

Determining the fluxes of ions (Pb^{2+} , Cu^{2+} and Cd^{2+}) at the root surface of wetland plants using the scanning ion-selective electrode technique

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Abstract

Background and aims Measuring specific ion fluxes from different regions of the root under practical physiological conditions is crucial for understanding metal uptake mechanisms by plants.

Methods We developed and tested a neutral carrier-based liquid-membrane Pb^{2+} and Cu^{2+} ion selective microelectrode (ISME) to investigate ion-transport processes along the roots of three common wetland plant species.

Results The Pb^{2+} and Cu^{2+} ISME exhibited a Nernstian response with Pb^{2+} and Cu^{2+} activities as low as 1.0 nM and 1.0 μM in deionized water and simulated soil

solution, respectively. *Phragmites australis* had a region of Cu^{2+} release for approximately the first 200 μm , while it exhibited Pb^{2+} and Cd^{2+} outward net flux up to the first 500 μm . Although in older sections of the root of *Phragmites australis* there were areas of influx of Cu^{2+} , Pb^{2+} and Cd^{2+} , the overall influx was much smaller than that of *Typha latifolia* or *Canna indica*. Such a reduced uptake and/or an increased efflux of metal ions across the root-cell plasma-membrane might explain the higher resistance of *Phragmites australis* to metals, at least in part.

Conclusions The Pb^{2+} and Cu^{2+} ISMEs are shown to permit detailed investigation of heavy-metal ion transport in plant roots, especially for plants used for phytoremediation.

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Introduction

A fundamental understanding of the micro-interfacial processes of metal ions in the rhizosphere is crucial for estimating the transfer of metals from soil to the food chain and for developing more efficient phytoremediation technologies to clean up metal-contaminated soil (Wenzel 2009). Although advances have been made in the past decade to unravel the multiple processes and interactions of heavy metals in the

rhizosphere of terrestrial plants, further research is required to better address the transport mechanisms.

Transport of metal ions into and out of tissues and cells has been studied using a variety of techniques. Results from these methods have provided more specific information on metal ion distribution and transport in plants. However, it is inherent to most techniques that average values are reported that relate to the entire sample (e.g. whole root system) and to the entire experimental period as a consequence of methodological limitations (relatively poor spatial and temporal resolution). Thus, these techniques may not be best suited for studying metal ion transport in specific localized regions of the root, such as the apex. Metal transport in the apex is likely to be a key factor in understanding the mechanisms of metal uptake.

An additional technique, known as the ~~scanning ion-selective electrode technique (SIET) or the microelectrode ion flux measurement (MIFE) technique~~ has provided a new opportunity to successfully address the issues raised above (Newman 2001; Newman et al. 1987; Shabala et al. 1997; Shabala and Newman 1997; Smith et al. 1994; Kochian et al. 1992; Pineros et al. 1998). This technique allows the measurement of selected ion net fluxes from different regions of the root under realistic physiological conditions, whilst optimally exploiting some of its key features (e.g. non-invasiveness, high spatial and temporal resolution). The method has already contributed to the functional characterization of transporter systems in the plant root (Newman 2001).

In general, it is currently possible to use the ~~SIET or MIFE system~~ to measure net fluxes of ions (such as H^+ , Ca^{2+} , K^+ , Na^+ , Cl^- , Mg^{2+} , NH_4^+ , NO_3^- , Cd^{2+}) as well as gaseous molecules (such as O_2 and NO) and other compounds (indoleacetic acid, IAA) in real time in various biological systems (Pineros et al. 1998; McLamore et al. 2009; Wu et al. 2010; Ramos et al. 2009; Mancuso et al. 2005; Mancuso et al. 2000). To our knowledge, only a Cd^{2+} liquid-membrane ion selective microelectrode (ISME) was routinely applied in the root system (Pineros et al. 1998; Farrell et al. 2005; Li et al. 2012), although several other heavy metal ions (e.g. Zn^{2+} , Cu^{2+} , Pb^{2+}) can also be measured using commercially available ionophores with suitable selectivities and response times (Papernik et al. 1996). It is important to determine the difference of patterns of ion fluxes among different heavy metals along the plant root, since contamination rarely occurs only for a single metal and is invariably due to complex mixtures.

The purpose of this study was to develop and test a neutral carrier-based Pb^{2+} - and Cu^{2+} -selective liquid-membrane microelectrode and to demonstrate its potential in studying Pb^{2+} and Cu^{2+} fluxes at the root surface. Wetland plants are natural absorbers of heavy metals and other nutrients. Removal of heavy metals and other pollutants from wastewater using wetland plants has been reported as a low cost and effective technology (Rai 2008). *Typha latifolia*, *Canna indic* and *Phragmites australis*, are well known hyperaccumulating emergent plants. There is evidence that these plants can accumulate Cu, Zn, Cr, Ni, Pb and Cd in their tissues (Ye et al. 1997a, b; Deng et al. 2004; Sasmaz et al. 2008). The knowledge about the abilities of different wetland plant species or tissues to absorb and transport metals under different conditions will provide insight into choosing appropriate plants for wetland phytoremediation systems. Thus, we studied the fluxes of these metals into cells of plant roots, including spatial aspects of heavy metal transport along roots of three common wetland plant species *Phragmites australis*, *Typha latifolia* and *Canna indica*, which are distributed widely in the tropics and in temperate regions (McNaughton et al. 1974; Taylor and Crowder 1984; Ye et al. 1992a, b; Ye 1995; Van der Werff 1991; Dunbabin and Bowmer 1992).

Materials and methods

Construction and characterization of Pb^{2+} and Cu^{2+} ISMEs

The construction of liquid-membrane ISMEs has been previously described in detail (Smith et al. 1994, 1999; Kühtreibe and Jaffe 1990). Various ionophore cocktails were prepared based on a Pb^{2+} ionophore and a Cu^{2+} ionophore previously described (Kamata and Onoyama 1991; Ceresa and Pretsch 1999). The composition of the cocktails included an ionophore, lipophilic salts, and a solvent. The lipophilic salts tested were sodium tetraphenyl borate (NaTPB) and tetradodecyl ammonium tetrakis(4-chlorophenyl) borate. The solvents tested were o-nitrophenol octyl ether (o-NPOE). All chemicals were obtained from Sigma-Aldrich (Shanghai, China).

Ion selective microelectrodes were pulled on a vertical micropipette puller (WD-2, Chengdu Instrument Factory, Chengdu, China) from 1.5 mm non-filamented borosilicate glass capillaries (TW150-3, World Precision Instruments Inc., Sarasota, FL USA)

to obtain tip diameters of approximately 3–5 μm , which were then silanized with dimethyldichlorosilane (Sinopharm Chemical Reagent Co. Ltd., Beijing) at 200 °C for 30 min, and stored at room temperature in a desiccator. The microelectrodes were first backfilled with a solution containing 0.1 mM PbCl_2 or CuCl_2 and 0.1 mM NaCl, and then front-filled with a 90–120 μm column of the appropriate Pb^{2+} or Cu^{2+} ionophore cocktail. Microelectrodes were connected through chlorided silver wires to the headstage of the signal amplifier. The reference electrode consisted of an Ag/AgCl wire in a glass micropipette (tip diameter ca. 50 to 100 μm) containing 0.5 M KCl in a 1% agar solution.

The ionophore cocktails were then tested in a series of tenfold dilutions of Pb^{2+} or Cu^{2+} in deionized (DI) water and simulated soil solution in the presence of electrolytes typically used for plant nutritive solutions to determine the Nernstian slope. The simulated soil solution contained 0.1 mM $\text{Ca}(\text{NO}_3)_2$, 0.1 mM MgSO_4 , 1.0 mM NaCl and 0.1 mM KNO_3 .

Cocktails that exhibited poor or no selectivity were discarded, while the ratio of ingredients was adjusted for cocktails that showed promise in order to improve selectivity and to increase the slope.

The approach of Bakker (Bakker 1996, 1997a, b) was used to evaluate the performance of the electrodes in the absence of the primary ion (Pb^{2+} or Cu^{2+}). Calibration curves for the individual interfering ions were generated by measuring the electrode millivolt outputs in a series of solutions containing varying activities of the potentially interfering ion ranging from 1×10^{-4} M and 1×10^{-2} M. After this, a similar calibration plot was then generated for varying primary ion (Pb^{2+} or Cu^{2+}) activities. The selectivity coefficients were then calculated using the formula (taking Pb^{2+} as example):

$$\log K_{\text{Pb},J}^{\text{pot}} = \frac{2F\{E_J - E_{\text{Pb}}\}}{2.303RT} + \log \left(\frac{\partial_{\text{Pb}}}{\partial_{\text{Z}_J}^{2/Z_J}} \right)$$

where J represents the interfering ion, $K_{\text{Pb},J}^{\text{pot}}$ is the selectivity coefficient, Z_J is the valency of the interfering ion tested, E_{Pb} and E_J are the electrode millivolt outputs in the testing solutions, ∂ is the activity of the interfering and primary ion, R is the gas constant, F is the Faraday constant, and T is the absolute temperature.

When applying pharmacological agents in experiments, it is necessary to consider the potential interference of these chemicals with the sensitivity and function

of the electrodes. In our study, various commonly used pharmaceuticals were selected to study their effects on the performance of the microelectrodes. The effect of the pharmaceuticals on the response of the microelectrodes was tested by calibrating with solution containing 50 μM 2, 4-dinitrophenol (DNP, a uncouplers), 50 μM Verapamil and 50 μM Nifedipine (Ca^{2+} channel blockers), 100 μM tetraethylammonium (TEA, K^+ channel blocker), bafilomycin (a proton-pump inhibitor), 100 μM ethylisopropylamiloride (EIPA, Na^+/H^+ exchange inhibitor), or 100 μM N-ethylmaleimide (NEM, a -SH inhibitor). All concentrations were selected on the basis of various literature reports indicating these concentrations are relevant from a physiological point of view.

Preparation and calibration of Pb^{2+} , Cu^{2+} and Cd^{2+} ISMEs

The glass microelectrodes described above with good performance and the ~~scanning ion-selective electrode technique~~ (SIET system, BIO-001A; Younger USA, LLC, MA, USA) were used for measuring ion fluxes. The Pb^{2+} ISME was backfilled with a solution containing 0.1 mM PbCl_2 and 0.1 mM NaCl, and front filled with an appropriate cocktail. The Cu^{2+} electrodes were backfilled with 0.1 mM CuCl_2 and 0.1 mM NaCl and front filled with an appropriate cocktail. The Cd^{2+} ISME was front filled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20,909, Sigma-Aldrich, St Louis, MO) and backfilled with 10 mM $\text{Cd}(\text{NO}_3)_2$ and 0.1 mM KCl.

The ISMEs were calibrated in solutions containing 5, 10 and 50 μM Cu^{2+} , Pb^{2+} or Cd^{2+} prior to the ion flux measurement. Only electrodes with Nernstian slopes >25 mV per decade were used. Details on fabrication and calibration of ISMEs have been described previously (Li et al. 2012; Ma et al. 2010).

Measurement of ion flux of an ion gradient source

An artificial ion flux source was used to generate a Pb^{2+} or Cu^{2+} gradient to test the efficiency of a self-referencing Pb^{2+} and Cu^{2+} ISMEs. As an artificial ion source a filamented microelectrode with a tip diameter of 10 μm , filled with a solution of 10 mM $\text{Pb}(\text{NO}_3)_2$ or $\text{Cu}(\text{NO}_3)_2$ in 1.0% agarose was used. These sources were placed in a Petri dish containing 50 μM Pb^{2+} or Cu^{2+} and were allowed to equilibrate overnight for a

steady state to be established with a range of known, steady Pb^{2+} or Cu^{2+} concentration gradients. The Pb^{2+} or Cu^{2+} -ISME was repeatedly vibrated along the direction away from the artificial ion flux source with amplitude of 10 μm at the positions of respectively 30 and 60 μm away from the source. Thus the voltage difference of Pb^{2+} or Cu^{2+} -ISME at each position were monitored for 5 min prior to the addition of 50 μM of interfering ions. Afterwards, the recording was restarted and then continued for 5 min for each interfering ion. The data recorded during the first 3 min were discarded due to the diffusion effects of stock addition. The ion flux was then calculated using Fick's first law of diffusion.

$$J = D \times \Delta C / \Delta x$$

where J is the ion flux in the x direction ($\text{pmol}/(\text{cm}^2 \text{ s})$); D is the ion/molecule diffusion constant (cm^2/s); $\Delta C/\Delta x$ is the ion concentration gradient (pmol/cm^4).

Scanning ion-selective electrode technique (SIET)

measurements of Pb^{2+} , Cu^{2+} and Cd^{2+} fluxes on the root surface of wetland plants

With the plant intact, the primary (longest) root was mounted horizontally in the measuring chamber, and loosely fixed in place with dental wax. After mounting the plant in the chamber, the chamber was placed on an inverted microscope in a Faraday cage and filled with 5 mL of measuring solution consisting of 10 μM of either $\text{Cu}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$ or $\text{Cd}(\text{NO}_3)_2$, and 0.1 mM KNO_3 , 0.1 mM $\text{Ca}(\text{NO}_3)_2$, 0.1 mM MgSO_4 , 1.0 mM NaCl , and 0.3 mM MES (solution pH = 6.0). Whereas the shoot was kept out of the bath solution, all the roots were immersed in the bath solution but were kept separate from the primary root. In our preliminary study, long time-course ion fluxes were recorded subjecting the plants to metal treatment for 30 min at a couple of specific root positions. The steady-state ions fluxes were first recorded for 10 min prior to the treatment. Then, a metal stock solution was slowly added to the test solution to yield a final metal concentration of 10 μM (Cd^{2+} , Cu^{2+} and Pb^{2+}). Afterward, flux recording was restarted and then continued for 30 min. The data recorded during the first 3 min were discarded due to the diffusion effects of stock addition. The results showed that the addition of metal induced a sudden influx of Cd^{2+} , Pb^{2+} and Cu^{2+} in all the plant species

(Fig. S1-S3), except for that at the root tip of *Phragmites australis*, where a significant and stationary ion efflux was observed. The influx rate decreased quickly in the first several minutes and then exhibited a stable and steady influx for the test time (Fig. S1-S3). This is consistent with the findings of Ryan et al. (1992). The higher influx of Cd^{2+} , Cu^{2+} and Pb^{2+} observed in the roots is possibly dominated by a stronger cell wall sorption (Redjala et al. 2009, 2010). Thus after immobilization of the roots in the measuring solution and equilibration for 10 min, the steady ions fluxes were then recorded across the root tips of the wetland plants. The reference electrode tip was placed in the bath solution and kept at a distance of at least 1 cm from any of the roots. Gradients of ions adjacent to the root were measured by moving the ISMEs using a computer-controlled stepper motor between two positions in a pre-set excursion of 30 μm at a programmable frequency of 0.3 Hz. The near pole was within 5 μm of the root surface.

To assess differences between the ion flux profiles in laboratory and field conditions, ion flux measurement was also conducted in the extract from the soil in the locations where the plants normally grow. Procedures of soil solution extraction and analysis are provided in Supplementary Material. The extracted solution was then used as base solution for ion flux measurement.

The flux data were obtained with the ASET software, which is part of the SIET system. Eventually, the raw data from all the measurements, including background-mV estimation of concentration and the microvolt difference estimation of the local gradient, were converted into net ion fluxes ($\text{pmol cm}^{-2} \text{ s}^{-1}$) using MageFlux, developed by the Xu-Yue company.

Plant materials and growth conditions

Seeds of the wetland plants *Phragmites australis*, *Typha latifolia* and *Canna indica* were collected from an uncontaminated site in the Yellow River Delta in Shandong Province, P. R. China in 2011, fully imbibed and chilled at 4 °C in the dark for 2 weeks. Prior to germination, the seeds were surface sterilized in 0.5% NaOCl for 15 min. The seeds were then germinated and cultivated in clean river sand irrigated by 20% strength Hoagland nutrient solution [composition: 1.0 mM $\text{Ca}(\text{NO}_3)_2$, 1.0 mM KNO_3 , 0.4 mM MgSO_4 , 0.2 mM KH_2PO_4 , 10 μM $\text{Fe}(\text{II})$ -EDTA, 9 μM H_3BO_3 , 0.2 μM ZnSO_4 , 0.1 μM CuSO_4 , 2 μM MnSO_4 , 0.02 μM

(NH_4)₆Mo₇O₂₄] (Hoagland and Arnon 1938) under glasshouse conditions for two months. The nutrient solution was changed every 2 d and the pH of the solution was adjusted to 6.0 using HCl or NaOH. The seedlings were kept in a glasshouse with a temperature of $25^\circ\text{C} \pm 5^\circ\text{C}$ and a 16/8 h day/night cycle, with a light intensity of $480 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ throughout the reported experiments. Plants were then grown in Hoagland nutrient solution for 2 weeks before being used for the flux studies. The length of the whole root system was about 5 to 10 cm. Individuals of similar shoot height and root length within the same species were then selected for the measurement of ion fluxes as described above.

Accumulation of Cu, Pb and Cd in tissues of wetland plants

For the measurement of metal content in plant, roots and shoot were separately harvested after exposure of the seedlings with Hoagland solutions containing $10 \mu\text{M}$ of Cd^{2+} , Cu^{2+} and Pb^{2+} in 1 L HDPE containers for 0, 7, and 14 d, respectively. Four seedlings were transplanted into each container. There were four containers for each treatment as replicates. The pH of the nutrient solution was adjusted daily to 6.0 using 0.1 M NaOH if necessary. All solutions were aerated continuously and refreshed every 2 d. The plant was washed for three times and then all samples were dried at 80°C for 72 h. The metal content of the plant tissues was analyzed following digestion with concentrated HNO_3 . ICP-MS (Agilent 7500i, Agilent Technologies Co. Ltd., Santa Clara, CA, USA) was used to determine metal concentrations in the digest. As certified reference materials for plant analyses we used GBW07605 tea leaves that were supplied by the State Bureau of Technical Supervision, People's Republic of China. The concentrations that were actually measured did not deviate more than 5% from the certified metal concentrations.

Results

Ionophore cocktails of Pb^{2+} and Cu^{2+} ISME

We characterized slopes and selectivity for Pb^{2+} cocktails containing 5% - 50% ionophore (Table S1). It was found that Pb^{2+} cocktails [composed of 10% w/w methylene-bis-N,N-diisobutyldithiocarbamate, 8% w/w

NaTPB, 3% w/w tetradodecyl ammonium tetrakis(4-chlorophenyl) borate, and 79% w/w o-NPOE] exhibited good performance in sensitivity and selectivity testing.

Different Cu^{2+} cocktails containing 5% - 50% ionophore were also prepared and tested and it was indicated that Cu^{2+} cocktails [composed of 10% w/w diphenyl thiocarbazon, 12% w/w NaTPB, 10% w/w tetradodecyl ammonium tetrakis(4-chlorophenyl) borate, and 68% w/w o-NPOE] exhibited good performance in sensitivity and selectivity testing (Table S2).

Calibration of Pb^{2+} and Cu^{2+} ISME ionophore cocktails

The calibration for the Pb^{2+} ISME over a wide range of solution Pb^{2+} activities in DI water is shown in Fig. 1. The slope of the calibration curve (29 mV/dec) was close to that predicted theoretically by the Nernst equation, indicating that the microelectrode maintained a high Pb^{2+} sensitivity over the range of concentrations tested (10^8 -fold) of Pb^{2+} activities in DI water. Because a defined solution containing electrolytes is required for normal root growth and function, we also evaluated the Pb^{2+} ISME sensitivity with a simulated soil solution as background. The electrode responded over a large range of Pb^{2+} activities with a simulated soil solution background (Fig. 1). The Pb^{2+} ISME showed a linear Nernstian

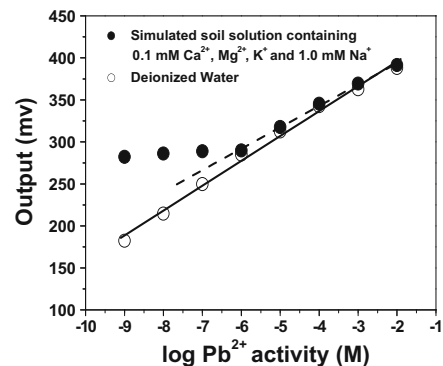


Fig. 1 Calibration curves for Pb^{2+} ISME in $\text{Pb}(\text{NO}_3)_2$ solutions of DI water or simulated soil solution. The electrode response (in mV) has an arbitrary origin. The solid and broken lines represent the linear regression lines for the experimental data obtained in DI water and simulated soil solution, respectively. The sensitivity (slope) is equal to 29 mV/dec ($r^2 = 0.997$) for the calibration in DI water and 26 mV/dec ($r^2 = 0.993$ excluding the three lowest points) for the calibration in stimulated soil solution. Points represent the averages of three and five different electrodes for the DI water and simulated soil solution background, respectively. Standard errors of the mean were in all cases smaller than the diameter of the symbol

response down to Pb^{2+} activities as low as $1.0 \mu\text{M}$ in the simulated soil solution, with a slope of 26 mV/dec from $1.0 \mu\text{M}$ to 100 mM Pb^{2+} activity.

From the calibration curves of the Cu^{2+} ISME, it is apparent that the electrode showed a linear response over a wide range of Cu^{2+} activities (10^9 -fold) in DI water (Fig. 2). The sensitivity of the electrode for Cu^{2+} (27 mV/dec) was close to that predicted theoretically by the Nernst equation. The electrode maintained sensitivity over a 10^6 -fold range of Cu^{2+} activities in simulated soil solution, with a slightly sub-Nernstian slope of 25 mV/dec (Fig. 2). In addition, the electrode showed a linear response over this range of activities, showing departure from linearity (i.e. loss of sensitivity) at activities lower than $1.0 \mu\text{M}$ Cu^{2+} due to the cumulative effect of physiological ion in simulated soil solution.

For the effect of pharmaceuticals on the performance of the microelectrodes, Fig. S4 shows a typical example of a drug test on both microelectrodes. With the exception of NEM, none of the drugs interfered with microelectrode function at the concentrations used in our experiments. Both Pb^{2+} - and Cu^{2+} selective microelectrodes showed a large shift in the calibration curve when $100 \mu\text{M}$ NEM was added to the solution.

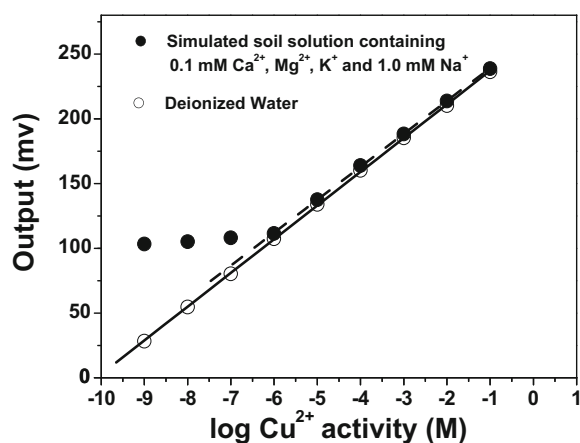


Fig. 2 Calibration curves for Cu^{2+} ISME in $\text{Cu}(\text{NO}_3)_2$ solutions of DI water or simulated soil solution. The electrode response (in mV) has an arbitrary origin. The solid and broken lines represent the linear regression lines for the experimental data obtained in DI water and simulated soil solution, respectively. The sensitivity (slope) is equal to 27 mV/dec ($r^2 = 0.999$) for the calibration in DI water and 26 mV/dec ($r^2 = 0.994$ excluding the three lowest points) for the calibration in stimulated soil solution. Points represent, respectively, the averages of three and five different electrodes for the DI water and simulated soil solution background. Standard errors of the mean were in all cases smaller than the diameter of the symbol

Selectivity of Pb^{2+} and Cu^{2+} ISME ionophore cocktails

The potential response curves of Pb^{2+} and Cu^{2+} ISME obtained according to Bakker's method toward the interfering ions are shown in Fig. S5. Most measured ions indeed show a near-Nernstian response behavior, with alkali metal ions being extremely discriminated. The selectivity coefficients of the Pb^{2+} and Cu^{2+} ISME are then calculated and compiled in Table 1 and in Table 2 for a series of interfering ions together with data of electrodes reported in literature. From the selectivity coefficients presented in Table 1, the Pb^{2+} ISME is in between 800 to 10^{12} times more selective for Pb^{2+} as compared to the other cations tested. Table 2 shows that the ions usually contained in nutrient solutions do not interfere with the Cu^{2+} ISME.

From Fig. S6, it can be seen that the Pb^{2+} or Cu^{2+} effluxes changes little at each point, which indicates that microelectrodes have a reliable stability. The presence of 0.05 mM Zn^{2+} , Cd^{2+} or $\text{Pb}^{2+}/\text{Cu}^{2+}$ in the test solution had no significant effects on the $\text{Pb}^{2+}/\text{Cu}^{2+}$ efflux at each positions of 30 and $60 \mu\text{m}$ away from the source, suggesting no interference of the heavy metal ions with microelectrode function at the tested concentrations in our experiment.

Localization of ion (Pb^{2+} , Cu^{2+} and Cd^{2+}) fluxes along the root apex of wetland plants

A preliminary experiment was carried out with an initial measurement at the root surface followed by $300 \mu\text{m}$ walking steps. The meristematic zone and the elonga-

Table 1 Potentiometric selectivity coefficients ($\text{Log}K^{\text{pot}}_{\text{Pb},j}$) for Pb^{2+} ion selective microelectrodes. (this work) and published earlier for electrodes with optimized detection limits (Lerchi et al. 1992; Ceresa et al. 2001)

Interfering Ion (J^{n+})	$\text{Log}K^{\text{pot}}_{\text{Pb},j}$ This work	Lerchi et al. 1992	Ceresa et al. 2001
Cd^{2+}	-2.9	1.2	-5.4
Cu^{2+}	-3.1	0.7	-3.7
Zn^{2+}	-11.78	-4.6	
H^{+}	-8.6		-3.5
Ca^{2+}	-11.19	-10.9	-12.3
Mg^{2+}	-12.27	-10.8	-12.4
Na^{+}	-8.51	-5.6	-6.3
K^{+}	-7.46	-5.3	-6.3

Table 2 Potentiometric selectivity coefficients ($\text{Log}K^{\text{pot}}_{\text{Cu},\text{J}}$) for Cu^{2+} ion selective microelectrode, (this work) and published earlier for the electrode with optimized detection limits (Shamsipur et al. 2001) and for that used in biological studies (Papeschi et al. 2000)

Interfering Ion (J^{n+})	$\text{Log}K^{\text{pot}}_{\text{Cu},\text{J}}$ This work	Shamsipur et al. 2001	Papeschi et al. 2000
Cd^{2+}	-10.0	-4.0	
Pb^{2+}	-8.4	-3.5	
Hg^{2+}	-2.5		
H^{+}	-9.4		
Ca^{2+}	-10.1	-4.1	-3.5
Mg^{2+}	-10.8	-10.8	-3.6
Na^{+}	-9.4	-4.0	-5.7
K^{+}	-8.2	-4.0	-7.4
NH_4^{+}	-2.2		-7.0

tion zone were found to exhibit higher ions influx rates at the root of *Typha latifolia* (Fig. S7). The net influx then decreased markedly after 900 μm from the root tips. A similar pattern of ions fluxes was also observed at different regions of the root of *Canna indic* and *Phragmites australis* (data not shown here). To further study spatial characteristics of ions flux in the apical region of these wetland plants, the net fluxes of Cd^{2+} , Cu^{2+} and Pb^{2+} in the region within 1000 μm from the root tips was monitored followed by 100 μm distance and the results are shown in Fig. 3. Generally, *Typha latifolia* exhibited higher metal ion influx rates than those of the *Canna indic* and *Phragmites australis*. As can be seen from Fig. 3, the Cd^{2+} and Pb^{2+} influxes in the region of cell division (at positions 100 and 200 μm from the root apex) of *Typha latifolia* were significantly higher than the influxes at positions further back from the apex (Fig. 3a). Moreover, at the more distal positions, after the region of maximum uptake, the Pb^{2+} and Cd^{2+} influx decreased quickly and reached a steadily decreasing influx. A similar flux profile was observed along roots of *Typha latifolia* and *Canna indica* for Pb^{2+} and Cd^{2+} (Fig. 3a, b).

Beginning at the root tip, the first 400 μm of both *Typha latifolia* and *Canna indica* showed a gradual increase in Cu^{2+} influx until the largest rate of Cu^{2+} influx was observed in the region of elongation (at approximately 400–600 μm behind the root tip) (Fig. 3a, b). Beyond this point, a decrease in Cu^{2+} flux at the root was observed (Fig. 3a, b). Whereas *Phragmites australis* had

a region of Cu^{2+} outward net flux at the root hair, and while it exhibited Pb^{2+} and Cd^{2+} outward net fluxes up to the first 500 μm (Fig. 3c), no ion release was observed in either *Typha latifolia* or *Canna indica*.

In general, the soil extract induced a decrease of metal influx rate, especially for plant species *Typha latifolia* and *Canna indica* at meristematic and elongation zones (Fig. S8). Metal efflux was even observed at the mature zone of *Phragmites australis*, besides the root apex (Fig. S8). Compared to the simple simulated soil solution, the soil extract contain significantly higher quantities of competitive divalent cations, e.g. Ca^{2+} and Mg^{2+} (Table S3). The presence of these ions may compete for the uptake sites on the plant cell thereby inhibiting the absorption and decreasing the internalization flux. According to our previous study (Li et al. 2012), Cd^{2+} uptake by halophytic species such as *Suaeda salsa* is regulated by Ca transporters or channels in root cell plasma membranes. This has been shown through the use of verapamil, a voltage-dependent Ca channel blocker, and La^{3+} , a voltage-independent Ca channel blocker, both of which inhibited Cd influx into roots.

Accumulation of Cu, Pb and Cd at tissue of wetland plant

Metal content in the shoot and roots of the three wetland plant species grown in culture solution containing 10 μM metal ions was measured at various times. In general, metal uptake by the plants increased along with exposure time, and most metals accumulated by plants were retained in the roots (Fig. 4). Only a small portion could be transported to the shoots, indicating their limited mobility once inside the plant. This is consistent with previous reports (Deng et al. 2004; Fitzgerald et al. 2003), whereas the exclusion of metals from above-ground tissues has been suggested as a metal tolerance strategy for wetland plant species (Taylor and Crowder 1983). The results also showed that metal accumulation by wetland plants differed among species. *Phragmites australis* always accumulated less metal in the roots as compared to *Typha latifolia* and *Canna indica*. The results showed the low metal accumulation in *Phragmites australis* was mainly due to the lower metal absorption rate as well as to the release of metal from the root as detected by using the [scanning ion-selective electrode technique \(SIET\)](#). Compared to *Phragmites australis*, *Typha latifolia* and *Canna indica* showed a

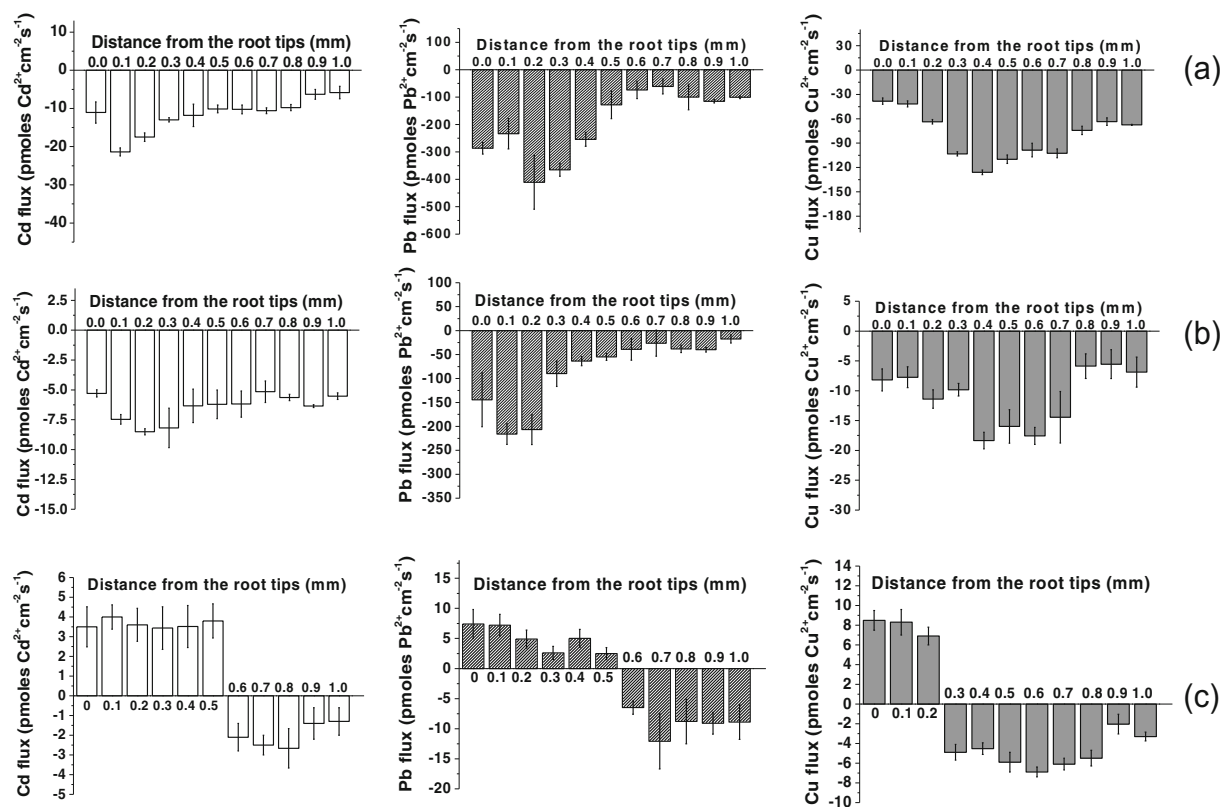


Fig. 3 Measurement of fluxes (outward positive) of Cd^{2+} , Pb^{2+} and Cu^{2+} (mean \pm standard error) across the root tips of three common wetland plant species, *Typha latifolia* (a) *Canna indic* (b) and *Phragmites australis* (c), using Cd^{2+} , Pb^{2+} and Cu^{2+} ion selective microelectrodes and the scanning ion-selective electrode

higher metal influx in the root regions, especially in the meristematic and elongation zone (Fig. 3).

Discussion

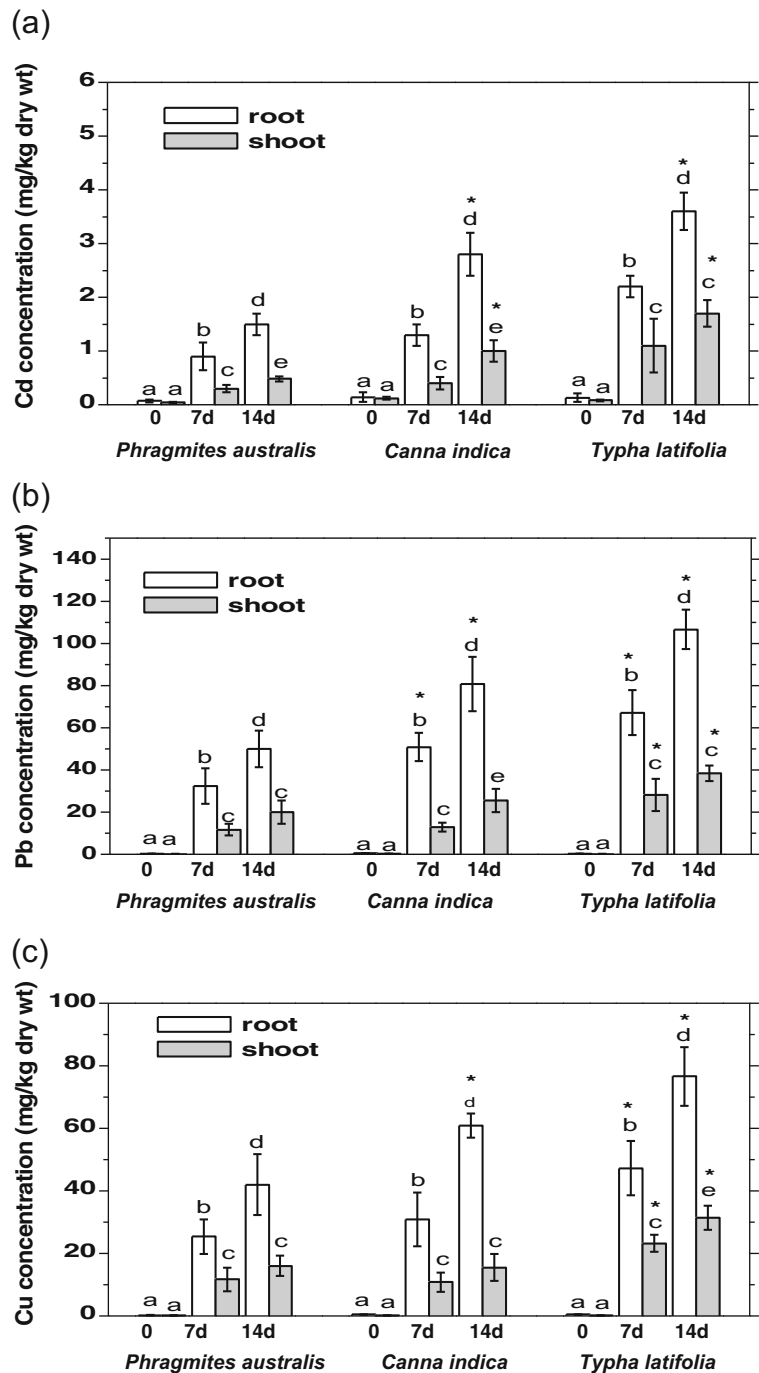
The ionophore cocktails used were generally based on those outlined in other papers that describe ISMEs (e.g. Pineros et al. 1998; Amman et al. 1987). We characterized slopes and selectivity for Pb^{2+} and Cu^{2+} cocktails containing different percentage of ionophore. Cocktails that exhibited poor or no selectivity were discarded, while cocktails that showed promise had the ratio of ingredients adjusted so as to improve selectivity and to increase slope. The ISMEs for Pb^{2+} and Cu^{2+} developed in this study showed high selectivity. In general, the selectivity coefficients found are comparable, albeit that the new microelectrodes in the present study are somewhat better for most of the alkali and alkaline earth metal ions that are relevant as background electrolytes in biological studies

technique. Flux measurements were carried out in 10 μM $\text{Cd}(\text{NO}_3)_2$, $\text{Pb}(\text{NO}_3)_2$ or $\text{Cu}(\text{NO}_3)_2$ at different positions along the root apex. Each mean is based on $n = 8$ values from replicate scans of four plants. Roots were scanned in segments of 100 μm

(Lerchi et al. 1992; Ceresa et al. 2001; Shamsipur et al. 2001; Papeschi et al. 2000). Time traces of the potential response of the Pb^{2+} and Cu^{2+} ISME were generated by addition of 1.0 mM K^+ and/or Mg^{2+} in 1×10^{-3} M and 1×10^{-4} M Pb^{2+} or Cu^{2+} respectively. As shown in Fig. S9, no significant potential drift was observed upon addition of 1.0 mM K^+ and/or Mg^{2+} , indicating a good robustness of the Pb^{2+} and Cu^{2+} ISME in the presence of physiologically relevant concentrations of K^+ and Mg^{2+} , both of which are important trace elements for plant nutrition. Therefore, the selectivity shown makes these electrodes functional for practical applications.

Calibration curves for Pb^{2+} and Cu^{2+} ISME with DI water or different concentrations of K^+ and/or Mg^{2+} as background were shown in Fig. S10. The electrode displayed a slight interference by K^+ and Mg^{2+} ions at low activities of the Pb^{2+} or Cu^{2+} ions (1×10^{-6} M) but no significant interference was noticed when the activities of the Pb^{2+} or Cu^{2+} in solution was high. The slope of the calibration curve is close to that predicted theoretically

Fig. 4 Concentrations of Cd (A), Pb (B) and Cu (C) in shoots and roots of *Phragmites australis*, *Typha latifolia* and *Canna indica* after exposure to 10 μ M metal ions (Cd, Pb or Cu) for 0, 7, and 14 d. Data are mean \pm SD ($n = 4$). Different letters in the same column of the same species show significant differences ($p < 0.05$) of metal concentrations in shoot/root. Significant difference of metal concentrations in shoot/root of the same exposure period among the species is indicated by an asterisk (*) ($p < 0.05$). The plants of *Phragmites australis* with low metal contents are regarded as controls



(28.5 mV/dec) by the Nernst equation. Thus, the electrode remains highly sensitive and selective for Pb^{2+} or Cu^{2+} under conditions that mimic the plant nutrition solution. Below Pb^{2+} or Cu^{2+} solution activities of 1×10^{-6} M, the electrode response was still linear but exhibited sub-Nernstian behavior. The Nernstian slopes obtained by the ISMEs in simulated soil solution (Fig. 1

and Fig. 2) extend the use of these electrodes in plant hydroponic growth solutions, without concern of interference, thus broadening the application of these electrodes.

Ion fluxes were mapped along root hairs of the primary root using the ISMEs and the ~~scanning ion-selective electrode technique~~. Functionally different root zones of the wetland plants were expected to exhibit

different ion flux responses. To test this hypothesis, the Pb^{2+} , Cu^{2+} and Cd^{2+} fluxes were measured in different regions along the root axis (approximately 100 μm increments) of these three wetland plants after exposure to the corresponding metal-containing nutrient solutions. The measurements were conducted in solutions containing 10 μM of each of the heavy metal ions, which are more environmentally relevant levels of the heavy metals. These concentrations are still in the linear Nernstian response ranges (Figs. 1 and 2).

The observed decrease in ion flux to the root surface with increasing distance from the root tip is consistent with the research of Pineros et al. (1998) who showed that the Cd^{2+} flux to the root of bread wheat (*Triticum aestivum* L) also decreased with increasing distance from the tip. Farrell et al. (2005) also observed that the mean net Cd^{2+} influx at the root surface of four durum wheat cultivars was greatest at a distance of 0.5–1.5 mm from the root tip and decreased with increasing distance from the tip. Our previous study also indicated that the Cd^{2+} influx in the rhizosphere of the Halophyte *Suaeda salsa* was greatest near the root tip (within 150 μm of the tip) (Li et al. 2012). Although similar patterns were observed for the wetland plants *Typha latifolia* and *Canna indica* used in the present study, the net Cd^{2+} flux was much greater than the flux observed for wheat (Pineros et al. 1998) and Halophyte (Li et al. 2012).

A release of metal ion from the root tip region can only occur if the heavy metal is being taken up in some other part of the root. It should be also kept in mind that it may be the oscillatory behaviour of Pb^{2+} , Cd^{2+} and Cu^{2+} fluxes from the root apex of *Phragmites australis* in this region. Shabala et al. (1997) have for instance reported that H^+ and Ca^{2+} ion fluxes around the elongation region of corn roots may oscillate. Our 30-min flux measurements show that oscillations are not present (Fig. S1–S3). Although in older sections of the root of *Phragmites australis* (beyond 200–500 μm from the apex) there were areas of uptake of Cu^{2+} , Pb^{2+} and Cd^{2+} (Fig. 3c), the overall influxes were much smaller than those of *Typha latifolia* or *Canna indica*. This is in accordance with the observation that *Typha latifolia* accumulated three times more cadmium than *Phragmites australis* in natural water ecosystems (Kozłowska et al. 2009). Deng et al. (2004) found that *Typha latifolia* accumulated significantly higher amounts of Cu, Cd, Pb and Zn in its roots and shoots than *Phragmites australis* in field conditions. Ye et al. (1997a, b) investigated Zn, Pb and Cd accumulation in

Phragmites australis and *Typha latifolia* under glass-house conditions and the results of these authors also indicated that *Typha latifolia* has higher accumulative capacities for Zn, Pb and Cd than *Phragmites australis*.

Heavy metal resistance may result from the ability to prevent uptake (avoidance) or from the ability to cope with high amounts of heavy metals in the tissues (tolerance). The observed significant metal outward net flux and relative low influx at the root tip of *Phragmites australis* indicated that this plant possess an exclusion mechanism to avoid metal toxicity. As an important means of detoxification, plants can actively reduce concentrations of unwanted metals in their cells by simply pumping out the metals. Such ATP-dependent efflux pumps were first found in roots of *Silene vulgaris* (van Hoof et al. 2001), but later also in roots of other plants (Migocka et al. 2011). The fact that *Phragmites australis* can withstand extreme environmental conditions (like industrially degraded habitats including the presence of toxic heavy metal contaminants such as Zn, Pb and Cd) indicates that it is resistant to heavy metals. Our results suggest that a reduced uptake and/or an increased release of metal ions across the root-cell plasma-membrane might at least in part explain the higher resistance of *Phragmites australis*.

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