

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

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19 ABTRACT

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

40

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

## 43 1. Introduction

44 *Juncus acutus* L., is a caespitose, halophytic rush, with a sub-cosmopolitan  
45 distribution, that inhabits coastal marshes and dune slacks encompassing a wide range  
46 of salinity (Fernández-Carvajal, 1982). Together with various other *Juncus* species, it  
47 has been proposed as a bio-tool for wetland restoration projects around the world  
48 (Sparks et al., 2013; Marques et al., 2011). In particular, it has potential for the  
49 remediation of metal pollution, since it shows great tolerance to excess metals and the  
50 capacity to accumulate large amounts of them in its tissues without serious symptoms of  
51 toxicity (Mateos-Naranjo et al., 2014; Santos et al., 2014; Christofilopoulos et al.,  
52 2016). Medas et al. (2017) have recently suggested that *J. acutus* is able to optimize its  
53 response to metal pollution by tuning different biomineralization mechanisms with the  
54 minerals and geochemical conditions of the site. Previous studies of metal accumulation  
55 and its effects on the performance *J. acutus* have focused on zinc (Mateos-Naranjo et  
56 al., 2014; Santos et al., 2014; Christofilopoulos et al., 2016; Medas et al., 2017),  
57 although recently interactions of Zn with Cr, Ni and Cd have also been assessed  
58 (Christofilopoulos et al., 2016).

59 Zinc is an essential element for plant metabolism (Kabata-Pendias and Pendias,  
60 2001). However, its excess can lead to various phytotoxicity effects on plant  
61 metabolism (Chaney, 1993), and specifically on halophytic species (Liu et al., 2016).  
62 The photosynthetic apparatus (i.e. Calvin cycle and photosystem functionality) is  
63 especially sensitive to this ion excess (Van Assche and Clijsters, 1986). Despite such  
64 potentially deleterious effects, *J. acutus* is regarded as Zn-hypertolerant, a feature  
65 attributable to a series of physiological and biochemical adaptations. In particular,  
66 Mateos-Naranjo et al. (2014) showed that carbon assimilation and the efficiency of PSII  
67 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 24/15°C; photon flux rat (400–700 nm), 35  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . 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  $25\pm 3$  °C, and a relative humidity of 40-60%, with  
98 natural day light (maximum quantum flux rate of 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). 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  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  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  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  or NaCl).  
113 Furthermore, pH of the solution was monitored and adjusted to 6.5 - 7.0. The entire  
114 solution (including  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  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 (60°C 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

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

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*

135 Gas exchange and chlorophyll fluorescence parameters were measured on the  
136 same sections of randomly selected, fully developed photosynthetic tillers ( $n = 10$ )  
137 using an infrared gas analyzer (LI-6400-XT, Li-COR Inc., NE., USA) and a modulated  
138 fluorimeter (FMS-2; Hansatech Instruments Ltd., UK), respectively. The following gas  
139 exchange parameters were recorded at a light flux density of  $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ,  
140 ambient  $\text{CO}_2$  concentration ( $C_a$ )  $400 \mu\text{mol mol}^{-1}$  air, leaf temperature of  $25 \text{ }^\circ\text{C}$  and  $50$   
141  $\pm 5 \%$  relative humidity: net photosynthetic rate ( $A_N$ ), stomatal conductance ( $g_s$ ),  
142 intercellular  $\text{CO}_2$  concentration ( $C_i$ ), and intrinsic water use efficiency ( $i\text{WUE}$ ). The  
143 saturation pulse method was used to determine the energy yields of the Photosystem II  
144 (PSII) reaction centers: maximum quantum efficiency of PSII photochemistry ( $F_v/F_m$ ),  
145 quantum efficiency of PSII ( $\Phi_{\text{PSII}}$ ; Genty et al., 1989) and non-photochemical  
146 quenching (NPQ). As described by Schreiber et al. (1986), a  $0.8 \text{ s}$  saturating actinic  
147 light pulse of  $15000 \mu\text{mol m}^{-2} \text{s}^{-1}$  was given, at dawn (stable,  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  ambient  
148 light) and midday ( $1700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), to photosynthetic tillers previously dark-  
149 adapted or exposed to light for  $30 \text{ min}$ .

150 Finally, the total chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoid  
151 ( $C_x+c$ ) contents of extracts obtained from randomly selected fully developed  
152 photosynthetic tillers ( $n = 5$ ), were determined with a Hitachi U-2001  
153 spectrophotometer (Hitachi Ltd., Japan), using three wavelengths ( $663.2$ ,  $646.8$  and  
154  $470.0 \text{ nm}$ ). For more details, see Mateos-Naranjo et al. (2008). Concentrations of  
155 pigments ( $\mu\text{g g}^{-1}\text{fw}$ ) were calculated according to Lichtenthaler (1987).

156

### 157 *2.5. Tissue ion concentrations*

158 Tiller and root samples taken from ten plants per treatment were dried at  $80^\circ\text{C}$  for  
159  $48 \text{ h}$  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 HNO<sub>3</sub>, 0.5 ml  
161 HF and 1 ml H<sub>2</sub>O<sub>2</sub>. 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 ( $iWUE$ ; GLM: salinity x Zn,  $p <$   
197  $0.05$ ). Thus, plants grown under saline conditions had consistently higher  $iWUE$  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<sub>Md and Pd</sub>: salinity x Zn,  $p < 0.05$ ; Fig. 3A, B).  $\Phi_{PSII}$   
204 values at dawn and at midday followed a similar pattern to those of  $F_v/F_m$  (GLM<sub>Md</sub>:  
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<sub>Md</sub>: 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 photoinhibition 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<sub>Chl *b* and  $C_{x+c}$</sub> :  
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

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

240

#### 241 **4. Discussion**

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

249 This experiment confirmed previous work that had demonstrated hypertolerance  
250 to Zn stress in *Juncus acutus* (Mateos-Naranjo et al., 2014). Thus, the concentration of  
251 Zn required to kill 50% of its tillers after 40 days of exposure ( $LC_{50}$ ; Paschke et al.,  
252 2000) was greater than our most severe treatment of 100 mM. However, elevated  
253 concentrations of Zn in the culture solution progressively affected plant development,  
254 and this was particularly reflected in a clear reduction of RGR and an increase in the  
255 percentage of dead tillers. These deleterious effects are consistent with previously  
256 described general responses of vascular plants to excess Zn (Vaillant et al., 2005;  
257 Mateos-Naranjo et al., 2008; Santos et al., 2014). Nevertheless, we found that Zn

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

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

308 effect cannot be attributed to alleviation of stomatal limitation, since  $g_s$  values did not  
309 vary between salinity levels in either Zn treatment. Therefore, differences in  $A_N$  value  
310 between NaCl levels et each specific Zn concentration treatment could be explained by  
311 non-stomatal limitations (Flexas and Medrano, 2002). In this regard, Perez-Romero et  
312 al. (2016) found that photosynthesis activity was more limited by mesophyll  
313 conductance ( $g_m$ ) than  $g_s$  in *Salicornia ramossisima* in response to Cd. Moreover,  $g_m$  has  
314 been widely implicated in photosynthetic responses patterns to salinity (Flexas et al.,  
315 2012). Hence, it is possible that  $A_N$  differences between salinity levels in *J. acutus*  
316 plants at the same Zn concentration could be linked with  $g_m$  variations; however this  
317 area requires further research. Another possibility relates to impairment of major  
318 carbon-assimilation enzyme activities, such as RuBisCO that may degrade the  
319 photosynthetic pathway under metal stress (Perfus-Barbeoch et al., 2002; Khan and  
320 Khan, 2014). A degree of metal tolerance has been demonstrated in the maintenance  
321 such enzyme functions (Ying et al., 2010; Pérez-Romero et al., 2016). Taking into  
322 account these issues, the higher  $C_i$  in *J. acutus* plants grown without NaCl addition  
323 suggests that differences in carbon assimilation between salinity treatments could have  
324 been linked to limitation in RuBisCO carboxylation capacity (Mateos-Naranjo et al.,  
325 2008, 2014).

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

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

352         Finally the greater tolerance to Zn in plants treated with NaCl was linked with a  
353 better water balance, an idea supported by the overall higher  $\delta$ WUE values. Thus, these  
354 plants would be able better to preserve the trade-off between CO<sub>2</sub> acquisition for growth  
355 and water loss, as indicated the higher  $A_N$  and the invariable  $g_s$  values compared with  
356 their counterparts not treated with NaCl. Han et al. (2013) also found a positive effect of  
357 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.

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383

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

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<i>Parameter</i>	<i>Na</i>	<i>Zn</i>	<i>Na x Zn</i>
RGR	<b>0.03*</b>	<b>0.00**</b>	0.06
Dead Tillers	0.07	<b>0.00**</b>	0.19
A <sub>N</sub>	<b>0.02*</b>	<b>0.00**</b>	<b>0.01*</b>
g <sub>s</sub>	0.30	<b>0.00**</b>	0.06
C <sub>i</sub>	<b>0.04*</b>	0.05	0.22
<sub>i</sub> WUE	<b>0.02*</b>	<b>0.00**</b>	<b>0.03*</b>
F <sub>v</sub> /F <sub>m</sub> , Md	<b>0.00*</b>	<b>0.00**</b>	<b>0.02*</b>
F <sub>v</sub> /F <sub>m</sub> , Pd	<b>0.00**</b>	<b>0.00**</b>	<b>0.02*</b>
Φ <sub>PSII</sub> , Md	0.09	<b>0.00**</b>	<b>0.02*</b>
Φ <sub>PSII</sub> , Pd	<b>0.01**</b>	<b>0.00**</b>	0.06
NPQ, Md	<b>0.01**</b>	<b>0.00**</b>	0.10
NPQ, Pd	0.63	0.51	0.62
Chl <i>a</i>	<b>0.00**</b>	0.88	<b>0.00**</b>
Chl <i>b</i>	0.06	0.57	<b>0.04*</b>
C <sub>x+c</sub>	<b>0.02*</b>	0.22	<b>0.04*</b>
[Zn] <sub>T</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[Zn] <sub>R</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[Na] <sub>T</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[Na] <sub>R</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[K] <sub>T</sub>	0.95	0.58	0.47
[K] <sub>R</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[Mg] <sub>T</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[Mg] <sub>R</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[Ca] <sub>T</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[Ca] <sub>R</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>
[P] <sub>T</sub>	<b>0.02*</b>	<b>0.00**</b>	<b>0.04*</b>
[P] <sub>R</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.01**</b>
[Mn] <sub>T</sub>	0.78	<b>0.00**</b>	0.88
[Mn] <sub>R</sub>	<b>0.00**</b>	<b>0.00**</b>	<b>0.00**</b>



**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  $\pm$  SE, n = 5.

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

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  $\pm$  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<sub>2</sub> concentration,  $C_i$  (C), and intrinsic water use efficiency,  $iWUE$  (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  $\pm$  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,  $\Phi_{PSII}$  (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  $\pm$  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<sub>x+c</sub> (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

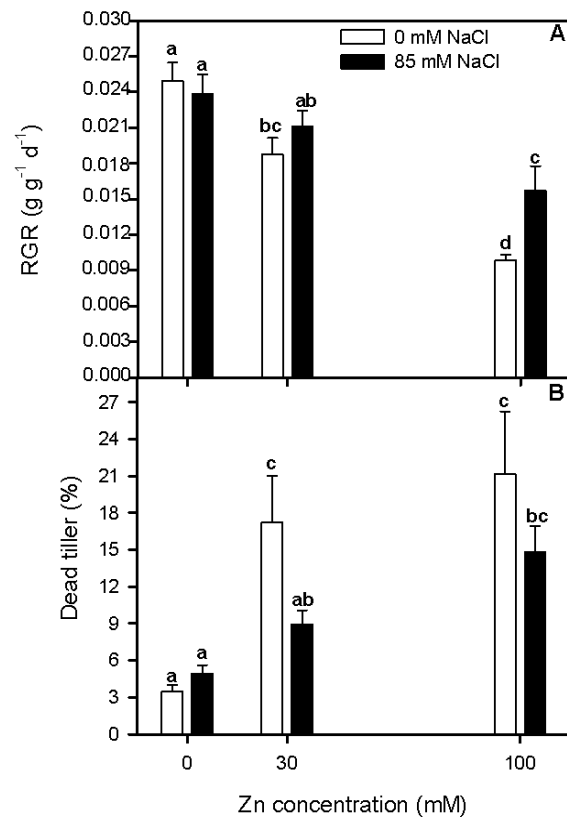


Fig. 2

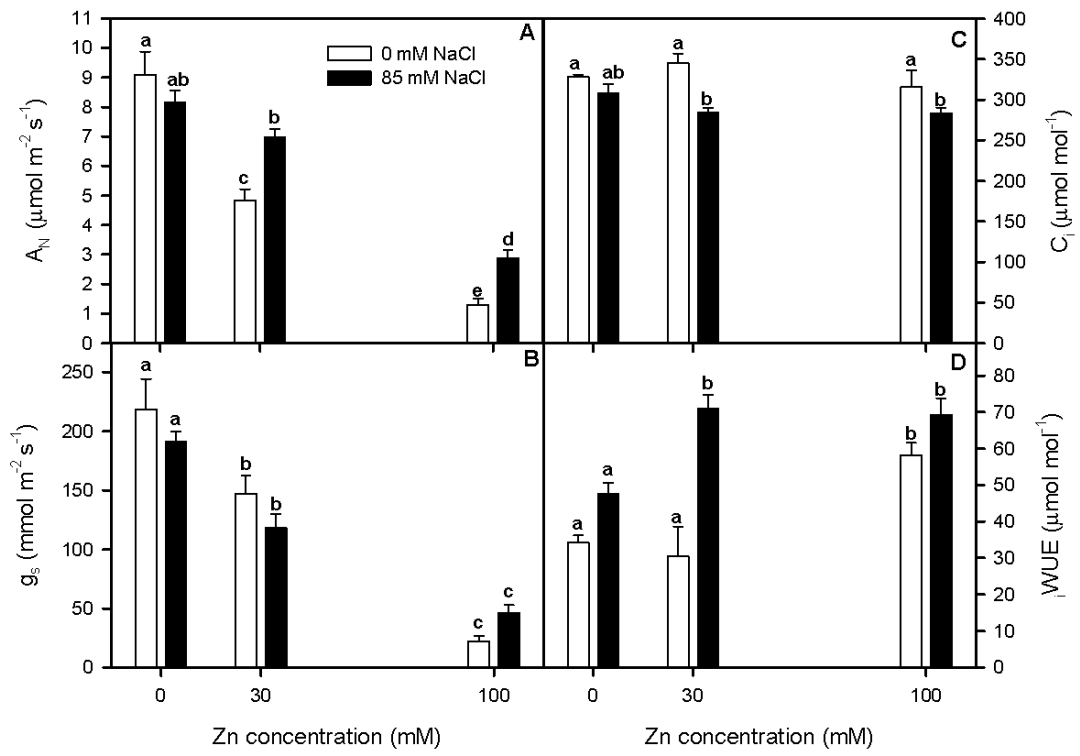


Fig. 3

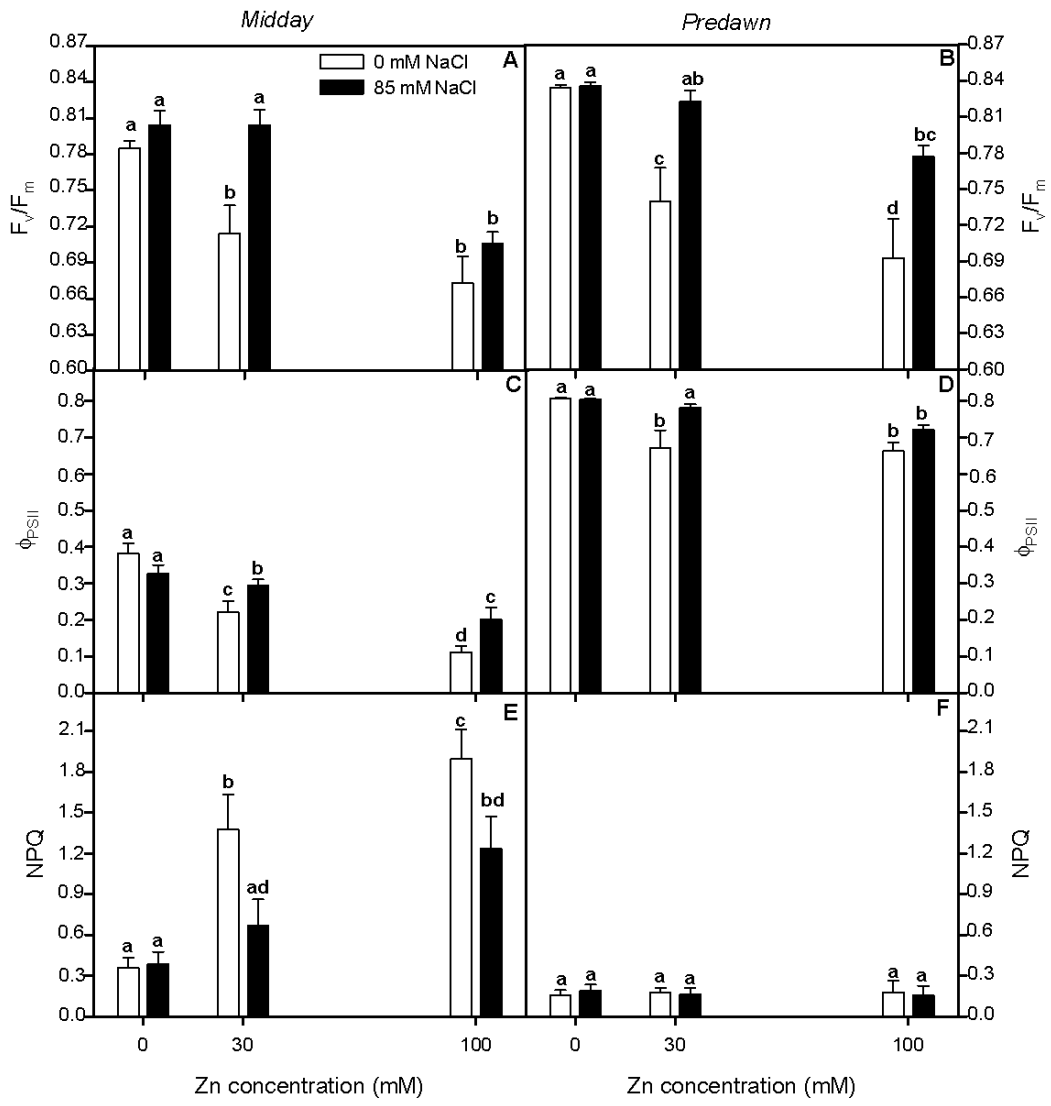


Fig. 4

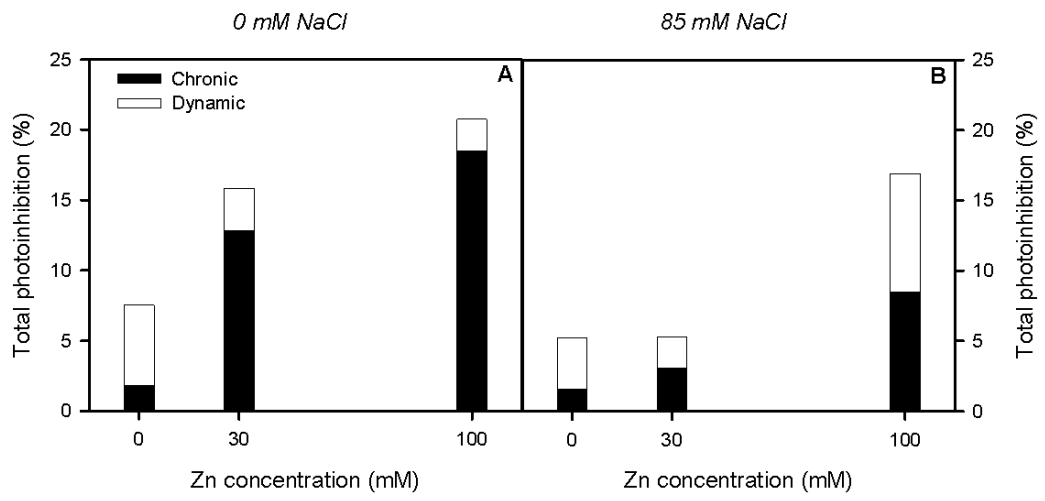


Fig. 5

