

## RESEARCH ARTICLE

# Species-specific functional trait responses in two species coexisting along a shore-to-inland dune gradient

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## Keywords

Carbon isotopic discrimination; coastal dunes; environmental gradients; leaf reflectance; nitrogen isotope composition; photochemical efficiency; water use efficiency.

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## ABSTRACT

- Coastal dunes are characterised by strong gradients of abiotic stress, typically increasing in severity from inland areas towards the shoreline. Thus, dune gradients represent unique opportunities to study intraspecific responses to environmental changes and to investigate which factors drive community change. This study aims to examine functional trait variation in two coexisting species in response to environmental changes along a dune gradient in NW Spain. Trait convergence was also investigated and compared between both ends of the gradient.
- We measured functional leaf traits related to plant efficiency in the use of light, water and nutrients, also possible stressors (salt content and pH) and availability of limiting resources (water and nutrients) in the soil.
- Most soil variables showed changes following a non-directional gradient. Differences in soil variables were site specific and depended on growth of the study species. Structural and functional traits depended on species and/or plant position on the gradient, except for effective quantum yield of PSII and leaf  $\delta^{15}\text{N}$ . The pattern of variation was mostly directional for reflectance indices related to leaf physiology. Multivariate analyses showed significant interspecific differences in the set of traits they exhibited along positions in the gradient. Species also differed in the combination of traits selected under given environmental conditions.
- Coexisting species display a specific set of traits that reflects different strategies to environmental stress. Our study highlights the overly simplistic nature of some previous studies that assume dune gradients are monotonically directional, without considering that these gradients may be differentially modified by species activity.

## INTRODUCTION

Coastal sand dunes occur at the interface between marine and terrestrial environments. These contrasting influences create highly stressful disturbance-prone environments, to hostile for most species to persist (Griffiths 2006; Miyanishi & Johnson 2020). The species that inhabit such habitats must endure the harsh conditions related to the combination of multiple stresses and disturbances occurring at different temporal and spatial scales (Franks 2003; Forey *et al.* 2008; Conti *et al.* 2017). The intensity of these factors and disturbances often varies gradually from the shoreline to inland locations, thus determining complex environmental gradients (Franks 2003; Griffiths 2006). These complex gradients determine distribution of species along the shoreline–inland coast gradient, rather than any specific environmental factor (Angiolini *et al.* 2013; Fenu *et al.* 2013). As a rule, the highest stress occurs at the coastal end of such gradients because of the increased severity of abiotic factors (high salt spray and soil salinity, low nutrient availability, sand burial, sand blasting). At the inland end of such gradients, the main hostile factors are related to competition (biotic) rather than physical factors (Houle 1996; Griffiths 2006; Lortie & Cushman 2007).

Therefore, the nature of the selective forces may change drastically over variations in the dune gradients. As a result, it is expected that species inhabiting the foredune develop very contrasted strategies to those of inland species. The former species would be restricted to foredunes because of lower competitive ability, whereas inland species would be unable to thrive in foredunes because of lower tolerance to the harsh abiotic conditions. Spatial variations in environmental factors, such as soil salinity (Kachi & Hirose 1979), soil water and nutrient content (Kachi & Hirose 1979), salt spray (Barbour 1978) and sand movement (Maun 1998) occur gradually over the dune gradient, and plant species are distributed into zones according to their tolerance the specific conditions (Hesp 1991; Fenu *et al.* 2013). This tolerance is a consequence of the numerous adaptations of coastal dune species: e.g. pubescence, epicuticular waxes (Ehleringer *et al.* 1976), leaf folding and rolling to minimize water loss, succulence and storage organs to control water supply (Maun 1994), accumulation of solutes (Khedr *et al.* 2003),  $\text{C}_4$  or CAM metabolism (Elhaak *et al.* 1997), special germination strategies (Maun 1994), plasticity in functional traits (Maun 1998; Ciccarelli *et al.* 2016), mycorrhizal interactions (Çakan & Karataş 2006), and differences in life-form (Çakan & Karataş 2006). Very few dune species are able to occupy the

entire dune gradient, with ecotypes that can coexist along the dune gradient (Crawford 2008), e.g. *Eryngium maritimum* L. and *Pancreatum maritimum* L., which are perennial herbs often found growing from shoreline to inland locations.

Geographic environmental gradients, where groups of species occur under different conditions, provide natural laboratories for the study of relative effects of taxonomy and environmental plasticity on variations in species functional traits (Asner *et al.* 2014; Turnbull *et al.* 2016; Fyllas *et al.* 2020). Studies comparing variations in functional traits across species and gradients may aid in identifying life history strategies (Reich *et al.* 2003; Adler *et al.* 2014; Fyllas *et al.* 2020). Such studies also contribute to better predicting vegetation dynamics under global change, assuming that contrasting sets of functional traits might result in different responses to increased temperatures and drought (Carnicer *et al.* 2013; Huxley *et al.* 2023). Dune gradients, in particular, provide a unique opportunity to study intraspecific responses to environmental changes in natural conditions, and to investigate abiotic factors that may drive plant adaptation (Ciccarelli & Bona 2022) and community change (Lortie & Cushman 2007). Moreover, coastal dunes also represent vulnerable systems, threatened by urban expansion, biological invasions, and rising sea levels as a result of climate change. Such changes may affect unique processes of these systems that are important for ecosystem functioning and the services that the ecosystem provides.

Previous studies in coastal dune systems reported that many severe stressors, e.g. salt spray, sand burial, swash inundation, drought, high light, wind exposure and nutrient deficiency are directional and increase or decrease consistently with distance from the shoreline (Kachi & Hirose 1979; Hesp 1991, 2002). However, microhabitat selection or habitat modifications by a species, spatial heterogeneity caused by landform and geomorphic processes, or interactions between factors (Houle 1997) disrupt directionality of environmental gradients.

Here, we (i) investigate changes in soil physicochemical characteristics along a dune gradient where *E. maritimum* and *P. maritimum* coexist. We hypothesized that species along dune gradients may differentially alter directionality of dune gradients as a result of their idiosyncratic biological activity. To test this hypothesis, we measured physicochemical variables along the dune gradient in bare soil and in soil surrounding the roots of *E. maritimum* and *P. maritimum*. These variables might explain variations in leaf physiology and determine species spatial patterns, since they can act as stressors (salt content and pH) or limit important resources (water and nutrients) for plant survival and growth. (ii) We assessed whether *E. maritimum* and *P. maritimum*, the two species that coexist in our study system along the whole 600-m long dune gradient, differ in leaf traits related to resource-use efficiency, i.e. light, water and nitrogen, which underpin differences in ecological strategies. As both species have different leaf morphology, phenology and life form, we expected significant interspecific variations in their responses to changes along the gradient. We also expected some concordance between variation in leaf functional traits and soil physicochemical variables, presumably consistent with gradients from the shoreline to the inland, since previous studies have reported that plant zonal distribution patterns in dune systems correlate with variables such as soil salinity (Kachi & Hirose 1979; Maccioni *et al.* 2021), soil water content (Kachi & Hirose 1979; Zunzunegui *et al.* 2022), salt spray (Du &

Hesp 2020), and sand movement (Maun 1998; Miller 2015). Finally, we examined (iii) species trait divergence at different positions along the gradient. We predicted lower trait variance at the shoreline end of the dune gradient, where environmental conditions are harsher than at the inland extreme of the gradient because of environmental filtering effects on community assembly (Meinzer 2003; Reich *et al.* 2003; Bucci *et al.* 2004).

## MATERIAL AND METHODS

### Species and location

We chose two perennial herbs, *Pancreatum maritimum* L. and *Eryngium maritimum* L. that coexist along a 600 m coast–inland gradient in the coastal dune system of Valdoviño (43°36′44″ N, 8°10′58″ W; Galicia, NW Spain). This gradient is part of the well-preserved dune system and lagoon of Valdoviño, a Natural Space in the General Protection Regime of the Spanish government, and part of the Natura 2000 network of the European Union. The lagoon (Ramsar listed in 1992), with a surface area of ca. 75 ha and average depth of 1.5 m, is fed by the rivers Vilar and Castro, being separated from the sea by an extensive system of rising dunes in which this study was carried out (Habitats 2110 “embryonic dunes” and 2130 “fixed coastal dunes with herbaceous vegetation/grey dunes”; Annex I of the Habitat Directive (92/43/CEE D). Geomorphologically, the site is characterized by schists framed by gabbros, amphibolites, orthogneiss, granite and migmatites as well as a large variety of sedimentary environments, with a detrital group, mainly sandy, associated with the beach systems and dune chains, and a series of fine sediments, clays and silts constituting sediments of the lagoon (Calvo & Macías 2000).

*P. maritimum* and *E. maritimum* were chosen as they are abundant and the only two species that coexist along the 600-m long gradient, from the shore to the terrestrial environment (hereafter shore-to-inland gradient) (Bermúdez 2013). The gradient has a slope of 4.2%, and orientation NNE (Thornthwaite’s classification), the climate is characterized as Humid IV Mesothermic II (Carballeira *et al.* 1983), with average temperature 13.0 °C and annual thermal oscillation of 9.5 °C in the coldest month and 18.3 °C in the warmest month. *P. maritimum* L. (Amaryllidaceae) is a cryptophyte with a bulbous stem and leaves that emerge from a basal rosette. The glaucous blue-green leaves are thick, long and narrow, with 3–15 hermaphroditic, funnel-shaped white flowers that blossom in late summer (August–October) in umbels at the top of reproductive stems. *E. maritimum* L. (Umbelliferae) is a perennial hemicryptophyte, with a long taproot to depths of 50 cm in sand or shingle. The leathery blue-green leaves are three-lobed, folded, stiff and waxy, with prickly borders. Reproductive stems range from 0.15 to 0.60 m in height, bearing inflorescences consisting of dense capitula with spiny bracts (Isermann & Rooney 2014).

### Sampling design

Four 20 × 8 m plots were established along a shore-to-inland gradient running perpendicular to the coastline. The plots were placed with the longest side parallel to the coast at different positions from the coast: the first starting on the foredune (0 m), and the remaining at distances of 200, 400 and 600 m from the foredune. Twelve mature plants of *P. maritimum* and

*E. maritimum* were randomly selected in each plot and used to measure instantaneous and time-integrated functional leaf traits, as described below.

### Leaf spectral reflectance

Spectral reflectance (300–1100 nm) of a fully expanded leaf was measured in 12 randomly selected mature individuals per species and plot and recorded with a portable spectroradiometer (Unispec, PP Systems, Haverhill, MA, USA). The spectral resolution (defined as the Full Width Half Maximum, FWHM) of the UniSpec instrument is ca. 10 nm with a  $\sim 3$  nm sampling interval (Raleigh resolution  $< 10$  nm; Bin size –diode array– 3.3 nm). Leaf spectral reflectance was calculated by dividing leaf spectral radiance by radiance of a reflective white standard (Spectralon Reflectance Standard; Labsphere, North Sutton, NH, USA). Reflectance data were then processed using AVICOL v.6 software (Gomez 2006) to calculate indices related to functional traits and physiological processes: chlorophyll index (CHL), measured as  $R_{750}/R_{550}$  (Lichtenthaler *et al.* 1996), where  $R_{750}$  and  $R_{550}$  are reflectance at 750 and 550 nm, respectively.  $R_{750}$  is relatively insensitive to chlorophyll content, whereas  $R_{550}$  is sensitive to variations in leaf chlorophyll content. This index is highly correlated with leaf chlorophyll content (Gitelson & Merzlyak 1997). Photochemical reflectance index (PRI),  $(R_{531} - R_{570}) / (R_{531} + R_{570})$  (Peñuelas *et al.* 1995), is a normalized index (from  $-1$  to  $1$ ) related to equilibrium of violaxanthin–zeaxanthin in the xanthophyll cycle. This is a light-dependent reaction associated with thermal dissipation of energy that decreases photochemical efficiency of PSII (Peñuelas *et al.* 1995). The normalized difference vegetation index (NDVI), computed as  $(R_{800} - R_{680}) / (R_{800} + R_{680})$ , is a normalized vigour index (Peñuelas *et al.* 1994; Gamon *et al.* 1995). The visible wavelength (680 nm) is near the red absorption bands of chlorophyll *a*, whereas the near infrared range (800 nm) is sensitive to variations in water content. NDVI has been correlated with leaf chlorophyll content – but is only reliable at low chlorophyll content (Lichtenthaler *et al.* 1996)–, and with photosynthetic efficiency, foliar nitrogen, phosphorus and potassium content (Gamon *et al.* 1995).

### Chlorophyll fluorescence

The effective quantum yield of photosystem II ( $\Phi_{PSII}$ ) was measured close to the centre of the leaf (the same leaves used to measure spectral reflectance), avoiding primary veins. This parameter is related to the fraction of absorbed light energy used for photochemistry, calculated from data obtained with a portable pulse-amplitude-modulated fluorometer (Mini-PAM; Heinz Walz, Effeltrich, Germany) as  $\Phi_{PSII} = (F'_m - F_t) / F'_m$  (Maxwell & Johnson 2000; Roháček 2002), where  $F_t$  is the minimum fluorescence in an illuminated environment, and  $F'_m$  is maximum intensity of fluorescence after a light-saturating pulse. Data were obtained at incident photosynthetic photon flux density (PPFD) of  $900 \mu\text{mol}\cdot\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1} \pm 21$  SE, using an external halogen lamp attached to the fluorometer. Measuring light and saturating light pulses ( $> 4000 \mu\text{mol}\cdot\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 0.8 s pulse length, actinic white light) were applied using a fibre-optic probe at an angle of  $60^\circ$  relative to the sample, and 12 mm from the leaf, using an Arabidopsis leaf clip holder 2060-B (Heinz Walz).

### Relative water content

Leaf relative water content (RWC) is the proportion of water stored in leaves in relation to saturation. RWC was measured in young, fully expanded leaves from 8 to 12 individuals per species and plot. Leaves were transported to the laboratory in hermetically sealed, Ziploc plastic bags. RWC was calculated as  $[(M_f - M_d) / (M_t - M_d)] \cdot 100$ , where  $M_f$  is leaf fresh mass,  $M_t$  is leaf turgid mass, measured after saturation of leaves on filter paper soaked with distilled water in covered Petri dishes for 20 h at  $4^\circ\text{C}$  in the dark (leaves were blotted dry with tissue paper before determination of  $M_t$ ), and  $M_d$  is dry mass of leaves after drying in an oven at  $80^\circ\text{C}$  to constant weight.

### Leaf mass per area

Leaf mass per area (LMA) measures leaf dry mass per unit of light-intercepting leaf area. Species with a high LMA are thicker, denser, or both (Wright *et al.* 2004). Leaf projected area per individual was measured in 8–12 individuals per species and plot, using a flatbed scanner (CanonScan LiDE 50; Canon, Tokyo, Japan) and processed using image analysis software (ImageJ 1.43j; National Institute of Health, USA). Samples were then dried in an oven at  $80^\circ\text{C}$  to constant weight and weighed to determine dry mass. LMA was calculated as the quotient between dry mass and area ( $\text{g}\cdot\text{m}^{-2}$ ).

### Stomatal density

The stomatal density (SD) of 8–12 individuals per species and plot was determined by counting the number of stomata in abaxial and adaxial leaf sides and dividing by leaf area of a young and fully expanded leaf. Leaf imprints were obtained by applying a thin layer of clear nail varnish to both sides of the leaf. Impressions were peeled off and mounted on slides and  $100\times$  micrographs captured, and stomata counted in three frames of  $500 \times 500 \mu\text{m}$  per slide. Stomatal density was expressed as number of stomata per  $\text{mm}^2$ .

### Leaf carbon and nitrogen content, and isotope composition

Two to five portions of mature leaves from eight randomly selected plants per species and plot were cleaned, dried at  $60^\circ\text{C}$  for  $> 5$  days, then ball-milled and sieved. Sub-samples of 1–2 mg homogenized fine powder (particle size  $< 500 \mu\text{m}$  diameter) were encapsulated in tin capsules. The molar  $^{15}\text{N}/^{14}\text{N}$  and  $^{13}\text{C}/^{12}\text{C}$  ratios were determined in an Isotope Ratio Mass Spectrometer (ThermoFinnigan MAT253; Bremen, Germany). Foliar isotope compositions of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) were expressed relative to the composition of a standard (Vienna Pee Dee Belemnite (VPDB) calcium carbonate for C, and air for N). The  $\delta$  values ( $\text{‰}$ ) were calculated as  $\delta = [(R_{\text{sam}}/R_{\text{std}}) - 1] \times 1000$ , where  $R$  is  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$  ratio in plant sample ( $R_{\text{sam}}$ ) and standard ( $R_{\text{std}}$ ), respectively. For analytical purposes, polyethylene (IAEA-C6) and  $(\text{NH}_4)_2\text{SO}_4$  (IAEA N1) were used as secondary international isotope standards for C and N, respectively. The  $\delta^{13}\text{C}$  values were transformed into carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) using the expression:  $\Delta^{13}\text{C} = (\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{plant}}) / (1 + \delta^{13}\text{C}_{\text{plant}})$  (Farquhar *et al.* 1989), assuming a  $\delta^{13}\text{C}$  air value of  $-8.0\text{‰}$  on the VPDB scale. Carbon and N content were determined

(expressed as percentage dry mass) in the same sample in an elemental analyser (FlashEA 1112 Series; ThermoFinnigan, Bremen, Germany) coupled to a mass spectrometer. Analyses were performed in the Instrumental Analysis Unit, University of A Coruña (Spain). There is usually a negative relationship between  $\Delta^{13}\text{C}$  and water use efficiency (WUE) (Yi & Yano 2022). In  $C_3$  plants,  $\Delta^{13}\text{C}$  generally lies between  $\sim 15\%$  (for plants with higher WUE) and  $25\%$  (or plants with lower WUE) (Dawson *et al.* 2002).

### Soil physicochemical variables

One soil sample of 5–10 cm topsoil in close contact with the roots (rhizospheric soil) of six plants of each species was collected from each plot. Six soil samples were also randomly collected from open spaces between plants, in the same plots at each of the four positions along the dune gradient. Samples were collected with a cylindrical steel sampler (5 cm diameter  $\times$  25 cm long). Samples were transported to the laboratory in airtight 120 ml plastic containers.

Soil salinity was determined as electrical conductivity in a 1:2.5 soil:water suspension, with an EC meter (524 Crison; Crison Instruments, Barcelona, Spain). Soil pH was determined in a similar soil:water suspension, with a pH meter (pH-metro 507; Crison Instruments). Different soil subsamples were used to quantify amount of inorganic phosphorus available for plants ( $P_{\text{soil}}$ ), using the Olsen method. The proportions of total soil carbon ( $C_{\text{soil}}$ ), nitrogen ( $N_{\text{soil}}$ ) and sulphur ( $S_{\text{soil}}$ ) were determined in finely ground ( $<2$  mm) soil subsamples ( $\sim 2$  mg). Subsamples were encapsulated in tin capsules and analysed (Department of Edaphology, University of Santiago de Compostela) by combustion at high temperature in a CHNS elemental analyser (LECO CHNS-932; LECO, St. Joseph, MI, USA).

### Statistical analysis

Effects of ‘species’ and ‘position’ on the dune gradient (0, 200, 400 and 600 m from shoreline to inland) on plant traits and soil variables were analysed by two-way ANOVA. The factor ‘Species’ consisted of two levels for plant traits (*E. maritimum*, *P. maritimum*), and three levels for soil variables (*E. maritimum*, *P. maritimum*, bare soil). Models were made using the ‘aov’ function in the ‘stats’ package of the R statistical software environment (R Core Team 2022). *P*-values were obtained using the ‘Anova’ function of the ‘car package’ (Fox 2019), with type III sum of squares. Post hoc comparisons between means were tested following the ANOVA, when factors with more than two groups were significant, using the ‘emmeans’ function from the ‘emmeans’ package, using the Bonferroni method. The significance threshold ( $\alpha$ ) was set to 0.05. All variables were checked for normality of residuals and homogeneity of variance prior to the analyses. Specifically, the CHL index and LMA were square-root transformed,  $N_{\text{soil}}$  and  $P_{\text{soil}}$  were  $\log_{10}$  transformed, EC was inverse transformed and RWC was transformed to the power of 3 to meet ANOVA assumptions (Zar 1984).

A two-way permutational multivariate analysis of variance (PERMANOVA) was used to detect significant spatial (four positions along the gradient) and species differences in plant traits and rhizosphere soil variables. Homogeneity of multivariate dispersion of groups defined by the species and positions along the gradient was tested using permutational analysis of

multivariate dispersions (PERMDISP). When the PERMANOVA showed spatial differences, pairwise *a posteriori* tests were performed to determine which pairs of positions differed. The analyses, based on Euclidean similarity matrices of normalized variables, were carried out using the PRIMER v7 software (Clarke & Gorley 2015), after 9999 permutations of raw data. Finally, we used IBM SPSS Statistics v. 25 (IBM 2023) for Principal Components Analyses based on a correlation matrix of ‘pseudo-species’ (each species was recoded into a set of dummy variables or ‘pseudo-species’ corresponding to each species in each of the positions along the dune gradient) by soil and plant traits to identify which traits best define the strategies of the studied species.

## RESULTS

### Leaf reflectance and chlorophyll fluorescence

Chlorophyll content, as estimated by the CHL index, differed significantly between species, and values were highest in *P. maritimum* at all sampling points along the gradient. Chlorophyll content also varied significantly along the gradient, with an overall decrease from the shore to the inland plots, especially in *P. maritimum* (Table 1, Fig. 1a). Photochemical efficiency (PRI) was higher in *P. maritimum* than in *E. maritimum* throughout the gradient (Table 1, Fig. 1b). PRI was also affected by positions of plants along the gradient. It increased significantly in the central gradient (200–400 m), there were no significant differences between plants at the most extreme positions (0 and 600 m), showing a non-directional trend. The species differed in the patterns of variation in the vigour index (NDVI) along the shore-to-inland gradient (Table 1; position  $\times$  species). NDVI for *E. maritimum* remained fairly constant along the gradient, whereas it increased steadily from shore to inland in *P. maritimum* (Fig. 1c). Finally, effective quantum yield of PSII ( $\Phi\text{PSII}$ ) showed no significant differences between species or among positions along the gradient (Table 1, Fig. 1d).

### Structural traits

Direct estimations of leaf water content found higher RWC in *P. maritimum* ( $88.7 \pm 0.5\%$  [mean  $\pm$  SE]) than *E. maritimum* ( $86.4 \pm 0.7\%$ ) (Table 1, Fig. 2a). RWC values varied significantly along the gradient, with mostly increasing values from shore to inland sites (Table 1, Fig. 2a). LMA was consistently higher (i.e. denser/thicker leaves) in *E. maritimum* ( $134 \pm 3 \text{ g}\cdot\text{m}^{-2}$  [mean  $\pm$  SE]) than in *P. maritimum* ( $114 \pm 3 \text{ g}\cdot\text{m}^{-2}$ ) along the entire gradient. Both species had very similar trends of variation along the gradient in LMA, which decreased from the shore to inland sites, reaching minimum values at 400 m from the foredune (Table 1, Fig. 2b: 0 m:  $134 \pm 4 \text{ g}\cdot\text{m}^{-2}$  [mean  $\pm$  SE], 200 m:  $124 \pm 4 \text{ g}\cdot\text{m}^{-2}$ , 400 m:  $112 \pm 5 \text{ g}\cdot\text{m}^{-2}$ , 600 m:  $120 \pm 4 \text{ g}\cdot\text{m}^{-2}$ ). Contrasting patterns of variation in stomatal density (SD) were found along the gradient. SD tended to increase steadily in *P. maritimum* from shore to inland sites and followed the opposite pattern in *E. maritimum*. Overall, this generated significant differences between the species in SD at 0, 200 and 400 m from the foredune – with higher SD in *E. maritimum* than in *P. maritimum* – but no differences between species at 600 m (Table 1, Fig. 2c).

**Table 1.** Results of factorial analysis of variance for the effects of position, species and their interaction on leaf traits: chlorophyll content (CHL), vigour (NDVI), photochemical efficiency (PRI and  $\Phi$ PSII), relative water content (RWC), leaf mass per area (LMA) stomatal density (SD), nitrogen ( $N_{\text{plant}}$ ) and carbon content ( $C_{\text{plant}}$ ) in leaves, nitrogen isotope composition ( $\delta^{15}\text{N}$ ) and carbon isotope discrimination ( $\Delta^{13}\text{C}$ ).

	position (df = 3)			species (df = 1)			position × species (df = 3)			error (df = 88)
	SS	F	P	SS	F	P	SS	F	P	
CHL	0.17	15.1	<b>&lt;0.001</b>	0.81	219.6	<b>&lt;0.001</b>	0.03	3	<b>0.033</b>	0.32
PRI	0.0057	11.8	<b>&lt;0.001</b>	0.0096	60.1	<b>&lt;0.001</b>	0.0008	1.6	0.185	0.014
NDVI	0.046	35.4	<b>&lt;0.001</b>	0.006	12.86	<b>&lt;0.001</b>	0.039	29.5	<b>&lt;0.001</b>	0.038
$\Phi$ PSII	0.0268	1.344	0.265	8 × 10	0.001	0.973	0.0009	0.045	0.987	0.5839

	position (df = 3)			species (df = 1)			position × species (df = 3)			error (df = 74)
	SS	F	P	SS	F	P	SS	F	P	
RWC	2.31 × 10 <sup>11</sup>	19.99	<b>&lt;0.001</b>	5.63 × 10 <sup>10</sup>	14.64	<b>&lt;0.001</b>	2.84 × 10 <sup>10</sup>	2.46	0.070	2.85 × 10 <sup>11</sup>
LMA	11.3	6.29	<b>&lt;0.001</b>	17.3	28.96	<b>&lt;0.001</b>	1.60	0.90	0.445	44.2

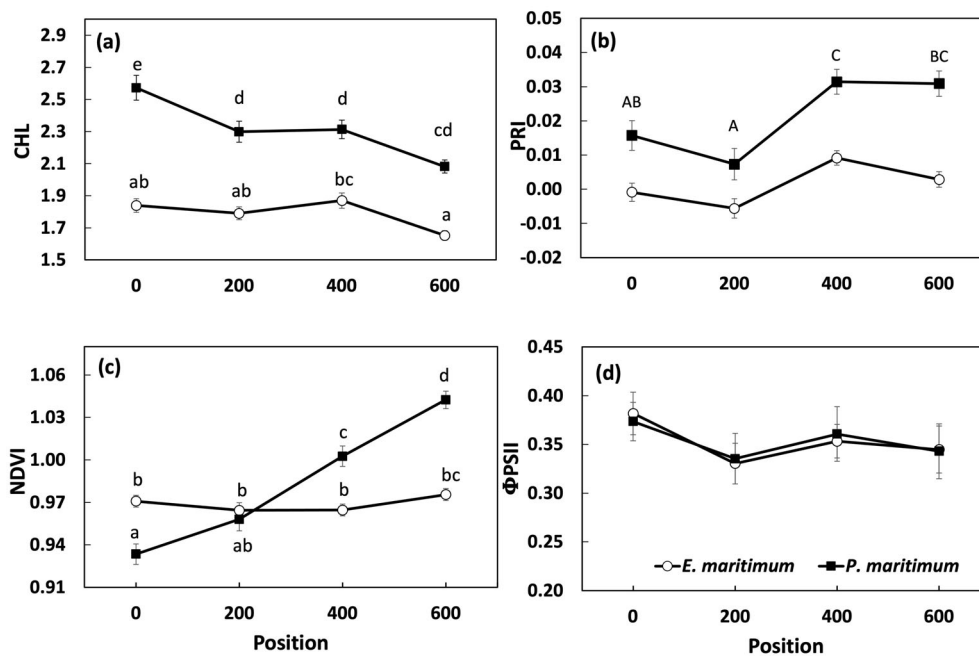
  

	position (df = 3)			species (df = 1)			position × species (df = 3)			error (df = 69)
	SS	F	P	SS	F	P	SS	F	P	
SD	131	0.04	0.990	108017	91.19	<b>&lt;0.001</b>	10407	2.93	<b>0.040</b>	81728

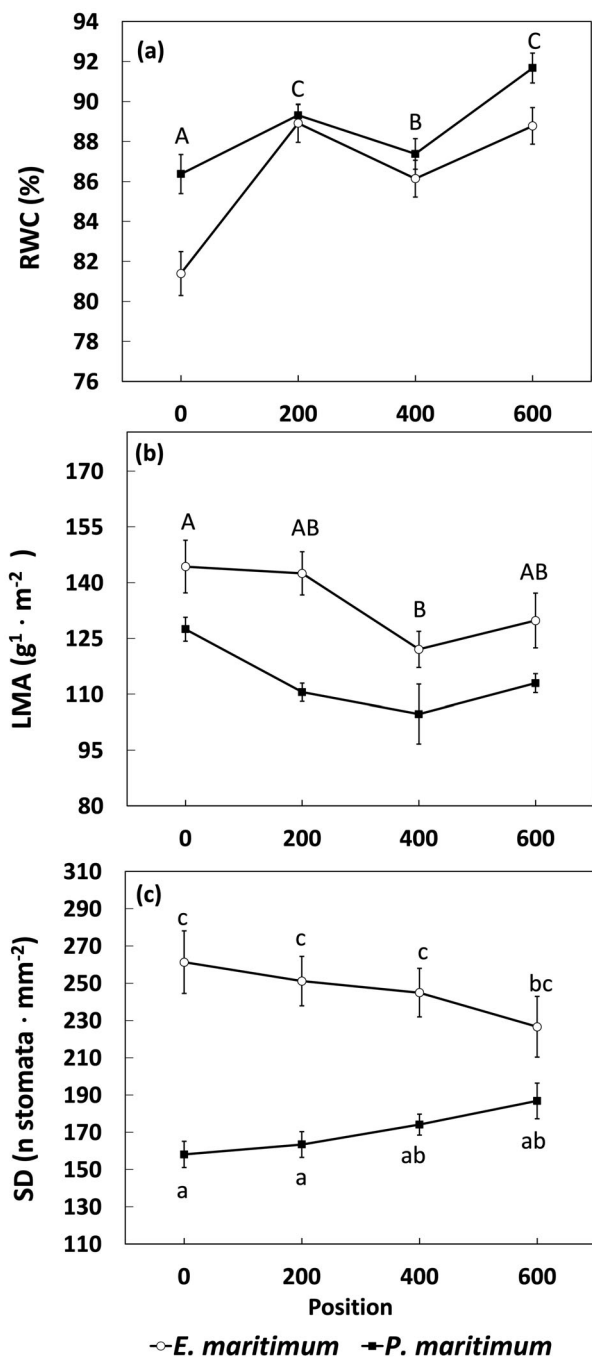
  

	position (df = 3)			species (df = 1)			position × species (df = 3)			error (df = 56)
	SS	F	P	SS	F	P	SS	F	P	
$N_{\text{plant}}$	0.4	0.24	0.865	1.06	1.94	0.169	18.23	11.12	<b>&lt; 0.001</b>	30.6
$\delta^{15}\text{N}$	2.227	0.519	0.671	0.007	0.005	0.946	10.71	2.496	0.069	80.05
$C_{\text{plant}}$	12	0.763	0.519	4	0.77	0.384	78	4.988	<b>0.004</b>	292
$\Delta^{13}\text{C}$	2.1	1.32	0.276	4.2	7.95	<b>0.007</b>	6.2	3.87	<b>0.014</b>	29.9

Significant differences ( $P \leq 0.05$ ) are shown in bold. df = degrees of freedom; SS = sum of squares of mean; F = F-value; P = P-value.



**Fig. 1.** Values of leaf functional traits based on leaf spectral reflectance and chlorophyll fluorescence measurements at different positions along the dune gradient: (a) chlorophyll index (CHL), (b) photochemical reflectance index (PRI), (c) normalized difference vegetation index (NDVI), and (d) effective quantum yield of photosystem II ( $\Phi$ PSII). Open circles represent mean ( $\pm$ SE,  $n = 12$ ) for *Eryngium maritimum* and solid squares represent mean ( $\pm$ SE,  $n = 12$ ) for *Pancratium maritimum*. In (a) and (c) different letters near mean values indicate significant differences between groups resulting from the position × species interaction ( $P < 0.05$ , Bonferroni test). In (b) there is no significant effect of position × species, and capital letters are used to indicate post hoc differences between PRI values at different positions ( $P < 0.05$ , Bonferroni test).



**Fig. 2.** Values of structural leaf traits of the studied species at different positions along the dune gradient: (a) relative water content (RWC), (b) leaf mass per area (LMA), and (c) stomatal density (SD). Open circles represent mean ( $\pm$ SE) for *Eryngium maritimum* and solid squares represent mean ( $\pm$ SE) for *Pancratium maritimum*. ( $n = 12$  for *P. maritimum* for LMA, RWC and for SD at 0 m;  $n = 9$  for *E. maritimum* for LMA and RWC at 200 and 400 m, and for SD at 0, 200 and 400 m;  $n = 8$  for *E. maritimum* for LMA and RWC at 600 m, and for SD at 0 and 600 m. In (a) and (b) there was no significant effect of position  $\times$  species; capital letters indicate post hoc differences between RWC and LMA at different positions ( $P < 0.05$ , Bonferroni test). In (c) different letters near mean values indicate significant differences between groups resulting from the position  $\times$  species interaction ( $P < 0.05$ , Bonferroni test).

### Nitrogen and carbon leaf content

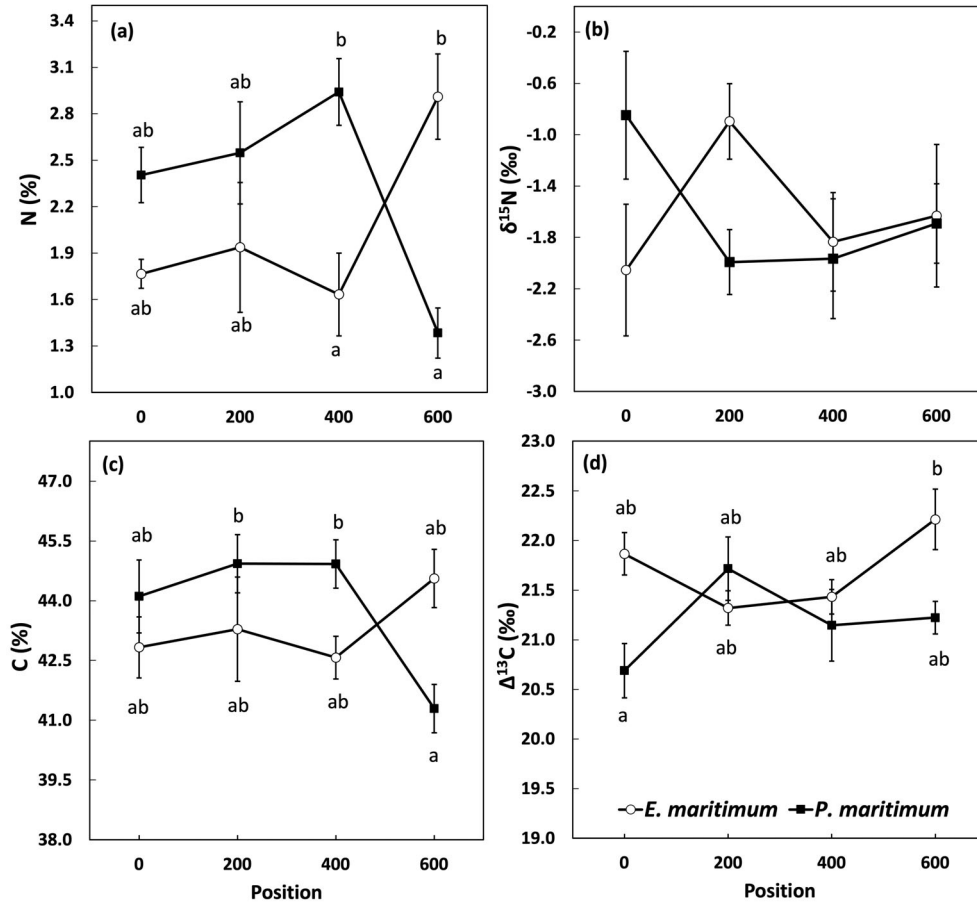
There were differences between species in the proportion of N in leaves; however, these differences depended on their position along the gradient (Table 1; position  $\times$  species). There were no differences in leaf N content between species at 0 and 200 m; however, there were significant differences between the species at 400 and 600 m, but with opposite patterns. Leaf N content in *P. maritimum* was higher at 400 m than in *E. maritimum*. From 400 to 600 m leaf N content in *P. maritimum* decreased significantly, whilst in *E. maritimum* it increased significantly. As a result, the leaf N content of *E. maritimum* was higher at 600 m than in *P. maritimum*. The variation in leaf C content followed a similar pattern to that found for leaf N content in both species (Table 1, Fig. 3c).

### Nitrogen isotope composition and carbon isotope discrimination

Leaf  $\delta^{15}\text{N}$  did not differ significantly between species or along the gradient (Table 1, Fig. 3b); however, there were significant differences in the WUE in species, as estimated by  $\Delta^{13}\text{C}$ , but these depended on position along the gradient. In particular, there were significant differences between the species when comparing the species at opposite ends of the gradient: WUE of *P. maritimum* was higher (lower  $\Delta^{13}\text{C}$ ) at 0 m than WUE of *E. maritimum* at 600 m (higher  $\Delta^{13}\text{C}$ ; Table 1, Fig. 3d).

### Soil physicochemical variables

The soil pH along the gradient differed significantly, depending on plant position, with the main differences between pH of bare soil and pH of the rhizosphere of both species, particularly at both ends of the gradient (Table 2, Fig. 4a). There were differences in EC of the different soil types along the gradient. EC for bare soil and rhizosphere of *E. maritimum* were similar along the gradient, but EC of the rhizosphere of *P. maritimum* was lower at 0 m than at 400 m, and values for *P. maritimum* at 0 m were also lower than those in bare soil at the same position (Table 2, Fig. 4b). Soil types did not differ significantly in C content except between the coastal end of the gradient (higher values) and 400 m from the foredune (lower values) (Table 2, Fig. 4c). Soil C content was mainly derived from carbonates ( $C_{\text{soil}}$  was mainly carbonates except 0.1% to 0.2% from other sources). The soil N content changed across the gradient, depending on soil origin: there were no differences between N content of the rhizosphere of *E. maritimum* and that of bare soil; however, N in the rhizosphere of *P. maritimum* decreased along the gradient from coast to inland positions, being significantly lower than in the other soil types at the inland end of 600 m (Table 2, Fig. 4d). The soil P content changed along the gradient differently for the soil types considered (Table 2). The P content was lower in bare soil than in the rhizosphere of both species, which had similar levels from 0 to 400 m, but at 600 m P content in the rhizosphere of *P. maritimum* increased strongly, being significantly higher than that in the rhizosphere of *E. maritimum* (Table 2, Fig. 4e). Soil S content also changed along the gradient depending on the soil types considered (Table 2). The S content remained fairly constant in the rhizosphere of both species but decreased steadily in bare soil from coast to the inland positions (Table 2, Fig. 4f).



**Fig. 3.** (a) Leaf nitrogen content, (b) nitrogen isotope composition ( $\delta^{15}\text{N}$ ), (c) leaf carbon content and (d) carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) at different positions along the dune gradient. Open circles represent mean ( $\pm\text{SE}$ ,  $n = 8$ ) for *Eryngium maritimum* and solid squares represent mean ( $\pm\text{SE}$ ,  $n = 8$ ) for *Pancratium maritimum*. Different letters near the mean indicate significant differences between groups resulting from the position  $\times$  species interaction ( $P < 0.05$ , Bonferroni test).

**Table 2.** Results of factorial ANOVA for the soil variables: total amount of nitrogen ( $N_{\text{soil}}$ ), total amount of carbon ( $C_{\text{soil}}$ ), C/N ratio, pH, electric conductivity (EC), available phosphorus ( $P_{\text{soil}}$ ), and sulphur ( $S_{\text{soil}}$ ) for position, species and their interaction.

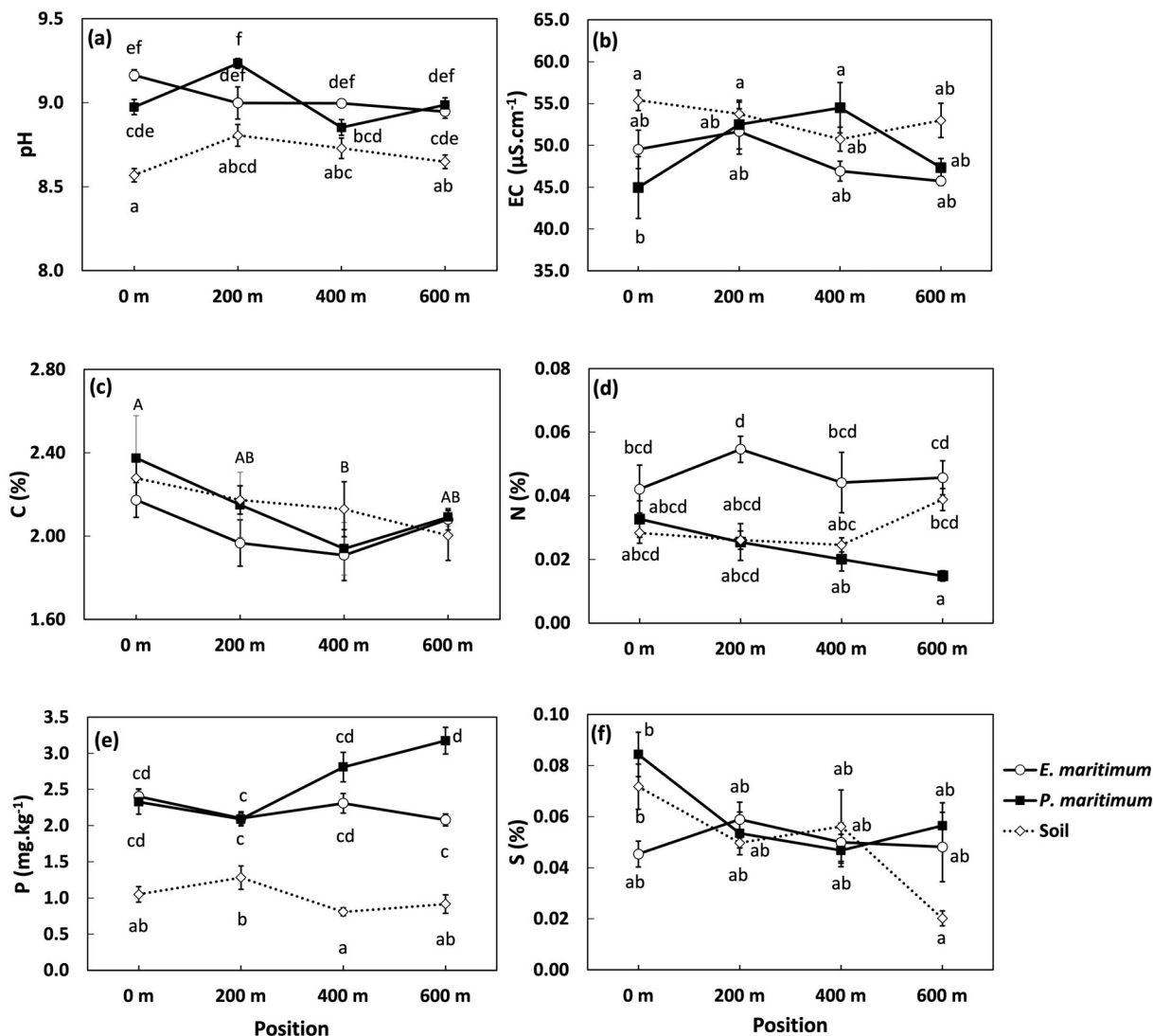
	position (df = 3)			species (df = 2)			position $\times$ species (df = 6)			error (df = 60) SS
	SS	F	P	SS	F	P	SS	F	P	
$N_{\text{soil}}$	0.087	1.02	0.391	1.273	22.32	<b>&lt;0.001</b>	0.487	2.85	<b>0.017</b>	1.712
$C_{\text{soil}}$	0.79	3.15	<b>0.031</b>	0.19	1.16	0.321	0.28	0.55	0.764	5.02
C/N	0.031	0.33	0.807	1.445	22.55	<b>&lt;0.001</b>	0.539	2.8	<b>0.018</b>	1.922
pH	0.3	6.13	<b>0.001</b>	1.8	57.7	<b>&lt;0.001</b>	0.5	5.79	<b>&lt;0.001</b>	0.9
EC	$2.33 \times 10^{-5}$	2.02	0.12	$5.12 \times 10^{-5}$	6.67	<b>0.002</b>	$6.92 \times 10^{-5}$	3.01	<b>0.012</b>	0.00023
$P_{\text{soil}}$	0.003	0.162	0.922	2.447	188.28	<b>&lt;0.001</b>	0.256	6.58	<b>&lt;0.001</b>	0.39
$S_{\text{soil}}$	0.006	4.59	<b>0.006</b>	0.002	1.92	0.155	0.008	3.02	<b>0.012</b>	0.026

Significant differences ( $P \leq 0.05$ ) are shown in bold. df = degrees of freedom; SS = sum of squares of the mean; F = F-value; P = P-value.

### Spatial and species differences in traits assemblages

The PERMDISP was homogeneous within group multivariate dispersions ( $F: 0.4189$ ;  $df: 1.44$ ;  $P$  (perm): 0.7364) for leaf traits and soil variables in the different positions along the dune gradient. However, the PERMDISP was non-homogeneous within

group multivariate dispersions for species ( $F: 6.808$ ;  $df: 1.46$ ;  $P$  (perm): 0.013). The PERMANOVA showed significant spatial and species differences in leaf traits and soil variables assemblages (Table 3). Pairwise comparisons between positions along the dune gradient showed significant differences for all pairs (maximum permutation  $P = 0.0016$ , for the 0–600 m comparison).



**Fig. 4.** Values of soil physicochemical variables along the dune gradient: (a) pH, (b) soil salinity, (c) total carbon content, (d) total nitrogen content, (e) available phosphorus, and (f) sulphur content. Open circles represent mean ( $\pm$ SE,  $n = 6$ ) for soil obtained from the rhizosphere of *Eryngium maritimum*, solid squares represent mean ( $\pm$ SE,  $n = 6$ ) for soil obtained from the rhizosphere of *Pancratium maritimum*, and open diamonds represent mean for bare soil ( $\pm$ SE,  $n = 6$ ). In (a), (b), (d), (e), and (f) different letters near the mean indicate significant differences between groups resulting from the position  $\times$  species interaction ( $P < 0.05$ , Bonferroni test). In (c) there was no significant effect of position  $\times$  species, and capital letters are used to indicate post hoc differences in total carbon content between the different positions ( $P < 0.05$ , Bonferroni test).

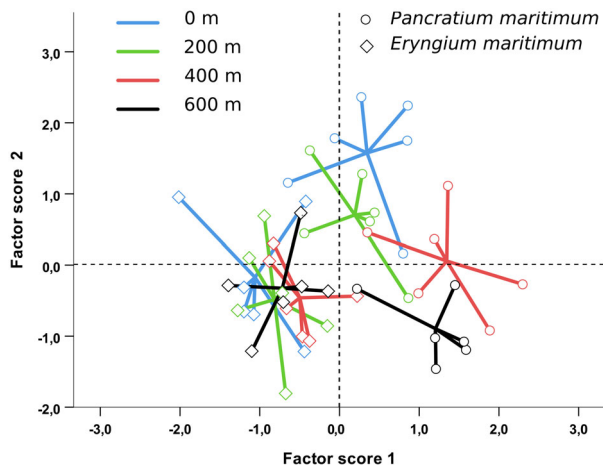
**Table 3.** Results of two-way permutational multivariate analysis of variance (PERMANOVA) showing the influence of position, species and their interaction on leaf traits and soil variables.

	df	SS	pseudo- $F$	$P$ (perm)	unique perms
Position	3	148.6	3,683	0.0001	9869
Species	1	220.0	16,357	0.0001	9935
Position $\times$ species	3	127.4	3,158	0.0001	9883
Residuals	47	538.0			

df = degrees of freedom; SS = sum of squares; Pseudo- $F = F$  value by permutation,  $P$  (perm) =  $P$ -values based on >9000 permutations (lowest possible  $P$ -value is 0.0001); Unique perms = number of permutations; St = stage; Tr = treatment.

### Principal components analysis

The three main components extracted by PCA accounted for 47.5% of total variance of the data matrix. The traits that loaded most heavily on PC1 were photochemical reflectance index and soil P content, both with positive scores, and soil N content, with a negative score. PC2 explained 15.3% of total variation with strongest, positive, scores for S soil content and CHL index. N and C leaf content showed the highest loading on PC3, which explained 11.6% of total variation (Table S1). The representation of the 'pseudo-species' and their centroids in the plane defined by the two principal components (Fig. 5) shows a clear segregation of species along the main component, with positive scores for *P. maritimum* and negative for *E. maritimum*. The second



**Fig. 5.** Location of *Pancratium maritimum* (Pm; O) and *Eryngium maritimum* (Em; ◇) on the PCA plane defined by the two principal components, accounting for 35.9% of total variation. Colours indicate plant position along the shore-to-inland gradient (blue: first sector, 0 m; green: second sector, 200 m; red: third sector, 400 m; black: fourth sector, 600 m). Plants located in the same sector of the gradient are connected to the centroid of the group by lines of the same colour.

component represents the shore–inland dune gradient, with positive scores for the more coastal positions along the dune gradient, and negative scores for the inland positions.

## DISCUSSION

Our study found that most of the studied soil variables, e.g. N content and pH, changed following a non-directional gradient. This contrasts with the general observation that most abiotic gradients in dune systems are directional. Moreover, some of these changes appear dependent on growth of the species, highlighting the complexity of environmental gradients in the dune ecosystem. We also found significant differences between the two species in structural and functional traits related to use of resources, suggesting they have evolved different strategies to cope with the harsh environmental conditions in coastal dune systems. In particular, *E. maritimum* had leaf traits indicative of a water-conserving strategy, while *P. maritimum* exhibited leaf traits indicating a higher capacity to capture light energy and higher productivity.

### Spatial differences along the dune gradient in trait assemblages

Our results provide evidence for differences in abiotic factors along the dune gradient that depend on which species (*E. maritimum* or *P. maritimum*) are growing in the soil. Environmental gradients in dune systems are classically described as directional, with consistent changes from shoreline to inland positions. Thus, it is expected that soil water availability increases while soil salinity decreases with increasing distance from the shoreline (Kachi & Hirose 1979). Other severe stressors, e.g. salt spray, sand burial, swash inundation, drought, high light intensity, wind exposure and nutrient deficiency, are reported to dominate in the foredune environment and to decrease with distance from the coast (Hesp 1991). However, our results indicate that some putative abiotic

gradients are unclear here (i.e. values of these abiotic factors are not significantly affected by position in the gradient) or change in a more complex way, such as non-directional (sequence of values does not increase or decrease steadily with distance from the shoreline). Soil C content decreased from 0 to 400 m and S content in bare soil also decreased from the shoreline to inland. In contrast to previous studies that found a correlation between increasing soil nutrient content and increasing distance from the beach (Hesp 1991; Lortie & Cushman 2007), we did not find such a relationship for soil N content along the dune gradient. For other abiotic factors, such as pH, when changes were detected along the gradient, these were non-directional. Interestingly, however, we found differences in physicochemical characteristics that were site-specific and depended on growth of the study species. For example, *P. maritimum* had lower soil N content than the other two soil types examined at the inland end of the gradient. Similarly, soil salinity (measured as EC) was lower around roots of *P. maritimum* than in bare soil at the shoreline end of the gradient, and both species were growing in soils with higher P and higher pH than bare soil at most positions along the gradient. Thus, the present results do not conform to general observations that most abiotic gradients in dune systems are directional (Kachi & Hirose 1979; Hesp 1991). Lortie & Cushman (2007) also found – in much shorter coastal dunes (200 m long) – a mixture of directional (soil nitrate) and non-directional (ammonium, pH) gradients. Our study highlights the complexity of such gradients that are also affected by the identity of the species growing in them and suggests further questions: are species selecting microhabitats or do they modify the soil in which they grow? Based on our data, we cannot conclusively say which of these processes is present. However, the variation in soil P might be reflecting the ability of the species to establish mycorrhizal associations and therefore potentially modifying the soil environment. At the elevated pH found in the dune system, P can be immobilized as insoluble carbonates (Kooijman *et al.* 2009). The increased available P in the rhizosphere of *P. maritimum* and *E. maritimum* in comparison to bare soil, particularly in inland sites, could be explained by the known ability of these species to establish mycorrhizal associations (Isermann & Rooney 2014; De Castro *et al.* 2018), which are common in this environment (Camprubi *et al.* 2011).

### Species differences in response strategies to abiotic changes in dune gradients

#### Structural traits

The patterns of variation along the dune gradient in the structural traits examined revealed significant spatial dependence for all traits. The pattern was only directional for stomatal density, which was influenced by species, the two having opposite SD patterns.

The LMA has been considered the main determinant of variations in growth patterns, at least for herbaceous plants (Poorter *et al.* 2009). In the species studied here, LMA decreased similarly from the coastal sites, where abiotic conditions were most stressful, towards inland sites, reaching a maximum at 400 m from the shoreline. A high LMA, i.e. a high photosynthetically active material per unit leaf area, may be beneficial for species subject to high levels of radiation, as experienced in dune habitats, thus reducing the risk of photoinhibition

(Sánchez-Vilas & Retuerto 2011). High LMA may be particularly advantageous at the shoreline, since high LMA is considered beneficial for water conservation and tolerance to high winds characteristic of such sites (Retuerto & Woodward 1992, 1993). *E. maritimum* had consistently higher LMA than *P. maritimum*, i.e. *E. maritimum* produces leaves with a higher mass per unit area, which suggests greater reliance on leaf structure for water stress tolerance. The presence of a bulb in *P. maritimum* increases resource storage, which may complement leaf adaptations in coping with water stress. Consistently, *E. maritimum* had higher stomatal density, a xeromorphic character, which suggests it should be less vulnerable to drought than *P. maritimum*. The higher stomatal density in *E. maritimum* may enhance adaptation to drought via more efficient control of water loss through changes in stomatal conductance. In fact, stomatal density has been positively related to stomatal conductance, net CO<sub>2</sub> assimilation rate and water use efficiency (Retuerto & Woodward 1993; Xu & Zhou 2008; Hasanuzzaman *et al.* 2023). The opposite trend in stomatal density, which decreased in *E. maritimum* and increased in *P. maritimum* from shoreline to inland sites, suggests contrasting strategies in the two species involving diffusion of CO<sub>2</sub> into the leaf and outward diffusion of water vapour (Hetherington & Woodward 2003).

The RWC was higher in *P. maritimum* than in *E. maritimum* leaves, consistent with lower WUE and lower LMA. The higher RWC in *P. maritimum* may be related to a higher capacity to accumulate proline (Khedr *et al.* 2003), an osmotically active amino acid that contributes to water retention and protection of proteins and membranes from effects of high salinity. However, the mean RWC values were > 80% in both species along the entire gradient. As this is much higher than the wilting point (Ludlow 1989), both species could presumably maintain leaf water potential for C acquisition and growth.

The lack of correspondence between the patterns of variation in structural traits and in soil physicochemical properties indicates that no physicochemical parameters explain the spatial variation in structural traits for either species. Each combination of trait and species appears to respond differently to a complex environmental gradient or to a single environmental factor that was not considered in this study. Under these conditions, it is very difficult to interpret the observed patterns of variation in terms of adaptation.

#### Functional traits

The greenness indices CHL and NDVI are related to leaf chlorophyll content (Sims *et al.* 2006), which is linked to the capacity of live vegetation to absorb photosynthetically active radiation (PAR). Therefore, the between-species differences in the spectral indices CHL and NDVI observed in this study indicate that *P. maritimum* generally had a higher capacity to capture light energy, a main determinant of plant productivity (Monteith & Moss 1977). Although CHL and NDVI have also been positively related to leaf N content (Gamon *et al.* 1995), we did not observe parallel variations in CHL and NDVI and leaf N content (in either species) along the dune gradient – especially at the inland end, where leaf N content was lower in *P. maritimum* (where NDVI were highest) than in *E. maritimum*. Additionally, it is important to recognize that NDVI and CHL are based on reflectance at different wavelengths, resulting in different sensitivities to leaf properties. In our study, the opposing trends observed for these indices in *P. maritimum*

might be influenced by leaf properties beyond chlorophyll content. Compared to the CHL index, NDVI seems to be particularly sensitive to changes in leaf structure and water content (Peñuelas *et al.* 1997). In particular, here, values of NDVI for *P. maritimum* align with the pattern for RWC, increasing from the coast to inland, which suggests that water content may have a strong influence on this index in this context.

The effective quantum yield of PSII ( $\Phi$ PSII) measures the proportion of light absorbed by chlorophyll used in photochemistry, and is positively correlated with radiation use efficiency (Maxwell & Johnson 2000; Roháček 2002). Although radiation use efficiency differs significantly between species and environments as a result of varying environmental constraints (Garbulsky *et al.* 2010), we found that the  $\Phi$ PSII did not differ between species and was not spatially dependent along the shore-to-inland gradient. The lack of variability in  $\Phi$ PSII suggests that, although leaves of both species differed morphologically, both types could maintain very similar photochemical efficiencies throughout the whole dune gradient. However, the two species differed in PRI, which, unlike  $\Phi$ PSII, exhibited clear directional spatial dependence along the gradient (increasing steadily from shoreline to inland sites). Thus, although our results do not support previous reports of close relationships between PRI and  $\Phi$ PSII (see review in Garbulsky *et al.* (2011)), they are consistent with other reports of widely varying relationships between PRI and  $\Phi$ PSII in different species (Peñuelas *et al.* 1995; Guo & Trotter 2004; Busch *et al.* 2009). PRI values are inversely related to dissipation of excess radiation energy as heat, a photoprotective mechanism that involves the xanthophyll cycle in thylakoid membranes (Peñuelas *et al.* 1995; Demmig-Adams *et al.* 1996; Gamon *et al.* 1997). Therefore, the correlation between PRI and  $\Phi$ PSII may be weak when the xanthophyll cycle sustains thermal dissipation of the light energy absorbed (Busch *et al.* 2009). PRI has also been inversely related to the ratio of total carotenoids to chlorophyll contents (Peñuelas *et al.* 1995) and the ratio between  $\beta$ -carotenes to chlorophylls (Filella *et al.* 2009), which may protect the leaves from photoinhibition (Gamon *et al.* 1997). According to Guo & Trotter (2004), interspecies differences in relationships between PRI and  $\Phi$ PSII are probably related to differences in size of the carotenoid pool relative to size of the total chlorophyll pool. Thus, lower PRI in *E. maritimum* than in *P. maritimum*, but similar  $\Phi$ PSII, can be explained by a low ratio of carotenoids to chlorophylls in *E. maritimum*. In general, plants with high saturating irradiance and CO<sub>2</sub> uptake rates are expected to need less photoprotection and therefore require a lower ratio of carotenoids to chlorophylls (Guo & Trotter 2004). Here, the studied species differed in the way they use light energy and in requirement for photoprotection, which was higher in *E. maritimum*, as indicated by lower PRI. This latter species also coped with excess radiation by alternative means related to leaf morphology, such as reducing the leaf surface area exposed to incident light by folding leaves and/or reflecting light via epicuticular waxes.

Nitrogen is a key element in pigment–protein molecules related to photon capture and transport to photosystems. It is also a component of key enzymes in photosynthetic reactions, such as Rubisco and related proteins (Evans 1989). Our results did not show any consistent relationship between soil and leaf N content and  $\delta^{15}\text{N}$  for either species. The lack of any relationship between soil N and leaf N may be explained by the fact

that soil N content was determined in soil samples from depths of 25 cm, whereas the plants may be exploiting soil nutrients at different depths (niche differentiation). Niche differentiation is also a possible explanation for the observed differences in leaf N content of the studied species. This finding does not conform to observations that coexisting species have similar mineral nutrient concentrations in their leaves (see Grime (2006) and references therein), and suggests that our study species may differ in their N demand, as well as in allocation to different structures and/or in ability to take up soil N. In this study, leaf N was also not related to changes in leaf  $\delta^{15}\text{N}$ , which is somewhat surprising as several field studies have reported correlations between the two leaf traits (Garten 1993; Johansson & Högberg 1994; Hobbie *et al.* 2000).

Although C isotope discrimination ( $\Delta^{13}\text{C}$ ) is a long-term integrator of ecophysiological processes, such as leaf conductance and photosynthetic capacity, it has mainly been used as a surrogate for potential WUE (Farquhar *et al.* 1989; Ehleringer 1993). In the present study, the  $\Delta^{13}\text{C}$  values suggest similar WUE in both species when measured at the same point in the gradient; however, when comparing plants at the two ends of the gradient, *E. maritimum* growing at 600 m had lower WUE (higher  $\Delta^{13}\text{C}$ ) than *P. maritimum* growing at 0 m. This may be the consequence of different strategies to cope with increasing water deficit (see RWC in Fig. 2a), which are magnified when comparing plants on the extremes of the gradient: it appears that *P. maritimum* had the lowest  $\Delta^{13}\text{C}$  at the foredune, while *E. maritimum* had the highest  $\Delta^{13}\text{C}$  when growing at the inland end of the gradient.

Overall, our PCA findings clearly suggest different multi-dimensional adaptation strategies of the two species to the changes associated with the shore-to-inland dune gradient. Both species clearly spatially segregate along the main component, suggesting different strategies in light use efficiency (PRI), and in the use of soil N and P. The species spatial arrangement along the second axis indicates that environmental changes associated with the shore-to-inland gradient induce more trait variation in *P. maritimum* than in *E. maritimum*, suggesting differences in species plasticity.

### Is convergence in leaf traits higher at the shoreline end of the dune gradient, with harsher environmental conditions, than at the inland end of the gradient?

The expectation of greater convergence in plant traits at the shoreline end of the dune gradient was not supported by results of the present study. Divergence in functional and structural traits found here – when present – was variable across the examined traits. In some traits, such as leaf N content and NDVI, species divergence was higher at the inland end of the dune gradient, while for traits such as CHL and stomatal density, divergence between the species was higher at the shoreline end. Our expectation of more convergence in structural and functional traits of species at the shoreline end of the dune gradient was based on previous reports suggesting that stress factors may constrain the range of plant responses in hostile environments, such as coastal sand dunes, thus producing functional convergence of species as predicted by the environmental filtering theory (Meinzer 2003; Reich *et al.* 2003; Zhao *et al.* 2022). However, in environments in which the main selective factors are related to competition, rather than to physical factors, limiting similarity has been suggested to be more

important in structuring communities, thus leading to trait divergence and consequent niche differentiation (Stubbs & Wilson 2004; Luo *et al.* 2021). However, in shoreline sites, the harsh and unpredictable disturbances could also cause divergence in functional traits, according to the disturbance filters proposed by Grime (2006). Other processes, such as facilitation and mutualism, can lead to either divergence or convergence of traits (Valiente-Banuet & Verdú 2007; Schöb *et al.* 2012; Verdú *et al.* 2021). Although it is commonly assumed that the severity of the abiotic factors decreases gradually from shoreline to inland sites (Houle 1996; Griffiths 2006; Lortie & Cushman 2007), the existence of comparable levels of convergence in traits of the studied species, at both ends of the dune gradient, suggest that processes operating in opposite directions, which generate trait convergence or divergence, may be equally important along the entire gradient. The lack of consistent directionality in the severity of most abiotic stress factors may explain the comparable levels of trait convergence at both ends of the dune gradient. Dune systems represent complex environments, where harsh abiotic conditions occur simultaneously with high heterogeneity and unpredictability at multiple spatial and temporal scales. The present results support the idea that there is no optimal configuration of traits for adaptation to such complex environments; instead, there are multiple suboptimal designs that allow the species to grow together while minimizing competitive interactions.

### Conclusions

Although many studies have assumed that species along dune gradients experience consistent and progressive directional changes in abiotic factors, our study provides clear evidence that some environmental gradients are not perceived as such or are perceived as non-directional variations. Changes in habitat suitability induced by species activity, as well as selection by species of microhabitats with spatial heterogeneity and complex interactions between factors (including mutualistic associations), may disrupt the way that species perceive supposedly directional abiotic gradients. The observed significant differences in structural and functional traits related to use of resources, such as light, water and nutrients, as well as in some soil characteristics, strongly suggest that the two studied species have evolved different strategies for survival in the harsh environment of coastal dune systems. This result could be expected, as the two species have very different evolutionary histories. Since they coexist in the whole dune gradient, we consider that alternative designs of similar fitness might evolve in the same environment. Lack of correspondence between patterns of variation in leaf traits and soil physicochemical variables suggests that species rarely respond to the action of a single environmental factor. Therefore, claims that plant responses along dune gradients are determined by a single environmental factor are often unfounded. Finally, processes that occur along the dune gradient and that induce convergence or divergence in species' traits are therefore of comparable importance.

### AUTHOR CONTRIBUTIONS

R.B. and R.R. designed the research and collected the data; R.B., J.S.V. and R.R. analysed the data and wrote the article. All authors read and approved the final manuscript.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Loading scores of plant traits and soil variables in the PCA and (%) of variance explained by the three main components.

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