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Potential for the Accumulation of PTEs in the Biomass of *Melilotus albus* Med. Used for Biomethane Production

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Abstract: In this paper, a possible use of white sweet clover (*Melilotus albus* Med.) for phytoremediation was assessed. The plants were grown on soils with naturally occurring concentrations of potentially toxic elements (PTEs). First, the content of PTEs was determined in plant biomass and in soil samples using: (a) Optical emission spectrometry with inductively coupled plasma to determine Sb, As, Cd, Cu, Ni, Pb, and Se, and (b) thermal decomposition, amalgamation, and atomic absorption spectrometry to determine Hg. The effectiveness of *Melilotus albus* Med. (*M. albus*) for phytoremediation was evaluated using the bioconcentration factor (BCF). The phytoextraction potential of *M. albus* was determined using bioaccumulation factor (BAC) and translocation factor (TF) values. The highest concentration of PTEs in roots was detected for zinc (10.56 mg/kg of dry weight, DW) and copper (5.128 mg/kg of DW). Similarly, the highest concentration in above-ground parts of the plant was detected for zinc and copper (12.638 and 4.0 mg/kg of DW, respectively). Although the values were relatively high, the effectiveness of the absorption of these PTEs by plant biomass from the soil was relatively very low. BAC and BCF were always lower than 1. On the other hand, the results suggested that *M. albus* effectively transports PTEs (only for Zn, Pb and Hg) from roots to shoots, because TF was always higher than 1. However, the accumulation of PTEs from soils with a natural abundance of PTEs was not excessive in comparison to conventional maize silage. Therefore, there is no potential risk of biomethane production in biogas plants when biomass from *M. albus* is used.

Keywords: white sweet clover; phytoremediation; phytoextraction; potentially toxic elements; pollution; heavy metals; bioaccumulation; industrial pollution; biomethane production; anaerobic digestion

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1. Introduction

There is currently a risk of farmland being contaminated with potentially toxic elements (PTEs) from various sources, e.g., from the chemical and engineering industries [1,2]. These substances represent a serious world-wide ecological problem with a negative effect on soil–plant ecosystems [3]. PTEs can be defined as a huge group of chemical substances (components of pesticides and fertilizers, including elements such as As, Cd, Pb, etc.). PTEs cause abiotic stress, which reduces plant growth. Some of the most significant PTEs are heavy metals (HM; Table A1) [1]. There is, however, a difference in the bioavailability and persistence of individual PTEs. If we, for example, compare some heavy metals and their compounds exhibiting toxicity with residues of active substances of pesticides, then differences can be found in their disintegration and mobility within the soil environment [1,2]. Organically based PTEs can be more easily incorporated into plant and animal biomass, for example. On the other hand, HMs (As, Cd, Pb, and Zn) and their compounds exhibit a longer half-life. Moreover, HM ions are characterized by limited mobility in the soils, primarily due to binding to soil particles [1–3]. The soil is contaminated with PTEs due to anthropogenic activities such as mining and metallurgical industries, agriculture, combustion processes, etc. [4,5]. High concentrations of PTEs are highly toxic and are considered as environment pollutants. PTEs that are taken for pollutants include As, Cu, Cd, Pb, Cr, Ni, Hg, and Zn [6,7]. Plants uptake PTEs, similarly to nutrients, mainly by their roots from the soil [3]. The uptake of PTEs by plants has no linear dependence on the total concentration of PTEs in the soil, but rather on their availability to plants, and differs in various plant species [6,8,9]. These PTEs stored in plant biomass may further enter the food chain and adversely affect the health of humans and animals. For example, Pb and Hg are absorbed from plant biomass in the digestive tract of mammals and further released into the bloodstream. Pb negatively affects the hematopoietic system (anemia occurs) and nervous system. Lead also causes renal disorders and liver dysfunction [9]. Hg binds to sulfanyl groups, hence uncompetitively inhibiting a number of enzymes. The danger of PTEs resides in the risk of their accumulation in tissues and of possible poisoning by repeated accumulation [8,9].

PTEs are accumulated particularly in roots while a much smaller amount is transported into the above-ground parts. Various plant species differ in their capacity of PTE accumulation, which is affected by root morphology [10]. Plants with a high number of fine roots can take up and accumulate a much greater amount of PTEs than plants with large-diameter roots. Heavy metals originating from non-natural sources represent abiotic stressors [11,12]. Certain types of plants are able to cope with the stress caused by the increased concentration of heavy metals in the soil by the process of phytoremediation [1,10–12]. Phytoremediation makes use of the notable ability of plants to concentrate elements and compounds from the environment and to metabolize various molecules in the plant tissues [12]. This process has a potential to eliminate pollutants from the environment [12]. According to Salt et al. [13], phytoremediation methods can be divided into categories such as phytoextraction, phytodegradation, rhizofiltration, phytostabilization, phytovolatilization, and rhizodegradation. In this study, the potential of *Melilotus albus* Med. for phytoextraction of PTE is researched. Phytoextraction is a method that is based on the accumulation of the molecules of heavy metals in the above-ground parts and sprouts of plants [14,15]. Figure 1 shows the individual phytoremediation methods and the significance of processes in the plants for their realization [14].

Hyperaccumulators represent a separate chapter. Hyperaccumulators are plants that can accumulate PTEs in their live tissues at levels which can be a hundred or thousand times higher than is common for a majority of plant species [16]. In hyperaccumulators, the process takes place without any influence on their growth and development [16,17]. Hyperaccumulators are able to translocate xenobiothics into aerial parts of the plant, so that the accumulation in shoots is higher than that in roots [16,17]. The concentration of PTEs in the shoots should also be higher than their concentration in the soil [17].

It has been shown that wastes from agricultural production can be used in biogas plants [18], which can ensure material reuse, energy recovery, and control of greenhouse gas emissions. The use of mixed cropping systems is nowadays very promising. These systems increase diversity, bring better protection against weeds, and reduce the loss of water from agricultural soils [19,20]. Moreover, the biomass from mixed cropping systems is suitable for biogas production [19,20]. The use of two or more crops in one cropping system may have a higher phytoremediation effect, which can subsequently influence the outcome of biogas production. Moreover, the use of various plant materials in anaerobic digestion can significantly affect the composition and utilization of digestate [21]. Supplementation with the resultant digestate resulted in increased yield and nutritional composition of various grown crops in further cultivation [21–23]. Therefore, harvesting technologies have recently been intensively studied [24].

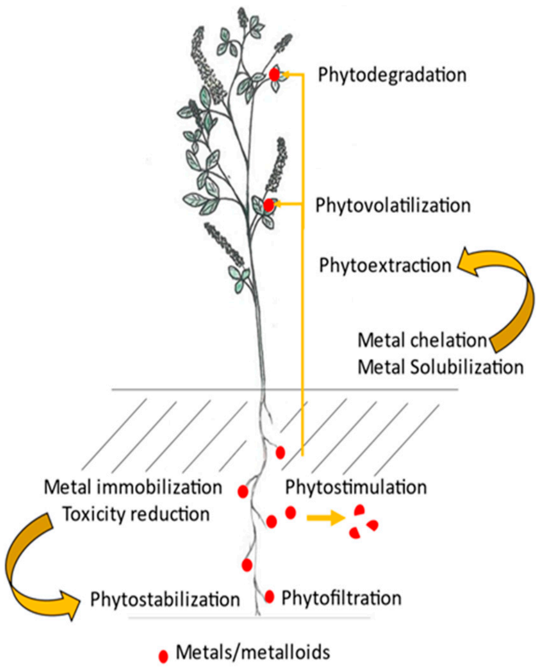

Figure 1. Processes used in phytoremediation of heavy metals—modified according to Ojuederie et al. [14].

However, reutilization of plants after the phytoremediation of PTEs can pose a risk of these pollutants being released back into the environment due to the decomposition of plant residues [14,16]. When these plant residues are used as a forage, PTEs can accumulate in the food chain and become a risk to human health. Therefore, many technologies for the removal of plant residues are used, such as pyrolysis [25]. Biochar from pyrolysis can be used as a sorbent of dyes or in agriculture [26]. Recently, the production of biomethane from phytoremediation of plant residues has been researched. This method may provide a possible compensation for the costly phytoremediation process [27]. In this paper, the possible effectiveness of white sweet clover for accumulation of PTEs (potential inhibitors of biomethane production) is assessed. White sweet clover is a legume, which means that it is able to assimilate nitrogen, thanks to the symbiosis with Rhizobium sp. microbes. Using legumes on arable land results in increased content of N in the soil through biological fixation [18–20]. This effect leads to the reduced need for mineral fertilizers. The reduced application of mineral N fertilizers is in line with the European Green Deal, which expects that mineral N fertilizers will be reduced by 20% by 2030 [28].

White sweet clover (*Melilotus albus* Med.) contains coumarin, a secondary metabolite that plays a role in defense against pathogens. There are also indications that coumarins are important in Fe deficiency responses and even in the induced systemic resistance of plants [29]. Coumarines have a negative impact on anaerobic digestion if the biomass containing them is used for biogas production [30]. This causes lower production of methane during anaerobic digestion. However, Popp et al. [31] found that feeding the biogas reactor microbiome with coumarin-rich feedstocks while maintaining coumarin concentrations below 0.5 g/L allows microbes to adapt to coumarins through structural and functional community reorganization and coumarin degradation [21].

The main goal of this study was to evaluate the ability of phytoextraction of various PTEs, using the tested crop *Melilotus albus* Med., Meba, on soils with naturally abundant PTEs. The partial goal was to find out whether, in the case of excessive accumulation of PTEs in the plant biomass, this biomass can be potentially toxic for further use, for example in biogas or silage production. We tested the following hypothesis: growing crops on agricultural soils exposed to excessive farming may result in the accumulation of PTEs in plant biomass with further harmful potential in the subsequent use of the biomass.

2. Materials and Methods

2.1. Sampling Area, Plant and Soil Sampling Procedure

The experiments were conducted on sites (Figure 2) with no direct addition of PTEs in 2019. The plots were in Vatín (49°31′03.3″ N 15°58′31.9″ E) and Troubsko (49°10′14.8″ N 16°29′50.2″ E). The two sites belong to research field stations; Vatín is under management of Mendel University in Brno and Troubsko is managed by Agricultural Research Ltd. White sweet clover (*M. albus*) was sown in an amount of 12.0 kg/ha, at a depth of 0.02 m, i.e., 5.85 million individuals/ha. The sowing rate was based on Rigal et al. [32]. Plants were not treated with any pesticide. When the adult white sweet clover was 1 year old, four samples of aerial biomass and four samples of underground biomass were collected at the beginning of butonization (formation of flower buds) at BBCH 51 [33]. Soil samples of 100 g in weight were then collected from four places on the plot, and were taken to the laboratory for the determination of some parameters of aerial biomass, underground biomass, and soil.

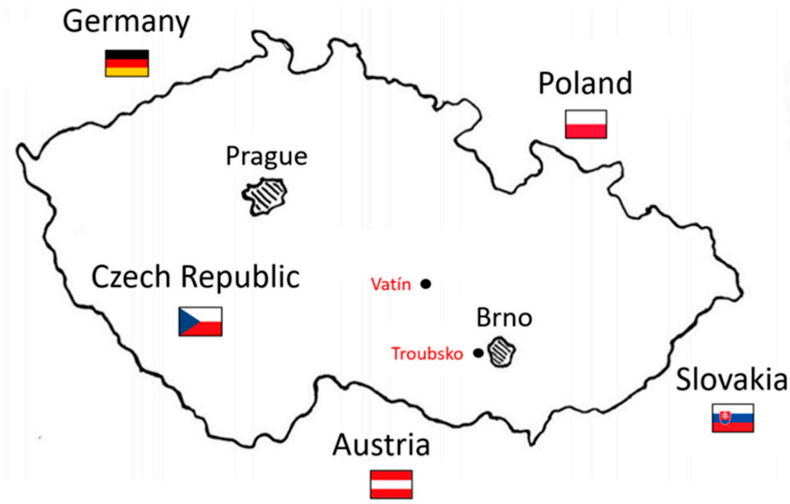

Figure 2. Localization of experimental sites in the Czech Republic.

Experimental plots in Vatín were located on Cambisol sandy loam, in the central part of Bohemian–Moravian Highland. Climatological parameters were as follows: a mean annual temperature of 7 °C and mean annual total precipitation amount of 658 mm (long-term mean 1981–2010), and the av. altitude was 540 m a.s.l. Experimental plots in Troubsko were located on Haplic Luvisol in the South Moravia region with an average altitude of 287 m a.s.l., mean annual temperature 8.9 °C, and total precipitation 525 mm (long term mean 1981–2010).

2.2. Determination of HM in Plant and Soil Samples, Data Processing, and Statistical Analysis

The selected parameters to be determined in the samples also included the concentration of Sb, As, Cd, Cu, Ni, Pb, Hg, Se, and Zn in mg/kg. These heavy metals (HM) were chosen because of their potential phytotoxic effect and negative impact on the process of methanogenesis. There is a danger that HM contained in plant biomass could be cumulated in the biogas plant fermentor where methanogenesis takes place. The analyses of plant and soil samples were made according to methods stipulated in ČSN EN ISO 11885 [34] for Sb, As, Cd, Cu, Ni, Pb, and Se, and ČSN 75,7440 [35] for determination of Hg. The determination of Sb, As, Cu, Cd, Ni, and Pb was carried out by optical emission spectrometry with inductively coupled plasma (ICP-OES), and determination of Hg was performed using thermal decomposition, amalgamation, and atomic absorption spectrometry.

The obtained data served to complete the calculation of the values of bioconcentration factor (BCF; Equation (1)), translocation factor (TF; Equation (2)), and bioaccumulation coefficient (BAC; Equation (3)) according to Ghazaryan et al. [1] and Amin et al. [36]:

$$\text{BCF} = \text{Concentration of element in root/soil,} \quad (1)$$

$$\text{TF} = \text{Concentration of element in shoot/root,} \quad (2)$$

$$\text{BAC} = \text{Concentration of element in shoot/soil,} \quad (3)$$

Exploratory data analysis according to Wilcox [37] and Janiga [38] was used in the assessment of measured data of respective parameters to verify homogeneity and normality of gathered data. The potential differences in HM concentration of plant and soil samples were analyzed using Statistica 12 (StatSoft, Dell, Round Rock, TX, USA) software. One-way ANOVA in combination with post hoc Tukey's HSD test was used to analyze the above differences. Differences between the individual groups of data were analyzed using a paired *t*-test. All statistical analyses were performed at level of significance $p < 0.05$.

3. Results and Discussion

3.1. Concentration of PTEs in the Soil and Plant Biomass

Table 1 shows that the highest PTE concentrations in the aerial biomass were those of zinc (average 12.638 mg/kg) and copper (average 4.0 mg/kg). As is the case with other microelements, Cu is to a certain extent beneficial to plants and animals but its high concentrations cause damage to cells [3,39,40]. Cu is essential for the vital functions of organs, as no physiological and biochemical processes can take place without it. In animals, these processes are necessary for the formation of pigments, elastin, and collagen. Cu also helps the transfer of iron from liver to bone marrow by which it participates in the process of hematopoesis [41]. One serious sign of a lack of Cu in animals, combined with other factors, is osteoporosis. This is a systemic disease of the skeleton, characterized by the focal or generalized loss of bone tissue with the ratio between organic and inorganic skeleton matter remaining preserved [42]. In plants, Cu represents an essential heavy metal, i.e., a microelement which most physiological processes could not do without. Cu in plants is a part of proteins, and is indispensable for the transfer of electrons and hence for the processes of photosynthesis and cellular respiration [3,8,43]. Plants can absorb it from the soil while humans and animals receive it in food [43]. Mikalajune et al. [44] experimented with red clover on plots contaminated with Cu and found that clover was able to absorb 4 mg/kg after two months, 12 mg/kg after four months, and 23 mg/kg after six months of being grown on the contaminated soil. The authors further tried to grow red clover on the soil contaminated with Zn. In this case, they found that clover absorbed 19 mg/kg after two months, 39 mg/kg after four months, and 47 mg/kg after six months of being grown on the contaminated soil. When we compare these values of Cu and Zn concentrations with values measured in the current research study (Table 1), *M. albus* apparently exhibited markedly lower Cu and Zn concentrations in its biomass than red clover did. In contrast, the lowest HM concentration in the aerial biomass was observed in Hg, which reached on average 0.003 mg/kg. The second lowest concentration was detected in Cd (0.075 mg/kg).

Table 1. Determination of selected PTEs in aerial biomass, underground biomass and in the soil (mg/kg).

	Shoot	**Root**	**Soil**
Sb	0.55 ± 0.08 c	0.60 ± 0.25 b	n.d.
As	0.62 ± 0.09 c	0.85 ± 0.14 b*	6.98 ± 0.73 c
Cd	0.08 ± 0.02 d	0.11 ± 0.04 c	n.d.
Cu	4 ± 0.54 b	5.13 ± 0.75 a	20.5 ± 1.31 b
Ni	0.53 ± 0.15 c	0.73 ± 0.17 b	19.43 ± 0.25 b
Pb	0.69 ± 0.28 c	0.66 ± 0.38 b	13.49 ± 6.155 b
Hg	0.003 ± 0.001 e	0.002 ± 0.001 d	0.042 ± 0.003 d
Se	0.45 ± 0.189 d	0.29 ± 0.199 b	n.d.
Zn	12.64 ± 3.34 a	10.56 ± 6.06 a	78.07 ± 5.09 a

Data represent mean ± standard deviation over at least 5 replications. Different letters indicate significant differences in the concentration of individual HMs in soil (ANOVA, $p < 0.05$, post hoc Tukey's HSD test). Symbol * indicates significant differences between HM concentrations in shoots and roots (pair *t*-test, $p < 0.05$). n.d. = not detected.

It follows from Table 1 that the highest concentration of microelement in the underground biomass was that of Zn (av. 10.560 mg/kg). Other high concentrations were those of Cu (av. 5.128 mg/kg) and arsenic (av. 0.848 mg/kg). Ghazaryan et al. [4] informed that in sweet yellow clover (*Melilotus officinalis*), higher concentrations of Cu intensively accumulate in roots and lower concentrations are transported to the aerial biomass. However, conclusions of their study are not fully in line with the data (Table 1) presented in the experiment because the concentration of PTEs was demonstrably higher in the root compared to that in the shoot only in *M. albus*, whereas *M. officinalis* probably deposited PTEs in its biomass evenly. The lowest concentration in the underground biomass was that of mercury (av. 0.002 mg/kg), and very low concentrations were observed also of cadmium (0.105 mg/kg). The difference between the highest concentration of zinc and the lowest concentration of mercury was more than 10.5 mg/kg. However, when we further compare the measured concentrations of HM in the plant biomass and in the soil with available permissible limits of HM concentration in water, soil, and plants according to WHO and FAO (Table A2), we find out that the concentration of measured HM was higher than limits for drinking water; this was expected as the limits are very strict. On the other hand, the soil content of HMs was slightly higher (by ca. 10–20%) in our experiment than the Hg and Zn limits set up by WHO. This indicates that the soil content of PTEs was slightly increased; the reason for this however was not found. When shoots and roots were compared separately, the contents of most HMs in plant biomass did not exceed the limits. Only in the case of Zn was the limit value of 0.6 mg/kg exceeded, which also had to do with the increased Zn content in the soil as mentioned above.

It further follows from Table 1 that the concentrations of monitored heavy metals differed in the shoot and root biomass. Concentrations of Zn, Se, Hg, and Pb were higher in the shoot biomass than in the root biomass. However, these differences were not significant (Table 1). Thus, the values indicate that *M. albus* stored PTEs in shoot and root biomass relatively evenly, and was not able to transport a significant amount of PTEs from root to shoot. This may be related to the characteristics of legumes which are generally not considered as crops suitable for phytoremediation [45]. It can be also noted that concentrations of Ni, Cu, Cd, As, and Sb were higher in the root biomass than in the shoot biomass. A significant difference was recorded only in the case of As. It can be deduced from this that in the white sweet clover, some of the monitored PTEs were transported more either into the shoot biomass or into the root biomass. Amounts of heavy metals in the soil environment are shown in Table 1. Eid et al. [46] studied the phytoremediation potential of nine native plants from Egypt growing in a sewage sludge dump in the Nile River delta. They confirmed that the ability of plants to accumulate PTEs highly depends on the plant species and on pollution in the location. Similar results were obtained by Vaverkova et al. [47], who studied the phytoremediation potential of various plants growing on landfills, in the landfill surroundings, or compost plants. The extent of PTE accumulation in *Cordus* shoots was dependent on the location and on the distance from the source of pollution (surrounding the landfill). Therefore, there is an assumption that the model plant (*M. albus*) does not exhibit increased capacity for taking up and then transporting PTEs within its biomass.

3.2. Accumulation and Translocation of PTEs

Data shown in Table 2 represent bioconcentration factors (BCF) in various PTEs. BCF determines the plants ability to concentrate xenobiotics present in the soil into the plant body. This shows which plants are suitable for the process of phytoremediation (BCF > 1) [12]. The presented results show that the BCF value for none of the observed PTEs was higher than 1. This indicates (Table 3) that *M. albus* does not effectively accumulate any of the observed PTEs in its roots. Similar results are shown in Ghazaryan et al. [4], where BCF of *M. officinalis* for Cu was 0.75 in the polluted soil. However, with the addition of fertilizer combined with EDTA, the BCF factors increased to 1.43. In the case of plants grown on a highly polluted dumping site in India, a generally higher BCF was determined for Cd [10,46]. Similarly, the BCF of Cd for *Plantago major* sampled close to a heavy traffic road was 23.33 and Cd was the most accumulated heavy metal [48].

Table 2. Translocation factor (TF), bioconcentration factor (BCF), and bioaccumulation factor (BAC) in *Melilotus albus* for the respective detected PTEs.

	BCF	BAC	TF
Sb	n.d.	n.d.	0.57 ± 0.19 a
As	0.15 ± 0.09 b	0.11 ± 0.003 b	0.39 ± 0.08 a
Cd	n.d.	n.d.	0.80 ± 0.31 a
Cu	0.26 ± 0.03 a	0.20 ± 0.04 a	0.79 ± 0.12 a
Ni	0.04 ± 0.01 c	0.023 ± 0.003 c	0.76 ± 0.25 a
Pb	0.03 ± 0.028 c	0.06 ± 0.02 d	1.51 ± 1.40 a
Hg	0.04 ± 0.02 c	0.08 ± 0.03 d	1.76 ± 0.90 a
Se	n.d.	n.d.	0.63 ± 0.28
Zn	0.16 ± 0.1 b	0.18 ± 0.06 a	1.39 ± 0.58 a

Data represent mean ± standard deviations over at least 5 replications. Different letters indicate significant differences in concentration of individual HM in soil (ANOVA, $p < 0.05$, post hoc Tukey's HSD test). n.d. = not detected.

Table 3. Phytoremediation potential of plants based on BAC, BCF, and TF according to Ghazaryan et al. [4].

	Low Accumulation	Accumulator
BAC	<1.0	≥1.0
BCF	<1.0	≥1.0
TF	<1.0	≥1.0

BAC = bioaccumulation factor, BCF = bioconcentration factor, TF = translocation factor.

Values of bioaccumulation factor (BAC) for *M. albus* can be found in Table 2. This parameter shows the efficiency of plants to accumulate xenobiotics in the above-ground biomass. Similarly, to BCF, a BAC > 1 indicates a plant species suitable for phytoextraction processes [43,44]. The BAC parameter was lower than 1 in all observed PTEs, which indicates a low ability of the species to accumulate these xenobiothics in their shoot tissue. Amin et al. [36] investigated the BAC of the legume *Cyamopisi tetragonoloba* and the non-fixing plant *Sesamum indicum* exposed to soils loaded with Pb. They observed that under the lowest loadings BAC and BCF exhibited the highest values (100 mg/kg). However, with the rising concentration of Pb in the soil, the observed parameters decreased. In case of the highest Pb concentration (1000 mg/kg), BCF and BAC decreased by 57.3 and 42.1%, respectively, when compared to 100 mg/kg. In the present study, the plants were grown in the soil without artificially added PTEs.

Table 2 shows the values of translocation factor (TF) which is used to evaluate the suitability of plants for phytoextraction processes. TF>1 indicates that plants are suitable for the phytoextraction of specific PTE [4]. Differences in TF among the individual PTEs were not statistically significant. PTEs are mostly represented by heavy metals that are absorbed by roots and subsequently transported via symplast (energy-dependent process) into the plant body [49,50]. Specifically, they can be built into the cell wall or transported into the vacuole [51,52]. However, they can be also transported into the above-ground body organs. This transport is conducted mainly via xylem, which belongs to the apoplast route [52]. This process is on the one hand highly dependent on the genetic makeup, but also on the environmental condition in which the plant grows. In our study, the highest TF values were found in Hg (1.764). The results suggest that *M. albus* can be used for the phytoextraction of Zn and Pb as well (TF values).

However, the ability of *M. albus* to phytoremediate Zn and Hg was disputed by Cukrowska et al. [53], where TF factors of *M. albus* for Hg were 0.56 and 0.51 in wet and dry seasons, respectively. These values suggests that *M. albus* is not suitable for phytoextraction of Hg. In another study, crops capable of the phytoremediation of Pb and Cd fodder mallow (*Malva verticillata* L.), rye (*Secale cereale* L. var. *multicaule* METZG. ex ALEF.), and white sweet clover (*Melilotus alba* MEDIC.) were determined [54]. White sweet clover showed the lowest ability to accumulate these PTEs in its tissues. In contrast, the present study shows that white sweet clover is suitable for lead phytoextraction according to TF values. Similarly, our study shows a suitability of white sweet clover for phytoextraction of Zn, which is not in agreement with Chan et al. [55], where yellow sweet clover (*M. officinalis*) showed a low TF for Zn (0.516). These differences in results can be attributed to the quality of the soil, because the ability of plants to translocate PTE to the above-ground parts of plants may depend on the soil fertility [1] and on the presence of organic acids [55]. Native plants of India grown on a highly polluted dumping site showed a higher TF rate than the reference plants [46].

However, the suitability of *M. albus* for phytoremediation and phytoextraction processes is questioned by the low BCF and BAC values in case of Pb, Hg, and Zn. The present results suggest that these PTEs are sufficiently transported from roots to shoots, but on the other hand, the low values of BCF and BAC indicate the low rate of their accumulation in roots and shoots. These results are in agreement with Pajuelo et al. [17] who reported the fact that many legumes are tolerant to the presence of PTEs because they belong to the group of heavy metal excluders which do not accumulate PTEs in shoots. Therefore, legumes are suitable for being grown on fields polluted by PTEs. For example, *M. officinalis* (species related to *M. albus*) also did not show a capacity to accumulate Cu in its above-ground biomass ([4]; Table 4). When the values of BCF, TAC, and TF are compared between the individual plant species, legumes apparently show the lowest ability to bind HM in their biomass (Table 4). In contrast, representatives from the families of *Asteraceae* and *Amaranthaceae* exhibit the values of BCF, TAC, or TF more than two times higher (Table 4). This is a very important precondition for growing legumes in soils with a higher content of PTEs to produce biomass for ensiling because when the plants of this family have a lower capacity to bind HM in their biomass, then the risk of HM getting into silage is lower, and the risk that the process of methanogenesis will be stopped or inhibited during silage degradation in the biogas plant is eliminated.

Table 4. The comparison of bioconcentration factor (BCF), bioaccumulation factor (BAC), and translocation factor (TF), in various studies and our study.

Publication	Plant Species	Sampling Location	Factor	PTEs					
				Cd	Cu	Ni	Pb	Hg	Zn
Our study	*Melilotus albus*	Agricultural soil	BCF	n.d.	0.26	0.04	0.03	0.04	0.16
			BAC	n.d.	0.2	0.023	0.06	0.08	0.18
			TF	0.8	0.79	0.76	1.51	1.76	1.39
Ghazaryan et al. [1]	*Melilotus oficinalis*	Mine	BCF	n.d.	0.75	n.d.	n.d.	n.d.	n.d.
			BAC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			TF	n.d.	0.08	n.d.	n.d.	n.d.	n.d.
	Amaranthus retroflexus		BCF	n.d.	0.29	n.d.	n.d.	n.d.	n.d.
			BAC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			TF	n.d.	0.37	n.d.	n.d.	n.d.	n.d.
Eid et al. [46]	*Amaranthus viridis*	Sewage sludge dump	BCF	1.07	0.49	0.55	1.25	n.d.	0.27
			BAC	2.59	0.62	1.85	2.03	n.d.	2.11
			TF	1.19	0.9	0.75	1.12	n.d.	1.02
	Bassia indica		BCF	0.9	0.59	0.52	1.29	n.d.	0.28
			BAC	0.83	1.52	2.95	0.9	n.d.	2.64
			TF	0.94	1.16	1.15	1.05	n.d.	1.3
	Conyza bonariensis		BCF	1.86	0.75	0.94	2.04	n.d.	0.34
			BAC	0.95	4.26	1.61	7.92	n.d.	1.04
			TF	8.23	1.09	0.58	0.91	n.d.	0.66
	Portulaca oleracea		BCF	1.36	0.86	0.66	1.75	n.d.	0.38
			BAC	0.74	1	2.06	0.71	n.d.	2.34
			TF	0.56	0.55	0.62	0.57	n.d.	0.99
	Rumex dentatus		BCF	1.64	0.73	0.81	2	n.d.	0.32
			BAC	0.81	1.04	3.72	0.63	n.d.	1.36
			TF	0.61	0.76	0.75	0.71	n.d.	0.8
	Solanum nigrum		BCF	2.72	1.04	1.02	2.79	n.d.	0.4
			BAC	5.19	2.11	1.12	4.62	n.d.	1.02
			TF	0.66	0.68	0.47	0.67	n.d.	0.66
	Lycopersicon esculentum		BCF	3.18	1.32	0.31	2.97	n.d.	0.45
			BAC	1.52	1.45	0.82	1.43	n.d.	0.89
			TF	0.29	0.38	0.65	0.32	n.d.	0.56
	Phragmites australis		BCF	1.43	0.81	0.67	1.74	n.d.	0.33
			BAC	0.97	1.18	1.92	1.04	n.d.	1.71
			TF	0.89	0.69	0.79	0.95	n.d.	0.73
Vaverkova et al. [47]	*Carduus*	Landfill ready for reclamation	BCF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			BAC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			TF	1.097	0.846	1.056	0.896	n.d.	1.003
		Compost plant	BCF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			BAC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			TF	0.974	0.462	0.863	1.099	n.d.	0.176
		Closed landfill area	BCF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			BAC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			TF	1.163	2.344	1.474	1.559	n.d.	0.71
		Landfill surroundings	BCF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			BAC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			TF	0.454	0.843	0.715	0.715	n.d.	0.581

n.d. = not detected.

M. albus is not able to accumulate the same amount as a hyperaccumulator, i.e., more than 10 ppm Hg; 100 ppm Cd and Se; 1000 ppm Co, Cr, Cu and Pb; 10,000 ppm Mn and

Zn. These results suggest that *M. albus* cannot be classified as a hyperaccumulator. Up to this point, 450 plant species have been classified as hyperaccumulators. The most common accumulated heavy metal is Ni and the least common are Mn, Cd, and Pb [56].

The use of plant residues after phytoremediation may be problematic due to the presence of PTEs. However, a trace level of some PTEs is necessary for the proper functioning of microbial metabolism because many of them, such as Cu, Mo, Fe, Ni, etc., are responsible for proper enzymatic function [57]. The presence of PTEs in higher concentrations can negatively affect processes of anaerobic metabolism [57,58]. Lin et al. [58] suggested that acid-forming bacteria are the most affected. The effect of PTEs on the production of methane depends on the type and amount of the metal. The inhibitory effect of Ni^{2+} on methane production was recorded only in concentrations above 30 mg/L, whereas the highest cumulative methane production was observed when the Ni^{2+} concentration was 4 mg/L [59]. The present study shows low effectiveness in the translocation of Ni to the shoot tissue (TF = 0.755), and even lower effectiveness in the accumulation of this heavy metal in tissues (BCF = 0.041; BAC = 0.003).

Similarly, low Cu concentrations (5 mg/L) had a stimulatory effect on methanogenesis. On the other hand, with the rising concentration of Cu, the methane production was decreasing [60]. Wu et al. [60] found that a half maximum inhibitory concentration (IC50) for Cu was 18.32 mg/L. Similarly, a Cu concentration of 13.5 mg/L caused reduced the methane production by 50% [61]. Different results were obtained when copper was added to the bioreactor with the residues of plants used for phytoremediation. A Cu concentration of 100 mg/kg not only promoted anaerobic digestion and required a shorter anaerobic digestion time, but also increased the methane content in biogas. Biogas production decreased when Cu in a concentration of 500 mg/kg was added [62].

Zinc is a microelement which is essential for many enzymes involved in anaerobic reactions [57]. This is why Zn has a common stimulatory effect on biomethane production [55]. Chan et al. [55] evaluated the effect of Zn supplementation on biogas production. The authors found that the concentration of Zn in the fermenter increased from 50 to 100 mg Zn^{2+}/L and positively affected methane yield. The total yield of methane increased by 30–65%.

4. Conclusions

In this study, the ability of *M. albus* Med. to accumulate PTE was assessed. This plant species was selected with regard to its use in sustainable agriculture, especially in growing systems of mixed culture of *Maize* and *Fabaceae* with the subsequent use for biomass production as an energy source in biogas stations. The results suggest that the *M.* albus Med. can absorb PTEs from the soil. The measured values thus indicated that the *M. albus* Med. would be able to accumulate heavy metals in its biomass at reduced efficiency, TF values did not exceed 1.0. On the other hand, there is a potential for the plant to resist abiotic stress caused by increased heavy metal concentration in the soil. The highest PTE concentrations in the plant biomass were those of zinc (av. 12.638 mg/kg in above-ground biomass and 10.560 mg/kg in underground biomass) and copper (av. 4.0 mg/kg in above-ground biomass and 5.128 mg/kg in underground biomass). The bioconcentration and bioaccumulation factors were always lower than 1. The plant can effectively translocate only Zn, Hg, and Pb from roots to shoots. The bioconcentration factor shows inefficiency of PTE accumulation in the shoots of *M. albus*, which leads to the conclusion that *M. albus* is not suitable for phytoremediation processes. The measured results showed that *M. albus* Med., Meba does not pose a danger to processes of anaerobic digestion in biogas plants by the excessive accumulation of PTEs in its biomass, when grown on soils comprising PTEs in naturally abundant concentrations. Therefore, its use in biogas production should not jeopardize biomethane production due to the high PTE concentration. Further research should focus on growing *M. albus* on soils with the increased content of PTEs, e.g., brownfields. Biomass would be then used for testing in the laboratory fermenter with the aim to analyze in details all processes in the fermenter.

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Appendix A

Table A1. List of abbreviations.

Abbreviations	Description
BAC	Bioaccumulation Factor
BBCH	Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie
BCF	Bioconcentration Factor
ČSN	Czech Technical Standard
DW	Dry Weight
FAO	Food and Agriculture Organization of the United Nations
HM	Heavy Metals
ISO	International Organization for Standardization
M. albus	*Melilotus albus* Med.
PTEs	Potentially Toxic Elements
TF	Translocation Factor
WHO	World Health Organization

Table A2. Permissible limits and targeted concentration of heavy metals.

Element	Permissible Limits of Heavy Metals in Water (mg/kg) WHO *	Targeted Value of Soil (mg/kg) WHO **	Permissible Limits of Heavy Metals in Plant (mg/kg) WHO **
As	0.05	1–30	1
Pb	0.05	85	2
Cd	0.005	0.8	0.02
Cr	0.05	100	1.30
Hg	0.001	0–0.03 ***	<0.03 ***
Zn	5.0	50	0.60
Cu	1.5	36	10

* WHO [63]. ** WHO [64]. *** Gworek et al. [65].

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