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Simultaneous measurements of nitrogen fixation in different plant tissues of the seagrass *Posidonia oceanica*

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ABSTRACT: The lack of simultaneous measurements of N_2 fixation associated with different plant tissues in seagrasses led us to investigate the temporal (seasonal and diurnal) variability of N_2 fixation rates associated with the different plant tissues (leaves, rhizomes and roots) of *Posidonia oceanica* along the Mallorcan coast (Mediterranean Sea) in areas with differing local nutrient regimes. Additional measurements were taken to quantify the activities of endophytic N_2 fixers in the roots. N_2 fixation in the different portions of the plants showed variability through the seasons, with generally higher activity associated with the leaves during summer (reaching up to 0.29 ± 0.00 µg N g^{-1} dry weight [DW] h^{-1} [mean ± SD] during the day) and higher activity associated with the roots (reaching up to 0.12 ± 0.02 µg N g^{-1} DW h^{-1}) during the day and night in winter. Root endophytic N_2 fixers also showed maximal rates (0.11 ± 0.07 µg N g^{-1} DW h^{-1}) during winter. N_2 fixation associated with the rhizomes remained low throughout the seasons (<0.01 µg N g^{-1} DW h^{-1}). N_2 fixation associated with the leaves can contribute up to 86% of total plant N_2 fixation during summer, while during the colder months, the belowground tissue parts play a more significant role in the plant's N_2 fixation. The rhizomes can contribute up to 67% of total N_2 fixation of the plant in autumn, because of their high biomass, while the roots can contribute up to 30% in winter.

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

1. INTRODUCTION

It is widely accepted that the availability of fixed nitrogen (N) is a major factor limiting the biological productivity in the open ocean (Sigman & Hain 2012) and in shallow coastal marine environments (Herbert 1999). Therefore, biological N fixation, i.e. the conversion of dissolved nitrogen gas (N_2) to organic N, is a very important process for the input of new fixed N and may enhance the productivity of N-limited ecosystems. In seagrass ecosystems, which are considered among the most productive ecosystems on Earth (Fourqurean et al. 2012), N_2 fixation as source of external N is being increasingly acknowledged as a significant contributing factor (Welsh 2000, Agawin et al. 2017), contributing >50% of plant N demand in tropical and subtropical seagrasses (*Posidonia oceanica*, Agawin et al. 2017; *Zostera capricorni*, O'Donahue et al. 1991; *Thalassia testudinum*, Patriquin 1972) and up to 12% in temperate seagrasses (*Z. noltii*, Welsh et al. 1996; *Z. marina*, McGlathery et al. 1998). Aside from the importance of N_2 fixation in contributing to the N requirements of seagrasses and maintaining their high productivity, the process is also important in the N cycle and budget at larger scales. In the Mediterranean Sea, coastal ecosystems

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are dominated by the endemic seagrass *P. oceanica*, which forms extensive meadows and plays key structural and functional roles in the region (Boudouresque et al. 2006, Montefalcone 2009), and N_2 fixation associated with this plant is suggested to be an important source of external N (Béthoux & Copin-Montégut 1986).

N_2 fixation has been reported to be associated with the different plant parts of seagrasses, from aboveground (phyllosphere, i.e. leaves) to belowground parts (rhizosphere, i.e. rhizomes and roots). Several studies have performed N_2 fixation measurements associated with the phyllosphere (Goering & Parker 1972, McRoy et al. 1973, Capone et al. 1979, Hamisi et al. 2009, Agawin et al. 2016, 2017), and the rates reported ranged from 0.003 to 302 µg N g^{-1} DW h^{-1}. N_2 fixation rates associated with the rhizosphere of seagrasses have also been measured (see review in Welsh 2000), and the rates, normally reported per area of sediment containing the roots and rhizomes, ranged from 0.001 to 5.83 mg N m^{-2} h^{-1} (Welsh 2000, Welsh et al. 1997). The wide variability of rates reported associated with both the phyllosphere and rhizosphere might be due to differences among seagrass species studied, among sampling seasons and environmental conditions, and differences in methodologies employed. The relative importance of N_2 fixation associated with different plant tissues of seagrasses remains to be properly assessed as (1) there is a lack of studies in which N_2 fixation is simultaneously measured in all plant parts using the same methodology and reported in the same units, and (2) in previous studies, often the temporal and spatial variability of the N_2 fixation rates have not been taken into account.

Previous studies reveal a plethora of N_2 fixers in the different plant parts of seagrasses. In the phyllosphere in tropical seagrass genera (i.e. *Thalassodendron*, *Thalassia*, *Cymodocea* and *Halodule*), classical microscopic techniques and recent molecular techniques have reported several N_2-fixing species of epiphytic cyanobacteria such as non-heterocystous *Oscillatoria* sp., heterocystous *Calothrix* sp. and unicellular cyanobacteria of the genus *Chroococcidiopsis* (Uku et al. 2007, Hamisi et al. 2009). In the phyllosphere of *P. oceanica*, molecular analysis of *nifH* genes (coding for the nitrogenase enzyme responsible for N_2 fixation) of epiphytic samples revealed a large number of *nifH* sequences representing a total of 15 phyla (see supplementary text and Fig. S1 in Agawin et al. 2017). The deeper, largely anoxic layers of the rhizosphere of seagrasses also have a mixed assemblage of heterotrophic N_2-fixing bacteria (Pereg et al. 1994, Bagwell et al. 2002). In the root zone of temperate (i.e. *Z. marina*) and tropical seagrasses (i.e. *T. testudinum* and *Syringodium filiforme*), N_2-fixing bacteria, belonging to the family *Vibrionaceae* were isolated (Shieh et al. 1989, Bagwell et al. 2002). In the rhizosphere of *P. oceanica*, recent molecular studies have revealed the presence of phylotypes belonging to the Desulfovibrio groups (Garcias-Bonet et al. 2009, Coma 2016), which can fix N_2 in the process of reducing sulphates. These different assemblages of N_2 fixers associated with different seagrass plant parts may have differential requirements regulating their growth and activity.

Previous studies suggest that different species of N_2 fixers may have differing sensitivities to environmental factors. For example, N_2 fixation is generally thought of as having a strong temperature dependence at the enzymatic level (Brauer et al. 2013), and marine N_2 fixation is generally associated with warmer, tropical and subtropical surface waters (Sohm et al. 2011). However, marine N_2 fixers have been reported to occur at lower temperatures (Holl et al. 2007, Agawin et al. 2014), suggesting that some N_2 fixers are not inhibited by low temperatures and that there are differences in optimal temperatures of different species of marine N_2 fixers. The light dependence of N_2 fixation can also vary among species. Some heterocystous (e.g. *Nostoc*, *Rivularia* and *Calothrix*; Jones 1992) and non-heterocystous N_2-fixing cyanobacteria (e.g. *Trichodesmium*; Rodriquez & Ho 2014) have known maximum N_2-fixing activities during the day, while some unicellular non-heterocystous cyanobacterial (e.g. *Gloeothece*; Bergman et al. 1997) and bacterioplanktonic N_2 fixers primarily fix N_2 during the night (Falcón et al. 2004). With regards to the role of the availability of dissolved inorganic nitrogen (DIN) in regulating marine N_2 fixation, it is assumed that the N_2 fixers are inhibited by the presence of high concentrations (≥1 µM) of NO_3^- and/or NH_4^+ (Knapp 2012) because of the additional energetic cost associated with assimilating N_2 gas relative to NO_3^- or NH_4^+ (Falkowski 1983). However, this is not always the case, as there is evidence that benthic N_2 fixers are less sensitive to elevated concentrations of NO_3^- or NH_4^+ (Knapp 2012). With regards to dissolved inorganic phosphorus (DIP) availability, N_2 fixation can be reduced with depleted DIP (Krauk et al. 2006), yet different species of marine N_2 fixers may have differing mechanisms to cope with DIP limitation, such as maximal DIP uptake rates, alkaline phosphatase activity (APA) and transcription of genes for DIP transporters and alkaline phosphatases (Sohm et al. 2011).

In seagrass ecosystems, the phyllosphere and rhizosphere have different regimes of the physico-chemical regulatory factors of N_2 fixation described above. The phyllosphere can be subject to more temperature fluctuations, more light availability and less availability of DIN, DIP and trace metals. In contrast, the rhizosphere may be subject to more stable temperatures, as well as to partial or total darkness and to porewaters rich in DIN and DIP. Subject to different physico-chemical regimes, the N_2 fixers associated with the phyllosphere and the rhizosphere may have contrasting growth and activity requirements and hence the temporal variability and the relative importance of N_2 fixation associated with the different plant parts of seagrasses may be different. Moreover, local nutrient regimes can vary among different sites, and thus spatial variability of N_2 fixation associated with the different plants parts can also be expected. Here, we investigated the temporal (seasonal and diurnal) variability of N_2 fixation rates associated with the different plant parts (leaves, rhizomes and roots) of *P. oceanica* along the Mallorcan coast (Mediterranean Sea) in areas with differing local nutrient regimes caused by different levels of anthropogenic impact. Additional measurements were performed to quantify the activities of endophytic N_2 fixers in the roots. The experiments conducted here allowed us to observe, for the first time, the ubiquitous occurrence of N_2 fixation in the different parts of the plant simultaneously and determine which environmental factors may be important in controlling N_2 fixation rates associated with *P. oceanica* meadows.

2. MATERIALS AND METHODS

2.1. Study sites and sampling

To assess the seasonal variability of N_2 fixation rates associated with *Posidonia oceanica*, measurements were conducted 6 times, representing the 4 seasons of the year with 2 overlapping spring and summer measurements in 2012 to 2013, in a 4 m depth seagrass meadow dominated by *P. oceanica* with co-occurring patches of *Cymodocea nodosa* in Alcanada (39.84° N 3.17° E, Alcudia Bay, Mallorca, Spain; Fig. 1). To assess the spatial variability of the N_2 fixation rates along the Mallorcan coast during the 2 summer periods, an additional 3 sites were studied at similar depths (Albufera, 39.83° N 3.14° E, Alcudia Bay; and Arenal, 39.50° N, 2.75° E, and Calanova 39.54° N, 2.60° E, both Palma Bay; Fig. 1). With the implementation of the European Union (EU) Water Framework Directive, the water masses around the Mallorcan coast are classified according to different types of substrate, coastal topography and level of anthropogenic impact the areas are receiving. The 4 sites (including Alcanada) represented 4 different trophic states of the water masses according to chlorophyll concentrations: Alcanada, ≤0.25 mg m^{-3}; Albufera, ≤31 mg m^{-3}; Arenal, ≤0.36 mg m^{-3} and Calanova, ≤0.42 mg m^{-3} (Moyà et al. 2009). The study site in Alcanada, Alcudia Bay, theoretically represents the most pristine site.

2.2. Quantification of N_2 fixation rates over a 24 h cycle

N_2 fixation rates were measured in the different plants parts of *P. oceanica* using the acetylene reduction assay (ARA) (Stal 1988, Capone 1993, Agawin et al. 2014). Careful collection of at least 24 shoots of *P. oceanica* with intact rhizomes and roots was performed using SCUBA in the 4 m depth seagrass meadow at each station immediately prior to each set of a 24 h cycle incubation experiments. To avoid desiccation of the plants, they were immediately transferred and maintained in a ~160 l container filled with seawater originating from the same sampling site where the plants were collected. The manipulation time of the plant parts was as short as possible before incubation. Each incubation experiment was

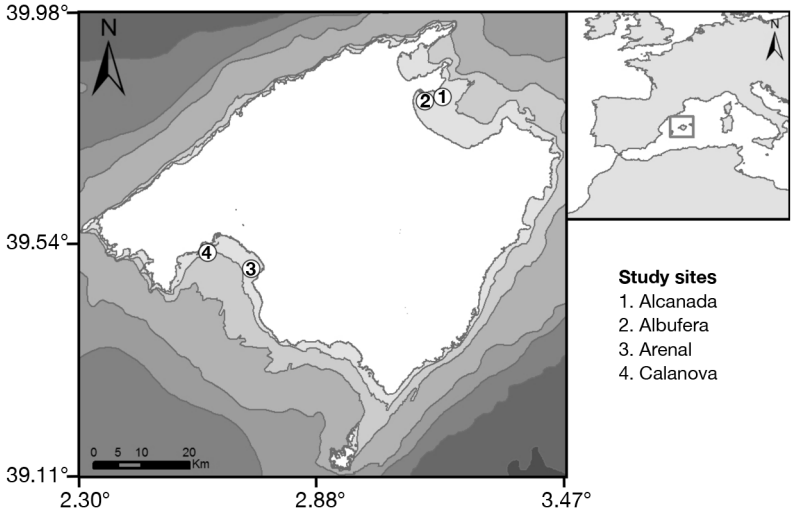

Fig. 1. Study sites in Mallorca, Spain. Grey zones correspond to isobaths (25, 50, 100, 200 m)

performed every ~3 h in a 24 h cycle, and a fresh set of plant materials was used each time.

For each incubation time, the second to the oldest leaf of each of 3 independent shoots was selected and cut into 5 cm segments from the top (oldest, heavily epiphytized leaf segment), middle (moderately epiphytized) and bottom (barely epiphytized youngest segment). From each of the 3 independent shoots, 5 cm piece of unrinsed rhizomes and roots were also selected. Additional 5 cm pieces of roots were selected from independent shoots and surface-sterilized to measure root endophyte N_2 fixation rates by a series of sterilization steps (i.e. 99% ethanol 1 min; 3.125% NaOCl 6 min; 99% ethanol 30 s; autoclaved GF/F filtered seawater final washing; Coombs & Franco 2003). The N_2 fixation rates measured with unsterilized roots include both the root surface N_2 fixers and the endophytic N_2 fixers. The wet weight of all plant materials was previously determined. Each plant part was inserted into its respective incubation vial. Leaves and roots were inserted into 10 ml gas chromatograph (GC) vials and the rhizomes into 50 ml Falcon centrifuge tubes. Each incubation vial or tube was humidified with 1 ml (for the GC vials) and 2.5 ml (for the Falcon tubes) sterilized GF/F filtered seawater from the same sampling site. All vials and tubes were capped with gas-tight septum ports. Vials and tubes containing the rhizomes and roots, respectively, were flushed with helium gas for 1 min to obtain anoxic conditions (to mimic the anoxic conditions in the sediment) and to avoid or minimize the negative effect of exposure of N_2-fixing cells to higher levels of O_2 when the plant parts are directly exposed to air. To avoid solubility and diffusion problems of acetylene that may arise when the plants are completely submerged in seawater, a volume of acetylene gas (final concentration 20% v/v) was then injected into each incubation vial or tube using gas-tight Hamilton syringes. The tubes containing the plant parts were incubated for 3 h underwater in the same *P. oceanica* meadow from which the plants originated.

Directly after the 3 h incubation time, 10 ml of headspace was taken using a gas-tight Hamilton syringe from the incubation vials or tubes, 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 acetylene were determined using a GC (model HP-5890, Agilent Technologies) equipped with a flame ionization detector (FID). The column was a Varian wide-bore column (ref. CP7584) packed with CP-PoraPLOT U (27.5 m length, 0.53 mm inside diameter, 0.70 mm outside diameter, 20 µm film thickness). Helium was used as carrier gas at a flow rate of 30 ml min^{-1}. Hydrogen and airflow rates were set at 30 ml min^{-1} and 365 ml min^{-1}, respectively. The split flow was used so that the carrier gas flow through the column was 4 ml min^{-1} at a pressure of 5 psi. Oven, injection and detector temperatures were set at 52°C, 120°C and 170°C, respectively. Ethylene produced was calculated using the equations in Stal (1988). In the calculations, acetylene was used as an internal standard, which circumvents inaccuracies due to gas losses during the experimental incubations, handling, storage and transport. The acetylene reduction rates were converted to N_2 fixation rates using a factor of 4:1 (C_2H_4:N_2 reduced; Jensen & Cox 1983) and reported per g dry weight of plant biomass incubated. The dry weight of the plant parts was determined by drying the plant parts at 60°C for 24 h (Short & Duarte 2001). The mean (±SD) detection limit for C_2H_4 production based on our method was 0.005 ± 0.003 nmol C_2H_4.

2.3. Data and statistical analyses

To determine the diurnal variability of the hourly rates of N_2 fixation and the effects of plant part, univariate ANOVA factor analysis and post hoc Tukey tests were used using the factors incubation time and plant part (top, middle and bottom portions of the leaves, rhizomes, roots and sterilized roots). The 2-way ANOVAs were performed independently for each season of the year and sampling site (Alcanada, Albufera, Arenal and Calanova). The assumption of homogeneity of variances was tested with Hartley's F_{max} test and our data met the assumption. Moreover, for each sampling site and season, approximate daily N_2 fixation rates for each plant part were calculated by (1) averaging the triplicate hourly N_2 fixation rates (N_x) at each time of day of incubation (T_x), (2) multiplying the average hourly rate N_x by the time interval to the next incubation time ($N_x \times [T_{x+1} - T_x]$), and (3) integrating all values of (2) over the 24 h incubation period. Pearson correlation and regression analyses were used to determine the relationships between the daily N_2 fixation rates of the different plant parts and *in situ* community N_2 fixation rates measured in parallel benthic chamber experiments (Agawin et al. 2017) and the variety of physico-chemical factors measured one time at a day at each sampling period or season in Alcanada, Alcudia Bay (Agawin et al. 2017). The normal distribution of the residuals of the linear regressions was checked using a histogram and P–P plot of standardized residuals,

and the residuals of the reported regression analyses were normally distributed. Statistical analyses were performed using the SPSS version 19 (IBM).

3. RESULTS

3.1. Diurnal variability of hourly N_2 fixation rates among plant parts

3.1.1. Alcanada, Alcudia Bay

At Alcanada, N_2 fixation rates differed significantly among plant parts with time of day during summer 2012 and winter 2012 (incubation time × plant part, $p < 0.05$; Table 1). During summer 2012, the heavily epiphytized top and middle portions of the leaves exhibited significantly higher (Tukey HSD test, $p < 0.05$) rates than the rest of the plant, reaching 0.29 ± 0.00 µg N g^{-1} DW h^{-1} (mean ± SD) during the day (Fig. 2A). In contrast, during winter 2012, root N_2 fixation rates were significantly higher (Tukey HSD test, $p < 0.05$) than in the rest of the plant, reaching 0.12 ± 0.02 µg N g^{-1} DW h^{-1} both during the day and night (Fig. 2C). However, the N_2 fixation rates did not differ significantly among plant parts with time of day during autumn 2012, spring 2013 or summer 2013 (incubation time × plant part, $p > 0.05$; Table 1) but generally differed with plant part (Table 1), with N_2 fixation of the top leaf exhibiting the highest rates during autumn 2012 (Tukey HSD test, $p < 0.05$; Fig. 2B) and summer 2013 (Tukey HSD test, $p < 0.05$; Fig. 2E), while endophyte (i.e. in sterilized roots) N_2 fixation exhibited significantly higher rates than the leaf portions during spring 2013 (Tukey HSD test, $p < 0.05$; Fig. 2D).

3.1.2. Albufera, Alcudia Bay

The N_2 fixation rates differed significantly among plant parts with time of day during the 2 summer sampling periods (2012 and 2013: incubation time × plant part, $p < 0.05$; Table 1) in Albufera. During summer 2012, the top leaf portions exhibited significantly higher rates than the rest of the plant parts, reaching 4.08 ± 0.54 µg N g^{-1} DW h^{-1} (mean ± SD) during the day (Tukey HSD test, $p < 0.05$; Fig. 3A). During summer 2013, the top leaf portions also exhibited significantly higher rates than the rest of the plant parts during the day, but during the night, N_2 fixation rates of root endophytes were significantly higher than those of all leaf portions (Tukey HSD test, $p < 0.05$; Fig. 3B).

3.1.3. Arenal and Calanova, Palma Bay

The N_2 fixation rates differed significantly among plant parts with time of day during the 2 summer sampling periods in Arenal (2012 and 2013: incubation time × plant part, $p < 0.05$; Table 1), but only dur-

Table 1. Results of univariate ANOVA of N_2 fixation rates. Effects of plant part (top, middle and bottom portions of the leaves, rhizomes, roots and sterilized roots) and different incubation times (along a 24 h cycle) at each of the sampling sites (Alcanada and Albufera in Alcudia Bay, Arenal and Calanova in Palma Bay) and sampling periods. *Significant at α = 0.05. In some experiments, some replicates or levels of time of day were missing

	Plant part			Incubation time			Incubation time × plant part			Error
	F	df	p	*F*	df	p	*F*	df	p	df
Alcanada, Alcudia Bay										
Summer 2012	18.79	5	<0.001*	5.38	5	0.001*	4.31	16	<0.001*	42
Autumn 2012	2.85	5	0.027*	0.83	6	0.551	0.95	16	0.513	40
Winter 2012	12.45	5	<0.001*	3.03	8	0.005*	2.56	33	<0.001*	81
Spring 2013	7.54	5	<0.001*	0.84	7	0.556	1.40	24	0.163	46
Summer 2013	5	5	<0.001*	2.07	7	0.060	1.37	31	0.143	64
Albufera, Alcudia Bay										
Summer 2012	15.25	5	<0.001*	4.59	4	0.006*	4.50	17	<0.001*	27
Summer 2013	7.5	5	<0.001*	9.95	7	<0.001*	3.21	34	<0.001*	80
Arenal, Palma Bay										
Summer 2012	11.34	5	<0.001*	5.03	7	0.001*	10.54	14	<0.001*	23
Summer 2013	11.87	5	<0.001*	4.24	7	0.001*	2.00	29	0.009*	75
Calanova, Palma Bay										
Summer 2012	1.85	5	0.130	0.65	5	0.664	1.33	17	0.233	34
Summer 2013	24.7	5	<0.001*	10.38	7	<0.001*	3.14	29	<0.001*	79

ing summer 2013 in Calanova (incubation time × plant part, $p < 0.05$; Table 1). In Arenal and Calanova during summer 2012, there was no clear diurnal pattern of N_2 fixation among plant parts (Fig. 3C,E), but during summer 2013, N_2 fixation associated with the roots at both sites were significantly higher than those associated with all leaf portions, reaching 0.41 ± 0.09 µg N g^{-1} DW h^{-1} during the day in Arenal and 0.42 ± 0.19 µg N g^{-1} DW h^{-1} in Calanova (Tukey HSD test, $p < 0.05$, Fig. 3D,F).

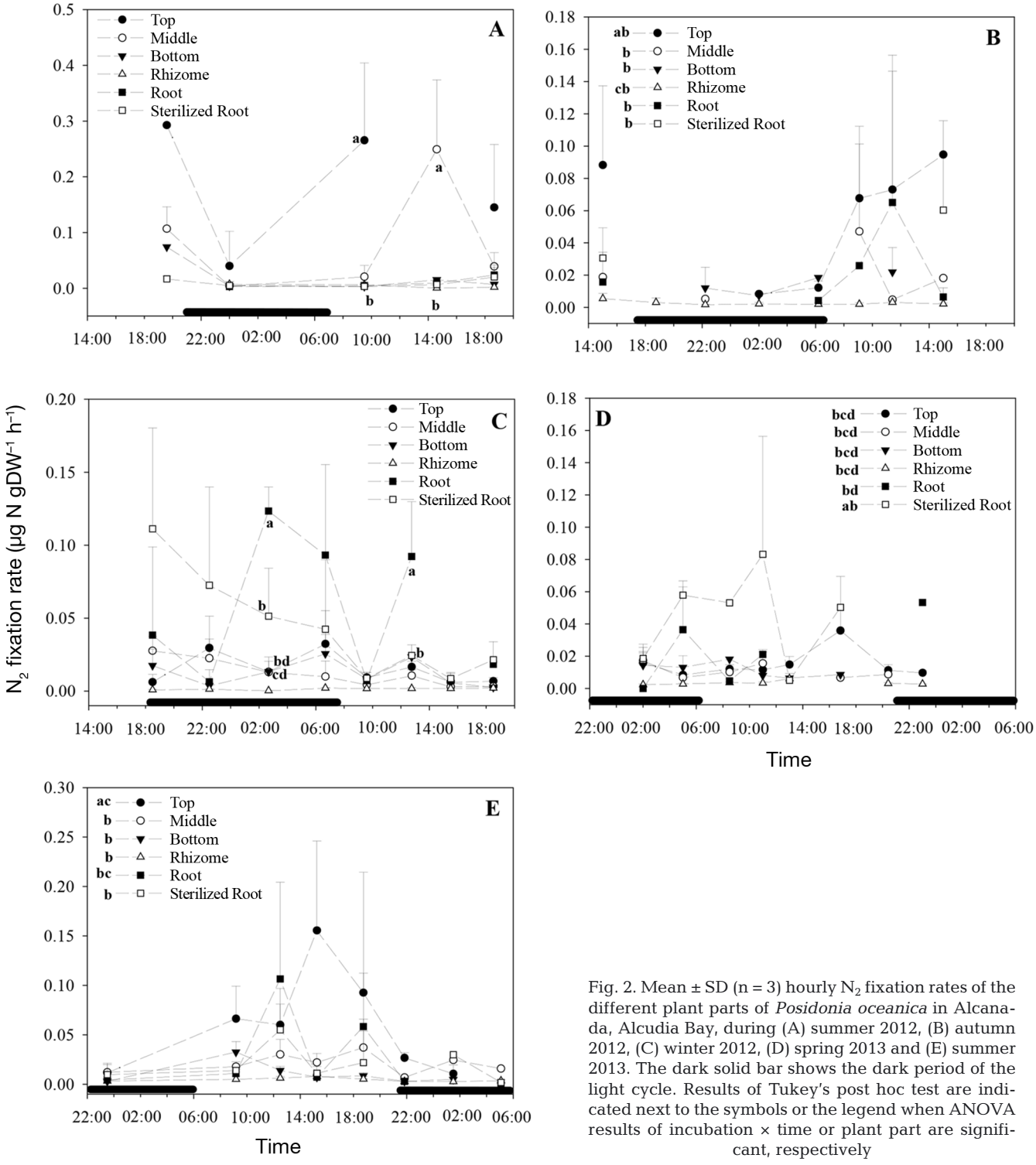

Fig. 2. Mean ± SD (n = 3) hourly N_2 fixation rates of the different plant parts of *Posidonia oceanica* in Alcanada, Alcudia Bay, during (A) summer 2012, (B) autumn 2012, (C) winter 2012, (D) spring 2013 and (E) summer 2013. The dark solid bar shows the dark period of the light cycle. Results of Tukey's post hoc test are indicated next to the symbols or the legend when ANOVA results of incubation × time or plant part are significant, respectively

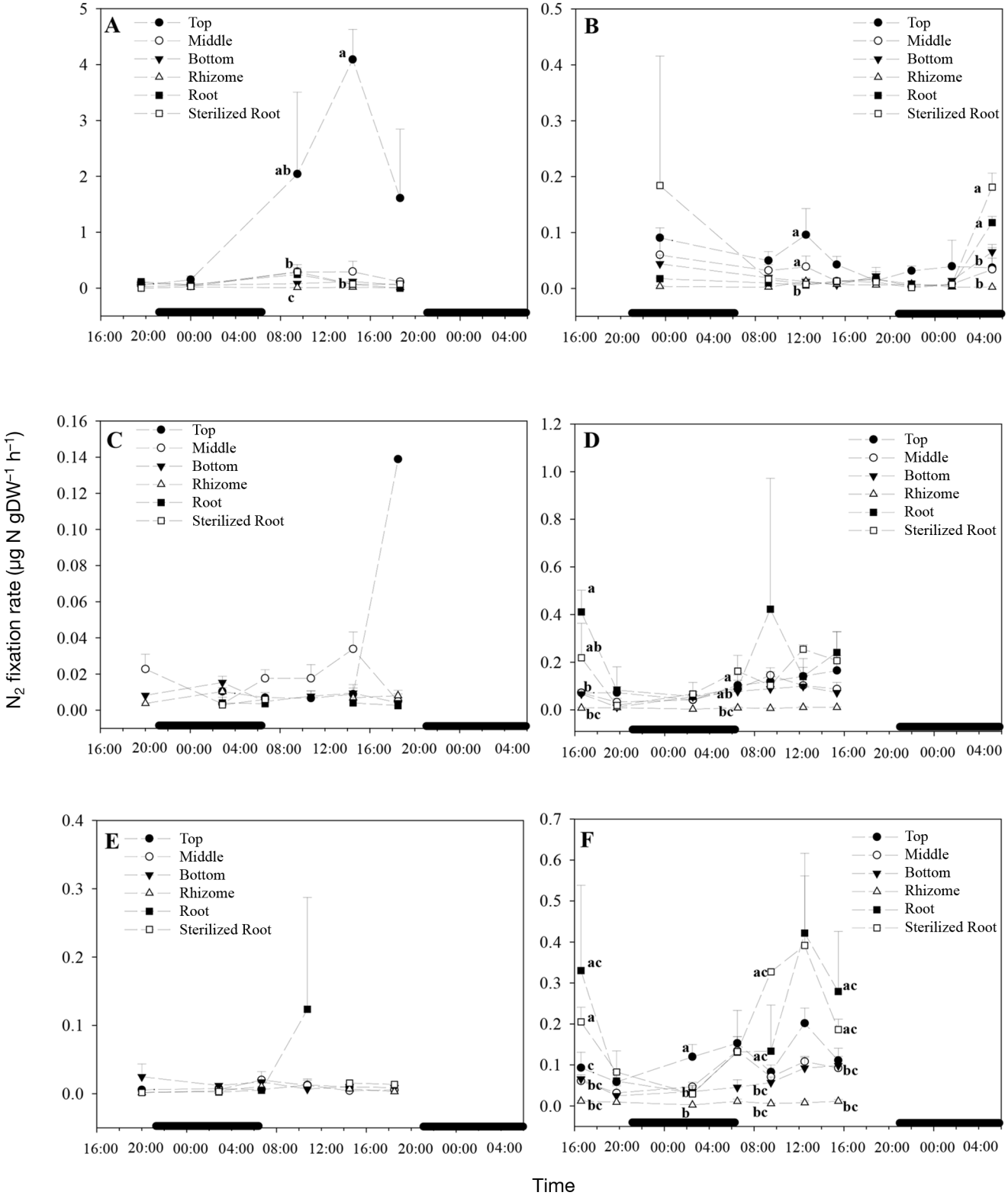

Fig. 3. Mean ± SD (n = 3) hourly N_2 fixation rates of the different plant parts of *Posidonia oceanica* in Albufera, Alcudia Bay, during (A) summer 2012 and (B) summer 2013; in Arenal, Palma Bay, during (C) summer 2012 and (D) summer 2013; and in Calanova, Palma Bay, during (E) summer 2012 and (F) summer 2013. The dark solid bar shows the dark period of the light
Results of Tukey's post hoc test are indicated next to the symbols when ANOVA results of incubation × time are significant

3.2. Integrated daily N_2 fixation rates and correlation with physico-chemical factors

Integrated daily N_2 fixation rates in Alcanada showed a general pattern of high rates for heavily epiphytized top portions of the leaves during summer and autumn, while activity associated with the roots and root endophytes was higher during winter and spring (Fig. 4). The N_2 fixation rates on rhizome surfaces remained low throughout all of the seasons (Fig. 4). Between-site differences in integrated daily N_2 fixation rates were more evident during the summer 2012 sampling, when the rates of all plant parts were higher in Albufera, followed by Alcanada, while low N_2 fixation rates were observed in the more impacted sites in Arenal and Calanova (Fig. 5A). However, during summer 2013, the daily N_2 rates in the different plant parts were similar among sites, but it should be noted that there were high N_2 fixation rates of root endophytes, particularly in Albufera, Arenal and Calanova (Fig. 5B).

Table 2 shows *in situ* community N_2 fixation rates measured in parallel benthic chamber experiments and some of the relevant physico-chemical environmental data of the water column (chlorophyll, temperature, salinity, dissolved O_2, total dissolved nitrogen [TDN] and total dissolved phosphorus [TDP] concentrations), porewater (TDN and TDP) and sediments (%N, C, P) of the 4 sampling sites (data taken from Agawin et al. 2017). Using data for Alcanada, Alcudia Bay, where seasonal sampling was performed, Pearson correlation and regression results showed daily N_2 fixation rates associated with the aboveground part of *P. oceanica* (the leaves in the different segment portions) to be significantly positively correlated with *in situ* community N_2 fixation rates ($r^2 = 0.43$, $p < 0.05$; Fig. 6) and water column TDN ($r^2 = 0.31$, $p < 0.05$) and ambient water temperature ($r^2 = 0.28$, $p < 0.05$; Fig. 7). However, the daily N_2 fixation rates associated with the roots and endophytes were significantly negatively correlated with porewater TDN ($r^2 = 0.51$, $p < 0.05$) and TDP ($r^2 = 0.83$, $p < 0.05$) and ambient temperature ($r^2 = 0.61$, $p < 0.05$), but positively correlated with sediment C content ($r^2 = 0.98$, $p < 0.05$; Fig. 8). In both the phyllosphere and rhizosphere plant parts, no significant correlations were noted with DIN or DIP (data not shown).

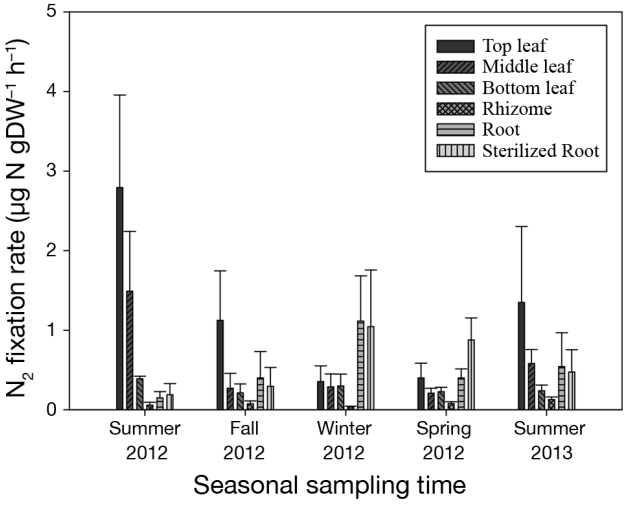

Fig. 4. Mean ± SD integrated daily N_2 fixation rates of the different plant parts of *Posidonia oceanica* in Alcanada, Alcudia Bay, through the seasonal sampling periods. SD is calculated by mathematical propagation of error from the original triplicate hourly N_2 fixation rates to the integrated daily N_2 fixation rate

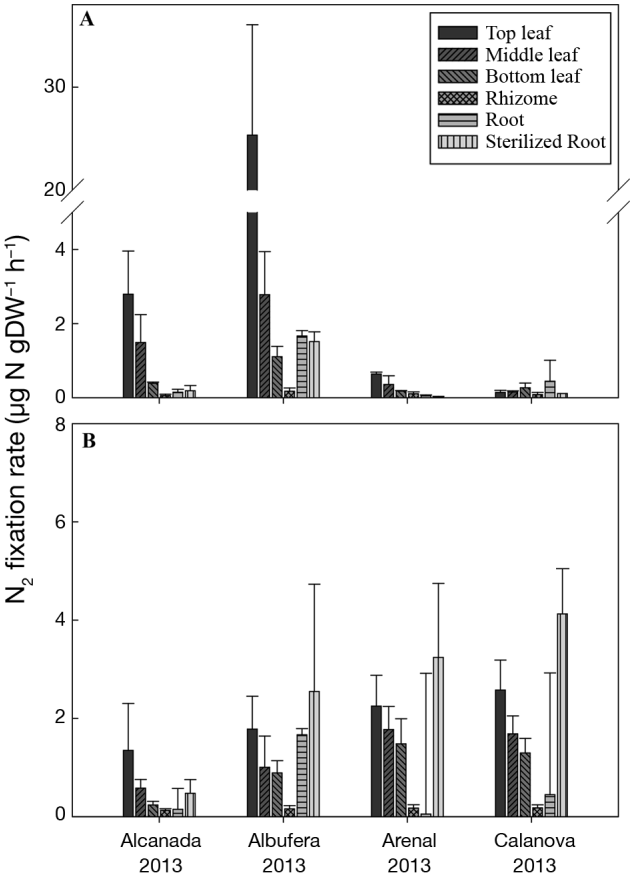

Fig. 5. Mean ± SD integrated daily N_2 fixation rates of the different plant parts of *Posidonia oceanica* at the 4 sites (Alcanada and Albufera in Alcudia Bay; Arenal and Calanova in Palma Bay) during the summers of (A) 2012 and (B) 2013. SD is calculated by mathematical propagation of error from the original triplicate hourly N_2 fixation rates to the integrated daily N_2 fixation rate

Table 2. Community N_2 fixation rates measured from parallel benthic chamber experiments and environmental data — chlorophyll, temperature, dissolved oxygen (O_2) and total dissolved nitrogen (TDN) and TDP from the water column; TDN and TDP from the porewater and % N, C and P in the sediment — at the sampling sites during the sampling periods (data from Agawin et al. 2017)

Sampling site	Community N_2 fixation rate (mg N m^{-2} d^{-1})	Water column					Porewater		Sediment		
		Chlorophyll (mg m^{-3})	Temp. (°C)	O_2 (mg l^{-1})	TDN (µM)	TDP (µM)	TDN (µM)	TDP (µM)	% N	% C	% P
Alcanada, Alcudia Bay											
Summer 2012	1.988	0.07	25.25	7.19	15.42	<0.60	73.31	5.02	<0.05	11.2	0.02
Autumn 2012	0.115	0.16	18.00	5.79	10.71	<0.60	–	<0.60	<0.05	–	–
Winter 2012	0.012	0.06	13.00	6.46	9.54	0.83	3.29	2.35	<0.05	11.50	0.02
Spring 2013	0.074	0.13	18.80	9.00	9.88	<0.60	83.37	3.11	<0.05	–	–
Summer 2013	0.842	–	23.50	4.45	8.39	<0.60	91.6	3.96	<0.05	11.35	0.02
Albufera, Alcudia Bay											
Summer 2012	2.204	0.34	25.50	4.90	5.70	15.96	6.16	68.04	0.28	12.90	0.03
Summer 2013	0.560	–	23.80	4.27	8.70	<0.60	–	<0.60	<0.05	10.72	0.02
Arenal, Palma Bay											
Summer 2012	0.196	0.10	24.25	6.29	4.55	<0.60	82.88	2.17	<0.05	11.59	0.02
Summer 2013	0.483	0.20	27.60	7.14	14.06	<0.60	83.57	9.69	<0.05	11.54	0.02
Calanova, Palma Bay											
Summer 2012	0.511	0.22	23.50	6.88	7.30	0.70	38.00	1.62	<0.05	11.57	0.01
Summer 2013	0.417	0.18	28.30	9.25	14.66	0.63	23.82	0.84	<0.05	11.70	0.01

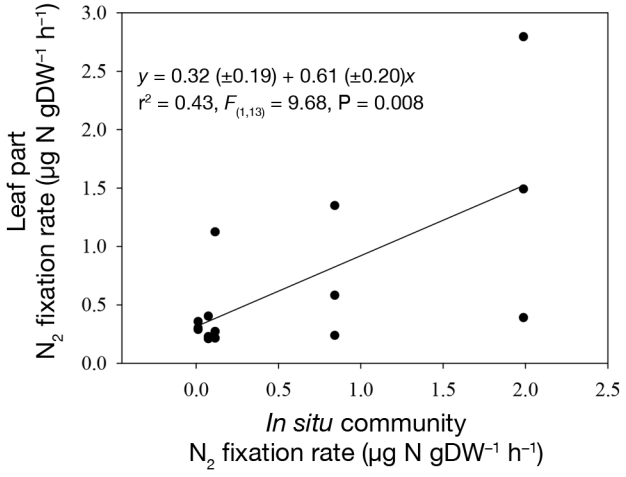

Fig. 6. Linear regression analysis between N_2 fixation rates associated with *Posidonia oceanica* leaf tissues and *in situ* community N_2 fixation rates in Alcanada, Alcudia Bay (Table 2). Results of the statistical analysis are indicated

3.3. Relative contribution of N_2 fixation associated with different plant parts

Taking into account the relative contribution of the different plant parts to overall plant biomass reveals that during summer, the leaves contribute the greater percentage to total N_2 fixation of the plant, especially during summer 2012, when they contributed up to 86.5% (Table 3). During the colder months, the belowground parts play a more significant role in plant N_2 fixation. The rhizomes, in spite of their low specific N_2 fixation rates, contribute significantly to total N_2 fixation (from 10.4 to 66.8%) because of their high biomass (Table 3). The roots clearly exhibit more significant contribution during the colder months (autumn 34.5% and winter 29.7%) than during summer (Table 3).

4. DISCUSSION

4.1. N_2 fixation rates with the different plant parts and their seasonal variability

This study reports, for the first time, the simultaneous measurement of N_2 fixation rates in all plant tissue parts of *Posidonia oceanica* (different portions of leaves, rhizomes and roots). The maximum rates measured here on the heavily epiphytized top portions of the leaves (0.29 ± 0.0 µg N g^{-1} DW h^{-1}) during summer in Alcanada, Alcudia Bay, were similar in magnitude to what has been measured at the same site during summer in 2010 (0.129 ± 0.104 µg N g^{-1} DW h^{-1}; Agawin et al. 2016) (Table 4). Among the sampling sites studied, the highest N_2 fixation rate measured associated with the leaves came from

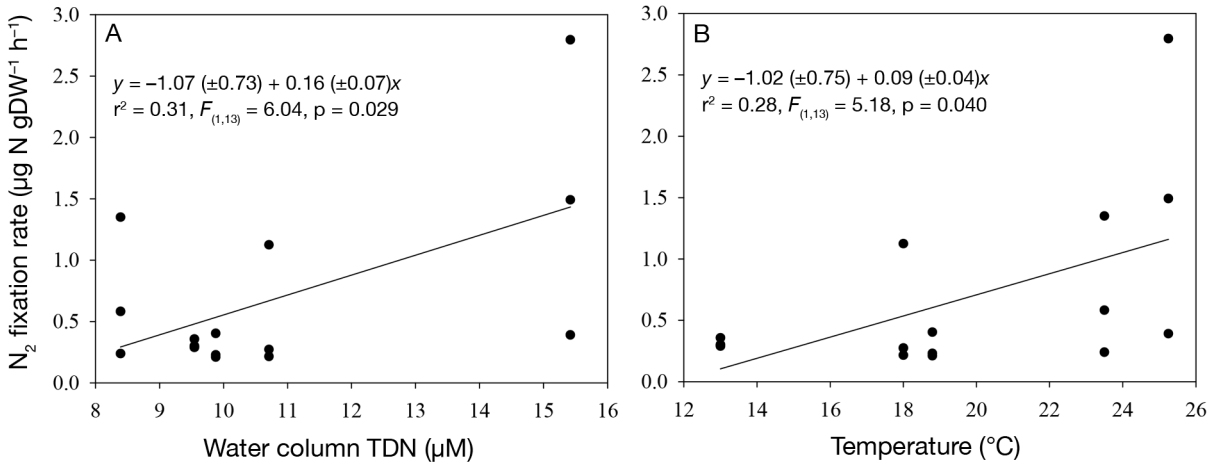

Fig. 7. Linear regression analyses between N_2 fixation rates associated with *Posidonia oceanica* leaf tissues and (A) water column total dissolved nitrogen (TDN) and (B) temperature in Alcanada, Alcudia Bay (Table 2). Results of the statistical analyses are indicated

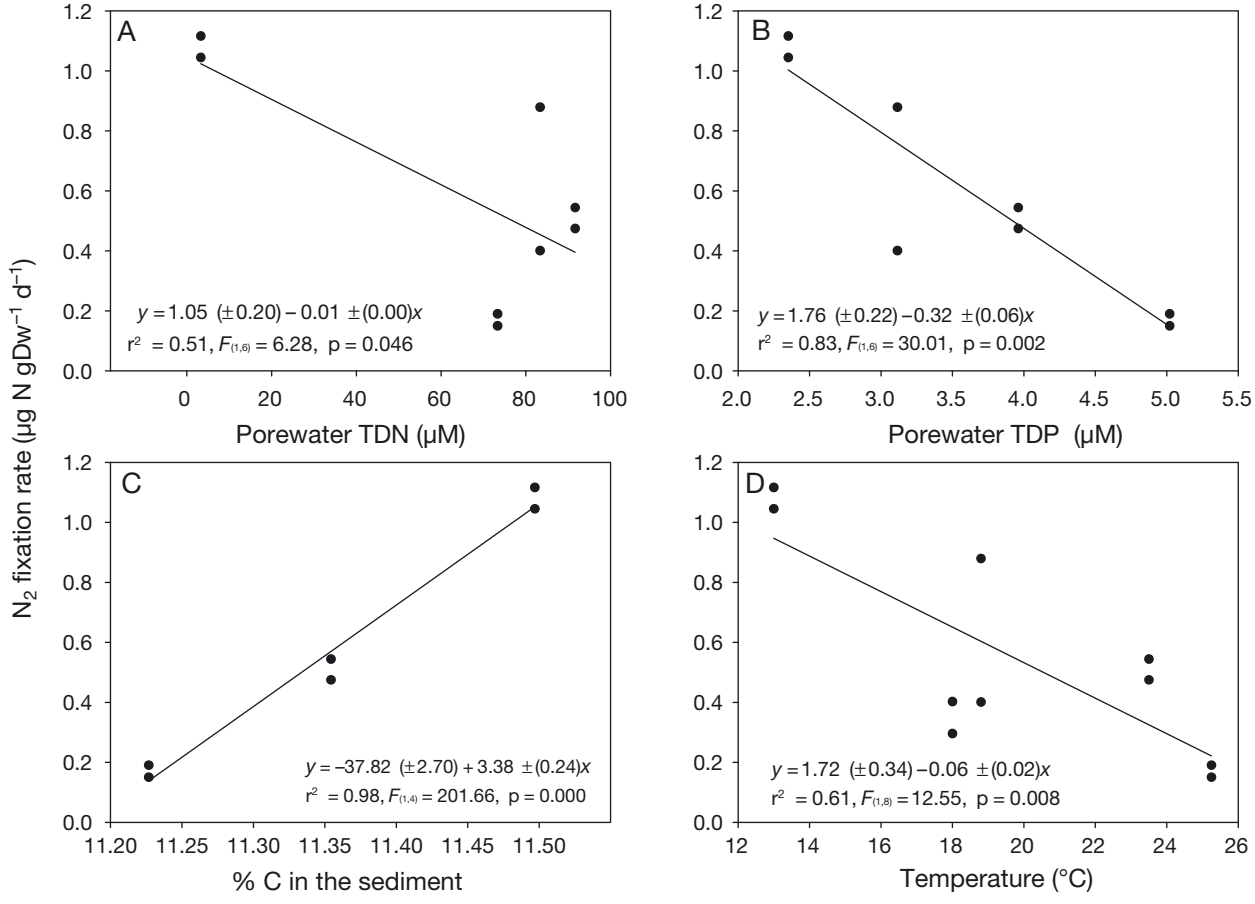

Fig. 8. Linear regression analyses between N_2 fixation rates associated with *Posidonia oceanica* root tissues and (A) porewater total dissolved nitrogen (TDN), (B) porewater total dissolved phosphorus (TDP), (C) % carbon in the sediment and (D) temperature in Alcanada, Alcudia Bay (Table 2). Results of the statistical analyses are indicated

Albufera, Alcudia Bay, which reached 4.1 ± 0.5 µg N g^{-1} DW h^{-1} during summer 2012. This rate is higher than the estimates from tropical seagrasses available from the literature, except for the exceptionally high rates of N_2 fixation reported by Goering & Parker (1972) (Table 4). The rates measured here using cut

Table 3. Relative contribution of N_2 fixation associated with different plant parts (leaves, rhizomes and roots) of *Posidonia oceanica* estimated in Alcanada, Alcudia Bay, during the different sampling seasons. The areal biomass of each plant part was measured in a parallel experiment using 0.02 m^2 sediment cores, where the biomass of rhizomes and roots was quantified at the top 10 cm. The daily N_2 fixation rate associated with the leaves was estimated as the average associated with the bottom, middle and top portions of the leaves. The percentage contribution of the different plant parts to total N_2 fixation is indicated in parentheses

Sampling date	Plant part biomass (g DW m^{-2})			Plant part N_2 fixation rate (µg N g^{-1} DW^{-1} d^{-1})			N_2 fixation associated with different plant parts (µg N m^{-2} d^{-1})		
	Leaves	Rhizomes	Roots	Leaves	Rhizomes	Roots	Leaves	Rhizomes	Roots
Summer 2012	482.9	1556.7	182.8	1.56	0.058	0.15	753.3 (86.5%)	90.3 (10.4%)	27.4 (3.1%)
Autumn 2012	12.5	412.5	49.3	0.53	0.075	0.402	6.6 (11.5%)	31.0 (53.9%)	19.8 (34.5%)
Winter 2012	413.2	2200.1	242.3	0.13	0.080	0.400	53.7 (16.4%)	176.0 (53.9%)	96.9 (29.7%)
Spring 2013	407.0	3325.6	46.3	0.28	0.080	0.401	114.0 (28.6%)	266.4 (66.8%)	18.6 (4.7%)
Summer 2013	422.5	2190.0	75.7	0.73	0.130	0.544	308.4 (48.7%)	284.2 (44.8%)	41.2 (6.5%)

segments of the leaves, which are significantly positively correlated with the *in situ* community N_2 fixation rates (Fig. 6) measured in parallel benthic chamber experiments (Agawin et al. 2017), imply the significance of phyllosphere-associated N_2 fixation in driving the patterns of community N_2 fixation in *P. oceanica* meadows.

Rhizome-associated N_2 fixation remained low throughout our study, consistent with results of Lehnen et al. (2016). In contrast, N_2 fixation rates associated with rhizomes in *Zostera noltii* were higher than those we measured in *P. oceanica*, implying that the significance of rates associated with rhizomes may depend on the seagrass species (Nielsen et al. 2001) (Table 5). N_2 fixation rates associated with roots in *P. oceanica* were found to be maximal in Arenal and Calanova, reaching 0.42 ± 0.19 µg N g^{-1} DW h^{-1} (Fig. 3D,F). This maximal rate associated with roots is higher than that reported for the tropical seagrass *Thalassia testudinum* and the temperate seagrasses *Z. noltii* and *Z. marina*, but lower than the maximal rate reported in *P. oceanica* in Fetovaia Bay (Table 5). Here, we also quantified, for the first time, N_2 fixation rates associated with *P. oceanica* root endophytes, although the presence of *nifH* gene sequences has been reported (but the activity not quantified; Garcias-Bonet et al. 2016). The N_2 fixation rates of root endophytes in *P. oceanica*, although exhibiting wide variability, can contribute totally or significantly to bulk N_2 fixation associated with the roots (Figs. 4 & 5). This is contrast with the findings in Smith & Hayasaka (1982) that surface sterilization of *Z. marina* roots reduced N_2 fixation rates by more than 99%, indicating that a major portion of the roots' N_2 fixers are on the surface.

The N_2 fixation rates associated with the different plant tissues of *P. oceanica* showed seasonal variability, with generally higher rates on the leaves during summer and higher rates associated with roots during winter in Alcanada, Alcudia Bay (Fig. 4).

4.2. Diurnal variability of N_2 fixation rates among plant parts

The diurnal variability of N_2 fixation activities among *P. oceanica* plant parts observed here indicates that each part may harbor a different community of N_2 fixers. In the phyllosphere, significantly high N_2 fixation activities were measured during the day, especially in summer. As O_2 can irreversibly deactivate the N_2-fixing enzyme, nitrogenase, the ability to fix N_2 of organisms found attached to *P. oceanica* leaves during the day may be dependent on their mechanisms to cope with O_2 evolution from daytime photosynthesis of the leaves and/or of the organisms themselves, in the case of phototrophs. 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 nitrogenase) that act as physical barrier to prevent O_2 from diffusing to the enzyme. For non-heterocystous cyanobacteria (e.g. *Trichodesmium* spp.), only 10–20% of their cells contain nitrogenase (Berman-Frank et al. 2003), providing spatial separation between the photosynthetic and N_2-fixing processes. Moreover, the cells containing nitrogenase contain active O_2-consuming systems (Gallon 2001). The phyllosphere of *P. oceanica* harbors both heterocystous and non-heterocystous cyanobacteria as well as anaerobic heterotrophic N_2-fixing bacteria (Agawin et al. 2016, 2017). For anaerobic heterotrophic N_2-fixing bacteria, the mechanisms on how they can reside and fix N_2 in oxygenated surrounding waters need to be investigated.

Table 4. Mean ± SD N_2 fixation rates (based on dry weight [DW] of seagrass leaf biomass) in the phyllosphere of *Posidonia oceanica* and of other seagrass meadows. nd: not detectable. Table updated from Agawin et al. (2016)

Location and seagrass species	N_2 fixation rate (μg N g^{-1} DW h^{-1})	Incubation conditions	Source
Alcanada		Present study	
Posidonia oceanica		Incubations every ~3 h for ~24 h of 5 cm leaf segments (qualitative epiphyte cover: bottom–top or few–many) during:	
	$3.0 \pm 0.0 \times 10^{-3}$ – 0.29	Summer 2012	
	$7.4 \pm 0.0 \times 10^{-3}$ – $9.5 \pm 0.0 \times 10^{-2}$	Autumn 2012	
	$2.6 \pm 0.2 \times 10^{-3}$ – $3.2 \pm 2.3 \times 10^{-2}$	Winter 2012	
	$8.0 \pm 1.0 \times 10^{-3}$ – $3.6 \pm 3.4 \times 10^{-2}$	Spring 2013	
	$2.8 \pm 0.0 \times 10^{-3}$ – 0.16 ± 0.09	Summer 2013	
Albufera			
P. oceanica	$2.7 \pm 0.2 \times 10^{-2}$ – 4.1 ± 0.5	Summer 2012	
	$6.5 \pm 2.0 \times 10^{-3}$ – $9.6 \pm 4.7 \times 10^{-2}$	Summer 2013	
Arenal			
P. oceanica	$4.1 \pm 0.6 \times 10^{-3}$ – 0.14 ± 0.00	Summer 2012	
	$1.2 \pm 0.0 \times 10^{-2}$ – 0.16 ± 0.06	Summer 2013	
Calanova			
P. oceanica	$6.6 \pm 0.5 \times 10^{-3}$ – $1.3 \pm 0.8 \times 10^{-2}$	Summer 2012	
	$2.5 \pm 0.6 \times 10^{-2}$ – $2.0 \pm 0.4 \times 10^{-1}$	Summer 2013	
Fetovaia Bay, Mediterranean Sea			Lehnen et al. (2016)[a]
P. oceanica	6.4×10^{-2} – 8.2×10^{-2}	Incubation for 1 d, rate per h is calculated by dividing by 24 h	
Bay of Alcudia, NW Mediterranean Sea			Agawin et al. (2016)
P. oceanica	$2.8 \pm 1.1 \times 10^{-3}$ – $1.29 \pm 1.04 \times 10^{-1}$	Daytime incubations of 5 cm leaf segments	
	$2.8 \pm 2.8 \times 10^{-3}$ – $1.18 \pm 1.32 \times 10^{-1}$	Nighttime incubations of 5 cm leaf segments (qualitative epiphyte cover: bottom–top or few–many); summer 2010	
	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 2012	
	$2.6 \pm 3.3 \times 10^{-1}$ – 2.53 ± 0.74	Whole-shoot phyllosphere *in situ* chamber incubations (multiple sampling in a span of ~ 24 h); spring 2012	
Dar es Salaam, Tanzanian coast, Western Indian Ocean			Hamisi et al. (2009)
Halodule uninervis	1.4×10^{-1} – 2.69	Light	
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 *H. uninervis* & C. *rotundata*	5.6×10^{-2} – 1.84	Day	
	1.26×10^{-2} – 9.74×10^{-1}	Night	
Florida Keys			McRoy et al. (1973)
Syringodium fiiforme	Trace	Light	
	0	Dark	
Thalassia testudinum	Trace	Light	
	0	Dark	
	0 – 2.4×10^{-2}	Light	
	0 – 5.6×10^{-2}	Dark	
Bimini Harbor, The Bahamas			
T. testudinum	5.01×10^{-1} – 1.37	Light	Capone et al. (1979)
	5.82×10^{-2} – 1.87×10^{-1}	Dark	
Redfish Bay, Texas			
T. 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)	

[a]Employed $^{15}N_2$ incorporation technique, whereas the rest of the studies cited employed the acetylene reduction assay

The pattern of root N_2 fixation activities — especially during winter, when they are significantly higher than those of the rest of the plant parts — shows high levels not only during the day but also during the night. Dissolved O_2 sediment in *P. oceanica* meadows rapidly diminishes at less than 6 cm depth (Holmer et al. 2003), and the roots of *P. oceanica* can extend deeper than 40 cm (Duarte et al. 1998); therefore, it is expected that the N_2 fixers associated with roots are subject to less O_2 or even anoxic conditions, and thus the issue of nitrogenase inactivation by O_2 may not be as problematic compared to that of N_2 fixers associated with the oxygenated phyllosphere. The peak root N_2 fixation exhibited during the day (Figs. 2A,B,E, 3D & F) also indicates the importance of root exudates (organic matter produced from aboveground during the day and transported to the roots) in stimulating the N_2 fixation activities of heterotrophs (Blaabjerg et al. 1998, McGlathery et al. 1998). However, the method that we employed here of separating the roots (and rhizomes) from their source of organic matter (leaf photosynthesis) may have underestimated their associated heterotrophic N_2 fixation activities and the effect on diel patterns. Our method may also have led to overestimation of root- (and rhizome-) associated heterotrophic N_2 fixation activities because of the release of dissolved organic carbon from the plant tissues due to leakage or physical damage (Welsh et al. 1996, Welsch 2000) involved in the cutting of root (and rhizome) pieces during the preparation of the plant parts for incubation.

4.3. Possible factors influencing N_2 fixation rates

4.3.1. Temperature

The finding that temperature is positively correlated with N_2 fixation rates on leaves of *P. oceanica* while it is negatively correlated with root-associated N_2 fixation suggests that the leaves and roots of *P. oceanica* have different compositions of N_2 fixers with differing optimum temperatures for N_2 fixation. The high N_2-fixing rates associated with the cyanobacteria-dominant phyllosphere of *P. oceanica* (Agawin et al. 2017) during summer, when temperatures can reach >23°C (present study; Table 2), corroborates with general unimodal temperature responses of nitrogenase enzymes (i.e. low activity below 15°C and temperature optima starting from 20°C; Ceuterick et al. 1978, Rainbird et al. 1983) and global distribution patterns of known N_2-fixing cyanobacteria (Falcón et al. 2005). Sulfate-reducing bacteria (SRB) are known to be associated with *P. oceanica* roots (Lehnen et al. 2016, Bertics et al. 2010). To date, there have been no studies on optimum temperature of growth and activity specific to N_2-fixing SRBs associated with the root tissue. In other seagrasses (*Z. marina*, *Z. noltii* and *Z. capricorni*), N_2 fixation rates measured in the rhizosphere with their associated sediments have high values in summer and low values in winter (O'Donohue et al. 1991, Welsh et al. 1996, McGlathery et al. 1998). However, here we have measurements of the highest N_2 rates associated with roots during winter, when temperature was minimal at 13°C, suggesting that N_2 fixers associated with the plant tissues in the rhizosphere may be adapted to function, and even have maximal activities, at low temperatures. This may be plausible because there are indeed N_2 fixers adapted to colder habitats (Prévost et al. 1987), but deeper investigation of the physiological basis of this is warranted. N_2 fixation in lower-temperature waters is one of the future directions of research in marine N_2 fixation (Sohm et al. 2011). Investigations on how their habitat (i.e. being buried in the sediment and being sheltered in the interior of the roots of *P. oceanica* in the case of the N_2-fixing endophytes) should also be explored.

4.3.2. Nutrients

The significant correlation of sediment organic C content and N_2 fixation associated with the roots of *P. oceanica* found here indicates that a major portion of N_2 fixers associated in the roots or in the sediment can indeed be heterotrophs and that their activity can be controlled by organic C availability in the sediment. The control of heterotrophic N_2 fixation associated with the rhizosphere in seagrasses by organic C availability has been demonstrated by enhanced activity upon addition of exogenous organic carbon sources to the sediment in *Z. marina* (McGlathery et al. 1998) and *Z. noltii* (Welsh et al. 1996), and positive correlations between the sediment depth profile of N_2 fixation and potential carbon sources (Welsh et al. 1996).

The effect of nutrient availability (i.e. N and P) on N_2 fixation activities in seagrass beds may depend on the habitat where the N_2 fixers are found and the concentration of these nutrients. DIN has long been recognized as an inhibitor of N_2 fixation (Falkowski 1983, Ramos et al. 1985, Martin-Nieto et al. 1991).

Table 5. Mean ± SD N_2 fixation rates (based on dry weight [DW] of seagrass root and rhizome biomass) in the rhizosphere of *Posidonia oceanica* and of other seagrass meadows. nd: not detectable

Location, species, part		N_2 fixation rate (μg N g^{-1} DW h^{-1})	Incubation conditions	Source
Posidonia oceanica	Alcanada		Incubation every ~3 h for ~24 h during:	Present study
Rhizomes		$4.4 \pm 1.9 \times 10^{-4}$ – $7.0 \pm 5.1 \times 10^{-3}$	Summer 2012	
		$2.0 \pm 1.0 \times 10^{-3}$ – $5.4 \pm 3.2 \times 10^{-3}$	Autumn 2012	
		$2.6 \pm 0.9 \times 10^{-4}$ – $2.1 \pm 0.4 \times 10^{-3}$	Winter 2012	
		$2.3 \pm 0.5 \times 10^{-3}$ – $6.0 \pm 3.2 \times 10^{-3}$	Spring 2013	
		$2.5 \pm 0.7 \times 10^{-3}$ – $7.9 \pm 0.7 \times 10^{-3}$	Summer 2013	
		$1.0 \pm 0.1 \times 10^{-2}$ – $1.8 \pm 1.6 \times 10^{-2}$	Summer 2012	
		$2.2 \pm 0.8 \times 10^{-3}$ – $1.2 \pm 0.6 \times 10^{-2}$	Summer 2013	
	Albufera	$3.7 \pm 0.2 \times 10^{-3}$ – $8.0 \pm 3.00 \times 10^{-3}$	Summer 2012	
		$2.9 \pm 0.8 \times 10^{-3}$ – $1.0 \pm 0.4 \times 10^{-2}$	Summer 2013	
	Arena	$1.4 \pm 0.5 \times 10^{-3}$ – $1.0 \pm 0.5 \times 10^{-2}$	Summer 2012	
		$2.1 \pm 1.0 \times 10^{-3}$ – $1.2 \pm 0.2 \times 10^{-2}$	Summer 2013	
	Calanova	$3.1 \pm 1.5 \times 10^{-3}$ – $2.4 \pm 1.3 \times 10^{-2}$	Summer 2012	
		$4.2 \pm 0.0 \times 10^{-3}$ – $6.5 \pm 8.0 \times 10^{-2}$	Autumn 2012	
		$6.4 \pm 0.8 \times 10^{-3}$ – $1.2 \pm 1.6 \times 10^{-1}$	Winter 2012	
Roots	Alcanada	$4.6 \pm 1.4 \times 10^{-3}$ – $5.3 \pm 0.0 \times 10^{-2}$	Spring 2013	
		$1.1 \pm 0.0 \times 10^{-3}$ – $1.06 \pm 0.9 \times 10^{-1}$	Summer 2013	
	Albufera	$6.3 \pm 0.2 \times 10^{-2}$ – $2.4 \pm 0.1 \times 10^{-1}$	Summer 2012	
		$5.4 \pm 2.3 \times 10^{-3}$ – $1.2 \pm 1.1 \times 10^{-1}$	Summer 2013	
	Arenal	$2.5 \pm 0.0 \times 10^{-3}$ – $4.1 \pm 2.8 \times 10^{-3}$	Summer 2012	
		$5.6 \pm 2.1 \times 10^{-2}$ – $4.1 \pm 0.9 \times 10^{-1}$	Summer 2013	
	Calanova	$2.3 \pm 0.2 \times 10^{-3}$ – $1.2 \pm 1.6 \times 10^{-1}$	Summer 2012	
		$3.1 \pm 1.8 \times 10^{-2}$ – $4.2 \pm 1.9 \times 10^{-1}$	Summer 2013	
P. oceanica	Fetovaia Bay, Mediterranean Sea		Incubation for 1 d, rate per h is calculated by dividing by 24 h	Lehnen et al. (2016)[a]
Rhizomes		nd	Late spring 2014	
Roots		1.75×10^{-1} – 1.69		
Zostera noltii	Arcachon Bay, N Atlantic Ocean		Summer 1997	Nielsen et al. (2001)
Rhizomes		~0.308		
Roots		~0.084		
Z. marina	Bellport Bay, N Atlantic Ocean		Summer 1980	Capone & Budin (1982)
Rhizomes		1.1×10^{-2} – 2.1×10^{-2}	Aerobic	
		3.9×10^{-1} – 4.6×10^{-1}	Anaerobic	
Roots		2.4×10^{-3} – 8.0×10^{-3}	Aerobic	
		1.2×10^{-2} – 1.8×10^{-2}	Anaerobic	
Thalassia testudinum	Florida Keys		Winter 1973	McRoy et al. (1973)
Rhizomes & roots		0 – 1.9×10^{-5}	Light; anaerobic	
		0 – 8.4×10^{-5}	Dark; anaerobic	

[a]Employed $^{15}N_2$ incorporation technique, whereas the rest of the studies employed the acetylene reduction assay

Although Knapp (2012) provided evidence that benthic N_2 fixers are less sensitive to elevated concentrations of NO_3^- (29 μM) or NH_4^+ (190 μM), we found a significant negative correlation between N_2 fixation associated with *P. oceanica* roots and sediment porewater TDN concentrations (<85 μM) in Alcanada, Alcudia Bay. Further studies are needed to investigate the concentrations of (TDN composed of DIN and DON), which can inhibit or totally shut down the functioning of different species of N_2 fixers, because most previous studies have dealt with inhibition by DIN (e.g. Knapp 2012), when DON (e.g. urea and amino acids) is also assimilated by N_2-fixing organisms (Benavides et al. 2017). The positive correlation

in the present study between N_2 fixation associated with the leaves and water column TDN may be plausible given that water column TDN had generally lower concentrations (up to 10× lower) than in sediment porewater (Table 2) and could also be due to the direct release of TDN as a consequence of N_2 fixation in the phyllosphere by cyanobacteria. It has been previously reported that a significant fraction of recently fixed N (as DIN and DON) can be directly released by cyanobacteria, releasing up to 80% of the N into the surrounding environment (Bronk et al. 1994, Ohlendieck et al. 2000, Mulholland et al. 2004).

P can be an important limiting nutrient for N_2 fixation based on evidence in some isolates of N_2 fixers (Krauk et al. 2006). The importance of P as a limiting nutrient for N_2 fixation can especially be pronounced in the Mediterranean, where P is considered to be severely limiting (Thingstad et al. 2005). Comparing the N_2 fixation rates among the sites studied here, the highest rates associated with the leaves were measured during summer 2012 in Albufera (Fig. 4), where TDP was highest in the water column (Table 2), indicating stimulation of N_2 fixation with higher TDP and that the N_2 fixers associated with the leaves may be able to use organic P sources utilizing alkaline phosphatases (Dyhrman et al. 2006). However, this pattern can be habitat-specific and may be dependent on the N_2-fixing community present and their P requirements, as well as the mechanisms they use to cope with P limitation or inhibition with excess P. For example, previous experiments have shown differing responses of different N_2-fixing species with DOP additions (Benavides et al. 2018). In the sediments of Alcanada, Alcudia Bay, where TDP concentrations are 8 times higher than in the water column (Table 2), the N_2 fixers may not be limited by P in the sediment, and may even be inhibited by high P concentrations, as indicated by the negative correlation between N_2 fixation associated with the roots and porewater TDP at this site. The suggested inhibition of N_2 fixation with high concentrations of P deserves further experimental study.

In summary, we measured simultaneous N_2 fixation in all parts of *P. oceanica* (different portions of leaves, rhizomes and roots). Each part may harbor a different community of N_2 fixers, which may allow them to exhibit different temporal patterns of N_2 fixation activity and different responses to environmental factors (e.g. temperature and nutrient limitation). Their response to environmental factors can be species-specific and may depend on the microhabitat (with specific environmental and nutrient regimes) they are subjected and adapted to.

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