A comparison of the rhizosphere-based method with DTPA, EDTA, CaCl_2 , and NaNO_3 extraction methods for prediction of bioavailability of metals in soil to barley

Mu-Hua Feng, Xiao-Quan Shan^{*}, Shuzhen Zhang, Bei Wen

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences,
Chinese Academy of Sciences, PO Box 2871, Beijing 100085, China

Received 29 July 2004; accepted 21 February 2005

The rhizosphere-based method is a preferred approach for evaluating bioavailability of metals in soil.

Abstract

A rhizosphere-based method was compared with DTPA, EDTA, CaCl_2 , and NaNO_3 extraction methods for the evaluation of bioavailability of heavy metals in soil to barley. The extractable amounts of Cr, Cu, Zn and Cd analyzed by the rhizosphere-based method correlated significantly with the metal content of barley roots. The extractable metals identified by DTPA, EDTA, CaCl_2 and NaNO_3 methods exhibited relatively poor or no correlation with the metal content of barley roots. The stepwise multiple regression equation of the rhizosphere-based method was the simplest one, as no soil properties needed to be entered, whereas the equations for the DTPA, EDTA, CaCl_2 and NaNO_3 extraction methods always require those variables. The most distinct feature of the rhizosphere-based method was that the proposed method was suitable for acidic, neutral and near alkaline soils. In contrast, the other extraction methods were restricted to soil types. In summary, the rhizosphere-based method is the most robust approach for evaluation of bioavailability of metals in soil to barley.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Bioavailability; Rhizosphere-based method; Moist rhizosphere soil; Low-molecular-weight organic acids; Heavy metals

1. Introduction

Bioavailability is defined as the extent to which living receptors are exposed to contaminants in soil or sediment (Ehlers and Luthy, 2003). The soil properties, metal speciation and plant species, especially soil–plant interactions, determine the bioavailability of metals in soils (Ehlerken and Kirchner, 2002). Various one-step extraction methods are frequently used for bioavailability evaluation because of simplicity and ease of operation.

Diethylenetriaminepentaacetic acid (DTPA) (0.005 M) (Haq et al., 1980; Norvell, 1984; Brun et al., 2001) and ethylenediamine tetraacetic acid (EDTA) (0.005–1 M) (Haq et al., 1980; Hammer and Keller, 2002; Chaignon et al., 2003) have been widely used due to their ability to form very stable, water-soluble and well-defined complexes with metal cations. Calcium chloride and NaNO_3 , soil background electrolyte solutions, are also frequently adopted as extractants for bioavailability prediction.

All the above methods are useful under defined conditions. However, no one method has been recognized as a universal approach for prediction of bioavailability (Sauvé et al., 2000). The common features

^{*} Corresponding author. Tel.: +86 10 62923560; fax: +86 10 62923563.

E-mail address: xiaoquan@mail.rcees.ac.cn (X.-Q. Shan).

of those extraction procedures are focused on metal fractions associated with certain soil geochemical phases, but all have ignored biological reactions in soil, especially soil–plant interactions in the rhizosphere zone. It is these reactions which determine the metal speciation, transformation, uptake by plants and accumulation in plants, thus determining overall metal bioavailability.

Low-molecular-weight organic acids (LMWOAs) are exudates by plant roots and products of fungi and bacteria activity (Fox and Comerford, 1990). Furthermore, there is increasing evidence that LMWOAs play an important role in supplying metals to roots, especially in metal deficient soils, and enhancing the uptake of metals by plants (Naidu and Harter, 1998; Cieřliński et al., 1998; Shan et al., 2002, 2003a). These studies have helped to clarify understanding of the geochemical behavior of the rhizosphere zone and the availability of metals operating at the soil–root interface.

There have been significant efforts applied in order to establish a more widely usable method for prediction of metal bioavailability in soil. In our previous study the total concentrations of acetic, lactic, malic and formic acids in rhizosphere soil and plant roots were found on average at sub-millimolar (Li et al., 2003). The effects of organic acids on metal release from soil (Shan et al., 2002) and on the uptake of metals by plants have been studied (Shan et al., 2003b). The influence of metal distribution among different soil colloidal fractions on bioavailability has been investigated (Wang et al., 2003). The relationship between metal fractions in soil solutions and metal contents of root apoplasm and symplasm has been examined (Wang et al., 2004a). Based on these studies the concept of a labile rhizosphere soil solution fraction has been recommended for prediction of bioavailability (Shan et al., 2003a).

Based on the above studies there is an understanding that many factors affect the bioavailability of metal to plants, and therefore traditional concepts of bioavailability, based on the simple cause–effect relationship between metals in soil fractions and metal accumulation by plants, must be refined. The new strategy is to mimic real field conditions as closely as possible and to integrate the combined effects of rhizosphere–plant interactions as a whole, with simulation of rhizosphere processes perceived as a key issue. The two critical parameters of the rhizosphere-based method are the use of (1) fresh moist rhizosphere soil and (2) LMWOAs as extractant in combination, in order to obtain labile metal fractions in soil solution, which have been shown to be correlated with metal contents of plants (Shan et al., 2003a).

The aim of the present study was to verify the preference of the rhizosphere-based method compared to the DTPA, EDTA, CaCl_2 , and NaNO_3 extraction methods in the prediction of bioavailability, by using

simple correlation analysis and stepwise multiple regression analysis. Emphasis of the comparison was put on the performance of suitability of those methods for different types of soils. A comparison was also made between the rhizosphere and the bulk soils for evaluation of metal bioavailability to barley.

2. Materials and methods

2.1. Soil sample

Fifteen A_p horizon cultivated soil samples were collected from different provinces of China, which represented typical Chinese soils with varying physical and chemical properties (Gong, 1999). Soil organic matter content (OM) was determined by the Walkley–Black procedure (Nelson and Sommers, 1982). Soil pH was measured in a 1:1 (w/v) soil to 0.01 M CaCl_2 solution. Cation-exchange capacity (CEC) was determined by the BaCl_2 method (Hendershot and Duquette, 1986). Amorphous iron oxides (amorphous-Fe) were determined by the ammonium-oxalate extraction method (Blakemore et al., 1987). Crystalline iron oxides (crystal-Fe) and manganese dioxides (MnO_2) were determined by the oxalate-ascorbic acid extraction method of Shuman (1982). The selected soil properties are presented in Table 1.

2.2. Pot experiments

The 15 soil samples collected were air-dried and ground to pass through a 1 mm nylon fiber sieve. Soil samples (0.5 kg per pot) were fertilized with 0.2 g each of N ($(\text{NH}_2)_2\text{CO}$) and P (KH_2PO_4) in solution, homogenized and incubated for 1 week before planting. Four pots were used to plant barley and for the control, respectively, for each soil. The same treatment procedure was used for control samples, but without plants. The control soil is referred to as bulk soil hereafter.

Barley (*Hordeum vulgare* L.) seeds were soaked in distilled water for 24 h, and then placed on wet filter paper to germinate. Seedlings were sown in each pot and thinned to 10 seedlings per pot after 2 weeks. The relative humidity of the soils was kept around 15% by watering with de-ionized water daily. Six weeks after planting, seedlings were harvested. Shoots and roots were first washed thoroughly with tap water and then with de-ionized water. Then the roots were cut off and washed in de-ionized water in an ultrasound-assisted bath at 35 W, 25 kHz for 15 min. Roots and shoots were dried in an oven at 65 °C for 2 days to constant weight.

Rhizosphere soil was obtained by firstly gently shaking off the loosely bound soil, while the rhizosphere

Table 1
Selected properties of soils

	Site	Taxonomy	pH	CEC (mol m ⁻³) ^a	OM (%) ^b	Fe		MnO ₂ (g kg ⁻¹)
						Amorphous (%) ^c	Crystalline (%) ^d	
North-east of China	Heilongjiang	Isohumisols	7.34	20.2	6.86	0.73	18.3	4.02
	Jilin	Isohumisols	5.65	14.0	2.56	0.70	26.6	6.58
Mid-China	Gansu	Isohumisols	6.55	41.2	0.96	0.26	21.9	3.27
	Neimenggu	Luvisols	6.32	56.8	3.81	0.26	13.1	3.09
	Shanxi	Luvisols	7.29	21.9	4.12	0.27	19.4	2.21
	Beijing	Cambisols	6.91	15.7	1.35	0.37	15.5	4.09
Eastern China	Shandong	Luvisols	6.75	18.0	0.98	0.47	16.7	4.74
	Jiangsu	Luvisols	6.37	22.3	0.68	0.47	16.1	3.62
	Anhui	Luvisols	5.16	25.0	2.09	0.66	18.0	3.54
	Hubei	Luvisols	6.67	22.8	0.92	1.04	32.9	5.25
	Fujian	Ferralsols	6.09	5.1	1.81	0.54	13.2	3.53
	Jiangxi	Ferrisols	4.70	14.2	0.80	0.29	24.4	1.90
Western China	Yunnan	Ferrisols	6.64	15.5	2.23	0.97	29.9	5.25
	Chengdu	Cambisols	6.50	42.6	1.03	0.54	22.0	4.82
	Xinjiang	Aridisols	7.44	74.0	0.90	0.26	14.1	4.14

^a CEC, cation exchange capacity.

^b OM, organic matter content.

^c Amor-Fe, amorphous iron oxide.

^d Cry-Fe, crystalline iron oxide.

soil adhering to the root system was isolated by more vigorous shaking or by hand. Moist rhizosphere soil or bulk soil was subjected to metal fractionation immediately or placed in a polyethylene bag and stored at -18°C prior to metal fractionation.

2.3. Rhizosphere-based method

The rhizosphere-based extraction method followed the protocol: 2.00 g of moist rhizosphere soils were mixed with 20 mL of combined organic acid solution of acetic, lactic, citric, malic and formic acids in 50 mL pre-cleaned plastic centrifuge tubes. The total concentration of these organic acids was 10 mM, and their molar ratio was 4:2:1:1:1. The mixture was shaken by an end-over-end shaker for 16 h, and then centrifuged at $3000\times g$ for 10 min. An aliquot of 5 mL of supernatant was removed immediately by a transfer pipette to a 10 mL volumetric flask and diluted to volume with 2% HNO₃. The extracts obtained were used for the determination of heavy metals. The soil moisture content was measured by drying soil at 65°C for 2 days. All extraction procedures were carried out in quadruplicate including blanks. The coefficients of variation of metal concentrations were less than 5% for each soil.

2.4. Comparison with other one-step extraction methods

In order to make a comparison, several other one-step extraction procedures were performed and the

detailed operating procedures were followed with the exception that moist rhizosphere soils were used. These one-step extraction methods are described briefly below:

DTPA extraction method (Lindsay and Norvell, 1978): 10.0 g of soil in 20 mL of 0.005 M DTPA + 0.01 M CaCl₂ + 0.01 M TEA, pH 7.3, shaking for 2 h;

EDTA extraction method (Wear and Evans, 1968): 2.00 g of soil in 20 mL of 0.05 M EDTA, shaking for 1 h;

CaCl₂ extraction method (Novozamsky et al., 1993): 2.00 g of soil in 20 mL of 0.01 M CaCl₂, shaking for 3 h;

NaNO₃ extraction method (Gupta and Aten, 1993): 8.00 g of soil in 20 mL of 0.1 M NaNO₃, shaking for 2 h.

2.5. Determination of heavy metals in soils, soil extracts, and plants

The contents of heavy metals in soils, soil extracts, and barley roots and shoots were determined by inductively coupled plasma mass spectrometry (ICP-MS, PQ3, Fisons Instruments, UK). The operating conditions were optimized and ¹¹⁵In was used as an internal standard. Soil and plant samples were decomposed with 3 mL of HNO₃/HClO₄/HF (1:1:1, v/v) mixture under high-pressure conditions (Zhang and Shan, 1997). For quality control, certified standard reference materials, flour and soil (GBW080001 and GBW08503), obtained from the National Research Center for CRM'S, China, were used, and good agreement was achieved between the data obtained by this method and the certified values.

2.6. Data analysis

Simple correlation analysis was performed using SPSS 11.5 for Windows (SPSS Inc., USA). Pearson correlation coefficients were determined by correlating metal contents in roots and shoots with extractable heavy metals from soils by various extraction methods.

Forward stepwise multivariate regression was also used to derive best-fit models of metal contents in plants as a function of soil properties. The variance inflation factor (VIF), the condition indices and the regression coefficient variance decomposition matrix were also assessed in order to evaluate the collinearity between independent variables. Collinearity becomes an issue when values of VIF exceed 10.0 and the condition index is over 30.

3. Results

3.1. Optimization of the rhizosphere-based extraction procedure

The concentrations of acetic, lactic, citric, malic and formic acids in the rhizosphere soils and barley roots ranged from several to several hundreds of μM (data not shown), thus a mixture of a total of 10 mM of acetic, lactic, citric, malic, and formic acids at the molar ratio of 4:2:1:1:1 was used as the extractant throughout this study.

To optimize the effect of extraction time on the extractable metals from soils at room temperature, moist rhizosphere soils from Beijing, Heilongjiang, and Jiangxi were used, with similar results being obtained. Taking the Heilongjiang soil as an example, the results are shown in Fig. 1. The extractable metals reached equilibrium after 12 h of extraction. The pH of soil solutions before and after extraction remained unchanged after an extraction time of 20 h. As a result, an extraction time of 16 h was chosen for the remainder of this study.

3.2. Comparison of the rhizosphere-based method with the DTPA, EDTA, CaCl_2 and NaNO_3 extraction methods for bioavailability using simple correlation analysis

The ranges of total heavy metal content in soils, barley shoots and roots are given in Table 2. Bio-availability of heavy metals in soil to barley was evaluated by correlating the metal content in soil fractions determined by the rhizosphere-based method and by the DTPA, EDTA, CaCl_2 and NaNO_3 extraction methods with the metal content in roots and shoots. The results are shown in Table 3. The extractable Cr and Cd exhibited correlation coefficients of 0.787 and 0.853

Fig. 1. Relationship between the extraction time and extractable metals from Heilongjiang soil by using 10 mM low-molecular-weight organic acids (LMWOAs) as extractant: □, Cr; ●, Ni; △, Cu; ▲, Zn; ○, Cd; +, Pb.

($p < 0.01$) for Cr and Cd, respectively, with the content of barley shoots, when the rhizosphere-based method was used. For the DTPA extraction method, a good correlation was obtained only between the extractable Zn and Zn of barley shoots ($r = 0.793$, $p < 0.01$). Using the EDTA extraction method, the extractable Zn and Pb exhibited correlations with the content of barley shoots of $r = 0.760$ ($p < 0.01$), and 0.658 ($p < 0.05$), respectively. However, very poor correlations were found for CaCl_2 and NaNO_3 extraction methods.

When the rhizosphere-based method was used, the extractable Cr, Cu, Zn, and Cd correlated significantly with the content of barley roots, with correlation coefficients of 0.745, 0.709, 0.719 and 0.732 ($p < 0.01$), respectively. When the DTPA method was used, a good correlation was obtained for Cu and Zn, with correlation coefficients of 0.726 and 0.717 ($p < 0.01$), respectively. For the EDTA extraction method, only the extractable Zn exhibited significant correlation with its content in roots, with correlation coefficients of 0.544 ($p < 0.05$). The CaCl_2 extractable Cr and Zn correlated with the content of barley roots, with coefficients of 0.533 and 0.573 ($p < 0.05$), respectively. For the NaNO_3 extraction method, there was no clear positive correlation between the extractable metal fractions in soil pools and metal content of barley roots.

There was no significant correlation between the soil Pb and Ni fractions and the metal content of barley roots when those single extraction methods were used for metal fractionation.

3.3. Comparison between the rhizosphere soils and the bulk soils for bioavailability

The relationship between extractable heavy metals from the rhizosphere soil (Table 3) and from the bulk soil (Table 4) by using five one-step extraction methods

Table 2

Total content of trace heavy metals in barley shoots, roots (mg kg⁻¹ DW), and in soils (mg kg⁻¹) (*n*=15)

Element	Content in shoot			Content in root			Content in soil		
	Range	Mean	% CV	Range	Mean	% CV	Range	Mean	% CV
Cr	0.273–0.844	0.555	40.6	1.68–10.4	4.81	44.8	4.15–76.1	42.4	38.6
Ni	0.242–0.828	0.625	27.3	4.14–15.7	8.09	38.3	10.0–95.4	38.5	52.4
Cu	8.35–14.1	10.8	15.2	7.30–27.4	12.4	14.8	31.9–51.0	18.2	47.2
Zn	25.1–66.2	38.2	30.5	11.6–62.3	32.8	51.4	38.5–510	182	66.0
Cd	0.0214–0.307	0.112	76.0	0.200–1.14	0.543	56.1	0.200–0.875	0.377	47.3
Pb	0.857–5.16	1.73	59.4	2.06–23.3	10.5	66.3	21.9–86.6	39.5	46.4

and the metal content in barley roots was examined. The extractable Cr, Cu, Zn and Cd from the rhizosphere and bulk soils exhibited significant correlation with the content in barley roots when the rhizosphere-based method was used. However, the correlation coefficients of the rhizosphere soils were greater than those of the bulk soils (Tables 3 and 4). For the DTPA extraction method, the extractable Cu, and Zn from the moist rhizosphere soils and the bulk soils had significant correlations with the Cu and Zn of roots, but the correlation coefficients of the rhizosphere soils were also better than those of the bulk soils. The EDTA extractable Zn from the rhizosphere soils had a good positive correlation with Zn content of barley roots. But when the EDTA extraction method was used for the bulk soils, no significant correlation was found for any of the metals investigated. The CaCl₂ extractable Cr and Zn from the rhizosphere soils showed positive correlation with the content of roots. However, the CaCl₂ and NaNO₃ extractable metals from the bulk soil had no significant correlation with metal content in barley roots. The above comparison between the rhizosphere soils and the bulk soils is convincing evidence that the use of rhizosphere soils is to be preferred to bulk soils for the prediction of bioavailability.

Table 3

Linear correlation coefficients (*r*) between the extractable metals from the rhizosphere soils by different extraction methods and metal content of barley shoots and roots (*n*=15)

	Element	RHIZO ^a	DTPA	EDTA	CaCl ₂	NaNO ₃
Shoots	Cr	0.787***	−0.164	−0.027	0.395	0.267
	Cu	−0.041	0.213	0.309	−0.085	0.191
	Zn	0.203	0.793***	0.760***	0.421	0.107
	Cd	0.853***	0.451	−0.052	−0.200	0.098
	Ni	0.180	−0.142	−0.464	−0.365	−0.010
	Pb	0.022	0.206	0.658**	0.179	0.173
Roots	Cr	0.745***	0.008	0.144	0.533**	0.329
	Cu	0.709***	0.726***	0.250	−0.016	0.301
	Zn	0.719***	0.717***	0.544**	0.573**	−0.271
	Cd	0.732***	0.362	0.092	−0.230	−0.038
	Ni	0.094	−0.026	0.149	−0.175	−0.423
	Pb	−0.447*	0.077	−0.006	−0.220	0.143

*, ** and *** indicate statistical significance at the probability level of *p*<0.1, *p*<0.05, and *p*<0.01, respectively.

^a RHIZO, rhizosphere-based method.

3.4. Comparison of suitability of the rhizosphere-based method with other one-step extraction methods for different types of soils

In order to demonstrate the preference of the rhizosphere-based method over other one-step extraction methods for predicting metal bioavailability in different types of soils, a total of 15 soils were divided into two groups based on soil pH. Soils of pH≤6.5 were referred to as acidic soils (*n*=7), and soils of pH >6.5 as neutral and near alkaline soils (*n*=8). Generally, barley roots extracted more heavy metals from the acidic soils than from the neutral and near alkaline soils. Similarly, all five one-step extraction methods extracted more metals from the acidic soils than from the neutral and near alkaline soils (data not shown).

A simple correlation analysis was performed between the extractable metals by different extraction methods from both the acidic and neutral and near alkaline soils, and the metal contents of barley roots, separately. The comparison results are shown in Table 5. The extractable Cr, Cu, Zn, and Cd from the acidic, neutral and near alkaline soils by the rhizosphere-based method correlated significantly with the content of barley roots. There was a good correlation between DTPA extractable Cu, Zn and Cd from the neutral and near alkaline soils and metal content of barley roots, but poor correlation for the acidic soils. For the DTPA extraction method, the correlation coefficients of the neutral and near alkaline soil (Table 5) were even better than those of all 15 soils together (Table 3). The EDTA

Table 4

Linear correlation coefficients (*r*) between the extractable metals from the bulk soils by different extraction methods and metal content of barley roots (*n*=15)

Element	RHIZO ^a	DTPA	EDTA	CaCl ₂	NaNO ₃
Cr	0.535**	0.128	−0.087	0.068	0.417
Cu	0.691***	0.687***	0.166	−0.139	0.026
Zn	0.638**	0.661***	0.269	0.333	0.012
Cd	0.606**	0.379	0.316	0.093	0.227
Ni	−0.313	−0.379	0.032	−0.150	−0.284
Pb	0.070	−0.034	0.397	−0.197	−0.064

*, ** and *** indicate statistical significance at the probability level of *p*<0.1, *p*<0.05, and *p*<0.01, respectively.

^a RHIZO, rhizosphere-based method.

Table 5

Linear correlation coefficients (r) between the extractable metals from acidic soils ($\text{pH} \leq 6.5$, $n=7$) and from neutral and near alkaline soils ($\text{pH} > 6.5$, $n=8$) by different extraction methods and metal contents of roots

Element	RHIZO ^a		DTPA		EDTA		CaCl ₂		NaNO ₃	
	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline
Cr	0.725*	0.732**	−0.005	0.019	0.195	0.104	0.739*	−0.581	0.454	−0.257
Cu	0.818**	0.789**	0.453	0.794**	0.723*	−0.364	0.196	−0.138	0.191	0.386
Zn	0.687*	0.727**	−0.694*	0.885***	0.836***	0.597	0.431	0.346	−0.111	−0.381
Cd	0.798**	0.880***	−0.501	0.958***	−0.350	0.005	−0.298	−0.150	0.268	−0.893***
Ni	0.283	−0.146	0.024	0.095	−0.101	0.306	−0.743*	−0.094	−0.565	−0.112
Pb	−0.532	−0.385	0.203	0.437	0.253	−0.323	−0.031	−0.371	−0.190	0.286

*, ** and *** indicate the statistical significance at probability level of $p < 0.1$, $p < 0.05$, and $p < 0.01$, respectively.

^a RHIZO, rhizosphere-based method.

extractable Cu and Zn exhibited good correlation with the content of Cu and Zn of barley roots for the acidic soils, but not for the neutral and near alkaline soils.

For neutral salt extractant CaCl₂, the extractable Cr had a good correlation with the content of barley roots for the acidic soils, but there was no correlation for the neutral and near alkaline soils. For NaNO₃, no positive correlation was observed for either the acid soils or for the neutral and near alkaline soils.

3.5. Multiple regression analysis for bioavailability

The stepwise multiple regression analyses for Cr, Cu, Zn, and Cd concentrations in roots as a function of extractable metals in soil solution fractions, soil pH, OM, CEC, amorphous-Fe and crystal-Fe and MnO₂ are listed in Table 6. For simplicity, the regression coefficients were omitted in the stepwise multiple regression equations, whereas the negative sign of the variables was maintained. The significance of coefficients, r , an

evaluation of the overall prediction of bioavailability, is also included in Table 6.

The simple correlation analysis indicated that there were no clear positive correlations of Ni and Pb concentrations in roots with the extractable Ni and Pb by all extraction methods (Table 3). The stepwise multiple regression analysis revealed the same results. Therefore, no discussion was given for Ni and Pb in detail afterwards.

Because the stepwise multiple regression equations for metals Cr, Cu, Zn and Cd were quite similar, we took the regression equations of Cr as representative. The stepwise multiple regression equation of the rhizosphere-based method was the simplest one with r of 0.745 ($p < 0.01$), to which no other soil property variables needed to be entered. The stepwise multiple regression equation of DTPA extractable Cr consisted of three steps. The first variable entered was Cr×OM with $r = 0.540$ ($p < 0.05$). The second variable entered was crystal-Fe and raised r to 0.650 ($p < 0.05$). The third variable entered was

Table 6

Stepwise multiple regression equations of metals in roots as a function of extractable metals in soil solution by different extraction methods and soil properties

Method	Regression step	Cr _{root}		Cd _{root}		Cu _{root}		Zn _{root}	
		Variables	r	Variables	r	Variables	r	Variables	r
RHIZO ^a	1	Cr	0.745***	Cd	0.732***	Cu	0.709**	Zn	0.719***
	2							OM	0.805***
DTPA	1	Cr×OM	0.540**	Cd×Amor-Fe	0.646***	(−)Cu×pH	0.777***	Zn	0.717***
	2	Cry-Fe	0.650**	Cd	0.760***	Amor-Fe	0.866***	Amor-Fe	0.825***
	3	Amor-Fe	0.730**	Amor-Fe	0.826***			(−)MnO ₂	0.860***
EDTA	1	Cry-Fe	0.467*	Cry-Fe	0.653***	Cu×Cry-Fe	0.478*	(−)Zn×Cry-Fe	0.680***
	2	Cr×OM	0.671**			(−)Cu×pH	0.580*	Zn	0.909***
NaNO ₃	1	Cr×OM	0.760***	(−)Cd×pH	0.673**	Cu×Cry-Fe	0.642**	Amor-Fe	0.612**
	2			Cry-Fe	0.871**			OM	0.711**
CaCl ₂	1	Cr×OM	0.815***	Cry-Fe	0.653***	Amor-Fe	0.453*	Amor-Fe	0.612**
	2	(−)CEC	0.880***	(−)Cd×Cry-Fe	0.727***			Zn×OM	0.716***
	3			Amor-Fe	0.796***			(−)CEC	0.780***

*, ** and *** indicate statistical significance at the probability level of $p < 0.1$, $p < 0.05$, and $p < 0.01$, respectively.

^a RHIZO, rhizosphere-based method; OM, organic matter content; Amor-Fe, amorphous iron oxide; Cry-Fe, crystalline iron oxide.

amorphous-Fe, which resulted in r of 0.730 ($p < 0.05$). The data from Table 6 for the DTPA extractable Cr can be presented in equation form as follows:

$$\begin{aligned} \text{Cr in barley root} = & -1978 + 39.7 \text{Cr} \times \text{OM} \\ & + 443 \text{ crystal-Fe} \\ & + 7986 \text{ amorphous-Fe} \end{aligned}$$

This suggests that $\text{Cr} \times \text{OM}$, crystal-Fe, and amorphous-Fe contributed to overall Cr availability. The $\text{Cr} \times \text{OM}$ interaction indicates that the DTPA extractable Cr was more available to plants at high organic matter content of soil. The similar ionic radius of Cr(III) to Fe(III) determines the affinity of Cr for Fe oxides and the speciation of Cr in soil (Agbenin, 2002), which was also responsible for bioavailability.

For EDTA extractable Cr, the first and second variables entered in the equation were crystal-Fe and $\text{Cr} \times \text{OM}$ with r of 0.467 ($p < 0.1$), and r of 0.671 ($p < 0.05$), respectively. The regression equations developed for the NaNO_3 and CaCl_2 extraction methods all required $\text{Cr} \times \text{OM}$ interaction as the first variable, and resulted in r of 0.760 and 0.815 ($p < 0.01$), respectively. The second variable entered in the equation for CaCl_2 extractable Cr was soil CEC with negative coefficient and resulted in r of 0.880 ($p < 0.01$).

4. Discussion

4.1. Bioavailability of metals in soils to barley

In our previous study it was demonstrated that acetic, lactic, malic and formic acids were dominant in plant roots and in the rhizosphere soil, ranging from several μM to several hundreds of μM , respectively (Li et al., 2003; Han et al., 2005). In order to simulate the content gradient of LMWOAs from root surface to soil solution (Cieřliński et al., 1998; Fox and Comerford, 1990) and to use a short-term extraction for simulation of long-term reaction in the rhizosphere environment, the use of a mixture of total 10 mM of acetic, lactic, citric, malic, and formic acids at the molar ratio of 4:2:1:1:1 as an extractant was proposed as reasonable.

Generally, the concentration of metals in shoots is lower than that in roots (Table 2), as reported in numerous studies (Cieřliński et al., 1998; Chaignon et al., 2003). The transport of metals from roots to shoots includes long-distance translocation in the xylem and storage in the vacuole of leaf cells, and these processes are affected by many factors (Yang et al., 1997) although the environmental stress of soil pollution is difficult to estimate in some cases (Cieřliński et al., 1998; Brun et al., 2001; Chaignon et al., 2003). Comparing the correlation between the metals in shoots and extractable metals in soil fractions to those between the metals in

roots and extractable metals (Table 3), it seemed that metals in shoots were not a good indicator of metal bioavailability, which is in agreement with the criticism of Chaignon et al. (2003). In the present study, roots were thoroughly washed with ultrasound-assisted washing, thus ensuring the removal of very fine soil grains adsorbed to the root epidermis (Wang et al., 2004a). When roots are washed with EDTA (Texier et al., 2000) or CaCl_2 solution (Lasat et al., 1996), there is a resulting desorption of root apoplastically bound metals (Wang et al., 2004a; Han et al., 2005). It was verified that both apoplastically and symplastically bound metals are the result of bioavailability (Wang et al., 2004a). Therefore, total root concentrations would be an indicator of bioavailability. Thus, the discussion was focused on the relationship between metals in roots and in soil pools.

4.2. Preference of moist fresh rhizosphere soil to bulk soil for bioavailability

The use of rhizosphere soil is logical for prediction of bioavailability of soil metals to barley compared with bulk soil (Tables 3 and 4), the reason being that the rhizosphere zone is a special microenvironment with different physical and chemical properties from bulk soil (Hammer and Keller, 2002). Low-molecular-weight organic acids produced by plant roots exudation and bacterial metabolism are involved in many processes operating in the rhizosphere, including nutrient acquisition, metal detoxification, and alleviation of anaerobic stress in roots (Jones, 1998). Through their acidification, chelating, and oxidation-reduction reaction in the rhizosphere, they can affect the speciation and transportation of heavy metals in the rhizosphere soil. Qin et al. (2004) demonstrated that various LMWOAs were able to influence the rate of heavy metals from different soils and increase the solubility of heavy metals through the formation of metal–organic complexes. Due to the effect of LMWOAs in the rhizosphere, the bioavailability of heavy metals in the rhizosphere is different from that in the bulk soil. Tu et al. (2004) indicated that root organic acids increased the release of arsenic from soil and increased its translocation to the fronds of fern. Further, a better correlation can be observed between the speciation and bioavailability of heavy metals in the rhizosphere soils than that in the non-rhizosphere soils (Wang et al., 2002). However, such influences depend on soil properties and plant species. Shuman and Wang (1997) studied the effect of rice variety on metal content in the rhizosphere and non-rhizosphere soil fractions; the results suggested that rice cultivars with high oxidizing power altered the forms of metals in rhizosphere so as to decrease their plant availability. Hammer and Keller (2002) found that plant *T. caerulescens*, and *S. viminalis* altered the heavy metal distribution patterns in different soil pools, which was related to their

bioavailability. All the above studies fully supported our result that the correlation coefficients of the rhizosphere soils were better than those of the bulk soils.

4.3. Preference of the rhizosphere-based method over other one-step extraction methods for bioavailability

The comparison using simple correlation analysis demonstrated that the rhizosphere-based method was better than other single extraction methods for the evaluation of bioavailability of Cr, Cu, Zn, and Cd in soils to barley roots (Table 3), although it failed to assess the bioavailability of Ni and Pb because Ni and Pb are non-essential elements to plants (Lee et al., 1997). The research of Pitchel et al. (1999) also suggested that none of the extractants reflected the uptake of Ni into the plants. Soil Pb was not correlated with plant accumulation because soil has rather high capacity for immobilizing Pb (Blaylock et al., 1997), thus Pb is generally unavailable to plants (Pitchel et al., 1999).

If one compares the stepwise multiple regression equations of the rhizosphere-based extraction method with those of other conventional single extraction methods, the conclusion can be drawn that the equation of the rhizosphere-based method is the simplest one. The extractable Cr, Cu, Zn, and Cd obtained by the rhizosphere-based extraction method account for about 70% of the variability in root metals (Table 6), and the addition of other soil variables provides less than significant improvement (see the second step of the extractable Zn regression equation, Table 6). In contrast, the DTPA, EDTA, CaCl_2 and NaNO_3 extraction methods required the addition of various soil variables for the different metals in the equations. Moreover, the most distinct feature of the rhizosphere-based method was that the suggested method was suitable for both acidic and neutral and near alkaline soils (Table 5), indicating that the rhizosphere-based method is a potentially universal approach for evaluation of metal bioavailability in soils to plants.

The success of the rhizosphere-based method could be ascribed to the use of fresh rhizosphere soil and LMWOAs as an extractant. The logic of fresh rhizosphere soil has been described above. It should be pointed out that LMWOAs are important plant exudates and microbial metabolites in the rhizosphere zone. The total concentration of LMOWAs and their ratio were chosen based on their abundance in the rhizosphere zone. Acetic and lactic acids are the highest concentrations of all five LMWOAs because these two organic acids are more likely to originate from rhizosphere bacteria (Jones, 1998) and are the most abundant carboxylic acids in soil solution (Strobel, 2001; Li et al., 2003). Bioavailability of soil metals is the result of a combination effect of root–soil–microbe interaction occurring in the rhizosphere soil zone, in

which LMWOAs are the primary factor involved in the metal adsorption by roots (Wang et al., 2004b; Han et al., 2005). For the rhizosphere-based method, complexation of metals by organic acids plays an important role in controlling metal solubility (Naidu and Harter, 1998; Cieřliński et al., 1998; Shan et al., 2002; Qin et al., 2004). Rhizosphere pH can also affect the extractability of LMWOAs from two aspects. On the one hand, pH changes the affinity of soils for heavy metals due to pH-dependent surface-charge density on soil colloids, on the other hand, the species of LMWOAs are also affected by the soil pH. Because different organic acids have different pK_a , the concentrations of organic ligands should be different at a given pH. The results obtained by our previous work (Shan et al., 2002) showed that the extractability of LMWOAs followed the order of complex stability of the organic acids with heavy metals, indicating that organic acids are the main controlling factor influencing extractability. Metals extracted by the rhizosphere-based method correspond to the mobile fractions in soil solution (Naidu and Harter, 1998), which are responsible for the short-term available pool (Cieřliński et al., 1998).

Although the DTPA extractable Cu and Zn had good correlation with those in barley roots (Table 3), the DTPA extraction method was only suitable for neutral and near-alkaline soils (Table 5) because the DTPA method was originally developed for the neutral and near-calcareous soils with insufficient transition metals (Lindsay and Norvell, 1978). When DTPA was applied to acidic soil, the buffering capacity of the soil solution might be exceeded (O'Connor, 1988), and considerable amounts of heavy metals occluded by Fe/Mn oxides and carbonates could be released (Hammer and Keller, 2002), which were not responsible for plant availability. This was evidence that DTPA was unsuitable for prediction of bioavailability of heavy metals in acidic soils to plants.

The EDTA extraction method was only suitable for acidic soils (Table 5). This was easily understood because the EDTA method was originally developed for acidic soils (Hammer and Keller, 2002). EDTA is a strong chelating reagent and can extract trace elements from soil geochemical phases including labile and non-labile fractions (Bermond et al., 1998). It resulted in relatively lower correlation coefficients between the EDTA extractable metals and metals in barley roots (Table 3).

The CaCl_2 and NaNO_3 extraction methods were only suitable for exchangeable metals (Hammer and Keller, 2002). Calcium is the dominant cation of soils and would reflect the differences in binding strength or solubility between different soils (Novozamsky et al., 1993). NaNO_3 would extract exchangeable metals mainly because its ionic strength is similar to that of soil solution and it does not affect the equilibrium

between soil solution and soil solid (Gupta and Aten, 1993). Although easily exchangeable metals can be extracted with NaNO_3 and CaCl_2 , NaNO_3 exerts a weak competition for the adsorption sites of organic matter and oxide surfaces (Novozamsky et al., 1993). This was the reason why no correlation coefficient was available for the NaNO_3 extraction method (Table 3). Hammer and Keller (2002) also suggested that plants seemed to take up heavy metals from pools rather than solely from NaNO_3 -extractable one. Overall, the suitability of CaCl_2 and NaNO_3 extraction methods were restricted to different types of soils. Soils with higher pH and higher cation exchange capacity generally sequestered more Cr (Stewart et al., 2003) due to the ineffective extraction of Cr by CaCl_2 from neutral and near alkaline soils (Table 5). This was consistent with the multiple regression analysis (Table 6). The negative coefficient of the second variable, soil CEC, entered in the multiple regression equation of the CaCl_2 extractable Cr meant that the extractable Cr was less available to roots for soil with high CEC. This observation was in agreement with the findings of Buchter et al. (1989). NaNO_3 extractable Cd was low at pH value > 5.5 (Naidu and Harter, 1998), a finding which is consistent with our results that the NaNO_3 extractable Cd correlated negatively with root Cd (Table 5).

5. Conclusion

The rhizosphere-based method has been suggested for the evaluation of bioavailability of soil metals to plants, with the use of fresh rhizosphere soils and low-molecular-weight organic acids as extractant being stressed. A systematic comparison was conducted, using both simple correlation analysis and multiple regression analysis between the rhizosphere-based method and the DTPA, EDTA, CaCl_2 and NaNO_3 extraction methods. The results indicated that the rhizosphere-based method was the most robust and it was able to properly predict the bioavailability of Cr, Cu, Zn, and Cd in soils to barley. The DTPA extraction methods were good for assessing the bioavailability of Cu and Zn, but the correlation coefficients of Cu and Zn were relatively poor. The EDTA extraction method was also able to assess the bioavailability of Zn, while the CaCl_2 extraction method can be used to assess the bioavailability of Cr and Zn. The most important feature of the rhizosphere-based method was that the method was suitable for both acidic and neutral and near alkaline soils. In contrast, the DTPA and EDTA extraction methods were suitable only for calcareous or for acidic soils, respectively. The CaCl_2 and NaNO_3 extraction methods were only suitable for exchangeable metals. Based on the aforementioned comparison, one can draw

the conclusion that the rhizosphere-based extraction method is a potentially universal approach for evaluation of bioavailability of heavy metals in soils to barley. However, additional field testing of the rhizosphere-based method for more plants needs to be evaluated, which is currently under progress.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (Grant nos. 20237010, 20177030). The authors thank Dr Douglas McLeod of the Association of Scottish Shellfish Growers for the improvement of the written English.

References

- Agbenin, J.O., 2002. The distribution and dynamics of chromium and nickel in cultivated and uncultivated semi-arid soils from Nigeria. *The Science of the Total Environment* 300, 189–199.
- Bermond, A., Yousfi, I., Ghestem, J.P., 1998. Kinetic approach to the chemical speciation of trace metals in soils. *Analyst* 123, 785–789.
- Blakemore, L.C., Searle, P.L., Daly, B.K., 1987. Methods for chemical analysis of soils. New Zealand Bureau scientific report no. 80. NZ Soil Bureau, Lower Hutt, New Zealand.
- Blaylock, M.J., Salt, D.E., Dushenkov, S., Zakharova, O., Gussman, C., Kapulnik, Y., Ensley, B.D., Raskin, I., 1997. Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. *Environmental Science & Technology* 31, 860–865.
- Brun, L.A., Mailler, J., Hinsinger, P., Pépin, M., 2001. Evaluation of copper availability to plants in copper-contaminated vineyard soils. *Environmental Pollution* 111, 293–302.
- Buchter, B., Davidoff, B., Amacher, M.C., Hinz, C., Iskandar, I.K., Selim, H.M., 1989. Correlation of ferundlich Kd and n retention parameters with soils and elements. *Soil Science* 148, 370–379.
- Chaignon, V., Sanchez-Neira, I., Herrmann, P., Jaillard, B., Hinsinger, P., 2003. Copper bioavailability and extractability as related to chemical properties of contaminated soils from a vine-growing area. *Environmental Pollution* 123, 229–238.
- Cieśliński, G., van Rees, K.C.J., Szmigielska, A.M., Krishnamurti, G.S.T., Huang, P.M., 1998. Low-molecular-weight organic acids in rhizosphere soils of durum barley and their effect on cadmium bioaccumulation. *Plant and Soil* 203, 109–117.
- Ehers, L.J., Luthy, R.G., 2003. Contaminant bioavailability in soil and sediment. *Environmental Science & Technology* 37, 295A–302A.
- Ehlken, S., Kirchner, G., 2002. Environmental processes affecting plant root uptake of radioactive trace elements and variability of transfer factor data. A review. *Journal of Environmental Radioactivity* 58, 97–112.
- Fox, T.R., Comerford, N.B., 1990. Low-molecular-weight organic acids in selected forest soils of the southeastern USA. *Soil Science Society of America Journal* 54, 1139–1144.
- Gong, Z.T., 1999. Chinese soil taxonomy. Chinese Academic Press, Beijing, China.
- Gupta, S.K., Aten, C., 1993. Comparison and evaluation of extraction media and their suitability in a simple model to predict the biological relevance of heavy metal concentrations in contaminated soils. *International Journal of Environmental Analytical Chemistry* 51, 25–46.

- Hammer, D., Keller, C., 2002. Changes in the rhizosphere of metal-accumulating plants evidenced by chemical extractants. *Journal of Environmental Quality* 31, 1561–1569.
- Han, F., Shan, X.Q., Zhang, J., Xie, Y.N., Pei, Z.G., Zhang, S.Z., Zhu, Y.G., Wen, B., 2005. Organic acids promote the uptake of lanthanum by barley roots. *New Phytologist* 165, 481–492.
- Haq, A.U., Bates, T.E., Soon, Y.K., 1980. Comparison of extractants for plant-available zinc, cadmium, nickel, and copper in contaminated soils. *Soil Science Society of America Journal* 44, 772–777.
- Hendershot, W.H., Duquette, M., 1986. A simple barium chloride method for determining cation exchange capacity and exchangeable cations. *Soil Science Society of America Journal* 50, 606–608.
- Jones, D.L., 1998. Organic acids in the rhizosphere – a critical review. *Plant and Soil* 205, 25–44.
- Lasat, M.M., Baker, A.J.M., Kochian, L.V., 1996. Physiological characterization of root Zn^{2+} absorption and translocation to shoots in *Zn* hyperaccumulator and nonaccumulator species of *Thlaspi*. *Plant Physiology* 112, 1715–1722.
- Lee, B.D., Carter, B.J., Basta, N.T., Weaver, B., 1997. Factors influencing heavy metal distribution in six Oklahoma benchmark soils. *Soil Science Society of America Journal* 61, 218–223.
- Li, Y.H., Huang, B.X., Shan, X.Q., 2003. Determination of low molecular weight organic acids in soil, plants, and water by capillary zone electrophoresis. *Analytical and Bioanalytical Chemistry* 375, 775–780.
- Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Science Society of America Journal* 42, 421–428.
- Naidu, R., Harter, R.D., 1998. Effect of different organic ligands on cadmium sorption by and extractability from soils. *Soil Science Society of America Journal* 62, 644–650.
- Nelson, D.W., Sommers, L.E., 1982. Total carbon, organic carbon, and organic matter. In: Page, A.L., et al. (Eds.), *Methods of soil analysis: Part 2*, second ed. ASA and SSSA, Madison, WI, USA, pp. 539–579.
- Norvell, W.A., 1984. Comparison of chelating agents as extractants for metals in diverse soil materials. *Soil Science Society of America Journal* 48, 1285–1292.
- Novozamsky, I., Lexmond, T.H.M., Houba, V.J.G., 1993. A single extraction procedure of soil for evaluation of uptake of some heavy metals by plants. *International Journal Environmental Analytical Chemistry* 51, 47–58.
- O'Connor, G.A., 1988. Use and misuse of the DTPA soil test. *Journal of Environmental Quality* 17, 715–718.
- Pitchel, J., Kuroiwa, K., Sawyer, H.T., 1999. Distribution of Pb, Cd, and Ba in soils and plants of two contaminated soils. *Environmental Pollution* 110, 171–178.
- Qin, F., Shan, X.Q., Wen, B., 2004. Effects of low-molecular-weight organic acids and residence time on desorption of Cu, Cd, and Pb from soils. *Chemosphere* 57, 253–263.
- Sauvé, S., Norvell, W.A., McBride, M., Hendershot, W., 2000. Speciation and complexation of cadmium in extracted soil solutions. *Environmental Science & Technology* 34, 291–296.
- Shan, X.Q., Lian, J., Wen, B., 2002. Effect of organic acids on adsorption and desorption of rare earth elements. *Chemosphere* 47, 701–710.
- Shan, X.Q., Wang, Z.W., Wang, W.S., Zhang, S.Z., Wen, B., 2003a. Labile rhizosphere soil solution fraction for prediction of bioavailability of heavy metals and rare earth elements to plants. *Analytical Bioanalytical Chemistry* 375, 400–407.
- Shan, X.Q., Wang, H.O., Zhang, S.Z., Zhou, H.F., Zheng, Y., Yu, H., Wen, B., 2003b. Accumulation and uptake of light rare earth elements in a hyperaccumulator *Dicroptheris dichotoma*. *Plant Science* 165, 1343–1353.
- Shuman, L.M., 1982. Separation soil iron and manganese oxide fractions for microelement analysis. *Soil Science Society of America Journal* 46, 1099–1102.
- Shuman, L.M., Wang, J., 1997. Effect of rice variety on zinc, cadmium, iron, and manganese content in rhizosphere and non-rhizosphere soil fractions. *Communication in Soil Science and Plant Analysis* 28, 23–36.
- Stewart, M.A., Jardine, P.M., Barnett, M.O., Mehlhorn, T.L., Hyder, L.K., McKay, L.D., 2003. Influence of soil geochemical and physical properties on the sorption and bioaccessibility of chromium (III). *Journal of Environmental Quality* 3, 129–137.
- Strobel, B.W., 2001. Influence of vegetation on low-molecular-weight carboxylic acids in soil solution – a review. *Geoderma* 99, 169–198.
- Texier, A.C., Andrès, Y., Illemassene, M., Le Cloirec, P., 2000. Characterization of lanthanide ion binding sites in the cell walls of *Pseudomonas aeruginosa*. *Environmental Science & Technology* 34, 610–615.
- Tu, S.X., Ma, L., Luongo, T., 2004. Root exudates and arsenic accumulation in arsenic hyperaccumulating *Pteris vittata* and non-hyperaccumulating *Nephrolepis exaltata*. *Plant and Soil* 258, 9–19.
- Wang, W.S., Shan, X.Q., Wen, B., Zhang, S.Z., 2003. Relationship between the extractable metals from soils and metals taken up by maize roots and shoots. *Chemosphere* 53, 523–530.
- Wang, Z.W., Shan, X.Q., Zhang, S.Z., 2002. Comparison between fractionation and bioavailability of trace elements in rhizosphere and bulk soils. *Chemosphere* 46, 1163–1171.
- Wang, W.S., Shan, X.Q., Wen, B., Zhang, S.Z., 2004a. A method for predicting bioavailability of rare earth elements in soils to maize. *Environmental Toxicology and Chemistry* 23, 767–773.
- Wang, Z.W., Zhang, S.Z., Shan, X.Q., 2004b. Effects of low-molecular-weight organic acids on uptake of lanthanum by barley roots. *Plant and Soil* 261, 163–170.
- Wear, J.I., Evans, C.E., 1968. Relationship of zinc uptake by corn and sorghum to soil zinc measured by three extractants. *Soil Science Society of America Proceedings* 32, 3–546.
- Yang, X.E., Baligar, V.C., Foster, J.C., Martens, D.C., 1997. Accumulation and transport of nickel in relation to organic acids in ryegrass and maize grown with different nickel levels. *Plant and Soil* 196, 271–276.
- Zhang, S.Z., Shan, X.Q., 1997. The determination of rare earth elements in soil by inductively coupled plasma mass spectrometry. *Atomic Spectroscopy* 18, 140–144.