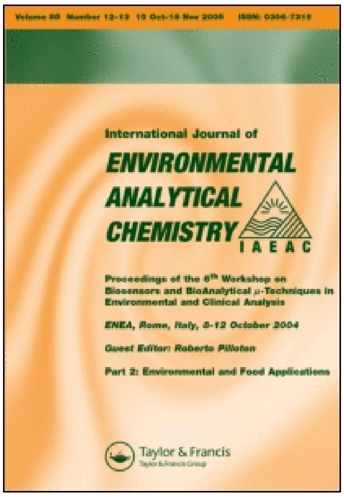

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UPTAKE OF HEAVY METALS BY LUPIN PLANTS IN ARTIFICIALLY CONTAMINATED SAND: PRELIMINARY RESULTS

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The aim of this study was to investigate the tolerance, uptake and accumulation of several metals of environmental interest by lupin plants. The effects of different metals on those parameters were evaluated individually as well as in groups, since the latter matches more closely real environmental conditions. The chemical form of some metals was also taken into consideration. Lupin plants were grown in different batches of sand artificially contaminated with Pb(II), Cd(II), Cr(III), Cr(VI), CH_3Hg^+ and Hg(II) (each metal at the $50\,mg\,L^{-1}$ level) or their combinations. After 4 weeks of growth, the results indicated that lupins were quite tolerant to Cd(II), Pb(II), Cr(III) and Hg(II) since contamination with those metals did not cause significant weight differences between metal-treated and control plants. On the other hand, the presence of Cr(VI) and CH_3Hg^+ induced severe signs of toxicity. Metal accumulation in lupins plants was influenced not only by the chemical form of the analyte but also by the co-presence of other metals. Metal concentration in the plants once harvested were found to be $4.9\,g\,kg^{-1}$, $2.3\,g\,kg^{-1}$, $0.4\,g\,kg^{-1}$ and $0.2\,g\,kg^{-1}$ for Cd(II), Hg(II), Pb(II) and Cr(III), respectively. Metals were preferentially accumulated in roots although a fast translocation to shoots was detected for Hg(II).

Keywords: Heavy metals; Soils; Lupin plants; Bioremediation

INTRODUCTION

Remediation is a promising approach to the decontamination of metal polluted sites, especially when large volumes of soil are involved. Conventional technologies were designed to operate in small, heavily contaminated sites. However, they are expensive procedures and may produce irreversible damage of flora and fauna [1]. Among the alternative technologies, phytoremediation is gaining more importance since it is a cost-effective and environmentally friendly technology. The so-called "green technology" consists in the use of plants, including trees, grasses and aquatic plants, to remove or to render innocuous a range of toxic pollutants present in soil, in water or even in the air [2]. Phytoremediation uses of the natural ability of plants to extract

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elements from soil and distribute them between root, stem, leaves, flowers and fruits, depending on the biological process in which the element is involved.

It is well established that the mineral composition of a plant strongly depends on the soil where it grows [3]. These differences can be partly attributed to plant's ability to absorb essential micronutrients and macronutrients from the soil. Plant roots absorb ions from a complex medium, containing not only the essential nutrient ions, which plants need for their development, but also a range of organic compounds and non-essential ions without a functional or structural role known till now [4]. Heavy metals such as Pb, Cd and Hg are included in the last group and have been responsible for several well-known disasters of public health [5–7]. Usually their presence, even at low concentration levels, is responsible for a wide variety of toxicity symptoms, although the essentiality or toxicity of a metal depends not only on the metal itself and its concentration level but also on its chemical form [8].

Certain plant species are potentially more suitable for phytoremediation. Special attention must be paid to those species, known as hyperaccumulators, which are not only able to grow in metalliferous soils but also to accumulate significantly high amounts of metals in their aerial parts. For example, *Thlaspi caerulescens* can accumulate Zn in shoots up to 3% dry matter (DM) [9]. However, these plants are small and slowly growing, therefore more suitable for theoretical investigation than for their use in decontamination processes. A good alternative to hyperaccumulators are those plants characterized by high biomass production and moderate capability to accumulate heavy metals. In this situation, special efforts should be directed to maximize the growth through the optimisation of agronomic practises as the total metal removed is directly related to the harvested biomass. A well known example is *Brassica juncea* [10].

Nowadays, one of the aims of phytoremediation is the screening of potential species capable of tolerating and accumulating high amounts of metals in their harvestable parts. Plants exhibiting multiple-tolerance are preferred since in environmental polluted sites more than one metal is usually involved [11]. Co-associations of some metals are commonly found in some industries such as Zn, Pb and Cd in Zn-smelters or Pb–Zn in urban soils.

The plants selected for the current study were lupins as they show several advantages such as adaptability to temperature changes and tolerance to poor soil quality [12]. They can grow in siliceous, sandy and acidic soils [13] and their symbiotic association with *Rhizobium* bacteria allows the reduction of atmospheric nitrogen into ammonium ion. These characteristics justify their cultivation all over the world, being Spain one of the richest countries in lupin flora.

The study shows two aspects related to the potential use of lupins in phytoremediation: the degree of tolerance and the capability of taking up and accumulating heavy metals in their vegetative parts. These parameters were evaluated under the influence of several metals of environmental concern such as Cd, Cr, Pb and Hg and their presence in various chemical forms and associations.

EXPERIMENTAL

Apparatus

An AGP-475 Radiber (Barcelona, Spain) growth chamber was used for the hydroponic studies. Plant digestion was carried out in a MPS 100 CEM (CEM Corp., Matthews, North Carolina, USA) microwave oven. A model 2380 flame atomic absorption spectrometer equipped with Pb, Cd, Cr and Hg hollow cathode lamps (Perkin–Elmer, Überlingen, Germany) and an air–acetylene burner was employed. Hg concentrations were determined by cold vapor-atomic absorption spectrometry (CV-AAS) with a flow injection analysis system (FIA) consisting of a peristaltic pump Gilson HP4, Tygon tubes for the peristaltic pump and a gas–liquid separator (Philips). The instrumental parameters were those recommended by the manufacturer.

Reagents

All reagents were of analytical grade or higher purity. Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Six stock solutions of $1\,g\,L^{-1}$ of each metal were separately prepared from suitable salts provided by Merck (Barcelona, Spain): Cd(II) from Cd $(NO_3)_2\ 2H_2O$, Cr(VI) from K_2CrO_4, Hg(II) from $HgCl_2$, Pb(II) from $Pb(NO_3)_2$, Cr(III) from $Cr(NO_3)_3$ and CH_3Hg^+ from CH_3HgCl. Six monoelemental solutions of each metal at $50\,mg\,L^{-1}$ level were prepared by diluting the stock solutions to appropriate volumes. Two multielemental solutions were also prepared: Cd(II), Cr(VI), Hg(II) (Mixture 1, M1) and Pb(II), Cr(III), CH_3Hg^+ (Mixture 2, M2). Both solutions contained each single element at a concentration of $50\,mg\,L^{-1}$. CH_3Hg^+ solutions were kept in the dark to avoid its degradation and transformation in other species. The sand was enriched with a Hewitt nutrient solution type. Sodium tetrahydroborate ($NaBH_4$) solution (3% w/v) was prepared by dissolving $NaBH_4$ powder (Aldrich) in deionized water and stabilised with 1% (w/v) sodium hydroxide. The solution was filtered before use to eliminate turbidity.

Plant Material

Lupinus albus seeds were provided by the Food Technology Department of CIT-INIA (Centro de Investigación y Tecnología–Instituto Nacional de Investigación Agraria). In order to avoid the appearance of fungi, the seeds were washed with a diluted solution of sodium hypochlorite for 15 min and rinsed with copious amounts of distilled water.

Sand Preparation

The inert sand was spiked with metals as follows: Batches of sand were exposed to different metal solutions (solutions containing the metals separately and solutions containing the combined metals, M1 and M2) for three days to reach the equilibrium between the sand and the solution. Then, the excess of solution was eliminated and finally, a defined volume of Hewitt's nutrient solution was added and completely mixed with the sand.

Metal Accumulation Procedure using Lupinus Seedlings

Lupin seeds were grown in seedlings (one seed per pot) containing 125 g of the different polluted sands (six batches containing each metal individually and two more containing the metallic mixtures: $M1 = Cd(II) + Cr(VI) + Hg^{2+}$ and $M2 = Pb(II) + Cr(III) + CH_3Hg^+$). The experiment was carried out in a growth chamber under controlled conditions of temperature (20°C) and light exposure (8 h per day). The seedlings were covered with plastic in order to maintain the humidity in the medium and to avoid excessive evaporation. Eight seedlings were used for each type of sand.

The plants were grown, harvested, divided in their different parts and those analyzed to evaluate metal uptake. A control group of seeds were grown in parallel in the absence of metals. The blank assay provides very useful information since it allows to establish the influence of each metal or group of metals during the growing of each set of plants.

Determination of Metal Content in Plants

After the desired period of time (3 and 4 weeks) the plants were harvested and their roots were gently washed with distilled water until the complete elimination of the remaining sand. Then, the weight of each plant as well as the length of the principal root and the stem were determined. Each plant was divided in different parts with a plastic knife obtaining three samples from each plant: the root, the stem and the leaves.

The samples were dried overnight at 65°C in an oven with forced air circulation, grounded in an agate mortar and kept in polyethylene bottles. Then, 100 mg of each sample were digested with 2 mL of concentrated HNO_3 in a microwave oven and appropriately diluted after complete digestion. Finally, the metal content was analysed using flame atomic absorption spectroscopy (FAAS) and the cold vapor technique (CV-AAS) for Hg. The analyses were done selecting the most sensitive wavelengths being 217.0, 357.9, 228.8 and 253.7 nm for Pb, Cr, Cd and Hg, respectively. The slit was fixed at 0.7 nm for the determination of all the elements.

RESULTS AND DISCUSSION

Metal Tolerance

After 4 weeks of growing in contaminated sand, the ability of lupins to tolerate metal stress was assessed using the index of tolerance (IT). The latter is calculated as the mean weight of a plant grown in the presence of a metal divided by the mean weight of a blank ratio, expressed as percentage [14]. Data in Fig. 1 shows the tolerance of lupin plants to grow in the different metal contaminated sands. An index of tolerance of 50%, which means 50% of optimum growth, is considered to be the minimum desired biomass production for plants growing in a metal contaminated site. The presence of Cd(II), Pb(II), Hg(II) and Cr(III) did not affect the biomass production, with indexes close to 100%. The presence of Cr(VI) caused the decrease of the IT to values about 60% together with an anomalous development in the length of the stem and in the root system. CH_3Hg^+ caused the inhibition of the germination process. However, in both mixed metal-contaminated sands, the simultaneous presence of other metals reduced to some extent the negative effect produced by CH_3Hg^+ and Cr(VI), which is of special interest in the case of the former. In the multiple metal contaminated sands, the IT obtained for M1 and M2 were 87.8% and 62.2%, respectively.

As far as the effect of individual metals on the root system is concerned, the presence of Cd, Pb and Hg(II) produced a significant reduction of the principal root length (around 50%) and an important development of the secondary root system (Fig. 2). CH_3Hg^+ and Cr(VI) caused dramatic symptoms of toxicity. Both elements inhibited the development of the principal and secondary roots, being particularly noticeable

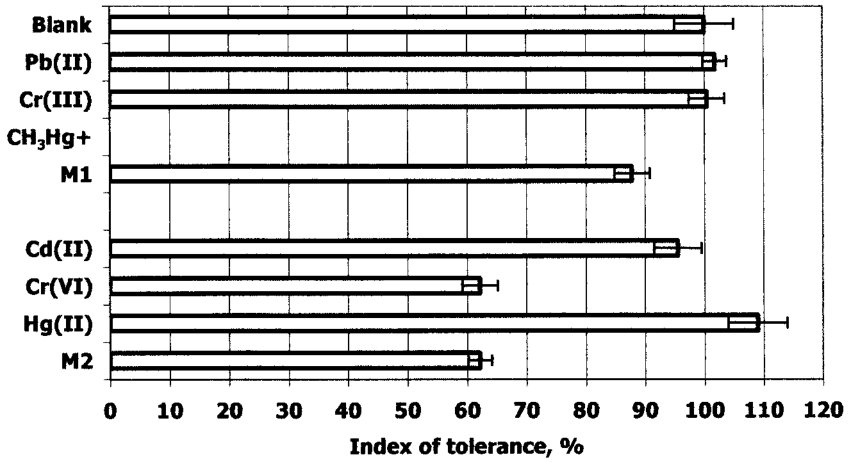

FIGURE 1 Tolerance index for lupin plants after a four-week growing period. M1 = Cd(II) + Cr(VI) + Hg^{2+} and M2 = Pb(II) + Cr(III) + CH_3Hg^+. Error bars represent ± s (s standard deviation for $n = 8$ plants).

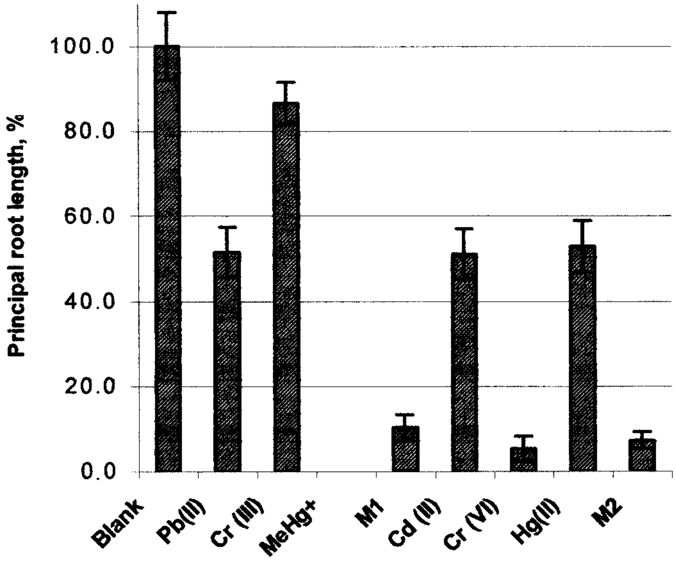

FIGURE 2 Root lengths of lupin plants after a four-week growing period. M1 = Cd(II) + Cr(VI) + Hg^{2+} and M2 = Pb(II) + Cr(III) + CH_3Hg^+. Error bars represent ± s (s standard deviation for $n = 8$ plants).

in the case of CH_3Hg^+. When the plants grew in mixed polluted sand, the effect on root morphology was similar, but much less pronounced. All seeds were germinated in the presence of various metals including CH_3Hg^+ (Fig. 3) but when CH_3Hg^+ was the only pollutant present, germination process was inhibited.

Related to plant length, no significant variations were noticed as a result of the presence of Cd, Pb, Cr(III) and Hg(II), (Fig. 4). On the other hand, Cr(VI) and CH_3Hg^+ were responsible for an important reduction in the length of lupins, especially

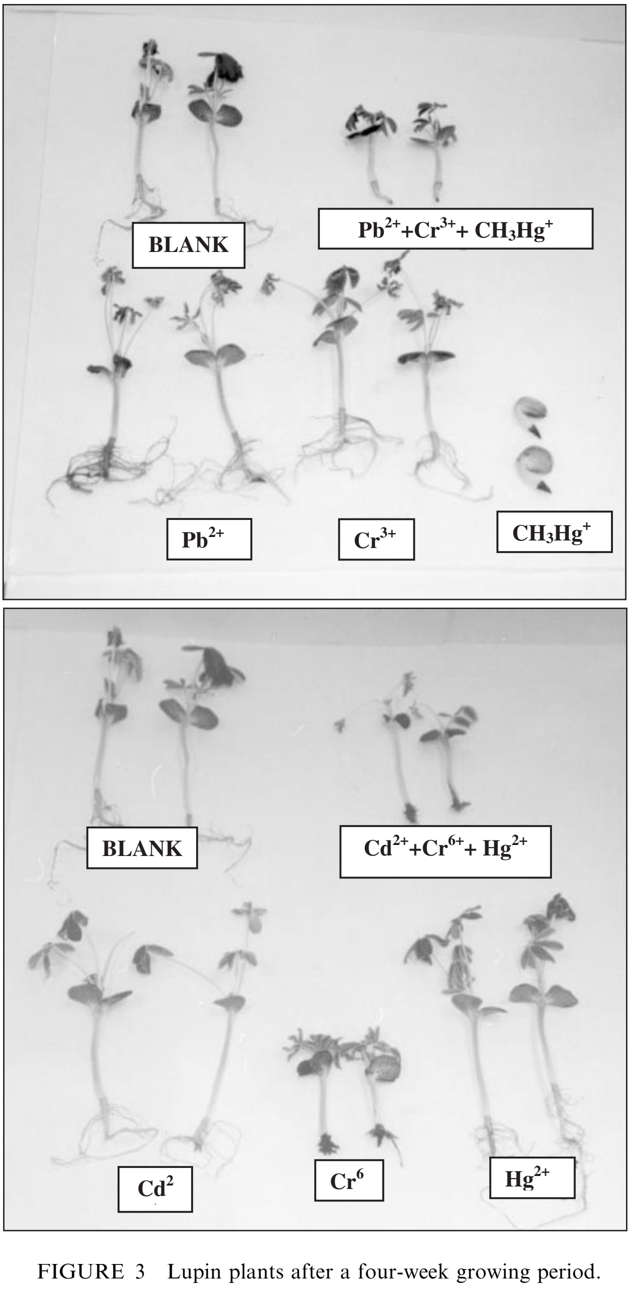

FIGURE 3 Lupin plants after a four-week growing period.

CH_3Hg^+, which led to a significant delay in plant development (after 3 weeks, the plants had not emerged over the top soil). In the presence of Cr(VI), lupins only reached 50% length of controls and those plants grown with Cd(II), Pb(II), Cr(III) and Hg(II). In contrast, the plants grown in both mixed metallic polluted sands, M1 and M2 containing Cr(VI) and CH_3Hg^+ respectively, showed larger length than those cultivated only in presence of one of them. Again, the negative effects of Cr(VI) and CH_3Hg^+ were

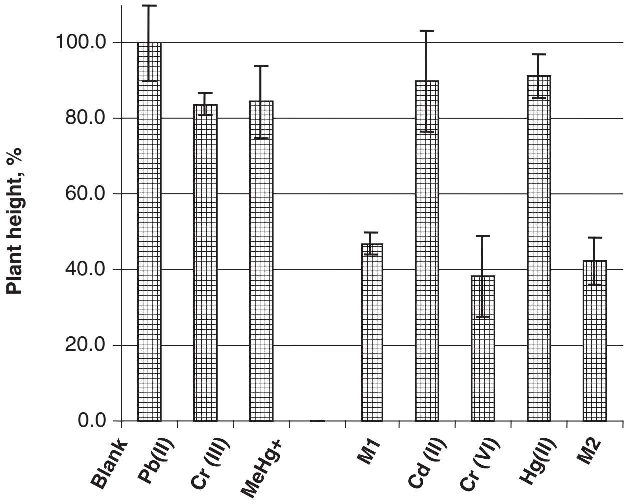

FIGURE 4 Plant heights of lupin plants after a four-week growing period. M1 = Cd(II) + Cr(VI) + Hg^{2+} and M2 = Pb(II) + Cr(III) + CH_3Hg^+. Error bars represent ± s (s standard deviation for $n = 8$ plants).

to some extent compensated by the presence of other metals. All these results indicate that lupin plants are tolerant to the presence of high amounts of Pb(II), Cd(II), Cr(III) and Hg(II), which make them potentially suitable for phytoremediation purposes. Both, Cr(VI) and CH_3Hg^+ caused severe signs of toxicity which makes lupins unsuitable for phytoremediation of these species. However, the presence of other metals decreases their negative influence.

The behaviour shown by lupin plants with different species of Cr and Hg emphasizes the importance of chemical speciation in plant–soil systems. The development and growth of a plant in a metal polluted soil depends not only on the total concentration of the heavy metals, but also their redox state, chemical species and type of soil (i.e.: organic matter, oxides of Fe and Mn, silicates, pH and CEC), which define the bioavailability of the metals. For instance, it is well known that Cr(VI) is toxic to plants while Cr(III) is essential. Nevertheless, several examples of plants that accumulate larger amounts of Cr(VI) than Cr(III) in shoots and roots and then die, have been reported [15].

Metal Accumulation and Translocation

Another problem to be faced in phytoremediation, along with tolerance, is related to metal accumulation and distribution in plants, especially concerning the translocation of metals from roots to shoots. In spite of the short interaction time between plant and sand, metals concentrations around $4.9\,g\,kg^{-1}$, $2.3\,g\,kg^{-1}$, $0.4\,g\,kg^{-1}$ and $0.2\,g\,kg^{-1}$ for Cd(II), Hg(II), Pb(II) and Cr(III), respectively, were found in lupins planted in sand contaminated with only one of these elements. These accumulation factors varied when lupins were grown in the mixed metal-contaminated sands. The co-presence of Cd(II), Cr(VI) and Hg(II) improved the Cd uptake until values of $8.48\,g\,kg^{-1}$ and notably decreased the Hg(II) uptake until a value of $0.45\,g\,kg^{-1}$.

In all cases the metal concentration found in the roots were significantly higher than those found in the shoots. The different mobility of metals through the plant can be

TABLE I Root/shoot concentration ratio for lupin plants after a four-week growing period

	Single metal-contaminated sand			*Mixed metal-contaminated sand*		
	[Root, mg kg^{-1}] × 10^{-2}	[Shoot, mg kg^{-1}] × 10^{-1}	*Root/Shoot ratio*	[Root, mg kg^{-1}] × 10^{-2}	[Shoot, mg kg^{-1}] × 10^{-1}	*Root/Shoot ratio*
Pb(II)	3.9 ± 0.4	5.3 ± 0.5	7.4	5.9 ± 0.5	4.8 ± 0.4	12
Cr(III)	1.6 ± 0.2	2.3 ± 0.2	7.0	3.0 ± 0.2	1.9 ± 0.1	16
CH_3Hg^+	–	–	–	4.8 ± 0.3	30 ± 2	1.6
Cd(II)	43 ± 3	59 ± 5	7.3	80 ± 6	51 ± 4	16
Cr(VI)	22 ± 2	17 ± 2	13	16 ± 2	14 ± 1	11
Hg(II)	19 ± 3	40 ± 5	4.8	2.1 ± 0.3	26 ± 2	0.81

Results expressed as mean value ± standard deviation ($n = 8$ plants).

related to the root/shoot ratio [15]. Table I summarizes the root/shoot concentration ratio after 4 weeks, which ranged from 0.81 for Hg(II) in the combined metal polluted sand to 16 for single Cd(II).

In a phytoremediation process not only the final metal concentration but also biomass production has to be considered. Hyperaccumulator species are characterized by containing high amounts of metal in shoots, but biomass production is usually not as high as other species, which concentrate moderate amounts of metal in their tissues. Therefore, it is necessary to express the results as amount of metal removed per plant when evaluating a species for purposes of phytoextraction.

Cd concentrations in root and shoot were higher than the concentrations found for the rest of the elements, either supplied alone or in the presence of other metals. However, the root/shoot ratio indicated poor translocation compared with the rest of the metals tested. On the other hand, inorganic mercury was rapidly translocated to the aerial parts of the lupins as it can be demostrated by the root/shoot values obtained: 4.8 and 0.81 in the absence and in the presence of Cd(II) + Cr(III), respectively. Although these metals decreased Hg accumulation significantly, their presence favoured the Hg transport across the plants. Based on the results of this study, lupins provide a promising starting point to remediate inorganic Hg polluted soils, since mercury phytoremediation has been limited to some assays with genetically modified plants, such as *Arabidopsis thaliana*, which reduces Hg(II) to Hg^0, providing volatile species [16].

Metal concentrations found in leaves were low and comparable to the values in stems except for inorganic mercury whose amount in leaves was comparable to roots (only in the mixed metal-contaminated sand). However, a higher mobility of species could be expected by increasing the growing time.

CONCLUSIONS

Lupin plants have shown a potential for phytoremediation of metal contaminated sites. Lupins are quite tolerant towards the presence of toxic metals such as: Pb(II), Cr(III), Hg(II) and Cd(II) and are specially suitable for inorganic mercury remediation because of the fast translocation of this analyte towards the aerial parts. The plant tolerance and metal uptake was strongly dependent not only on the metal itself (and chemical form in case of Hg and Cr) but also depends on the concomitant elements in the polluted sand.

Further research must be done in order to study the uptake in sites polluted with a mixture of several metals and to enhance the transport of the metals through the stem of the plants along with establishing the possible transformation processes of metal species in plants.

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