:768–779

Welsh DT, Bourguès S, de Wit R, Herbert RA (1996) Seasonal variations in nitrogen fixation (acetylene reduction) and sulphate-reduction rates in the rhizosphere of *Zostera noltii*. Mar Biol 125:619–628

Wilson K (1987) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. John Wiley & Sons, New York, NY, p 2.4.1–2.4.5

Zehr JP, Jenkins BD, Short SM, Steward GF (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ Microbiol 5:539–554

Editorial responsibility: Just Cebrian, Dauphin Island, Alabama, USA

Submitted: March 3, 2016; Accepted: April 28, 2016
Proofs received from author(s): May 27, 2016