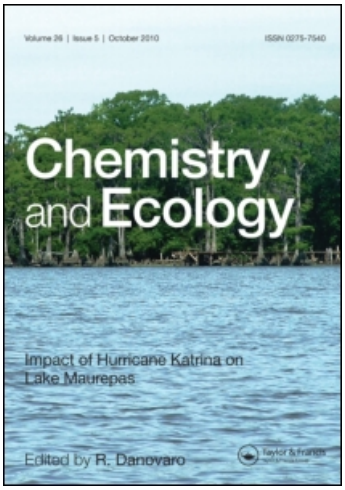

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EDTA reduces lead accumulation in *Symphytum officinale* L. (comfrey) roots

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EDTA reduces lead accumulation in *Symphytum officinale* L. (comfrey) roots

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The addition of EDTA in phytoextraction studies has been reported to increase heavy metal accumulation in above-ground parts or to have no negative impact on the overall (root/shoot) accumulation levels in terrestrial plants. At a purely quantitative level, this study assessed the phytoextraction potential of a previously untested high-biomass terrestrial plant, *Symphytum officinale* L. (comfrey), in the presence of Pb and EDTA. In this hydroponic-based study, we report a small increase in shoot accumulation of Pb with EDTA but, conversely, the presence of EDTA in the nutrient medium markedly reduced the overall quantity of Pb in the plant root by at least 80%. The loss does not appear to be explained by EDTA acting alone, increased transport of Pb to the shoots, or anionic charge repulsion of the $[PbEDTA]^{2-}$ complex. The elusive action and negative effect of EDTA on Pb accumulation in *S. officinale* provides additional reasons towards a growing trend away from the use of EDTA as a chelating agent in phytoextraction.

Keywords: EDTA; lead; phytoremediation; *Symphytum officinale* L.

1. Introduction

Lead (Pb) is ranked the number one heavy metal pollutant and number two of all hazardous substances by the Agency for Toxic Substances and Disease Registry [1]. Phytoremediation is a potential remediation method that uses plants to remove or render harmless pollutants like Pb [2]. Phytoextraction (which focuses on shoot accumulation of pollutants) and rhizofiltration (which focuses on root accumulation of pollutants) are the two most applicable methods for soil and water-based phytoremediation, respectively.

For Pb, there are presently no known natural hyperaccumulator plants. Consequently, in the past decade an alternative strategy has been to apply chelating agents, such as ethylenediamine tretraacetic acid (EDTA), to soils. These compounds are considered to enhance heavy metal accumulation in above-ground harvestable shoots of high biomass plants by increasing the release of Pb from soil particles the solubility of Pb within the plant, and, consequently its transport from roots to shoots [3,4]. In this way, artificial hyperaccumulation of Pb (defined as at least

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10,000 mg kg^{-1} d.w. Pb in shoots) has been reported in plants such as *Brassica juncea* [5], *Zea mays* and *Pisum sativum* [4]. However, the value of chelation techniques in phytoremediation remains controversial.

The use of chelating agents to solubilise heavy metals can spread contamination through leaching [6]. Moreover there are concerns about the persistence of EDTA in the environment [7]. To alleviate these concerns, better management of chelating agent application and more environmentally compatible chelating agents are being investigated. For example, incremental addition as opposed to single high concentration applications of EDTA not only reduced leaching but also increased shoot accumulation of Pb [8,9]. The use of biodegradable chelating agents like ethylenediaminedissuccinate (EDDS) has also shown to be less toxic to *Brassica rapa* (as measured by shoot dry weight and effect on mycorrhizal fungi) However, with even the most effective EDDS treatments, leaching of Pb was still problematic [9].

In previous studies [10], we showed that the endogenous polyphenol content of roots in the high biomass plant *Symphytum officinale* L. (comfrey or knitbone) plays a role in Pb accumulation, which may be important for rhizofiltration. It has a somewhat larger root system (based on approximate rooting depth, up to 2.5 m) than other reported phytoremediation plants such as *Brassica juncea* (up to 1.2 m) and *Pisum sativa* (up to 0.65 m). Importantly, this would provide a larger surface area for remediation. However, the Pb accumulation potential of *S. officinale* for phytoextraction purposes with the addition of chelating agents such as EDTA and alternatives such as N-[2 acetamido] iminodiacetic acid (ADA), are unknown. The objective of the present short-term bench-scale hydroponic study is to obtain this information. Although, for environmental reasons, there is currently a movement away from the use EDTA [11], it remains the most studied chelating agent across plant species. Its use in this study therefore allows us to quantitatively compare the levels of Pb accumulation by *S. officinale* in the presence and absence of a chelating agent that has been shown to be generally favourable for Pb accumulation.

2. Materials and methods

2.1. *Whole plant accumulation of Pb in the presence and absence of EDTA*

Three-month-old *S. officinale* plants were grown from root cuttings in a growth room (16 h photoperiod at 26.5 μmol m^{-2} s^{-1}, 22 °C) in North-end sand supplemented with Nutricote 8-9-month slow-release fertiliser (Yates New Zealand Ltd., Auckland, New Zealand) at 5 g L^{-1} of sand.

Plants were then transferred to 300 mL low phosphate (0.005 mM KH_2PO_4) Huang and Cunningham nutrient solution (LPHC solution) for one week and then transferred to the fresh LPHC solution containing 500 μM $Pb(NO_3)_2$, with or without 500 μM EDTA [12]. The control solution was unmodified LPHC solution. All solutions were adjusted to pH 4.5. After 7 days, plants were harvested for Pb analysis. Prior to Pb analysis, Pb bound to the root surface was desorbed with LPHC solution containing 1 mM EDTA (pH 4.5) for 30 min and rinsed with nanopure H_2O.

2.2. *Investigation of the negative effect of EDTA on Pb accumulation*

As unexpectedly lower Pb accumulation in roots was noted in the presence of EDTA, the next two experiments were conducted to gain an insight to the factors that may have contributed to this effect. Two possibilities that were hypothesised to reduce Pb accumulation were:

(1) Presence of EDTA in the free state: pre-treatment of root segments with EDTA prior to Pb treatment

The most abundant roots in *S. officinale* are the fine lateral roots, which branch from the fleshier main root. Six mesh-like 'tea bags', each containing 1.2 g (fresh weight, f.w.) of 3–4-month-old lateral roots segments were prepared. Three bags were pre-treated with 50 mL of LPHC solution with 500 μM EDTA and three without. After 18 h, the bags were rinsed in dH_2O. One bag from each pre-treatment was placed separately in 50 mL of the following treatment solutions: LPHC containing (i) no Pb or EDTA (control solution), (ii) 500 μM $Pb(NO_3)_2$, or (iii) 500 μM $Pb(NO_3)_2$ with 500 μM EDTA. After 24 h, roots were washed in dH_2O and used for Pb analysis as described. In total, the experiment took 3 days (18 h for pre-treatment and 24 h for treatment). A 7-day test (72 h for pre-treatment and 96 h for treatment) was also performed. All solutions were aerated and at pH 4.5. Prior to Pb analysis, roots were rinsed with nanopure H_2O.

(2) Anionic repulsion caused by the 2- charge of the PbEDTA complex: whole plant accumulation of Pb in presence and absence of N-[2 acetamido] iminodiacetic acid (ADA), a chelating agent that forms a neutrally charged Pb complex

In trials similar to Pb accumulation in whole plants (above), plants were treated with 500 μM $Pb(NO_3)$ in the presence of either 500 μM EDTA or 500 μM ADA. In this case, the treatments were conducted at the optimal pH for ADA, pH 7.0. Prior to Pb analysis, roots were rinsed with nanopure H_2O.

2.3. *Analysis of metal content*

Roots and/or shoots were freeze-dried, ball milled to a fine powder, re-dried and then ashed at 495 °C for 18 h. The ash was dissolved with 2 mL of 10% HNO_3 (v/v), then diluted to 2% HNO_3 (v/v) with nanopure dH_2O. The concentration of Pb was determined by flame-atomic-absorption spectrometry (AAS, model GBC Avanta Σ) with analytical grade $Pb(NO_3)_2$ as the standard.

2.4. *Statistical analysis*

Experiments were replicated three times and analysed using one-way ANOVA (Statistix, Analytical Software, Tallahassee, FL) after testing for homogeneity of variance (F-max). Post-hoc comparison of means was performed using the Tukey test. Values are means ± SE of three replicates. Values not sharing the same cased letter within an organ type (root/shoot) are significantly different ($p \leq 0.05$).

3. Results and discussion

3.1. *Whole plant accumulation of Pb in the presence and absence of EDTA*

For reasons of environmental safety, the scientific community is tending to move away from the use of EDTA in phytoremediation [11], but EDTA is still the most tested and quantitatively the most efficient chelating agent for phytoextraction. Thus we decided to start with EDTA as a chelating agent to assess, on a purely quantitative and indicative level, how our plant would compare relative to other tested plants. Hydroponics were used to allow a comparison to be made with other work and, importantly, to avoid creation of significant quantities of contaminated solids.

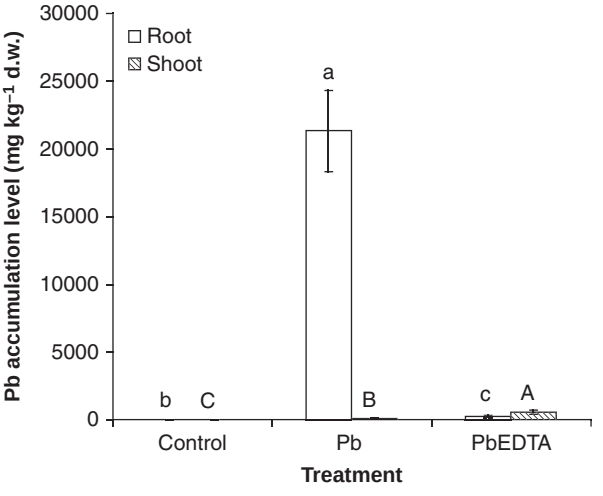

Figure 1. Pb accumulation level in 3-month-old *Symphytum officinale* shoots and roots of whole plants treated for 7 days in low phosphate Huang and Cunningham nutrient solution (pH 4.5) containing 0 (control), or 500 μM $Pb(NO_3)_2$ with or without 500 μM EDTA solution.

In the 500 μM $Pb(NO_3)_2$ treatments without EDTA, most of the Pb accumulated in the root; a mean of 21,319 mg kg^{-1} (dry weight, d.w.) of Pb accumulated in the root, compared to a mean of just 71 mg kg^{-1} (d.w.) in the shoot (Figure 1). The Pb accumulation level in shoots was at least seven times higher in the presence of 500 μM EDTA (558 mg kg^{-1} d.w. $p \leq 0.05$). Surprisingly, the addition of EDTA reduced Pb levels in the roots by at least 88% ($p \leq 0.05$) and this reduction was not accounted for by increased transport to the shoots.

Under the conditions of this study, *S. officinale* does not appear to translocate Pb in the shoots significantly, even in the presence of EDTA. An overall maximum shoot accumulation level of 558 mg kg^{-1} (d.w.) Pb is well below the 10,000 mg kg^{-1} d.w. desired threshold for phytoextraction that has been reported in *Brassica juncea* [5], *Zea mays* and *Pisum sativum* [4] and *Sesbania drummondii* [13] under similar hydroponic conditions.

3.2. *Investigation of the negative effect of EDTA on Pb accumulation*

When EDTA was added to the Pb-containing matrix, we observed an unaccounted loss of Pb accumulation in roots, that has not been widely noted. This has important implications for phytoextraction because, perceivably, if the concentration of Pb in the roots of chelated Pb-treated plants were higher, more Pb would accumulate in the shoots.

Some articles only publish the Pb levels in the shoot [3,4,9,14], but in others it was reported that EDTA increased root accumulation of Pb [8,15]. Slight decreases in root Pb accumulation that were not accounted for by translocation to the shoot were also present in experimental data by [5]. However, the small amount lost is likely to be due to the loss of PbEDTA from the root surface, which can be removed by washing (with dH_2O or EDTA prior to Pb analysis) in their experiments. We therefore tested two hypotheses (i and ii below) for reduction in Pb accumulation.

(1) Presence of EDTA in the free state

Lead accumulation in the root segments treated with 500 μM $Pb(NO_3)_2$ (unchelated Pb) increased over time (from the 3-day to the 7-day test), reaching over 80,000 mg kg^{-1}. Pre-treatment with 500 μM EDTA had no effect on Pb accumulation (Figure 2(a)). The opposite trend was observed for the 500 μM PbEDTA treatments, where Pb accumulation decreased with time (Figure 2(b)).

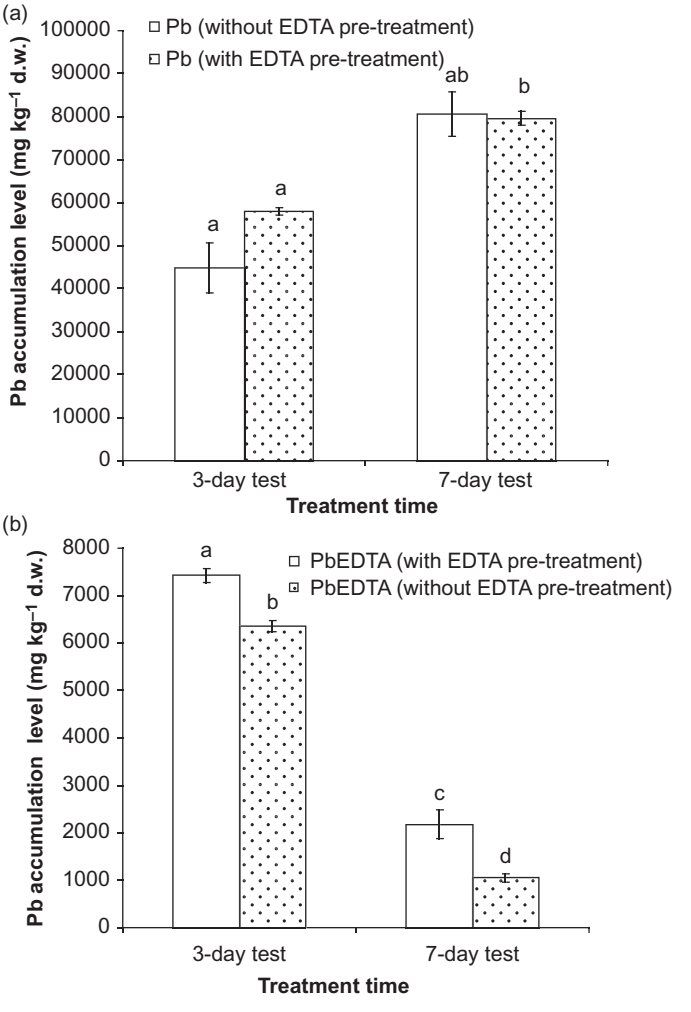

Figure 2. (a) Pb accumulation level in *Symphytum officinale* lateral root segments after the 3- and 7-day tests with $Pb(NO_3)_2$. In the 3-day test, lateral root segments were pre-treated for 18 h in low phosphate HC nutrient solution (pH 4.5), with or without 500 μM EDTA, followed by 24 h in low phosphate HC nutrient solution (pH 4.5) with 500 μM $Pb(NO_3)_2$. The 7-day test was the same, except the treatment duration was extended to 72 h for the EDTA pre-treatment and 96 h for the Pb treatment. (b) Pb accumulation level in *Symphytum officinale* lateral root segments after the 3- and 7-day tests with PbEDTA. In the 3-day test, lateral root segments were pre-treated for 18 hr in low phosphate HC nutrient solution (pH 4.5), with or without 500 μM EDTA, followed by 24 h in low phosphate HC nutrient solution (pH 4.5) with 500 μM $Pb(NO_3)_2$ and 500 μM EDTA. The 7-day test was the same, except the treatment duration was extended to 72 h for the EDTA pre-treatment and 96 h for the respective Pb treatment.

Regardless of EDTA pre-treatment, the level of Pb in PbEDTA treated root (Figure 2(b)) was at least six times lower at day 3, and 37 times lower at day 7, than in the unchelated Pb treatments (Figure 2(a)). The EDTA pre-treatment of roots exacerbated this effect, which suggests that EDTA itself may slightly change the root uptake ability of chelated complexes. Another possibly is that the unchelated Pb ions damaged the roots and allowed greater accumulation in roots compared with PbEDTA as proposed for sorghum plants [16]. However, this does not explain why there was a loss in Pb accumulation over time with PbEDTA in root segments. Perhaps, as suggested by [17], intact live roots are required for uptake of PbEDTA complexes. Thus they observed an increase in Pb uptake with intact whole seedlings of *Brassica juncea* but a decrease in root segments. However, our root segment and whole plant data showed the same trend: a reduction in root accumulation of Pb with PbEDTA compared with unchelated Pb.

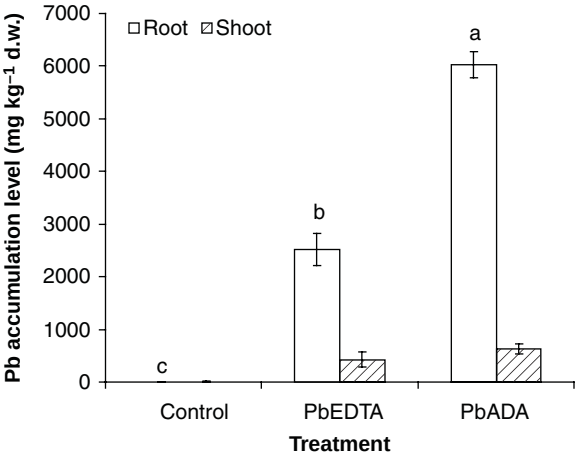

Figure 3. Lead accumulation levels in 3-month-old *Symphytum officinale* roots and shoots of whole plants treated for 7 days in low phosphate Huang and Cunningham nutrient solution (pH 7.0) containing 0 (control), 500 μM $Pb(NO_3)_2$ with or without 500 μM EDTA or ADA.

(2) Anionic repulsion caused by the 2- charge of the PbEDTA complex

Alternatively, our trends may be related to repulsion of the charged $[PbEDTA]^{2-}$ complex. Dekock and Mitchell (1957) showed that trivalent ions (e.g. Fe^{3+}) form EDTA complexes with no charge or a single negative charge and can be taken up into plants, whilst divalent cations (e.g. Cu^{2+}) form EDTA complexes with two negative charges and are repelled [18].

To directly determine if charge-based repulsion of the $[PbEDTA]^{2-}$ complex was involved, a neutrally-charged chelated Pb complex was tested. ADA is an analog of iminodiacetic acid (IDA) and has a similar ability to solubilise Pb in the soil as EDTA [19]. In the soil system, ADA forms neutrally-charged chelated Pb complexes $[PbADA]^0$ at equimolar concentrations of the metal ion and the chelator [20].

However, although more than twice as much Pb accumulated in roots with ADA treatments than with EDTA treatments, after 7 days the average Pb accumulation level (6023 mg kg^{-1} d.w., Figure 3) was still at least 70% below the level accumulated from unchelated treatments (compare results in Figure 2 and Figure 3). There was no difference in Pb levels in the shoots (approximately 600 mg kg^{-1}) between these two chelating agents (Figure 3).

4. Conclusion

Overall, our data indicate that, whilst the Pb accumulation potential of *S. officinale* shoots was increased in the presence of EDTA, it was minimal.The addition of chelating agents at similar concentrations to other studies for improving the overall chelate-assisted phytoextraction potential was also quite the opposite for *S. officinale* relative to other reports. The presence of EDTA and ADA markedly reduced the Pb accumulation ability of the roots. Although ADA slightly improved Pb accumulation compared to EDTA, neither can be said to increase Pb accumulation; both reduced Pb accumulation relative to treatments without such chelating agents.

Under the conditions of this study, the results suggest that EDTA itself and charge repulsion of the $[PbEDTA]^{2-}$ complex may play a small role in its reduction, but not a pivotal or overriding role. This reduction was not accounted for by translocation to the shoots as both the root and shoot weights were approximately the same. The main reason(s) for the reduction of

Pb accumulation in *S. officinale* roots appear to be complex and are still unclear. Moreover the mechanism underpinning this reduction, whether it is entirely due to effects at root level or due to effects at shoot level, is currently unknown. Whilst more studies (e.g. different metals, duration of treatment, further replications, soil-based studies, root physiology studies assessing root secretions or anatomical changes to the root) to consolidate and further explain the results of this study are recommended, the presently elusive action and negative effect of EDTA on Pb accumulation in *S. officinale* may provide quantitative reasons, in addition to the environmental concerns, towards a movement away from the use of EDTA as a chelating agent in phytoextraction.

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