1   **Growth and photosynthetic responses to copper stress of an invasive**

2   **cordgrass, *Spartina densiflora***

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1   **Abstract**

2   *Spartina densiflora* Brongn., is found in coastal marshes of south-west Spain, growing

3   in sediments with between 300 – 3000 mg Cu kg^{-1} total soil DW (450-4500 mg Cu kg^{-1}

4   supposing that the soil porosity is 0.5). An experiment was designed to investigate the

5   effect of copper from 0 to 5000 mg kg^{-1} (64 mmol l^{-1}) on the photosynthetic apparatus

6   and the growth of *S. densiflora*. We also determined total ash, copper, calcium,

7   magnesium and phosphorous concentrations, as well as C/N ratio. *S. densiflora* survived

8   to concentrations as high as 320 mg Cu kg^{-1} DW in leaves, although excess of Cu

9   diminished water use efficiency and Ca-, Mg- and P-uptake. Also, quantum efficiency

10   of PSII, net photosynthetic rate, stomatal conductance and pigment concentrations

11   declined with increasing external Cu. Finally, the decline in the photosynthetic function

12   resulted in a biomass reduction of between 50 and 80% (for 600 and 5000 mg Cu kg^{-1},

13   respectively).

14

15   *Keywords*: Copper; Growth rate; Invader; Photochemistry; Photosynthesis;

16   Photosynthetic pigments; Salt marshes; *Spartina densiflora*; Tissue analysis

1   **1. Introduction**

2

3   Copper is an essential micronutrient for plant growth, participating in important

4   biological reactions as an enzymatic cofactor and electron carrier in the photosynthetic

5   and respiratory processes (Andrade et al., 2004) but, when in excess, it becomes highly

6   toxic (Dewez et al., 2005). The excess of copper reduces plant growth as well as

7   photosynthetic and respiratory activities (Nalewajko and Olaveson, 1995). The

8   photosynthetic apparatus is particularly susceptible to this cation, resulting in a decrease

9   in the activity of photosystem II and electron transfer rates (Fernades and Henriques,

10   1991; Mallick and Mohn, 2003). Toxic concentrations of Cu may eventually build up in

11   certain estuarine and shallow coastal marine habitats near mining and industrial hot

12   spots (Nielsen and Nielsen, 2005).

13   The austral cordgrass, *Spartina densiflora* Brongn. (Poaceae), is invading salt

14   marshes as far apart as southern Europe (Tutin, 1980; Mateos-Naranjo et al., 2007),

15   North Africa (Fennane and Mathez, 1988) and North America (Kittelson and Boyd,

16   1997). This species has proven to be a vigorous invader and ecosystem engineer that

17   spreads by clonal growth and prolific seed production (Mateos-Naranjo et al., 2008a). In

18   the salt marshes of the joint estuary of the Tinto and Odiel rivers (SW Spain), one of the

19   most polluted areas by heavy metals in the world (Ruiz, 2001; Sáinz and Ruiz, 2006) on

20   account of the intense mining activity and waste from industrial zones, *S. densiflora*

21   grows over sediments with between 300 – 3000 ppm Cu per total soil DW (Nelson and

22   Lamothe, 1993; Sáinz et al., 2002), and with the greatest mobility and bioavailability

23   (Morillo et al., 2004). Other species of the *Spartina* genus have been shown to have a

24   great capacity to accumulate metals, such as copper, from the sediments. The

3

1 physiological impact of such bioaccumulation, nonetheless, has not been thoroughly

2 studied (Nybakken, 1988).

3   The main objective of the present study was to evaluate the effects of elevated

4 copper levels on *S. densiflora* growth and photosynthesis. The specific objectives were

5 to: (1) analyze the growth of plants in experimental copper treatments ranging from 0 to

6 64 mmol l^{-1} Cu; (2) ascertain the extent to which the effects on the photosynthetic

7 apparatus (PSII chemistry), on gas exchange parameters and on photosynthetic

8 pigments determine plant performance; and (3) examine the possible role of

9 concentrations of mineral matter (ash), calcium, magnesium, phosphorous and nitrogen

10 in explaining the copper effects on growth.

1   **2. Material and methods**

2

3   *2. 1. Plant material*

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5   Seeds of *S. densiflora* were collected in December 2006 from the Odiel Marshes

6   (Huelva, SW Spain), and subsequently stored at 4ºC (in darkness) for three months.

7   After the storing period, seeds were placed in a germinator (ASL Aparatos Científicos

8   M-92004, Madrid, Spain) and subjected to an alternating diurnal regime of 16 hours of

9   light (photon flux rate, 400-700 nm, 35 μmol m^{-2} s^{-1}) at 25ºC and 8 hours of darkness at

10   12ºC, for a month. The seedlings were planted in individual plastic pots (11 cm of

11   diameter) filled with pearlite and placed in a glasshouse (during spring 2007) with

12   minimum-maximum temperatures of 21-25ºC, 40-60% relative humidity and natural

13   daylight (minimum and maximum light flux: 200 and 1000 μmol m^{-2} s^{-1}). The pots were

14   carefully irrigated with 20% Hoagland's solution (Hoagland and Arnon, 1938) as

15   necessary. All the pots received the same irrigation.

16

17   *2.2. Stress treatments*

18

19   In April 2007, after a month of seedling cultures, the pots were allocated to five Cu

20   treatments in shallow trays (ten pots per tray, with one tray per Cu treatment): 0, 9, 15,

21   47 and 64 mmol l^{-1} Cu, in the same glasshouse. Cu treatments were established by

22   combining 20% Hoagland's solution and $CuSO_4 \cdot 7H_2O$ of the appropriate concentration.

23   The control, 0 mmol l^{-1} Cu treatment, had exactly 0.0005 mmol l^{-1} of Cu, since

24   Hoagland's solution contains a small amount of Cu as an essential trace nutrient. Cu

25   concentrations were chosen to cover variations recorded by Nelson and Lamothe (1993;

5

1   300-3000 mg Cu kg^{-1} per total soil DW, i.e. 450-4500 mg Cu kg^{-1} supposing that the

2   soil porosity is 0.5) and Sáinz et al. (2002) in the salt marshes of the joint estuary of the

3   Tinto and Odiel rivers.

4   At the beginning of the experiment, 3 L of the appropriate solution were placed in

5   each of the trays down to a depth of 1 cm. During the experiment, the levels in the trays

6   were monitored and they were topped up to the marked level with 20% Hoagland's

7   solution (without additional $CuSO_4 \cdot 7H_2O$) as a way to limit the change in the Cu

8   concentration due to water evaporation of the nutritive solution. In addition, the entire

9   solution (including $CuSO_4 \cdot 7H_2O$) was changed on a weekly basis (The total duration of

10 the experiment was 30 d)

11 The possibility of adding NaCl to the culture medium was disregarded, since the

12 absence of salt has been proven to affect neither the photosynthetic function of *S.*

13 *densiflora* nor its growth. In fact, Mateos-Naranjo et al. (2008b) found the highest rates

14 of net photosynthesis and leaf elongation in distilled water.

15

16 *2.3. Growth*

17

18 At the beginning and at the end of the experiment (i.e. after 30 d of treatment), four and

19 six entire plants (roots and leaves), from each treatment, respectively, were dried at

20 80ºC for 48 h and then weighed. Dried, ground samples were ignited in lidded, ceramic

21 crucibles and ash weights were recorded; the furnace temperature was raised slowly

22 over 6 h to 550ºC and this temperature was maintained for a further 8 h. Also, both

23 before and after the copper treatment, the number and height of all fully developed

24 tillers were measured.

25 The relative growth rate (RGR) in ash-free dry mass of whole plants was calculated

1 by using the formula:

2

3 $RGR = (\ln Bf - \ln Bi) \cdot D^{-1}$ $(g\ g^{-1}day^{-1})$

4

5 where Bf = final dry mass (an average of the four plants from each treatment dried at

6 the beginning of the experiment), Bi = initial dry mass and D = duration of experiment

7 (days).

8   Leaf elongation rate (LER) was measured in random leaves (n = 12, per treatment;

9 two measurements per plant) by placing a marker of inert sealant at the base of the

10 youngest accessible leaf. The distance between the marker and the leaf base was

11 measured after 24 h (Ewing et al., 1995).

12

13 *2.4. Determination of copper, calcium, magnesium, phosphorous and nitrogen*

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15 In accordance with protocols of Redondo-Gómez et al. (2007), at the end of the

16 experiment, leaf and root samples were dried at 80ºC for 48 h and ground. Leaves and

17 roots were carefully washed with distilled water before any further analysis. Then 0.5 g

18 samples (in triplicate), taken from a mixture of the leaves or the roots belonging to the

19 six plants used for each treatment, digested with 6 ml HNO_3, 0.5 ml HF and 1 ml H_2O_2.

20 Ca, Mg, P and Cu were measured by inductively coupled plasma (ICP) spectroscopy

21 (ARL-Fison 3410, USA). Total N and C concentrations were determined for undigested

22 dry samples by means of an elemental analyzer (Leco CHNS-932, Spain).

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24 *2.5. Chlorophyll fluorescence*

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1 Chlorophyll fluorescence was measured in random fully developed penultimate leaves

2 (n = 12, two measurements per plant) using a portable modulated fluorimeter (FMS-2,

3 Hansatech Instrument Ltd., England) after 7 and 30 d of treatment. Light- and dark-

4 adapted fluorescence parameters were measured at dawn (stable, 50 µmol m^{-2} s^{-1}

5 ambient light) and at midday (1600 µmol m^{-2} s^{-1}).

6 　　The plants were dark-adapted for 30 minutes, using leaf–clips exclusively designed

7 for this purpose. The minimal fluorescence level in the dark-adapted state (F_0) was

8 measured using a modulated pulse (<0.05 µmol m^{-2} s^{-1} for 1.8 µs) which was too small

9 to induce significant physiological changes in the plant. The data stored were an

10 average taken over a 1.6 second period. Maximal fluorescence in this state (F_m) was

11 measured after applying a saturating actinic light pulse of 15000 µmol m^{-2} s^{-1} for 0.7s.

12 The values of the variable fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency

13 of PSII photochemistry (F_v/F_m) were calculated from F_0 and F_m. This ratio of variable

14 to maximal fluorescence correlates with the number of functional PSII reaction centres,

15 and dark adapted values of F_v/F_m can be used to quantify photoinhibition (Krivosheeva

16 et al., 1996).

17 　　The same leaf section of each plant was used to measure light-adapted parameters.

18 Steady state fluorescence yield (F_s) was recorded after adapting plants to ambient light

19 conditions for 30 minutes. A saturating actinic light pulse of 15000 µmol m^{-2} s^{-1} for 0.7

20 s was then used to produce the maximum fluorescence yield (F_m') by temporarily

21 inhibiting PSII photochemistry.

22 　　Using fluorescence parameters determined in both light- and dark-adapted states, the

23 following were calculated: quantum efficiency of PSII ($\Phi_{PSII} = (F_m' - F_s)/ F_m'$), which

24 measures the proportion of the light absorbed by chlorophyll associated with PSII that is

25 used in photochemistry (Maxwell and Johnson, 2000); and non-photochemical

1 quenching (NPQ = (F_m – F_m') / F_m'; Redondo-Gómez et al., 2006), which is linearly

2 related to heat dissipation (Maxwell and Johnson, 2000).

3

4 *2.6. Gas exchange*

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6 Gas exchange measurements were taken on random fully expanded leaves (n = 10, a

7 measurement per plant and four extra measurements taken randomly) 7 and 30 days

8 after the treatment, by using an infrared gas analyzer in an open system (LI-6400, LI-

9 COR Inc., Neb., USA). Net photosynthetic rate (A), intercellular CO_2 concentration

10 (C_i) and stomatal conductance to CO_2 (G_s) were determined at ambient CO_2

11 concentration of 360 µmol mol^{-1}, temperature of 20/25ºC, 50 ± 5% relative humidity

12 and a photon flux density of 1000 µmol m^{-2} s^{-1}. A, C_i and G_s were calculated using

13 standard formulae of Von Caemmerer and Farquhar (1981). The water use efficiency

14 (WUE) was calculated as the ratio between A and transpiration rate [mmol (CO_2

15 assimilated) mol^{-1} (H_2O transpired)].

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17 *2.7. Photosynthetic pigments*

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19 At the end of the experiment period, photosynthetic pigments in fully expanded

20 penultimate leaves (n = 10, a measurement per plant and four extra measurements taken

21 randomly) from each treatment were extracted using 0.05 g of fresh material in 10 ml of

22 80% aqueous acetone. After filtering, 1 ml of the suspension was diluted with a further

23 2 ml of acetone and chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoids (C*x*+*c*)

24 contents were determined with a Hitachi U-2001 spectrophotometer (Hitachi Ltd,

25 Japan), by making use of three wavelengths (663.2, 646.8 and 470.0 nm). The

1   concentrations of pigments ($\mu g\ g^{-1}$ fwt) were obtained by calculation, using the method

2   of Lichtenthaler (1987).

3

4   *2.8. Statistical analysis*

5

6   Statistical analysis was carried out using Statistica v. 6.0 (Statsoft Inc.). Pearson

7   coefficients were calculated to assess the correlation between different variables. Data

8   were analyzed using a one- and two-way analysis of variance (*F*-test). Data were first

9   tested for normality with the Kolmogorov-Smirnov test and for homogeneity of

10   variance with the Brown-Forsythe test and arc-sine transformed (Fv/Fm at dawn and net

11   photosynthetic rate data) in order to normalize the error distribution for ANOVA.

12   Significant test results were followed by Tukey tests for identification of important

13   contrasts. Differences between measurements of fluorescence at dawn and midday were

14   compared by the Student test (*t*-test) to investigate whether Cu concentration affected

15   the sensitivity of plants to photoinhibition.

1 **3. Results**

2

3 *3.1. Determination of copper, calcium, magnesium, phosphorous and C/N ratio*

4

5 There was a marked decrease in the mineral (ash) content of leaves (but not of the roots)

6 with increasing external Cu concentration ($r = -0.76$, $p < 0.01$). The ash content of roots

7 dropped when exposed to 15 mmol l^{-1} Cu (Fig. 1A).

8   Tissue copper concentrations were greater in the roots than in leaves (two-way

9 ANOVA, $p < 0.001$), and increased with external Cu concentration ($r = 0.89$, $p <$

10 0.0001; $r = 0.98$, $p < 0.0001$, for root and leaf, respectively; Fig 1B). In contrast, leaf

11 and root Ca, Mg and P concentrations diminished with increasing Cu concentration

12 (Figs. 1C-E).

   Fig. 1   Carbon/nitrogen (C/N) ratio was higher in the leaves than in the roots of *S.*

14 *densiflora* (Fig. 1F). Both root and leaf C/N ratios tended to increase with external Cu

15 concentration, although leaf ratio dropped sharply when exposed to the highest Cu

16 concentration.

17

18 *3.2. Chlorophyll fluorescence*

19

20 The fluorescence parameters presented in Figure 2 correspond to plants after 30 d of

21 treatment. Fluorescence parameters after 7 d treatment did not show any difference with

22 the control and are not presented.

23   Values of Fv/Fm diminished, either at dawn or midday, with increasing Cu

24 concentration (dawn: $r = -0.85$, $p < 0.001$; midday: $r = -0.68$, $p < 0.001$; Fig. 3A).

25 Furthermore, Fv/Fm values were significantly higher for the control at both dawn and

11

1   midday (ANOVA, $p < 0.0001$ for each) because of higher values of Fm (data not

2   presented) and lower values of F_0. Fv/Fm was lower at midday, except for 64 mmol l^{-1}

3   Cu, and the reductions resulted again mainly from the fact that values of Fm were lower

4   at midday than at dawn (*t*-test, $p < 0.05$; Fig. 2A). On the other hand, the minimal

5   fluorescence (F_0) increased with increasing Cu concentration at both dawn and midday

6   ($r = 0.80$, $p < 0.001$; $r = 0.82$, $p < 0.0001$, respectively; Fig. 2B), while Fm remained

7   steady. F_0 was inversely correlated with Fv/Fm at both dawn and midday ($r = -0.95$, $p <$

8   0.05; $r = -0.93$, $p < 0.05$, respectively).

9   Quantum efficiency of PSII (ΦPSII) decreased with increasing Cu concentration,

10   either at dawn or midday ($r = -0.88$, $p < 0.0001$; $r = -0.75$, $p < 0.0001$; Fig. 2C). ΦPSII

11   values were significantly higher at midday than at dawn.

12   Non-photochemical quenching (NPQ) did not vary with Cu concentration at dawn,

13   whereas at midday it increased up to 47 mmol l^{-1} Cu, and then it declined with the

14   further increases in Cu concentration (Fig. 2D).

Fig. 2

16   *3.3. Gas exchange and pigment concentration*

17

18   The gas exchange parameters presented in Figure 3 correspond to plants after 30 d of

19   treatment. Net photosynthetic rate (A) after 7 d treatment did not show any differences

20   with the control (c. 15 µmol m^{-2} s^{-1}; $p > 0.05$) and hence it is not presented here.

21   Figure 3A shows that A declined sharply with the copper concentration of 15

22   mmol l^{-1} and then stabilized at the highest concentrations. There was a strong linear

23   relationship between A and RGR ($r = 0.91$, $p < 0.05$), and ΦPSII ($r = 0.89$, $p < 0.05$).

Fig. 3

     Stomatal conductance (Gs) showed the same pattern as A (Fig. 3B). In contrast,

25   intercellular CO_2 concentration (Ci) increased significantly with Cu (Fig. 3C). Water

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1   use efficiency (WUE), on the other hand, decreased significantly with Cu concentration

2   (Fig. 3D).

3   Pigment concentrations decreased with Cu concentration, showing the same

4   pattern as A and Gs (Fig. 4).

Fig. 4

6   *3.4. Growth*

7

8   Relative growth rate (RGR) was inhibited by Cu excess, as proves the fact that the

9   maximum value was recorded at the control (ANOVA, $p < 0.001$; Fig. 5A). In addition,

10   Cu treated plants showed chlorosis, especially at the two highest Cu concentrations.

11   RGR decreased with increasing Cu concentration ($r = -0.68$, $p < 0.0001$), and was

12   directly correlated with leaf elongation rate (LER; $r = 0.89$, $p < 0.05$), number of tillers

13   ($r = 0.95$, $p < 0.05$) and mean height of tillers ($r = 0.99$, $p < 0.001$; Figs. 5A-D).

Fig. 5

1 **4. Discussion and conclusions**

2

3   *Spartina densiflora* demonstrated a high tolerance to copper stress, since all plants

4   survived even at concentrations as high as 64 mmol l^{-1} of Cu during 30 d. Paschke and

5   Redente (2002) determined the lethal concentrations of copper (LC50, substrate copper

6   concentration that kills 50% of plants) for six grass species used in restoration activities

7   in concentrations close to 5 mmol l^{-1} after 60 d of treatment. In our glasshouse

8   experiment, we found that Cu levels were much higher in *S. densiflora* below- (72-1480

9   ppm Cu DW) than above-ground (10-320 ppm Cu DW) parts. A similar trend was

10   recorded for *Spartina maritima* (Reboredo, 1985) and *Spartina anglica* (Otte et al.,

11   1991) under field conditions. Luque et al. (1999) found Cu concentrations c. 24 mg kg^{-1}

12   DW in young leaves of *S. densiflora* sampled from Odiel salt marshes. In this site, 30

13   and 200 mg Cu kg^{-1} DW have been quantified in young and old leaves of this species,

14   respectively; and 150 and 600 mg Cu kg^{-1} DW for young and old leaves, respectively,

15   from the Tinto Marshes (Huelva, SW Spain; unpublished data). The lower Cu

16   concentration in leaves of *S. densiflora*, compared to that present in roots, could be

17   accounted for by the development of such mechanisms as compartmentation, which

18   could control the ion transport into leaves, thereby improving its tolerance to heavy

19   metals. The sequestering of metals in tissues or cellular compartments, which are less

20   sensitive to metals, has been described as a tolerance mechanism (Weis and Weis,

21   2004), entailing a restriction of the upward movement into the shoots (an avoidance

22   mechanism) and the translocation of excessive metals into the leaves (Verkleij and

23   Schat, 1990). Nevertheless, with excessive levels of soil Cu, this element may be

24   translocated from the roots and accumulated in the tops of the plant (Kabata-Pendias

14

1   and Pendias, 2001), which could explain the increase of Cu concentration recorded in

2   leaves of *S. densiflora* with increasing external copper.

3   On the other hand, roots of *S. densiflora* could also act as a 'barrier' to the uptake

4   or transport of Cu, since leaf concentration hardly increased when the external

5   concentration exceeded 9 mmol l^{-1} Cu. In this regard, Verkleij and Schat (1990) showed

6   that metal-chelating substances may be present in plant exudates, which act to decrease

7   metal uptake, and consequently toxicity. In other species of *Spartina*, some authors have

8   observed the formation of an iron plaque on the roots, with the ensuant accumulation of

9   metals in the rhizosphere (Doyle and Otte 1997), this plaque being present as well in *S.*

10   *densiflora* (Mateos-Naranjo et al., 2008b). On the other hand, the metal excess in the

11   root zone may inhibit water uptake, thus explaining the recorded decline of water use

12   efficiency.

13   Due to the significant functions of Cu in enzymes and its variable valence, ions

14   with an affinity to proteins and other compounds similar to that of Cu may have

15   antagonistic interrelationships, a fact which could be the explanation for the decrease in

16   ash content (Kabata-Pendias and Pendias 2001). However, a rise in ash concentration

17   was recorded in roots of *S. densiflora* at the highest Cu concentration, which could be

18   explained by the strong accumulation of this metal, as the results of internal metal

19   concentrations confirmed. In our experiment, the presence of Cu affected the

20   concentration of the macroelements, Ca, Mg, N and P. An excess of Cu inhibits the

21   activity of phosphatase, thereby diminishing the availability of P (Tyler, 1976). Lin and

22   Wu (1994) found that in *Lotus purshianus*, an excess of Cu reduced the concentration of

23   P in both root and leaf tissues. Cu-Ca interactions are highly complex and apparently

24   cross-linked with the range of pH in the growth media (Kabata-Pendias and Pendias,

25   2001), and Mg is replaced in the chlorophyll by Cu (Küpper et al., 2002). Llorens et al.

1 (2000) also reported an enhancement of Cu, in respect to N, due to changes in nitrogen

2 metabolism with a reduction of total nitrogen in plant tissues. However, a rise in N

3 concentration was recorded in leaves of *S. densiflora* at the highest Cu concentration. In

4 this regard, Kabata-Pendias and Pendias (2001) explained that the concentrations of the

5 two elements are relative to the formation of strong protein complexes with Cu.

6 Furthermore, it is possible that *S. densiflora* leaves became less rigid at high Cu levels,

7 since Fernandes and Henriques (1991) reported that the high sensitivity of diatoms to

8 Cu results from competition between Cu and $Si(OH)_4$ for the same transport site.

9   Excessive Cu accumulated in plants tissue can be toxic to plants, affecting several

10 physiological and biochemical processes (Balsberg Pahlsson, 1989). Ouzounidou

11 (1994) explained that the marked impact of copper could be attributed to an overall

12 inhibition of photosystem II (PSII), due to chloroplast membrane leakage; this may be

13 associated with a substantial stress response. In this manner, F_v/F_m decreases are more

14 permanent than increases of dynamic inhibition 'ΦPSII'; the latter usually relaxes as

15 light intensity is lowered. F_v/F_m did show a significant reduction at midday compared to

16 dawn values. At midday, the reduction in F_v/F_m values indicated that *S. densiflora*

17 experienced photodamage at the highest light flux. ΦPSII decreased as a consequence of

18 the decrease in photochemical quenching (qP) and the increase in NPQ (up to 47 mmol

19 l^{-1}), which indicates that the plants dissipated light as heat, in this way protecting the

20 leaf from light-induced damage (Maxwell and Johnson, 2000). Nonetheless, at the

21 highest Cu concentration, NPQ diminished, indicating that excess levels of Cu affected

22 this mechanism associated with the adaptation to high light irradiances (Demmig-

23 Adams, 1998).

24   The decline of F_v/F_m in the presence of copper resulted from an increase in F_0,

25 while Fm was maintained at a stable level. F_0 increased despite the decline of

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1   chlorophyll concentrations, these results being similar to results obtained by Ralph and

2   Burchett (1998). The higher values of F_0 indicated an impact on the PSII reaction

3   centre, as well as to reduction in the transfer of energy from the collecting antennas to

4   reaction centres (Ralph and Burchett, 1998). Padinha et al. (2000) also found that

5   photosynthetic efficiency was lower in plants of *Spartina maritima* from polluted sites.

6   On the other hand, Cu had a negative effect on net photosynthetic rate (A) and

7   stomatal conductance (Gs), without direct relation between both parameters since there

8   was not a reduction in intercellular CO_2 concentration (Ci). Burzynski and Zurek (2007)

9   found that lower Gs in cotyledons of *Cucumis sativus* treated with copper did not

10   influence A, as Ci remained unchanged, and was high. The decrease of A by Cu was

11   attributed to the influence of this metal on the activities of photosynthetic carbon

12   reduction cycle enzymes (PGK and GAPDH were inhibited by Cu), as well as loss of

13   chlorophyll pigments (Ouzounidou, 1994). Burzynski and Klobus (2004) suggested that

14   Cu initially destroys the photosynthetic carbon reduction cycle and subsequently

15   influences the photosynthetic electron transport. In the case of *S. densiflora*, the

16   decrease in pigment concentrations or increase in its degradation, and consequent

17   negative effect on photosynthetic electron transport, could lead as well to a decline in

18   the photosynthetic function. The substitution the central magnesium ion in the

19   chlorophyll by Cu may also damage the chlorophyll synthesizing system (Küpper et al.,

20   2002). In this respect, we recorded a decrease of Mg, whereas copper content increased

21   in the leaf tissue.

22   The decline in the photosynthetic function resulted in a biomass reduction of *S.*

23   *densiflora* which was apparent in the RGR of ash-free dry mass, number of tillers, leaf

24   elongation rate and mean height of tillers. Thus, the growth of *S. densiflora* was

25   inhibited by leaf tissue concentrations above 0.03 mg g^{-1} dry mass.

1   *Spartina densiflora* survived to concentrations up to 320 mg Cu kg^{-1} DW in

2   leaves, when it was treated with 5000 mg Cu l^{-1} (64 mmol l^{-1}), despite the fact that

3   concentrations of Cu between 20 and 100 mg Cu kg^{-1} DW in mature leaf tissue are

4   considered excessive or toxic (Kabata-Pendias and Pendias, 2001). Copper inhibited

5   water use efficiency, Ca-, Mg- and P-uptake, quantum efficiency of PSII, net

6   photosynthesis, stomatal conductance, and pigment synthesis, this resulting in a decline

7   of growth.

8

9   **Acknowledgements**

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1 Fig 1. Ash (A), total copper (B), total calcium (C), total magnesium (D) and total

2 phosphorous (E) concentrations, and C/N ratio (F) for leaves (○) and roots (●) of

3 *Spartina densiflora* in response to treatment with a range of Cu concentrations after a

4 month. Values represent mean ±SE, n = 3.

5
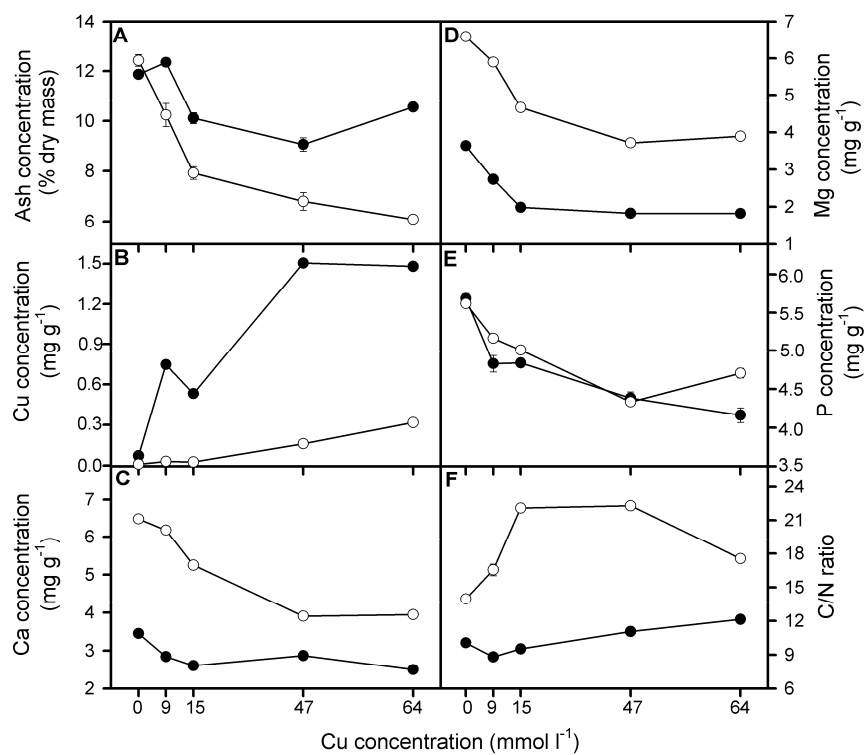
6 Fig 2. Maximum quantum efficiency of PSII photochemistry, F_v/F_m (A), minimal

7 fluorescence, F_0 (B), quantum efficiency of PSII, ΦPSII (C), and non-photochemical

8 quenching, NPQ (D), at mid-day (●) and at dawn (○) in randomly selected, fully

9 expanded penultimate leaves of *Spartina densiflora* in response to treatment with a

10 range of Cu concentrations for a month. Values represent mean ±SE, n = 12.

11

12 Fig 3. Net photosynthetic rate, A (A), stomatal conductance, Gs (B), intercellular CO_2

13 concentration, Ci (C), and water use efficiency (D) in randomly selected, fully expanded

14 penultimate leaves of *Spartina densiflora* in response to treatment with a range of Cu

15 concentrations for a month. Values represent mean ±SE, n = 10.

16

17 Fig 4. Chlorophyll *a*, chl *a* (A), Chlorophyll *b*, chl *b* (B), and carotenoid, C*x+c* (C)

18 concentrations in randomly selected, fully expanded penultimate leaves of *Spartina*

19 *densiflora* in response to treatment with a range of Cu concentrations for a month.

20 Values represent mean ±SE, n = 10.

21

22 Fig 5. Growth analysis of *Spartina densiflora* in response to treatment with a range of

23 Cu concentrations over a month. Relative growth rate (A), leaf elongation rate (B),

24 number of tillers (C), and mean height of tiller (D). Values represent mean ±SE, n = 6.

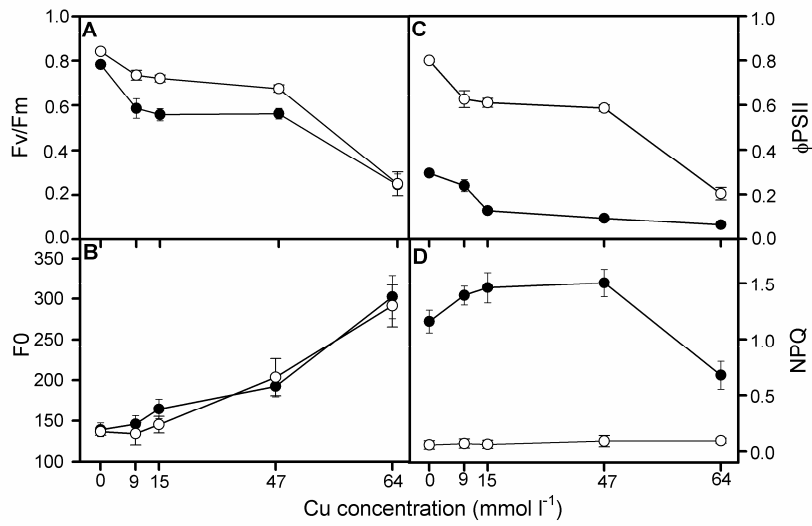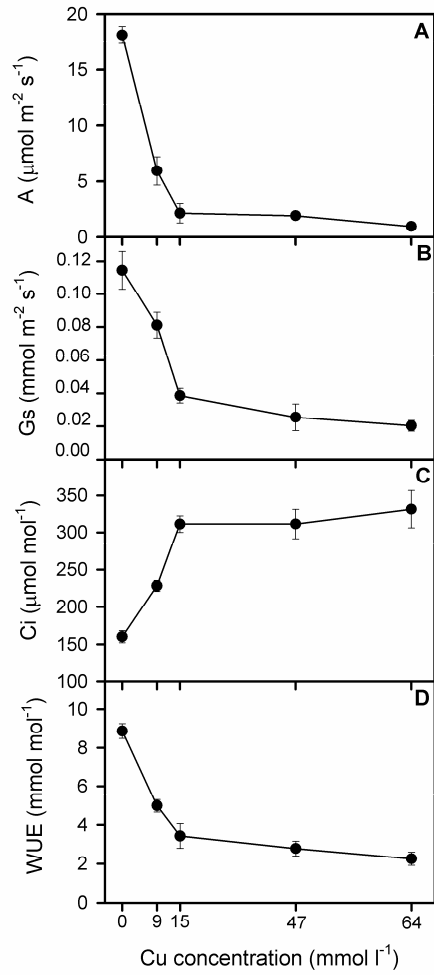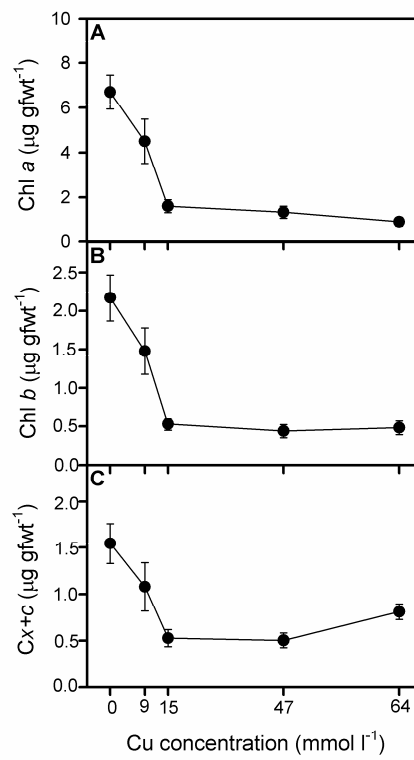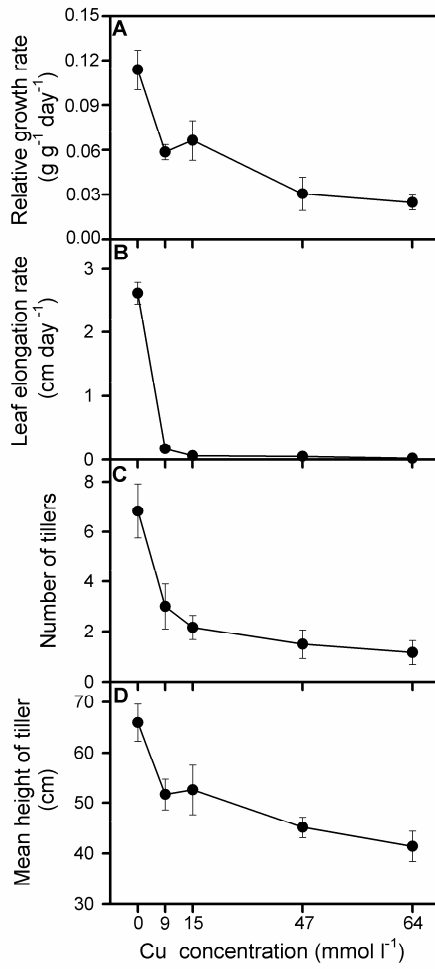25 The analysis was carried out on an ash-free dry mass basis.

1

2 Fig.1

1   Fig.2

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