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Van Puijenbroek, M.E.B., Teichman, C., Meijdam, N., Oliveras, I., Berendse, F., Limpens, J. (2017) Does salt stress constrain spatial distribution of dune building grasses *Ammophila arenaria* and *Elytrigia juncea* on the beach? *Ecology and Evolution*

The study consist of three parts

1. Field transplant experiment with two dune building grasses
2. Soil salinity measurements in the field
3. Glasshouse experiment with two dune building grasses

Field transplant experiment with two dune building grasses

We conducted a field experiment to assess the plant growth of *Ammophila arenaria* and *Elytrigia juncea* along five transects from beach to dune (Fig. 1) on the Hors on Texel, a barrier island in the Netherlands (coordinates: 52°59'51.97"N, 4°44'04.83"E). The Hors is a wide dissipative beach with much hydrodynamic reworking of the sand, which results in a high transport potential and opportunity for dunes to develop. Due to relatively storm free periods, many dunes have been able to develop on the Hors in the last 20 years. Within each transect, we selected four locations representing different stages of dune development, zone (I) the non-vegetated zone above the mean high water line, 0.78–1.1 m NAP (NAP refers to Amsterdam Ordnance Datum, which is equal to mean sea level near Amsterdam); (II) zone with *E. juncea* occurring, 1.17–1.19 m NAP; (III) zone with both species co-occurring, 1.42–1.94 m NAP; and (IV) zone where *A. arenaria* is dominant, 2.06–3.17 m NAP. At each location, we established six plots of 50 × 50 cm. The minimum distance between the plots was 2 m. Three treatments were randomly assigned to the plots: monoculture of *A. arenaria*, monoculture of *E. juncea*, and mixed culture of *A. arenaria* and *E. juncea*. In each plot, we planted 20 plants; in the mixed culture, we planted 10 plants of each species. The plants, consisting of one shoot, were collected from the same site and stored outside in plastic bags with moist sand for a maximum of 2 weeks until planting.

After planting, we standardize the leaf height between species and plots by clipping the leaves until the leaves were 3 cm long. We established the experiment in the end of March 2014. We measured the number of dead and alive leaves for a fixed subplot of 30 × 30 cm within each plot in May–October 2014, March, August 2015 and August 2016. Within the each subplot we also measured the plant height 5 times. From July 2014 we also measured the number of tillers within the subplot and the number of tillers for the whole plot.

All plants in the non-vegetated zone (zone I) were pulled out of the plots shortly after the start from the field experiment, preventing inclusion in our analyses. Two additional plots in the zone with only *E. juncea* (zone II) were destroyed in September 2014. We excluded these plots from this time point onward.



Figure 1 Overview of the field site on the Hors at Texel. The dots indicate the different zones where we conducted the field transplantation experiment and measured the soil salinity. The star in the map indicates the location of the field site in the Netherlands. Zone I is the non-vegetated zone, zone II the zone with only *E. juncea* occurring, zone III the zone with both *E. juncea* and *A. arenaria*, and in zone IV *A. arenaria* is dominant.

Dataset: Fieldexperiment_Texel_data.csv

Collum name	Information
ID	ID number of vegetation plot
Area	Location of the field experiment Hors, on Texel, the Netherlands
Transect	Number of transect, 1 is the transect to the east, 5 the transect most to the west
Location	Location of the plot, for the numbers see Fig. 1
Wrack	Whether wrackline material was added to the plot at the start of the experiment, had no effect of growth and was therefore discarded as a treatment
Plant	Whether a plant was present in the plot, we had non-vegetated control plots
Type	Whether it was a monoculture or a mixed culture plot.
Species	Which species were planted. A = <i>Ammophila arenaria</i> , E = <i>Elytrigia juncea</i>
Plot	Number of the plot within a location
Date	Date of measurement
Month	Month of measurement
A_leaves_alive	Number of leaves of <i>Ammophila arenaria</i> alive inside the 30 cm x 30 cm subplot
A_leaves_d	Number of leaves dead <i>Ammophila arenaria</i> inside the 30 cm x 30 cm subplot
E_leaves_alive	Number of leaves of <i>Elytrigia juncea</i> alive inside the 30 cm x 30 cm subplot
E_leaves_d	Number of leaves dead <i>Elytrigia juncea</i> inside the 30 cm x 30 cm subplot
Height_1	Plant height in cm
Height_2	Plant height in cm

Height_3	Plant height in cm
Height_4	Plant height in cm
Height_5	Plant height in cm
A_tillers_subplot	Number of alive tillers of <i>Ammophila arenaria</i> inside the 30 cm x 30 cm subplot
E_tillers_subplot	Number of alive tillers of <i>Elytrigia juncea</i> inside the 30 cm x 30 cm subplot
A_tillers_all	Number of alive tillers of <i>Ammophila arenaria</i> in the whole plot
E_tillers_all	Number of alive tillers of <i>Elytrigia juncea</i> in the whole plot

Soil salinity measurements in the field

We measured the soil salinities at the locations where we established our field experiment. At each location we took soil samples from 5 depths (5, 10, 25 & 50 cm). The samples were taken back to the lab and dried at 105 °C. The dried soil samples were diluted on a 1:5 mass basis with distilled water. The electrical conductivity of this solution was measured and multiplied with a factor 17 to derive the EC at saturated conditions (EC_e) (Shaw, 1994). When there was groundwater at the sampling depth, we measured groundwater salinity directly in the field with the same instrument as used in the lab. The groundwater depth ranged between 44 cm to > 75cm below beach surface, depending on location and transect. The measurements were performed on 12, 13 and 14 August 2015. While 12 and 13 August were dry, there was precipitation (15mm) in the early morning of the 14 August which slightly reduced the soil salinity of one of the five transects, increasing the error bars per location.

To explore whether the soil salinity on Texel is comparable to other beaches along the Dutch coast, we complemented our data with soil salinity measurements on two additional beaches: the Hondbossche duinen (HD), in North Holland (coordinates: 52°44'34.31"N, 4°38'33.14"E; date: September 2015) and on Terschelling, another barrier island (coordinates: 53°24'30.31"N, 5°17'29.25"E, measured in June, August, and November 2015). Both beaches are dissipative beaches, however they have a smaller beach compared to the Hors. The HD is an artificial created mega-nourishment and has the smallest beach width, whereas the beach on Terschelling has a much wider beach width. On HD we measured soil salinity at the upper beach and dune foot, 1.9 m – 2.5 m NAP, and on Terschelling we measured at the upper beach, 1.9 – 2.3 m NAP.

Dataset: Field_soil_salinity_data.csv

Column	Information
Month	Month measured
Year	Year measured
ID	ID number of measurement
Area	Area where we measured
Transect	Transect of the field experiment on Texel, Fig. 1
Location	Location within a transect of the field experiment on Texel, Fig. 1
depth	Depth of soil salinity measurements
EC _e	The EC _e in mS/cm

Glasshouse experiment with two dune building grasses

Plant material

We collected 600 rhizomes equally divided over both *A. arenaria* and *E. juncea*, from the vicinity of our field transplantation experiment on the Hors, Texel. The rhizomes were stored in plastic bags with moist sand in a fridge (c. 4 °C) for three weeks until planting. Just before planting we standardized the rhizomes by cutting all of them to similar length (20 cm), it was not possible to standardize the number of nodes on each rhizome. The range in node number was for *A. arenaria* 6 – 11 and for *E. juncea* 8 – 24. The rhizomes were planted in 196 experimental pots (10 l volume) filled with 14 kg soil which

consisted of a mixture of (calcareous) sandy river soil and organic matter (3:1 volume mixture) and 1 litre of water. Three rhizomes of one species were planted in each experimental pot, about 5 cm below the soil surface. All pots were watered every week to keep the soil moisture content constant, no additional nutrients were provided during this initial phase. Shoots emerged from the rhizomes 1 to 4 weeks after the planting. Four weeks after the planting of the rhizomes, treatments were randomly assigned to all pots where tillers had developed. We ended up with 192 pots for the main experiment (see experimental design below), leaving four pots with living tillers to verify the experimental treatments. The greenhouse climate for both preparation phase and experiment was set to 20°C at day and 15°C at night, standard humidity (about 50 %). Natural light was supplemented by SON-T 400W lamps to guarantee 16 hours day length.

Experimental design

A total of 192 experimental pots were used in this experiment with a full factorial design of two factors: soil salinity (six different levels) and salt spray (with/without). For each treatment we had eight replicates, which were distributed over eight replicate blocks. Within each block the treatments were repeated for each species: *A. arenaria* and *E. juncea*. The position of the experimental blocks was randomized three times during the experiment to control for potential variation in light conditions within the greenhouse. For the salt spray treatment the plants were initially sprayed five times from all sides at 70 cm distance with either distilled water or water with 3.5% NaCl concentration (Sykes and Wilson, 1988). After 14 weeks we increased the spraying treatment by spraying ten times from all sides, to ensure that all leaves were sprayed. While spraying, waterproof cardboard was used to shield the other pots from the spraying. For the soil salinity treatment six different saline solutions were prepared with 0%, 0.25%, 0.5%, 0.75%, 1.0%, and 1.5% salt concentration (corresponding to 0, 42.8, 85.6, 128, 171, 214 mM NaCl, and 0.28, 6.0, 11.1, 16.2, 20.2, 33.90 mS/cm EC). The soil salinity treatments were based on the range of soil salinities we found in the field. To ensure there was no effect of salt spray on the soil salinity we applied once every week first the salt spray treatment and then the soil salinity treatment. Since the provided salt can accumulate in the soil, excess saline solution was supplied to the experimental pots to a set weight (16 kg). At this pot weight about one third of the saline solution drained from the pots, preventing accumulation of salt at concentrations higher than the treatment (Poorter et al., 2012; Sykes and Wilson, 1989). The saline solution was directly applied to the soil, to prevent a change in the salt spray on the leaves. Nutrients were added to the different saline solutions in the form of 2.5% Hoagland's solution, to ensure sufficient nutrients for plant growth. This low amount of nutrients represents the field conditions, since dunes are very nutrient poor (Maun, 2009). The plants were harvested after 25 weeks of the start of the treatments, which is more or less similar to the length of the growing season of the two dune building species.

Plant growth

Plant growth was measured by counting the number of shoots, leaves (alive, dead) and the height of longest leaf for each experimental pot. Shoots were defined as an individual stem with leaves. Leaves were considered dead when they had no green tissue left. All variables were measured weekly during the first 12 weeks of the experiment and again during week 18 of the experiment. For nine out of the 192 pots all plants died during the experiment, all corresponding to *A. arenaria*. No pots with *E. juncea* experienced mortality, however one experimental pot was planted erroneously with *A. arenaria* and was excluded from the analysis.

We harvested the experiment per block by collecting the whole plant after which we divided it into two fractions: the shoot (including both dead and alive leaves) and root biomass. The roots were carefully separated from the soil by gently rinsing them with flowing tap water. Biomass of both fractions was determined after drying the material at 40° C for three days.

Measurements of gas exchange and stomatal conductance

We measured CO₂ gas exchange and stomatal conductance to explore the mechanisms behind the biomass response. From week 21 to week 24 (May 1 – 21 2015), we measured the leaf photosynthesis (CO₂ net exchange) with a cross-calibrated LI-6400 portable photosynthesis system (LI-Cor, Inc, Lincoln, NE, USA) from single leaves of all plants in four randomly selected blocks. The CO₂ net exchange (Asat) was measured under ambient CO₂ concentrations of 400 ppm and photosynthetically active radiation (PAR) flux density at or near 2000 μmol m⁻² s⁻¹. Measurements were made from 08:30 to 12:00

h during the day (CET time) to minimise the risk of declines in gas-exchange rate as a result of stomatal closure, source–sink inhibition or other causes during the afternoon (Pérez-Harguindeguy et al. 2013).

The CO₂ net exchange (Asat) and stomatal conductance were calculated with the following equations from Caemmerer & Farquhar (1981).

$$1. \quad \text{Asat} = (F(C_r - C_s)/100S) - C_s E$$

$$2. \quad g_{sw} = 1/((1/g_{tw}) - (k_f/g_{bw}))$$

Asat is the photosynthesis in $\mu\text{mol m}^{-2} \text{s}^{-1}$, F molar flow rate of air ($\mu\text{mol s}^{-1}$), C_r and C_s are the sample and reference CO₂ concentrations ($\mu\text{mol CO}_2 \text{ mol air}^{-1}$), S is leaf area (cm^{-2}) and E is the transpiration ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$). g_{sw} is the stomatal conductance in $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$, g_{tw} is the total conductance ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$), g_{bw} the boundary layer conductance ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$) and k_f is calculated by $k_f = (K^2 + 1)/(K+1)^2$, where K is the stomatal ratio (estimate of the ratio of stomatal conductance of one side to the leaf to the other side).

Water use efficiency (WUE) was calculated as the ratio between the net CO₂ exchange and the stomatal conductance. During the harvest we measured the Specific Leaf Area (SLA), for the four blocks we we measured the single leaf gas exchange. The SLA was measured by scanning five fresh undamaged leaves with a leaf scanner (Li-3100 Area Meter) and weighing the dried leaves (dried at 40 °). For each of these five leaves we measured the leaf thickness.

Plant chemical analyses

We measured the concentrations of nitrogen, phosphor, potassium (K) and sodium (Na) in the harvested shoot biomass of all plants in a subset of four randomly selected blocks. The concentrations of plant nutrients N, P and K were measured to explore if nutrient limitation could explain the plant biomass at higher soil salinity (Colmer and Flowers, 2008; Rozema et al., 1983). Concentrations of Na were measured to explore whether ionic stress played a role in explaining the treatment effect (Munns and Termaat, 1986). The harvested shoot biomass, which includes dead and alive biomass, was first gently rinsed with distilled water to remove any residual salt spray. The dried shoot material (70 °C) was pulverised and digested with H₂SO₄, salicylic acid, H₂O₂ and selenium. Subsequently N and P concentrations were measured colorimetrically using a continuous flow analyser (SKALAR SAN plus system, The Netherlands). K and Na were measured by flame atomic emission spectroscopy (AES) (Walinga et al., 1989).

Dataset: Glasshouse_growing_data.csv

Column	Information
ID	ID number of the pots, A = Ammophila arenaria , E = Elytrigia juncea
Code1	Full code of the Pots
Code2	Code without species
Species	Species planted
Salt_spray	Salt spray treatment present or not
Salinity	Salinity treatment level, 1= 0%, 2=0.25%, 3=0.5%, 4=0.75%, 5=1.0%, 6=1.5%
Block	Blocks which the pots were situated
Date	Date of measurements
Days	Amount of days since the start of the treatments
Leaves_alive	Number of leaves alive in pot
Tillers_alive	Number of tillers alive in pot
Max_height	Maximum height of plants
Leaves_dead	Number of dead leaves in pot

Dataset: Glasshouse_harvest_data.csv

Column	Information
ID	ID number of the pots, A = Ammophila arenaria , E = Elytrigia juncea
Code1	Full code of the Pots
Code2	Code without species
Species	Species planted
Salt_spray	Salt spray treatment present or not
Salinity	Salinity treatment level, 1= 0%, 2=0.25%, 3=0.5%, 4=0.75%, 5=1.0%, 6=1.5%
Block	Blocks which the pots were situated
Dead_leaves	Biomass of the dead leaves in gram, only measured for Ammophila arenaria
Shoot_biomass	Total shoot biomass (including dead and alive) in gram.
Root_biomass	Root biomass in gram
Total_biomass	Total biomass in gram
SR_ratio	The shoot to root ratio, shoot biomass divided by the root biomass
SLA	Specific leaf area cm/gr
L_thickness	Thickness of the leaf in mm
Photo	Photosynthesis of the plant in $\mu\text{mol m}^{-2} \text{s}^{-1}$
Stom_Con	Stomatal conductance of the plant in $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$
WUE	The water use efficiency ($\mu\text{mol/mol}$)
Nitrogen	Nitrogen concentration in %
Phosphor	Phosphor concentration in %
Potassium	Potassium concentration in %
Sodium	Sodium concentration in %
Soil_salinity_pots	The soil salinity of the pots, measured as the ECe in mS/cm