Hyperaccumulation of lead using Agrostis tenuis

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Abstract

In recent years the quest for a circular economy approach and the upcycling of secondary raw materials have been pushed in the global political agenda. Increased interest has been taken by the recovery of materials from sludges, brines, contaminated waters and other media, such as "mining" of waste. Contaminated soils have an interesting role in this process, and various methodologies have been developed using chemical, bacteriological and pyrometallurgical cleaning procedures. However, these procedures all involve the movement of high volume of materials and the disruption of the industrial landscape; furthermore, they often require the use of hazardous solvents and high energy processes. This work proposes to identify less impactful methods aimed at the recovery of metals from mining areas while preserving the landscape and avoiding environmental impacts such as the increase of CO_2 for transport and increase hazard through use of solvents, this takes particular importance in areas of industrial heritage status. In particular, this work focuses on the use of *Agrostis tenuis*, an autochthonous species in mining areas of the UK, as a "mining tool" for the removal of lead. The selection of both the hyperaccumulator and the metal in this study are derived from the evaluation of the most common contamination in mining areas and the widespread prevalence of this hyperaccumulator and its resilience in highly contaminated abandoned mines. Aside from its presence within the mining areas, making it an autochthonous plant, *Agrostis tenuis* is selected for its visual morphology. Being a short grass, *Agrostis* does not change the visual appearance of the mining sites, most of which in the UK have a *historic landscape* status.

Introduction

Hyperaccumulator plants can accumulate high concentration of contaminants (especially metals) in their shoots without showing significant signs of toxicity (Ebbs et al., 1997; Thijs, Langill and Vangronsveld, 2017); the term was coined by Brooks and co (1977) to indicate plants that uptake large amounts of heavy metals from the soil, a behaviour contrary to that of excluder plants. In quantitative terms, the level of metal concentration achievable by hyperaccumulators is metal-dependent. A plant is classified as hyperaccumulator if it achieves: 10,000 mg/kg dry weight for Mn and Zn concentrations, 100 mg/kg dry weight for Cd, and 1,000 mg/kg dry weight for As, Cu, Co and Ni (Asad et al., 2019). Aside from the "high" metal concentration that these plants can achieve, another intrinsic feature is the ability to translocate the metals from their roots system to their shoots (Asad et al., 2019). The main characteristics of plant hyperaccumulators are detailed by Ernst (2005) as:

- a well branched root system;
- high transfer/efficient translocation from root to shoot;
- high capacity of metal accumulation in the aerial part of the plant without hindering growth;
- low transfer of the metals to the seeds of the plant.

These characteristics make hyperaccumulators the ideal candidates for phyto-remediation and even phyto-mining (Thijs, Langill and Vangronsveld, 2017). In particular, for phyto-mining, these plants remove metals from the abandoned substrates and concentrate them in the harvestable parts of plants. Overall, phyto-mining or phyto-extraction has been proposed as an environmentally friendly and low-cost technology for decreasing the heavy metal content of contaminated soils, creating an effective alternative to chemical/engineering based solutions (Ebbs et al., 1997; Lombi et al., 2003; Robinson et al., 2006; Asad et al., 2019). The hyperaccumulation capacity is considered selective, i.e. only one metal at a time can be hyperaccumulated by a specific plant hyperaccumulator. However, studies such as the one by Van Der Ent et al. (2018) indicated that some hyperaccumulators can intake high quantities of multiple metals simultaneously, with preference for one or the other depending on the pH of the soils.

This mechanism has been studied thoroughly for plants accumulating nickel, where approximately 350 taxa are known to accumulate between 1,000 and 38,000 mg/kg of dry leaf biomass (Reeves, 1992); Ni hyperaccumulators constitute 70% of the known hyperaccumulator species (Van Der Ent et al., 2018). However, not all metals have the same level of scientific interest and significantly fewer taxa have been identified for the hyperaccumulation of lead. Studies of herbaceous species growing in mining areas have indicated that lead accumulates 600 times more in some of the grass species (Yanquan *et al.*, 2005). In comparison, studies showing the accumulation in bushe-like plants indicated a much lower accumulation ability (Yanqun et al., 2004). In Wales, UK, *Agrostis tenuis* has been recorded in mining areas as resistant to lead and zinc poisoning (Bradshaw, 1952; Gregory and Bradshaw, 1965), particularly at the Welsh sites of Goginan mine and Parys Mountain – where the relative concentration of lead was estimated at 3,250 and 1,600 ppm respectively, this is in comparison for instance, with the 6,000 ppm of the mines in Yunnan (Robinson et al., 2006). *Agrostis* fits well with the characteristics of an hyperaccumulator, however its accumulation ability is limited to the first few centimetres of soil.

In the UK, around 200,000 sites have been identified as potentially at risk of soil contamination (Crane et al., 2017), but the problem is also recognised worldwide with an extent of 3.5 million sites identified as being potentially at risk of contamination in the EU and half a million recognised as highly contaminated and requiring remediation (Mahar et al., 2015), hence the challenge is wide spread with the levels and combination of contaminants being highly variable.

Examples of the level of contamination present at ancient mining sites are given by the assessments of the ancient mining activities in Wales where a lead concentration of 4 wt.% in the Frongoch and 0.9 wt.% in the Parys Mountain mine (Crane et al., 2017) was discovered.

Environmental Agency data (McGrath and Zhao, 2006) indicates a maximum concentration of lead in fine loamy sediments in close proximity to ancient metal mines reaching 16,338 ppm and averaging 3,500 ppm in coarse and fine silty sediments, fundamentally evidencing extensive diffusion of lead contamination due to water percolation through open adits and leachate from tailings: enriching the loamy and silty sediments of waterbeds in proximity of the mines. However, as these sediments have polymetallic contaminants, the recovery of the metals are extremely difficult using present methods (Ernst, 2005).

The literature indicates that 14 species of hyperaccumulators have been identified for lead (Mahar et al., 2015), with the four main species being: *Betula occidentalis* (1,000 mg/kg d wt) (Koptsik, 2014), *Brassica juncea* (10,300 mg/kg d wt) (Ernst, 2005), *Medicago sativa* (43,300 mg/kg d wt) (Ernst, 2005) and *Thlaspi rotundifolium* (8,200 mg/kg d wt)(Wenzel and Jockwer, 1999); however, it is always best practice to evaluate the presence of autochthonous species in abandoned mining areas to ensure the avoidance of environmental issues arising due to the use of exotic species becoming invasive in the long term (Li, 2006). For this study, the correspondence of lead contamination and the presence of grass hyperaccumulators and in particular *Agrostis* in areas such as the discharges at Cwmystwyth mine (Ceredigion, Wales) shows a potential route for the use of this common taxon for the phytoextraction of metals. *Agrostis* is known for the multi-selective accumulation of Cd, Cu, Mn, Ni, As, Pb and Zn (Gregory and Bradshaw, 1965; Li, 2006), a summary of the types of *Agrostis* species and the metals accumulated is presented in Table 1. In particular, *Agrostis* grown on Pb-rich soil shows the preferential formation of chloropyromorphite ($Pb_5(PO_4)_3Cl$) in the roots of the plant (Cotter-Howells, Champness and Charnock, 1999). The formation of this compound within the roots indicates a way forward for the stabilization of the metal contaminant in the soil and for this specific task *Agrostis tenuis* Sibth is already commercially available (Prasad and De Oliveira Freitas, 2003). Although other plants species like *Thlaspi alpestre* and *Minuartia verna* have been recorded as establishing successful populations on metal rich soils such as "the acidic [soils] in Central Wales and Snowdonia", the almost consistent proliferation of *Agrostis tenuis* increases its attractiveness for use in lead bioremediation (Jowett, 1964). The prevalence of *Agrostis tenuis* and *stolonifera* was also observed near the metal refineries at Prescot, where heavy metal aerial pollution and a Cu concentration in the soil of 4,000 ppm was detected. The *Agrostis* species were shown to evolve increasing metal tolerance by selecting for metal tolerant genotypes that could thrive. It was observed that although a large number of plant species were present around Prescot before pollution, the *Agrostis* species were the only genus able to develop the genetic variability required to survive in such heavily polluted conditions within a relatively short period of time (sometimes within a single generation) (Wu, Bradshaw and Thurman, 1975).

Overall, *Agrostis* shows great capabilities to adapt and increase its accumulation capacity under stress from soil pollution, exhibiting a major advantage as a versatile hyperaccumulator in soils displaying different metal mixing (association of metals) and metal concentrations (ratio and absolute concentration of the different metals) (Austruy, et al., 2013).

Furthermore, the direct formation of lead salts as a way to store and effectively avoid the toxicity of the metal by *Agrostis* implies that a simple removal method of the lead compound from the harvested biomass is feasible, indicating a route for effective removal of the contaminant from the soils. Of course, this route would be viable only if the salts are present in highest concentration in the harvestable shoots of the plants. This is specifically the focus of this paper and the results below can be used to indicate the level of concentration of lead in the shoots of commercially available *Agrostis tenuis* (as used in this study) and their composition with the aim of extracting metal salts (from the soil) and stabilising them within the plant. This could be a method not only for remediating soils contaminated by industrial and mining activities, but also tap into a secondary raw material source that can lessen the demand for virgin metals and the environmentally damaging activities linked to their sourcing.

In summary, *Agrostis* offers strong benefits as a versatile hyperaccumulator and includes environmental benefits for the preservation of the scenic historic landscapes where often ancient mines and abandoned industrial sites are located. The height and type of plant (generic grass) are non-invasive and supports the idea of preservation of the landscape character. Furthermore, *Agrostis* grows fast and it is easy to harvest using routine agricultural machinery, while allowing for fast removal of the metal-rich biomass for further treatment/use.

Table 1
Reference to Agrostis spp. as hyperaccumulator in the literature, indicating the type of Agrostis, the metal/s targeted and the type of experimental setting. When “Literature” is indicated it means that the authors did not perform an experiment but they are reporting previous data from the literature themselves.

Agrostis type	Metal extracted	Reference	Type of trial
Capillaris	Cd, Cu, Mn, Ni, Pb, Zn	(Ernst, 2005)	In situ
Castellana; Tenerrima	As	(Asad et al., 2019)	Literature
Tenuis; Stolonifera	Pb, Zn	(Li, 2006)	Literature
Stolinifera; Capillaris	As, Cd, Cu, Pb, Zn	(Pérez-de-Mora et al., 2006)	In situ; Containers
Spp.	As; Cd; Cu; Pb; Zn	(Boshoff et al., 2014)	In situ
Capillaris	Pb	(Rodríguez-Seijo et al., 2016)	In situ
Capillaris	As, Pb, Cu, Cd, Sb	(Austruy et al., 2013)	Containers
Spp.	Cu; Zn; Pb	(Mahar et al., 2015)	Literature
Tenuis	Zn; Pb	(Wenzel and Jockwer, 1999)	Literature
Capillaris; Canina	As; Cu; Pb; Sb	(Bech et al., 2016)	Literature
Durieui	Pb	(Fernández et al., 2017)	In-situ
Capillaris	Pb	(Miransari, 2011)	Literature
Capillaris	Pb	(Chaney et al., 1997)	In-situ
Capillaris	Pb	(Tangahu et al., 2011)	Containers
Capillaris; Gigantea	Cu	(Lange et al., 2017)	Literature

Materials And Methods

Agrostis tenuis commercially available seeds (REF 6-1836) were planted in off the shelve multi-purpose compost (Verve). The seedlings were kept within a controlled environment with a temperature of 22 ±

0.5°C using cycles of 12 hours of light (T5 light wave, 240V AC ~ 50Hz, 216W 1A) and 12 hours of darkness.

Two experiments were performed synchronously:

In experiment 1, 500 g of soil was prepared and 3 g of seeds were evenly distributed in the soil. 10 ml of 0.1 M of $Pb(NO_3)_2$ were administered every other day until the 50th day to monitor the plant growth. These plants were designated as Pb-CU-AT. 4 replicates of this experiment were running concurrently.

In experiment 2, 500 g of soil was prepared and enriched with 30 g of $Pb(NO_3)_2$, delivered via an acidified solution of the salt. 3 g of seeds were evenly distributed in the soil and the plant growth was monitored up to 50 days. The choice of 30g is to reflect the approximate concentration of Pb found in the mine of Cwmystwyth as analysed by the authors. These plants were designated as Pb-EN-AT. This experiment was done in 3 replicates.

A *blank experiment* was also run to ascertain the baseline behaviour in the absence of any external contamination. Here, 500 g of soil was prepared and seeded with 3 g of seeds. They were designated as AT.

In the course of the experiments, a portion of the plants were harvested after 20days to ascertain the Pb concentration in the aerial portion of the biomass.

Analyses of the harvested plants were carried out using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), X- Ray Fluorescence (XRF), Scanning Electron Microscopy coupled with Energy Dispersive X-Ray Spectroscopy (SEM-EDX), Transmission Electron Microscopy (TEM) and Fourier Transform Infra-Red spectroscopy (FTIR). The ICP-OES utilised is located within the facilities of the Institute of Health, Medicine and Environments (IHME) at Brunel University, while all the other analytical techniques utilised for this work are housed within the Experimental Techniques Centre in Brunel University.

Soil pH calculation was carried out using a Hanna Instrument ™ Hi 2550-02 pH benchtop meter. The methodology was a standard soil sampling procedure where 10 g of oven dried soil (333k) was mixed with 100 ml of deionised water (pH 7.4) and then allowed to sit for 30 minutes (Carter, Gregorich and Gregorich, 2007). The pH was then carried out after previously calibrating the pH meter with standard buffer solutions of pH 4, pH 7 and pH 10.

SEM analyses were performed using a Zeiss™ Supra VP35 Field Emission Gun SEM equipped with an EDAX Ametek™ Octane Super EDS detector. Imaging was done on the Smart-SEM software.

To carry out SEM analysis, small sections of the harvested plants were placed on an aluminium stub covered with conductive carbon tape. The samples were then sputter coated for 120 seconds with a thin layer of Au under vacuum using a Quorum™ sputter coater.

TEM-EDS analyses were performed to analyse the potential intracellular accumulation of Pb within *Agrostis tenuis*. The sample was prepared for TEM analysis using the following procedure: the harvested plant was first flash frozen in liquid nitrogen (≈ 77K) and then freeze dried for 12h at ≈ 218K using a ScanVac Coolsafe, afterwards the freeze-dried samples were cut into smaller sections and fixed with 5 mL of 2% glutaraldehyde solution for 12 h at 277K. The fixed biomass was then washed in deionised water 3 times before being immersed in a 5 mL 30% ethanol solution for 15 minutes. The biomass was then transferred to a 5 mL 50% ethanol solution, and then a 5mL 70% ethanol solution for a further 15 minutes, and finally a 100% ethanol solution for 30 minutes. The ethanol dehydrated biomass was then imbedded in a propylene oxide: agar Low viscosity resin (1:1) for 12 h. The mix was then exchanged for pure resin and polymerised in an oven at 333K for 24h. Ultrathin sections of the embedded samples were obtained using an RMC Power Tome PC ultramicrotome and then floated onto TEM copper grids.

The sections were examined using a Jeol 2100 TEM at 80kV, with energy dispersive X-Ray spectroscopy (EDS) carried out using a Thermo Scientific™ Ultra dry I detector. The software used for imaging was the Gatan microscopy suite® GM3 software, and for EDS analysis, the Thermo Scientific™ NSS EDX analysis software was used.

Elemental analysis using XRF was done. Before testing, samples were dried at 333K for 48h and then ground using a pestle and mortar. For soil matter, 10g of the dried sample was collected and mix thoroughly with 0.5ml of 10% PVA solution. The mixed sample was then loaded into an XRF pellet forming die. The die was then pressed with a pressure of 10 ton using a Specac hydraulic press. A solid pellet with a diameter of 30mm is formed which is then dried at 60^{o}C overnight. This sample is ready to be analysed by XRF.

For plant material, 0.5-2g of dried sample was used as it was.

XRF analysis was carried out using a Rigaku NEX DE benchtop XRF (60kV) in Helium. The prepared soil and plant samples were placed in XRF cups, backed by a mylar film, before undergo examination using a Fundamental analysis profile. Pre-calibration of the XRF was carried out using the reference calibration standard MCA R-2128. Results obtained were checked using ICP-OES analysis.

Similar to the XRF analysis, for ICP-OES, the samples were dried and then homogenised using a pestle and mortar. For soil matter, 10 mL of nitric acid (> 68%) was added to 0.5g of soil in a MARS microwave Xpress plus vessel (TFM with an inner Teflon lining). These samples then underwent digestion in a MARS microwave using a power setting of 1200-1800w, with a ramp time of 5 minutes and 30 seconds and a hold time of 4 minutes and 30 seconds, reaching a temperature of 175^{o}C. On completion of digestion, the samples were centrifuges for 10 minutes at 3000rpm.

For plant material (shoot), 10 mL of nitric acid (> 68%) was added to 0.5g of sample. These samples then underwent digestion in a MARS Xpress plus vessel using a power setting of 290-1800w, with a ramp time of 20 minutes and a hold time of 10 minutes, reaching a temperature of 200^{o}C. On completion of digestion, the samples were centrifuges for 10 minutes at 3000rpm.

The centrifuged samples were placed in a tube and diluted to a volume of 15 mL. Calibration using Pb metal standard solution diluted to 0.05, 0.1, 0.5, 1, 2, 5, 10 mg/L was carried to ensure $r^2 > 0.995$. Finally, sample analysis was done using the Perkin Elmer® Optima 5300DV ICP-OES.

FTIR analysis was used to analyse the chemical changes in *Agrostis tenuis* before and after treatment with Pb were carried out on the samples. The samples examined were dried in air overnight and analysed using a Perkin Elmer® spectrum one FTIR. A spectrum was collected in the range 3000cm^{-1} to 500cm^{-1} at 30 accumulations.

Results

Soil pH analysis

The pH values of the soil samples were found to be fairly acidic with figures of 4.8, 5.4 and 4.5 recorded for AT, Pb-CU-AT and Pb-EN-AT respectively. It appears that the addition of $Pb(NO_3)_2$ leads to some alteration in the pH properties of the soil which could directly be due to the expected acidic profile of lead nitrate salts that could in turn affect the soil microbiome, eliciting a biotic response.

Plant growth analysis

The growth of the plant was monitored by measuring the overall plant height. This involved measuring the visible portion of the plant shoot above the soil to the tallest part of the blade.

The growth progression of the plant sown on soil intentionally imbued with lead nitrate (Pb-EN-AT & Pb-CU-AT) and without any metal addition (AT) shows that there is a difference in the growth profile. It appears that without the inclusion of any contaminant, AT on average was able to reach a maximum height of 18 ± 2.0cm after 50days of growth. This was in contrast to Pb-CU-AT (14.6 ± 3.2 cm) and Pb-EN-AT (8 ± 3.0 cm), both of which showed a decrease in the maximum achievable height after a 50day period —the latter experiencing a drop of 57%, against a 22% of the former.

Aside from the height attainable being affected, the apparent toxicity of Pb is evident in the different germination and subsequent growth behaviour of the experiments as seen in Fig. 2. The inhibition in the germination of seeds can be witnessed when comparing the amount of plant cover between Pb-CU-AT and Pb-EN-AT. Although there is still growth in both instances, the plant cover of Pb-CU-AT is more robust than in Pb-EN-AT which is likely due to the different starting levels of Pb in the potting medium.

The tolerance indices (TI) – a measure of a plant’s ability to tolerate a given stressor– was calculated using the average length of the shoot, as the growth parameter, and were shown to both be < 1.

$$TI = \frac{[Growthparameter]Pbcontaminatedsoil}{[Growthparameter]Controlsoil}$$

Pb-CU-AT and Pb-EN-AT had values of 0.78 and 0.43 respectively. TI values < 1 are thought to indicate that the plant is stressed, thus experiencing a reduction in biomass.

Further observation following harvesting of only the aerial part and allowing the regrowth has yielded interesting results with regards to improved growth (Fig. 1b) and TI. The new mean shoot lengths for Pb-EN-AT and Pb-CU-AT are 20.0 ± 3.6 cm (previously 8 ± 3.0 cm) and 22.4 ± 2.0 cm (previously 14.6 ± 3.2 cm) respectively. Though the TI had previously returned values < 1, suggesting that both Pb-EN-AT and Pb-CU-AT had experienced a decrease in biomass on exposure to Pb, in this regrowth, there appears to be an improvement in the TI of the experiments: Pb-EN-AT (1.0) and Pb-CU-AT (1.1). This improvement in the tolerance seemingly within a 50day period is an excellent result and demonstrates the adaptability of *Agrostis tenuis* and consequently its suitability as a soil remedial tool.

SEM-EDS analysis

The morphology of the roots and shoot of the plants were imaged using SEM. The shoot of *Agrostis tenuis* shows a typical cuticular surface found in grass species, with regular folding having cuticular and epicuticular wax particulates scattered across its shoot façade. EDS analysis reveals a generally high carbon content, confirming their organic identity as possible waxes and roots exudates. Additionally present are Mg, Si, P, S, Cl and K, all of which are not out of place in a plant undergoing normal metabolic functions.

Variations in the external characteristics of both the roots and shoots of Pb-EN-AT and Pb-CU-AT appear to be almost visually absent. The roots system, however, does seem to exhibit some disparity with the density of root hairs. We see foreign particulates scattered across its surface (highlighted by the red boxes in Fig. 3b&d) – despite the initial rinsing of the roots. Some of these sits on the surface while others appear to be more intimately bonded to its surface. Elemental analysis shows that these particulate matters contain a significant amount of lead as part of their composition (Fig. 3f).

This leads one to suspect that the particulates present on the root structure of Pb-EN-AT are formed as a direct result of the interaction with lead ions. Similarly, in Pb-CU-AT, more particulates are found on the roots (Fig. 3d) meaning that the precipitation of lead containing compounds are uniform between Pb-CU-AT and Pb-EN-AT, thus reinforcing the extensive role the rhizosphere plays in triggering this soil remediation process. On the shoots, we also see Pb bearing particulates (Fig. 3e).
Closer analysis of the EDS appears to show that Pb is incorporated into a matrix different from the form it was delivered as — $Pb(NO_3)_2$. In particular, lead-bearing particulates detected also embedded in the shoots show a composition corresponding to chloropyromorphite.

TEM-EDS analysis

Small sections of *Agrostis tenuis* were examined after extensive preparatory measures detailed in the methodology section. The TEM micrographs (Fig. 4a & b) confirms the accretive ability of *Agrostis tenuis* when exposed to heavy metal contaminants. In this case, we can see the sequestering of lead as dark contrast regions within the cellular structure of *Agrostis tenuis*. One can see quasi-spherical lead nanoparticles lying next to the cell wall and what appears to be the actual cystol of the cell.

Closer inspection of these particulates show that they are elongated, a phenomenon also witnessed in *Salvinia minima*, primarily in the lead particles found in the plant cystol. Elemental analysis, using EDS, confirms the presence of Pb and other potential elements that co-precipitate with it.
The signals from Si, C, O (naturally present) and Cu (from the TEM grid) can be discounted. This would leave P, Pb, Cl and Ca as the relevant elements to consider as making up the composition of the particles accumulated within the plant cell. These particles show a size of < 30 nm and their elemental composition established by EDS analysis can be seen as a positive identification of the precipitation of the mineral chloropyromorphite as the ideal lead stabilising form. This also supports the EDS analysis of the particulate seen in Fig. 3. The presence of the calcium recorded could be due to the formation of calcian pyromorphite.

Elemental analysis using XRF

From the results (Fig. 5), it appears that the storing potential of the plants are quite obvious. Interestingly, the uptake abilities showed variation between the samples, Pb-CU-AT and Pb-EN-AT. Whilst one of the samples, Pb-EN-AT, started with the highest amount of Pb enrichment, the amount concentrated in the shoot was several orders of magnitude lower than the sample that started with a low but increasing amount of Pb added to it (Pb-CU-AT) indicating the ability of adjusting to a changing environment.
To evaluate the phytoremediation potential and suitability of *Agrostis tenuis* for use as a hyperaccumulator, the bioaccumulation coefficient (BAC) was calculated

$$BAC = \frac{Concentration in plant (shoot)}{Concentration in soil}$$

Plants having a BAC of > 1 are thought to be better adapted for phytoextraction of metal contaminants to within their plant tissue.

After the first harvest, the experiments indicate a BAC value > 1 in the AT (1.09), while the BAC values for Pb-CU-AT and Pb-EN-AT are calculated at 0.3 and 0.002, respectively. However, at the second harvest Pb-CU-AT's BAC double to 0.59, while Pb-EN-AT increases one order of magnitude to 0.01. Despite both values still being below 1, this increase indicates a quick ability of Agrostis to increase its accumulation capacity.

FTIR analysis

The most prominent bands across all of the FTIR spectra seen in Fig. 6 are those that could be ascribed to the presence of the cellular wall structure and a robust plant cuticular layer. With regards to the plants that had been fed on a diet consisting of lead (Pb-CU-AT and Pb-EN-AT) and on a lead-free diet (AT), very little perturbation of the chemical bonds appears to have occurred.

Discussion

From the results obtained thus far, it is apparent that *Agrostis tenuis* is not only able to survive in the presence of heavy metal contamination but is in fact able to successfully thrive. Starting with the height profile of *Agrostis tenuis* grown in the absence of Pb (AT) against those grown in a Pb medium (Pb-CU-AT and Pb-EN-AT), one of the limitations known to hyperaccumulators, i.e., a lag in growth rate becomes quite obvious (Tangahu et al., 2011). This is a common feature noted amongst hyperaccumulators and indeed in plants in general that have been exposed to heavy metals above a certain threshold. Toxic effects such as: a reduction in the population of useful soil microorganisms that would normally assist in the breaking down of organic matter to release essential nutrient for plant growth, a substitution of essential cations with heavy metals, inhibition of enzymic activities and proliferation of damages rendered by the increase in oxidative stress due to the presence of the heavy metals could all account for inhibition in the growth rate witnessed in Pb-CU-AT and Pb-EN-AT and are all perfectly within previously observed behavioural pattern (Van Ginneken et al., 2007; Chibuike and Obiora, 2014). It seems as though the issue of the lagging growth rate might have less to do with the soil chemistry, at least in terms of the pH which appeared to show that both the plant grown in a soil free of lead and that grown with lead had similar values. It appears that the initial concentration of lead in the soil is the most important factor impacting growth; thus, while the Pb-CU-AT experiment shows plants ability to adapt in spite of the continuously increasing pollution, growing at almost the same rate as within the clean compost, Pb-EN-AT shows a lower germination rate, and also a limited final height. Indeed, this inhibition of germination in plants exposed to lead, in addition to other traits like roots elongation, chlorophyll production, seedling development, cell division etc, are just some of the characteristic traits that show the toxic aftermath from exposure to heavy metal contaminants (Amin et al., 2018). However, this effect appears to be mitigated within the second cycle of growth (see Fig. 1b) which confirms the high adaptability of this plant to stressful conditions.

Additionally, the appearance of lead-based deposits within the plant structure satisfies the phytoextraction function of using *Agrostis tenuis* and confirms its suitable for the decontamination of Pb polluted sites. From the particles within the cellular structure of *Agrostis tenuis* (Fig. 4), one can see that there appears to be a preference for lead storage closer to the plant cell wall. This is in contrast to what was seen in the case of the plant species *Salvinia minima* (Castro-Longoria et al., 2014), where *Salvinia minima* was shown to accumulate more lead within the cell wall structure (middle lamella, primary and secondary wall) and less in the actual cystol of the cell. In the case of *Aspergillus* species, it was thought that this preference for accumulation in the cell wall was due to an ionic interaction; the negative factions present in the cell wall (carboxylate groups) acted to attract the positively charged lead ions to the cell wall structure (Pavani, Sunil Kumar and Sangameswaran, 2012). Although accumulation in the cell wall is one of the many mechanism plants have to cope with heavy metal stress, usually its first defence, aggregation in the cystol or rather within a vacuole does appear to occur (Fig. 3; Fig. 4a & b) and is thought to be linked to the presence of phytochelatins (Estrella-Gómez et al., 2009; Fahr et al., 2013; Castro-Longoria et al., 2014). The existence of these lead-based deposits (Fig. 3 and Fig. 4) in the shoot means that *Agrostis tenuis* was able to take up Pb via its root system and translocate it to its aerial portion, thus satisfying the description of phytoextraction, which is “the uptake/absorption of contaminants by plants into the above ground portion of the plants (shoots) that can be harvested …” (Tangahu et al., 2011). The movement to the aerial portion, a trait beneficial to the soil clean-up process, is likely assisted by an evapotranspiration process which again is thought to be more enhanced in hyperaccumulator due to their ability to maintain a metal “shoot-to-root concentration” of above one (Tangahu et al., 2011), though as we have seen this condition is not fully achieved within one life cycle of the plant. SEM showed that there were significant Pb containing particulate primarily on the roots (Fig. 3), this appears to be quite similar to what occurred in *Phyllostachys pubescens* (Liu et al., 2015) and *Eichhornia crassipes* (Baruah, Hazarika and Sarma, no date), both of which showed morphological magnification of the uptake and localization of Pb in the root and shoot system. The appearance of these inclusion both in the roots and shoots in the aforementioned instances and within *Agrostis tenuis* shows that the plants are able to store this metal extracellularly (Fig. 3) and intracellularly (Fig. 4).

Elemental analysis further substantiates the accumulating abilities of the *Agrostis tenuis* used in this study. While there does appear to be some latent Pb content in the starting soil AT (< 3.2 μg/g), the amount remains lower than the mean concentration of Pb found in urban soils in the UK (110 mg/kg [μg/g]), from soils in London gardens (654 mg/kg[μg/g]) and also lower than the proposed safe levels (600 mg/kg) in areas frequented by children (Madhavan, Rosenman and Shehata, 1989; Ross et al., 2007). The same goes for the plant tissue, which here has been evaluated as having a value (3.5 μg/g) that is lower than mean urban herbage values for the UK (8.6 mg/kg[μg/g]).

Furthermore, the ability of the plants to store in multiple locations might have caused some underestimation in the results (Fig. 5), as only the shoot was used to ascertain the amount of Pb in the biomass. From electron microscopy (Fig. 3), we are able to see lead bearing particulates in/on the roots indicating that not all lead was readily bio-available and one part of the accumulated lead was still stored in the roots and not yet translocated in the shoots; more than one growth cycle of the plant might be required to obtain a full translocation. Indeed, the case of Phyllostachys pubescens (Liu et al., 2015) showed that the calculated bioaccumulating factor was significantly augmented by the higher amount of lead accumulated in the roots. The same could be said for water hyacinth where the lead content in the roots were higher than in the petioles or leaves (Malar et al., 2016). The indications gathered by these experiments mean that in one life cycle *Agrostis tenuis* accumulates Pb when subjected to low concentration in the soil (AT), and adapts to higher concentrations when these are slowly increased (Pb-CU-AT) although it has a slower start when the initial concentration is high (Pb-EN-AT). Regardless, the added benefit of leaving the root without harvesting is to ease the regeneration of the plants after the aerial portion has been harvested, thus continuing the remediation process and potentially increasing it through time since the adaptability of the plant is already noticeable within one cycle, as seen after comparing Pb-EN-AT and Pb-CU-AT. The improvement in the TI for Pb-EN-AT and Pb-CU-AT from < 1 to > 1 in the second growth cycle, shows the development of tolerance in the presence of Pb contamination (Audet and Charest, 2007; Amin et al., 2018). This improvement is also highlighted in the BAC values for both Pb-EN-AT and Pb-CU-AT which both underwent very significant improvements after the first harvest. It is highly probable that the high concentration in the soil of Pb-EN-AT is not readily bioavailable within the first cycle of growth hence limiting the accumulation ability of the plant. This could be confirmed by the high volume of salt particles visible surrounding the roots of the plants for both Pb-CU-AT and Pb-EN-AT in Fig. 3. While the Pb-bearing particles visible in the figure are changing their nature from nitrate to a more bio-available form, they do not have enough time to be accumulated by the plants. Further cycles of accumulation need to be trialled and examined in order to correctly establish the BAC of the plants under evaluation, and this can be established by investigating plants growing naturally in polluted sites such as Agrostis capillaris L exposed to Pb pollution due to shooting activities (Rodríguez-Seijo et al., 2016), where after examination of 13 different sites it was found that 5 of the sites exhibited BAC values > 1, with 9 sites showing phytostabilising abilities as indicated by a bioconcentration factor (BCF) also > 1. Even though the phytoextraction abilities of *Agrostis capillaris L* exceeds that of the samples studied here, the initial amount of Pb present in the soil needs to be considered. Rodríguez-Seijo et al (2016) found Pb soil concentration of 402–724 mg/kg (µg/g), due to shooting activities, these are significantly smaller than those utilised in this paper (> 9,697µg/g). Thus, while the samples used here display BAC values that would lump them in a category as moderate accumulator plants (Gawryluk, Wyłupek and Wolański, 2020), the reality is that the excessive amount of soil Pb (in our paper) when compared to Pb content in literature implies that the actual performance of *Agrostis tenuis*, here, moves it quite comfortably from a moderate tier to an above average tier.

The process of metal accumulation within *Agrostis tenuis* appears to involve the transformation of the intentionally inoculated lead salt, $Pb(NO_3)_2$, into the less bioavailable form pyromorphite, which is incidentally one of the more stable forms of lead salts with a solubility (K_{sp}) of -84.4 and a slightly higher solubility of >-76.8 when Cl is exchanged for -OH and -F (Traina and Laperche, 1999). Amongst some of the other common means of lead remediation of contaminated sites, one method of remediation is the intentional addition of phosphate to amend the soil. The premise behind this process involves the use of a source of phosphate (e.g. rock phosphate, phosphoric acid, apatite etc.) to form stable lead phosphate phases, reducing free Pb. Usually, the rapid and preferential formation of pyromorphite above all other types of phosphates occurs due to thermodynamics. The higher stability of pyromorphite in comparison to other lead phosphate phases (e.g. plumbogummite and tsumebite) results in the eventual transformation into pyromorphite (Ryan et al., 2001; Rhee, Hillier and Gadd, 2012) or calcian pyromorphite seen in Charterhouse mine (Kampf, Steele and Jenkins, 2006). Thus, the formation of pyromorphite in mine-waste, though preferred, does not readily occur unless with the presence of an ample source of phosphorus – specifically in the form of phosphates. As we have demonstrated, *Agrostis tenuis* grown without the additional seeding of the soils with phosphate has been shown to capture quite a significant amount of Pb in the form of chloropyromorphite. It helps that *Agrostis* plant roots are capable of exuding phosphatase enzymes that are capable of converting organic phosphorus to phosphates (within their rhizosphere) which can then be taken up by the plant; also, the presence of soil organism around the rhizosphere is capable of converting organic phosphorus to phosphates (Cotter-Howells and Caporn, 1996). All of this makes the use of *Agrostis tenuis* quite self-sufficient, or at least without the problem of intentionally adding phosphates which normally involves prior acidification of the soil to promote the dissolution of Pb and P sources; this could inadvertently cause the leaching of other metal contaminants in the soil (Rhee, Hillier and Gadd, 2012).

As long as lead is not just sequestered but housed in a relatively stable form (pyromorphite), half of the problem of having Pb in a bioavailable format within the soil is eliminated; the transformation through the plant exuding phosphatase enzymes requires time, which is confirmed by the fact that the *Agrostis* can cope with an incremental addition of aliquots of lead salts (Pb-CU-AT), while it has a slower reaction to a larger amount of lead salt being present directly in the soil (Pb-EN-AT).

Conclusion

The literature confirms *Agrostis* spp. as a highly adaptable hyperaccumulator in a variety of metal combinations but particularly associated with the accumulation of lead. This study focuses on the evaluation of the behaviour of *Agrostis tenuis* as lead hyperaccumulator, specifically on the speed of adaptability to high lead concentration and how and where the lead-bearing particles are stored in the plant. In order to achieve these objects, three experiments were performed on *Agrostis tenuis* in the following conditions:

- [AT] the plant was sown and grown on a commercially available multi-purpose compost;
- [Pb-CU-AT] 10ml of 0.1 M of $Pb(NO_3)_2$ was added to the soil on alternating days for a 50day growth period;
- [Pb-EN-AT] 30 g of $Pb(NO_3)_2$ was added to the soil on the sowing day for 50day growth period.

Overall, the pH of the three soils were similar: AT (4.8), Pb-CU-AT (5.4) and Pb-EN-AT (4.5). They are well within the range of healthy growth of *Agrostis* spp. We observe a slight acidification for Pb-EN-AT due to the addition of the lead nitrate salt in an acidic solution to the soil; an increase in pH is visible in Pb-CU-AT potentially due to the reaction of the plants to the incremental addition of lead nitrate in the acidic solution triggering phosphatase enzymatic reaction.

The growth of the plants shows (Fig. 1a) a lagging effect in the high lead concentration soil of Pb-EN-AT, while Pb-CU-AT and AT show a similar growing curve. The elevated adaptability of *Agrostis tenuis* is visible in the second growth cycle (Fig. 1b) here the curve becomes significantly similar, especially for Pb-EN-AT.

Lead bearing nanoparticles (~ 30 nm) are visible through TEM analyses (Fig. 4) in the shoots of Pb-EN-AT and Pb-CU-AT, indicating the ability to translocate lead-bearing particles from the roots to the shoots, storing them close to the cellular walls in a chloropyromorphite form (Cotter-Howells, Champness and Charnock, 1999) – this mineral form is less bio-available and can be present as a non-hazardous compound within the plant itself (Ryan et al., 2001; Rhee, Hillier and Gadd, 2012).

The evaluation of the quantity of lead sequestrated in the shoots of the plants within a first harvest cycle and the consequent BAC calculated shows a very interesting scenario. While in the low concentration AT (control) experiment, the plants accumulate all the available lead, in Pb-CU-AT and Pb-EN-AT. Three phenomena can explain these data that seem to indicate a very low capacity of phytoextraction by *Agrostis*:

- the plant needs time to adapt to the stressful environment,
- the plant needs time to transform the lead in the soil to a bioavailable form in order to sequestrate it,
- the plant needs time to translocate the metal to the shoots.

These phenomena seem to take place all at the same time and the experiments conducted thus far are unable to indicate the most predominant amongst the aforementioned occurrences. Where aliquots of lead nitrate are added to the soil (Pb-CU-AT), the plant’s enzymes react by generating phosphates to transform the nitrate into the bioavailable phosphate form, this is visible in the change of nature of the lead-bearing particles in close proximity to the root system (Fig. 3f). Observing the lead-bearing particles, in close proximity to the root, we can see that a larger volume is present in the Pb-EN-AT (Fig. 3d), indicating that the plant is attempting to gather the lead; the transformation rate is slow in comparison with the overall lead concentration. This appeared to have been resolved within the second regrowth cycle (Fig. 1b), where a growth similar to Pb-CU-AT was witnessed, indicating the improved capability to cope in the high metal stress environment. The data points towards the ease of adaptability of this species, *Agrostis tenuis*, and consequently its importance as a hyperaccumulator in different environments.

Declarations

Ethics approval and consent to participate We confirm that no ethical approval was required for the topic treated in this paper Consent for publication We confirm that all authors consent to the publication of this paper which has not been sent elsewhere for publication Availability of data and materials All the data and materials discussed in this paper are owned by the authors Competing interests The authors confirm that there are no competing interests in the publication of this paper Funding No external funding were used to produce the data presented in this paper Authors' contributions Lorna Anguilano (corresponding author) and Uchechukwu Onwukwe have produced the data and the manuscript text. Aghis Dekhli, Susanna Venditti, Danny Aryani have produced part of the experiments of hyperaccumulation. Alan Reynolds has provided expertise on types of hyperaccumulator and methodology. Acknowledgments The authors acknowledge the support of Dr. Ashley Howkins of the Experimental Techniques Centre (ETC). The authors likewise express their gratitude for the help conferred by Ms. Nicola Beresford, Mr. James Clayton and Ms. Marta Straszkiewicz from the Institute of Health, Medicine and Environments (IHME) of Brunel University. The authors also acknowledge the commercialisation department within Brunel Research Support and Development Office for funding part of this project.

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Figures

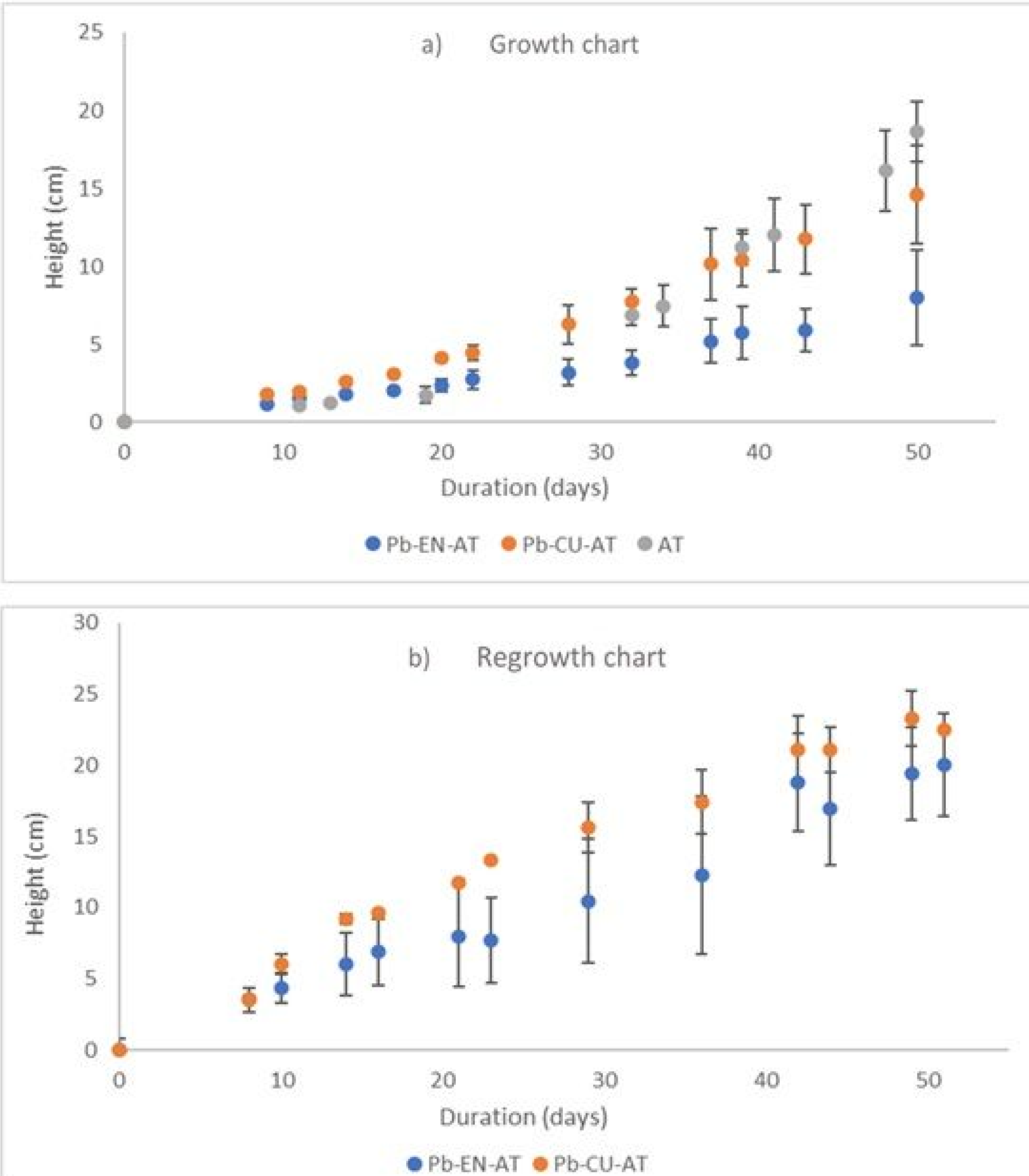

Figure 1

Growth chart of Pb-EN-AT, Pb-CU-AT and AT over a 50day a) and a regrowth chart of Pb-EN-AT and Pb-CU-AT, after 1st harvest, covering a 50day period b).

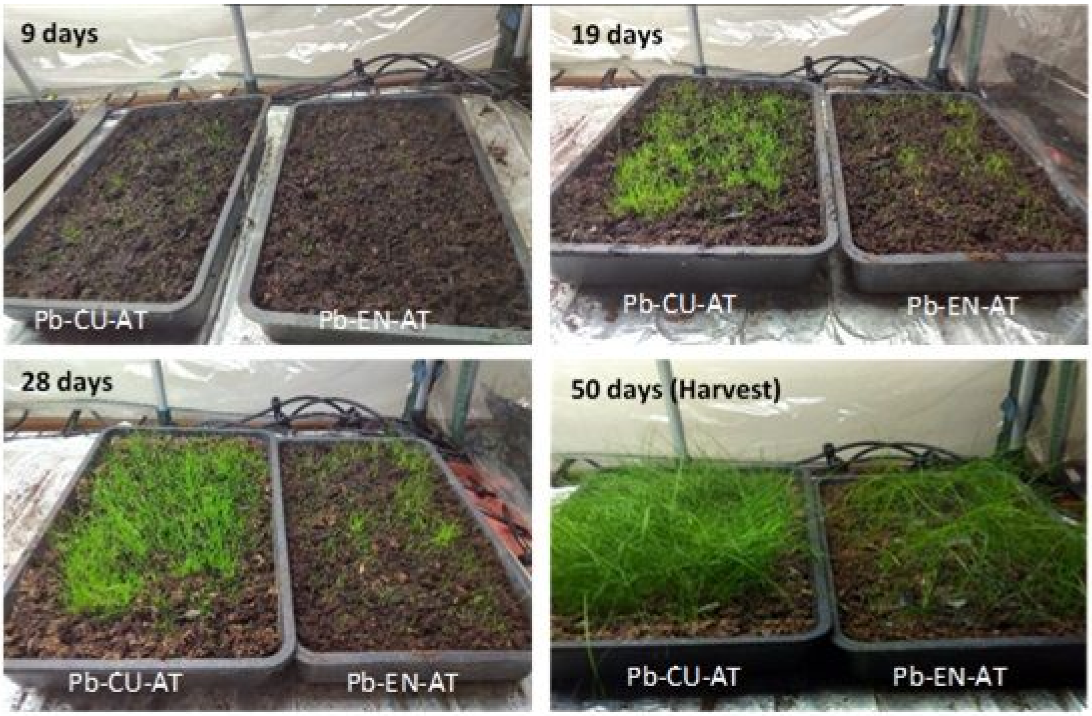

Figure 2

Pictures showing the shoot growth progress of experiment 1(Pb-CU-AT) and experiment 2 (Pb-EN-AT) over 50 days

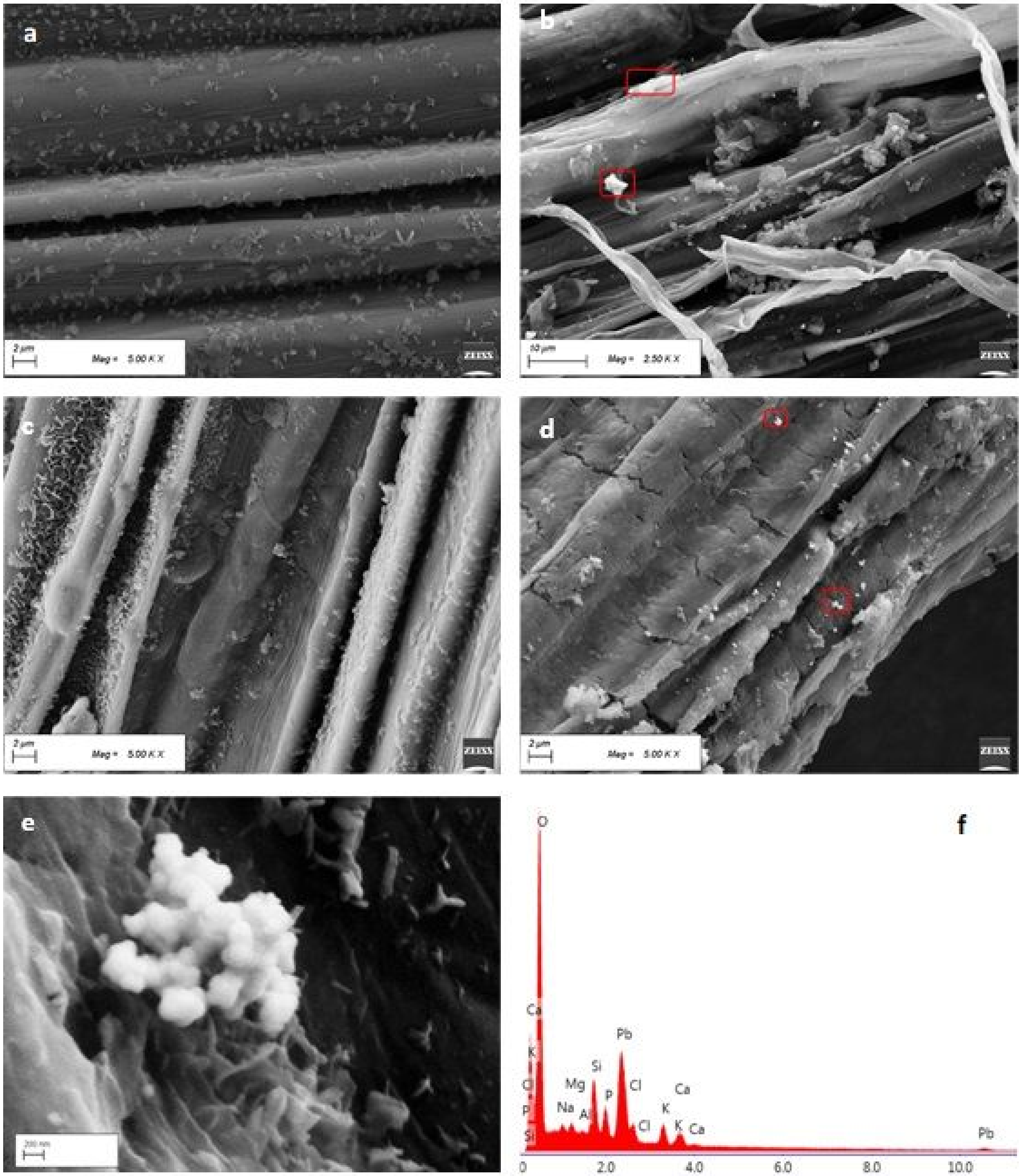

Figure 3

SEM micrographs showing the surface of the shoots (left) and roots (right) of Pb-CU-AT (a&b) and Pb-EN-AT (c&d), with a typical EDS showing the presence of Pb from the highlighted particulates in the micrographs (f); a close up of the Pb bearing particulate is seen in (e)

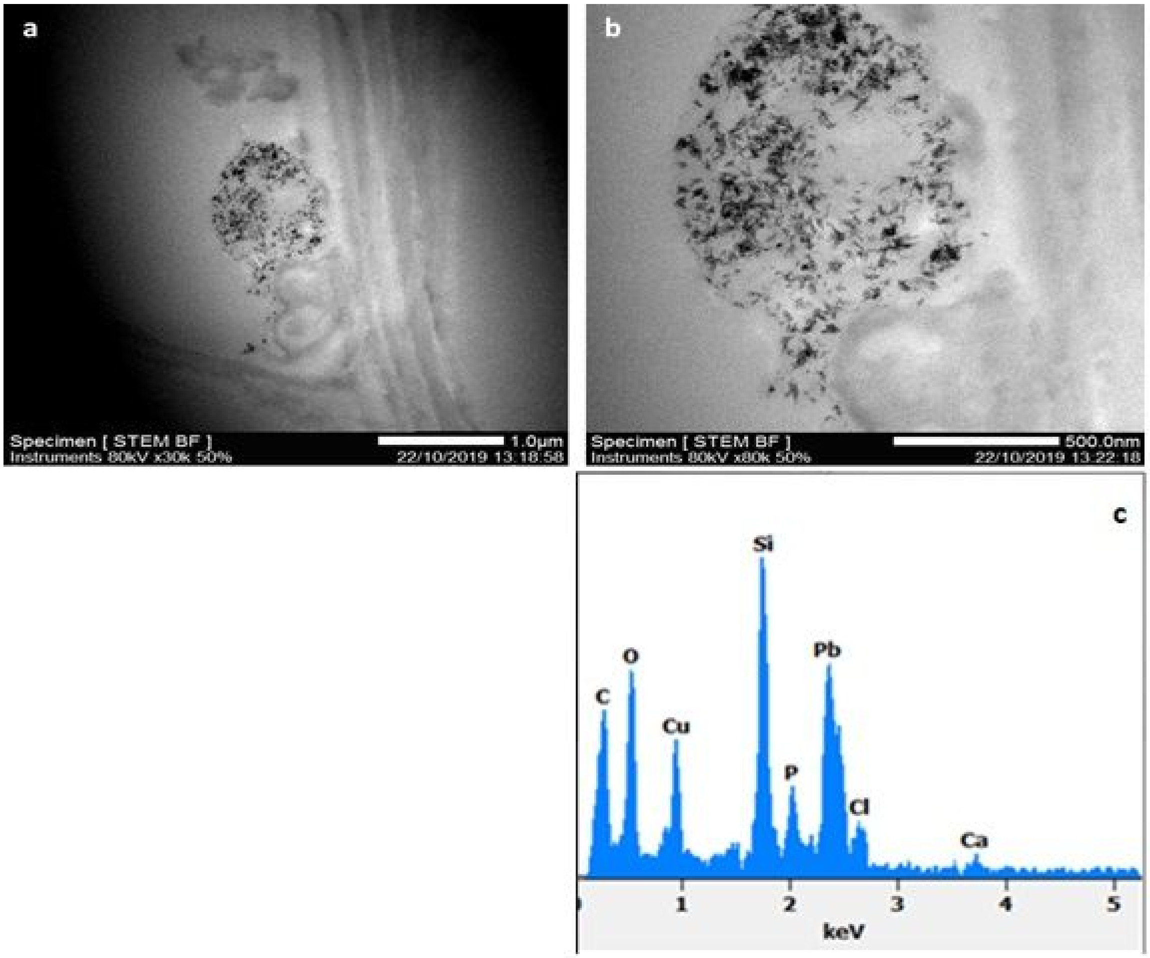

Figure 4

TEM micrographs of the shoot section from the Agrostis tenuis plant exposed to lead nitrate during growth. (a -c). Sections of Pb-AT showing internal localisation of lead nanoparticles close to the plant cell wall; EDS spectrum of the selected area from (b) is shown in (c)

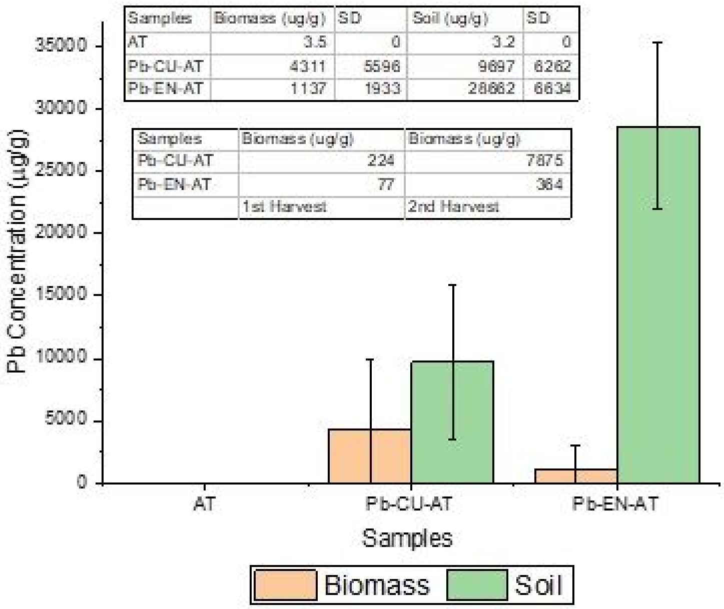

Figure 5

Bar chart showing the overall elemental analysis result of AT, Pb-CU-AT and Pb-EN-AT after sampling in the 20day period. Inset, table of values of the overall elemental analysis result(top) and a second table showing the values across the 1st and 2nd harvest cycle (below).

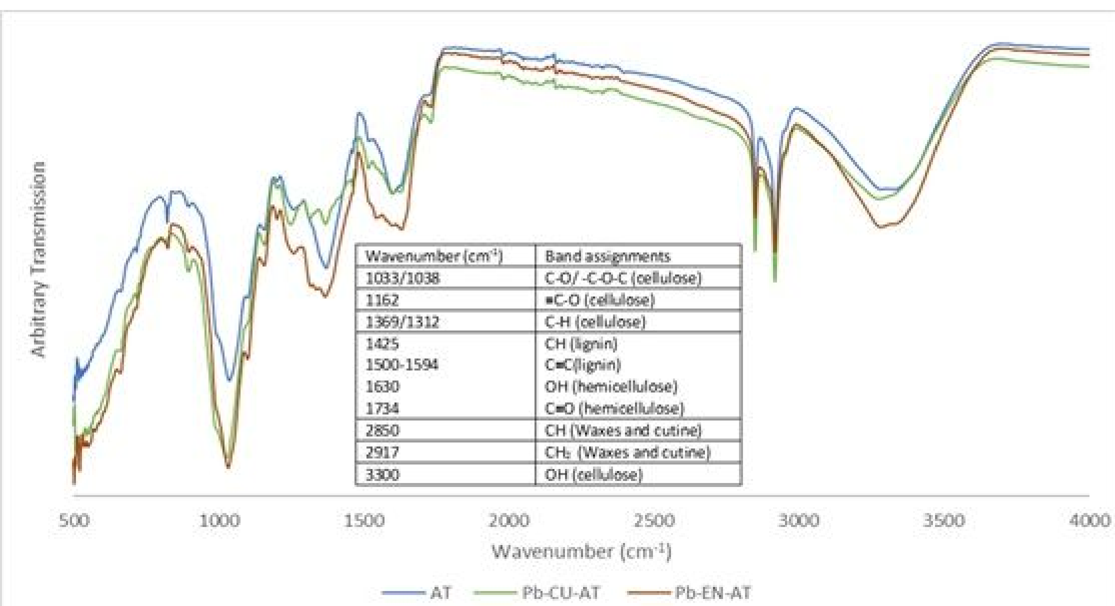

Figure 6

FTIR spectra of the shoot section of AT, Pb-CU-AT and Pb-EN-AT with inset of infrared band assignments (Alonso-Simón et al., 2011; Traoré, Kaal and Martínez Cortizas, 2018; Chakradhari et al., 2019)