International Journal of Phytoremediation: Vol. 5, No. 1, pp. 13–23 (2003)

# Optimizing Phytoremediation of Heavy Metal-Contaminated Soil by Exploiting Plants' Stress Adaptation

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## ABSTRACT

Soil phytoextraction is based on the ability of plants to extract contaminants from the soil. For less bioavailable metals, such as Pb, a chelator is added to the soil to mobilize the metal. The effect can be significant and in certain species, heavy metal accumulation can rapidly increase 10-fold. Accumulation of high levels of toxic metals may result in irreversible damage to the plant. Monitoring and controlling the phytotoxicity caused by EDTA-induced metal accumulation is crucial to optimize the remedial process, i.e. to achieve maximum uptake. We describe an EDTA-application procedure that minimizes phytotoxicity by increasing plant tolerance and allows phytoextraction of elevated levels of Pb and Cd. *Brassica juncea* is tested in soil with typical Pb and Cd concentrations of 500 mg $kg^{-1}$ and 15 mg $kg^{-1}$, respectively. Instead of a single dose treatment, the chelator is applied in multiple doses, that is, in several small increments, thus providing time for plants to initiate their adaptation mechanisms and raise their damage threshold. *In situ* monitoring of plant stress conditions by chlorophyll fluorescence recording allows for the identification of the saturating heavy metal accumulation process and of simultaneous plant deterioration.

**KEY WORDS:** Pb and Cd accumulation, phytoextraction, plant stress tolerance, multiple dose chelator application, chlorophyll fluorescence monitoring.

1522-6514/03/$.50

## I. INTRODUCTION

Phytoremediation is a promising, environment-friendly method for large-scale cleanup of contaminated soil and water (Cunningham and Ow, 1996; Dushenkov *et al*., 1997; Bañuelos *et al*., 1997). It has been used successfully to remove toxic metals from sites where their concentration posed a risk to human health (Gatliff, 1994). Soil phytoremediation is based on the ability of particular plants to extract contaminants efficiently (that is, in large concentration) from the soil and accumulate them in the above-ground parts for subsequent harvesting and removal from the site. This process is repeated until the contaminant concentration drops below desired levels. Depending on initial and final concentrations and site conditions, this approach may take several years. Increasing the efficiency of each crop will significantly reduce the time and cost for the completion of the procedure. For some heavy metals (e.g., Pb) the uptake rate is greatly increased by adding a chelating agent to the soil to mobilize the contaminants for uptake into plants (Blaylock *et al*., 1997; Epstein *et al*., 1999). The accelerated remediation process in a fully developed crop takes place within a short time period (3 to 6 days) during which the uptake rate and the final concentration of the contaminants in the plants reach their maximum (Salt *et al*., 1997; Vassil *et al*., 1998; Barocsi *et al*., 2000; Richter *et al*., 1998). The process is so efficient that in certain species heavy metal accumulation increases 10-fold within days in the presence of a chelator (Barocsi *et al*., 2000). Efforts have been made to optimize the remediation process by altering or replacing different technological parameters (Bañuelos *et al*., 1997; Salt *et al*., 1997; Vassil *et al*., 1998). However, the amending procedure itself is similar in most cases. At present, the generally followed procedure is to add a synthetic chelator, usually EDTA (ethylenediamintetraacetic acid), to the soil along with acetic acid in a single treatment (Blaylock *et al*., 1997). Light irrigation is provided after the amendment application. As a result of the increased heavy metal load due to the accelerated uptake, the plants are irreversibly damaged and eventually die. This happens as soon as the heavy metal concentration exceeds the damage threshold of a plant and, simultaneously, saturates the plant tissues.

### A. Theoretical Basis

The approach, which we describe here, focuses on applying the total amount of chelator in multiple dose treatments. The success of the process depends on its optimum performance during the few critical days. This is because most of the metal uptake occurs in a short period following amendment application, and a second amendment can be done only after the next crop matures. The new procedure effectively increases the maximum amount of heavy metals removed from the soil at a cost of slightly prolonging the treatment period, which is negligible in the context of the 2 to 3 month crop cycle. The results that led to the proposed optimization method have been gained from phytoremediation field tests carried out since 1997 at agricultural sites in the Upper-Silezia region of Poland and verified by greenhouse experiments. The tests confirmed the potential for phytoremediation at these sites that are heavily contaminated with Pb and Cd (average concentrations: [Pb]=500 mg $kg^{-1}$ soil dry weight and [Cd]=15 mg $kg^{-1}$ soil d.w.; Richter *et al*., 1998).

The following observations and assumptions have been used to develop the optimization technique. EDTA is particularly efficient at mobilizing Pb (Epstein *et al*., 1999; Salt *et al*., 1997). Both the uptake rate and the final Pb content in plant tissues are proportional to the EDTA concentration applied (Vassil *et al*., 1998). The uptake rate is the highest in the first 2 to 3 days following a single, high-dose amendment application. At high EDTA concentrations (≥4 mmol $kg^{-1}$ soil d.w.), Pb and Cd uptake by plants saturate within 2 days because plants exhaust quickly (short resistance phase) and cannot tolerate heavy metal stress any longer (Figure 1, solid curve). To avoid this, the total amount of EDTA is evenly distributed and applied in several, lower dose increments. We assumed that a saturation level could be identified for reduced EDTA doses as well. In this case, it is possible that slower uptake may lead to an adaptation to heavy metal stress that will subsequently result in enhanced tolerance. One effect of heavy metal accumulation is that it irreversibly reduces photosynthetic activity (Moustakas *et al*., 1994). On the other hand, it induces reversible effects as well by blocking enzymes and generating active oxygen. The adaptation process can activate the antioxidant system (Gallego *et al*., 1996; Koricheva *et al*., 1997) or a range of cellular detoxification mechanisms (Hall, 2002). By properly inducing stress adaptation, the plant damage threshold can be raised, which will allow accumulation of elevated levels of toxic metals (Figure 1, dashed curve). It was expected to take only a few more days for a plant to reach its threshold

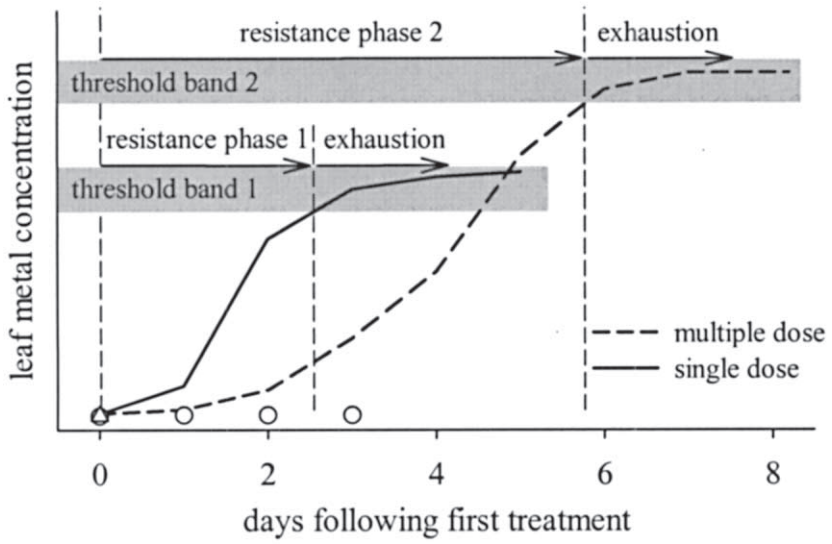


**FIGURE 1.** Model for increasing toxic metal accumulation by exploiting plant stress tolerance. Solid curve: single, high-dose amending of chelator (Δ) results in quick heavy metal accumulation and plant exhaustion. Dashed curve: predicted prolongation of resistance phase and increase of accumulation by applying the same amount of chelator in several, lower dose increments (O).

at reduced EDTA doses with the above- mentioned benefits. Subsequent field and greenhouse experiments have validated these expectations.

## II. MATERIALS AND METHODS

### A. Field Measurements

The field measurements were conducted between June 7-14, 2000 at field sites in Bytom, near Katowice, Upper-Silezia, Poland. A $3 \times 4$ m$^2$ subset of a plot planted with Indian mustard (*Brassica juncea* cv. 426308 originated from Phytotech Inc. — later Edenspace Systems Corporation) was reserved for this experiment, while the rest of the area was used for other experimental purposes. All the tests were carried out on matured (abloom) plants.

Four identical areas of the experimental plot were marked as subplots for investigating different amendment application time schedules. Each soil treatment consisted of amending EDTA solute in water followed by acetic acid solution. A control subplot (no EDTA applied) and three subplots with different schedules of EDTA treatments were monitored for 6 days. The cumulative EDTA volume, reached after subsequent treatments, was set so that its total value was 4 mmol kg$^{-1}$ soil d.w. for each of the three amended subplots. The amendment concentration is adjusted for the volume of a 10-cm layer of soil. The concentration of the acetic acid was equal to the EDTA concentration. All treatments were done between 9 to 10 am. Subplot *A* received a single dose of 4 mmol EDTA kg$^{-1}$ soil d.w. on day 1. Subplot *B* received two doses of 2 mmol EDTA kg$^{-1}$ soil d.w. on days 1 and 2. Subplot *C* was treated three times with 1.3 mmol EDTA kg$^{-1}$ soil d.w. each on days 1, 2 and 3. Leaf samples were collected on days 1, 2, 3, and 6 for chemical analysis.

Field experiments consisting of three replicate sets with similar protocols were repeated during July 11-16, 2001.

### B. Greenhouse Experiments

Two sets of experiments were carried out in 2000 that resulted in the same tendency. The protocol for the July 2000 field tests was based on the results of the June 2000 greenhouse tests. A second set of experiments was started on September 20, 2000. We extended the treatment and monitoring period to 10 to 12 days, which proved sufficient for thoroughly monitoring the remediation process.

Four experimental 1000-ml pots were each filled with 500 mg d.w. of "Florasca B" soil (pH 6.8). Each pot was seeded with three *B. juncea* (cv. 426308) seedlings, and then placed outdoors. During the growth period, fertilizer was applied twice by irrigating the soil with Hoagland solution on the third week, immediately after germination, and at the beginning of the sixth week. Soil Pb concentration of 1000 mg kg$^{-1}$ soil d.w. was accomplished by mixing $PbCO_3$ into the dry soil before seeding. The treatment period began when the plants budded on the ninth week. Each treatment used a set molar volume of EDTA solute in 100 ml of water and was followed immediately by irrigation with 100 ml of 2% acetic acid in water. One pot (*Lc*) was used for control (no EDTA applied). For the other three pots, the total volume of chelator was set to 4 mmol kg$^{-1}$ soil d.w. applied in different increments

over time. Pot *L4* was treated on the first day of the treatment period with the total 4 mmol EDTA $kg^{-1}$ soil d.w. Pot *L2* was amended twice on days 1 and 5 with 2 mmol EDTA $kg^{-1}$ soil d.w. each. Pot *L1* was treated four times on days 1, 3, 5, and 7 with 1 mmol EDTA $kg^{-1}$ soil d.w. each.

The phytoremediation process was monitored through quantitative Pb analysis of leaf disks, complemented with *in situ* chlorophyll fluorescence analysis of the same disks to monitor photosynthetic activity (Lichtenthaler and Rinderle, 1988; Sgardelis *et al.*, 1994; Snel and van Kooten, 1990; Valentini *et al.*, 1994).

### C. Chemical Analysis

To enhance the accuracy of the element analysis, six identical leaf disks, with a diameter of 13 mm, were taken from each subplot/pot at each sampling. Two hundred miligram samples were dried at 90°C, were digested in Teflon® bowls, with the addition of 1-1 ml concentrated $HNO_3$ and $H_2O_2$ in equal parts, under pressure at 130°C for 45 min. The water-clear contents of the bombs were filtered through Whatman paper, no. 42, and brought to a volume of 10 ml with twice-distilled water. Each such solution was analyzed for Cd and Pb by atomic absorption spectrometer (ICAP 61, Thermo Jarrel Ash, Franklin, MA, USA).

### D. Fluorescence Analysis

A computerized, field-portable chlorophyll fluorometer, CFM-636973 (Barocsi *et al.*, 2000), was used to record the fluorescence decay parameter *RFd* (Lichtenthaler and Rinderle, 1988). This parameter had been proven in previous tests to strongly correlate with the Pb concentration of leaf samples (Richter *et al.*, 1998). The fluorometer excited the leaves at 635 nm and measured their fluorescence at 690 and 735 nm.

## III. RESULTS

In the field tests, by approximately 50 h after the first treatment, all subplots had been exposed to the total EDTA dose (4 mmol $kg^{-1}$ soil d.w.). Plants in subplot *C*, treated three times, showed little phytotoxicity (Figure 2c), while those in subplot *A*, exposed immediately to the maximum EDTA dose, completely deteriorated (Figure 2a). In this latter group, all the leaves dried completely with a minimal loss of pigments. Compared with control, there was no visually observable reduction of leaf area. Plants in subplot *B* with 2-dose treatment (Figure 2b) showed intermediate toxicity symptoms. As seen from Figure 3, application of the highest EDTA dose in a single treatment (subplot *A*) resulted in the steepest increase of the plants' heavy metal content within 24 h. Later, however, the [Pb]–time and [Cd]–time functions behaved exactly as predicted, namely, the rate of concentration change declined to zero. In subplots *B* and *C*, the heavy metal concentrations increased in proportion to the doses of EDTA applied. For subplot *B*, the plant deterioration started approximately 48 h after the start of treatments simultaneously with a sharp increase of the rate of accumulation, while plants in subplot *C* remained intact through the end of day 3. Compared with *control* (8.0 mg $kg^{-1}$), the final [Pb] increased to 364 mg $kg^{-1}$, 624

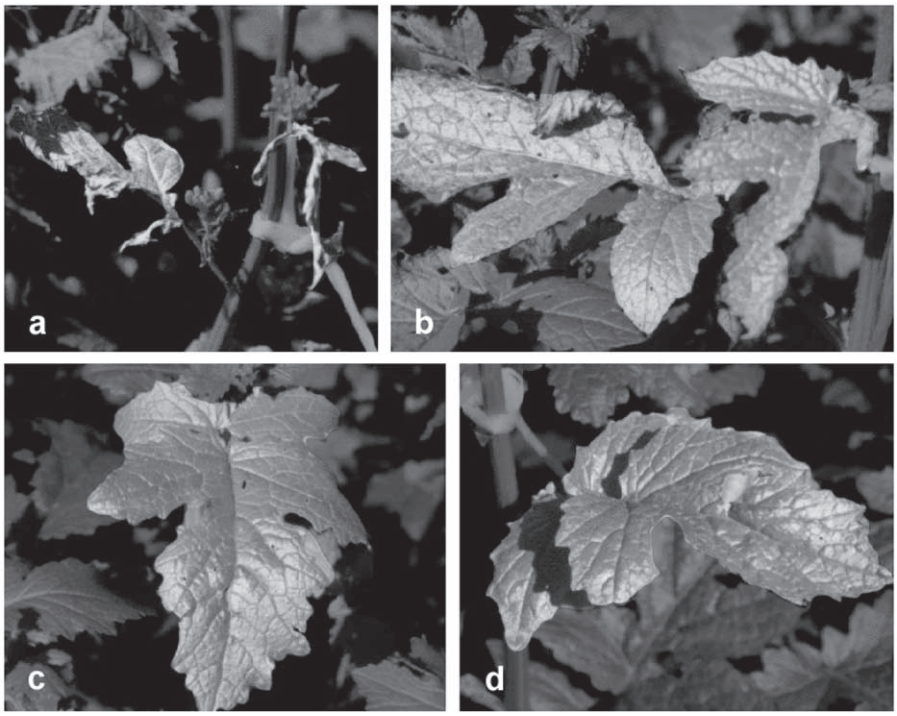


**FIGURE 2.** View of field plants exposed for 50 hours to different EDTA treatments. (**a**) Subplot *A*: 1 × 4 mmol EDTA $kg^{-1}$ soil d.w. (**b**) Subplot *B*: 2 × 2 mmol EDTA $kg^{-1}$ soil d.w. (**c**) Subplot *C*: 3 × 1.3 mmol EDTA $kg^{-1}$ soil d.w. (**d**) *Control*: no EDTA applied.

mg $kg^{-1}$ and 771 mg $kg^{-1}$ for subplots *A, B,* and *C,* respectively. The final [Cd] values are 2.8 mg $kg^{-1}$, 23.8 mg $kg^{-1}$, 38.9 mg $kg^{-1}$, and 41.8 mg $kg^{-1}$ for *control*, *A, B,* and *C,* respectively.

We observed that uptake was most intense during daylight, when transpiration was greatest (t=0 refers to 9:00 am in all diagrams). In separate greenhouse experiments, we studied the temporal change of daylight transpiration at different EDTA concentrations (1 to 5 mmol $kg^{-1}$ soil d.w.) applied in single dose treatments. We found an increase of stomatal conductance in the first 24 to 48 h after EDTA application from 9 mmol $H_2O$ $m^{-2}s^{-1}$ up to 35 to 370 mmol $H_2O$ $m^{-2}s^{-1}$ (higher value corresponding to higher EDTA concentration). During the next 3 days, it declined to 12 to 42 mmol $H_2O$ $m^{-2}s^{-1}$.

It was also shown in these preliminary experiments that the EDTA doses applied did not influence the plants in the absence of soil Pb and Cd.

During the September 2000 greenhouse experiments, the monitoring period was prolonged in order to reach saturation of the plants (Figure 4 top). The [Pb]–time functions were identical to those predicted theoretically (compare to Figure 1). The rise in the plants' damage threshold was clearly demonstrated in all three treatments.

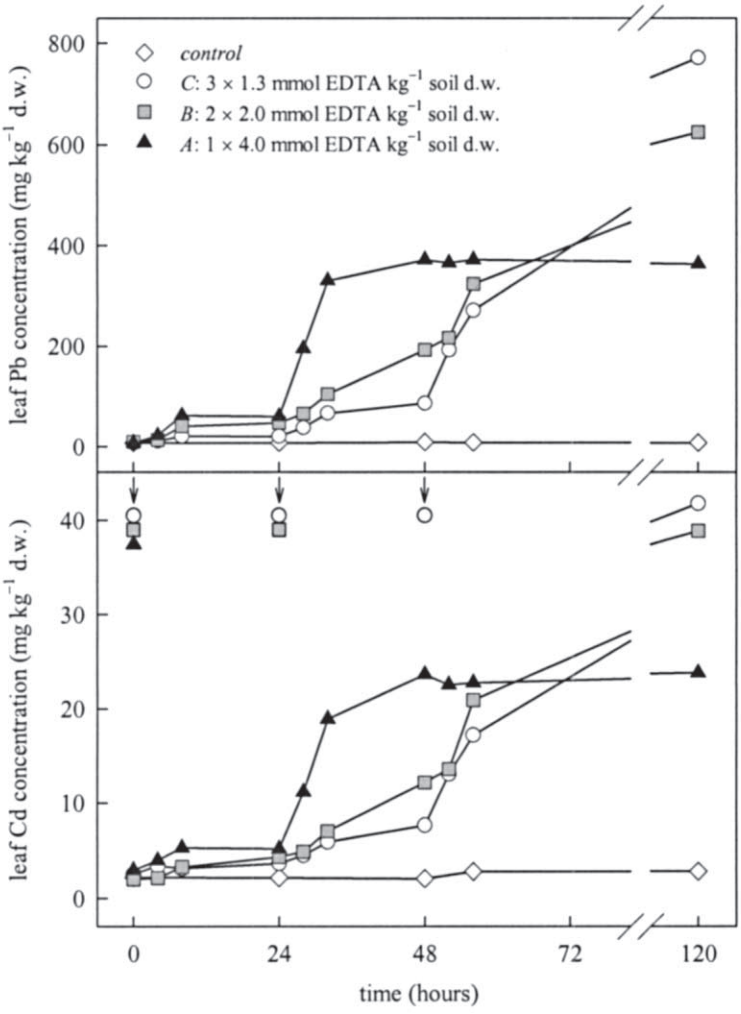


**FIGURE 3.** Pb (top) and Cd (bottom) concentrations obtained from field tests vs. time. Markers with vertical arrows show the treatment times; the time of the first measurement (9:00 am) is considered zero. *A*, *B*, and *C* are references of subplots with different EDTA treatments.

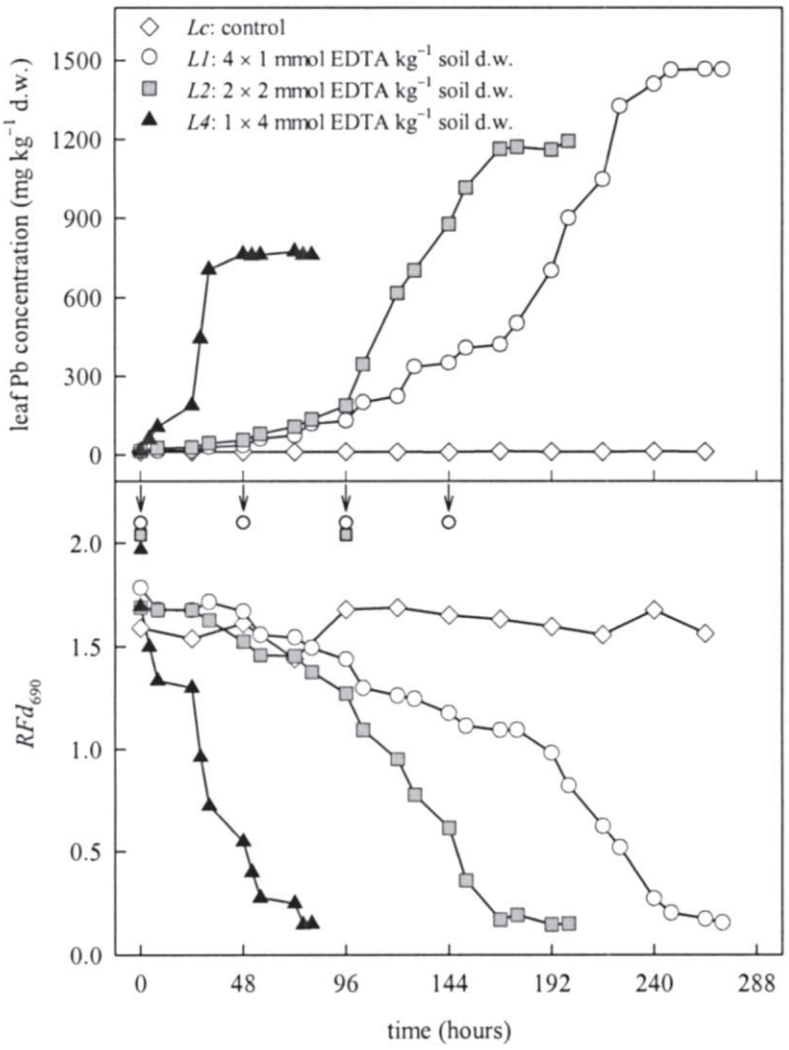


**FIGURE 4.** Time dependence of Pb concentration (top) and *RFd* (bottom) for the greenhouse tests. Markers with vertical arrows show the treatment times; the time of the first measurement (9:00 am) is considered zero. *L1*, *L2*, and *L4* are references of subplots with different EDTA treatments; *Lc* refers to control (no EDTA applied).

The amount of accumulated Pb increased by reducing the EDTA dose applied at a given time and, simultaneously, increasing the number of treatments. As seen in Figure 4 (bottom), plant deterioration could be followed directly by recording the temporal change of *RFd* values, denoted as *RFd*(*t*), confirming the strong influence of Pb accumulation on photosynthetic activity (Barocsi *et al.*, 2000; Moustakas *et al*., 1994; Sgardelis *et al*., 1994). The saturation of *RFd*(*t*) correlated well with saturation of Pb uptake by plants. Figure 5 plots the *RFd*–[Pb] function for all three treated subplots where the saturation effect is even more remarkable. An extremely strong correlation of $r^2$=0.96 was found for [Pb]–*RFd* pairs under no saturation (Figure 5, dashed curve). All three curves depart the fitted inverse trend curve at saturation. In addition, the curve for subplot *L1* (four-step treatment) deviates slightly in the other direction (increase of *RFd*), showing the presence of plant stress tolerance.

## IV. DISCUSSION

Efficient phytoextraction of metals (Anderson *et al*., 1998) relies on several optimized conditions and parameters (Blaylock *et al*. 1997; Kramer *et al*., 1996). First, an effective plant species, with the potential to hyperaccumulate metals and the ability to produce large amounts of harvestable biomass, must be selected that is suitable for the climate, soil type, contamination, and other characteristics of the target site. For Pb and Cd, in particular, a chelator must be chosen that is cost effective and capable of inducing hyperaccumulation in the specific soil/metal/plant

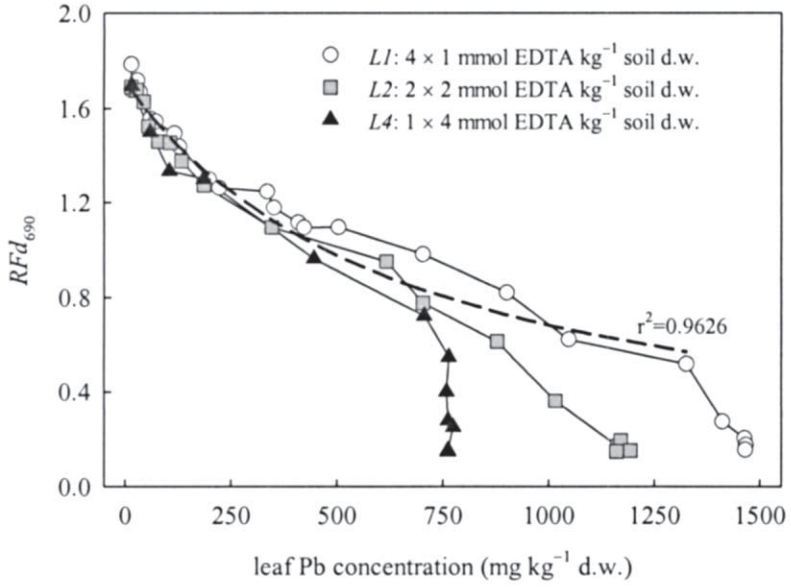


**FIGURE 5.** *RFd*–[Pb] curves (marked lines) and the inverse fitting function (dashed line) for the greenhouse tests.

scenario. This work focused on the optimization phase of the phytoremediation process by using a new approach for the application of synthetic chelators. It was demonstrated that, for *B. juncea* grown in Pb-contaminated soil, the optimization of the treatment procedure can be achieved by applying the total amount of the chelating agent (EDTA) in multiple, time-delayed steps. At each step, the chelator dose was kept low to allow the plants to initiate tolerance mechanisms. The metal accumulation thus can be higher, due to the elevation of the plant's damage threshold and saturation level, and plant death is avoided. Although the remediation process is prolonged and multiple treatments are required, the final concentration of Pb taken up per crop is much higher than after a single treatment. The final accumulated Pb amount was doubled with multiple dose treatments, which translates to a 50% reduction in cleanup time, assuming that this effect will be expressed in subsequent crops.

A computerized chlorophyll fluorometer (Barocsi *et al*., 2000) was utilized to monitor *in situ* the temporal change of *RFd*. Since *RFd*(*t*) has a high degree of correlation with the accumulated [Pb], it can assist in adjusting the optimum treatment schedule and determining the optimal harvest time. Furthermore, the observation of the increased transpiration suggests that watering plays a major role in the effectiveness of the remediation process. The recording of *RFd*(*t*) can help in adjusting the correct level of irrigation by identifying early saturation of heavy metal uptake in plants.

## ACKNOWLEDGMENTS

The project was supported by the National Energy Technology Laboratory of the US Department of Energy under a cooperative agreement with Florida State University. The cooperation of Central European Advanced Technologies (CEAT) Ltd., Hungary, is gratefully acknowledged. The development of the chlorophyll fluorometer was supported by the Hungarian Committee for Research and Development (OMFB) and the gy. Bekesy Research Fund. We thank A. J. Todd and Z. Puskar for their constructive comments.

## REFERENCES

Anderson, C.W.N., Brooks, R.R., Stewart, R.B., and Simcock, R. 1998. Harvesting a crop of gold in plants. *Nature* **395**, 553–554.

Bañuelos, G.S., Ajwa, H.A., Mackey, B., Wu, L., Cook, C., Akohoue, S., and Zambrzuski, S. 1997. Evaluation of different plant species used for phytoremediation of high soil selenium. *J. Environ. Qual*. **26**, 639–646.

Barocsi, A., Kocsanyi, L., Varkonyi, S., Richter, P., Csintalan, Z., and Szente, K. 2000. Two-wavelength, multipurpose, truly portable chlorophyll fluorometer and its application in field monitoring of phytoremediation. *Meas. Sci. Technol*. **11**, 717–729.

Blaylock, M.J., Salt, D.E., Dushenkov, S., Zakharova, O., Gussman, C., Kapulnik, J., Ensley, B.D., and Raskin, I. 1997. Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. *Environ. Sci. Technol*. **31**, 860–865.

Cunningham, S.D. and Ow, D.W. 1996. Promises and prospects of phytoremediation. *Plant Physiol.* **110**, 715–719.

Dushenkov, S., Vasudev, D., Kapulnik, Y., Gleba, D., Fleisher, D., Ting, K.C., and Ensley, B. 1997. Removal of Uranium from Water Using Terrestrial Plants. *Environ. Sci. Technol.* **31**, 3468–3474.

Epstein, A.L., Gussman, C.D., Blaylock, M.J., Yermiyahu, U., Huang, J.W., Kapulnik, Y., and Orser, C.S. 1999. EDTA and Pb-EDTA accumulation in *Brassica juncea* grown in Pb-amended soil. *Plant Soil* **208**, 87–94.

Gallego, S.M., Benavides, M.P., and Tomaro, M.L. 1996. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Plant Sci.* **121**, 151–159.

Gatliff, E.G. 1994. Vegetative remediation process offers advantages over traditional pump-and-treat technologies. *Remed. Summer* **4(3)**, 343–352.

Hall, J.L. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.* **53**, 1-11.

Koricheva, J., Roy, S., Vranjic, J.A., Haukioja, E., Hughes, P.R., and Hanninen, O. 1997. Antioxidant responses to simulated acid rain and heavy metal deposition in birch seedlings. *Environ. Pollut.* **95**, 249–258.

Kramer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A.J.M., and Smith, J.A.C. 1996. Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379**, 635–638.

Lichtenthaler, H.K. and Rinderle, U. 1988. The role of chlorophyll fluorescence in the detection of stress conditions in plants. *Crit. Rev. Anal. Chem.* **19/I**, 29–85.

Moustakas, M., Lanaras, T., Symeonidis, L., and Karataglis, S. 1994. Growth and some photosynthetic characteristics of field-grown *Avena sativa* under copper and lead stress. *Photosynthetica* **30**, 389–396.

Richter, P.I., Barocsi, A., Csintalan, Z., Kuperberg, J.M., and Szdzuj J. 1998. Monitoring soil phytoremediation by a portable chlorophyll fluorometer. *J. Field Anal. Chem.* **2**, 241–249.

Salt, D.E., Pickering, I.J., Prince, R.C., Gleba, D., Dushenkov, S., Smith, R.D., and Raskin, I. 1997. Metal accumulation by aquacultured seedlings of Indian mustard. *Environ. Sci. Technol.* **31**, 1636–1644.

Sgardelis, S., Cook, C.M., Pantis, J.D., and Lanaras, T. 1994. Comparison of chlorophyll fluorescence and some heavy-metal concentrations in Sonchus spp and Taraxacum spp along an urban pollution gradient. *Sci. Total Environ.* **158**, 157–164.

Snel, J.F.H. and van Kooten, O. 1990. The use of chlorophyll fluorescence and other non invasive spectroscopic techniques in plant stress physiology. *Photosynth. Res.* (Special Issue) **25**, 146–332.

Valentini, R., Cecchi, G., Mazzinghi, P., Scarascia Mugnozza, G., Agati, G., Bazzani, M., De Angelis, P., Fusi, F., Matteucci, G., and Raimondi, V. 1994. Remote sensing of chlorophyll a fluorescence of vegetation canopies. II. Physiological significance of fluorescence signal in response to environmental stresses. *Remote Sens. Environ.* **47**, 29–35.

Vassil, A.D., Kapulnik, Y., Raskin, I., and Salt, D.E. 1998. The role of EDTA in lead transport and accumulation by Indian mustard. *Plant Physiol.* **117**, 447–453.