Salinity alleviates zinc toxicity in the saltmarsh zinc-accumulator *Juncus acutus*

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ABTRACT

The potential importance of *Juncus acutus* for remediation of Zn-contaminated lands has been recognized, because of its Zn tolerance and capacity to accumulate Zn. Since it is also a halophyte, the extent to which salinity influences its Zn tolerance requires investigation. A factorial greenhouse experiment was designed to assess the effect of NaCl supply (0 and 85 mM NaCl) on the growth, photosynthetic physiology and tissue ions concentrations of plants exposed to 0, 30 and 100 mM Zn. Our results indicated that NaCl supplementation alleviated the effects of Zn toxicity on growth, as Zn at 100 mM reduced relative growth rate (RGR) by 60% in the absence of NaCl but by only 34% in plants treated also with NaCl. This effect was linked to a reduction in Zn tissue concentrations, as well as to overall protective effects on various stages in the photosynthetic pathway. Thus, at 85 mM NaCl plants were able to maintain higher net photosynthesis (A_N) than in the absence of added NaCl, although there were no differences in stomatal conductance (g_s). This contributed to preserving the trade-off between CO_2 acquisition and water loss, as indicated by higher intrinsic water use efficiency ($_i$WUE). Hence, A_N differences were ascribed to limitation in the RuBisCO carboxylation, manifested as higher intercellular CO_2 concentration (C_i), together with dysfunction of PSII photochemistry (in term of light harvest and energy excess dissipation), as indicated by higher chronic photoinhibition percentages and variations in the photosynthetic pigment profiles in presence of Zn under non-saline conditions.

Keywords: Chlorophyll fluorescence; Gas exchange; Halophyte; Photoinhibition; Salinity; Zn-stress.

1. Introduction

Juncus acutus L., is a caespitose, halophytic rush, with a sub-cosmopolitan distribution, that inhabits coastal marshes and dune slacks encompassing a wide range of salinity (Fernández-Carvajal, 1982). Together with various other *Juncus* species, it has been proposed as a bio-tool for wetland restoration projects around the world (Sparks et al., 2013; Marques et al., 2011). In particular, it has potential for the remediation of metal pollution, since it shows great tolerance to excess metals and the capacity to accumulate large amounts of them in its tissues without serious symptoms of toxicity (Mateos-Naranjo et al., 2014; Santos et al., 2014; Christofilopoulos et al., 2016). Medas et al. (2017) have recently suggested that *J. acutus* is able to optimize its response to metal pollution by tuning different biomineralization mechanisms with the minerals and geochemical conditions of the site. Previous studies of metal accumulation and its effects on the performance *J. acutus* have focused on zinc (Mateos-Naranjo et al., 2014; Santos et al., 2014; Christofilopoulos et al., 2016; Medas et al., 2017), although recently interactions of Zn with Cr, Ni and Cd have also been assessed (Christofilopoulos et al., 2016).

Zinc is an essential element for plant metabolism (Kabata-Pendias and Pendias, 2001). However, its excess can lead to various phytotoxicity effects on plant metabolism (Chaney, 1993), and specifically on halophytic species (Liu et at., 2016). The photosynthetic apparatus (i.e. Calvin cycle and photosystem functionality) is especially sensitive to this ion excess (Van Assche and Clijsters, 1986). Despite such potentially deleterious effects, *J. acutus* is regarded as Zn-hypertolerant, a feature attributable to a series of physiological and biochemical adaptations. In particular, Mateos-Naranjo et al. (2014) showed that carbon assimilation and the efficiency of PSII were not affected by high concentrations of Zn in the culture solution. Furthermore,

68 Santos et al. (2014) found that maintenance of the functionality of its photosynthetic
69 apparatus was linked with its ability to overcome oxidative damage produced by excess
70 Zn uptake, through the modulation of its antioxidant enzymatic machinery and efficient
71 dissipation of the cellular redox potential consequent on Zn incorporation into
72 chlorophyll molecules. These studies however, did not take account of the potential
73 interaction of Zn with other important factors characteristic of marshes ecosystems,
74 particularly salinity. It has been demonstrated that the accumulation of sodium in
75 another halophyte, *Spartina densiflora*, can mitigate its responses to Zn-induced stress
76 (Redondo-Gómez et al., 2011). Hence knowledge of the extent to which salinity might
77 modulate the physiological responses of *J. acutus* to excess Zn is necessary for a
78 realistic assessment of its metal toxicity thresholds and its potential for the remediation
79 of zinc-polluted saltmarshes.

80 This study employed a factorial experiment which aimed to: (1) investigate the
81 influence of NaCl on the growth responses of *J. acutus* plants exposed to different Zn
82 concentrations; (2) determine the extent to which this influence could be accounted for
83 by impacts on its photosynthetic apparatus, both in terms of carbon assimilation and
84 efficiency of light-energy use, and (3) assess the nutrient and Zn accumulation patterns
85 consequent on the joint effects of treatment with elevated NaCl and Zn.

86

87 **2. Material and Methods**

88 *2.1. Plant material*

89 Seeds of *Juncus acutus* were collected in December 2013 from different
90 individuals (n = 20) randomly selected from a well-established population in Doñana
91 National Park (Huelva, SW Spain). The seeds were transported to the laboratory for

92 germination in a germination chamber (ASL Aparatos Científicos M- 92004, Madrid,
93 Spain) under the following conditions: photoperiod, 16/8 h light/darkness; temperature,
94 24/15ºC; photon flux rat (400–700 nm), 35 μmol m^{-2} s^{-1}. Germinated seedlings were
95 immediately transferred to individual plastic pots (12 cm in depth, 0.5 L total volume)
96 filled with perlite and placed in a glasshouse (University of Seville, Greenhouse
97 Service) at controlled temperature of 25±3 ºC, and a relative humidity of 40-60%, with
98 natural day light (maximum quantum flux rate of 1000 μmol m^{-2} s^{-1}). Pots were irrigated
99 with nutrient solution (Hoagland and Arnon, 1938) before the onset of the experimental
100 treatments.

101

102 *2.2. Zn and NaCl experimental stress treatments*

103 In June 2014, pots containing the *J. acutus* plants were randomly assigned to
104 three Zn treatments (concentrations of 0, 30 and 100 mM) in factorial combination with
105 two NaCl concentrations (0 and 85 mM) for 40 days. Zn and NaCl concentrations were
106 established by combining Hoagland´s solution with appropriate amounts of
107 $ZnSO_4 \cdot 7H_2O$ and NaCl, respectively. Thus, at the beginning of the experiment, the pots
108 were placed in plastic trays containing appropriate solutions to a depth of 1 cm (10
109 replicate pots per stress treatment combination). In order to avoid changes of Zn and
110 NaCl concentration caused by water evaporation from the nutrient solution, levels in the
111 trays were monitored continuously throughout the experimental and topped up to the
112 marked level with Hoagland's solution (without additional $ZnSO_4 \cdot 7H_2O$ or NaCl).
113 Furthermore, pH of the solution was monitored and adjusted to 6.5 - 7.0. The entire
114 solution (including $ZnSO_4 \cdot 7H_2O$ and NaCl) in the trays was renewed weekly and their

115 positions were changed randomly every 2 days to avoid effects of environmental
116 heterogeneity inside the glasshouse.

117 After 40 days of exposure to the stress-inducing treatments, measurements of
118 growth, gas exchange, chlorophyll fluorescence, photosynthetic pigment concentrations
119 and tissue ion concentrations were made.

120

121 *2.3. Growth measurements*

122 Four plants from each treatment were harvested at the beginning of the
123 experiment and a further ten at the end. Plants were divided in roots and shoots and
124 these biomass fractions were oven dried (60ºC for 48 h) and then weighed. In addition,
125 the number of dead tillers was recorded at the end of the experiment.

126 The relative growth rate (RGR) of whole plants was calculated using the formula:

127

128 $RGR = (\ln B_f - \ln B_i) \cdot D^{-1}$ (g g^{-1} day^{-1})

129

130 where B_f = final dry mass, B_i = initial dry mass (the mean of the four plants from
131 each treatment sampled at the beginning of the experiment) and D = duration of
132 experiment (days).

133

134 *2.4. Photosynthetic physiology*

Gas exchange and chlorophyll fluorescence parameters were measured on the same sections of randomly selected, fully developed photosynthetic tillers (n = 10) using an infrared gas analyzer (LI-6400-XT, Li-COR Inc., NE., USA) and a modulated fluorimeter (FMS-2; Hansatech Instruments Ltd., UK), respectively. The following gas exchange parameters were recorded at a light flux density of 1500 µmol photons m^{-2} s^{-1}, ambient CO_2 concentration (C_a) 400 µmol mol^{-1} air, leaf temperature of 25 °C and 50 ± 5 % relative humidity: net photosynthetic rate (A_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and intrinsic water use efficiency ($_i$WUE). The saturation pulse method was used to determine the energy yields of the Photosystem II (PSII) reaction centers: maximum quantum efficiency of PSII photochemistry (F_v/F_m), quantum efficiency of PSII (Φ_{PSII}; Genty et al., 1989) and non-photochemical quenching (NPQ). As described by Schreiber et al. (1986), a 0.8 s saturating actinic light pulse of 15000 µmol m^{-2} s^{-1} was given, at dawn (stable, 50 µmol m^{-2} s^{-1} ambient light) and midday (1700 µmol photons m^{-2} s^{-1}), to photosynthetic tillers previously dark-adapted or exposed to light for 30 min.

Finally, the total chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoid (C*x*+*c*) contents of extracts obtained from randomly selected fully developed photosynthetic tillers (n = 5), were determined with a Hitachi U-2001 spectrophotometer (Hitachi Ltd., Japan), using three wavelengths (663.2, 646.8 and 470.0 nm). For more details, see Mateos-Naranjo et al. (2008). Concentrations of pigments (µg g^{-1}fw) were calculated according to Lichtenthaler (1987).

2.5. Tissue ion concentrations

Tiller and root samples taken from ten plants per treatment were dried at 80ºC for 48 h and ground, according to the protocols of Mateos-Naranjo et al. (2011). Then,

160 triplicate 0.5 g samples from each specific tissue were digested in 6 ml HNO3, 0.5 ml
161 HF and 1 ml H_2O_2. Ca, Mg, K, P, Na and Zn concentrations in the digests were
162 measured by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, USA).

163 *2.6. Statistical analysis*

164 Statistical tests were performed in the software package Statistica v. 6.0 (Statsoft
165 Inc.). Generalized linear models (GLM) were used to analyze the interactive effects of
166 Zn and NaCl concentrations (as categorical factors) on the growth and physiological
167 parameters (as dependent variables) of *J. acutus* plants. Multiple comparisons were
168 analyzed by a LSD (post hoc) test. Before statistical analysis Kolmogorov-Smirnov and
169 Brown-Forsythe tests were used to verify the assumptions of normality and
170 homogeneity of variances, respectively. Differences between tiller and root ion
171 concentrations were compared by the Student test (t-test).

172

173 **3. Results**

174 *3.1. Effects of Zn and NaCl on growth*

175 There were significant effects of both zinc and salinity on the RGR of *Juncus*
176 *acutus* but no significant interactions (Table 1, GLM: salinity, $p < 0.05$; Zn, $p < 0.01$).
177 Thus, in non-saline conditions RGR decreased 25% and 60% in plants grown at 30 and
178 100 mM Zn, respectively, compared to control; however, growth was much less
179 affected by Zn in plants exposed to 85 mM NaCl (i.e. 11% and 34% for 30 and 100 mM
180 Zn, respectively; Fig. 1A). Similarly, the percentage of dead tillers increased sharply
181 with Zn concentration (GLM: Zn, $p < 0.01$), but this increase was less acute in plants
182 grown in saline conditions (GLM: salinity, $p = 0.07$; Fig. 1B).

183

184 *3.2. Effects of Zn and NaCl on photosynthetic physiology*

185 There were significant effects of salinity and Zn treatments on net photosynthetic
186 rate (A_N) after 40 d of treatment (Table 1, GLM: salinity, $p < 0.05$; Zn, $p < 0.01$ and
187 salinity x Zn, $p < 0.01$). Thus A_N decreased progressively with increasing Zn
188 concentration in plants grown at both NaCl concentrations. However, plants exposed to
189 saline conditions maintained higher CO_2 assimilation rates at both increased
190 concentrations of Zn than their non-saline counterparts (Fig. 2A). Very similar trends
191 were recorded for stomatal conductance (g_s) but salinity did not significantly affect the
192 responses to Zn (GLM: salinity x Zn, $p = 0.06$; Fig. 2B). In contrast, salinity
193 significantly reduced the intercellular CO_2 concentration (C_i) (GLM: salinity, $p < 0.05$),
194 whereas Zn concentration per se did not. However, C_i values were reduced at the high
195 salinity only in the presence of excess (30 or 100 mM) Zn (Fig. 2C). Salinity and Zn
196 had synergistic effects on intrinsic water use efficiency ($_i$WUE; GLM: salinity x Zn, $p <$
197 0.05). Thus, plants grown under saline conditions had consistently higher $_i$WUE but the
198 difference was only significant at 30 mM Zn (Fig. 2D).

199 Chlorophyll fluorescence parameters were also affected by the combination of Zn
200 and salinity treatments. F_v/F_m values, both at dawn and midday, tended to decrease with
201 increasing Zn concentration in plants grown in non-saline conditions. However, in
202 plants exposed to salinity, this effect was less marked and only evident at the highest Zn
203 concentration treatment (Table 1,$GLM_{Md\ and\ Pd}$: salinity x Zn, $p < 0.05$; Fig. 3A, B). Φ_{PSII}
204 values at dawn and at midday followed a similar pattern to those of F_v/F_m (GLM_{Md}:
205 salinity x Zn, $p < 0.05$; Fig. 3C,D), except that the differences in predawn values were
206 minimal. NPQ values at midday increased markedly with Zn concentration, both in the
207 absence and presence of salinity, but this effect was substantially stronger in the absence

208 of salinity (Table 1, GLM_{Md}: salinity, $p < 0.01$ and Zn, $p < 0.001$; Fig. 3E). Predawn
209 NPQ did not show any response to Zn or salinity, with values c. 0.15 in all cases (Fig.
210 3F).
211 The percentage of chronic photoinhibition increased progressively with increasing
212 Zn concentration at both NaCl concentrations (Fig. 4A,B). However, this increment was
213 more acute in plants grown under non-saline conditions. The percentage of dynamic
214 photoinhition did not vary with salinity or Zn treatments, except in plants grown at the
215 highest Zn concentration and 85 mM NaCl, which showed a greater percentage
216 inhibition than in the other treatments (Fig. 4A,B).
217 The concentration of chlorophyll a (Chl *a*) was decreased by excess Zn in the
218 growth medium, although this reduction was entirely mitigated by salinity (Table 1,
219 GLM: salinity x Zn, $p < 0.01$; Fig. 5A). Chlorophyll b (Chl *b*) and carotenoid (C*x*+*c*)
220 concentrations did not show any response to excess Zn in plants grown in the absence of
221 salinity, but they increased in those exposed to both Zn and salinity ($GLM_{Chl\ b\ and\ Cx+c}$:
222 salinity x Zn, $p < 0.01$; Fig. 5B,C).
223
224 *3.3. Effects of Zn and NaCl on tissue ion concentrations*
225 Tissue ion concentrations were greater in roots than in tillers, except for K in all
226 specific treatments and for P in plants grown at 100 mM Zn + 0 mM NaCl, 0 mM Zn +
227 85 mM NaCl and 30 mM Zn + 85 mM NaCl, (t-test, $p < 0.05$; Table 2). In addition,
228 there were significant effects of salinity and Zn treatments on tissue ion concentrations
229 except for K and Mn tiller concentrations (Table 1). Thus Zn concentrations increased
230 markedly with the concentration of Zn in the growth medium in both roots and tillers,
231 but this increment was more acute in the absence of NaCl addition (GLM: salinity x Zn,
232 $p < 0.01$; Table 2). Furthermore, tissue Na concentrations were considerably greater

under saline conditions and tended to increase with the Zn concentration. Except for roots in presence of NaCl, where Na concentration showed a reduction with Zn augmentation (GLM: salinity x Zn, $p < 0.01$; Table 2). On the other hand, overall the concentrations of Mg, Ca, P and Mn in tillers and roots, and K in roots decreased with the increase of the concentration of Zn in the growth medium at both saline levels (Table 2). In general, the concentrations of these elements were significantly lower in plants grown with NaCl supplementation (Table 2).

4. Discussion

Understanding the effects of high metal concentrations on tolerant species and the thresholds for phytotoxicity is essential for the design and development of effective methodologies for environmental remediation. Similarly important is knowledge of possible interactions between metals, and between metals and other important environmental factors that may limit species distribution; in estuarine ecosystems interactions with salinity are relevant to the future use of halophytes that can cope with the growing problem of metal pollution of salinized lands (Kholodova et al., 2010).

This experiment confirmed previous work that had demonstrated hypertolerance to Zn stress in *Juncus acutus* (Mateos-Naranjo et al., 2014). Thus, the concentration of Zn required to kill 50% of its tillers after 40 days of exposure (LC_{50}; Paschke et al., 2000) was greater than our most severe treatment of 100 mM. However, elevated concentrations of Zn in the culture solution progressively affected plant development, and this was particularly reflected in a clear reduction of RGR and an increase in the percentage of dead tillers. These deleterious effects are consistent with previously described general responses of vascular plants to excess Zn (Vaillant et al., 2005; Mateos-Naranjo et al., 2008; Santos et al., 2014). Nevertheless, we found that Zn

toxicity was partially counterbalanced by addition of NaCl to the growth medium, such that salinity-treated plants were able to maintain a higher RGR than their non-salinity treated counterparts. In addition, they reduced toxicity, as indicated by lower percentages of dead tillers at both 30 and 100 mM Zn. Therefore, the results suggest that salinity increases the tolerance of *J. acutus* to the toxic effects of high concentrations of Zn. This interaction is consistent with results for species not recognized as hypertolerant to Zn: Redondo-Gómez et al. (2011) demonstrated that the addition 170 mM NaCl to a growth medium with 1 mM Zn diminished the damage caused by metal excess in *Spartina densiflora*, and Han et al. (2013) reported similar amelioration of the effects of 100 μM Zn by the addition of 50 mM NaCl to the growth medium with in *Kosteletzkya virginica*.

The mechanisms by which NaCl supplementation could enhance plant tolerance to elevated metal concentrations are not clear. Effects on metal uptake and translocation, and the resulting nutrient uptake balance have been described in certain estuarine species (Fitzgerald et al., 2003; Kadukova and Kalogerakis, 2007; Han et al., 2013). Redondo-Gómez et al. (2011) found that NaCl supplementation increased Zn accumulation in *S. densiflora* tissues compared with non-salinized plants, but this was accompanied by an overall improvement in nutrient uptake. Similar modifications in mineral content were recorded in *Kosteletzkya virginica* tissues in response to salinity and Zn (Han et al. 2013), but in that case NaCl addition acted through a modification of Zn distribution rather than a decrease in plant Zn uptake capacity. In contrast, we found that although tissues Zn concentrations in *J. acutus* increased markedly with the external concentration in accordance with previous studies, this increase was progressively lower as tissue Na concentration increased in response to NaCl supplementation. Furthermore, salinity hindered the uptake of most nutrients in the

283 highest Zn concentration. These discrepancies may be ascribed to the severity of stress
284 imposed, since a maximum concentration of 100 mM Zn was used in the present study
285 whereas Redondo-Gómez et al. (2011) and Han et al. (2013) used only 1 mM and 100
286 μM, respectively. Reduced nutrient concentrations with the progressive accumulation of
287 Na in roots and shoots have been found previously in other halophytes (Redondo-
288 Gómez et al., 2007, 2010).

289 Notwithstanding the nutritional imbalance induced by Na accumulation, the
290 lower concentrations of Zn in the tissues of plants grown in the presence of NaCl could
291 help to explain their higher tolerance. Excess Zn accumulated in the tissues is likely to
292 be toxic, affecting a variety of physiological and biochemical processes (Kabata-
293 Pendias and Pendias, 2001). However, despite such reductions in tissue Zn
294 concentration in *J. acutus*, it must be acknowledged that concentrations were still
295 greater than the toxicity threshold for plants generally (Kabata-Pendias and Pendias,
296 2001). Consequently, other mechanisms must be involved in the ameliorative effect of
297 NaCl on Zn toxicity in *J. acutus*.

298 Metal hypertolerance has been associated with various ecophysiological
299 adaptations to metalliferous environments (Evangelou et al., 2004; Mateos-Naranjo et
300 al., 2014; Santos et al., 2014). In particular, Mateos-Naranjo et al. (2014) indicated that
301 Zn hypertolerance in *J. acutus* was linked with its capacity to maintain carbon
302 assimilation and the efficiency of PSII even at Zn concentration of 100 mM. In contrast
303 we found a clear deleterious effect of Zn at this concentration on the photosynthetic
304 apparatus in the present experiment; this discrepancy may be attributable to different
305 experimental and measurement conditions. Although A_N (along with g_s) decreased
306 considerably with increasing Zn concentration, plants grown at 85 mM NaCl were able
307 to maintain higher A_N values than their non-saline counterparts. However, this positive

effect cannot be attributed to alleviation of stomatal limitation, since g_s values did not vary between salinity levels in either Zn treatment. Therefore, differences in A_N value between NaCl levels et each specific Zn concentration treatment could be explained by non-stomatal limitations (Flexas and Medrano, 2002). In this regard, Perez-Romero et al. (2016) found that photosynthesis activity was more limited by mesophyll conductance (g_m) than g_s in *Salicornia ramossisima* in response to Cd. Moreover, g_m has been widely implicated in photosynthetic responses patterns to salinity (Flexas et al., 2012). Hence, it is possible that A_N differences between salinity levels in *J. acutus* plants at the same Zn concentration could be linked with g_m variations; however this area requires further research. Another possibility relates to impairment of major carbon-assimilation enzyme activities, such as RuBisCO that may degrade the photosynthetic pathway under metal stress (Perfus-Barbeoch et al., 2002; Khan and Khan, 2014). A degree of metal tolerance has been demonstrated in the maintenance such enzyme functions (Ying et al., 2010; Pérez-Romero et al., 2016). Taking into account these issues, the higher C_i in *J. acutus* plants grown without NaCl addition suggests that differences in carbon assimilation between salinity treatments could have been linked to limitation in RuBisCO carboxylation capacity (Mateos-Naranjo et al., 2008, 2014).

On the other hand, the greatest photosynthetic tolerance to Zn-induced stress under saline conditions was associated with the highest integrity and functionality of the photochemical apparatus of *J. acutus*. It is known that Zn is concentrated in chloroplasts and interacts with the PSII donor, inhibiting the photosynthetic fixation of CO_2 and the Hill reaction (Prasad and Strzalka, 1999). In addition, Monnet et al. (2001) indicated that the destruction of antenna pigments would affect the efficiency of PSII. Our results revealed that F_v/F_m and Φ_{PSII} values were affected by elevated Zn and this effect was

more acute in plants grown in absence of NaCl, suggesting that NaCl alleviates Zn-induced, excess-light photoinhibition. Furthermore, under non-saline conditions and in presence of Zn, NPQ values were higher, which indicates that more of the absorbed energy would have been dissipated as heat and would not taken the photochemical pathway (Flexas et al., 2012). In line with our results, Padinha et al. (2000) and Mateos-Naranjo et al. (2008) also found that Zn stress affected the PSII photochemistry of the halophytes *Spartina maritima* and *S. densiflora*, respectively. Damage to photosynthetic components may lead to an increase of photoinhibition (Werner et al., 2002), a phenomenon that affects photosynthetic productivity and, consequently, plant growth (Melis, 1999). This fact could contribute to explaining our growth data, since chronic photoinhibition percentage increased in presence of Zn under non-saline conditions, whereas this increased photoinhibition was ameliorated under saline conditions, although less so in plants exposed to 100 mM Zn. However, these plants showed a greater dynamic photoinhition percentage compared to other treatments, which would indicate an overcompensation effect of the excess of energy fixed, through thermal dissipation mechanisms, thereby protecting the leaf from light-induced damage (Maxwell and Johnson, 2000). In addition, the benefit of NaCl supplementation to photosynthetic-pigment concentration in the presence of Zn could contribute to explaining its positive effects on the photosynthetic apparatus efficiency of *J. acutus.*

Finally the greater tolerance to Zn in plants treated with NaCl was linked with a better water balance, an idea supported by the overall higher $_{i}$WUE values. Thus, these plants would be able better to preserve the trade-off between CO_2 acquisition for growth and water loss, as indicated the higher A_N and the invariable g_s values compared with their counterparts not treated with NaCl. Han et al. (2013) also found a positive effect of NaCl supplementation on water relations, in *Kosteletzkya virginica,* under Zn excess.

358 This beneficial effect could be linked with the key role of Na accumulation in plant
359 osmotic adjustment (Shabala et al., 2009). Hence, it is possible that the higher Na
360 concentration in tissues of *J. acutus* under saline conditions and the reduction in g_s in
361 the presence of Zn might help to alleviate any water stress ascribed to Zn toxicity.

362

363 **5. Conclusions**

364 We may conclude that the presence of NaCl in the growth medium, at
365 concentrations representative of estuarine environments, considerably reduces the
366 deleterious effects of elevated Zn concentrations on the growth and development of *J.*
367 *acutus*. This beneficial effect was largely mediated by the reduction of Zn levels in *J.*
368 *acutus* tissues, together with an overall protective effect on its photosynthetic apparatus,
369 manifested as improved carbon harvesting, functionality of the photochemical apparatus
370 (PSII) and photosynthetic pigment concentrations. Furthermore, amelioration by NaCl
371 was linked with the maintenance of a more advantageous water balance. These
372 ecophysiological characteristics would enhance the fitness and competitive ability of *J.*
373 *acutus* in zinc-polluted estuaries and saltmarshes, providing a tolerant bio-tool for the
374 management and restoration metal pollution in salinized lands.

375

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501

Table 1. Generalized linear model (GLM) results for the growth, physiological and tissues ions concentration of *J. acutus* plants in response to Zn and NaCl concentration (as categorical variables) and its interaction. * Significance level 95% and ** Significance level 99%. Md (midday), Pd (predawn), T (tiller and R (root).

Parameter	*Na*	*Zn*	*Na x Zn*
RGR	**0.03***	**0.00****	0.06
Dead Tillers	0.07	**0.00****	0.19
A_N	**0.02***	**0.00****	**0.01***
g_s	0.30	**0.00****	0.06
C_i	**0.04***	0.05	0.22
$_i$WUE	**0.02***	**0.00****	**0.03***
$F_v/F_{m,\ Md}$	**0.00***	**0.00****	**0.02***
$F_v/F_{m,\ Pd}$	**0.00****	**0.00****	**0.02***
$\Phi_{PSII,\ Md}$	0.09	**0.00****	**0.02***
$\Phi_{PSII,\ Pd}$	**0.01****	**0.00****	0.06
$NPQ_{,\ Md}$	**0.01****	**0.00****	0.10
$NPQ_{,\ Pd}$	0.63	0.51	0.62
Chl *a*	**0.00****	0.88	**0.00****
Chl *b*	0.06	0.57	**0.04***
C*x*+*c*	**0.02***	0.22	**0.04***
$[Zn]_T$	**0.00****	**0.00****	**0.00****
$[Zn]_R$	**0.00****	**0.00****	**0.00****
$[Na]_T$	**0.00****	**0.00****	**0.00****
$[Na]_R$	**0.00****	**0.00****	**0.00****
$[K]_T$	0.95	0.58	0.47
$[K]_R$	**0.00****	**0.00****	**0.00****
$[Mg]_T$	**0.00****	**0.00****	**0.00****
$[Mg]_R$	**0.00****	**0.00****	**0.00****
$[Ca]_T$	**0.00****	**0.00****	**0.00****
$[Ca]_R$	**0.00****	**0.00****	**0.00****
$[P]_T$	**0.02***	**0.00****	**0.04***
$[P]_R$	**0.00****	**0.00****	**0.01****
$[Mn]_T$	0.78	**0.00****	0.88
$[Mn]_R$	**0.00****	**0.00****	**0.00****

Table 2. Ion concentration in tiller and roots of *Juncus acutus* treated with a range of Zn concentration in combination with 0 mM and 85 mM NaCl, after 40 days. Values represent mean ± SE, n = 5.

Treatments								
		Tiller concentration						
Zn (mM)	**NaCl (mM)**	**Zn (mg Kg^{-1})**	**Na (mg g^{-1})**	**K (mg g^{-1})**	**Mg (mg g^{-1})**	**Ca (mg g^{-1})**	**P (mg g^{-1})**	**Mn (mg Kg^{-1})**
0	**0**	32.3 ± 0.5^{a}	0.97 ± 0.1^{a}	29.9 ± 0.1^{a}	3.54 ± 0.3^{a}	5.89 ± 0.1^{a}	2.95 ± 0.2^{a}	35.5 ± 0.6^{a}
30	**0**	304.6 ± 1.4^{b}	1.84 ± 0.2^{b}	29.4 ± 0.3^{a}	3.19 ± 0.2^{a}	4.97 ± 0.2^{b}	2.39 ± 0.1^{b}	27.3 ± 0.3^{b}
100	**0**	611.7 ± 0.8^{c}	3.65 ± 0.2^{c}	30.2 ± 0.1^{a}	3.04 ± 0.1^{a}	4.09 ± 0.2^{c}	2.50 ± 0.2^{b}	23.9 ± 0.4^{c}
0	**85**	36.9 ± 0.6^{a}	7.75 ± 0.5^{d}	28.9 ± 0.5^{a}	3.31 ± 0.3^{a}	4.20 ± 0.2^{c}	2.89 ± 0.5^{a}	32.7 ± 0.2^{a}
30	**85**	248.5 ± 0.5^{d}	6.96 ± 0.1^{e}	29.7 ± 0.2^{a}	3.06 ± 0.2^{a}	3.85 ± 0.4^{c}	2.82 ± 0.2^{a}	27.3 ± 0.4^{b}
100	**85**	412.3 ± 1.1^{e}	8.41 ± 0.3^{d}	30.9 ± 0.3^{a}	2.83 ± 0.1^{b}	3.71 ± 0.3^{c}	2.52 ± 0.3^{b}	22.9 ± 0.1^{c}
		Root concentration						
0	**0**	87.3 ± 0.7^{a}	1.48 ± 0.1^{a}	28.9 ± 0.2^{a}	5.48 ± 0.2^{a}	15.84 ± 0.5^{a}	3.36 ± 0.5^{ab}	39.3 ± 0.2^{a}
30	**0**	2122.6 ± 1.3^{b}	2.92 ± 0.2^{b}	24.5 ± 0.3^{b}	4.60 ± 0.2^{a}	17.36 ± 0.2^{a}	4.25 ± 0.5^{a}	39.9 ± 0.4^{a}
100	**0**	2479.0 ± 0.3^{c}	5.78 ± 0.4^{c}	16.8 ± 0.4^{c}	4.07 ± 0.1^{b}	8.54 ± 0.2^{b}	2.39 ± 0.2^{b}	33.5 ± 0.1^{b}
0	**85**	58.4 ± 1.2^{d}	21.17 ± 0.2^{d}	20.2 ± 0.4^{b}	4.87 ± 0.2^{a}	13.27 ± 0.6^{a}	2.63 ± 0.1^{b}	33.5 ± 0.1^{b}
30	**85**	1455.4 ± 2.2^{e}	16.14 ± 0.3^{e}	20.5 ± 0.2^{b}	3.62 ± 0.1^{b}	8.67 ± 0.2^{b}	2.71 ± 0.1^{b}	27.5 ± 0.2^{c}
100	**85**	1969.2 ± 1.1^{f}	13.95 ± 0.4^{f}	17.3 ± 0.3^{c}	3.41 ± 0.2^{c}	4.89 ± 0.1^{c}	2.67 ± 0.1^{b}	26.4 ± 0.2^{c}

Different letters indicate means that are significantly different from each other

Figure legends

Fig. 1. Relative growth rate, RGR (A) and percentage of dead tillers (B) in *Juncus acutus* plants in response to a treatment with a range of Zn concentration with (●) and without (○) NaCl addition, after 40 days. Values represent mean ± SE, n = 10. Different letters indicate means that are significantly different from each other (LSD test, $P < 0.05$).

Fig. 2. Net photosynthetic rate, A_N (A), stomatal conductance, g_s (B), intercellular CO_2 concentration, C_i (C), and intrinsic water use efficiency, $_i$WUE (D) in randomly selected, fully developed photosynthetic tiller of *Juncus acutus* treated with a range of Zn concentration with (●) and without (○) NaCl addition, after 40 days. Values represent mean ± SE, n = 10. Different letters indicate means that are significantly different from each other (LSD test, $P < 0.05$).

Fig. 3. Maximum quantum efficiency of PSII photochemistry, F_v/F_m (A,B), quantum efficiency of PSII, ΦPSII (B,C), and non-photochemical quenching, NPQ (D,E), at midday and predawn in randomly selected, fully developed photosynthetic tiller of *Juncus acutus* treated with a range of Zn concentration with (●) and without (○) NaCl addition, after 40 days. Values represent mean ± SE, n = 10. Different letters indicate means that are significantly different from each other (LSD test, $P < 0.05$).

Fig. 4. Total chronic and (●) and dynamic (○) photoinhibition percentage in randomly selected, fully developed photosynthetic tiller of *Juncus acutus* treated with a range of Zn concentration at 0 mM (A) and 85 mM (B) NaCl concentration, after 40 days. Values represent absolute percentage per each specific treatment.

Fig. 5. Chlorophyll a, Chl *a* (A), chlorophyll b, Chl *b* (B) and carotenoids, C*x*+*c* (C) concentrations in randomly selected, fully developed photosynthetic tiller of *Juncus acutus* treated with a range of Zn concentration with (●) and without (○) NaCl addition, after 40 days. Values represent mean ± SE, n = 5. Different letters indicate means that are significantly different from each other (LSD test, $P < 0.05$).

Fig. 1

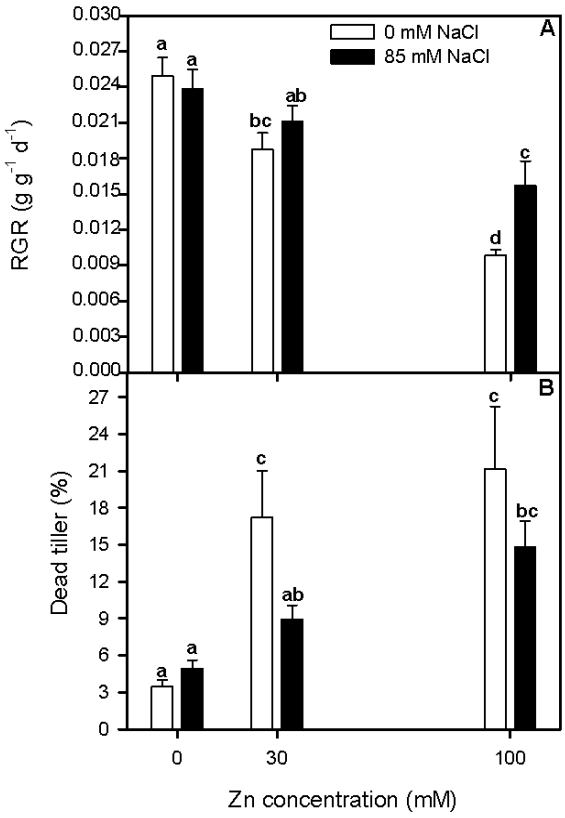

A
0 mM NaCl
85 mM NaCl
RGR (g g-1 d-1)
0.030
0.027
0.024
0.021
0.018
0.015
0.012
0.009
0.006
0.003
0.000
a
a
bc
ab
d
c
B
Dead tiller (%)
27
24
21
18
15
12
9
6
3
0
a
a
c
ab
c
bc
0
30
100
Zn concentration (mM)

Fig. 2

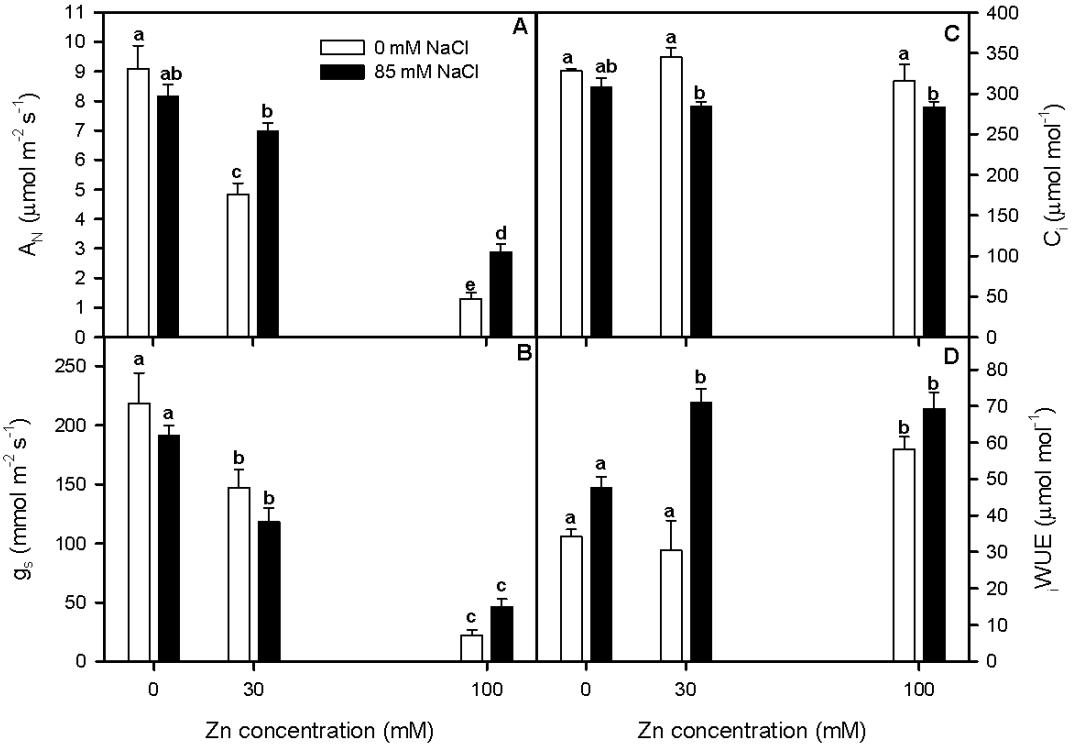

A
B
C
D
0 mM NaCl
85 mM NaCl
A_N (μmol m^{-2} s^{-1})
g_s (mmol m^{-2} s^{-1})
C_i (μmol mol^{-1})
$_i$WUE (μmol mol^{-1})
Zn concentration (mM)
Zn concentration (mM)

Fig. 3

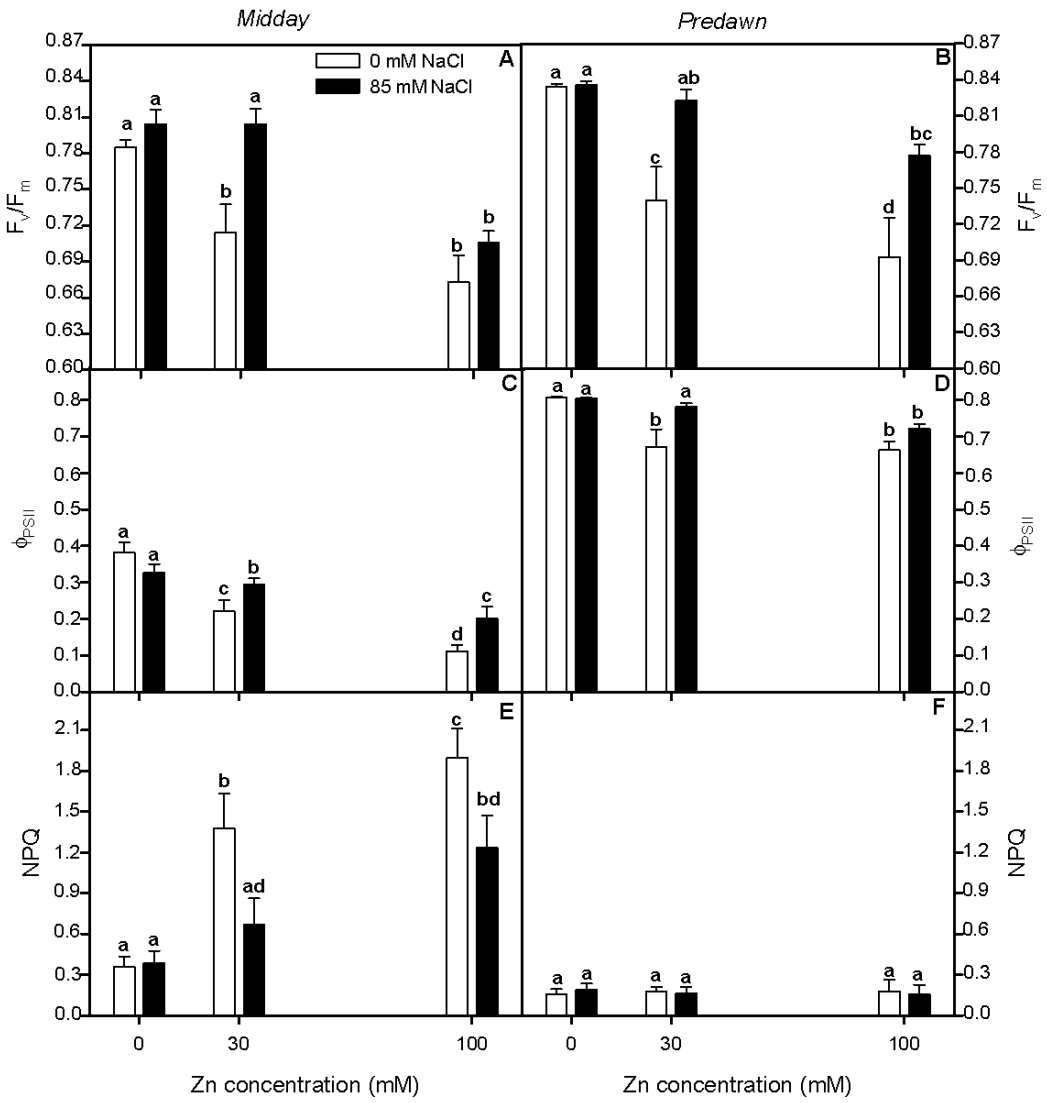

Midday
Predawn
0 mM NaCl
85 mM NaCl
A
B
C
D
E
F
F_v/F_m
ϕ_{PSII}
NPQ
Zn concentration (mM)
Zn concentration (mM)

Fig. 4

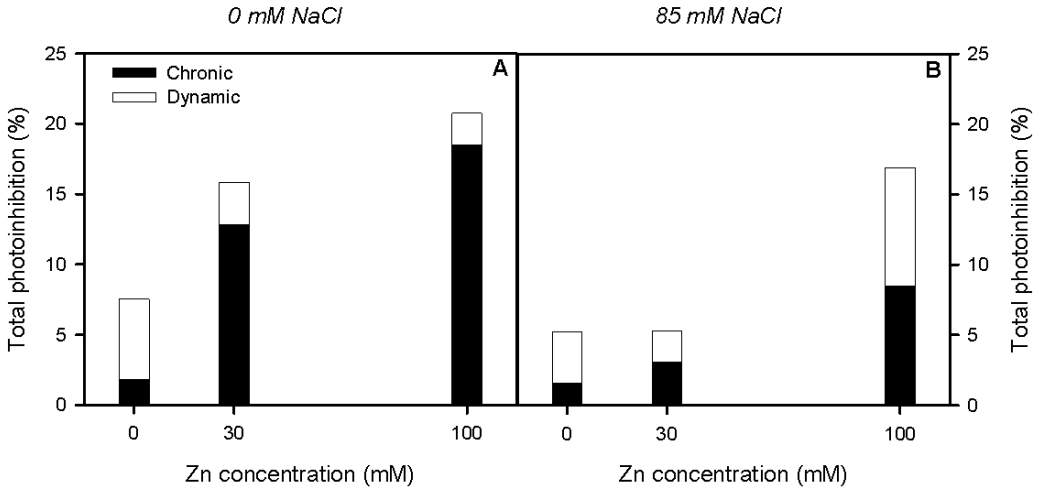
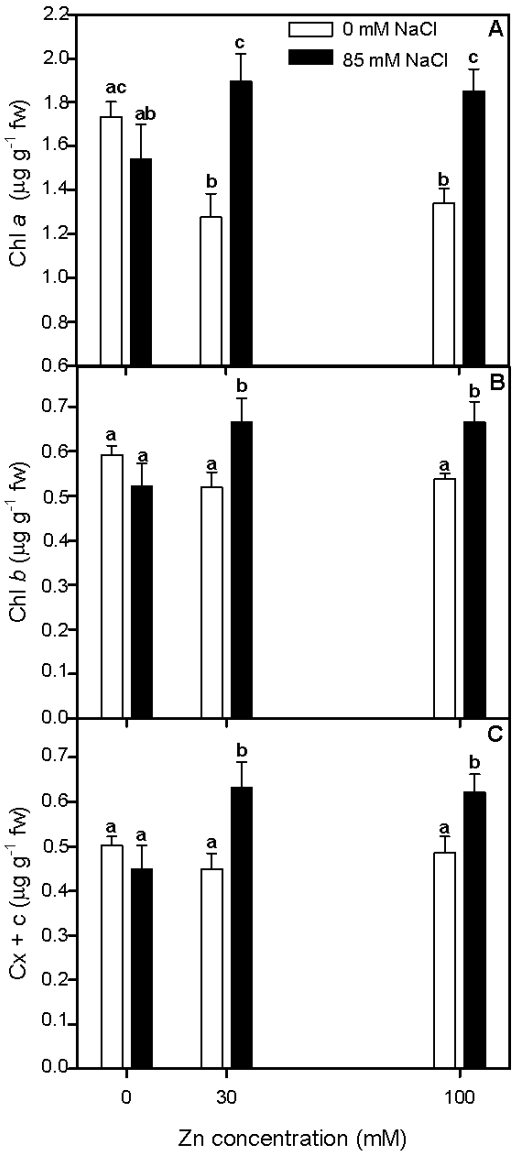

0 mM NaCl
85 mM NaCl
Chronic
Dynamic
A
B
Total photoinhibition (%)
Total photoinhibition (%)
0
5
10
15
20
25
0
30
100
Zn concentration (mM)
Zn concentration (mM)

Fig. 5


0 mM NaCl
85 mM NaCl
A
B
C
Chl a (µg g-1 fw)
Chl b (µg g-1 fw)
Cx + c (µg g-1 fw)
ac
ab
b
c
a
0
30
100
Zn concentration (mM)