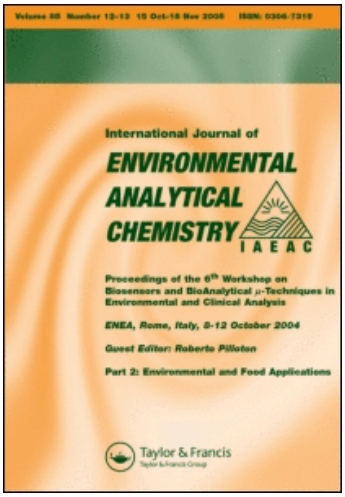

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Comparison of Different Cleaning Procedures of Root Material for Analysis of Trace Elements

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COMPARISON OF DIFFERENT CLEANING PROCEDURES OF ROOT MATERIAL FOR ANALYSIS OF TRACE ELEMENTS

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When plant roots are analyzed for trace elements, the cleaning procedure used can affect the analytical accuracy in two ways. First, with inadequate cleaning of the root surface, high levels of trace elements in the soil can contaminate the roots. Second, elements present in the root can be leached into the wash solution. In this study, nine different cleaning solutions were tested on *Typha latiofolia* (common cattail) for both of these effects. The results of this investigation suggested that there is no universal washing procedure for plant root samples. The best procedure was found to be soaking the root three hours in a solution containing 1% detergent (Alconox) made up in deionized water followed by rinsing five times with deionized water. Other reagents (deionized water, 1% detergent with 1 M $MgCl_2$, 0.1 M HCl, 0.01 M EDTA, 1 M $MgCl_2$ alone, 1 M NaOAc/HAc) and procedures (ultrasonification) were either less effective at cleaning the surface or leached trace elements from the root itself. On balance, any of the following three washing reagents were acceptable: 1% Alconox, 0.01 M EDTA or 1 M $MgCl_2$.

KEY WORDS: Roots, plants, trace elements, cleaning, metals.

INTRODUCTION

Ingestion of foods of plant and animal origin, together with occupational exposure, are the primary route of entry of trace elements into humans[1]. Soils are known to be a major repository of trace elements. Plant analysis provides a direct means of integrating some of the complicated soil-plant mechanisms which govern the uptake of trace elements from soils[2]. Thus, it is very important to understand the soil-plant interrelationships. The capacity of plants to accumulate trace elements depends on several factors, such as plant species; plant parts and age; ion interactions; and soil and climatic conditions. The intensity of soil exploration by roots will have a large influence on the total supply of trace elements that can move to the plant tissue. The various transfer pathways of trace elements from soils to plants have been the objective of research in numerous agricultural, toxicological, and environmental studies. However, as previously discussed[3], very little effort has been devoted to the optimization of the preparation of plant samples for chemical analysis.

Concentrations of trace elements in soils are generally several orders of magnitude greater than in the plants[1]. Therefore, the risk of potential contamination is very high when determining concentrations of trace elements in root material. A large diversity of methods for cleaning the roots have been commonly reported in the literature. The most common cleaning procedures are: washing the roots with water[4,5]; washing with mild

acid[6]; washing with detergent and EDTA[2]; washing with detergent and mild acid[7]; wet sieving with deionized water[8]; scrubbing and peeling[9]; washing with $Ca(NO_3)_2$[10]; washing with dithionite-citrate-bicarbonate[11]; and freeze-drying of roots and cleaning in an ultrasonic bath[12]. The diversity in the sample preparation, particularly washing with different solutions, makes comparison of results from different studies difficult.

There are two practical problems which should always be addressed in the selection of a cleaning procedure for plant root samples collected for multi-element analysis: root contamination by soil particles and leaching of trace elements by different washing solutions. The same four requirements recommended for a washing procedure for plant leaf samples could be adapted for root samples: a) remove surface contamination efficiently; b) internal concentrations remain unchanged; c) the procedure uses cheap, readily available reagents; and d) no special techniques or instruments are required[13].

Frequently, the concentrations of trace elements in plant roots are expressed without specifying the washing procedure[14–21]. The main objective of this study was to address the diversity of methods for washing root samples reported in recent literature. In this study different methods of cleaning the roots were tested to determine the most efficient one. In order to better understand the soil-root relationship, trace elements in different soil fractions in which the plants were growing were also determined. This study is a continuation of the effort to unify the methods employed in the preparation of plant material prior to analysis[3].

EXPERIMENTAL

Plants: sample preparation and analysis

In this study, to maximize the continuity between samples, only one plant species, *Typha latiofolia* (the common cattail), was used throughout the experiment. The plant is perennial, with pithy cylindrical stems and coarse rhizomes. Large quantities of whole plants were collected within an area of 1 m^2 at a marshy area of Hamilton Harbour in Lake Ontario. The area was defined by U.S.–Canada International Joint Commission as an Area of Concern with different contaminants in water and bottom sediments. The objective of collecting the samples at a contaminated area was to assure elevated levels of different trace elements in the plant material. It was expected that any decrease in the trace elements concentrations caused by the different washing procedures used in the experiment would remain above the detection limits of the analytical method used in the study. The plants were washed in the field with lake water immediately after collection. Collected plants were placed in plastic bags and transported to the laboratory where the roots were separated from the above-ground biomass (stems and leaves). To obtain maximum homogenization, the roots were cut into small pieces and thoroughly mixed. The mixture was divided into nine portions of similar weight. Each portion was used in different sample preparation procedures (shown in Table 1).

Each subsample of root material was washed by one of the following washing solutions: 1) deionized water; 2) deionized water in ultrasonic bath (5 minutes, change water, additional 10 minutes of ultrasoning); 3) 1% detergent (Alconox) in DDW; 4) diluted acid solution (0.1 N HCl); 5) 0.01 M EDTA; 6) 1 M $MgCl_2$ (pH = 7); and 7) 1 M NaOAc (adjusted to pH 5 with acetic acid). Two subsamples were washed with a combination of the previous cleaning procedures: 8) detergent (1%) in DDW + HCl

Table 1 Washing procedures tested in this study. All the washing procedures were followed by five rinses with doubly distilled water.

Fraction code	*Description of washing procedures*
1-DDW	soaked in distilled water for three hours
2-UB	washed in ultrasonic bath for 5 min., change water, additional 10 min. of ultrasoning.
3-Det	soaked in detergent solution (1% Alconox) in DDW for 3 h.
4-HCl	soaked in acid solution 0.1 N (HCl) for 3 h.
5-EDTA	soaked in 0.01 M EDTA solution for 3 h.
6-$MgCl_2$	soaked in (1 M, pH = 7) $MgCl_2$ solution for 3 h.
7-NaOAc	soaked in 1 M NAOAc solution (adjusted pH to 5 with acetic acid) for 3 h.
8-Det + HCl	washing by method 3-Det, followed by 30 sec. rinse by deionized water and wash with method 4-HCl
9-Det + $MgCl_2$	washing by method 3-Det, 30 sec. rinse by deionized water followed by wash with method 6-$MgCl_2$

(0.1 N); 9) detergent (1%) in DDW + $MgCl_2$ (1 M). Samples were soaked in a washing media for approximately 3 hours followed by five repeated rinses with double distilled water. Care was taken not to rub the plant material during any of the washing steps. In the washing procedures, which used more than one washing agent, a 30-second deionized water rinse separated the two washes.

After washing, the samples were dried at 70°C in an oven to a constant weight. The dried samples were homogenized and pulverized to approximately 177 μm in a Wiley mill equipped with stainless steel blades. The digestion of the samples was carried out by concentrated HCl:HNO_3 (1:3) (*aqua regia*). The acids were added to Teflon beakers containing 0.2 to 0.5 g samples with subsequent mixing. All samples were allowed to degas at room temperature overnight to prevent a vigorous reaction during heating. The Teflon beakers were covered with Teflon lids to protect the samples from contamination while allowing gas to escape. The samples were digested in a microwave oven (Floyd, Inc. Model RMS 150). The microwave digestions followed a five stage scheme: a) 3 minutes at 25 psi, b) 3 minutes at 50 psi, c) 3 minutes at 75 psi, d) 5 minutes at 100 psi, and e) 5 minutes at 130 psi.

Blanks were collected from the final rinse and analyzed simultaneously with the samples to detect any possible remaining contamination or leaching of soluble elements during the washing. The presence of titanium (Ti) was used as a control in the cleaning procedure. According to Kabata-Pendias and Pendias[22], concentration of Ti greater than few tenths of $\mu g.g^{-1}$ (dry weight) indicates contamination of the plant material by soil or sediment. Potassium, whose concentration in plants is greater than in sediments, was analyzed in all the samples as an indicator of leaching. Losses of K can be due to the loss of the K contained in the plant cells after rupture of their walls[23].

Sediments: sample preparation and analysis

Sediment samples (± 15 cm depth) were collected from the same location as the plants. The samples were thoroughly mixed, placed in plastic containers, transported to the laboratory, and freeze dried. Trace elements in the exchangeable-, carbonates-, and organic-fractions, were determined in the sediment samples using modifications of the extraction procedure of Tessier *et al.*[24]. The following extractants were employed: magnesium chloride (1 M, pH 7.0) for the exchangeable fraction; sodium acetate (1 M adjusted to pH 5.0 with acetic acid) for the carbonate fraction; and a chelating agent (EDTA, 0.01 M) for the organic-bound fraction. The total concentration of trace elements in the sediments was determined by acid digestion with *agua regia* + HF (4:1) in an oven microwave.

Determination of major and trace elements (Al, Ba, Ca, Cr, Cu, Fe, K, Mn, Pb, Si, Sr, Ti, and Zn) in both plants and sediments, was carried out by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using an Jobin Yvon Model 74. The standard solutions consisted of high purity concentrations of 0.5 and 5 $mg.L^{-1}$ of the trace elements in a solution of 2% HNO_3 (Delta Scientific Laboratory Products, Canada). To avoid clogging of the ICP-AES, before the analysis all samples were centrifuged at 5,000 rpm for 20 minutes. Certified reference materials of the National Institute of Standards and Technology [apple leaves—SRM 1515; citrus leaves—SRM 1572; orchard leaves—SRM 1571, and Buffalo River sediment—SRM 2704] were used in the quality control. Subsamples of the certified reference materials were digested with the same mixtures used for the samples. Statistical analysis of obtained data was carried out using the Statistical Analysis System[25].

RESULTS AND DISCUSSION

The sediment is the main source of different trace elements to the roots. It is well known that adsorption of trace elements to sediments varies with redox potential and concentrations of complex forming ligands[1]. Consequently, in order to obtain information on the availability of different elements to the roots, it is important to investigate the chemical forms of the elements in the sediments. Table 2 summarizes the

Table 2 Total concentrations of trace elements in the sediments and percentage extracted by different reagents.

	Al (%)	*Ba* µg/g	*Ca* (%)	*Cr* µg/g	*Cu* µg/g	*Fe* (%)	*Mn* µg/g	*Pb* µg/g	*Si* (%)	*Sr* µg/g	*Zn* µg/g
Total concentration	5.2	364	3.3	43	125	4.7	421	225	1.4	161	368
% extracted with:											
$MgCl_2$ (1 M, pH 7) exchangeable	< 1	4	24	26	5	< 1	13	4	< 1	17	7
NaOAc (1 M, pH 5) carbonates	< 1	2	< 1	1	8	< 1	22	9	< 1	25	14
EDTA (0.01 M) organic-bound	< 1	< 1	7	1	31	< 1	17	37	< 1	5	23

total concentration of the investigated elements in the sediments and the percentage extracted by different reagents. The concentrations of most elements in the sediments were comparable with those reported at other contaminated areas[26,27]. The results indicate that three of the major elements in the sediments, Al, Fe, and Si, were strongly bound to the sediment particles and < 1% of these elements was easily available to the plant roots. On the other hand, between 21 and 36% of the total concentrations of Ca, Cr, Cu, Mn, Pb, Sr, and Zn in the sediments was weakly adsorbed or bound to carbonates and organic matter. Due to the high sorption capacity of organic matter for Cu, Pb, and Zn (Table 2), a complete removal of these elements from the root surface would require the use of chelating agents, such as EDTA and detergent. Relatively high concentrations of available Mn in the sediments indicates that it plays an important role in the adsorption of other elements. Trace elements can co-precipitate with Mn oxides and form coatings on the root surface[28–30]. Removal of the coatings of the root surface is fundamental before the analysis of roots to accurately estimate the concentrations of trace elements in the root tissue.

Figure 1 shows the concentrations of Ti and K in the roots cleaned with the different washing solutions. Titanium, although present in the soils, is known not to be assimilated by plants to any great extent. Levels of Ti in the roots were used as a control of the cleaning procedures. Subsamples of the root material washed with (1%) detergent (solution #3) presented the lower levels of Ti (Figure 1). However, the Ti concentration in all the subsamples was significantly lower than the levels in the sediment samples (861 $\mu g.g^{-1}$). The average concentration of K in the sediments was 0.4%, considerably lower than the concentrations in the root material (Figure 1). Losses of K in the roots after a washing procedure can be due to the loss of K contained in the plant cells after rupture of their walls[23]. The lower concentrations of K in the roots after being washed with solutions #4 (HCl), #7 (NaOAc), and #8 (detergent + HCl) (Table 3), suggested some degree of leaching. When the root material was washed with detergent and $MgCl_2$ together (solution #9), a significant decrease in the K concentrations was observed. Consequently, this washing solution was considered not adequate and the results will not be presented here.

Table 3 summarizes the concentrations of trace elements in roots washed with eight different procedures. The washing solution #7 (NaOAc) proved to be very inadequate. It presented statistically significant greater concentrations of all trace elements determined in the roots. Cleaning the roots with double distilled water or in an ultrasonic bath (procedures #1 and #2, Table 3) proved to be insufficient to remove sediment particles. Washing solutions #4 and #8, which employ HCl, are not suitable for trace elements analysis because they have shown some degree of leaching of the root surface. The results suggest that even small concentrations of detergent were effective in the cleaning. The purpose for using detergent is to break the surface tension. Similar results for Ba and Cr were obtained with all the washing procedures tested in this study, suggesting that these two elements are not very sensitive to the washing method employed.

Similar concentrations of elements in roots were obtained after washing with solutions: #3 (detergent), #5 (EDTA), and #6 ($MgCl_2$), which performed very well giving similar cleaning results. Consequently, due to its best performance and lack of evidence of leaching, we recommend the use of either (1%) detergent, (0.01 M) EDTA, or (1 M) $MgCl_2$ solution for cleaning plant roots. However, each of these washing solutions has shown to be less efficient for removing some trace elements. For instance, detergent did not appear to be very efficient for removing Ca and Sr. EDTA solution was not very efficient removing the adsorbed Zn. On the other hand, $MgCl_2$ was very inefficient in removing Pb adsorbed on the roots. Therefore, several factors may affect the choice

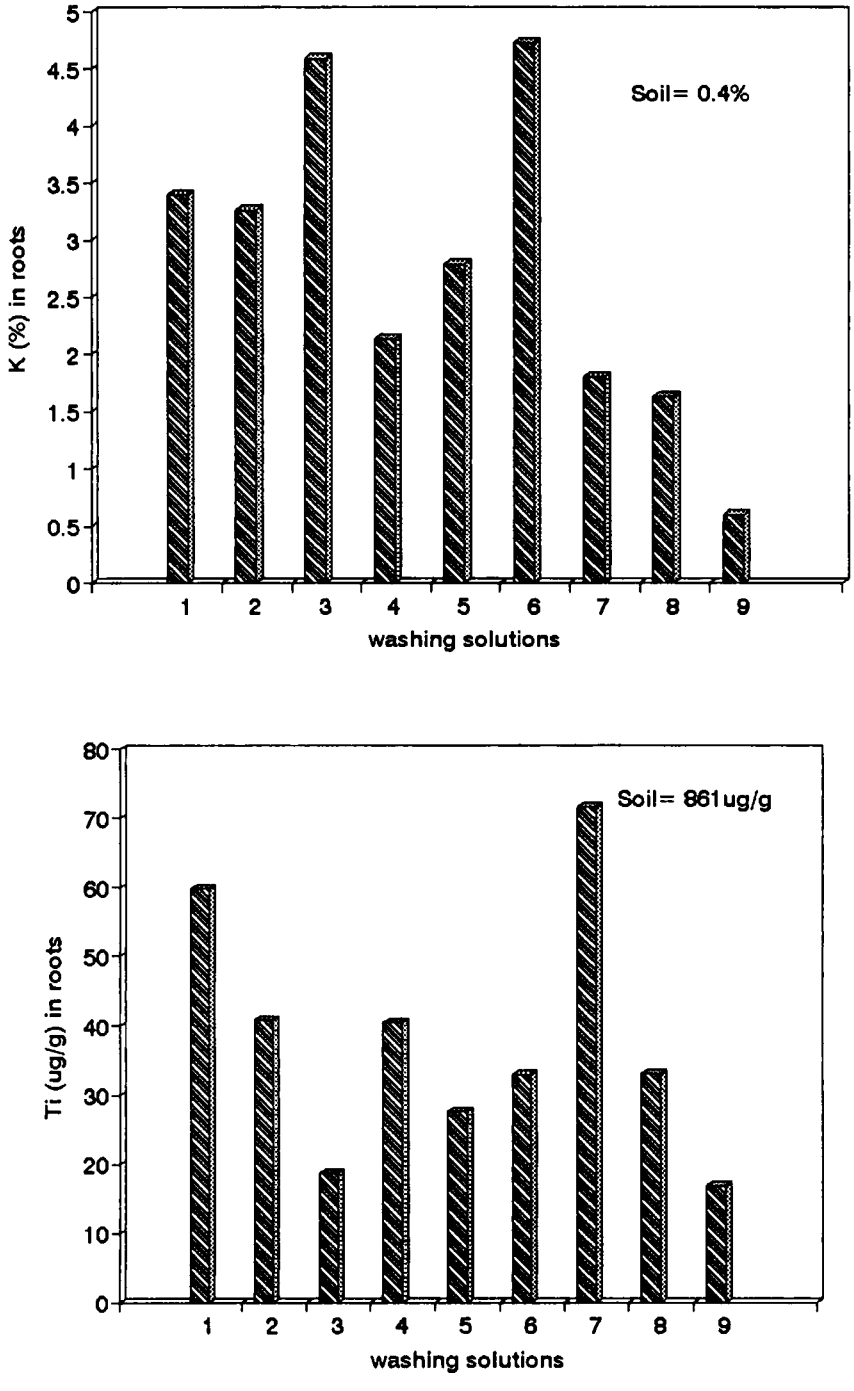

Figure 1 Concentration of Ti and K in root system (washing solutions as described in Table 1).

Table 3 Concentration of trace elements (median and standard deviation) in root material after washing with eight different procedures. Procedures with same letter are not statistically different ($p < 0.005$). All concentrations are expressed in $\mu g.g^{-1}$ except for Al, Ca, Fe, and Si that are in %.

	1-DDW	*2-U.B.*	*3-Det.*	*4-Hcl*	*5-EDTA*	*6-$MgCl_2$*	*7-NaOAc*	*8-Det + HCl*
Al	0.17 ± 0.05 b	0.06 ± 0.03 c	0.07 ± 0.01 c	0.15 ± 0.02 b	0.11 ± 0.01 c	0.07 ± 0.01 c	0.32 ± 0.02 a	0.1 ± 0.01 c
Ba	37 ± 10 b	23 ± 10 b	26 ± 2 b	32 ± 2 b	33 ± 4 b	26 ± 1 b	60 ± 5 a	26 ± 4 b
Ca	1.11 ± 0.1 a	1.02 ± 0.2 a	0.7 ± 0.05 b	0.46 ± 0.04 c	0.53 ± 0.04 c	0.37 ± 0.02 c	0.85 ± 0.05 b	0.64 ± 0.08 b, c
Cr	5 ± 3 b	4 ± 1 b	5 ± 2 b	4 ± 1 b	5 ± 1 b	3 ± 1 b	12 ± 3 a	4 ± 1 b
Cu	17 ± 4 b	54 ± 8 a	9 ± 1 b	13 ± 1 b	16 ± 2 b	19 ± 3 b	32 ± 5 a, b	17 ± 3 b
Fe	0.5 ± 0.04 c	0.7 ± 0.13 b	0.44 ± 0.07 c	0.43 ± 0.04 c	0.43 ± 0.03 c	0.43 ± 0.08 c	0.94 ± 0.09 a	0.5 ± 0.04 c
Mn	310 ± 30 b	510 ± 120 a	210 ± 20 c	160 ± 10 c	160 ± 30 c	243 ± 41 c	456 ± 47 a	221 ± 45 c
Pb	39 ± 8 b	56 ± 11 a, b	18 ± 4 c	20 ± 13 c	11 ± 4 c	42 ± 10 b	76 ± 2 a	21 ± 1 c
Si	0.41 ± 0.1 a	0.63 ± 0.2 a	0.12 ± 0.04 c	0.25 ± 0.01 b	0.13 ± 0.03 c	0.11 ± 0.05 c	0.69 ± 0.17 a	0.17 ± 0.04 b, c
Sr	49 ± 5 b	59 ± 36 a	39 ± 3 c	25 ± 2 d	38 ± 7 c	27 ± 4 d	49 ± 5 b	36 ± 4 c
Zn	105 ± 11 b	53 ± 41 c	54 ± 6 c	54 ± 3 c	93 ± 12 b	63 ± 8 c	137 ± 13 a	62 ± 13 c

of the cleaning procedure for plant roots. Selection of elements to be determined, plant species under investigation, and environmental differences are among these factors. The last two variables, although not taken into account in this study, should be tested in the future.

The analytical accuracy was assessed by analysis of four certified reference materials (Table 4). The values obtained for the SRM reference standards were generally within the certified ranges. The recovery values obtained for Zn in the different plant reference materials, however, was at the low end of the certified ranges. The detection limits of this study, defined as that concentration equivalent to 3x standard deviation (n = 19) obtained from all the blank samples, are shown in Table 4. All the root and sediment samples analyzed in this study were well above the detection limits.

Table 4 Mean recovery of trace elements in certified reference material of the National Institute of Standards and Technology. Average of five replicates.

	Ca (%)	*Cu* μg/g	*Fe* μg/g	*Mn* μg/g	*Pb* μg/g	*Sr* μg/g	*Zn* μg/g
SRM-1515	1.53 ± 0.02	5.6 ± 0.24	83 ± 5	54 ± 3		25 ± 2	12.5 ± 0.3
Apple leaves recovery (%)	92	89	99	96	n.a.	100	88
SRM-1572	3.15 ± 0.1	16.5 ± 1	90 ± 10	23 ± 2	13.3 ± 2.4	100 ± 2	29 ± 2
Citrus leaves recovery (%)	101	109	104	102	106	95	90
SRM-1571	2.09 ± 0.03	12 ± 1	270	91 ± 4	45 ± 3	37	25 ± 3
Orchard leaves recovery (%)	97	110	93	93	104	92	85
SRM-2704	2.6 ± 0.03	98.6 ± 5	4.1 ± 0.1	555 ± 19	161 ± 17	130	438 ± 12
River sediment recovery (%)	98	99	92	96	111	92	98
Detection limits ($\mu g.L^{-1}$)	8	3.6	4.9	1.8	15.5	7.6	2.0

CONCLUSIONS

Due to the much higher concentrations of trace elements in sediments than in plant tissue, it is very difficult to obtain a fully contamination-free analysis of root systems. The results obtained in this study show that there is no universal washing procedure for root systems but there are better suited methods as well as inadequate procedures. The low concentrations of some of the elements determined after washing the roots with HCl as described in this study may be due to leaching of the root surface. On the other hand, some washing procedures such as using detergent, EDTA or $MgCl_2$, as described in this study, have proved to yield a negligible leaching but an intensive removal of surface contaminants from the roots. The efficacy of the different washing procedures varied from element to element. On balance, any of the following three washing reagents were acceptable: 1% Alconox, 0.01 M EDTA or 1 M $MgCl_2$. An effort should be made to standardized the cleaning procedures for the different plant species and trace elements analyzed. This will enable comparison of results from similar plant materials obtained from different working groups. Quantification of Ti and K in the root samples should be implemented as indicators of potential contamination and leaching, respectively. The different washing procedures should be tested for each specie under investigation. Variations due to plant species and environmental differences between sites can be expected and should be studied.

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References

1. D. C. Adriano, "*Trace Elements in the Terrestrial Environment*" (Springer-Verlag, New York, 1986), pp. 533.
2. J. E. Richards, in: *Soil Sampling and Methods of Analysis*, (M. R. Carter, ed. Lewis Publishers, Florida, 1993), pp. 115–139.
3. J. M. Azcue and A. Mudroch, *Intern. J. Environ. Anal. Chem.*, **57,** 151–162 (1994).
4. C. Chih-Yu and C. Chou, *Soil Sci. Nutr.*, **37,** 659–669 (1991).
5. R. Salim, M. Al-Subu and A. Douleh, *J. Environ. Sci. Health*, **A27,** 1619–1642 (1992).
6. R. Carrillo and L. J. Cajuste, *J. Environ. Sci. Health*, **A27,** 1771–1778 (1992).
7. D. Y. Boon and P. N. Soltanpour, *J. Environ. Qual.*, **21,** 82–86 (1992).
8. J. J. Alberts, M. T. Priece and M. Kania, *Estuarine, Coastal and Shelf Science*, **30,** 47–58 (1990).
9. M. F. Babcock, D. W. Evans and J. J. Alberts, *Sci. Total Environ.*, **28,** 203–214 (1983).
10. I. M. McKenna, R. L. Chaney and F. M. Williams, *Environ. Pollut.*, **79,** 113–120 (1993).
11. M. L. Otte, S. J. Bestebroer, J. M. van der Linden, J. Rozema and R. A. Broekman, *Environ. Pollut.*, **72,** 175–189 (1991).
12. M. C. Freitas, R. Cornelis, F. Corte and L. Mees, *Sci. Total Environ.*, **130/131,** 109–120 (1993).
13. J. R. Porter, *Environ. Pollut.*, **12,** 195–202 (1986).
14. F. C. Lidon and F. S. Enriques, *Soil Sci.*, **154,** 44–49 (1992).
15. M. A. Arthur, G. Rubin, R. E. Schneider and L. H. Weinstein, *Environ. Toxicol. Chem.*, **11,** 541–547 (1992).
16. M. A. Arthur, G. Rubin, R. E. Schneider and L. H. Weinstein, *Environ. Toxicol. Chem.*, **11,** 1301–1306 (1992).
17. A. Molina-Diaz, J. J. V. Sagrista and I. P. Reguera, *Intern. J. Environ. Anal. Chem.*, **45,** 219–228 (1991).
18. I. M. McKenna, R. L. Chaney, S. Tao, R. M. Leach and F. M. Williams, *Environ. Reser.*, **57,** 73–87 (1992).
19. S. K. Jain, P. Vasudevan and N. K. Jha, *Water Res.*, **24,** 177–183 (1990).
20. V. W. D. Chui, S. Y. Lam-Leung, M. Cheung and V. K. C. Wu, *Environ. Internat.*, **18,** 201–210 (1991).
21. V. Antoniou and N. Zantopoulos, *Bull. Environ. Contam. Toxicol.*, **48,** 515–519 (1992).
22. A. Kabata-Pendias and H. Pendias, "*Trace Elements in Soils and Plants*" (CRC Press, Inc., Boca Raton, 1986).
23. M. Thompson and J. N. Walsh, "*Handbook of Inductively Coupled Plasma Spectrometry*" (Blackie and Son, Ltd., London, 1989). 2nd ed., pp. 319.
24. A. Tessier, P. G. C. Campbell and M. Bisson, *Anal. Chem.*, **51**(7), 844–851 (1979).
25. SAS, "*SAS User's Guide, 5th ed. Statistica*" (Analysis Systems Institute Inc., Cary, NC. 1985) pp. 1288.
26. W. Salomons and U. Forstner, "*Chemistry and Biology of Solid Waste*" (Springer-Verlag, Berlin, 1988), pp. 305.
27. R. J. Allan and A. J. Ball, *Water Poll. Res. J. Canada*, **25,** 387–487 (1990).
28. M. L. Otte, J. Rozema, L. Koster, M. S. Haarsma and R. A. Broekman, *New Phytol.*, **111,** 309–317 (1989).
29. L. St-Cyr and A. A. Crowder, *Soil Sci.*, **149,** 191–198 (1990).
30. C. Vale, F. M. Catarino, C. Cortesano and M. I. Cacador, *Sci. Total Environ.*, **97/98,** 617–626 (1990).