Ecocycles 2015
Scientific journal of the European Ecocycles Society

Ecocycles 1(2): 22-34 (2015) ISSN 2416-2140
DOI: 10.19040/ecocycles.v1i2.34

ORIGINAL ARTICLE

Tissue kinetics of heavy metal and boron accumulation, in the recovery of industrial sewage sludge by superplants

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Abstract - The overall pollution in the planet is reaching unbearable limits for humanity. Never before mankind had come to adopt measures agreed between countries in the magnitude of current ones. Among the measures taken to alleviate the problem are increasing green alternatives. This paper aims reporting how green alternative using Genetically Modified Organisms (GMOs) which have proven over last decade to be by far the best overall tool for combating pollution, operate in more detail. A kinetic study of heavy metals (Ni, Cu, Zn, Cd, Pb, and Hg) and B accumulation by plants grown in sludge collected from a treatment plant of industrial sewage is carried out. Summarizing: the results investigated in this work confirm again the capacity and usefulness of this tool as a solution to global pollution problems, studying another specific case which is particularly interesting since provides a real solution for a common problem: industrial water sludge. In this manner, substantiating the strategy designed in a scientific project (triggered by Doñana's disaster) granted by UE funds in 2000, and presented in Hanover's Universal Exposition. Glossing and discussing its positive implementation during years to check out the so called superplants against pollution.

Keywords - keywords: global pollution, decontamination, *Nicotiana glauca*, mining, Cd, Pb, industrial sewage sludge

Received: December 16, 2015 Accepted: December 22, 2015

Introduction

In a world where climate change is contributing to leading human being to massive poisoning and catastrophes such as earthquakes and tsunamis, it is perhaps extremely urgent to find ways to cope and to end pollution problems. Among the different aspects affecting environmental pollution, industrial sludge generation has become one of the most important issues. This is because today's society bases its standards of living, in the industry potential and development. Until now, such an industrial development had not incorporated the economic costs of pollution in its financial accounts. However, at present there is no other option.

There is great interest from the industry in general, for transferring sludge to Agriculture. It is supposed that could be, a residual value for industrial processes, turning a problem into a benefit. In this sense the sludges have a large quantity of organic matter and nutrients, aspects which are excellent features for agriculture. However industrial activity, uses in most of its processes, elements which are toxic at certain concentrations and inevitably tend to accumulate into sludge. Therefore, sludges show a specific major problem for their use in agriculture which is their containing pollution. It can be argued that certain industry sectors seek, sometimes, dodging decontamination costs, thus transferring all or part of the problem to Agriculture. This attitude has become complacent in some environmental administration officials. The reason is because the problem is global and has been generated mostly in the past. Public administration behaves as if it were economically delicate to require an absolute rigor in the implementation of environmental legislation due to possible unemployment which could be generated. The list of pollutants in the industry is huge, but can be approximately divided into two groups: organic and inorganic. Among organics the most dangerous are pesticides by its very aggressive effect (Sánchez-Bayo F, 2011), however shortly in life time, because of its easy degradation. Factors inevitably linked, since their aggressiveness is based on its easy decomposition, understanding by decomposition also its high chemical reactivity. This is the reason why man has no other alternative that using natural live competition (mainly animal such insect) and natural products to control pests (Dayan and Duke 2014). In contrast to inorganics, heavy metals are also on top of toxicity since they are typically found in concentrations producing same type of problem (electronic decompensation of the cell) but over a longer period of time. For example when a photon of high energy or a pesticide enters the cell, chemical radicals are formed, that is mainly because unpaired electrons are produced. The final problem created to cell is no other that an electronic unbalance. Actually heavy metals are indestructible. As an example of industrial sludge generation, contamination in fishery may be due to a variety of chemicals used in most factory processes (Sciortino and Ravikumar 1999)

There are different ways to decontaminate industrial sludges by means of traditional methods which are based on chemistry and physics mainly (Salado et al. 2010). Accordingly, the aim of this work was to deepen into the knowledge of an alternative tool to classical techniques and hence demonstrate its capability as today's best instrument possible to remediate industrial sewage sludge. This technology is based conceptually (Aguilar, López-Moya and Navarro-Aviñó 2009) upon plants which has emerged in last decade as highly useful. Especially auspicious is the one based in plants which were naturally (Darwinian) selected from contaminated soils, and later modified genetically. Some of these mentioned plants, which have also been studied in this work, behaved previously as hyperaccumulators (1-5%, dry weight). Plants which were obtained by inserting a phytochelatin synthase in those *Nicotiana glauca* individuals previously chosen on Darwinian selection from contaminated soils (Gisbert et al. 2008; Gisbert et al. 2003). This hyperaccumulation was demonstrated in mining soils containing total concentrations of heavy metals about 20,000 ppm (Martínez et al. 2006). Since it is logical to think that storage activity for an hyperaccumulator can be diminished or annulled when it is growing in a substrate bearing low metal concentration (under EU limits) this study aims to determine whether these plants are able to maintain their usefulness in industrial sewage sludge containing comparatively highly reduced concentration of heavy metal and boron.

As pollution in the world affects increasingly to biosphere in general and specifically to an essential good for humanity such as water, it is very important to position scientists themselves and solve the water problem as a priority. Since 2006 European and non-European scientists, under EU shelter, have joined forces to address this problem in a very efficient but respectful way to environment (Schröder et al. 2007). To address this water problem it must be solved, not only the difficulty of its pollution, but it will inevitably necessary to resolve the question of sludge generated in water treatment plants. This work has addressed this latter issue, focusing on heavy metals and B pollution. Besides also aims to answer the following questions. Can these plants decontaminate sludges within a short time? Can therefore be used as a feasible solution to sewage problem? How long it would take? Which are the crucial features in the accumulation process for plants growing in sewage sludge?

To answer these questions it is imperative to understand several aspects related to accumulation kinetics. Indeed accumulation kinetics of heavy metals in a plant depends on a number of factors, which mainly are: net driving force generating intensity for global accumulation within the plant (suction force to accumulate), heavy metals concentration in the substrate where plant is grown, substrate characteristics in which heavy metals are present (acid soils, lime, slurries, aqueous, etc) organic matter concentration underground, and alive population in place (life environment where plants grow) time lasted since plant starts contact to contaminated environment up to samples collection for analysis, competition or not, among metal in mixture to enter into plant through the same systems, weather in site (temperature, wind, light amount, etc.) and exposure to other known and unknown contaminants (eg. existing solvents which can alter metal chemistry or pollutants coming from the air). Some of these factors are not easily controlled, for example, specific conditions of living matter in soil, specific daily climate and unknown contaminants. Therefore the study of heavy metal accumulation in plants growing in sludge coming from an industrial sewage treatment plant is a complex problem where many factors are involved, especially when performed outdoors. To minimize the number of influencing factors, this work was carried out in greenhouse, under strictly controlled conditions. However, keeping in mind that the feasibility to face water problem will most likely require working mainly outdoors.

Materials and methods

Plant material assay. Plant material was handled as described in (Martínez *et al.* 2006) with minor modifications. T3 modified lines seeds corresponding to lines 6, 12, 17, and 18, and wild type seeds were germinated in Petri dishes containing 6 g l^{-1} agar, Murashige and Skoog Basal Salt Mixture (Murashige and Skoog 1962) and 10 g l^{-1} sucrose at pH 5.7 buffered with 0.25 g l^{-1} MES (2-[N-mor-pholino]ethanesulfonic acid). Ten days after (when the first leaves were developed) plantlets were transplanted to 5 kg sludge/sand pots diluted 50% (v/v). Regarding Genetically Modified Organisms (GMOs) 3 out of 4 lines were chosen to design the graphics. Since authors were comparing 3 GMOs lines and a wt line line 17 was discarded for calculations and plotting, in order to simplify extension and understanding of graphics. Therefore only are showed graphics for lines 6, 12 and 18. Previously sludge was dehydrated in oven at 35º during 24 hours. Plants were grown in plant tissue culture growth chamber (25 C, 17 C, day and night 16 h light at 120 lmol m^2 s^{-1} photon flux density), then transferred to greenhouse pots containing the mixture sludge-sand. Pots were covered with film for a few days to obtain better acclimatization conditions. Plants were irrigated using only water, on a daily basis during the first month, and every 7 day thereafter. Before harvesting, plants were grown alternatively 3 weeks and 3 months. When plants were collected, modified and wild type lines were divided into roots, stems and leaves and fresh weight was determined. Roots were water washed, then $CaCl_2$-HCl (pH 3.8) for 10 min, and finally by deionized water. Stems and leaves were washed with deionized water. Samples were lyophilized to obtain dehydrated material. Stems, leaves and roots after dried were analyzed for heavy metal contain.

Metal analysis. Plant samples were analyzed for their heavy metals and boron content by Inductively Coupled Plasma Mass Spectrometry (ICP- MS) analysis according previous experience (Martínez *et al.* 2006). The analytical sludge samples were performed by an external company, a partner company of the Ministry of environment, controlled by National Accreditation Body (ENAC), responsible for accrediting calibration laboratories tests. Trials 163 / LE268, LE629, LE630, applying ICP-MS test and Atomic Absorption Spectrometry (AAS) techniques specifically for Hg in the remaining sludge.

Statistical analysis. Average and **s**tandard deviation values were calculated for at least three individuals of wild type genetic line and for at least three individuals of each three GMO lines when compared to study differences due to gen insertion. Statistics were calculated for each different tissue: roots, stems and leaves and for total heavy metals and B accumulation in whole plants as showed for instance in Fig 2. Errors bars are not showed in some of the Figures in order to improve the visibility of differences.

Results

1. Pattern of accumulation profile in the different plant tissues

1.1. Pattern root to leaves at initial time t0 (3 weeks)

1.1.1. Similarities in pattern root to leaves found for the different lines at t0

At initial time (Fig1A) **Ni, Cu, Zn and Cd** follow a similar tissue accumulation pattern **leaves > roots > stems**. However, differently B follows mostly the scheme **leaves> stems> roots**. Interestingly in all the cases leaf is the tissue where maximum accumulation is observed. This is irrespective to the heavy metal initial concentration in sludge (Table 1, Fig 1A). From these two facts two conclusions can be inferred: first, plants at time t0 (3 weeks) can phytoextract (leaves > roots) to aerial part, and second, extraction capacity follows a linear progression in regard to initial metal concentration in sludge: the higher initial concentration the higher the accumulation at t0 collection. For instance at t0, accumulation, in terms of global plant assimilation (throughout whole plant) follows the order (Table 2): Zn (65 as initial concentration in sludge, and 178.67 as average of all tissues), and then obtaining average of wt and GMOs > Cu (30, 27.03) > Ni (25, 2.24) > Pb (37, 1.51) > Hg (<0.75, 1.06) > Cd (<2, 0.61). Pb and Hg have to be considered included in a slightly different group, since both are first, very heavy, and second, are almost next in Periodic System (atomic numbers 82 and 80, respectively). However, Pb in some aspects is also a

Table 1. Sludge composition. Essential parameters and heavy metal concentrations determined in the sludge before planting. Limits regulated by UE for industrial soil and Agricultural sludge are also shown to compare toxicity levels studied in the sludge.

Composition	*sludge*	*EU sludge*[b]	*EU soil*[c]
pH	6.9		
humidity (%)	9.1		
ratio C/N	7.35		
Ca (%)	11.20		
Mg (%)	1.13		
N (%)	2.96		
K (%)	0.55		
P (%)	0.25		
OM (%)	37.5		
Fe (g kg^{-1})	17		
Hg (mg kg^{-1})[a]	< 0.75	10	0.5
Cu (mg kg^{-1})[a]	30	1000	50
Pb (mg kg^{-1})[a]	37	750	70
Zn (mg kg^{-1})[a]	65	2500	150
Ni (mg kg^{-1})[a]	25	37	50
Cd (mg kg^{-1})[a]	< 2.0	10	1
Cr (mg kg^{-1})[a]	27	1000	60

[a] European Union limits (mg kg^{-1}) for agricultural soils (pH 6-7): Cu 50-140; Pb 50-300; Zn 150-300; Ni 30-75; Cd 1-3; Cr 100-150, Hg 1-1.5
[b] European Union limits (mg kg^{-1}) maximum proposed in the sludge
[c] European Union limits (mg kg^{-1}), maximum proposed for agricultural soils (pH 6-7)

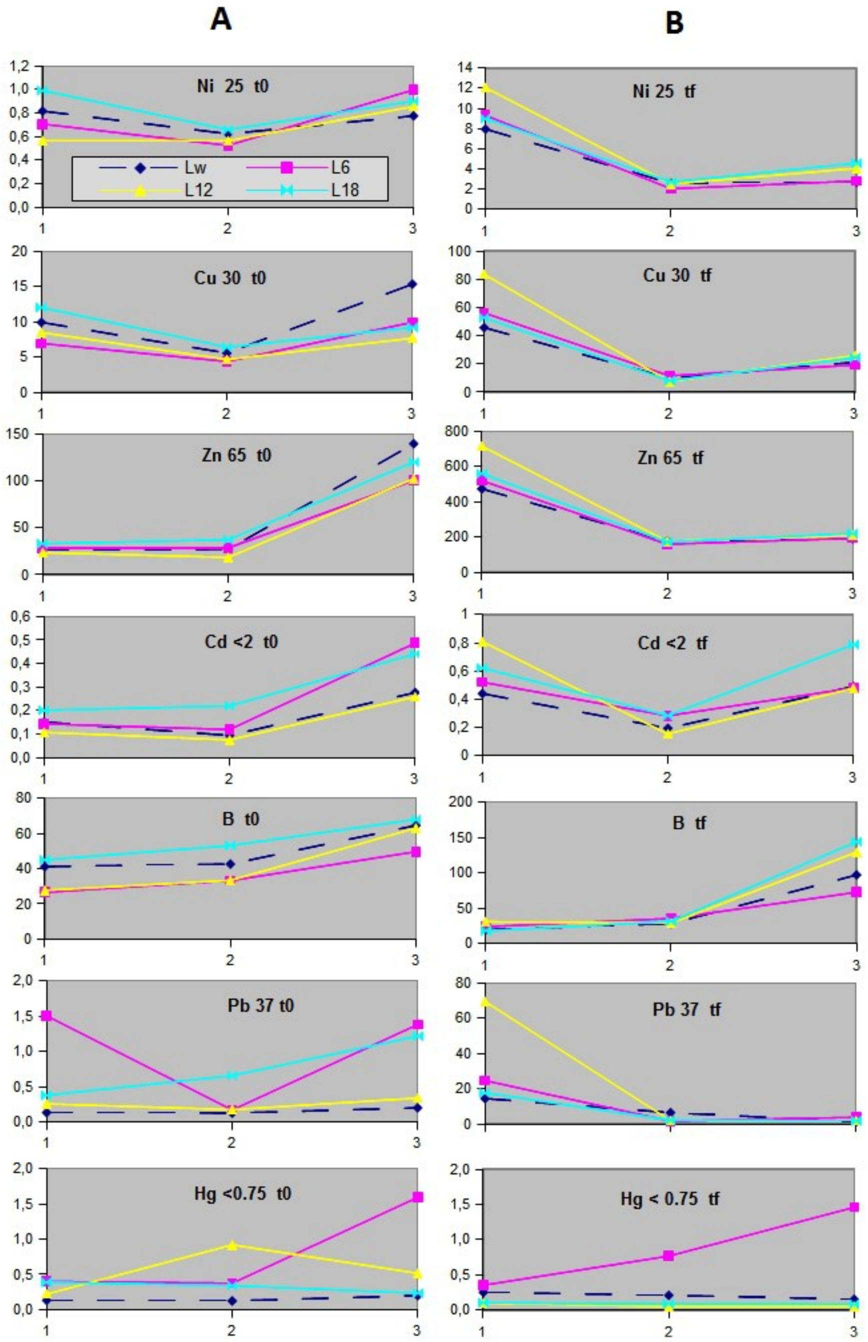


Figure 1. Metal and B accumulation pattern root to leaves. 1A Y axis indicates element concentration at initial time (t0, 3 weeks) determined (ppm) in X axis for roots (1), stems (2) and leaves (3). Title inside rectangles shows element initial sludge concentration except B (unknown). Wild type and 3 GMO lines are compared. Reader must be aware that scale changes in graphics. **1B. Pattern of metal accumulation from root to leaves at the final time (tf, 3 months)**, Reader must be aware scale changes in graphics. Particularly when comparing t0 versus tf

remarkable exception, most likely since its mobility is extremely small due to its big mass, its small volatility and its extraordinary capability to bind anions. Differently, Hg shows an extraordinary low melting point (for a heavy metal), is very mobile and more important, very volatile. Regarding B missing on the list, unfortunately there is no data about its initial concentration. In the other hand, Cr was not studied because it was not available the right technology to discern Cr^{+3} and Cr^{+6}, which can produce the opposite effect on human health (Borrás *et al.* 2008).

Table 2. Total accumulated concentration (ppm) **in plants for heavy metals and B.** Average (AVG) concentrations are presented as set values for wt and GMOs only for t0. For tf accumulation differences are too distant to establish an average

	t0			tf	
Metal-B	wt	GMO	AVG (wt, GMO)	wt	GMO
Ni	2.22	2,26	2.24	13.25	16.36
Cu	30.83	23,23	27.03	77.02	96.61
Zn	193.24	164.10	178.67	838.42	974.20
Cd	0.52	0.69	0.61	1,13	1,47
Pb	1,00	2,03	1,51	22.16	41.69
Hg	0.46	1,67	1,06	0.62	1,03
B	148.44	133,31	140.88	145.08	171.65

This t0, possible linear relationship, for initial metal in sludge versus initial metal accumulated in plant, is especially important since this could indicate absence of a significant threshold concentration, from in which, the plant begins to phytoextract metals. This means that, although in some cases there is small presence of metal in sludge, plant tends to accumulate it already, working as remediator. In general, it would be reasonable to consider that a plant species only uses high accumulation mechanisms, at high concentrations, that is, when metal concentration produces stress and not at lower concentrations since these mechanisms may require important amount of energy regarding expense in steady state. However this is not the present case. Therefore metals are transported from roots to leaves, producing higher concentration in leaves as it should be expected from an hyperaccumulator plant species. Consequently these plants have the ability to extract metals from sludge, decreasing hence their concentrations on it (data not shown). In fact when ratio total accumulated concentration in plants versus initial metal concentration inside sludge is calculated, which is equivalent to calculate BCF (bioconcentration factor: ratio of the concentration of a particular chemical element in a living organism to its chemical concentration in the surrounding media, Table 3), the following classification is obtained at t0: Zn (2.97 wt and 2.52 GMOs), Cu (1.03, 0.77), Hg (0.61, 2.22), Cd (0.26, 0.34), Ni (0.09, 0.09), Pb (0.03, 0.05). In the case of Pb it seems like there is a certain threshold for high transport into tissues, since BCF is much smaller compared to rest of metals, however when plants were previously tested in highly contaminated soils, it was demonstrated that Pb reached at the end, concentrations similar to those of other metals, especially for GMOs (Martínez *et al.* 2006). Pointing to Pb as a case of slow kinetics instead of low phytoextraction.

1.1.2. Differences in pattern root to leaves found for the different lines at t0

Accumulation profile for Boron (Fig 1A) differs mainly by the fact that its increment follows the pattern stem> root. The stem is a tissue (from a functional point of view) serving to communicate roots to leaves and flowers. Therefore, normally functions as a connection channel. This is true as long as the metal (nutrient or toxin) is "metabolized" correctly since obviously some contaminants flow in an uncontrolled and unpredictable way. This explains why the stem (from a functional point of view) is usually the plant tissue showing lowest metal accumulation, since metals tend to accumulate first into root (as a gateway), and then pass quickly through stem (pushed usually by hydraulic force) to finally accumulate into leaves. One possible explanation for this differential accumulation pattern for B might be based on a capacity, especially high for the plant species analyzed in this work, to accumulate B. Thus it could be argued that at t0, B transport from roots to stems is comparatively faster than for the rest of metals. However, obviously, accumulation kinetics of a particular species for a given metal depends not only on the specific plant ability to accumulate the metal, but also on its initial concentration. Therefore it can be not conclusive to argue about the exact accumulation capacity for B since the initial concentration in sludge is unknown. However, what is really known is the total accumulated concentration at t0 (Table 2, 148.44 wt, 133.31 GMOs). Such accumulation in absolute numbers indicates that at t0, B ranks in second place behind Zn, metal which was shown to be hyperaccumulated by GMOs in previous work (Martínez *et al.* 2006). Therefore, pointing to B as a possible hyperaccumulated element. Another marked difference, as mentioned above, is due to specific Pb and Hg physical and chemical characteristics since both elements show different kinetics of accumulation respect to other metals. Hence in view of the results obtained at t0 it could be possible to hypothesize for this experiment about three metal groups: not extremely toxic metals which comprises Zn, Cu, Ni, and Cd (although cataloged extremely toxic, its chemistry is closer to those divalent metals and its concentration is < 2, but judging by the initial accumulation in plants, probably much smaller), extremely toxic metals which comprehends Pb and Hg, and a group comprised only by B.

1.2. Pattern root to leaves at final time tf (3 months)

Table 3. BCF wt versus GMO for each metal at t0 and tf. BCF depends beyond specific extractive power of each individual plant, at least on two other facts: elapsed time t0 to tf, and negative interaction effect which each metal exerts to energetic level for each specific plant individuals (toxicity). Which depends on its specific concentration in these specific conditions resulting in specific interaction each metal undergoes relative to other metals in substrate (in synergistic or competitive interaction). In consequence BCF is a useful but relative indicator of plant accumulation capability for each individual.

	BCF			
	t0		tf	
Metal	wt	GMO	wt	GMO
Ni	0,09	0.09	0,53	0,65
Cu	1,03	0.77	2,57	3,22
Zn	2,97	2,52	12,90	14,99
Cd	0,26	0,34	0,56	0,74
Pb	0,03	0,05	0,60	1,13
Hg	0,61	2,22	0,31	1,37

1.2.1. Similarities found for the different lines at tf

Again, it would hypothetically be possible classify patterns into the same three groups (Fig 1B): Ni, Cu, Zn and Cd in one group, Pb and Hg in another group, and a third pack comprised exclusively by B. All these metals Ni, Cu, Zn and Cd follow a similar accumulation pattern, in the way roots >> stems < leaves, but now roots>leaves, while at t0, roots < leaves. However at tf, the most relevant fact is the observation of the largest metal amount accumulated in all tissues and for all plant individuals over trial time. Still as for t0, lingers positive ratio leaves/stem, therefore metals remain transported from stems to leaves as it is expected for accumulator plants bearing high extraction capacity. That kind of plants not only phytoextract from media but also tend to accumulate metals in leaves what makes further decontamination easier. This fact is clearly shown for Ni, Cu, Zn, B and Cd, although interestingly in this last case the ratio leaf/stem is higher than for the mentioned metals what is remarkable since Cd is very toxic metal and therefore usually not highly accumulated in plant tissues. From these two mentioned facts: higher total plant accumulation and specific metal tissue distribution (stem<roots and leaves>stem) it can be inferred three conclusions. First it is possible that at tf, the stem still can function as fastest transport tissue, and therefore where least accumulation is observed. Stated in another words, due to a large metal accumulation throughout whole plant, comparatively to t0, and particularly in roots, it could be expected metal flow sufficiently reduced in stem, tending to collapse, since higher metal flow is pushing from roots. If collapsed that could reduce comparatively flow from stem to leaves. However since concentration is higher in leaves there must still be an intense force which is driving metals to leaves. Second, plants at tf can phytoextract to leaves since leaves>stem or when in some cases leaves>=stem then roots=stems, this indicates that metal still is relatively easily transported and mainly to leaves. A third conclusion which supports above statement, is consequence of the high extractive capacity observed, taken in terms of bioconcentration factor BCF at tf (see Table 3). Specifically BCF is in most cases higher than unit, what necessarily means, that there is an effective phytoextraction implementation for all different species (wt and GMO) and for all metals analyzed. However, in absolute terms BCF of GMOs values for Ni (0.65) and Cd (0,74) only when rounded reach the unit, indicating that not all concentrations in sludge are matched inside the plant at a specific time.

1.2.2. Differences found in the pattern of accumulation for the different lines at tf

Among all the elements studied B (Fig 1B) shows the most conserved accumulation pattern in the way leaves>roots while for most of metals leaves<roots. Interestingly, Pb tf visual comparison is misleading, since judging by pattern profile seems that Pb accumulation has decayed. However, both wt and GMOs, multiplied by 20 overall accumulated concentration in plant (Table 2): wt (1.0 in t0 versus 22.16) GMOs (2.03 vs 41.69). This means that plants actually accumulate Pb in remarkable amount, especially at tf. Although this is mainly due to a large increase in accumulation in roots for both species: wt and GMOs (Fig 1B). Similarly to that of Pb it is shown Hg tf pattern (except a genetically modified line which shows very contrasting profile), however differently, Hg total accumulated concentration ratio, remains barely unchanged (Table 2).

2. wt versus GMOs: comparative accumulation analysis
2.1. wt-GMOs comparative accumulation analysis at t0

There is higher concentration of elements inside GMOs for most tissues analyzed (Table 2, Fig 1A, Fig 2 gray bars), although when comparing entire plant accumulation, values for Cu, Zn and B show a slight difference favoring wt. There are two specific cases regarding leaves in which wt plant shows higher metal accumulation (Fig 1A): Cu and Zn. Both can be explained assuming that Cu and Zn are oligoelements which have regular presence in living organisms and therefore generate low toxicity compared to other metals existing in sludge. Consequently wt plants might also have metabolizing, and sufficiently effective, detoxification systems, when compared with those provided by modified species, at t0. Therefore this could explain why wt plants do not show in these concentrations for those metals and for a short period of time, a very clear difference. It is therefore not surprising that accumulation ratio GMOs/wt, shows higher differences at concentrations much higher than those observed initially in sludge, as reported in previous experiments (Martínez *et al.* 2006).

2.2. wt-GMOs comparative accumulation analysis at tf

At time tf three conclusions can be drawn. First, practically all the modified lines, and in practically all the tissues, accumulate higher concentration for each specific element (Fig 2 red bars, Fig 1B, Table 2) except in the case of Hg, which requires special attention since taking whole plant accumulation two out of three GMO lines, accumulate less than wt (Fig 2, red bars). Second, for wt and GMO species the extraction capacity follows a positive progression versus initial metal concentration in soil, that is, the higher initial concentration the higher global accumulation except for the small swap on the list Cu-Pb (Fig 1C, 1D, Table 2). Third, regardless the initial concentration and specific element, greater total accumulation in GMOs is observed. Therefore it could hypothesize about a threshold absence regarding two main aspects. There is probably no threshold concentration to activate accumulation mechanisms. There is also no clear threshold concentration since it is possible any time to observe differences favoring GMOs.

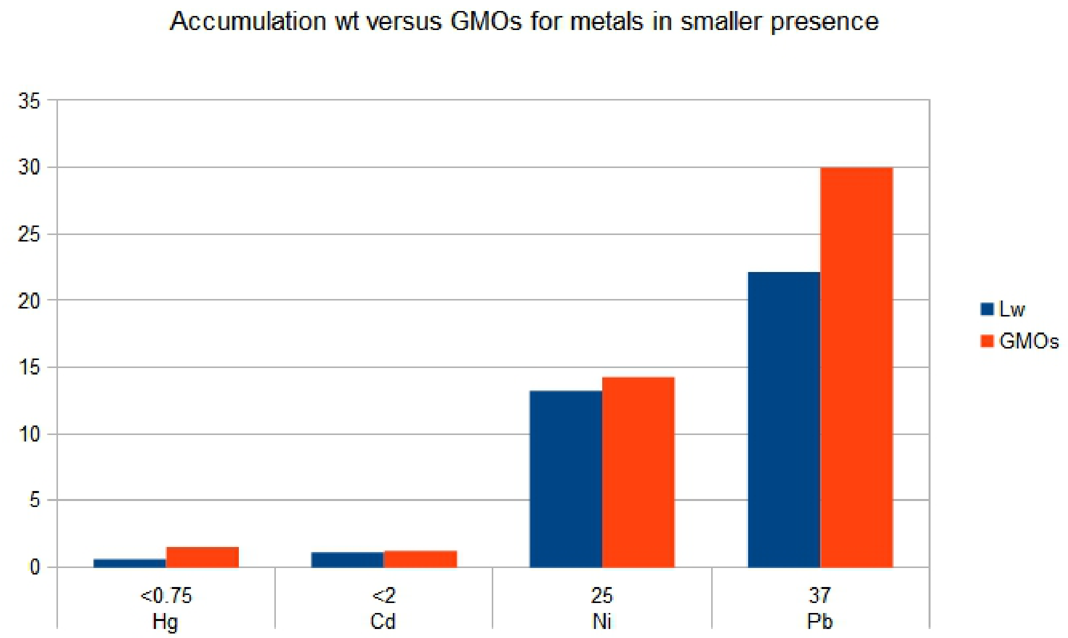
2.3. wt-GMOs comparative accumulation analysis at tf/t0

Modified lines accumulate higher metal and B amount than wild plants, for all the individual lines, particularly at tf (Fig 2 red bars, Table 2). Same at t0 except for Cu and B. However differences in accumulation wt versus GMO are small at t0 (Table 2), probably since metal concentration in sludge is low (below UE limits, Table 1). Regarding Ni, Cu, Zn and Pb accumulation at tf, all modified lines follow the same accumulation pattern L6 < L18 < L12 (Fig 2), while Cd and B share the same accumulation pattern, L6 < L12 < L18. Interestingly these modified lines might serve as valuable tools for Pb decontamination in sludge, since both wild species and modified lines at tf multiply by 20 initial t0 accumulation, while accumulation ratio GMO/wt of 2, remains unchanged at both tf and t0 (Fig 2, Table 2). When individual lines are investigated, L12 shows an exceptional Pb accumulation. The GMO accumulation profile measured by the ratio tf/t0 follows the order (Table 4, Fig 3B): Pb > Zn > Ni > Cu > Cd > B > Hg. Wt follows same order (Table 4, Fig 3A), except Hg > B.

These results provide two important conclusions. On one hand, confirm previously published results concerning heavy metal and B accumulation (Gisbert *et al.* 2003; Martínez *et al.* 2006) indicating that these specific GMOs show almost the same but enhanced physiology as wild species. In addition, these results support the proper execution of the experiment described in this article since these data point again to same conclusions. Remarkably these basic qualities for wt species showed that total Pb accumulation in leaves from t0 to tf increases 6,02 times consistent again with previous reports (Gisbert *et al.* 2003; Martínez *et al.* 2006) thus indicating that Pb may be mobilized from sludge to plant leaves despite its low mobility (Table 4, Fig 3A). Moreover, genetic modification doubles Pb roots accumulation as comparing both tf/t0 (111.19 versus 52.89) and what is more important, the quotient tf/t0 for GMOs in root tissue shows increased accumulation by over a hundred in three months.

For Hg although at t0 (Fig 2) GMOs clearly display greater total Hg accumulation than wt species, surprisingly there is an overall drop of metal at the end of the trial (Table 2), except for one of the lines (L6, Fig 2), which exhibits a slight overall Hg increase at tf. This phenomenon can be inherently explained assuming that different lines (except L6) lose Hg through probably leaves due to its extremely high volatility. Indeed, although wt shows an overall Hg increase at the end of the trial (Fig 2, Table 2) actually displays less Hg at tf in leaves than stem (Fig 1B, Fig 3A). Regarding overall trial envisage when compared metal incremented concentration within the plants (tf/t0), there is a clear increase for all metals analyzed, except this mentioned drop of mercury for GMO (Table 2). This means that plants accumulate metal over time similarly in all the cases except B and Hg, coherently to observed accumulation pattern at tf: roots > leaves > stems. Besides there is an increase of metal concentration inside root tissue (Fig 3A) except scarcely for B and Hg. This observation might indicate that at trial end, the aggregation in root tissue is the limiting step regarding accumulation changes inside the plant.

Therefore the overall results show that at t0 (after 3 weeks) there is a different distribution pattern for the studied heavy metals in different tissues. Indeed, is the aerial tissue, specifically the leaf tissue, the one showing a comparatively greater accumulation of heavy metal and B. It stands to reason that plant species showing high capacity to accumulate heavy metals, will display a fast heavy metal flow to the aerial part. Consequently, roots will have a relatively minor accumulation. In contrast, at tf, after 3 months plant growth, there is a greater accumulation in root tissue since heavy metals flow must be increasing as the plant becomes higher do to different aspects as for example hydraulic driving force from plant. Coherently at tf the leaves accumulate relatively lower concentration compared to roots, because at end of test heavy metal amount accumulated inside root is very high



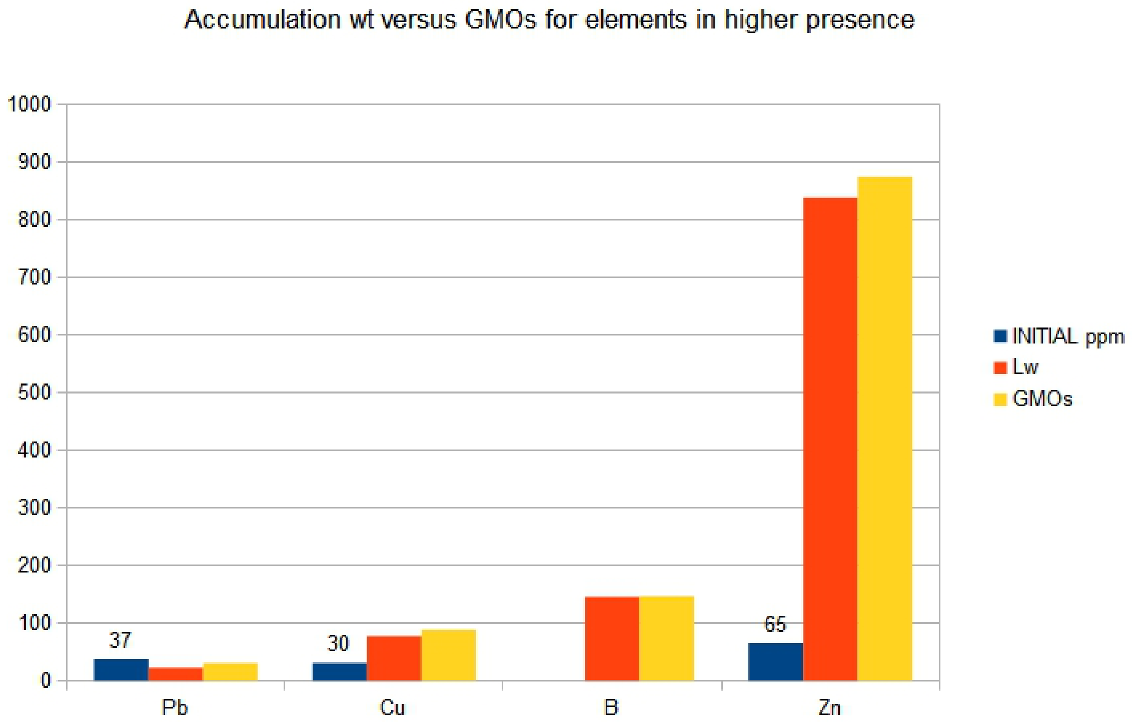


Figure 1C. Comparative accumulation wt (Lw) versus GMOs at tf, for 4 metals group having less concentration (except Cu) in plant. Fig 1D. Comparative accumulation wt versus GMOs, for the group of metals having higher concentration in plant. Pb is repeated in this group to better show differences among two groups. Standard deviation is showed for at least three individuals of each line as plotted in Figure 2 as total sum of different tissues analyzed. Here bars are eliminated to improve clarity.

compared to initial t0. Therefore although high flow of elements continues to leaves it is comparatively low. Consequently the root tissue due to a large accumulation factor becomes the limiting step for metal arrival to aerial

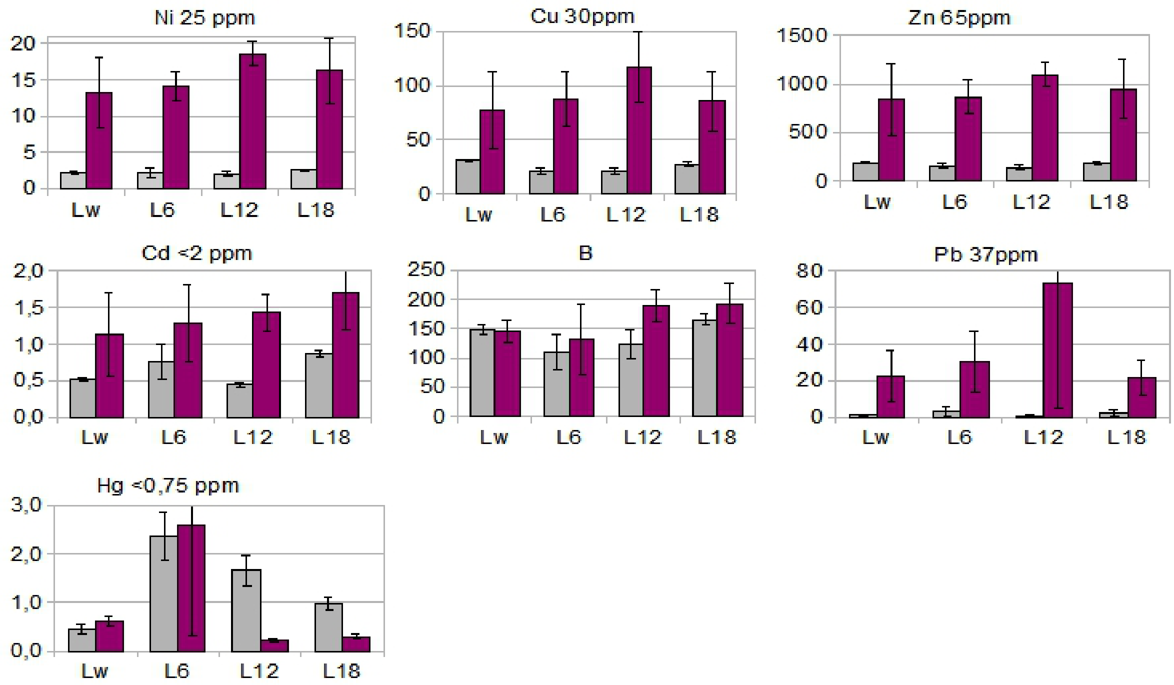


Figure 2. Total heavy metal and B accumulation at t0 and tf for wt and GMO specific lines. **Y axis indicate element concentration (ppm) at initial t0 and tf (bars next). X axis indicates specific group of plants.** Title shows initial concentration in the sludge. t0= 3 weeks; tf= 3 months. Bars represent average value and its corresponding standard deviation.

part of the plant. Understanding by "limiting step" the bottle neck where all the elements have to be gathered first, since it is not investigated whether there is any “malfunctioning” transport from roots to shoots or either it is just the regular kinetics for the plants in these specific conditions.

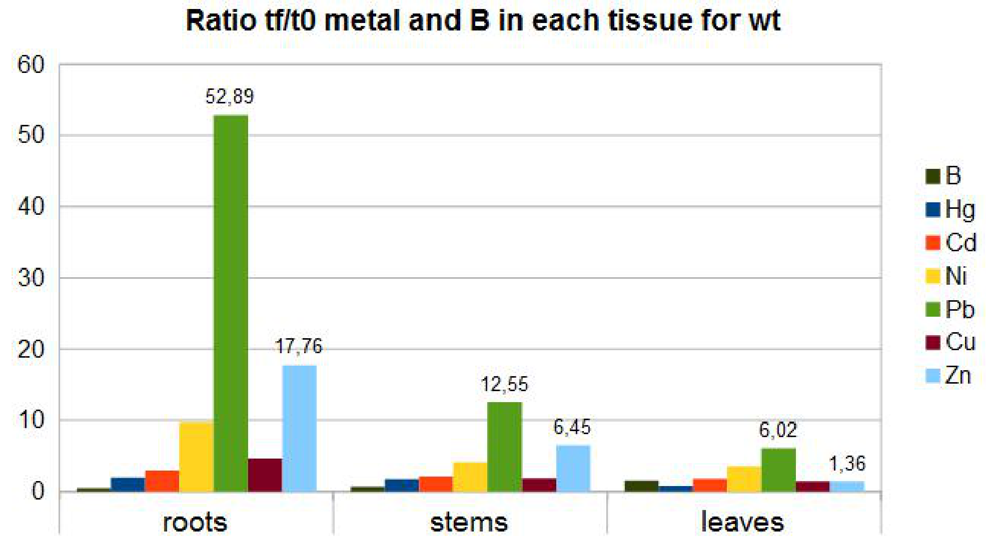
Discussion

This work aims to investigate at least three critical aspects. First, find out whether genotypes which were previously tested in mining soils (bearing physical, chemical and biological structure naturally formed), and showing extraordinary extractive power (Martínez *et al.*

Table 4. Ratio tf/t0 for wt and GMOs in different tissues. Ratio is a measure of incremented accumulation in plants. Therefore stands for a measure of average accumulation speed from one point to another, that is how in these specific conditions metals and B have incremented transport, regarding two group of species (GMO and wt). To take conclusions it is coherent to assume that the plant response will depend on how toxic is metal at the beginning and when toxicity is lowering during trial course (internal detoxification). Consequently speed can be lower when metal is per se less toxic for plant. Total, is a virtual ratio calculated just to compare.

Ratio of heavy metal and B accumulation tf/t0 for wt and GMO

tf/t0 GMO	Ni	Cu	Zn	B	Cd	Pb	Hg
roots	14.51	7,45	21,75	0,80	4,73	111.19	0.54
stems	4,14	1,86	6,54	0,84	1,89	7,89	0.79
leaves	4,20	2,66	1,94	1,88	1,53	3,33	0.7
total	22,85	11,97	30,23	3,52	8,15	122,38	1,80
tf/t0 wt	Ni	Cu	Zn	B	Cd	Pb	Hg
roots	9,73	4,62	17,76	0,47	2,90	52,89	1,91
stems	4,12	1,79	6,45	0.66	2,05	12,55	1,68
leaves	3,50	1,38	1,36	1,51	1,77	6,02	0.78
total	17,35	7,79	25,57	2,64	6,72	71,46	4,37



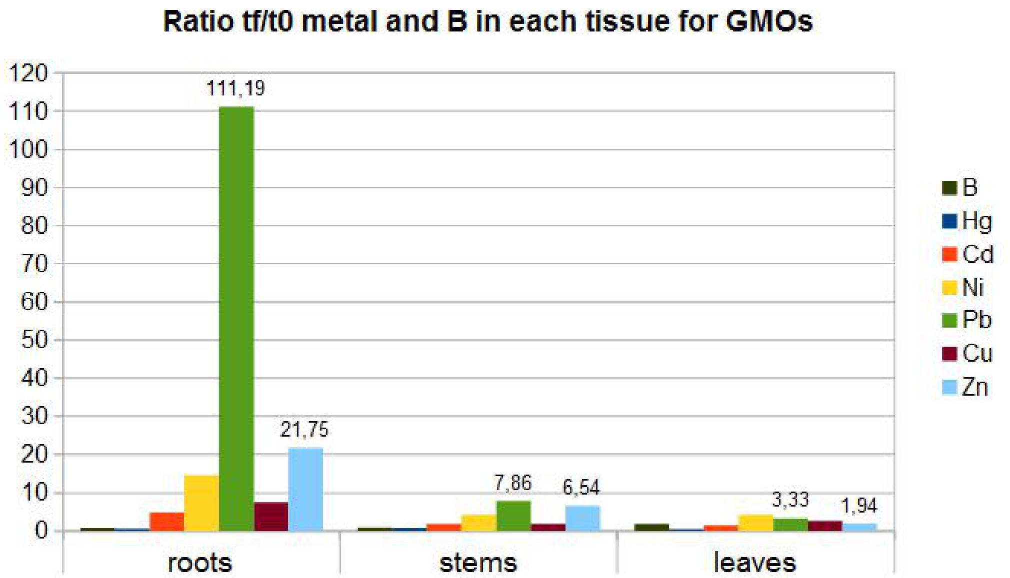


Figure 3. Tissue accumulation wt versus GMOs at t0 and tf.·**A** Ratio for wt. **B**. Ratio for GMOs. Y axis indicate ratio value. X axis indicates specific tissue (stems as a functional tissue).

2006) are also physiologically effective in an extremely different environment such as industrial sludge (artificial soils, no physical, chemical and biological structure, but dry matter). Second, given that tested species evidenced high phytoextraction capability at extremely high heavy metal concentrations (about 20,000 ppm) would they also show an efficient extraction in a substrate containing thousands of times smaller concentrations? In other words are those hyperaccumulator genotypes still useful at low heavy metal concentrations or will simply exclude them? Third, investigate accumulation time (kinetics) in order to achieve a better understanding of the speed at which these genotypes can clean up a contaminated sludge. Understanding by "decontamination" reducing industrial contaminant concentration from sludge to amounts safe enough for most living species under those circumstances in which they could possibly be present in Nature. Therefore, understanding decontamination beyond legal terms. In this way it is important to know whether the genotypes tested are candidates to be used commercially in main areas of decontamination, for instance: natural soils with high concentrations of metals (mining), contaminated soil (industrial or not) and industrial sludge.

Being the untransformed genotype a plant species which grows naturally in arid areas, including deserts, and whose individuals were chosen by Darwinian selection

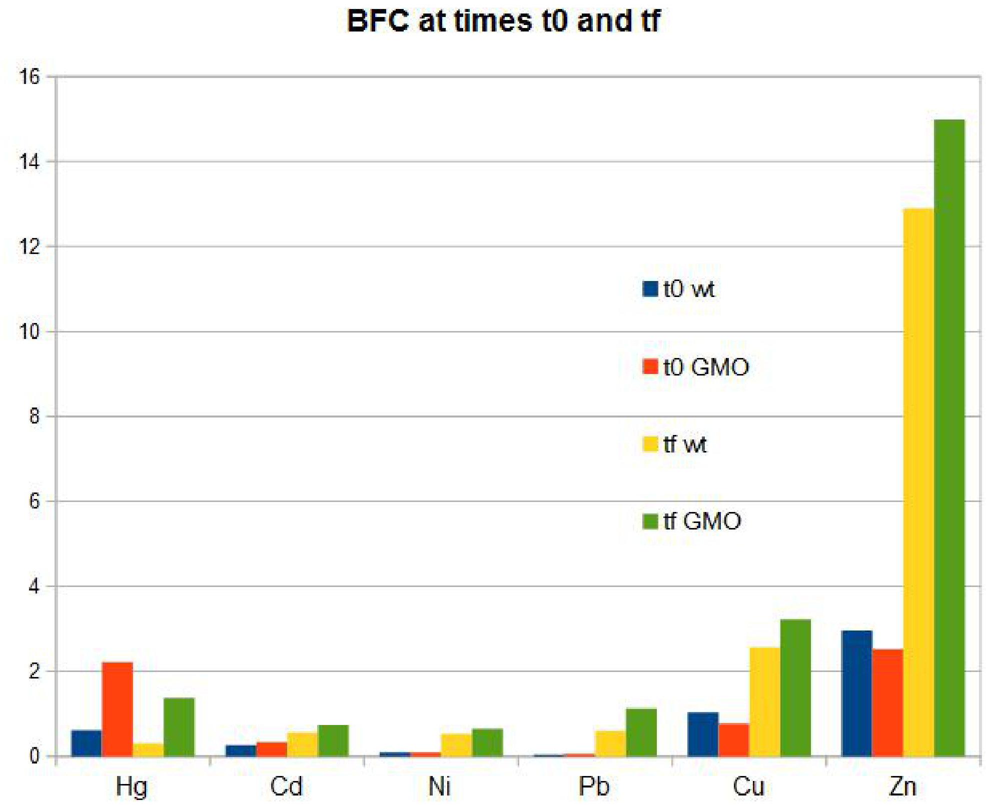


Figure 4. Power extraction in BCF terms. Each block compares, start and end times for a specific metal and for both groups of species (wt and GMO). Consequently indicating whether the metal decays in its accumulation or increases, comparing at same time wt versus GMOs.

(Gisbert *et al.* 2008) the tested genotypes in fact also survive and grow in industrial sludge which is a very different substrate respect to those tested in previous studies (for example, Gisbert *et al.* 2003; Martínez *et al.* 2006). Indeed different genotypes were grown in this case in media composed mainly by sludge extracted from a treatment plant of industrial sewage. Therefore results shown in this paper demonstrated usefulness of investigated genotypes also in sludge, answering positively to first question, but also to the second question. Sludge is slightly acidic, showing an organic matter (OM) of about 37% (Table 1) and small heavy metal concentrations, in which grown plants exhibit accumulation values consistent to results previously observed in different experiments, although as expected, lower in absolute numbers. For example it is observed, as in previous mining soils experiments (Gisbert *et al.* 2003; Martínez *et al.* 2006), a large storage capacity for metals such as Zn, Cu, Pb and Cd, keeping in mind that initial concentrations in sludge are much lower. Thus absolute values are not comparable.

Interestingly, currently tested genotypes in this work showed high heavy metal accumulation, even when initial concentrations were very small compared to those of mining soils. That is, if genotypes have a threshold response to start activating mechanisms which force accumulation then this threshold will be lower than those concentrations present in the sludge. Obviously GMOs have permanently activated expression of incorporated phytochelatin (*TaPCS1*) under 35S promoter control, but the collateral activations that this overexpression could involve are unknown. In this sense it is observed that tf/t0 accumulation ratio is greater than 1 in all cases, except for B and Hg in some tissues (Fig 2, Table 4). Probably because in these conditions, both elements do not represent a serious toxicity to the genotypes used at least along all trial. For the rest of heavy metals, an obvious multiplier effect is obtained.

The accumulation kinetics for a plant species, depends on several factors including (Navarro-Aviñó, Aguilar, and López-Moya 2007; Navarro-Aviñó, 2008; Navarro-Aviñó, Aguilar and López-Moya. 2008): media characteristics, suction force exerted by plant for each specific metal (engine of accumulation), initial metal concentration, time interval in which plant has been growing in contaminated media, and other metals presence, producing competence or benefits entrance in root (or by other tissue) for an specific metal.

Concentrations of each element in different tissues (Fig. 1A, B) indicate that plants behave as accumulators since, plant individuals, first produce a fast metal rise in leaves at t0. This means that metals undergo a rapid "suction" and a fast passage into plant aerial part, while at tf occurs greater accumulation in roots than in leaves. However at same time overall concentration in plants has increased in all tissues. This implies that at the end time (3 months), root concentration is higher than inside leaves, just the opposite to that observed at initial time. Nevertheless when study was conducted for the same species in mining soils (Martinez et al. 2006), plants, accumulated much more, in aerial part than in root tissue after 6 months growth (final time). One possible explanation might be not only a huge heavy metal initial concentration but also increased flow concentration to plant, which could remain higher during all assay development. This could be, among other facts, because the heavy metal gradient established from substrate to aerial part of plant, is much lower for plants grown in sludge, at all times, compared with the potential gradient, any time generated, in mining soils. In addition, metal concentration in sludge is therefore relatively less toxic to plant, while at the same time plants reduce the concentration of metal in sludge. Thus decreasing toxic effect means that for plant, maintaining an active remediation system which demands energy expenditure, would then become an energetically inefficient plant process.

From a practical point of view the most important goal was to determine whether GMOs are the best tools available in regard to wt (main global rival), and above all to know exactly the ability of tested genotypes to decontaminate sludge. In this regard it is remarkable that GMOs, consistent with previous work, produced a greater accumulation in virtually all cases (Fig 2). Besides, both wt and GMOs, multiply overall initial accumulation in plant at the end of the assay (Table 2). In the other hand, assuming BCF to be a good indicator of the ability to remediate a contaminated environment, BCF results greater than 1 in most cases, rendering unequivocal outcomes in this aspect (Table 3, Fig 4). Finally is observed greater GMO total accumulation compared to wt individuals in a way entirely consistent and coherent to outcomes obtained in previous experiments, regardless specific metal and initial concentration in sludge (Fig 1C, D; Table 2). Therefore collecting all facts in this work it can be concluded that there is probably no threshold in either case. There is no threshold concentration for plant species to activate the accumulation mechanisms at least in concentration range tested in this assay. There is also probably no threshold for showing GMO higher accumulation versus wt, at least in concentration range tested in this assay, and as far as time interval taken be long enough since Ni, Zn and Cu showed higher BCF only at t0 but not at tf (Table 3, Fig 4).

As a conclusion: the results presented in this report rise the answer that these GMO lines are able to keep a high rate of metal accumulation in soils containing metal concentrations that are at least one hundred times lesser than some of the ones tested in previous experiments. As final human health remark, this work establishes a plain confirmation of plants utility, for which these plants are also capable of removing heavy metals and other elements from sewage sludge. Heavy metals which are recently proposed, after carefully weighting and deeply analyzing a batch of clinical data collected all over the world, to be the direct cause of a variety of specific cancer types (Borrás, López-Moya, and Navarro-Aviñó 2008). Finally, fulfilling entirely the main objective raised in the Introduction section of going deeper into knowledge for evidencing a green alternative, the results showed in this work, coherently complement research previously carried out. Indeed these genotypes are also able to cope with organic contaminants as crude petroleum, gasoline, light gas oil, diesel, mixture of used car oils and detergents (Navarro-Aviñó, 2009; Navarro-Aviñó, 2012a). Besides GMO individuals were also able to cope for instance with high salinity (Mediterranean Sea concentration) better than wt individuals (Navarro-Aviñó, 2007), heavy metals in sludge from textile industry (containing industrial organics), radioactive Cs, and phytoremediation in aquatic media (Navarro-Aviñó, 2012b). Therefore to the authors knowledge this is possibly (as data suggest) the best tool capable and feasible (short enough in time) to remediate sewage sludge and highly contaminated soils and water, as well as many other pollutants, in a naturally integrated manner. In this manner completing the idea embodied in a project, back to year 2000 presented in Hannover Universal Exposition about the generation of “superplants” as a necessary tool to combat today's pollution. Consequently these type of “superplants” might be a real alternative contributing to have a better biosphere environment and a better human health, precisely in a very critical situation for health in Earth planet.

References

Aguilar I, López-Moya JR, Navarro-Aviñó JP. 2009. The enforceability of the phytoremediation in the world. In: Navarro-Aviñó JP eds. Phytoremediation: The Green Salvation of the World. Ontario (Canada): Research Singpost Press. p. 243-275.

Borrás R, López-Moya JR. Navarro-Aviñó JP. 2008. Health Implications: Trace Elements in Cancer. In: Prasad M. N. V., eds. Trace elements as contaminants and nutrients: consequences in ecosystems and human health. John Wiley & Sons, Inc. p. 495-521. http://dx.doi.org/ 10.1002/9780470370124.ch20

Gisbert C, Almela C, Vélez D, López-Moya JR, de Haro A, Montoro R, Serrano R, Navarro-Aviñó JP. 2008. Identification of As accumulation plant species growing on highly contaminated soils, International Journal of Phytoremediation 10: 185-196. http://dx.doi.org/10.1080/15226510801997457

Gisbert C, Ros R, de Haro A, Walker DJ, Bernal MP, Serrano R, Navarro-Aviñó JP. 2003. A plant genetically

modified that accumulates Pb is specially promising for phytoremediation, Biochemical and Biophysical Research Communications 303 (2): 440-445. http://dx.doi.org/10.1016/S0006-291X(03)00349-8
Dayan FE, Duke SO. 2014. Natural compounds as next-generation herbicides. Plant Physiol 166 (3): 1090-1105. http://dx.doi.org/10.1104/pp.114.239061
Martínez M, Bernal MP, Almela C, Vélez D, García-Agustín P, Serrano R, Navarro-Aviñó JP. 2006. An engineered plant that accumulates higher levels of heavy metals than *Thlaspi caerulescens*, with yields of 100 times more biomass in mine soils. Chemosphere 64 (3): 478-485. http://dx.doi.org/10.1016/j.chemosphere.2005.10.044
Murashige T, Skoog FA. 1962. Revised medium for rapid growth and bio-essays with tobacco tissue cultures. Physiol. Plant. 15: 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x
Navarro-Aviñó JP. 2007. Method for improving salinity tolerance. PCT/ES/2008/000662; US 20110126312A1.
Navarro-Aviñó JP. 2009. Método de recuperación de espacios degradados mediante uso de especies vegetales genéticamente modificadas. Method for the recovery of degraded areas using genetically modified plant species. Patent PCTES2009070080, US20120020736.
Navarro-Aviñó JP. 2012a. Fitorremediación intensificada de espacios degradados por sustancias tóxicas. Patent P201100381.
Navarro-Aviñó JP. 2012b. Método de recuperación integral de lagos naturales y espacios fluviales contaminados. P201200695.
Navarro-Aviñó JP. 2012b. Método de recuperación integral de lagos naturales y espacios fluviales contaminados. P201200695.
Navarro-Aviñó JP, Aguilar I, López-Moya JR. 2007. Aspectos bioquímicos y genéticos de la tolerancia y acumulación de metales pesados en plantas. Ecosistemas 16(2): 10-25.
Navarro-Aviñó JP. 2008. Toxicity and accumulation of heavy metals in plants. La toxicidad y la acumulación de los metales pesados en plantas. Lifescienceslab 1: 61-68.
Navarro-Aviñó JP, Aguilar I, López-Moya JR. 2008. Basic mechanisms of heavy metals accumulation in plants. In: Navarro-Aviñó JP eds. Phytoremediation: The Green Salvation of the World. Ontario (Canada): Research Signpost Press. p. 167-196. http://dx.doi.org/10.2174/978160805121210063
Salado R, Vencovsky D, Daly E, Zamparutti T, Palfrey R. 2010. Environmental, economic and social impacts of the use of sewage sludge on land. European Commission, Interim report DG Environment. First Interim Report No. DG ENV.G.4/ETU/2008/0076r.
Sánchez-Bayo F, 2011. Impacts of agricultural pesticides on terrestrial ecosystems. In: Sánchez-Bayo, F, van den Brink PJ, Mann RM, eds. Ecological Impacts of Toxic Chemicals. Bentham Science Publishers p. 63-77. http://dx.doi.org/10.2174/9781608051212110101
Schröder P, Navarro-Aviñó JP, Azaizeh H, Golan Goldhirsh A, DiGregorio S, Komives T, Langergraber G, Lenz A, Maestri E, Memon A, Ranalli A, Sebastiani L, Smrcek S, Vanek T, Vuillemier S, Wissing F. 2007. Using phytoremediation technologies to upgrade waste water treatment in Europe, Environmental Sciences and Pollution Reports 14(7): 490-497. http://dx.doi.org/10.1065/espr2006.12.373
Sciortino JA, Ravikumar R. 1999. Potential pollutants, their sources and their impacts. In: Sciortino JA, Ravikumar, R, eds. Fishery Harbour Manual on the Prevention of Pollution. Bay of Bengal Programme: FAO Corporate document repository. Fisheries and Aquaculture Department. BOBP/MAG/22.