*Annals of Botany* **123: 121–132, 2019**
doi: 10.1093/aob/mcy148

# Salinity decreases Cd translocation by altering Cd speciation in the halophytic Cd-accumulator *Carpobrotus rossii*

Miaomiao Cheng[1] • Peter M. Kopittke[2] • Anan Wang[1] • Caixian Tang[1]

[1] Department of Animal, Plant and Soil Sciences, Centre for AgriBioscience, La Trobe University, Victoria, 3086, Australia
[2] School of Agriculture and Food Sciences, The University of Queensland, Queensland, 4072, Australia

## ABSTRACT

**Background and Aims:** Salt has been shown to affect Cd translocation and accumulation in plants but the associated mechanisms are unclear. This study examined the effects of salt type and concentration on Cd uptake, translocation and accumulation in *Carpobrotus rossii*.

**Methods:** Plants were grown in nutrient solution with the same Cd concentration or $Cd^{2+}$ activity in the presence of 25 mM $NaNO_3$, 12.5 mM $Na_2SO_4$ or 25 mM NaCl for ≤ 10 d. Plant growth and Cd uptake were measured, and the accumulation of peptides and organic and Cd speciation in plant tissues were analyzed.

**Key Results:** Salt addition decreased shoot Cd accumulation by > 50% due to decreased root-to-shoot translocation, irrespective of salt type. Synchrotron-based X-ray absorption spectroscopy revealed that after 10 d, 61-94% Cd was bound to S-containing ligands (Cd-S) in both roots and shoots but its speciation was not affected by salt. In contrast, Cd in the xylem sap was present either as free $Cd^{2+}$ or complexes with carboxyl groups (Cd-OH). When plants were exposed to Cd for ≤ 24 h, 70% of the Cd in the roots was present as Cd-OH rather than Cd-S. However, NaCl addition decreased the proportion of Cd-OH in the roots within 24 h by forming Cd-Cl complexes and increasing the proportion of Cd-S. This increased Cd-S complexes by salt were not due to changes in glutathione and phytochelatin synthesis.

**Conclusions:** Salt addition decreased shoot Cd accumulation by decreasing Cd root-to-shoot translocation due to the rapid formation of Cd-S complexes (low mobility) within the root, without changing the concentrations of glutathione and phytochelatins.

**Key words:** Cd uptake, organic acids, phytochelatins, phytoextraction, sodium salt, Synchrotron, XANES

## INTRODUCTION

Cadmium (Cd) is one of the most environmentally toxic pollutants. It can accumulate in plant tissues to harmful levels in the diets of animals and humans without being toxic to the plant itself. This Cd enters the environment mainly through the application of a range of compounds, including sewage sludge as a soil amendment, wastes from incinerators and other industrial activities and phosphate fertilizers (Nicholson *et al.*, 1994). Among the various approaches for remediation, phytoextraction using plants to remove Cd from contaminated soils is an environmentally friendly and cost-efficient approach (Mahar *et al.*, 2016).

In some areas, high levels of salinity are another important environmental stress. An estimated 20-50% of all irrigated lands worldwide are affected by excess salt, with NaCl being the salt of greatest interest (Pitman and Läuchli, 2002). High concentrations of bioavailable Cd are associated with high levels of soluble salts in coastal or semi-arid areas because of mining or urbanization (US Environmental Protection Agency, 2000; Panta et al., 2014; Lutts and Lefèvre, 2015). In these saline soils, most glycophyte Cd-hyperaccumulating species are not suited for growth (Ghnaya *et al.*, 2007; Lutts and Lefèvre, 2015). As a result, the influence of NaCl on Cd accumulation in plants, particularly in halophytes, is of increasing interest (Lutts and Lefèvre, 2015). It has been reported that the presence of NaCl in Cd-containing solutions improves the growth of halophytes but decreases the Cd concentration in the plant tissues (Chai *et al.*, 2013; Ghnaya *et al.*, 2007; Lefèvre *et al.*, 2009; Wali *et al.*, 2015). This decrease in tissue Cd concentration may be due to a dilution effect, with NaCl alleviating the Cd-induced growth reduction (Ghnaya *et al.*, 2007; Wali *et al.*, 2015). NaCl may also decrease root Cd uptake via the decreased activity of $Cd^{2+}$ in solutions associated with the formation of $CdCl_n^{2-n}$ complexes (Smolders and McLaughlin, 1996). However, these previous studies are in contrast with others showing that the effect of NaCl on Cd accumulation is concentration-dependent. For example, NaCl increased the concentration of Cd in tissues of the halophyte *Spartina alterniflora* at 1 mM Cd but decreased the Cd concentration at 3 mM Cd (Chai *et al.*, 2013). Furthermore, both NaCl and KCl decreased the concentration of Cd in the leaves of *Atriplex halimus* but $NaNO_3$ increased it (Lefèvre *et al.*, 2009). Thus, the apparent discrepancies between previous studies suggest that the effect of salt on Cd accumulation in plants not only differs between plant species but also between salt types.

*Carpobrotus rossii*, a halophytic succulent plant species, has been shown to have potential for phytoextraction of Cd in soils with high salinity (Zhang *et al.*, 2016). Our previous study has shown that the addition of NaCl to Cd-containing solutions significantly reduced Cd accumulation in shoot tissues, particularly by decreasing Cd translocation from the root to shoot even when the activity of $Cd^{2+}$ in nutrient solution was maintained constant (Cheng *et al.*, 2018). These observations for *C. rossii* differ from those reported previously for *Arabidopsis thaliana*, *Solanum nigrum* and *Beta vulgaris*, where NaCl increased Cd concentrations in both shoots and roots (Smolders and McLaughlin, 1996; Xu *et al.*, 2010). Regardless, the mechanism whereby NaCl reduces the translocation of Cd to the shoots in *C. rossii* remains unclear.

The chemical speciation of Cd plays an important role in influencing its mobility within plant tissues. To moderate $Cd^{2+}$ concentrations in the symplast, free $Cd^{2+}$ can be regulated by binding to phytochelatins (PCs) and glutathione (GSH), with Cd having a high affinity for thiol groups (Clemens *et al.*, 2002; Clemens, 2006). These Cd-S complexes can be subsequently transported into vacuoles and potentially transformed to inactive forms for storage. In addition, some free $Cd^{2+}$ ions in the cytosol can be anti-ported into the vacuole and then weakly bound to simple organic acids (Clemens, 2006). Our previous study (Cheng *et al.*, 2016) showed that the majority of the Cd in both the roots and shoots of *C. rossii* was bound to thiol groups, but these Cd-S complexes were not involved in the root-to-shoot translocation. Rather, the Cd in the xylem sap was present either as free $Cd^{2+}$ ions or complexed with carboxyl groups. Thus, it is possible that NaCl alters Cd speciation within the plant tissues, with high levels of NaCl known to alter the composition of plant cells and the concentration of inorganic and organic compounds in plant tissues (Munns and Tester, 2008). For example, using a sequential extraction procedure, Wali *et al.* (2015) found that NaCl changed Cd chemical form in the tissues of *Sesuvium portulacastrum* by increasing the proportion

of Cd bound to pectates, proteins and chloride, with a concomitant increase in Cd tolerance and translocation. However, the sequential extraction can cause experimental artifacts in regard to the Cd speciation. In this regard, synchrotron-based X-ray absorption spectroscopy (XAS) is potentially useful as it allows for *in situ* analyses of metal speciation in hydrated plant tissues. To the best of our knowledge, no study has provided direct (*in situ*) analyses of changes in Cd speciation in plants under dual stresses of Cd and salinity, which is needed to understand the detoxification and accumulation of Cd in halophytic plant species.

This study examined the effect of salt type and concentration on Cd uptake, translocation and accumulation in *C. rossii*. Specifically, it examined whether changes in Cd concentrations were associated with concomitant changes in Cd speciation within those tissues. We hypothesized that 1) the addition of $NaNO_3$ and $Na_2SO_4$, like NaCl, would decrease tissue Cd concentrations; and 2) the decrease in Cd accumulation in shoots by salts would be associated with changes in the levels of physiologically-relevant ligands with a concomitant change in Cd speciation within the plant tissues.

## MATERIALS AND METHODS

### *Plant growth*

The plants of *Carpobrotus rossii* (Haw.) Schwantes (Aizoaceae) were grown in a controlled glasshouse with a 14-h photoperiod at 18-25 °C and 45-65% relative humidity in Victoria, Australia (Cheng *et al.*, 2018). Uniform cuttings (two nodes each cutting) were cut from mother plants and washed with tap water. Then, the cuttings were transplanted to 5-L of continuously-aerated solution containing the following nutrients (μM): $MgSO_4$ 200, $KH_2PO_4$ 10, $K_2SO_4$ 600, $Ca(NO_3)_2$ 600, FeNaEDTA 20, $H_3BO_3$ 5, $MnSO_4$ 1, $CuSO_4$ 0.2, $Na_2MoO_4$ 0.03, $ZnSO_4$ 1. The plants were grown in a controlled-environment growth room with day/night temperatures of 20/18 °C and 50% relative humidity. A 14-h photoperiod with a light intensity of 400 μmol $m^{-2}$ $s^{-1}$ was supplied. The solutions pH was maintained at ca. 6.0 with 1 M KOH and were renewed every 6 d. After rooting and acclimation for two weeks, four rooted cuttings with similar size were weighted and transferred to each of new pots containing the treatment solution described under Experiments 1 and 2 (Cheng *et al.*, 2018). Four additional cuttings were washed with deionized water, oven-dried (80 °C), and weighed to determine their initial weight.

### *Experiment 1*

This experiment aimed to examine the effect of salt type on the uptake, translocation and speciation of Cd in plants after exposure for 10 d. It consisted of four salt treatments (control, 25 mM $NaNO_3$, 12.5 mM $Na_2SO_4$ and 25 mM NaCl) and three Cd concentrations (Table S1). The three Cd levels used were 1) 0 μM (control), 2) a constant Cd concentration of 15 μM (the $Cd^{2+}$ activity varied from 3.2 to 10.9 μM depending upon the salt concentration) and 3) a constant $Cd^{2+}$ activity of 10.9 μM (total Cd concentration varied from 15 to 50 μM depending upon the salt type) (Table S1). The concentration of NaCl (25 mM) was selected because this concentration had no impact on plant growth but significantly decreased Cd accumulation in *C. rossii* in our previous experiment (Cheng *et al.*, 2018). The corresponding concentrations of $NaNO_3$ (25 mM) and $Na_2SO_4$ (12.5 mM) were chosen such that the concentration of Na remained constant. Each treatment was replicated three times and arranged randomly. The speciation of Cd in the nutrient solutions (Table S1) was calculated using the GEOCHEM-EZ program using the default log *K* values for Cd (Table S2) (Shaff *et al.*, 2010). Following their initial growth in basal solutions, plants were grown in the treatment solutions for a further 10 d. Solution pH was buffered with 2 mM MES [2-(N-morpholino) ethanesulphonic acid] and maintained to 6.0 with 1 M KOH. The nutrient solutions had the same composition as the basal nutrient solution. The only exception was that KOH, not $K_2SO_4$, was added as K in the treatment solutions (final K concentration 1210 μM). The solutions were aerated continuously, and renewed every three days.

Ten days after the plants were grown in the treatment solutions, xylem sap was collected as described by Cheng *et al.* (2016). Then, plants were separated into leaves, stems and roots, and

fresh weight recorded. The roots were divided to three parts, with first two parts immersed in ice-cold 20 mM $Ca(NO_3)_2$ for 15 min, washed with deionized water, and frozen in liquid nitrogen. Then the first subsample was stored at -80 °C for later analyses of Cd speciation using synchrotron-based XAS, while the second subsample was freeze-dried for analyses of peptides and organic acids. The third subsamples was immersed in 20 mM $Na_2$-EDTA for 15 min, washed with deionized water, weighed, and oven-dried (80 °C) for chemical analysis. Similarly, after washing with deionized water, shoots were divided into three parts. The relative growth rate (RGR) was calculated according to the equation

$$RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$$

where $W_1$ and $W_2$ were the dry matter at the beginning and the end of the treatment period, and ($t_2$-$t_1$) was the treatment duration (Hunt, 1990).

*Experiment 2*

This experiment studied the effects of NaCl on Cd uptake and translocation within 24 h. It consisted of three treatments and three replicates, with all three treatments having 10.9 µM calculated $Cd^{2+}$ activity. The three treatments were 1) 15 µM Cd with no added salt, 2) 50 µM Cd with 25 mM NaCl [Cd + low salt (LS)], and 3) 85 µM Cd with 50 mM NaCl [Cd + high salt (HS)] (Table S1). The cuttings were prepared as for Experiment 1, and then grown in the same controlled-environment growth room, with samples collected 6, 12 and 24 h after treatment. Plant sample preparation followed the same procedure as outlined for Experiment 1. The Cd concentrations in shoot tissues were too low to permit later analysis using synchrotron-based XAS.

*Analyses of Cd concentration in plant tissues*

Oven-dried tissue samples from Experiments 1 and 2 were digested using concentrated $HNO_3$ in a microwave digester (Multiwave 3000, Anton Paar) after being ground. Elemental concentrations in the digestion solutions and the xylem saps (0.5 ml mixed with 2.5 ml of 5% $HNO_3$) were determined by an inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 8000, MA, USA).

*Cadmium speciation by X-ray absorption spectroscopy (XAS)*

The speciation of Cd in the plant tissues were examined *in situ* at the XAS beamline of the Australian Synchrotron (Victoria, Australia). Details of this beamline, as used for the analysis of Cd in plant tissues have been provided previously (Cheng *et al.*, 2016). For Experiment 1, the spectra of the plant tissues were only collected for the various treatments where the $Cd^{2+}$ activity was maintained constant (i.e. T9-T11, Table S1). For Experiment 2, spectra of the root tissues were collected from all three treatments. The frozen plant tissues (1-2 g) were ground by pestle in an agate mortar filling with liquid nitrogen. The xylem sap was collected from plants and transferred to the Australian Synchrotron immediately before analysis. Both X-ray absorption near edge structure (XANES) and extended absorption fine structure (EXAFS) spectra were collected using a 100-element solid-state Ge detector, with the beam size adjusted to ca. 2.5 mm by 0.2 mm. We had previously assessed beam damage in hydrated plant samples at this beamline, with no damage evident under the current experimental conditions using the cryostat (Cheng *et al.*, 2016).

The XANES and EXAFS spectra were also collected from Cd-containing standard compounds. Our previous study showed that the spectra of compounds with S-containing ligands (such as Cd-GSH and Cd-PC) were difficult to separate using this approach (Cheng *et al.*, 2016). In addition, the spectra of compounds where the Cd is complexed by various carboxyl groups (e.g. Cd-organic acids and Cd-polygalaturonate) were also similar to each other and visually indistinguishable from that of aqueous $Cd(NO_3)_2$ (Cheng *et al.*, 2016). Thus, in the present study, we only prepared four Cd-containing standard compounds. The three aqueous standards were (i) 1 mM $Cd(NO_3)_2$, (ii) 1 mM of $Cd(NO_3)_2$ with 5 mM GSH, and (iii) 1 mM of $Cd(NO_3)_2$ with 100 mM NaCl. Hereafter, the spectra for Cd complexed by GSH are referred to as 'Cd-S' (compounds with S-containing ligands) and aqueous $Cd(NO_3)_2$ are referred to as 'Cd-OH' (Cd either complexed by various carboxyl groups

or present as the free aqueous $Cd^{2+}$ ion). Standards were prepared using 10 mM stock solutions of $Cd(NO_3)_2$ together with 50 mM GSH or 1 M NaCl. Solution standards were then mixed with 30% glycerol, and pH (other than 1 mM $Cd(NO_3)_2$) was adjusted to ca. pH 6 using 0.1 M NaOH. The results using GEOCHEM-EZ modeling indicate that 99% of Cd was $Cd^{2+}$ in the $Cd(NO_3)_2$ standard, and 81.4% with $Cl^-$ in the Cd-NaCl standard. No constant was available in GEOCHEM-EZ for GSH. A solid standard of $Cd_3(PO_4)_2$ was also analyzed after being diluted to 100 mg Cd $kg^{-1}$ using cellulose.

For all XANES and EXAFS analyses, spectra were energy-normalized using the reference energy of the Cd foil, and duplicate spectra for each sample were merged using Athena (version 0.9.22) (Ravel and Newville, 2005). Linear combination fitting (LCF) was performed for the XANES data using Athena and the fitting energy range was –30 to +100 eV relative to the Cd K-edge.

*Analyses of peptides and organic acids in plant tissues*

Four peptides, including GSH, phytochelatin 2 (PC2), phytochelatin 3 (PC3) and phytochelatin 4 (PC4), and three organic acids (citrate, malate and succinate) in plant materials were extracted and analyzed using high performance liquid chromatography mass spectrometry (Agilent 6460 Triple Quadrupole LC/MS) (Cheng *et al.*, 2018).

*Statistical analysis*

One-way analysis of variance was performed to examine for significance ($P \leq 0.05$) of treatment differences using GenStat v. 11 (VSN international).

**RESULTS**

*Experiment 1: Plant growth*

After 10-d treatment, all plants visually appeared to be healthy other than plants in the solutions containing 15 μM Cd in the absence of added salt (T5) which were visibly smaller (Table 1). In solutions without Cd, neither $Na_2SO_4$ nor NaCl influenced plant biomass or RGR, but the addition of $NaNO_3$ increased shoot biomass by 11% ($P<0.05$) compared to the control (T1). In comparison, the addition of 15 μM Cd (T5) in the absence of added salt decreased shoot biomass by 46%, root biomass by 68% and RGR by 57% compared to the control. However, salt addition to these Cd-containing solutions markedly alleviated the Cd-induced growth reduction, with RGR increasing by 56-88% (Table 1). The magnitude of this growth improvement was similar for all three salts ($NaNO_3$, $Na_2SO_4$ and NaCl).

*Experiment 1: Cd concentration and content in plants*

The concentrations of Cd in tissues of plants growing without added Cd (T1-T4) were under the detection limit (<0.03 μg $g^{-1}$) and were not shown. For the remaining treatments, concentrations of Cd were highest in the root (0.79-1.1 mg $g^{-1}$), followed by the stem (0.19-0.50 mg $g^{-1}$), leaf (0.06-0.38 mg $g^{-1}$) and xylem sap (6-25 mg $L^{-1}$) (Fig. 1a-d).

The addition of salt did not impact upon the concentration of Cd in the root tissues, with concentrations in all salt-containing treatments (T6-T11) being similar to that in the salt-free treatment (T5) (Fig. 1a). The only exception was the $Na_2SO_4$ treatment at 15 μM Cd (T7) where the root Cd concentration was 25% lower than in the salt-free treatment. Despite the concentration in the roots being similar irrespective of treatment, the addition of salts decreased the concentration of Cd in xylem sap by 65-75% at 15 μM Cd (T6-T8, Fig. 1b). Accordingly, tissue Cd concentrations in these treatments (T6-T8) were 40-60% lower in stems and 70-80% lower in leaves compared to the salt-free treatment (T5) (Fig. 1c,d). Next, we compared treatments where the calculated $Cd^{2+}$ activity was maintained constant in order to determine whether the decrease in shoot Cd concentrations could be attributed simply to an increase in ionic strength and a concomitant decrease in $Cd^{2+}$ activity. However, similar trends were also observed when plants grown in the treatments with the same $Cd^{2+}$ activity (T9-T11) although the extent of salt effect was smaller.

Specifically, the addition of $NaNO_3$, $Na_2SO_4$ and NaCl decreased the Cd concentration in the xylem sap by 60%, 64% and 30%, and in the leaf tissues by 65%, 85% and 69%, respectively, compared to the salt-free treatment (T5).

The addition of $NaNO_3$, $Na_2SO_4$ or NaCl with 15 μM Cd (T6-T8) decreased Cd accumulation in shoots by 55%, 70% and 68% compared to solutions with Cd alone (T5) (Fig. 1e). Similarly, when plants were grown in solutions at a constant $Cd^{2+}$ activity (T9-T11), the addition of $NaNO_3$, $Na_2SO_4$ and NaCl decreased shoot Cd accumulation by 48%, 66% and 52%, respectively. In contrast, salt addition actually increased Cd accumulation in the roots. Accordingly, all the three salts significantly decreased the Cd translocation factor (the ratio of Cd content in the shoots and the roots) by 66-91%, with the magnitude of this decrease being greater for $Na_2SO_4$ and NaCl than for $NaNO_3$ (Fig. 1f). This marked decrease in the translocation factor was observed regardless of whether Cd was maintained at the same concentration (T6-T8) or the same calculated $Cd^{2+}$ activity (T9-T11).

*Experiment 1: Cd speciation in plant tissues using XAS*

We examined the XANES spectra for the standard compounds. Clear differences could be seen between the spectra of $Cd_3(PO_4)_2$, Cd-OH, Cd-Cl and Cd-S, especially for Cd-S. Specifically, the various standard compounds could be identified based on (i) slight shifts in the energy corresponding to the white line peak, being 26,720.8 eV for Cd-S, 26,720.9 eV for $Cd_3(PO_4)_2$, 26,721.1 eV for Cd-Cl, and 26,721.5 eV for Cd-OH, (ii) differences in the magnitude of this white-line peak, and (iii) shifts in a spectral feature being at 26,762 eV for Cd-OH, at 26,765 eV for $Cd_3(PO_4)_2$, at 26,768 eV for Cd-Cl, and 26,769 eV for Cd-S (Fig. 2).

We then compared the XANES spectra of the root and shoot tissues, and examined whether salt type affected Cd speciation in those tissues. As expected (Cheng *et al.*, 2016), the spectra for both the root and shoot tissues were comparatively featureless (flat) and visually similar to that of Cd-S, with the spectral feature being at 26,769 eV (corresponding to Cd-S) (Figs 2, S1a). Furthermore, these spectra were visually similar irrespective of salt type, indicating that salt type did not alter Cd speciation within these tissues. Indeed, LCF predicted that the majority (62–94%) of Cd in the roots, stems, and leaves was associated with S-containing ligands, with the proportion of Cd-S being higher in leaf tissues (83-94%) than in roots (70-84%) and in stems (62–79%) (Table 2).

In comparison, the spectra of the xylem sap clearly differed, being similar to that of Cd-OH with a spectral feature at 26,763 eV (Figs 2, S1b). The only exception to this was the xylem sap for the NaCl treatment (T11) where the white line peak was at 26,721 eV and the spectral feature was at 26,766 eV (corresponding to Cd-Cl) (Figs 2, S2). This difference was also observed for the EXAFS spectra, with the spectrum of the xylem sap from the NaCl-treated plants (T11) being similar to that of the Cd-Cl standard (Fig. S3). Furthermore, LCF subsequently predicted that Cd-OH was the dominant form (89-94%) in the xylem sap of all treatments except for plants grown in solutions with NaCl where the majority of Cd was present as Cd-Cl (Table 2).

*Experiment 1: Concentrations of GSH, PCs and organic acids in plant tissues*

Compared to the control (T1), the addition of salts in the absence of Cd did not affect the concentration of GSH in plant tissues (Fig. 3a-c). However, the addition of Cd in the absence of salt decreased GSH concentrations in stems and roots by 49% ($P<0.05$), and tended to decrease GSH in the leaf ($P=0.08$). The addition of salts to Cd-containing solutions had little effect on the GSH concentrations in the tissues, expect for the $Na_2SO_4$ treatment at 15 μM Cd (T7), compared to the treatment with Cd alone (T5).

The concentrations of PCs in the plants grown without Cd were below the detection limit (<0.18, 0.13 and 0.10 nmol $g^{-1}$ DW for PC2, PC3 and PC4, respectively) and are therefore not presented. For the remaining treatments (T5-T11), PC3 was the dominate PCs in all the tissues, and the three PCs showed a similar pattern in response to salt and Cd additions (Fig. 3d-f). Compared to the treatment with Cd alone (T5), the addition of salt significantly decreased the concentration of PCs in aerial tissues, especially in the leaf (T6-T11), but did not affect the concentrations of PCs in roots

except for the 25 mM NaCl and 15 μM Cd treatment (T8), irrespective of salt type and solution $Cd^{2+}$ activity.

Overall, in the absence of Cd, $Na_2SO_4$-treated plants had higher concentrations of organic acids compared to the control (T1) but NaCl-treated plants had lower concentrations (Fig. S5). Exposure to Cd decreased the concentrations of organic acids in aerial tissues, especially in stem, but had little effect on the concentration in roots. Compared to the Cd-alone treatment (T5), salt addition to Cd-containing solutions significantly increased the levels of organic acids in shoots, with the strongest effect observed for $Na_2SO_4$. In contrast, the concentration of organic acids in roots was only increased by the addition of $NaNO_3$ and $Na_2SO_4$ but not NaCl.

*Experiment 2: Cd concentration and content in plants*

At the same calculated $Cd^{2+}$ activity in solution (10.9 μM), the addition of 25 mM NaCl increased root tissue Cd concentrations by 23% after 24-h exposure, while the addition of 50 mM NaCl increased root tissue Cd concentrations by 27% after 12-h exposure and by 52% after 24 h (Fig. 4a). However, the opposite trend was observed regarding the concentration of Cd in the shoots; the addition of 25 mM NaCl decreased the concentration of Cd in shoot tissues by 19% after 24-h exposure ($P$>0.05). Moreover, the addition of 50 mM NaCl significantly decreased the shoot Cd concentration by 47% after 6-h exposure and by 45% after 24-h exposure (Fig. 4b). However, the addition of NaCl had little impact on Cd accumulation in whole plants, with the exception of the 50 mM NaCl treatment where Cd accumulation was 23% lower after 24-h exposure (Fig. 4c).

As observed for Experiment 1, the addition of NaCl to solutions with the constant $Cd^{2+}$ activity significantly decreased the Cd translocation factor by 17-63%, 20-25% and 54-148% after exposure for 6, 12 and 24 h, respectively, with 50 mM NaCl having a greater effect than 25 mM NaCl (Fig. 4d).

*Experiment 2: Cd speciation in plant tissues using XAS*

The XANES spectra of the root tissues grown in solutions containing Cd alone were similar for all three periods (6, 12, and 24 h), but differed substantially from the spectra of roots exposed to Cd for 10 d (Figs 2, 5a). Indeed, the spectra of those roots exposed to Cd for ≤24 h were visually similar to that of the Cd-OH standard, with the spectral feature being at ca. 26,763 eV.

The effect of NaCl addition on Cd speciation in roots was then examined within 24 h. After 6 h, the spectra for the roots were visually similar regardless of NaCl treatment, being similar to the Cd-OH standard (Figs 5a, S4a). However, these spectra for the root tissues grown in NaCl-containing solutions appeared to differ over time, with increasing exposure time (≤24 h) decreasing the magnitude of the white peak line together with a shift in the feature from ca. 26,760 eV to 26,770 eV (Fig. 5a). After 12 h, the magnitude of the white peak line was lower in the roots of plants treated with 25 mM NaCl than that of other plants, with a shift in the feature from ca. 26,763 eV (corresponding to Cd-OH) to 26,768 eV (corresponding to Cd-Cl) (Figs 5a, S4b). In a similar manner, after 24 h, the addition of NaCl changed the spectra of the root tissues at both 25 and 50 mM (Figs 5a, S4c). For the spectra of NaCl-treated roots, the magnitude of the white peak line was lower together with a shift in the feature from ca. 26,760 eV to 26,770 eV. Thus, it appeared that the addition of NaCl for 12 or 24 h resulted in a shift in speciation from Cd-OH to Cd-Cl. This was also in accordance with the EXAFS spectra, with an apparent shift from the peak at k = 3.5 $Å^{-1}$ to 3.7 $Å^{-1}$ following the addition of 25 mM NaCl for 12 h (Fig. 5b, c).

We also used LCF to predict the speciation of the Cd in the roots, with 70% of Cd present as Cd-OH in the roots of plants grown in the absence of added salt for ≤24 h. In contrast, upon the addition of 25 mM NaCl, the proportion of Cd-OH decreased due to the increased formation of Cd-S. Indeed, the amount of Cd predicted to be present as Cd-S increased from 28% after 6 h to 61% after 24 h (Table 3).

*Experiment 2: Concentrations of GSH, PCs and organic acids in plant tissues*

The GSH concentrations in the roots of NaCl-treated plants increased by 70% within 24-h exposure, while the concentration in the roots exposed to the Cd-alone treatment did not change significantly (Fig. 6a). In contrast, increasing the exposure time, especially after 12 h, increased the concentrations of PCs in roots with the magnitude of increase being similar for all roots (Fig. 6b, d). Moreover, the addition of NaCl did not affect the concentration of GSH or PCs in roots at any exposure time. The only exception was that after 24-h exposure to Cd, 50 mM NaCl increased the concentrations of GSH, PC2, PC3 and PC4 in the roots by 28%, 32%, 37% and 45%, respectively. In comparison, the concentration of organic acids in root tissues decreased slightly with exposure time, especially after 24 h exposure (Fig. S6). Different with the effect on peptides, NaCl addition did not affect the concentration of organic acids in root tissues within 12 h, but decreased the concentration by 15-35% after 24 h exposure.

## DISCUSSION

*Salinity decreased Cd accumulation in plant shoots*

In all Cd-containing solutions, the addition of any of the three salts (NaCl, $Na_2SO_4$, and $NaNO_3$) improved plant growth and decreased shoot Cd concentration after 10-d growth, irrespective of whether the Cd concentration or the $Cd^{2+}$ activity was maintained constant (Table 1; Fig. 1). However, this decreased Cd concentration in shoots of *C. rossii* was not caused by a 'dilution effect'. Rather, the amount of Cd accumulated in both the shoot and whole plants was significantly lower in salt-treated plants (Fig. 1), with this being consistent with our previous observation in this same plant species for NaCl. However, this differs from previous observations in the halophyte *A. halimus* where, NaCl decreased but $NaNO_3$ increased leaf-tissue Cd concentrations (Lefèvre *et al.*, 2009). Although these authors did not discuss possible reasons for this increase in Cd concentration upon exposure to $NaNO_3$, it is clear that the addition of $NaNO_3$ (but not NaCl or KCl) greatly decreased shoot dry weight and water content (Lefèvre *et al.*, 2009). Thus, the increased Cd concentration in leaf tissues of *A. halimus* caused by $NaNO_3$ (Lefèvre *et al.*, 2009) might be related to toxicity under dual stresses of $NaNO_3$ and Cd. In comparison, in our present study, all three salts similarly alleviated the deleterious effect of Cd on plant growth. It is unclear whether the decreased Cd accumulation in *C. rossii* by salts was the consequence of decreased Cd uptake by roots and/or Cd translocation from roots to shoots.

Interestingly, salt addition did not alter the Cd content of the whole plant but decreased the translocation factor markedly after 6 or 12 h (Fig. 4), confirming that salt addition reduced Cd translocation from root to shoot comparatively rapidly without influencing Cd uptake. Similar observations that NaCl decreased Cd root-to-shoot translocation in *Nicotiana tabacum* and halophyte *Kosteletzkya virginica* were also reported (Han *et al.*, 2012; Zhang *et al.*, 2013), although NaCl was proposed to enhance Cd translocation in plants through the formation of $CdCl^+$ which is of increased mobility within plants (Clarke *et al.*, 2002; Ozkutlu *et al.*, 2007; Wali *et al.*, 2015).

*Salinity decreased translocation of Cd*

To understand if this salt-induced decrease in Cd root-to-shoot translocation was due to a change in Cd speciation within the plant, we examined the effect of salts on Cd speciation using synchrotron-based XAS and the concentrations of physiologically relevant ligands. The majority of Cd was associated with S-containing ligands in both the roots and shoots after 10-d exposure to Cd, which is consistent with previous findings (Cheng *et al*., 2016). Determination of the peptides showed that thiols from PCs, particular PC3, contributed up to 98% of total thiol groups (sum of GSH and PCs) in those tissues, confirming the important role of PCs in Cd accumulation and complexation. However, the addition of Na salts did not alter Cd speciation in either the shoots or the roots when examined after 10-d exposure (Fig. 2; Table 2), although the salts greatly decreased the concentrations of PCs but increased organic acids in plants (Figs 3, S5). In our study, the molar ratio of PC-SH:Cd was greater than 4 in all roots and shoots irrespective of salt addition, which greatly exceeds the expected values of 2-4 for the Cd-PC complexes (Salt *et al.*, 1997; Rauser, 1999; Vázquez *et al.* 2006). Furthermore, the molar ratio for the COOH:Cd was greater than 7:1,

indicating that all the relevant ligands were over-saturated in the tissues of *C. rossii* and not all of them were bound to Cd *in vivo* (Salt *et al.*, 1995; Salt *et al.*, 1997).

The finding that Cd translocated as Cd-OH in xylem sap was consistent with previous study (Cheng *et al*., 2016). However, an interesting exception was that the Cd in the xylem sap of NaCl-treated plants was present as Cd-Cl rather than as Cd-OH as observed for the other treatments (Fig. S2; Table 2). Presumably, this formation of Cd-Cl in the xylem sap of NaCl-treated plants is due to the elevated concentration of Cl in these plants. Regardless, it seemed that Cd speciation within the plant tissues after 10-d exposure could not explain the earlier observations that salts altered the tissue concentrations of Cd.

We then considered tissue Cd speciation of plants grown in solutions for ≤24 h. Interestingly, in the roots of these plants grown with Cd alone, Cd speciation changed depending on the duration of Cd-exposure. For plants exposed to Cd for only 24 h (Experiment 2), the majority of Cd in the root was as Cd-OH rather than Cd-S whilst the majority of Cd in plants was associated with S-containing ligands after 10-d exposure (Experiment 1). Similar observations were reported previously for 5-day-old seedlings of *Brassica juncea*, with 65% of Cd associated with O after 6-h exposure but with 60% associated with S after exposure to Cd for 48 h (Salt *et al.*, 1997). These findings suggest that O-containing ligands (such as the pectin of the cell wall or simple organic acids) play an important role in the detoxification of Cd during the initial stages when the production of PCs is limited or when exposure to lower levels of Cd (Wagner, 1993). However, similar to *B. juncea*, with increasing exposure time and with increased Cd accumulation, chelation of Cd with S-containing compounds becomes the dominant detoxification mechanism in *C. rossii*.

Unlike the effect of salt on roots exposed to Cd for 10 d, NaCl addition altered Cd speciation in the roots of these shorter-term treatments (≤24 h). It was evident that increasing the exposure time from 6 to 24 h increased the proportion of Cd-S in the roots from 27.8 to 61.4% at 25 mM NaCl and from 22.0 to 35.2% at 50 mM NaCl, with the proportion being constant during the period in the roots exposed to Cd alone (Fig. 5a; Table 3). It is not clear why the conversion from Cd-OH to Cd-S was slower in solutions containing 50 mM NaCl. However, it is possible that the proportion of Cd associated with the cell wall was higher in the roots at 50 mM than at 25 mM NaCl. Meychik *et al*. (2005) observed that the concentrations of polygalacturonic acid groups in cell walls was higher for plants of halophyte *Suaeda altissima* grown at 250 mM NaCl than those at 0.3 mM NaCl. Similarly, Mariem *et al.* (2014) found that halophyte *S. portulacastrum* grown at 200 mM NaCl contained proportionally more Cd bound to the cell walls than plants grown at 0.09 mM NaCl. However, in our study, it was not possible to distinguish between the free-$Cd^{2+}$ ion, Cd associated with the cell wall, and Cd complexed with organic acids.

It was hypothesized that the increasing formation of Cd-S in plant roots over 24 h was due to increased synthesis of PCs, since salinity may increase antioxidant defense, particular for the halophytes, and thus increase GSH concentration shortly (Xu *et al*. 2010; Lutts and Lefèvre, 2015). However, NaCl addition did not increase the concentration of GSH or PCs in roots (except for 50 mM after 24 h) although the magnitude of the increase in GSH concentration with time in the NaCl-treated roots was greater (Fig. 6). This differs from the observation that salt increased the concentrations of both GSH and PCs in *A. thaliana* (Xu *et al*. 2010). The lack of NaCl effects on the concentrations of PCs might be due to the high efficiency of PC synthesis in *C. rossii* within short-time exposure to Cd. It was evident that the concentration of thiols from PCs in the roots was 3-fold higher than the concentration of GSH after 6-h and increased by 12-fold after 24 h, regardless of NaCl treatment, suggesting that GSH was not the limiting factor for the synthesis of PCs in plant tissues. Considering the tissue Cd concentrations, the molar ratio of PC-SH:Cd was smaller in the roots of NaCl-treated plants (T3-T7) than in the plants treated with Cd alone (T5-T8) within 24-h exposure. This could not explain the higher proportion of Cd-S complexes in the roots of NaCl-treated plants. Therefore, the present results imply that the increased Cd-S complexes by salt in *C. rossii* did not result from an increased accumulation of GSH or PCs.

It is unknown why NaCl addition increased the proportion of Cd-S in the roots after 24 h, which requires further study. One possibility is that the $Na^+$ in plant roots receiving NaCl might compete

for the cation/$H^+$ antiporter with $Cd^{2+}$ to move across the tonoplast and/or change the electrochemical potential difference for $H^+$, which energizes the pumping of $Cd^{2+}$ and $Na^+$ into the vacuole (Jones and Gorham, 2002; Flowers and Colmer, 2008; Munns and Tester, 2008). To maintain the low level of $Cd^{2+}$ in the cytoplast, more $Cd^{2+}$ shifted to bind with S-containing groups in the cytoplast, then transported to the vacuole and stored as inactive Cd-complexes with high-molecular-weight compounds (Clemens *et al.*, 2002; Clemens, 2006).

Given that the complexes of Cd-S are not transported from roots to shoots via the xylem (Table 2), the decreased Cd root-to-shoot translocation and shoot accumulation in NaCl-treated plants could be attributed to the increased Cd retention in roots in the form of Cd-S within 24 h after Cd treatment. In addition, the decreased Cd translocation by salt could also be partly attributed to other possible reasons such as the distribution of Cd inside root cells (Clemens, 2006; Mariem *et al.*, 2014), expression of Cd transporter AtHMA4 that mediated the xylem loading of Cd and/or activity of $Cd^{2+}/2H^+$ antiporters that transported the free $Cd^{2+}$ ions into the vacuole (Clemens, 2006; Xu *et al*., 2010), or the changes of leaf transpiration (Liu et al., 2010), but these hypotheses require to be further tested.

*Cd-Cl complexes formed within plant tissues*

In the roots of NaCl-treated plants for ≤ 24 h, up to two-thirds of the Cd was present as Cd-Cl (Figs 5, S4; Table 3). To our knowledge, this study provides the first direct evidence for the presence of Cd-Cl complexes within plant tissues. Other studies have shown that the formation of Cd-Cl complexes within the bulk nutrient solution increases the concentrations of Cd in plant tissues when the $Cd^{2+}$ activity was maintained in bulk solutions (Smolders and McLaughlin 1996; Xu *et al*. 2010). Regardless, in our present study, it remains unknown whether these Cd-Cl complexes were taken up by the plant from the bulk nutrient solution or whether they formed *in planta*. We contend that the Cd-Cl complexes were more likely formed *in planta* rather than being taken up directly by roots. Firstly, if the roots had taken up Cd-Cl complexes directly, NaCl-treated plants would be expected to have a higher Cd accumulation since the solutions had the same $Cd^{2+}$ activity (10.9 μM) but at least three-fold higher total Cd concentration (15 μM compared to 50 and 85 μM, Table 1) (Smolders and McLaughlin, 1996). Secondly, high proportions of Cd-Cl complexes were only found in root tissues after 12-h exposure, with the majority of the root Cd being present as Cd-OH after 6-h exposure. Thus, the substantial shift in Cd speciation from Cd-OH (6 h) to Cd-Cl (12 and 24 h) in NaCl-treated plants was more likely to be the consequence of the subsequent accumulation of Cl within the plant tissues (particularly the vacuoles) rather than the direct uptake of Cd-Cl complexes.

Regardless, the question remains, why did Cd-Cl complexes form in the shorter-term but Cd-S complexes form in the longer-term? We propose that for plants exposed to Cd for ≤24 h, the comparatively rapid accumulation of Cl within the plant tissues results in the formation of Cd-Cl complexes. However, in the longer term (10 d), Cd-S complexes form due to their higher stability constants. Indeed, Cd has similar stability constants for malate (1.3-2.4), succinate (1.0-2.8) and Cl (1.3-2.8), but higher stability constants for citrate (1.0-4.5) and thiol groups (7.0-12) (Martell and Smith, 1974).

## CONCLUSION

To our knowledge, this is the first study to provide direct analyses of Cd speciation and relevant ligands in plants under dual stresses of Cd and salinity. We found that salt addition decreased Cd translocation in *C. rossii* by forming Cd-Cl complexes and increasing the proportion of Cd-S in root tissues within 24 h. However, the increased Cd-S complexes were not due to the increased synthesis of GSH or PCs in plant tissues. Further work should focus on the mechanisms of the increased Cd-S complex in salt-treated plants, which could provide important information for improving phytoremediation efficiency and decreasing Cd intake by food crops in Cd-contaminated saline soils.

**ACKNOWLEDGEMENTS**
This research was undertaken on the XAS beamline at the Australian Synchrotron (AS162/XAS/10855), Victoria, Australia. We thank Drs. Peter Kappen and Bernt Johannessen (Australian Synchrotron) for their advice on use of XAS, Prof. Peng Wang for the valuable suggestions to the experimental design, Mr. James O'Sullivan for helping with the XAS analyses, and Dr. Zhiqian Liu for assistance with the LC-MS. Funding from the Australian Research Council for a Future Fellowship for P.M.K. (FT120100277) is acknowledged.

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**Table 1.** Dry weight of shoots and roots, and relative plant growth rate of *Carpobrotus rossii* grown in different treatments for 10 d. Error bars represent ± SEM of three replicates. Means with the same letter did not differ significantly (Duncan's test, $P<0.05$) (Experiment 1).

| Treatment | | | | Dry weight (g plant$^{-1}$) | | |
|---|---|---|---|---|---|---|
| Code | Salt (mM) | Cd (μM) | $Cd^{2+}$ activity (μM) | Root | Shoot | Relative growth rate (mg g$^{-1}$ d$^{-1}$) |
| T1 | 0 | 0 | 0 | 0.25 a | 1.81 b | 94.2 ab |
| T2 | 25 mM $NaNO_3$ | 0 | 0 | 0.26 a | 2.07 a | 105.1 a |
| T3 | 12.5 mM $Na_2SO_4$ | 0 | 0 | 0.24 a | 1.75 b | 98.0 ab |
| T4 | 25 mM NaCl | 0 | 0 | 0.24 a | 1.90 ab | 87.3 b |
| T5 | 0 | 15 | 10.9 | 0.08 d | 0.97 d | 39.8 e |
| T6 | 25 mM $NaNO_3$ | 15 | 7.4 | 0.14 cd | 1.26 c | 74.9 c |
| T7 | 12.5 mM $Na_2SO_4$ | 15 | 3.9 | 0.22 ab | 1.04 cd | 73.3 c |
| T8 | 25 mM NaCl | 15 | 3.2 | 0.17 bc | 1.09 cd | 74.4 c |
| T9 | 25 mM $NaNO_3$ | 22 | 10.9 | 0.10 cd | 1.12 cd | 67.7 cd |
| T10 | 12.5 mM $Na_2SO_4$ | 42 | 10.9 | 0.11 cd | 1.02 cd | 62.1 d |
| T11 | 25 mM NaCl | 50 | 10.9 | 0.13 cd | 1.10 cd | 67.3 cd |

**Table 2.** The predicted speciation of Cd in various tissues of *Carpobrotus rossii* grown for 10 d in solutions with the same $Cd^{2+}$ activity (10.9 µM), as calculated using linear combination fitting (LCF) of the K-edge XANES spectra (Experiment 1).

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Salt | Cd | | Cd-OH | | |
| Code | (mM) | (µM) | R-factor | (%) | Cd-S (%) | Cd-Cl (%) |
| *Root* | | | | | | |
| T5 | 0 | 15 | 0.001316 | 23.2 (0.9) | 76.8 (2.7) | - |
| T9 | 25 mM $NaNO_3$ | 22 | 0.001027 | 15.7 (0.8) | 84.3 (2.5) | - |
| T10 | 12.5 mM $Na_2SO_4$ | 42 | 0.000943 | 29.8 (0.8) | 70.2 (2.6) | - |
| T11 | 25 mM NaCl | 50 | 0.001235 | 19.2 (0.9) | 80.8 (2.8) | - |
| *Xylem sap* | | | | | | |
| T5 | 0 | 15 | 0.000357 | 89.3 (1.1) | 10.7 (1.8) | - |
| T9 | 25 mM $NaNO_3$ | 22 | 0.000816 | 93.6 (3.3) | 6.4 (0.8) | - |
| T10 | 12.5 mM $Na_2SO_4$ | 42 | 0.000335 | 91.9 (5.2) | 8.1 (0.5) | - |
| T11 | 25 mM NaCl | 50 | 0.000101 | 24.9 (3.4) | - | 75.1 (0.7) |
| *Stem* | | | | | | |
| T5 | 0 | 15 | 0.000418 | 38.1 (0.5) | 61.9 (1.4) | - |
| T9 | 25 mM $NaNO_3$ | 22 | 0.000585 | 38.8 (3.3) | 61.2 (0.6) | - |
| T10 | 12.5 mM $Na_2SO_4$ | 42 | 0.000369 | 30.2 (0.5) | 69.8 (2.1) | - |
| T11 | 25 mM NaCl | 50 | 0.000490 | 21.4 (0.6) | 78.6 (2.0) | - |
| *Leaf* | | | | | | |
| T5 | 0 | 15 | 0.000384 | 11.7 (0.5) | 88.3 (2.1) | - |
| T9 | 25 mM $NaNO_3$ | 22 | 0.000927 | 11.8 (1.2) | 88.2 (0.8) | - |
| T10 | 12.5 mM $Na_2SO_4$ | 42 | 0.001401 | 17.3 (1.4) | 82.7 (1.0) | - |
| T11 | 25 mM NaCl | 50 | 0.000748 | 5.7 (0.7) | 94.3 (1.0) | - |

The values in brackets show the percentage variation in the calculated values. The goodness of fit is indicated by the R-factor. $R\ factor = \Sigma_i\ (experiment - fit)^2 / \Sigma_i\ (experimental)^2$, where the sums are over the data points in the fitting region.

**Table 3.** The predicted speciation of Cd in the roots of *Carpobrotus rossii* grown for ≤ 24 h in solutions with the same $Cd^{2+}$ activity (10.9 μM), as calculated using linear combination fitting (LCF) of the K-edge XANES spectra (Experiment 2).

| Treatment | | | R-factor | Cd-OH (%) | Cd-S (%) | Cd-Cl (%) |
|---|---|---|---|---|---|---|
| Code | NaCl (mM) | Cd (μM) | | | | |
| Cd | | | | | | |
| 6h | 0 | 15 | 0.002689 | 71.7 (6.2) | 28.3 (1.4) | - |
| 12h | 0 | 15 | 0.001856 | 69.5 (6.6) | 30.5 (1.2) | - |
| 24 h | 0 | 15 | 0.002303 | 70.1 (1.3) | 29.9 (2.0) | - |
| Cd + LS | | | | | | |
| 6h | 25 | 50 | 0.002436 | 72.2 (1.3) | 27.8 (4.0) | - |
| 12h | 25 | 50 | 0.000526 | - | 36.4 (3.5) | 63.6 (0.9) |
| 24 h | 25 | 50 | 0.004027 | - | 61.4 (2.5) | 38.6 (9.8) |
| Cd + HS | | | | | | |
| 6h | 50 | 85 | 0.003031 | 78.0 (1.5) | 22.0 (4.5) | - |
| 12h | 50 | 85 | 0.002435 | 82.8 (1.3) | 17.2 (2.3) | - |
| 24 h | 50 | 85 | 0.001513 | - | 35.2 (5.5) | 64.8 (1.6) |

The values in brackets represent the percentage variation in the calculated values. The goodness of fit is indicated by the R-factor. $R\ factor = \Sigma_i\ (experiment - fit)^2 / \Sigma_i\ (experimental)^2$, where the sums are over the data points in the fitting region.

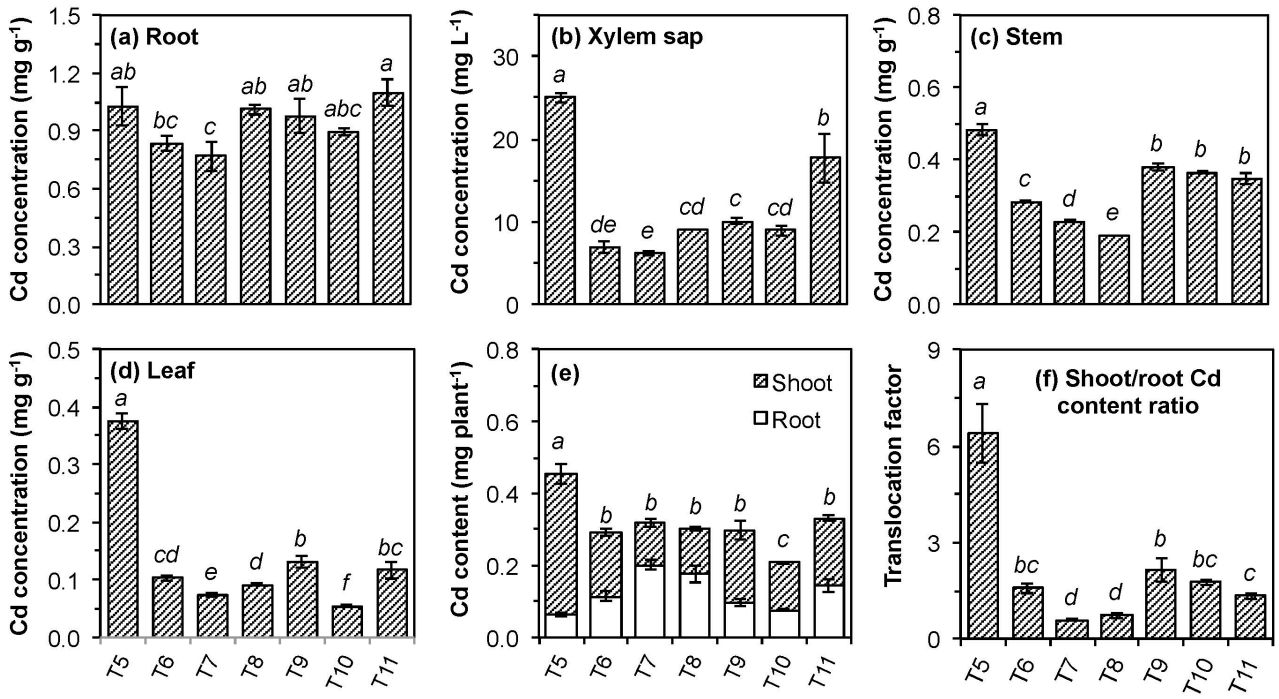


**Figure 1.** Concentrations of Cd in roots (a), xylem saps (b), stems (c) and leaves (d), total Cd content (e), and Cd translocation factor (e) of *Carpobrotus rossii* grown in different treatments for 10 days. T5: 15 μM Cd; T6: 15 μM Cd + 25 mM $NaNO_3$; T7: 15 μM Cd + 12.5 mM $Na_2SO_4$; T8: 15 μM Cd + 25 mM NaCl; T9: 22 μM Cd + 25 mM $NaNO_3$; T10: 42 μM Cd + 12.5 mM $Na_2SO_4$;

T11: 50 µM Cd + 25 mM NaCl. Error bars represent ± SEM of three replicates. Means with the same letter did not differ significantly (Duncan's test, $P<0.05$) (Experiment 1).

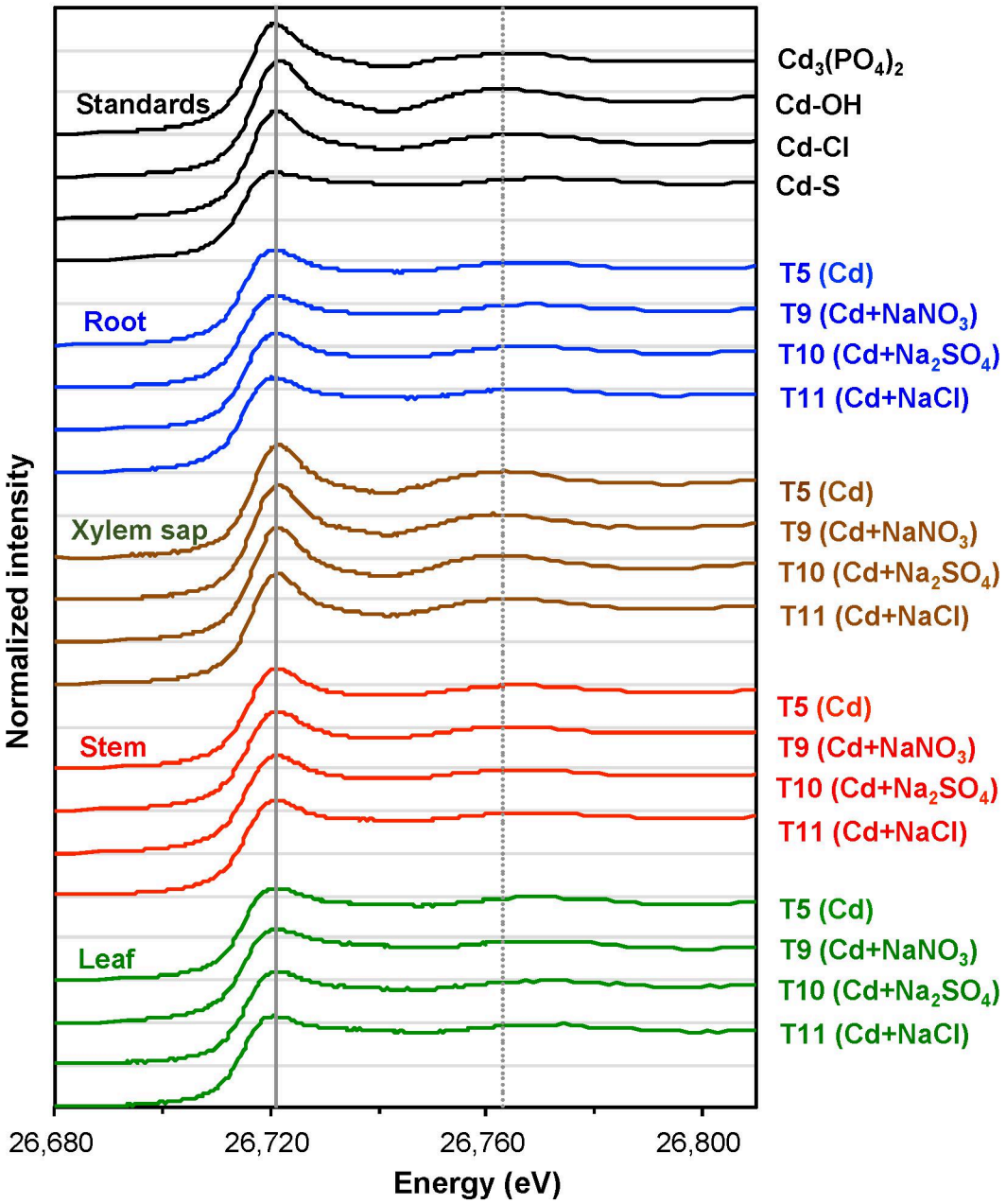


**Figure 2.** Nomalized Cd-edge XANES spectra of the Cd standards and various tissues of *Carpobrotus rossii* grown 10 d in solutions with the same $Cd^{2+}$ activity (10.9 µM). T5: 15 µM Cd; T9: 22 µM Cd + 25 mM $NaNO_3$; T10: 42 µM Cd + 12.5 mM $Na_2SO_4$; T11: 50 µM Cd + 25 mM NaCl. The horizontal grey lines represent a value of 1 for each of the normalized spectra, while the vertical grey lines represent white-line peak at 26,721.5 eV (solid line) and the spectral feature at 26,762 eV (dotted line) for Cd-OH standard (Experiment 1).

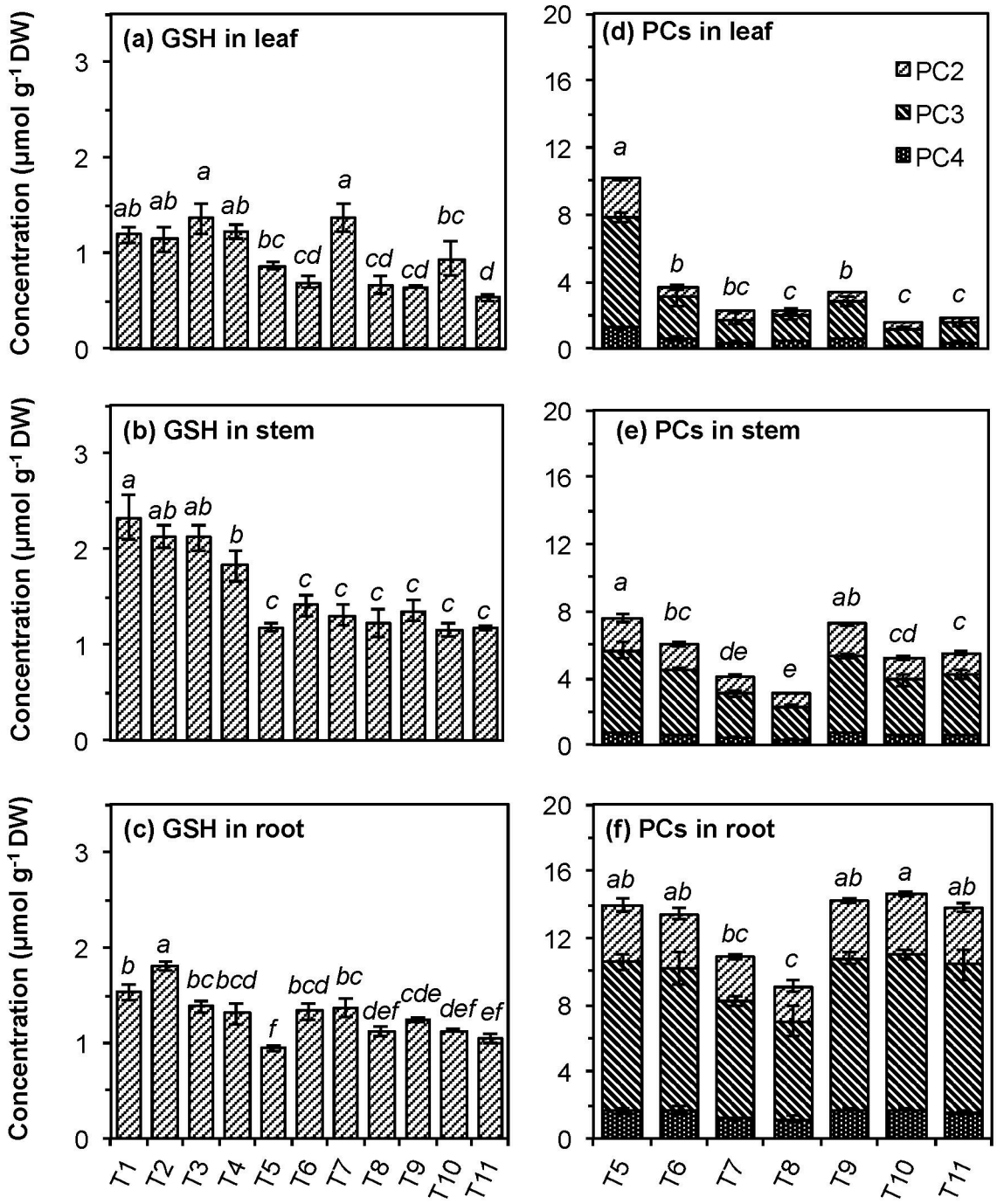


**Figure 3.** Concentrations of GSH (a, b, c) and PCs (d, e, f) in leaves (a, d), stem (b, e), and roots (c, f) of *Carpobrotus rossii* grown in different treatments for 10 d. T1: control; T2: 25 mM $NaNO_3$; T3, 12.5 mM $Na_2SO_4$; T4: 25 mM NaCl; T5: 15 µM Cd; T6: 15 µM Cd + 25 mM $NaNO_3$; T7: 15 µM Cd + 12.5 mM $Na_2SO_4$; T8: 15 µM Cd + 25 mM NaCl; T9: 22 µM Cd + 25 mM $NaNO_3$; T10: 42 µM Cd + 12.5 mM $Na_2SO_4$; T11: 50 µM Cd + 25 mM NaCl. Error bars represent ± SEM of three replicates. Means with the same letter did not differ significantly (Duncan's test, $P<0.05$) (Experiment 1).

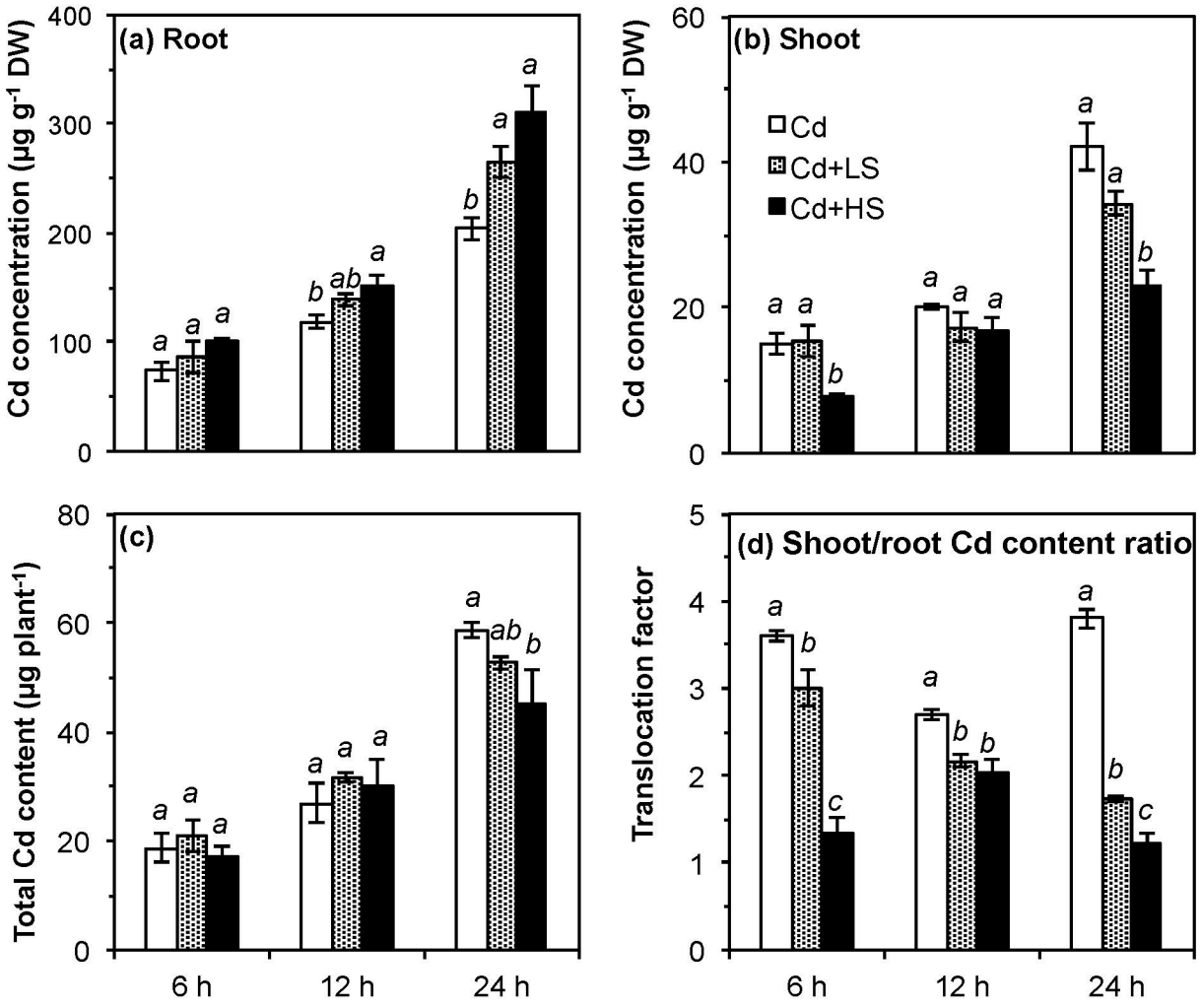


**Figure 4.** Concentrations of Cd in roots (a) and shoots (b), total Cd content (c), and translocation factor (d) of *Carpobrotus rossii* grown in solutions with same $Cd^{2+}$ activity (10.9 µM) for 6, 12 and 24 h. Cd: 15 µM Cd; Cd +LS (low salt): 50 µM Cd + 25 mM NaCl; Cd +HS (high salt): 85 µM Cd + 50 mM NaCl. Error bars represent ± SEM of three replicates. Means with the same letter did not differ significantly at each harvest time (Duncan's test, $P<0.05$) (Experiment 2).

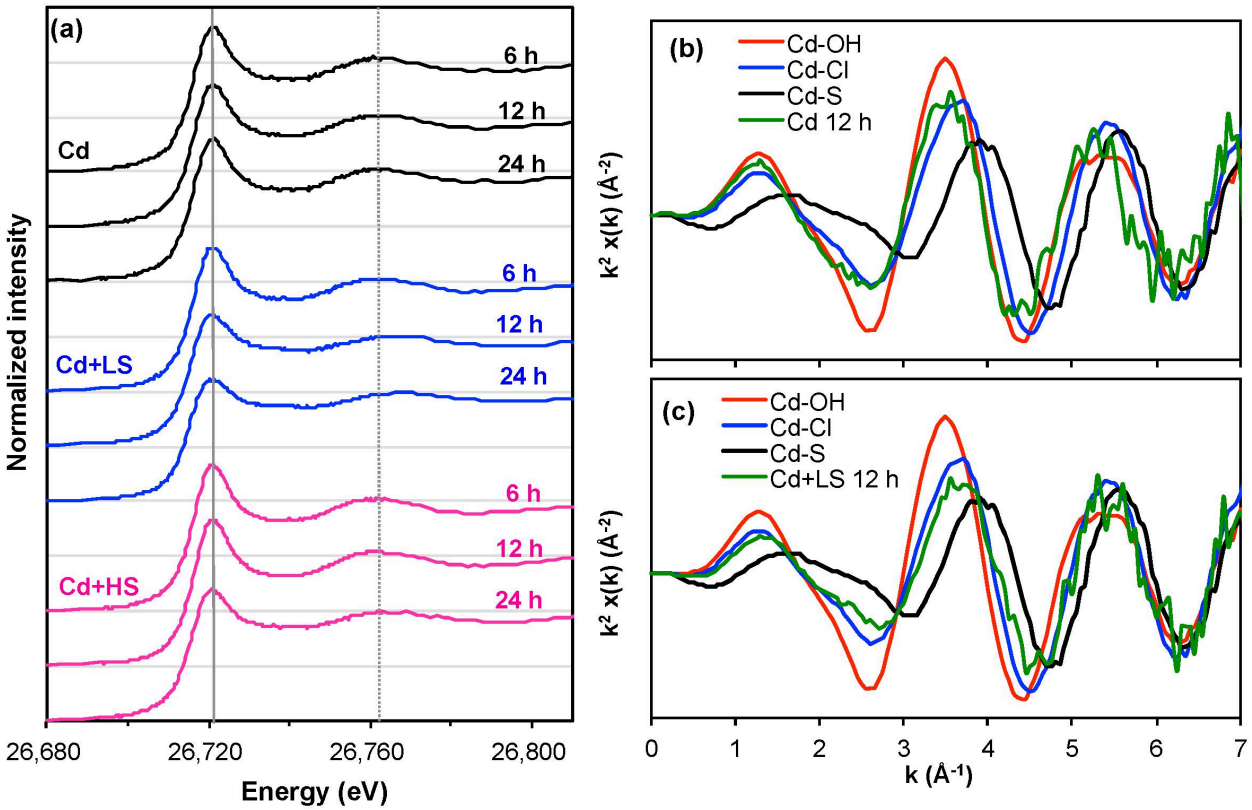


**Figure 5.** Nomalized Cd-edge XANES spectra of the roots of *Carpobrotus rossii* grown in solutions with the same $Cd^{2+}$ activity (10.9 µM) for 6, 12 and 24 h (a), and $k^2$-weighted EXAFS spectra of standards plus roots of plants exposed to Cd (b) and Cd +LS (b) after 12 h. Cd: 15 µM Cd; Cd +LS (low salt): 50 µM Cd + 25 mM NaCl; Cd +HS (high salt): 85 µM Cd + 50 mM NaCl. The horizontal grey lines represent a value of 1 for each of the normalized spectra, while the vertical grey lines represent white-line peak at 26,721.5 eV (solid line) and the spectral feature at 26,762 eV (dotted line) for Cd-OH standard (Experiment 2).

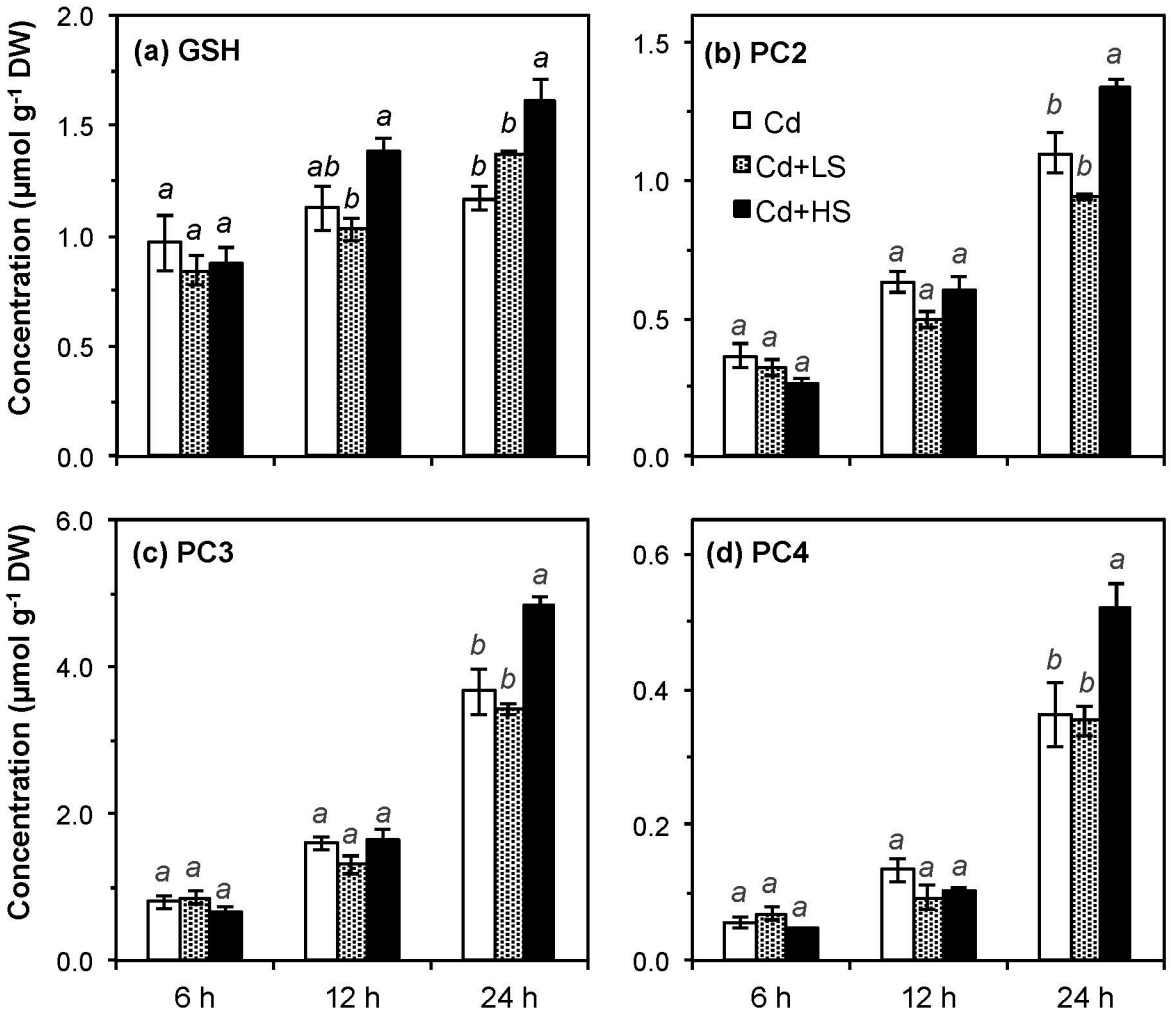


**Figure 6.** Concentrations of GSH (a), PC2 (b), PC3 (c) and PC4 (d) in roots of *Carpobrotus rossii* grown in solutions with the same $Cd^{2+}$ activity (10.9 µM) for 6, 12 and 24 h. Cd: 15 µM Cd; Cd +LS (low salt): 50 µM Cd + 25 mM NaCl; Cd +HS (high salt): 85 µM Cd + 50 mM NaCl. Error bars represent ± SEM of three replicates. Means with the same letter did not differ significantly at each harvest time (Duncan's test, $P<0.05$) (Experiment 2).

**Supporting information**

# Salinity decreases Cd translocation by altering Cd speciation in the halophytic Cd-accumulator *Carpobrotus rossii*

Miaomiao Cheng[1], Peter M. Kopittke[2], Anan Wang[1] and Caixian Tang[1]

[1] Department of Animal, Plant and Soil Sciences, Centre for AgriBioscience, La Trobe University, Victoria, 3086, Australia

[2] School of Agriculture and Food Sciences, The University of Queensland, Queensland, 4072, Australia

**Table S1.** The composition of nutrient solutions used for Experiments 1 and 2. Only the salts and Cd concentrations are shown, with basal nutrients added at the concentrations listed in the *Materials and Methods*.

| | Experiment 1 | | | | | | | | | | | Experiment 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment code → | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | Cd | Cd+LS | Cd+HS |
| Salt form | - | $NaNO_3$ | $Na_2SO_4$ | NaCl | - | $NaNO_3$ | $Na_2SO_4$ | NaCl | $NaNO_3$ | $Na_2SO_4$ | NaCl | - | NaCl | NaCl |
| Salt concentration (mM) | - | 25 | 12.5 | 25 | - | 25 | 12.5 | 25 | 25 | 12.5 | 25 | - | 25 | 50 |
| Cd (μM) | - | - | - | - | 15 | 15 | 15 | 15 | 22 | 40 | 50 | 15 | 50 | 85 |
| $Cd^{2+}$ activity (μM) | - | - | - | - | 10.9 | 7.4 | 3.9 | 3.2 | 10.9 | 10.3 | 10.9 | 10.9 | 10.9 | 10.9 |
| Cd speciation (%) | | | | | | | | | | | | | | |
| $Cd^{2+}$ | - | - | - | - | 94.2 | 93.6 | 52.7 | 41.1 | 93.9 | 53.0 | 41.4 | 94.2 | 41.4 | 28.7 |
| $CdSO_4^0$ | - | - | - | - | 3.3 | 1.5 | 45.6 | 0.7 | 1.5 | 45.8 | 0.7 | 3.3 | 0.7 | 0.3 |
| $CdCl^+$ | - | - | - | - | 0 | 0 | 0 | 56.8 | 0 | 0 | 57.0 | 0 | 57.0 | 70.4 |
| $CdNO_3^+$ | - | - | - | - | 0.2 | 2.6 | 0.1 | 0.1 | 2.6 | 0.1 | 0.1 | 0.2 | 0.1 | 0 |
| $CdEDTA^{2-}$ | - | - | - | - | 2.3 | 2.3 | 1.6 | 1.3 | 2.0 | 1.1 | 0.9 | 2.3 | 0.9 | 0.6 |

The speciation of Cd in the bulk nutrient solution was calculated using GEOCHEM-PC. -, no addition or not applicable

**Table S2**. The default reaction constants (log *K* values) used in the GEOCHEM-EZ to calculate the speciation of Cd in the nutrient solutions.

| | *log K* |
|---|---|
| $Cd^{2+} + H_2O \rightleftharpoons CdOH^+ + H^+$ | -9.8 |
| $Cd^{2+} + 2H_2O \rightleftharpoons Cd(OH)_2^0 + 2H^+$ | -20.19 |
| $Cd^{2+} + 3H_2O \rightleftharpoons Cd(OH)_3^- + 3H^+$ | -33.5 |
| $2Cd^{2+} + H_2O \rightleftharpoons Cd_2OH^{3+} + H^+$ | -8.7 |
| $Cd^{2+} + Cl^- \rightleftharpoons CdCl^+$ | 2.0 |
| $Cd^{2+} + 2Cl^- \rightleftharpoons CdCl_2^0$ | 2.6 |
| $Cd^{2+} + 3Cl^- \rightleftharpoons CdCl_3^-$ | 2.4 |
| $Cd^{2+} + 4Cl^- \rightleftharpoons CdCl_4^{2-}$ | 2.5 |
| $Cd^{2+} + SO_4^{2-} \rightleftharpoons CdSO_4^0$ | 2.5 |
| $Cd^{2+} + PO_4^{3-} \rightleftharpoons CdPO_4^-$ | 15.6 |
| $Cd^{2+} + EDTA^{4-} \rightleftharpoons CdEDTA^{2-}$ | 18.2 |
| $2Cd^{2+} + EDTA^{4-} \rightleftharpoons Cd_2EDTA^0$ | 21.6 |
| $Cd^{2+} + NO_3^- \rightleftharpoons CdNO_3^+$ | 0.3 |
| $Cd^{2+} + 2NO_3^- \rightleftharpoons Cd(NO_3)_2^0$ | 0.01 |

**Results**

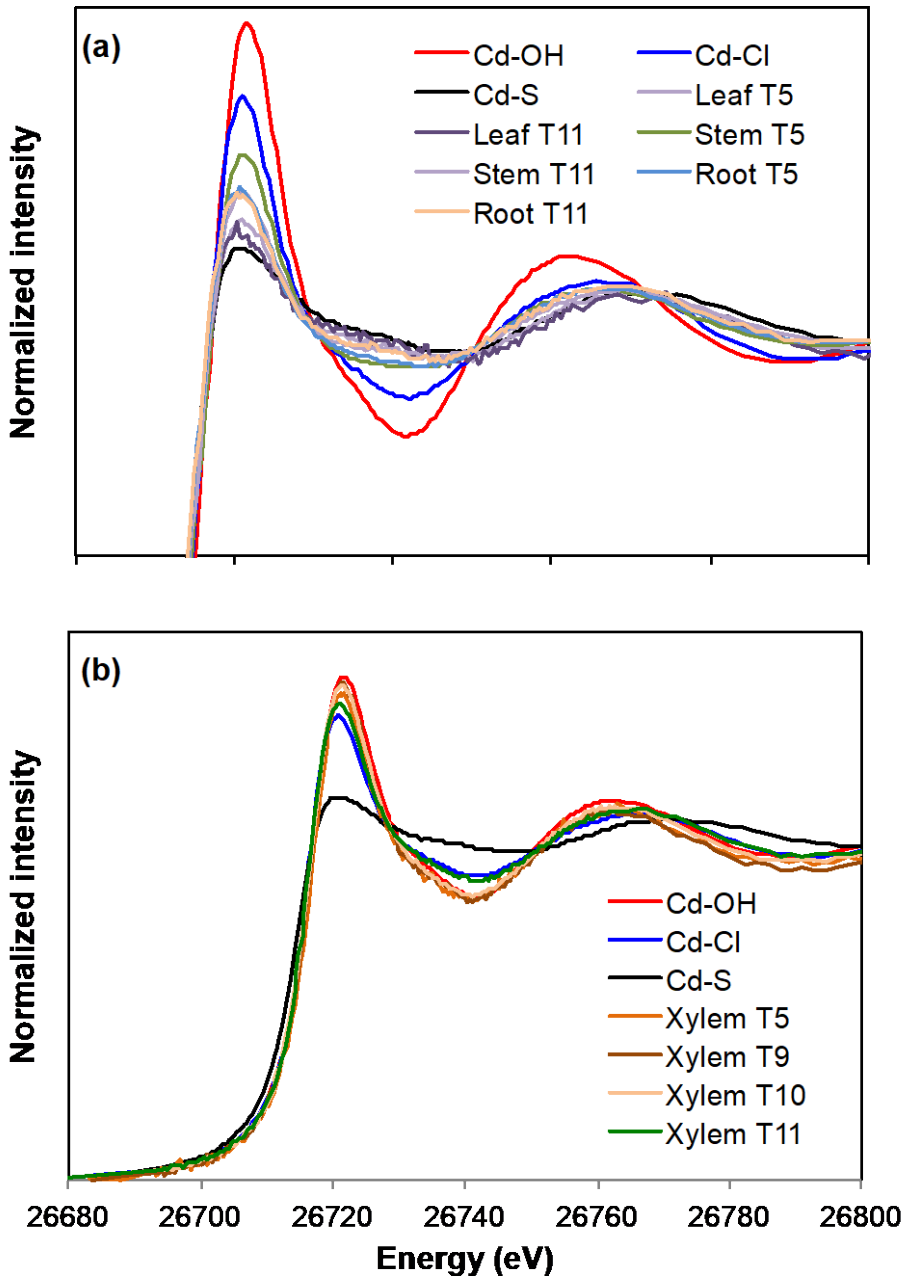


**Figure S1.** Nomalized Cd-edge XANES spectra of the leaf, stem and root (a) and xylem sap (b) of *Carpobrotus rossii* grown in different treatments for 10 d in solutions with the same $Cd^{2+}$ activity (10.9 µM). T5: 15 µM Cd; T9: 22 µM Cd + 25 mM $NaNO_3$; T10: 42 µM Cd + 12.5 mM $Na_2SO_4$; T11: 50 µM Cd + 25 mM NaCl (Experiment 1).

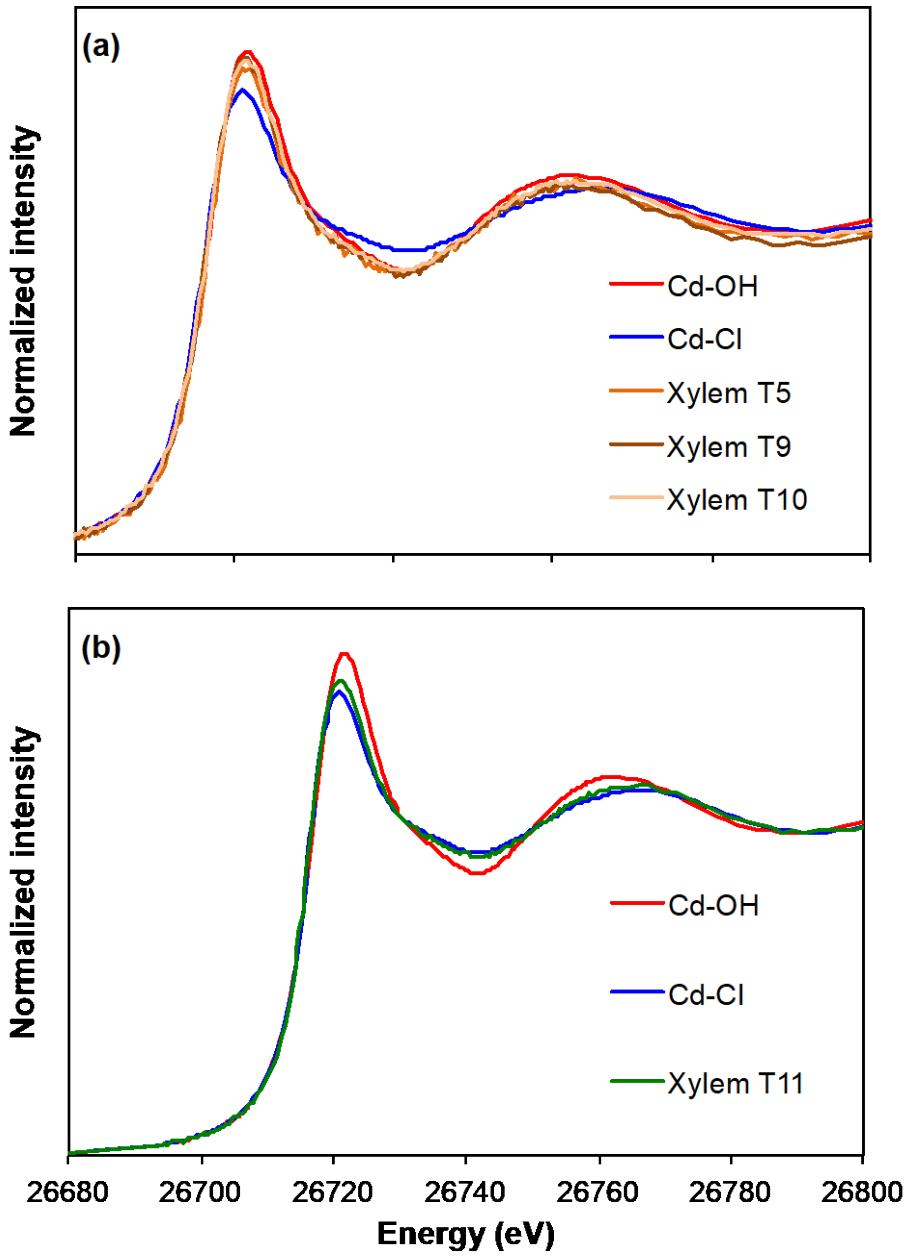


**Figure S2.** Nomalized Cd-edge XANES spectra of the Cd standards and the xylem sap of *Carpobrotus rossii* grown in different treatments for 10 d in solutions with the same $Cd^{2+}$ activity (10.9 µM). T5: 15 µM Cd; T9: 22 µM Cd + 25 mM $NaNO_3$; T10: 42 µM Cd + 12.5 mM $Na_2SO_4$; T11: 50 µM Cd + 25 mM NaCl (Experiment 1).

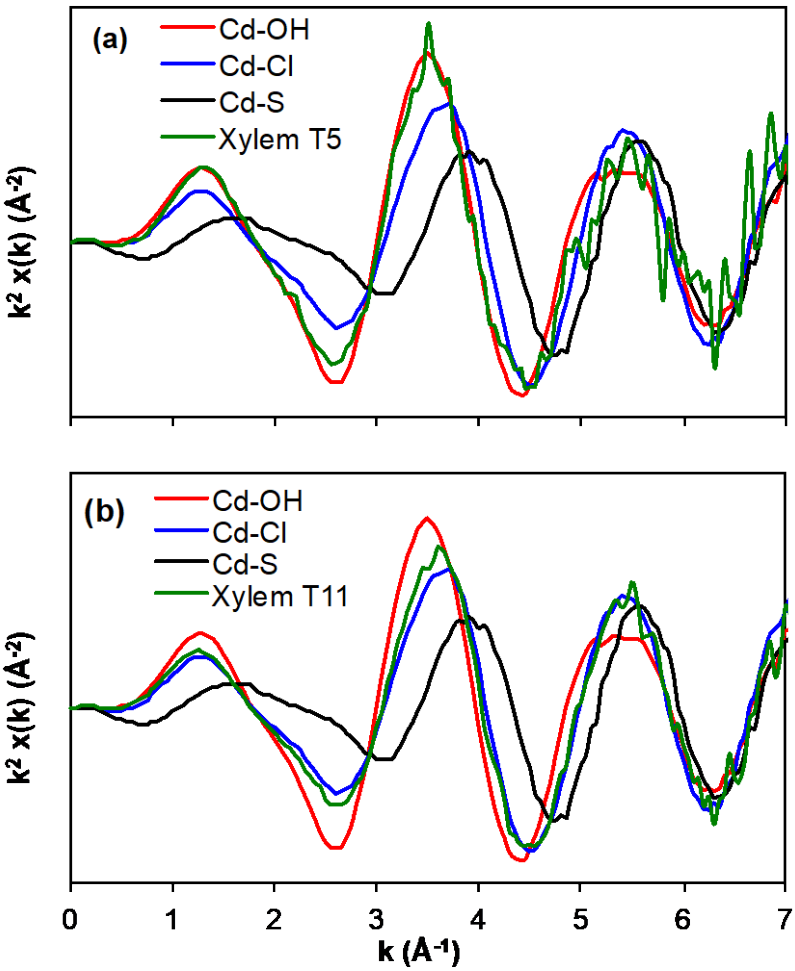


**Figure S3.** The $k^2$-weighted EXAFS spectra of Cd standards and the xylem sap of *Carpobrotus rossii* grown for 10 d in solutions with 15 µM Cd (T5, a), and in solutions with 50 µM Cd and 25 mM NaCl (T11, b) (Experiment 1).

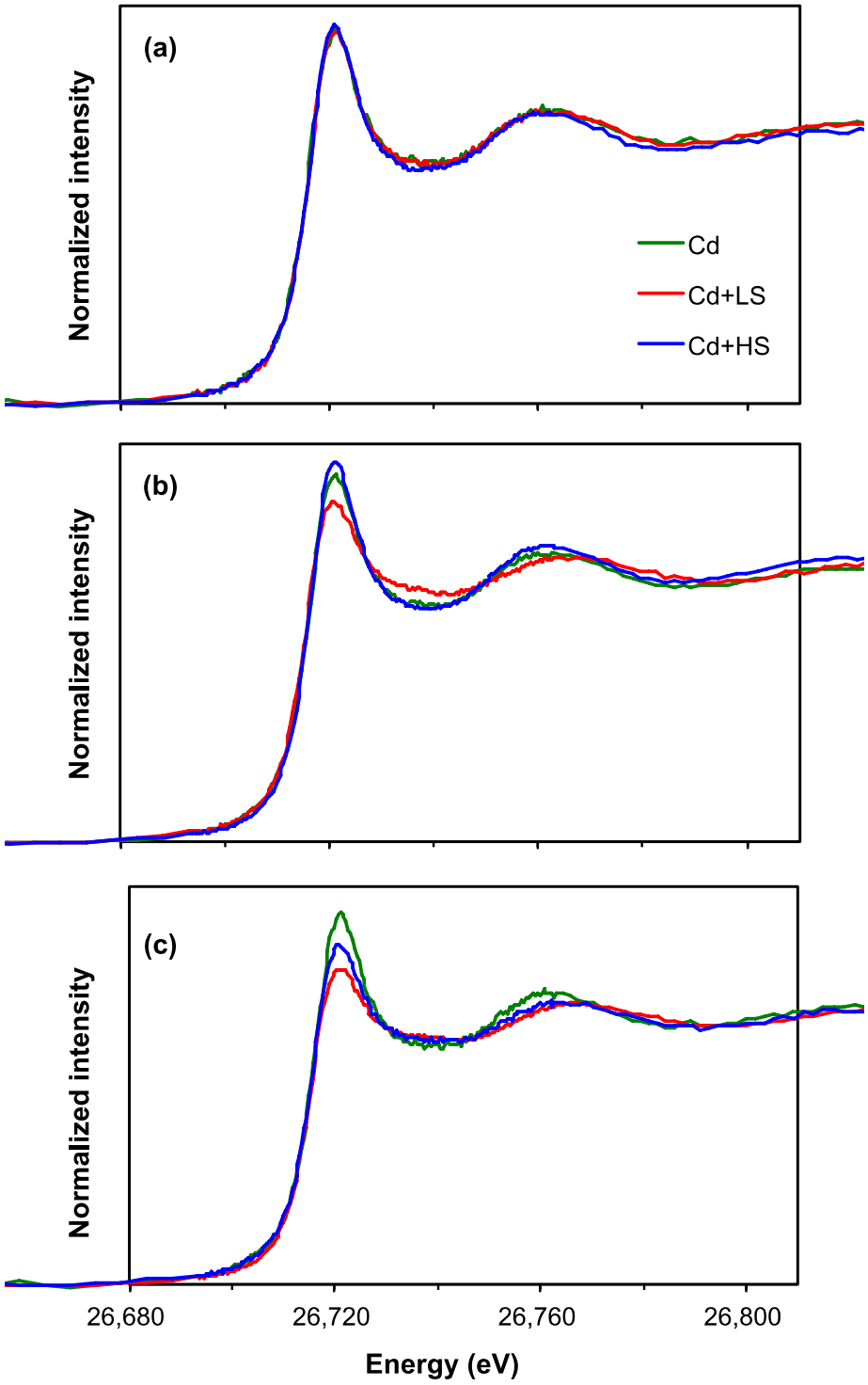


**Figure S4.** Nomalized Cd-edge XANES spectra of the roots of *Carpobrotus rossii* grown in solutions with the same $Cd^{2+}$ activity (10.9 µM) for 6 (a), 12 (b) and 24 h (c), Plants were either grown with 25 mM NaCl (LS, low salt) or with 50 mM NaCl (HS, high salt). Cd: 15 µM Cd; Cd +LS: 50 µM Cd + 25 mM NaCl; Cd +HS: 85 µM Cd + 50 mM NaCl (Experiment 2).

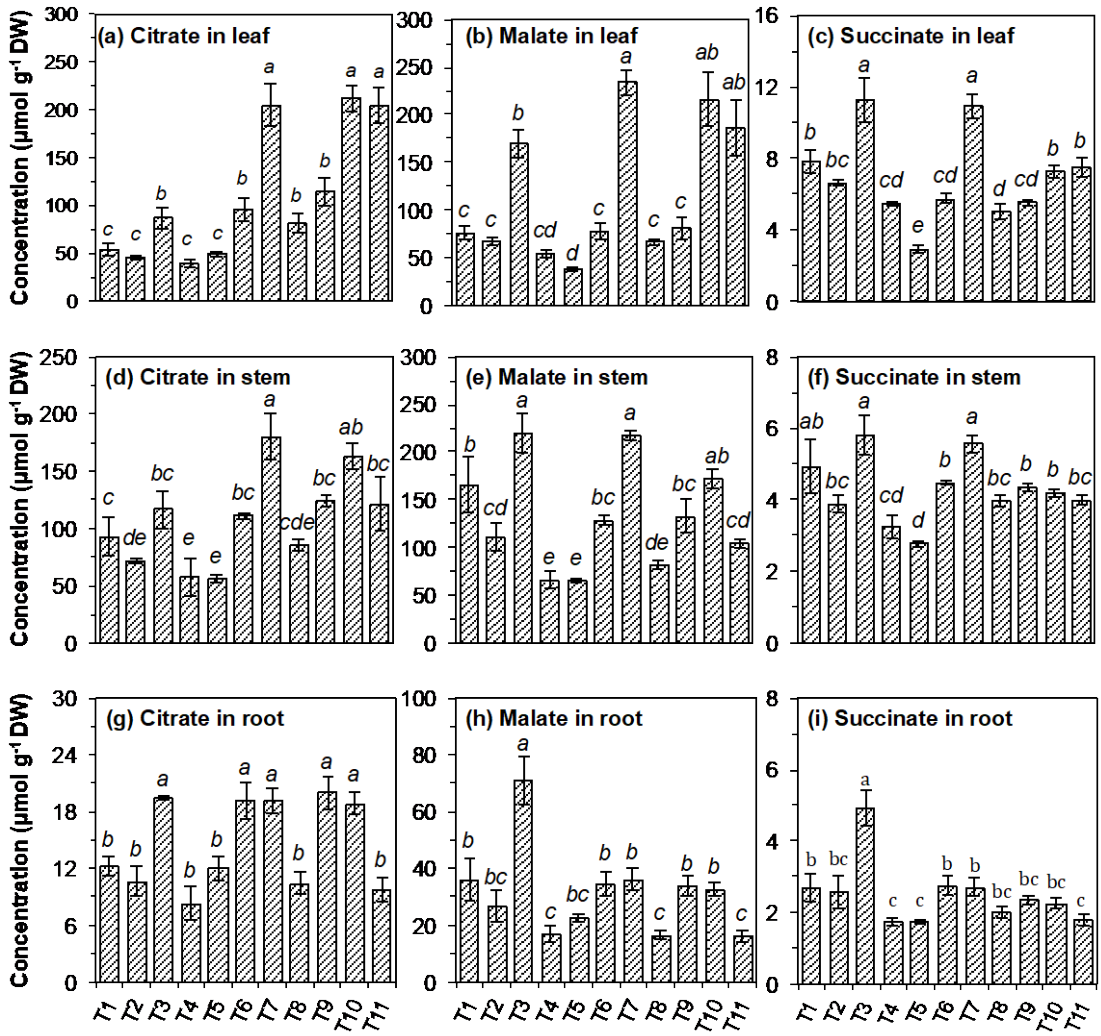


**Figure S5.** Concentrations of citrate (a, d, g), malate (b, e, h) and succinate (c, f, i) in leaves (a, b, c), stem (d, e, f), and roots (g, h, i) of *Carpobrotus rossii* grown in different treatments for 10 d. T1: control; T2: 25 mM $NaNO_3$; T3: 12.5 mM $Na_2SO_4$; T4: 25 mM NaCl; T5: 15 μM Cd; T6: 15 μM Cd + 25 mM $NaNO_3$; T7: 15 μM Cd + 12.5 mM $Na_2SO_4$; T8: 15 μM Cd + 25 mM NaCl; T9: 22 μM Cd + 25 mM $NaNO_3$; T10: 42 μM Cd + 12.5 mM $Na_2SO_4$; T11: 50 μM Cd + 25 mM NaCl. Error bars represent ± SEM of three replicates. Means with the same letter did not differ significantly (Duncan's test, $P<0.05$) (Experiment 1).

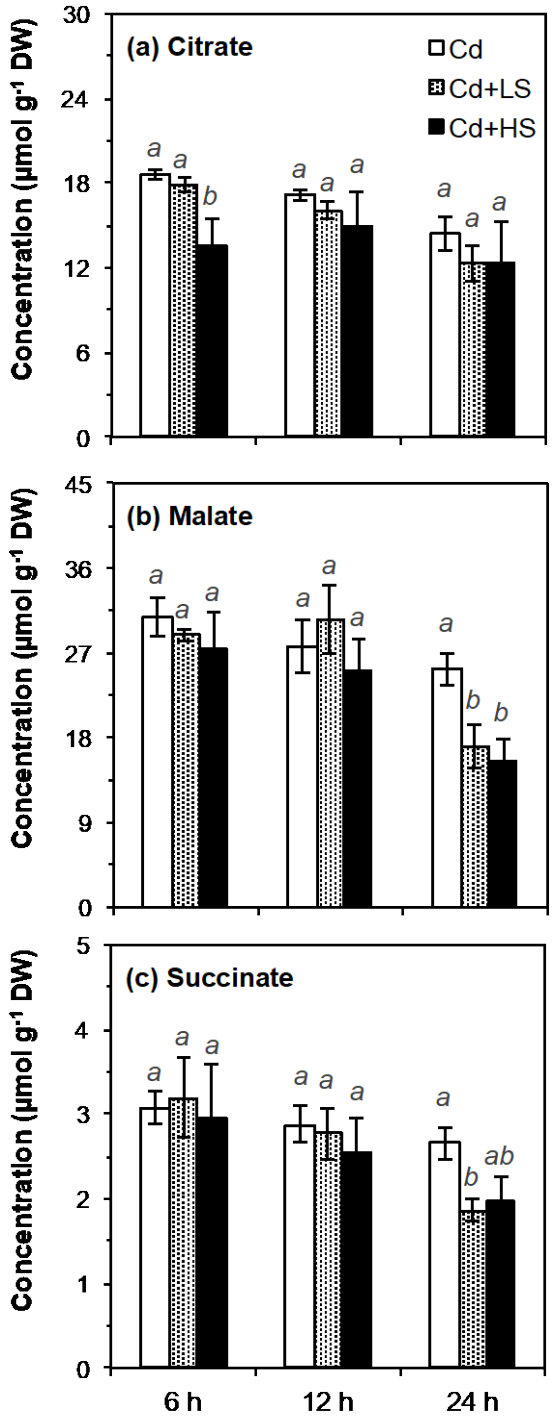


**Figure S6.** Concentrations of citrate (a), malate (b) and succinate (c) in roots of *Carpobrotus rossii* grown in solutions with the same $Cd^{2+}$ activity (10.9 µM) for 6, 12 and 24 h. Cd: 15 µM Cd; Cd +LS (low salt): 50 µM Cd + 25 mM NaCl; Cd +HS (high salt): 85 µM Cd + 50 mM NaCl. Error bars represent ± SEM of three replicates. Means with the same letter did not differ significantly between the treatments at each harvest time (Duncan's test, $P<0.05$) (Experiment 2).