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Gazala M Alhdad, Christian Zörb, Mohammed J Al-Azzawi, Tim Flowers

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Is the reduced growth of the halophyte *Suaeda maritima* under hypoxia due to toxicity of iron or manganese?

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Highlights

- Elevation, determines the flooding and hypoxia experienced by saltmarsh plants
- Plants of *Suaeda maritima* at lower elevations are smaller than those higher up the marsh
- We grew *S maritima* in flooded soil and hypoxic culture solution
- Hypoxia, reduced growth and increased manganese and iron in shoots and roots
- We showed Mn was unlikely to be toxic, but Fe could reach toxic concentrations

ABSTRACT

For most plants, submergence in water is a rare occurrence, but for plants that grow on salt marshes flooding with seawater may be a twice-daily event. This is the case for plants of the halophyte *Suaeda maritima*, growing at low elevations on salt marshes. These plants are, however, smaller than those growing at higher elevations, where flooding is less frequent and the soil better drained. We investigated whether the reduced growth brought about by flooding with saline water was a consequence of toxicity of manganese or iron. Seedlings of *S. maritima* were grown both in a solid medium (a mixture of salt-marsh mud and sand) that was either submerged twice a day or continuously flooded with half-strength seawater and in a hydroponic solution where the oxygen concentration was adjusted by bubbling with nitrogen or air. Hypoxia, reduced the growth of plants in both solid and liquid media and resulted in increases in manganese and iron in the shoots and roots. Experiments in culture

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solution showed that elevated levels of manganese were unlikely to be toxic, but that iron did reach toxic concentrations in flooded plants.

Keywords: Suaeda maritima; Salinity; Halophyte; Waterlogging; Metal toxicity

1. Introduction

Suaeda maritima is a plant that grows in both the upper and lower regions of salt marshes, although plants are larger on upper than lower elevations (Wetson, 2008, Wetson and Flowers, 2010). The hypoxic conditions that exist in the lower marsh compared to the normoxic conditions of the upper marsh (Colmer et al., 2013) are likely to result in reduced ATP production, as oxygen is in poor supply to the roots. *S. maritima* has no aerenchyma to facilitate diffusion of oxygen from the shoots (Hajibagheri et al., 1985, Wetson, 2008), although it does accumulate high concentrations of lactate in both normoxic and hypoxic conditions (Colmer et al., 2013, Wetson et al., 2012). Reduced ATP supply could reduce the uptake of ions that determine the growth rate (Yeo and Flowers, 1986) and so reduce growth. There is also the possibility that hypoxia influences the bioavailability and accumulation of metal ions, leading to deficiency or toxicity, which might explain the difference in growth between upper and lower elevations of a salt marsh - the subject of this paper.

Coastal salt marshes are heavily influenced by daily tidal inundations that waterlog the soil for different lengths of time depending on elevation. Waterlogging affects the availability of micronutrients for plants, as periodic and prolonged flooding of soil results in biological and chemical processes that are very different from those that happen in well-drained and aerated soils. When a soil is flooded, oxygen diffuses from the air into the soil around 10,000 times more slowly than in well-drained soil, so the concentration of oxygen can decrease to very low levels (Ponnamperuma, 1972), reducing the redox potential of the soil and altering its elemental profile. Once oxygen is depleted, respiring soil microbes use nitrates as electron acceptors, followed by oxides of manganese, then iron and then sulphate. The conversion of Mn (IV) and Fe (III) oxides to Mn (II) and Fe (II) oxides, increases the solubility of both elements with a sharp decline in redox potential (Ponnamperuma, 1972). The end result of changes in oxidation state in the soil is a significant increase in soluble Fe^{2+} and Mn^{2+}, even at high pH (see Millaleo et al., 2010) with potential consequences for plant growth.

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The concentration of Mn in agricultural soils is highly variable (by some 40 fold; Nagajyoti et al., 2010) with values, on a soil water basis, ranging from 20 nM to 72 µM (Mansfeldt, 2004; Goss et al., 1992). The concentrations of Fe in aerobic soils at normal pH values (pH 5 to 7) are very low (in the nM range; see Marschner, 1986) and can limit the growth of plants. However, high external concentrations of both elements (Mn^{2+} and Fe^{2+}, the forms in which plants take up Mn and Fe) are toxic (Millaleo et al., 2010, Marschner, 1986). Poor aeration in salt-marsh soils leads to high, potentially toxic, concentrations of both Fe (Otero et al., 2009) and Mn (Otero et al., 2009, Singer and Havill, 1985). For example, at low elevation of a salt marsh in southern Brazil, Mn reached concentrations of about 300 µM, 10 to 20 cm below the surface of a zone dominated by *Spartina alterniflora*; Fe concentrations were about 200 µM in the same zone (Otero et al., 2009). In salt marshes from N. Carolina, Fe concentrations ranged from about 20 to 700 µM (Adams, 1963). Such high concentrations could lead to reduced growth directly due to Mn or Fe toxicity or as a consequence of the costs of adapting to such high concentrations.

Unfortunately, the literature does not provide a consensus on the effects of changed Mn and Fe concentrations on the growth of salt-marsh species. Cooper (1984) reported that the shoot dry weights of *Plantago maritima, Armeria maritima and Juncus gerardii* were reduced by Mn concentrations greater than 250 µM (the results for *Salicornia europeaea, Puccinellia maritima, Triglochin maritima, Aster tripolium,* and *Festuca rubra* were less clear). *S. europeaea* and *A. tripolium* have been reported sensitive to Mn concentrations greater than 160 µM in solution culture, but in the absence of salt (Singer and Havill, 1985). Singer and Havill (1993) later claimed that although Mn concentrations were relatively high in the upper 1 cm of salt-marsh soils and that salt-marsh species have considerable tolerance to Mn, the concentration did not correlate with elevation or species distribution. Whether plants of *S. europaea, P. maritima, J. gerardii* or *A. maritima* were grown under flooded or drained conditions had little effect on the Fe or Mn concentrations in their shoots (Rozema and Blom, 1977). Adding NaCl (170 mM) to the culture solution in which *P. maritima* and *A. tripolium* were grown reduced the uptake of Mn (Singer and Havill, 1993), a result in line with the finding that halophytes sampled from salt marshes had lower Fe and Mn concentrations than plants from non-saline habitats (Gorham and Gorham, 1955). Data in the literature do not answer the question of whether high Mn or Fe concentrations might be responsible for differences in growth of *S. maritima* between upper and lower elevations of salt marshes.

We investigated the effects of external Fe and Mn concentrations on the growth of *S. maritima* and its content of these elements under normoxic and hypoxic conditions, using

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both a soil-based medium and a hydroponic solution (for details see below) in order to elucidate metal bioavailability and its consequences for *S. maritima* growing on the varying conditions of a salt marsh. We examined the hypothesis that the accumulation of Mn and Fe in *S. maritima* plants growing in hypoxic conditions, characteristic of the lower marsh, was sufficient to result in toxicity and so reduce growth relative to plants growing more aerobic conditions.

2. Materials and methods

2.1. Plant material, germination and initial growth of seedlings

Seeds of *S. maritima* from Cuckmere Haven, East Sussex (UK National Grid Reference 551400098500, TQ515978 were germinated in plastic trays containing silver sand irrigated with half-strength nutrient solution (Stout & Arnon, 1939; Supplementary Table 1) and grown for four weeks, in a growth chamber (Weiss 2400E/+5 JU-Pa-S; Weiss Technik, Gmbh, Reiskirchen-Lindenstruth, Germany) with a 16 h photoperiod at 200 μmols m^{-2} s^{-1} and 22 ^{o}C and 60% relative humidity; during the dark period, the temperature was 17 ^{o}C and the relative humidity 70%.

2.2. Plant growth

Since in the majority of previous research on *S. maritima*, plants have been grown hydroponically at pH values below 7, this practice was continued in some of the experiments described in this study. Plants were grown in a half-strength culture solution (Stout and Arnon, 1939) made up in a dilution of an artificial seawater (Harvey, 1966), in order to provide the necessary nutrients that are low in seawater (N and P) while maintaining the ratios of the major ions (Cl, Na, Mg and Ca) present in natural seawaters. In order to investigate the effects of hypoxia, some of the solutions contained 0.1% agar. Preliminary tests showed that a 0.1% agar solution more effectively simulated the situation in waterlogged soils and in the rhizosphere, as compared to N_2 flushed or non-flushed agar-free nutrient solutions (see also Wetson, 2008). We recognise that there may be a contrast with plants growing in natural saltmarsh soils, but attempting to grow plants at a high pH with hydroponics, means that many micronutrients precipitate from solution, so that the solution

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has to be changed daily or other ways found of supplying micronutrients, such as by foliar spray (Singh et al., 2002). Experiments were also conducted in a medium based on a natural salt-marsh soil for comparative purposes (see below). All experiments were repeated at least once with representative data being presented here.

2.3. Experiment 1: The effect of aerobic and hypoxic conditions on growth and trace metal contents under controlled conditions in a growth cabinet

Seeds were germinated as described above. Plants were transplanted at 4 weeks into nutrient solution in artificial seawater diluted to 350 mM Na^+containing agar (see below) and grown in black plastic-lidded beakers (500 ml, 15 cm high and 7 cm diameter). There were 15 plants per treatment (5 beakers per treatments; 3 plants per beaker), each plant being suspended through a hole in the lid and held in place with non-absorbent cotton wool. Agar-nutrient solution was prepared by dissolving 10 g of agar (Sigma, Plant Cell Culture A 1296) in 2 L of distilled water and autoclaving at 120 °C for 15 minutes. After cooling, this solution was added to 7.39 L of full-strength artificial seawater, then distilled water was added to make a final volume of 10 L, so producing 350 mM Na^+ with 0.1% w/v agar. The solution was stirred thoroughly to avoid lumps of agar forming. For normoxic treatments, compressed air was bubbled through the solution to obtain good aeration in the solution prior to filling the beakers (pre-bubbled). For hypoxic treatments, nitrogen gas was bubbled through the solutions to reduce oxygen to less than 0.5 mg L^-. Gas was not bubbled through the solutions during the eight weeks of treatment as this can damage the roots (Wetson, 2008), but the solutions were changed twice a week.

Plants were harvested after 8 weeks in the Weiss cabinet with one of two treatments.

(a) Normoxic nutrient solution (pre-bubbled with air) with 350 mM Na^+ (350 N).

(b) Hypoxic nutrient solution (pre-bubbled with N_2) with 350 mM Na^+ (350 H).

Oxygen concentrations were recorded before and after changes of the culture solution with an oxygen meter (HI 9142 oxygen meter, HANNA Instruments); pH values and electrical conductivity (EC) were measured before growth medium solutions were renewed.

2.4. Experiment 2: The effect of flooding in soil-based system on ion uptake in S. maritima plants grown in a glasshouse

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Plants were grown in pots in a mixture of sand and estuarine mud for 8 weeks in a system of tanks in a glass-house where they could be flooded for different periods of time under semi-controlled conditions, as described by (Alhdad et al 2013). The mud was collected from an estuarine marsh at Shoreham, East Sussex (TQ206060). The mud was mixed in a large trough with equal volumes of half-strength seawater (collected from the sea as described in Alhdad et al 2013) and washed silver sand. After thorough mixing by hand and removal of any large shells or debris, pots were filled with this mixture and left to drain overnight before the *Suaeda* seedlings were transplanted. Stout and Arnon (1939) culture solution, made up in half-strength fresh seawater, was pumped for one hour twice daily to simulate tides. Plants in one set of pots were flooded twice daily, simulating normal tidal exposure, while the other set of pots remained continuously flooded. Electrical conductivity (EC) and pH in the tanks was measured every 2 d: the average EC was 29.6 ±0.03 dS m^{-1} and the average pH 8.2.±0.01. The minimum day-time temperature in the glass house was 24 °C and at night 17.0 °C (16/8 h light/darkness); the relative humidity ranged between 60-75%.

Direct measurements of the degree of oxygenation of soil could not reliably be made with the oxygen sensor because of the likelihood of damage to the delicate membrane by the pressure of soil particles during its insertion into the soil (Wetson, 2008). Consequently, redox potential (Eh) was used as an index of soil oxygenation. Readings were taken using a Combined Redox Electrode with a platinum rod and a Calomel reference electrode ORP meter (CMPTRII/DWGI806, Thermo Electron Corporation, Fife, Scotland) attached to a portable meter (HI 9025 HANNA Instruments). The redox state (Eh) of the growth medium surrounding the roots was measured at three depths: 1 cm, ~4 cm and ~8 cm below the surface.

2.5. Experiments 3 and 4: the response of Suaeda maritima to varying concentrations of Fe and Mn under aerobic and hypoxic conditions (culture solution experiments)

These experiments were performed to compare the effect of aeration and hypoxia at different concentrations of iron (Fe added as FeEDTA, ethylenediaminetetraacetic acid Fe(III) sodium salt) and manganese (Mn added as $MnSO_4$) in half-strength Stout & Arnon nutrient solution, with 350 mM Na^+ in artificial seawater under normoxic and hypoxic conditions.

Four-week-old plants were transferred to black plastic boxes, (2 L; 10 plants per box and 3 boxes per treatment) and suspended with non-absorbent cotton wool through holes in a

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lid. The solutions (containing agar and pre-bubbled with air or N_2 as described above) were changed at weekly intervals; pH and EC values were recorded weekly before and after solutions were renewed. These experiments were carried out in the controlled environment chamber (Weiss 2400E/+5 JU-Pa-S growth cabinet; Weiss Technik, Gmbh, Reiskirchen-Lindenstruth, Germany) in the same conditions as those in which the seed were germinated (see 2.1). The boxes were topped up to a constant level with distilled water to replace evapotranspiration losses throughout the experiment. For the hypoxic boxes, water was bubbled with nitrogen gas before use. Plants were harvested after Fe and Mn toxicity appeared in the treatments with high concentrations; for Fe after 10 d of treatment, and for the Mn experiments, the plants were harvested after 21 d.

Four concentrations of Fe were used for Experiment 3 (13.6 µM, 262 µM, 514 µM, 1.01 mM Fe) and five of Mn for Experiment 4 (3.35 µM, 250 µM, 1 mM, 5 mM, 10 mM Mn) in either normoxic or hypoxic solutions, making eight treatments in Experiment 3 and ten in Experiment 4.

2.6. Fresh and dry weight determination

After harvesting, the shoots were carefully rinsed with distilled water, patted dry with paper towels and quickly weighed for determination of fresh weight. The roots were gently rinsed under running tap water to remove agar and then washed three times with distilled water. Dry mass was determined after 72 h in an oven at 80°C.

2..7 Nutrient analysis

Leaf and root samples were collected and dried at 80°C for 24 h before elemental analysis. After crushing, a sample (50 mg DW of each) was ashed at 550°C for 4 h, dissolved in 70% concentrated nitric acid (0.5 ml), heated for five minutes and diluted with distilled water to a final volume 20 ml. All ions were measured by ICP-MS, performed on an Agilent 7500ce ICP-MS; the data was acquired in helium gas collision mode with a He flow of 4.5 ml min^{-1}. RF Power was 1500W and the spray chamber was cooled to 2°C.

2.8. Statistical analysis

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Data were analysed by ANOVA using SPSS v 18. Different letters above the bars on the graphs or after figures in tables indicate a significant difference in means from post-hoc Tukey tests.

3. Results

3.1. Experiment 1: growth, Fe and Mn in Suaeda maritima plants grown under normoxic and hypoxic conditions, in artificial seawater

The growth of *S. maritima* was investigated in nutrient solutions (Experiment 1) where conditions ranged from good aeration by pre-bubbling with air, to severe hypoxia using pre-bubbled stagnant agar solution in artificial seawater (350 mM Na^+). Shoot dry weight was higher (1.3 times) in normoxic than in hypoxic condition (Fig 1). The solutions were not bubbled with gas during the experiment to avoid injury to the roots (Wetson, 2008) and although the O_2 concentration in the normoxic treatments decreased between changes of culture solution, the concentration was at least 7 times that in the hypoxic treatments, which hardly changed between renewals (Table 1). The overall activities of nutrients in the normoxic and hypoxic solutions did not differ (the activities estimated using Visual Minteq version 3) were, in mM: Ca, 5.41; Cl, 275.74; Cu, 0.07; Fe, 0.01; K, 8.08; Mg, 22.79;; Na, 274.29; NO_3, 4.99; SO4, 6.62; and in µM: Mn, 1.76 and Zn, 0.22). The main differences (100% or more) between the normoxic and hypoxic solutions were changes (increase +, decreases -, in parentheses) $Cu(OH)_4^{2-}$ (+112%); $Mn(OH)_4^{2-}$ (+151%); $Mn_2(OH)^{3+}$ (+100%); $Zn(OH)_4^{2-}$ (+151%).

Table 1 here

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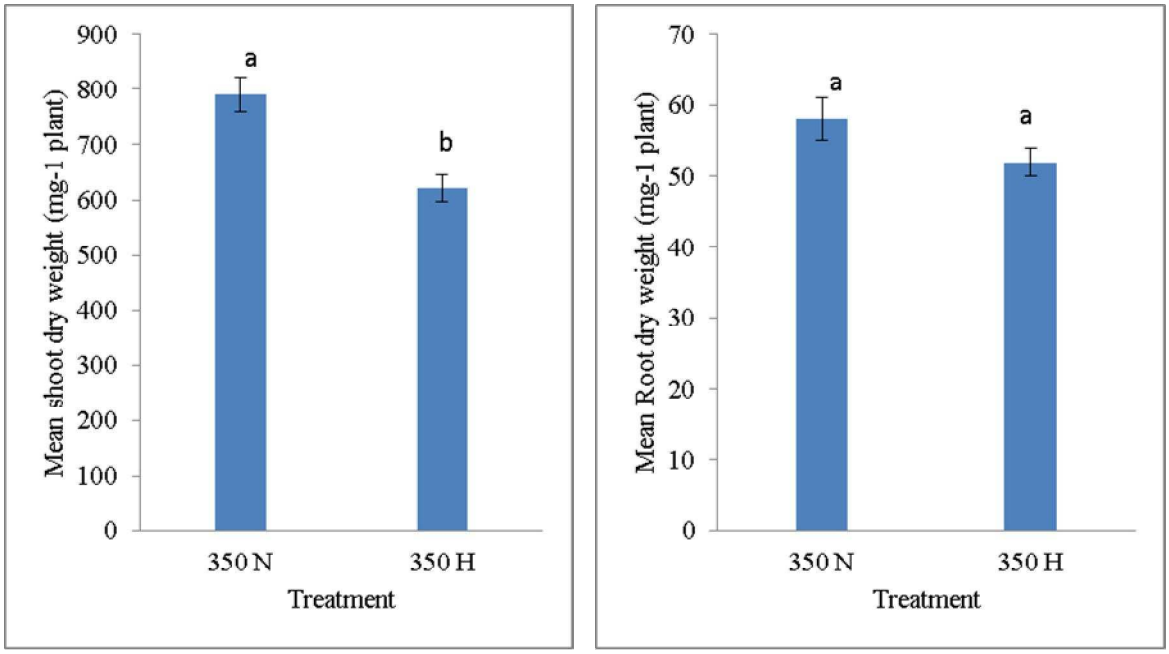

Fig 1

3.1.1. *Shoot and root trace elements*

Under normoxic conditions, the concentration of Mn was almost three times higher in the shoots than in the roots (Table 2) and the shoots contained the majority of this element (ca 40 times the quantity in the roots (Table 2). By way of contrast, the concentration of Fe was about 40 times higher in the roots than the shoots, with the roots containing most of the Fe (three times that in the shoots).

Under hypoxic conditions, Mn and Fe concentrations increased in the shoots when compared to normoxic values – by 1.4 and 2.0 times, respectively. The most dramatic effect of hypoxia was to increase the concentration of Mn in the roots to 12.5 times those in normoxic roots. Root Fe concentration fell to 80% of the normoxic value.

Table 2 here

3.2. Experiment 2: Fe and Mn in Suaeda maritima grown under drained and flooded conditions in the greenhouse

In order to evaluate whether the effects seen at pH 5.5-6.0 in culture solution were similar to those at the pH of seawater (8.0 to 8.3; Harvey, 1966), growth was determined in a medium composed of salt-marsh mud and sand (50% sand, necessary to adjust the hydraulic

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conductivity) with two flooding regimes in half-strength fresh seawater at pH 8. Plants in one set of pots were flooded twice daily, simulating normal tidal exposure, while the other set of pots remained continuously flooded.

3.2.1. Salinity, pH, redox values and growth

The salinity, as judged by the electrical conductivity of the medium, was lower in the mud/sand mixture (about 30 dS m^{-1} than in the culture solution (about 40 dS m^{-1} in the high salt treatment) and the pH was significantly higher in the mud/sand (8.2) than in the culture solution (5.0 – 5.6) (compare the values in Tables 1 and 3).

Table 3 here

Mean redox values (Eh) showed the contrasting redox state of the growth medium in drained and flooded conditions (Table 3). In the flooded growth medium the Eh values were more negative at all depths, especially at the base of the growth medium, than in the drained growth medium. The effects of flooding on the plants were more dramatic in the solid medium than in the culture solution. Plants grown in drained conditions in the soil had three times the dry weight of those in the flooded conditions (Table 3), whereas in the culture solution plants were just 1.3 times higher in normoxic as opposed to hypoxic solutions (Fig 1).

3.2.2. Mn and Fe, concentrations in the shoot

Shoot Mn and Fe concentrations were three times greater in flooded shoots than in shoots grown in drained conditions These changes were similar to, but of greater magnitude, than those seen in the culture solution (where the increases were 1.4, 2.0 and 1.5 times, respectively; see above).

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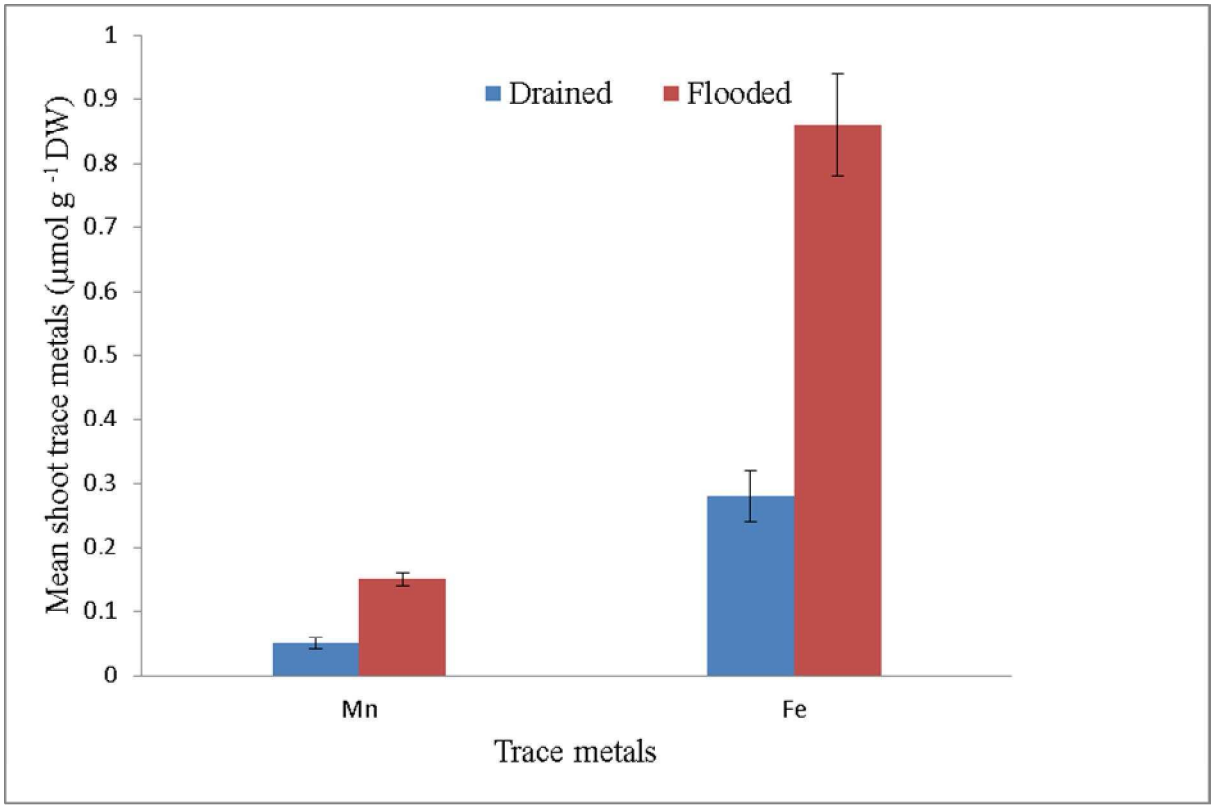

Fig 2

3.3. Experiments 3: the response of *Suaeda maritima* to varying concentrations of Fe under aerobic and hypoxic conditions

3.3.1 Plant biomass

Since the previous experiments showed that Fe concentrations were increased in the shoots by flooding (in hydroponics and in the sand/mud mixture), in this experiment the growth of *S. maritima* was investigated in nutrient solutions where conditions ranged from good aeration by pre-bubbling with air, to severe hypoxia using pre-bubbled stagnant agar solution, in artificial seawater containing 350 mM Na^+ and different concentrations of Fe (as EDTA; 13.6, 264, 514 µM, and 1.0 mM Fe). With the lower concentrations of Fe (13.6 µM, 262 µM, 514 µM), symptoms of toxicity (yellow colour) began after 7 d of treatment. In the highest Fe concentration used (1 mM Fe in the growth medium, a very high Fe concentration; luxury concentrations for crops are about 200 µM Fe-EDTA, see Discussion below; data not included in Figure 3), the seedlings died after 24 h.

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Shoot dry weight decreased with increasing Fe concentration in the growth medium (Fig 3) but under hypoxia the overall decrease was lower than under normoxic conditions, primarily because of the better growth of normoxic shoots in the absence of additional Fe (viz. at 13.6 µM Fe). Root dry weight was significantly higher in normoxic than hypoxic conditions until the Fe concentration exceeded 500 µM.

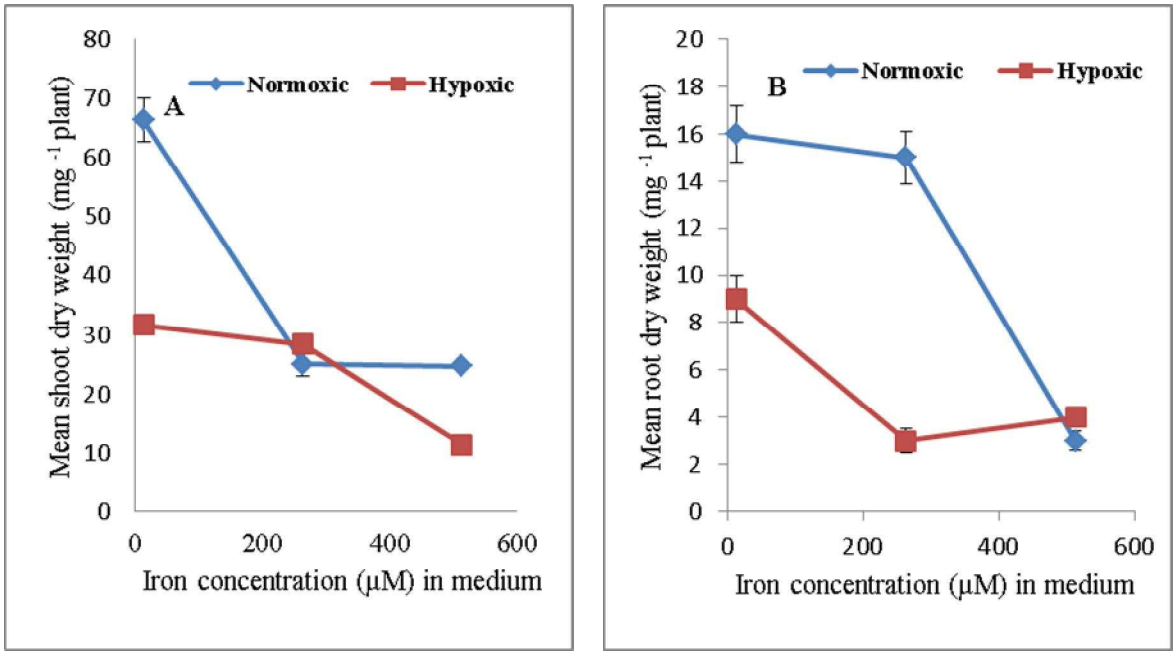

Fig 3

3.3.2. Fe and Mn concentrations in the shoot and root

Analysis of the data showed that shoot Fe concentrations increased under both normoxic and hypoxic conditions as the Fe concentration in the growth medium increased (Fig 4A; $P < 0.001$). Shoot Mn, on the other hand, decreased as the external Fe increased but only under hypoxic conditions (Fig 4B). In general, shoot Mn and Fe and were higher in hypoxic than normoxic conditions ($P < 0.001$). The response of the root concentrations of Mn and Fe to increases in the external Fe concentration was similar to those of the shoots: Fe increased but Mn decreased under hypoxic conditions or showed little change with higher oxygen supply (Fig. 4C, D, $P < 0.001$). Changes in shoot and root contents in response to increases in external concentrations of Fe followed a similar pattern to that of the changes in Fe concentrations (data not presented).

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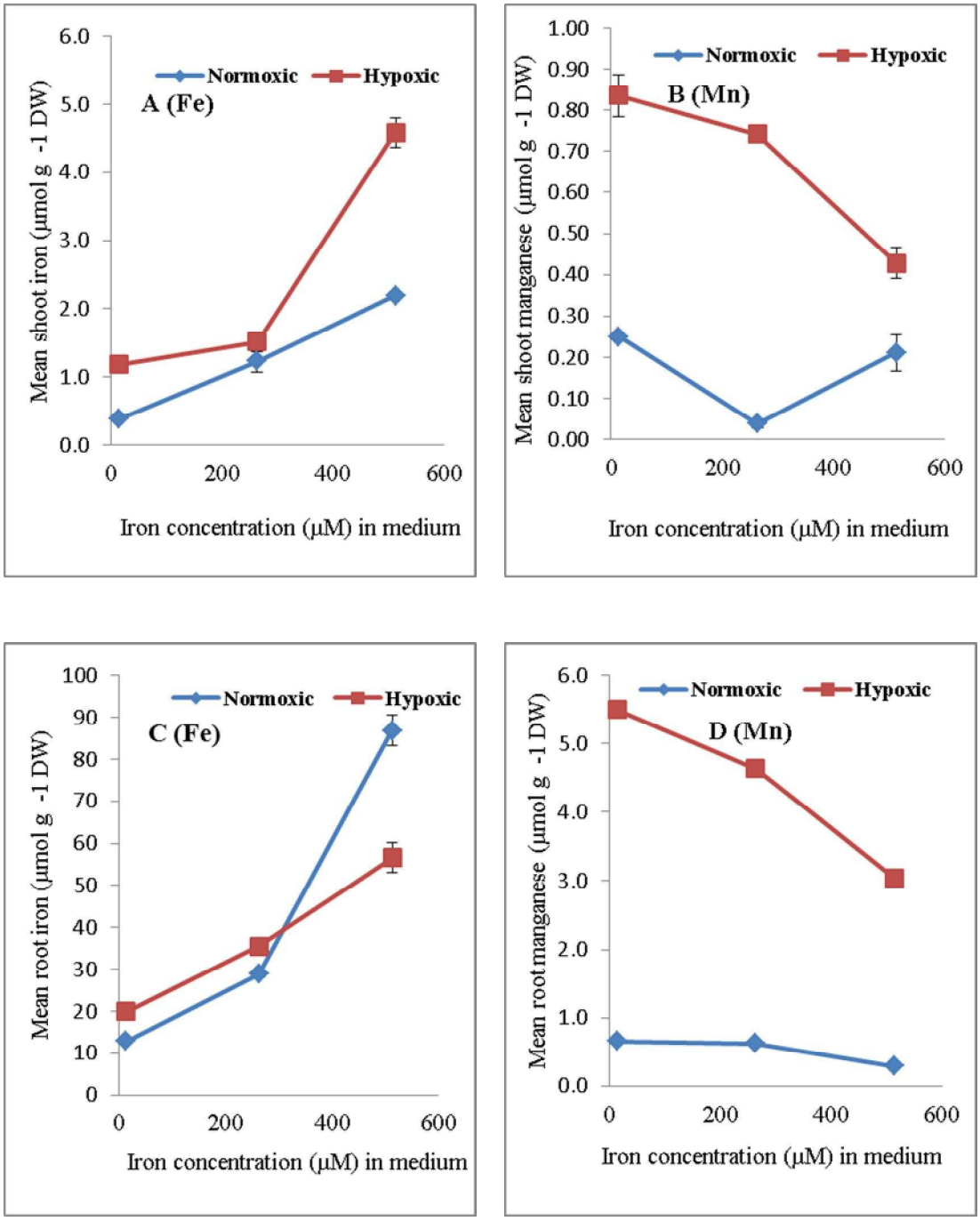

Fig 4

3.3.3.Shoot and root growth and internal Fe concentration

Combining the data on growth with that of Fe concentrations in the plant, revealed shoot growth (Fig 5 A&B) declined strongly with increasing internal (shoot and root) Fe concentrations. Root growth also declined with increasing Fe concentration, with the relationsships between mean root growth (y, mg^{-1} plant) and root Fe concentration (x, µmol g^{-1} dry weight) being y = -0.18x + 19 (R^2=0.98) under normoxic conditions and y = -0.13x + 10 (R^2=0.52) under hypoxic conditions, confirming its toxicity.

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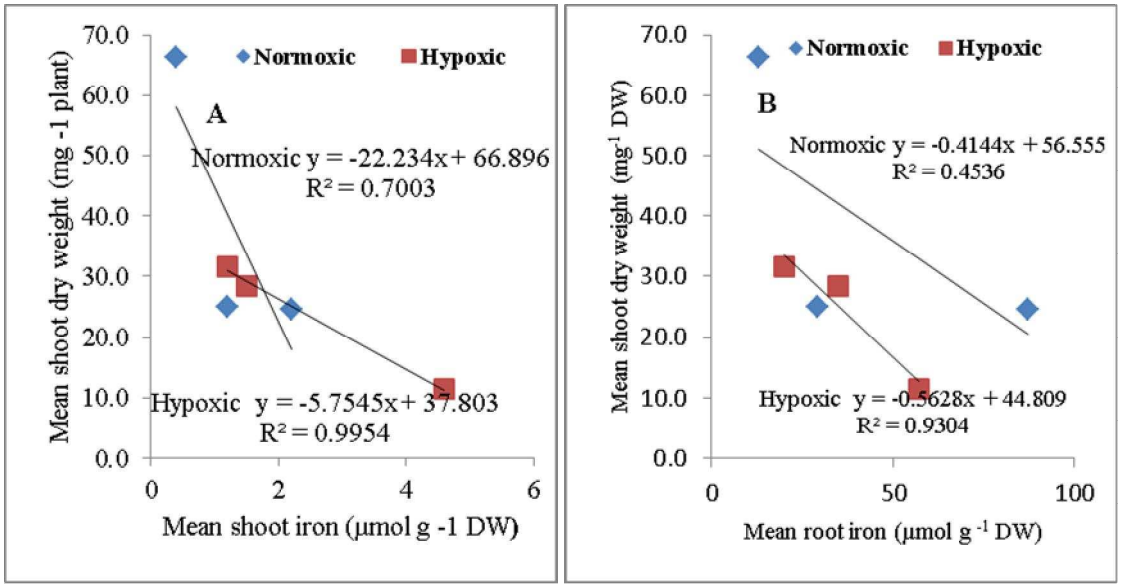

Fig 5

3.4. Experiment 4: the response of *Suaeda maritima* to varying concentrations of manganese under normoxic and hypoxic conditions

In this experiment, the growth of *S. maritima* was investigated in nutrient solution where conditions ranged from good aeration (pre-bubbled with air) to severe hypoxia (pre-bubbled stagnant agar solution) in artificial seawater containing 350 mM Na^+ and different concentrations of $MnSO_4$ (3.35 and 250 µM, 1, 5, and 10 mM).

3.4.1. Shoot and root dry weight

Analysis of the data showed that shoot and root dry weight were significantly affected by Mn concentration ($P < 0.001$), oxygen concentration (shoot dry weight ($P < 0.001$), root dry weight ($P < 0.05$)), and their interaction, ($P < 0.001$). As shown in Fig 6, shoot and root dry weights were maximal in 1 mM Mn then decreased. Under normoxic conditions, plants had a greater shoot dry weight than those grown in the hypoxic conditions.

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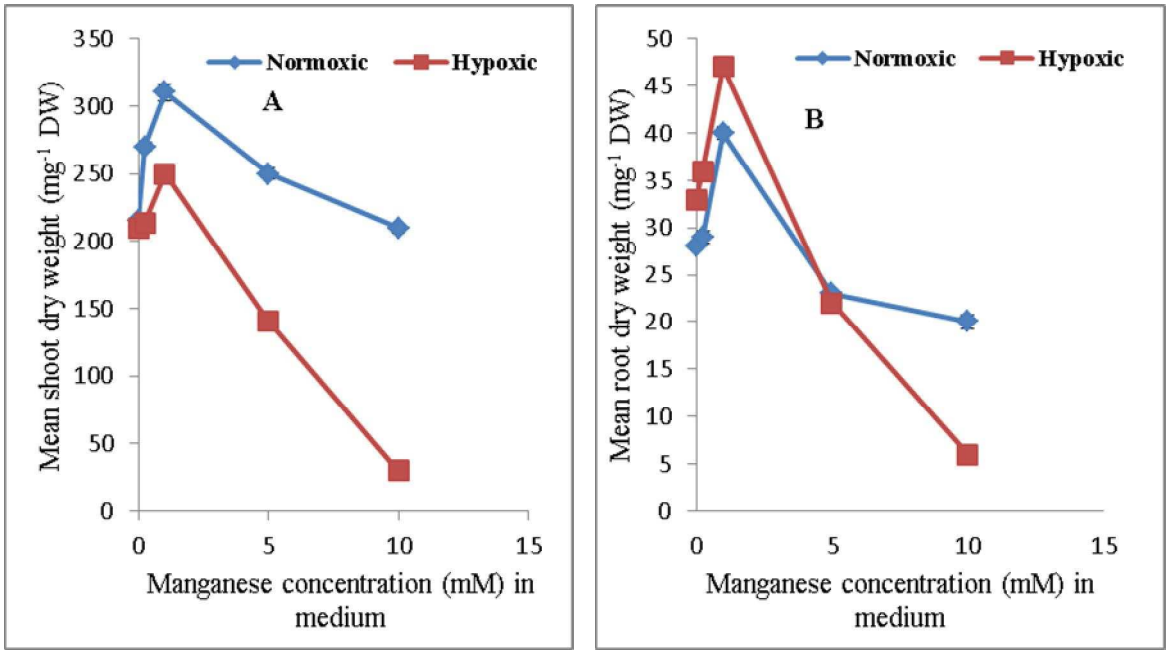

Fig 6

3.4.2. Mn and Fe concentrations in the shoot and root

Increasing the external Mn concentration increased the shoot Mn concentration under both normoxic and hypoxic conditions (Fig 7A). However, there was a dramatic difference in the response of shoot Fe concentrations depending on the oxygen concentration. Under hypoxic conditions Fe concentrations rose above those present at the optimal Mn concentration for growth (1 mM), but fell under normoxic conditions (Fig 7B). The patterns of change in the concentrations of Mn and Fe in the roots as the external Mn increased were rather different from changes seen in the shoots (compare Figs 7 A and C with B and D). In the optimal Mn concentration for growth (1 mM), root Mn concentrations were similar in normoxic and hypoxic conditions (Fig 7C). As the external Mn concentration increased to 5 mM, the concentrations of both Mn and Fe increased, with the increase in Mn, being greater under normoxic conditions and that of Fe being greater in hypoxia (Fig 7 C and D).

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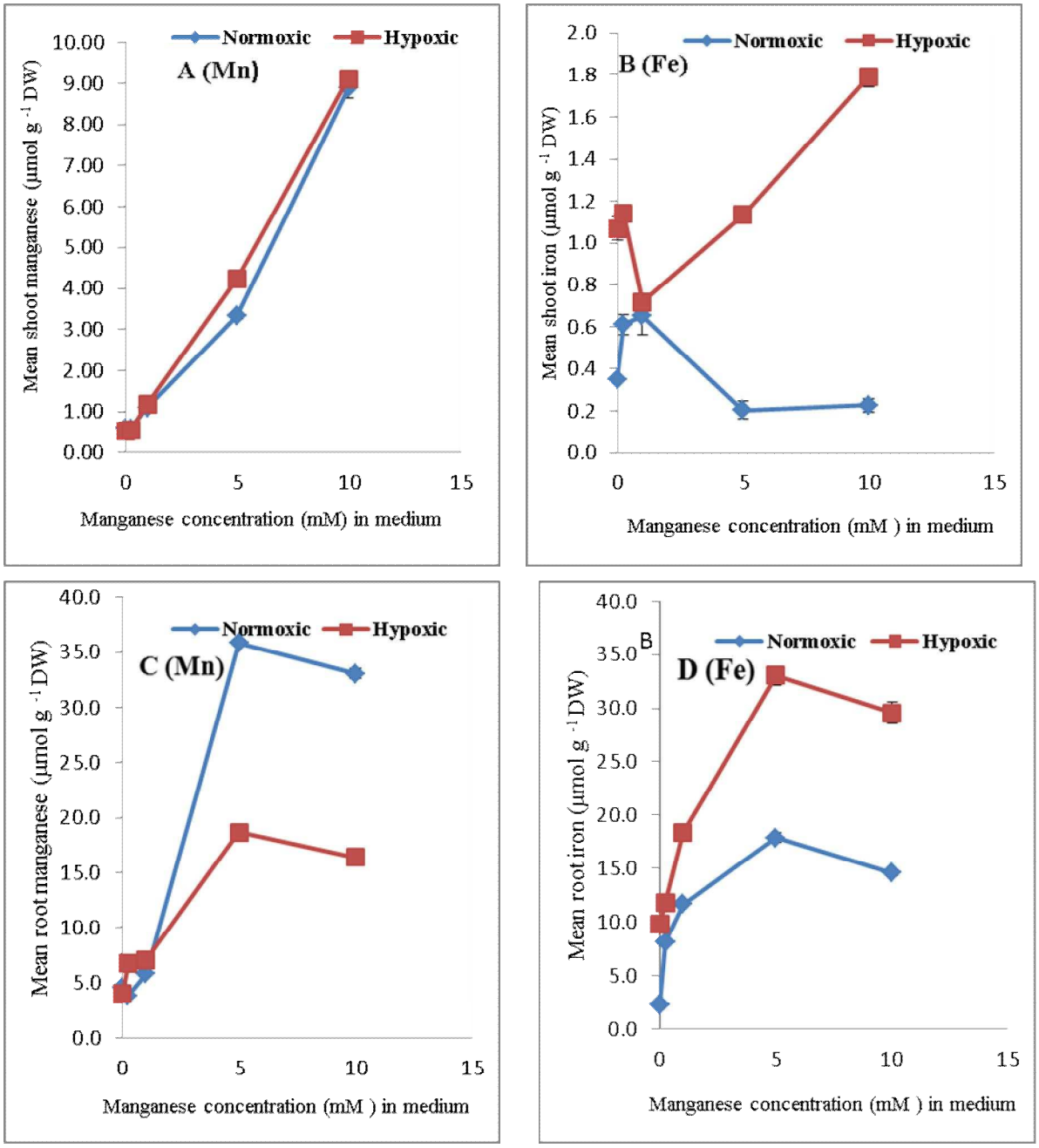

Fig 7

3.4.3. Shoot and root growth and internal Mn concentration

It is clear that plant growth was more sensitive to Mn concentration under hypoxia in nutrient solution than when root oxygen supply was more abundant. There was a strong negative relationship between shoot manganese and shoot dry weight in hypoxic conditions (R^2 = 0.94), and a weak negative correlation between shoot manganese and shoot dry weight in normoxic conditions (R^2 = 0.25) (Fig 8A). The relationships between shoot dry weight and root manganese concentrations were similar, but less good (Fig 8B). Root growth also

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decreased with increasing root Mn concentration, with the relationships between mean root growth (y, mg^{-1} plant) and root Mn concentration (x, µmol g^{-1} dry weight) being y = -0.36x + 34 (R^2=0.54) under normoxic conditions and y = -1.8x + 49 (R^2=0.58) under hypoxic conditions.

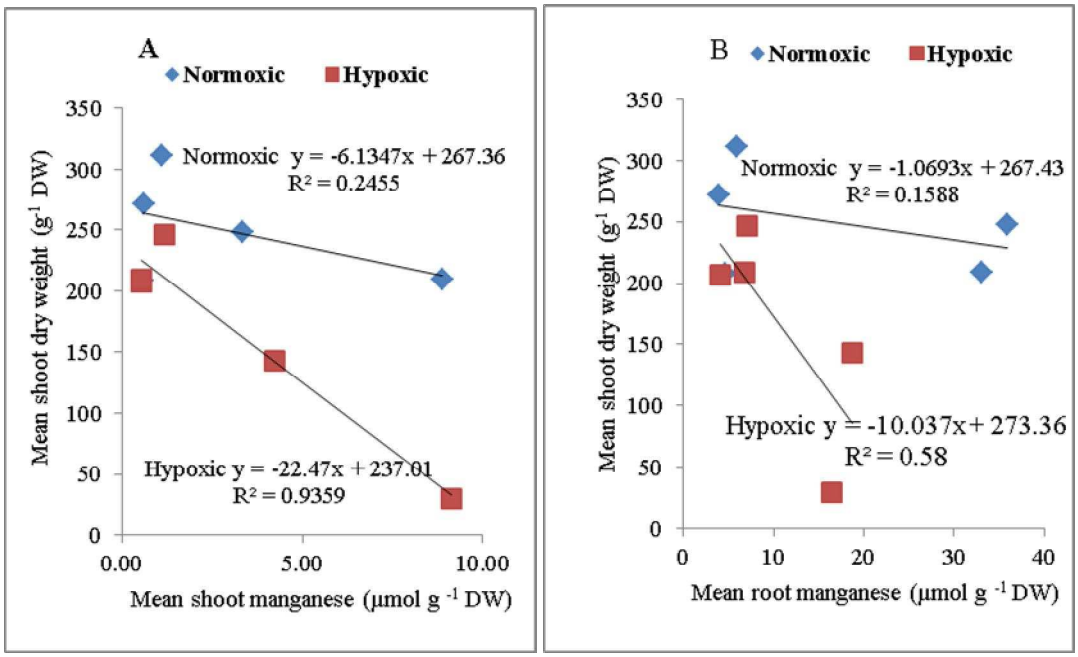

Fig 8

4. Discussion

It is clear that decreased oxygen supply decreased the plant biomass, whether the plants were grown in culture solution (shoot weight was 1.3 times higher in normoxic than in hypoxic conditions) or in the mud/sand mixture (where shoot dry weight was three times higher in drained than flooded conditions, Fig. 1). These findings are consistent with measurements made on plants growing on a Sussex salt marsh where the dry weight of plants growing at an upper elevation was 1.5 times that of plants growing 0.6 m lower down the salt marsh (3.8 ± 0.1 g per plant as opposed to 2.6 ± 0.2 g per plant; Alhdad et al., 2013) and similar to the findings of Al-Zahrani (1990) and Wetson et al. (2012). Our data are at variance with those on a saltmarsh population of *Suaeda salsa* (Syn *S. maritima* subsp. *salsa*), where growth was hardly reduced by waterlogging in the presence 200 mM NaCl (Song et al 2011). However, growth of an inland population of the same species was reduced by saline (200 mM NaCl) waterlogging (Song et al 2011), consistent with the conclusions

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drawn by Barrett-Lennard and Shabala (2013) that of 13 halophytes for which they tabulated data, nine showed reduced growth in response to waterlogging under saline conditions (four benefitted from combined stresses). We do not know if the greater effect of flooding in the solid than the liquid medium in our experiments was due to a difference in oxygen concentrations as we were unable to measure this parameter in the solid medium. However, the redox potentials measured in the mud (about -300 to -400 mV) are consistent with severe hypoxia or even anoxia (see Wetson and Flowers, 2010).

Hypoxia not only reduced growth, but also brought about changes, sometimes dramatic, in the concentrations of Fe and Mn within the plants. Hypoxia increased shoot Fe concentrations, which were similar in the two media (0.3 - 0.9 µmol g^{-1}DW or 17 - 50 µg g^{-1}dry weight), by three fold for plants growing in solid medium (Fig 2) and twofold for plants in solution culture (Table 2); root Fe concentration fell in liquid medium (Table 2; to 80% of the normoxic value; there are no values for roots from plants in the solid medium). Unlike Fe, shoot Mn was considerably higher in plants grown at the low pH (ca. 5) of the liquid medium (0.58 µmol g^{-1} dry weight or 32 µg g^{-1}DW under normoxic conditions, Table 2) than in plants grown under normoxic (drained) conditions at the higher pH (ca. 8) of the solid medium (0.05 µmol g^{-1} dry weight or 3 µg g^{-1}dry weight, Fig 2). However, hypoxia increased the concentration by 40% in the liquid medium (Table 2) and three fold (Fig 2) in the solid medium; the concentration of Mn in the roots of solution-grown plants increased to 12.5 times those in normoxic roots (Table 2). As the greater effect of hypoxia on growth in solid than liquid medium was associated with greater increases in Fe and Mn, the question we asked is whether the reduction in growth could be correlated with either deficiency or toxicity of iron or manganese, metals whose concentration in the soil is highly dependent on its redox status.

Plants grew optimally in 1 mM Mn (Experiment 4, Fig 6), when the shoot Mn concentration was 1.2 µmol g^{-1} dry weight (66 µg g^{-1}dry weight) and the root Mn 7.1 µmol g^{-1} dry weight (390 µg g^{-1}dry weight) in plants grown in both normoxic and hypoxic conditions; Fig 7). In neither solution-grown or mud/sand-grown plants of Experiments 1 and 2 did reducing the oxygen (and reducing the growth) supply increase the Mn concentration beyond these values: the concentration of Mn in the shoots of solution-grown plants under hypoxia was 0.81 µmol g^{-1} dry weight and 2.5 µmol g^{-1} dry weight in the roots (Table 2). In the solid medium (Experiment 2) shoot Mn was 0.15 µmol g^{-1} dry weight (Fig 2) under flooded conditions. Although we were unable to measure the Mn concentration in the roots of plants grown in the solid medium, on the basis of the root: shoot ratio of Mn in solution-grown plants this would not have been

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greater than 0.05 µmol g^{-1} dry weight. Comparison of the activities in the normoxic and hypoxic solutions and the drained and flooded soils using Visual MINTEQ did not reveal any obvious differences in activities that would have driven differences in ion uptake. The majority of Mn in the solution existed as the chloride; hypoxia increased the activity of $Mn(OH)_4^{2-}$ by 151%, but the activity in the hypoxic solution was vanishingly small at 3×10^{-32} M. Concentrations of around 1 µmol g^{-1} dry weight in the shoots or 2.5 µmol g^{-1} dry weight in the roots should not have had any negative effect on shoot dry weight (Figure 9A). Consequently, it is unlikely that the decrease in growth under hypoxia on the salt marsh would be due to Mn toxicity or deficiency. In fact the Mn concentration in the shoots of the plants grown in solid medium (0.05 µmol g^{-1} dry weight in drained and 0.15 µmol g^{-1} dry weight in flooded conditions, 2.7 and 8.4 µg g^{-1} dry weight, respectively; Figure 2) are well below those of plants growing under optimal Mn (Figure 7A) or those plants listed in Table 4 and suggest that the plants growing in solid medium could be Mn deficient. Even though hypoxia caused a dramatic increase in Mn concentration in Experiment 4, it was only at higher Mn concentrations (around 5 µmol g^{-1} dry weight and above in the shoots) that there was a reduction in shoot and root dry weight and then this was more pronounced under hypoxia than under normoxic conditions (Fig 8A).

It is clear that plant growth was more sensitive to Mn concentration under hypoxia than when root oxygen supply was more abundant. There was a strong negative relationship between shoot manganese and shoot dry weight in hypoxic conditions ($R^2 = 0.94$), and a weak negative correlation between shoot manganese and shoot dry weight in normoxic conditions ($R^2 = 0.25$; Fig 8). It is not clear why this should be, but it is notable that during Mn treatment, there was a marked difference in the shoot Fe concentration between hypoxic and normoxic treatments (Fig 7): under hypoxia, shoot Fe rose (to almost 2 µmol g^{-1} dry weight in 10 mM Mn), while there was little effect on shoot Mn between plants growing in different oxygen regimes (Fig 7A).

The Mn concentration occurring in plants is variable – between 0.5 and 9 µmol g^{-1} dry weight, accumulating predominantly in the shoots (Millaleo et al., 2010). Mn concentrations in the shoots of salt-marsh and strand line plants vary between 0.4 and 1.1 µmol g^{-1} dry weight (Table 4). Cooper (1984) found no major effects of concentrations of Mn up to 10 mM on shoot or root biomass of eight halophytes growing in saline conditions, except for *Salicornia europea* whose growth declined at 10 mM external Mn. From a review of the literature between 1975 and 2009, Kopittke et al (2010) reported Mn the least toxic of Pb, Hg, Cu, Cd, As, Co, Ni, Zn and Mn in solution culture experiments: median toxic

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concentration of Mn in the solution were 47 µM, much lower than the optimal concentration for the growth of *S. maritima* under saline conditions. In wheat it is the ratio of Mn to Mg rather than the absolute concentration of Mn that is important (Goss et al., 1992) and since the Mg activity in the artificial seawater we used was about 23 mM, this may have had a mitigating effect on any Mn toxicity (no brown specks, symptoms of Mn toxicity, were visible on the leaves; data not shown).

Hypoxia increased Fe concentration in the shoots of culture-solution grown plants to 0.8 µmol g^{-1} dry weight (45 µg g^{-1} dry weight; Table 2) and to 0.86 µmol g^{-1} dry weight in plants in the solid medium (48 µg g^{-1} dry weight; Fig 2): under both conditions growth was reduced by hypoxia and the associated increase in shoot Fe. In solution culture, additional Fe always reduced plant growth (Fig. 3), regardless of the oxygen supply: shoot Fe concentrations rose as high as 4.6 µmol g^{-1} dry weight (257 µg g^{-1} dry weight; Fig 4A). Concentrations of Fe above 1 µmol g^{-1} dry weight in the shoots were associated with a decrease in shoot dry weight in the experiment where Fe was added to the culture solution (Fig 5A). The reduced growth seen in both liquid and solid media under hypoxia (Experiments 1 and 2) is consistent with iron toxicity although Fe concentrations in the shoots of *S. maritima* were at the lower end of those tabulated for halophytes (Table 4): although low, there were no apparent deficiency symptoms.

Table 4 here

As far as we are aware the concentration of Fe causing toxicity has rarely been studied in halophytes (Rozema et al, 1985 noted that 10 mM Mn was more toxic than 1 mM Fe, but did not publish effects of either element on growth). For *Suaeda maritima*, Fe reduced growth at all added concentrations (Fig 3) and these were lower than the concentrations of Mn that reduced growth. An Fe concentration of 272 µM reduced growth of *S. maritima* in comparison with growth in the presence of 27 µM Fe in saline (340 mM NaCl) or nonsaline aerated culture solution (Hajibagheri 1984). Although the sensitivity to the Fe and Mn under hypoxia, expressed as the slopes of the regression lines relating shoot growth to concentration (g dw loss / µmol; Figures 5 and 9), were similar for the shoots, root growth was very much (almost 20 times) more sensitive to Fe than Mn. However hypoxia, whether imposed in solution culture or in the mud/sand mixture did not raise the Fe concentrations in roots or shoots to concentrations that should be toxic.

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Thus although hypoxia has dramatic effects on the mineral nutrition of *S. maritima*, there is no unequivocal case for micronutrient toxicity, although the evidence we present suggest growth reduction is consistent with iron toxicity. Hypoxia would result in an increase in the concentration of Fe^{2+} and hence availability to the plant, However, there is likely a complex of factors that reduce growth under hypoxic conditions (Irfan et al 2010) and the more so at higher than lower pH. Most likely, growth is reduced through a combination of effects related to the reduced availability of ATP under hypoxic conditions. While these plants metabolise significant quantities of lactate (Wetson et al., 2012), ATP supply is presumably still reduced from normoxic conditions. The poorer growth of plants at high pH may have resulted from neutralisation of proton efflux and hence effects on ion transport (as well as reducing growth, hypoxia reduced the Na^{+} concentration in the shoots by approximately 24%; data not shown), whether through direct effects or the integrity of the cytoplasm and the ability to maintain ion compartmentation.

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Appendix Supplementary data

Supplementary data are available at *****

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Figure Legends

Fig. 1 Shoot dry weight (left panel) and root dry weight (right panel) of *Suaeda maritima* plants after 8 weeks growth, under different levels of oxygen: normoxic nutrient solution (350 N) and stagnant agar solution (350 H) in (350 mM $Na^{+)}$ in a growth chamber. Error bars are SE (n = 14). Letters above bars indicate significant difference in means from post-hoc Tukey tests.

Figure 2 Shoot manganese and iron concentrations of *Suaeda maritima* plants (12 weeks old at harvest) grown for 8 weeks under controlled conditions in the glasshouse. Plants were grown in mud and 50% sand at two heights and irrigated with Stout and Arnon culture solution in half-strength fresh seawater. The plants in the 'drained' treatment were subjected to flooding twice daily and those in the flooded treatment were continuously submerged . Error bars SE (n = 16).

Fig. 3. Shoot (A) and root (B) dry weight of *Suaeda maritima* plants after 10 days growth, under different concentrations of iron in normoxic and hypoxic (stagnant agar) nutrient solution, in the growth chamber. Error bars are SE (n = 14); where they are not visible standard errors were smaller than the size of the symbols used. Note the different scales on the ordinates.

Fig. 4. Shoot (A and B) and root (C and D) concentrations of Fe (A and C) and Mn (B and D) in *Suaeda maritima* after 10 days growth in different concentrations of Fe, in normoxic nutrient solution and hypoxic (stagnant agar) solutions, in the growth chamber. Error bars are SE (n = 6). Note the different scales on the ordinates.

Fig. 5. Relationship between shoot (A) and root (B) Fe and shoot dry weight in *S. maritima* plants, after 10 d growth in 350 mM Na^+ artificial seawater, in varying concentrations of Fe and oxygen.

Fig. 6. The mean shoot (A) and root (B) dry weight of *Suaeda maritima* plants (7 weeks old at harvest), after 3 weeks growth in hydroponic solutions containing 350 mM Na^+ (artificial seawater and half strength Stout &

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Arnon culture solution), in varying Mn concentrations, in both oxygenated (normoxic, pre-bubbled with air) and hypoxic (stagnant agar) nutrient solutions. Error bars are SE (n = 14). Note the different scales on the ordinates.

Fig. 7. The shoot (A and B) and root (C and D) concentrations of Mn (A and C) and Fe (B and D) in *Suaeda maritima* after 3 weeks growth in hydroponic solutions containing 350 mM Na^+ (artificial seawater and half strength Stout & Arnon culture solution) and varying Mn concentrations, in both normoxic (pre-bubbled with air) and hypoxic (stagnant agar) nutrient solution. Error bars are SE (n = 6). Note the different scales on the ordinates.

Fig. 8. Relationship between shoot (A) and root (B) Mn and shoot dry weight in *S. maritima* plants, after 3 weeks growth in 350 mM Na^+ artificial seawater, in varying concentrations of Mn.

Tables

Table 1

The pH and electrical conductivity (EC) of the medium used for growing *S. maritima* plants and the oxygen concentrations before and after renewing the solutions: normoxic medium (350 N), and hypoxic medium (350 H) in artificial seawater (350 mM Na^+), in a growth chamber at 20 ^oC. Different letters indicate significant differences between means in post-hoc Tukey tests at a significance level of $P < 0.05$, (n=40).

			Treatment	
			350 N	350 H
Oxygen concentration	µM	Before*	253±3.4a	12.5±1.3b
		After*	71.9±0.3b	9.38±0.3a
EC	dS m^{-1}	Before*	41.6±0.6b	41.5±0.6b
pH		Before*	5.0±0.08a	5.2±1a

*****Before – fresh solution before use
*After – after the plants had grown in the solution for eight weeks

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Table 2 Concentrations (µmol g^{-1} DW) and contents (µmol $plant^{-1}$) of Mn and Fe in shoots and roots of *Suaeda maritima* plants after 8 weeks growth under different levels of oxygen in normoxic nutrient solution and hypoxic (stagnant agar solution) in artificial seawater (350 mM Na^+), in a growth chamber. Letters following the SE (n = 6) indicate significant difference in means from post-hoc Tukey tests.

	Concentrations (µmol g^{-1} dry weight)		Contents (µmol $plant^{-1}$)	
	Normoxic	Hypoxic	Normoxic	Hypoxic
Shoots				
Mn	0.58±0.07 a	0.81±0.04 a	458±24 a	503±17 a
Fe	0.4±0.01 a	0.8±0.02 b	316±18 a	497±32 b
Roots				
Mn	0.2±0.006 a	2.5±0.3 a	11.6±4 a	130±32 b
Fe	17±0.8 a	13±0.2 b	986±43 a	676±36 b

Table 3 Dry mass and mean redox values (Eh; mV) recorded at three depths for 30 seconds, after one hour of flooding, in growth medium composed of a mixture of sand and estuarine mud, in which *Suaeda maritima* was grown. Measurements were taken at the top, middle and base of the growth medium, in drained and flooded pots, in the glasshouse tank system. Means are ± SE (n = 18), and include the electrical conductivity (EC) and pH in the tanks, which was measured every 2 d.

Conditions	Drained	Flooded
Dry mass (g / plant)	2.1±0.02	0.7±0.04
Eh Top (mV)	- 63.3 ±3.5	- 116.6±4
Eh Middle (mV)	- 183.4±4	- 286.6±4.1
Eh Base (mV)	- 276.2±8.3	- 380.5±7.3
EC Drained and flooded (dS m^{-1}))	29.6±0.1	
pH Drained and flooded pH	8.2±0.02	

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Table 4. Mn and Fe concentrations (µmol g^{-1} dry weight) in the shoots of some halophytes.

	Fe (µmol/g dw)	Mn (µmol/g dw)	
Agrostis stolonifera	0.34	0.44	Rozema and Blum 1977
Aster tripolium	1.25, 2.87	0.73, 1.09	Gorham and Gorham 1955; Rozema et al 1985
Elytrigia pungens	3.76	0.91	Rozema et al 1985
Festuca rubra ssp. litoralis	5.01	3.09	Rozema et al 1985
Glaux maritima	9.67	0.91	Rozema et al 1985
Halimione portulacoides	3.58	2.73	Rozema et al 1985
Honkenya peploides	0.72	0.36	Gorham and Gorham 1955
Juncus gerardii	0.82	0.44	Rozema and Blum 1977
Juncus maritimus	3.94	2.00	Rozema et al 1985
Limonium binervosum	3.94	0.36	Gorham and Gorham 1955
Limonium vulgare	3.58, 3.76	0.73, 0.91	Gorham and Gorham 1955; Rozema et al 1985
Limonium humile	3.40	0.36	Gorham and Gorham 1955
Plantago maritima	3.04	1.27	Rozema et al 1985
Salicornia perennis	0.54	0.36	Gorham and Gorham 1955
Salicornia stricta	1.22	0.55	Gorham and Gorham 1955
Silene maritima	1.07	1.27	Gorham and Gorham 1955
Spartina anglica	5.01	2.18	Rozema et al 1985
Spergularia media	7.70	1.09	Rozema et al 1985
Triglochin maritima	1.79	1.46	Rozema et al 1985
Average	3.19	1.11	
Max	9.67	3.09	
Min	0.34	0.36	