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Hylotelephium maximum from Coastal Drift Lines Is a Promising Zn and Mn Accumulator with a High Tolerance against Biogenous Heavy Metals

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Abstract: Heavy metal tolerance and accumulation potential are the two characteristics most important for plant use in phytoremediation technologies. Therefore, the aim of the present study was to characterize the tolerance of *Hylotelephium maximum* from coastal drift line vegetation against the biogenous heavy metals Cu, Zn, and Mn and its metal accumulation potential in controlled conditions. Plants were propagated vegetatively and cultivated in an automated greenhouse in a vegetative state (Experiment 1; Cu, Zn, and Mn) and in flowering-inducing conditions (Experiment 2; Mn gradient). In Experiment 1, total shoot biomass was negatively affected only by Mn at 1.0 g L^{-1}, but root growth was significantly inhibited by all metals at this concentration. Plants accumulated 250 mg kg^{-1} Cu, 3200 mg kg^{-1} Zn, and >11,000 mg kg Mn^{-1} in their leaves. In Experiment 2, only new shoot growth was significantly suppressed at 0.5 g L^{-1} Mn. At the highest concentrations, shoot biomass progressively declined at the level of inhibition of flower and stem growth. Visual toxicity symptoms of Mn appeared 2 weeks after full treatment on leaves of 2.0 g L^{-1} treated plants as black dots along the main veins and spread over the leaf surface with time. The maximum Mn accumulation capacity was reached in leaves (15,000 mg kg^{-1}), together with a high translocation factor and bioconcentration factor. The obtained results suggest that the particular accession of *H. maximum* has very good potential for practical phytoremediation purposes.

Keywords: copper; Crassulaceae; heavy metal tolerance; heavy metal toxicity; manganese; metal accumulation; zinc

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1. Introduction

Plant tolerance to high soil heavy metal concentrations is a critical feature for their use in environmental remediation practice [1]. Phytoextraction is an emerging phytoremediation technology, allowing for relatively inexpensive, efficient, and environment-friendly removal of chemical contamination from soils and waters by means of plants [2,3]. However, wide practical implementation of phytoextraction measures is hindered due to several reasons, the lack of biological knowledge on appropriate plant species being one of the most critical among them. Thus far, many studies have been focused on species with an extreme capacity to accumulate one or two specific metals, designated as hyperaccumulators [4].

It is usually argued that species tolerance against metals is a physiological prerequisite for heavy metal hyperaccumulation [5]. However, tolerance and hyperaccumulation are physiologically and genetically distinct traits [6]; therefore, these characteristics need to be assessed independently [7]. For the needs of practical phytoextraction, even plants with relatively low tolerance and high accumulation may be useful, and vice versa.

In contrast to "obligate hyperaccumulators" found on metalliferous soils, "facultative hyperaccumulators" may have evolved the ability to tolerate and accumulate metal ions due to indirect selection on a basis of common physiological mechanisms [8]. As an example, plants from saline habitats are potential candidates of such facultative hyperaccumulators due to common physiological mechanisms involved in protection against and/or detoxification of NaCl and heavy metals [9,10]. Thus far, mostly obligate halophytes have been used as potential heavy metal accumulators [11,12], but recent studies suggest that other wetland species are promising models for phytoextraction studies [13–16]. In addition to sea-affected coastal wetlands, coastal drift line habitats are promising places for potential metal-tolerant plant species, as drift line vegetation often comprises clearly non-halophytic, ruderal, or alien species with potential tolerance-related characteristics [17,18].

Several essential plant micronutrients can be classified as potentially toxic heavy metals, including Cu, Zn, and Mn [19]. The average concentration (physiological requirement) of Cu in plant tissues is in the range of 5–20 mg kg^{-1} [20]; that of Mn is 1–800 mg kg^{-1} [21]; and that of Zn, 1–2 mg kg^{-1} [22]. Above these ranges, these biogenous heavy metals possess potential toxicity for plants. Belonging to transition metals, Cu, Zn, and Mn are involved in various enzymatic reactions as cofactors and activators [23], as well as in critical processes of photosynthesis [24,25]. In contrast to Zn, which is usually in a stable state as Zn^{2+}, Cu and Mn can occur in different redox states and can therefore participate in electron transfer reactions involving photosynthesis, respiration, scavenging of reactive oxygen species, etc. [26]. The toxicity of these transition metals is due to the same reason that they are essential, and if not present in a chelated form, they can act as pro-oxidants [27,28]. For more detail, readers are requested to turn to recent specialized reviews on the tolerance and accumulation of Cu [29], Zn [30], and Mn [31].

Several taxonomically related species of Crassulaceae from the genera *Sedum* and *Hylotelephium* have been characterized as heavy-metal-tolerant accumulators. Two *Sedum* species were isolated from metal-contaminated soil around mining sites. *Sedum alfredii* is a Zn and Cd hyperaccumulator, with both hyperaccumulating and non-hyperaccumulating ecotypes found in nature [32]. The species can also accumulate relatively high concentration of Pb, as it was found around old Pb/Zn mines [33]. Another Zn/Cd hyperaccumulator, *Sedum plumbizincicola*, accumulates both metals predominantly in shoots, reaching concentrations of 7010 and 18,400 mg kg^{-1} for Cd and Zn, respectively [34]. *Sedum lineare* appeared to be tolerant to Cu, accumulating a high concentration of Cu in tissues [35]. *Sedum sediforme* was shown to be able to grow on soils with 16,800 mg kg^{-1} extractable Cu, accumulating 639 and 406 mg kg^{-1} Cu in roots and shoots, respectively [36]. Additionally, other *Sedum* and *Hylotelephium* species were analyzed in respect to their Cd phytoextraction potential [37]. Among them, *Hylotelephium spectabile* has emerged as a potential Cd accumulator with large between-population differences in phytoextraction potential [38]. The species accumulated a high concentration of Cd (603 mg kg^{-1}) in leaves when cultivated in hydroponics [39].

Thus far, there are no studies describing the heavy metal tolerance and accumulation potential of *Hylotelephium maximum* (L.) Holub (syn. *Hylotelephium telephium* subsp. *maximum*; formerly known as *Sedum maximum* Hoffm.). The general distribution of the species in Europe is associated with woodland, steppe, rocky bedrock, and marginal habitats [40]. In particular, *H. maximum* is a characteristic species of the European protected habitat 6120* Xeric sand calcareous grasslands [41]. On the practical side, both *Sedum* and *Hylotelephium* species are widely used for green roof construction, where tolerance to pollutants is especially important [42,43].

The aim of the present study was to characterize the tolerance of *Hylotelephium maximum* from coastal drift line vegetation against the biogenous heavy metals Cu, Zn, and Mn and its metal accumulation potential. It was hypothesized that *H. maximum* plants could tolerate relatively high concentrations of various heavy metals through common protection mechanisms and localized metal accumulation in aboveground parts.

2. Results

2.1. Experiment 1

Treatment of *H. maximum* plants with Cu, Zn, and Mn in the form of respective sulphate salts (Figure S1) led to a concentration-dependent increase in substrate EC (Figure 1). The decrease in EC with time reflected the depletion of soluble minerals by growing plants. The increase in substrate EC in some treatments in week 4 was due to fertilization in the previous week.

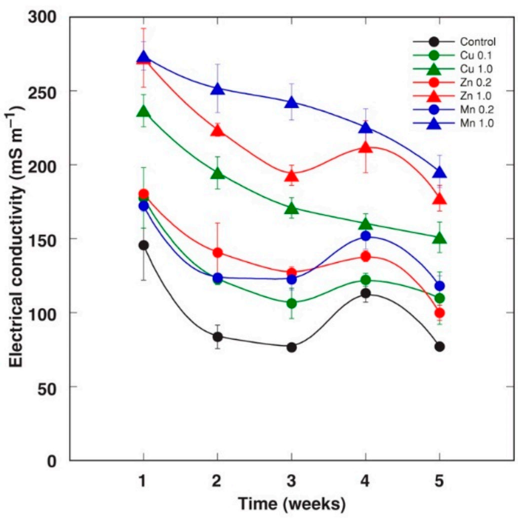

Figure 1. Effect of treatment with different concentrations of Cu, Zn, and Mn on changes in substrate electrical conductivity in containers with *H. maximum* (Experiment 1). Data are means ± SE from 5 replicates, each with 4 measurements, for each point.

At a lower treatment dose (0.1 or 0.2 g L^{-1}), all metals significantly decreased the biomass of old leaves of *H. maximum* plants, and the growth of new leaves was stimulated only with Zn and Mn (Figure 2A). Cu treatment at 1.0 g L^{-1} significantly decreased the mass of old leaves and stimulated the growth of new ones; Zn had no effect at this concentration, but Mn at 1.0 g L^{-1} inhibited the growth of all leaves. Stem growth was relatively less affected by treatments, as it was significantly stimulated only by 0.2 g L^{-1} Zn and inhibited by 1.0 g L^{-1} Mn treatment (Figure 2B). Root growth was significantly inhibited by all metals at 1.0 g L^{-1} (Figure 2B). Total shoot biomass was negatively affected only by Mn at 1.0 g L^{-1}.

The leaf chlorophyll concentration during the experiment showed only minor non-significant changes, with the exception of 1.0 g L^{-1} Mn treatment, where a significant increase was evident within the first two weeks after full treatment (Figure 3A). The chlorophyll *a* fluorescence parameter, Performance Index, showed significant stimulation relative to the control plants within weeks 2 to 3 in plants treated with 1.0 g L^{-1} Cu and Mn as well as decreased values for 1.0 g L^{-1} Zn at 1 and 4–5 weeks after the full treatment (Figure 3B).

The *H. maximum* plants showed clear organ-specific accumulation patterns with metal-dependent differences (Figure 4). In Cu-treated plants, an identical concentration of Cu (>250 mg kg^{-1} in 1.0 g L^{-1} treatment) was accumulated in the roots and old leaves, but the stems had a significantly lower concentration (Figure 4A). Cu was efficiently excluded from young leaves. The accumulation patterns of plant parts for Zn (Figure 4B) and Mn (Figure 4C) were similar, but the accumulation potential for Mn was significantly more pronounced (>11,000 mg kg^{-1}) than that for Zn (>3000 mg kg^{-1}). Leaves had similar high metal concentrations irrespective of age, with significantly lower levels in stems followed by roots.

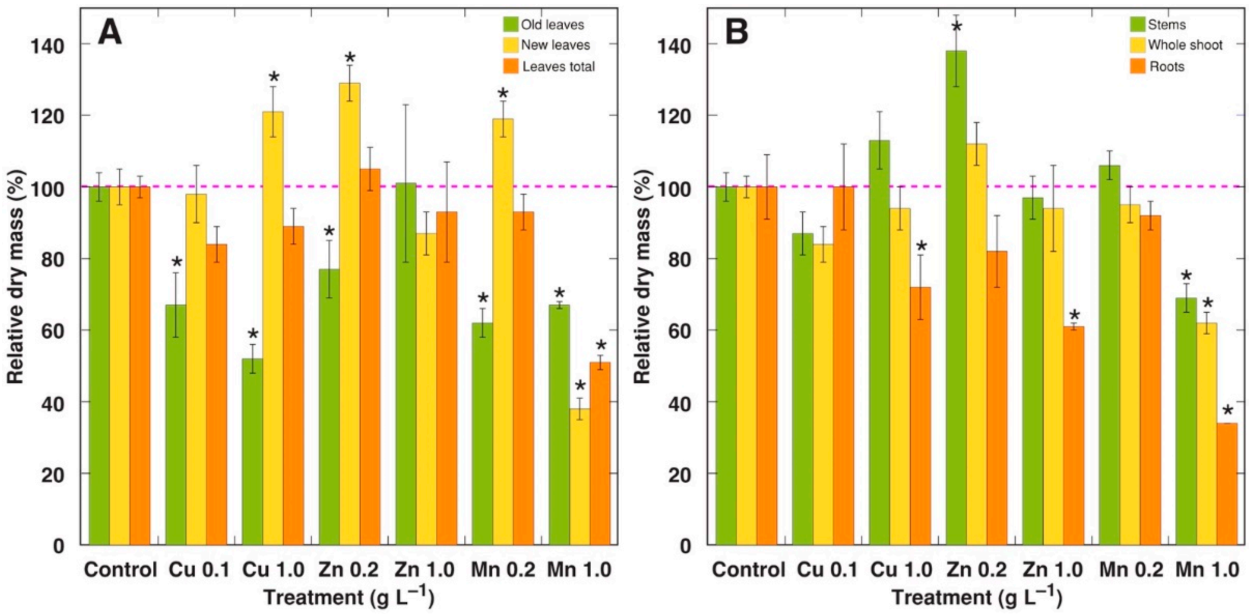

Figure 2. Effect of treatment with different concentrations of Cu, Zn, and Mn on relative dry mass of different parts (**A**: old leaves, new leaves, leaves total; **B**: stems, whole shoot, roots) of *H. maximum* plants (Experiment 1). Data are means ± SE from 5 replicates. Note: * indicates statistically significant difference from the respective control ($p < 0.05$).

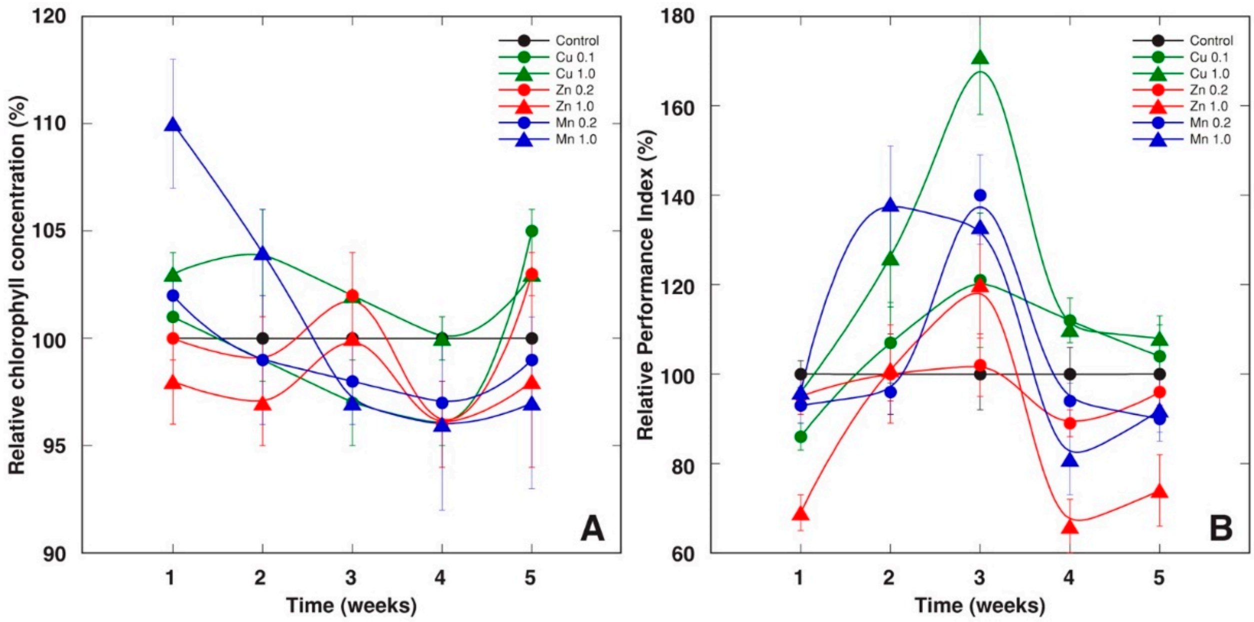

Figure 3. Effect of treatment with different concentrations of Cu, Zn, and Mn on changes in relative chlorophyll concentration (**A**) and Performance Index (**B**) in leaves of *H. maximum* (Experiment 1). Data are means ± SE from 5 replicates, each with 2 measurements, for each point.

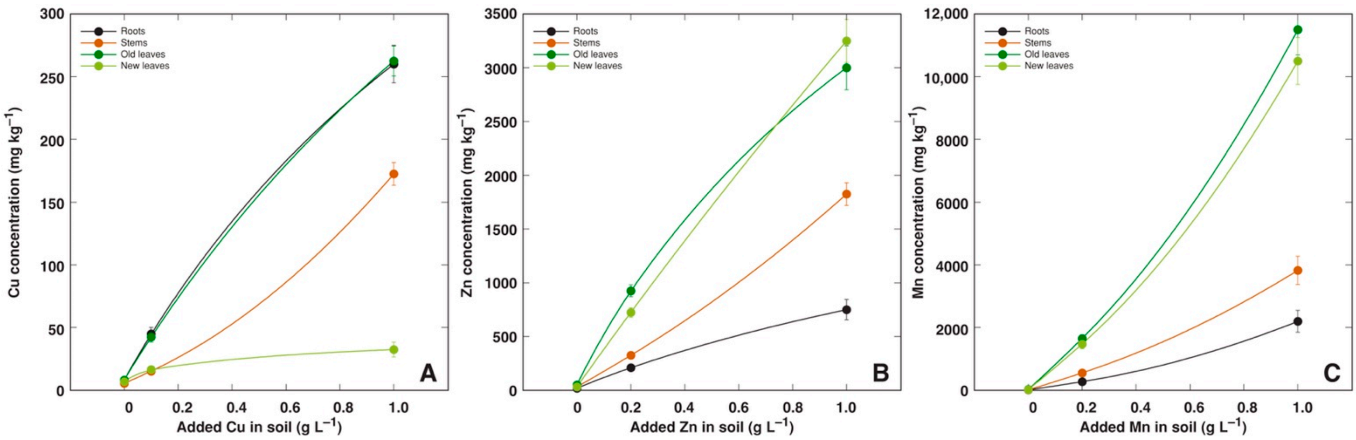

Figure 4. Effects of treatment with Cu on Cu concentration (**A**), treatment with Zn on Zn concentration (**B**), and treatment with Mn on Mn concentration (**C**) in different parts of *H. maximum* plants (Experiment 1). Data are means ± SE from 3 replicates for each point.

2.2. Experiment 2

Due to the extremely high capacity for Mn accumulation in the leaves of *H. maximum* revealed in the first experiment, an additional experiment was performed to confirm the Mn treatment effect after a longer time (eight weeks after full treatment) and over a wider range of concentrations (0.02 to 2.0 g L^{-1}) in the form of a sulphate salt (Figure S2). The treatment resulted in a concentration-dependent increase in substrate EC, with significant decreases during cultivation only for 0.5 to 2.0 g L^{-1} treatments (Figure 5), possibly reflecting more efficient removal of electrolyte-active substances by the respective plants.

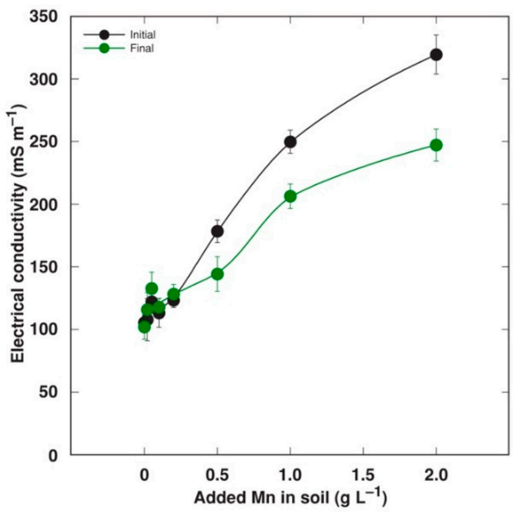

Figure 5. Effect of treatment with different concentration of Mn on initial (week 1) and final (week 9) substrate electrical conductivity in containers with *H. maximum* (Experiment 2). Data are means ± SE from 5 replicates, each with 4 measurements, for each point.

No significant effect of Mn treatment was evident up to 0.2 g L^{-1}, except for an increase in flower biomass, but at 0.5 g L^{-1} Mn, only new shoot growth was significantly inhibited (Figure 6). At the highest concentrations (1.0 and 2.0 g L^{-1}), the biomass of new shoots progressively declined, and there was a significant inhibition of flower, stem, and whole shoot growth. However, leaf growth was not significantly affected, and root growth was not significantly affected even at the highest Mn concentration (Figure 6B).

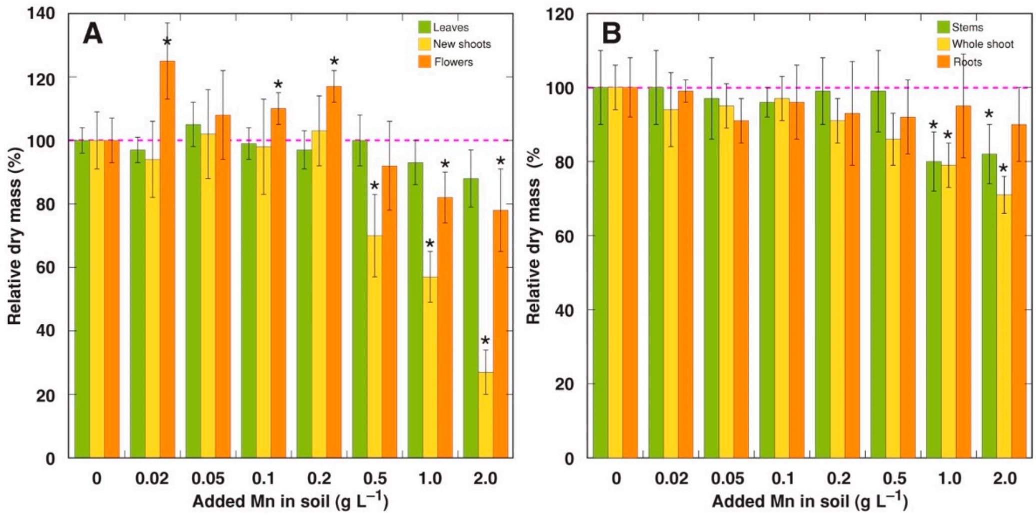

Figure 6. Effect of treatment with different concentrations of Mn on relative dry mass of different parts (**A**: leaves, new shoots, flowers; **B**: stems, whole shoot, roots) of *H. maximum* plants (Experiment 2). Data are means ± SE from 5 replicates. Note: * indicates statistically significant difference from the respective control ($p < 0.05$).

The first visual toxicity symptoms of Mn appeared 2 weeks after full treatment on the leaves of 2.0 g L^{-1} treated plants as black dots along the main veins (Figure 7B). With time, the dots spread over the leaf surface, covering the whole leaf 6–7 weeks after full treatment. The same pattern of symptoms was also evident on leaves of 1.0 g L^{-1} treated plants at 5 weeks and of 0.5 g L^{-1} treated plants at 8 weeks (Figure 8). At this time, dark lesions appeared also on the stems of 2.0 g L^{-1} treated plants (Figure 8).

Figure 7. Typical visual toxicity symptoms on leaves of *H. maximum* plants treated with 2.0 g L^{-1} Mn (**right**) in comparison to control plants (**left**) two weeks after full treatment (Experiment 2).

Figure 8. Typical visual toxicity symptoms on leaves of *H. maximum* plants treated with 0.5 (**left**), 1.0 (**middle**), and 2.0 (**right**) g L^{-1} Mn eight weeks after full treatment (Experiment 2).

Changes in the chlorophyll *a* fluorescence parameter Performance Index did not directly reflect the degree of treatment with Mn (Figure 9). Only one week after full treatment, the Performance Index was significantly decreased in the leaves of plants treated with 0.02 to 0.5 g L^{-1} Mn, with a maximum decrease in both the 1.0 and 2.0 g L^{-1} treatments. Later (weeks 4–6), the Performance Index values stabilized close to the control level, except for plants treated with 2.0 g L^{-1} Mn, where there was a significant increase in the parameter.

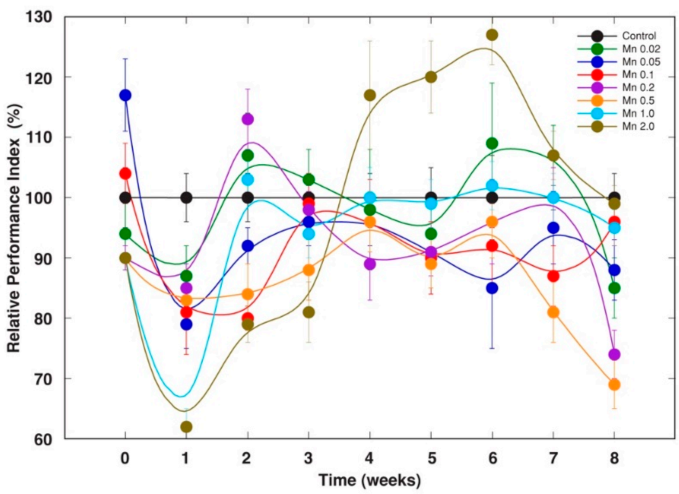

Figure 9. Effect of treatment with different concentrations of Mn on changes in relative Performance Index in leaves of *H. maximum* (Experiment 2). Data are means ± SE from 5 replicates, each with 2 measurements, for each point.

Different parts of *H. maximum* plants accumulated significantly different concentrations of Mn (Figure 10A). The highest concentration was in leaves, reaching 15,000 mg kg^{-1} in the 2.0 g L^{-1} treatment. Newly developed shoots and flowers accumulated about half of the Mn concentration found in leaves. In contrast, roots and stems accumulated equal concentrations of Mn, being only 10–15% in comparison to that in leaves. Due to the high relative proportion of leaves, the majority of Mn was accumulated in leaves (about 78%, Figure 10B). Preferential accumulation of Mn in aboveground parts in comparison to roots was indicated by the translocation factor >1 (Figure 10C). The translocation factor was the highest in leaves, reaching values of 10, but was only around 4 in flowers and 3 in new shoots. However, it was around 1 in stems. Similarly, the bioconcentration factor was the highest in leaves, increasing up to more than 9 at 1 g L^{-1} soil Mn concentration (Figure 10D). Values of the factor in the range of 2–4 were observed in new shoots and flowers but were only around 1 in roots and stems.

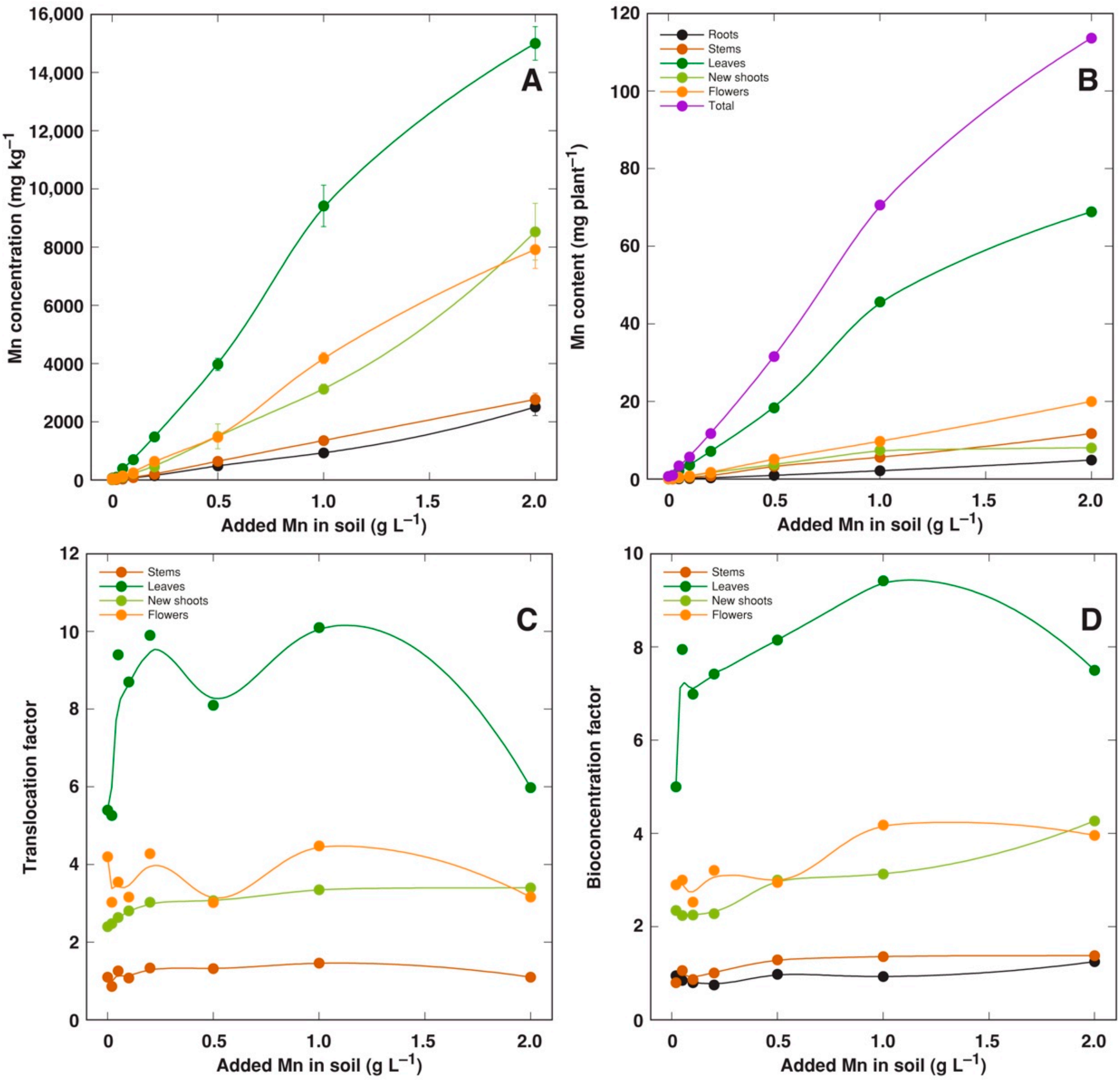

Figure 10. Effect of treatment with different concentrations of Mn on Mn concentration (**A**), Mn content (**B**), translocation factor for Mn (**C**), and bioconcentration factor for Mn (**D**) in different parts of *H. maximum* plants (Experiment 2). Data are means ± SE from 3 replicates for each point.

3. Discussion

Plants that accumulate metals most efficiently in natural conditions are designated as hyperaccumulators [45]. Both the slow growth as well as the small biomass of individual plants of most hyperaccumulator species significantly diminish their phytoextraction efficiency in field conditions, especially for large-scale phytoremediation activities [46]. In addition, requirements for specific environmental conditions can limit the broad use of these species. Consequently, new candidate species with an enhanced phytoextraction ability need to be found. Usually, hyperaccumulator species are found growing natively on soils with especially high concentrations of a particular metal ion or through chemical analysis of herbarium materials [47,48]. In contrast to the isolation of highly specific metal-accumulating plant species from particular contaminated places such as mining sites, a promising approach is to search for local native species able to accumulate a wider range of target metals. Salt-adapted species (halophytes) have been shown to be promising candidates for phytoremediation purposes [49]. Furthermore, instead of focusing on obligate halophyte species, several coastal habitats can be selected as targets for the research of both salt-resistant and metal-resistant plant species with a high potential for metal accumulation [18]. The accession of *H. maximum* used in the present study was found growing in a drift line habitat on a coast of the Baltic Sea with relatively high substrate salinity (as shown by soil electrical conductivity measurements, mentioned in the Materials and Methods), indicating the presence of putative tolerance mechanisms against osmotic stress and ion toxicity. It was found that *H. maximum* plants are extremely tolerant to high substrate concentrations of Cu, Zn, and Mn salts and have high potential for accumulation of these metals in aboveground parts.

These biogenous heavy metals differentially affected the growth of different parts of *H. maximum*. For example, Cu decreased the biomass of old leaves but stimulated the growth of new leaves at the higher concentration, and total shoot biomass was unaffected (Figure 2). Zn also inhibited the growth of old leaves at the lower concentration, but growth of new leaves was stimulated. While *H. maximum* plants at the vegetative stage showed a decrease in shoot biomass by 40% at 1.0 g L^{-1} Mn (Figure 2), the total shoot biomass of plants at the flowering stage decreased only by 30% at 2.0 g L^{-1} Mn, with no visible stem growth inhibition, but the development of new shoots was significantly suppressed (Figure 6).

Critical tissue toxicity levels in plant tissues are 300 to 600 mg kg^{-1} for Zn [50,51], above 20 to 30 mg kg^{-1} for Cu [52], and above 200 mg kg^{-1} for Mn [53]. As no toxicity symptoms were evident above internal metal concentrations of 250, 3000, and 4000 mg kg^{-1} in *H. maximum* leaves for Cu, Zn, and Mn, respectively, for at least five weeks, the species can be characterized as highly tolerant to these heavy metals. Even the Zn hyperaccumulator *S. alfredii* showed visual toxicity symptoms (brown and wilted leaves) when exposed to high Zn levels (1000 and 2500 µM) [54].

A decrease in photosynthesis-related parameters is a common symptom of the toxicity of heavy metals [10,55]. Chlorophyll concentration was not significantly negatively affected by either treatment in *H. maximum* (Figure 3A). In contrast, the photochemistry of photosynthesis, as indicated by changes in the chlorophyll *a* fluorescence parameter Performance Index, was negatively affected by Mn only at the early stages after treatment, especially for plants growing with 1 and 2 g L^{-1} Mn (Figure 9). However, no clear concentration-dependent effects were evident later (Figure 9). This most likely reflects the operation of efficient Mn-sequestration strategies in leaves, involving oxidation and complexation with organic ligands [31].

Visual symptoms of toxicity appeared only for Mn-treated plants. Usually, chlorosis of young leaves and the appearance of dark spots on mature leaves are mentioned as toxicity symptoms of excess Mn [31], which is related to the fact that Mn is a relatively immobile element [56]. However, only black spots were seen in the case of *H. maximum* (Figures 7 and 8), as chlorophyll concentration was not significantly affected (Figure 3). The initial appearance of Mn toxicity symptoms in close proximity to major leaf veins (Figure 7) could be associated with the transport of Mn by xylem tissues and deposition in adjacent cells. The appearance of purple-colored necrotic regions has been noted as a characteristic Mn toxicity response on soybean leaves, containing deposits of phenolic substances and Mn oxides [57]. Complex formation between phenolic substances and metals is suggested as one of the adaptive strategies for heavy metal tolerance [58]. It seems that the appearance of dark spots can only be used as an indication of Mn deposition instead of as a sign of Mn toxicity, especially in tolerant plant species such as *H. maximum*.

It is important to compare the tolerance indices of *H. maximum* with those of known Mn hyperaccumulators. Most known Mn hyperaccumulators are tropical woody species [59], but recently, several herbaceous species have also been characterized as Mn hyperaccumulators. *Celosia argentea* from a Mn tailing wasteland accumulated 20, 228, 8872, and 2823 mg kg^{-1} Mn in leaves, stems, and roots, respectively, at 600 mg L^{-1} Mn; however, the toxicity symptoms at this concentration were rather severe as leaves were wilting and abscising already after 10 days of exposure [60]. Another Mn hyperaccumulator, *Polygonum hydropiper*, showed significantly reduced growth already at 0.5 mmol L^{-1} Mn in hydroponics, but the maximum leaf Mn concentration (37,491 mg kg^{-1}) was achieved only with 4 mmol L^{-1} Mn treatment [61]. It is important to note that *P. hydropiper* plants at the lowest growth inhibitory concentration already had significantly increased H_2O_2 and malondialdehyde concentrations in leaves, pointing to deleterious effects due to reactive oxygen species.

Accumulation of heavy metals in aboveground parts is considered one of the strategies of heavy metal tolerance in plants [62]. In *H. maximum*, the Cu accumulation potential was identical in roots and old leaves, but both Zn and Mn were preferably accumulated in leaves irrespective of their age (Figure 4). Tissue-specific metal accumulation patterns are an interesting feature of many metal-accumulating species. The Zn hyperaccumulator *Sedum plumbizinicola* accumulated a five-fold higher concentration of Zn in shoots than in roots, but young leaves had a two-fold higher concentration than mature leaves [34]. No differences in respect to leaf age were found in the present study with *H. maximum* for both Zn and Mn (Figure 4). However, stems had significantly less capacity for the accumulation of Cu, Zn, and Mn, and both the translocation factor and bioconcentration factor for Mn were the lowest in plant stems (Figure 10). Usually, more Zn accumulates in younger leaves due to the relatively high mobility of this element [63].

Most Cu-tolerant plants are Cu excluders, accumulating these ions predominantly in root tissues [64], with the concentration rarely increasing above 100 mg kg^{-1} [65]. As an exception, another Crassulaceae species, *Crassula helmsii*, accumulated >9000 mg kg^{-1} Cu in shoot tissues [66]. While the hyperaccumulation threshold for Cu is currently defined as 300 mg kg^{-1} [45], *H. maximum* accumulated only >250 mg kg^{-1} Cu both in roots and old leaves (Figure 4A). Similarly, *Cyamopsis teragonoloba*, a candidate for the decontamination of slightly Cu-polluted soil, accumulated identical concentrations of Cu (about 155 mg kg^{-1}) in roots, leaves, and stems at 300 mg kg^{-1} Cu in soil [67]. Furthermore, the ability of *H. maximum* to accumulate Cu in leaves seems to be exceptional among *Sedum* and *Hylotelephium* species, as *Sedum rubrotinctum* was able to achieve 600 mg kg^{-1} Cu in roots, but the Cu concentration in shoots was only 10 to 15 mg kg^{-1} [68]. However, *Sedum lineare* could tolerate 500 mg kg^{-1} Cu in soil, leading to 65% inhibition of growth and accumulation of 1788 mg kg^{-1} Cu in tissues [35]. It is interesting to note that the Cd/Zn hyperaccumulator *S. plumbizincicola* had a relatively high shoot Cu concentration (254 mg kg^{-1}) when grown in soil containing 369 mg kg^{-1} Cu [69].

Meanwhile, the hyperaccumulation threshold for Zn is defined as 3000 mg kg^{-1} and that for Mn remains 10,000 mg kg^{-1} [45], and both values were exceeded in the leaves of *H. maximum* (Figures 4B,C and 10A). In hyperaccumulating plants such as *Thlaspi caerulescens*, Zn is stored mostly in the vacuoles of leaf epidermal cells [70]. The Zn hyperaccumulating ecotype of *S. alfredii* has a high ability for Zn remobilization by phloem transport and distribution to phloem-surrounding mesophyll cells of new leaves [71]. In the present study, *H. maximum* plants accumulated equal concentrations of Zn in both old and new leaves, not supporting the existence of a remobilization mechanism, but it was significantly lower in stems (Figure 4B). In *S. alfredii*, most Zn is accumulated in the cell walls of leaf and stem cells, but the remaining soluble fraction of Zn could represent the part complexed and stored in cell vacuoles [72].

Among the functional indicators of highly metal-accumulating plant species, both the translocation factor and bioconcentration factor are frequently used [73]. While the translocation factor characterizes the potential to preferentially store a particular metal in aboveground parts, the bioconcentration factor shows the ability of plant to accumulate the metal in respect to its concentration in soil. Both factors need to be >1 in order for the particular plant to be efficiently used in metal phytoextraction [3]. The extremely high values of translocation factor (10) and bioconcentration factor (9) for Mn in the leaves of *H. maximum* plants pointed to the promising phytoextraction efficiency of the species. Similarly, the coastal ecotype of the wetland species *Rumex hydrolapathum* had high potential for the accumulation of both Mn and Zn in leaves, as evident by high values of both translocation and bioconcentration factors, but the absolute concentration of these metals did not reach the threshold levels for hyperaccumulation [74]. However, in contrast to *H. maximum*, *R. hydrolapathum* plants accumulated metals predominantly in older leaves, followed by the induction of senescence and accompanied by the formation of new leaves with limited metal accumulation potential. These differences clearly point to different growth strategies of the two perennial species.

From an ecological point of view, hyperaccumulators are defined based on whether the plants accumulate the element in question above some threshold concentration when growing in natural habitats [45]. This idea is not very useful in respect to facultative accumulators, such as *H. maximum* in the present study. Thus, the concept of metal hyperaccumulation seems to be somehow artificial and has been seriously debated within recent years [7]. From the point of view of use in practical phytoextraction, the precise definition of "hyperaccumulator" is irrelevant; instead, large biomass production and accumulation of the metal in question are the two points that matter. In field conditions without any treatment, *S. alfredii* could remove 32.7 kg ha^{-1} Zn, reaching a maximum tissue concentration of 6279 mg kg^{-1} [75]. *S. alfredii* is relatively small and a low-biomass plant, producing only 5.5 t ha^{-1}; therefore, the phytoextraction potential of *H. maximum* for Zn could be comparable to that of *S. alfredii*, even with an approximately three-fold lower shoot Zn concentration. Given the higher ability for Mn accumulation, the phytoextraction potential of *H. maximum* for Mn could be even several times higher. *H. maximum* accumulated as much as 110 mg Mn per plant, which is a remarkable amount when considering this ability in field conditions. For example, with a planting density of 25 *H. maximum* plants per m^2, this could potentially lead to the removal of 27.5 kg ha^{-1} Mn. Among other practical benefits, *H. maximum*, as a perennial species, easily forms new shoots after mowing/clipping, which is an important feature for biomass removal in phytoremediation and regrowth of aboveground biomass.

Biological interactions in the rhizosphere are thought to be critical in the performance of any plant species in their native habitats, including metal hyperaccumulators [76]. Therefore, it can be suggested that in field conditions growing in metal-contaminated soil, *H. maximum* can benefit from mycorrhizal symbiosis or free-living, plant growth-promoting soil bacteria. It has already been shown that arbuscular mycorrhizal fungi enhance both the absorption and stabilization of Cd by *S. alfredii* [77].

While ecotypes from contaminated sites usually have a higher metal tolerance and accumulation ability [78], some species show these traits persistently. Initially, *Typha latifolia* plants from sites with different degrees of contamination with Zn showed comparable degrees of tolerance when growing in identical conditions [79]. Similarly, the accumulation ability for Zn and Cd in *Arabidopsis halleri* was shown to be a constitutive characteristic irrespective of origin from metallicolous or non-metallicolous soils [80]. *H. maximum* has not been shown to be specifically associated with a metallicolous or saline soil, and the particular accession did not show high salinity tolerance and ion accumulation potential in controlled conditions (Ievinsh, unpublished results). Therefore, it is reasonable to suggest that the tolerance to biogenous heavy metals and the Zn and Mn accumulation potential found are constitutive traits at the species level. However, this hypothesis needs to be tested in the future.

4. Materials and Methods

Hylotelephium maximum (L.) Hoffm. plants were found growing natively in drift line vegetation 10 m from a coastline on the coast of the Kalmar Strait (the Baltic Sea) near Ekerum Camping (island of Öland, Sweden), N 56°47′39.6024″ E 16°33′35.1540″. The soil electrical conductivity near the plant roots was 867 ± 34 mS m^{-1}, the sea water electrical conductivity was 9.6 ± 0.2 mS cm^{-1}, and the Na^+ concentration in the sea water was 9850 ± 50 mg L^{-1}. One flowering shoot was collected in August; placed in a sealed polyethylene bag; and, after 6-week storage at 4 °C, used for the establishment of a stock culture of soil-grown plants by vegetative propagation using single node explants with leaves.

Two separate vegetation pot experiments with *H. maximum* plants were performed. During the first experiment, short-term tolerance (5 weeks after the full treatment) against several biogenous heavy metals (Cu, Zn, and Mn at two concentrations each) and potential of metal accumulation were tested for the plants in their active vegetative growth phase. During the second experiment, a wider concentration range of substrate Mn was tested for a longer time period (9 weeks after the full treatment) using flowering-induced plants.

Plant propagation for experiments from stock plants was started in February. Nodal explants were planted in trays with moist quartz sand in sealed 48-liter plastic boxes and kept under moderate light (photon flux density of photosynthetically active radiation, 100 µmol m^{-2} s^{-1} at the plant level; 16-h photoperiod). After the formation of adventitious roots, plants were transplanted to 1.2-liter plastic containers filled with 1 L of substrate consisting of commercial garden soil (Biolan, Eura, Finland) and quartz sand (Saulkalne S, Saulkalne, Latvia), 1:1 (*v*/*v*). Plants were placed in an automated greenhouse (HortiMaX, Maasdijk, The Netherlands). Supplemented light was provided by Master SON-TPIA Green Power CG T 400 W (Philips, Amsterdam, The Netherlands) and Powerstar HQI-BT 400 W/D PRO (Osram, Munich, Germany) lamps (photon flux density of photosynthetically active radiation 380 µmol m^{-2} s^{-1} at the plant level) for a 16-h photoperiod. Day/night temperature was 23/16 °C, and relative air humidity was maintained at 60 to 70%. Substrate moisture was kept at 40–50% using deionized water.

For Experiment 1, uniform *H. maximum* plants with 2–3 newly formed leaf pairs were randomly distributed in seven groups used for treatments, with five plants per treatment. Sulfate salts of Cu, Zn, and Mn were used, each at two doses (0.1 and 1.0 g L^{-1} Cu, 0.2 and 1.0 g L^{-1} Zn, and 0.2 and 1.0 g L^{-1} Mn, indicated as the amount of the respective metal applied to 1 L of substrate), plus untreated control plants. Treatment with salts dissolved in deionized water was performed gradually within a week at the beginning of March. Plants were fertilized after three weeks using Yara Tera Kristalon Green fertilizer (Yara International, Oslo, Norway). A stock solution was created containing 150 g L^{-1}, and each plant received 250 mL of working solution made from 50 mL stock solution per 7 L^{-1} of deionized water.

For Experiment 2, uniform plants with 3–4 newly formed leaf pairs were randomly distributed in eight groups used for treatments, with five plants per treatment. Sulfate salts of Mn were used at seven doses (0.02, 0.05. 0.1, 0.2, 0.5, 1.0, and 2.0 g L^{-1} Mn, indicated as the amount of the respective metal applied to 1 L of substrate), plus untreated control plants. Treatment with salts was performed gradually within two weeks in the first half of May. Plants were fertilized after three and six weeks using Yara Tera Kristalon Green fertilizer as described above.

During plant cultivation, non-destructive analyses of leaf chlorophyll concentration and chlorophyll *a* fluorescence were performed weekly. The two largest photosynthetically most relevant leaves from adjacent nodes per plant were selected for analysis. Leaf chlorophyll concentration was measured using a CCM-300 chlorophyll meter (Opti-Sciences, Hudson, NH, USA). Chlorophyll *a* fluorescence was measured in leaves dark-adapted for at least 20 min using a Handy PEA fluorometer (Hansatech Instruments, King's Lynn, UK). Fluorescence data analysis was performed with PEA Plus software (Hansatech Instruments, King's Lynn, UK). A multiparametric fluorescence indicator and the Performance Index

Total, combining information on the status of both photosystems as well as the electron flow between the two systems on an absorption basis, were used for characterization of the photochemical efficiency of photosynthesis [44].

The total soluble salt concentration during cultivation was estimated as electrical conductivity (EC) using a HH2 meter equipped with a WET-2 sensor (Delta-T Devices, Burwell, UK). Measurements were performed at four sides of each container.

At termination of the experiments, plants were harvested and separated into individual parts (stems, leaves, new shoots, and inflorescences). For Experiment 1, the leaves present on the plants during the treatment period were designated as "old leaves", and the ones formed after the treatment were designated as "new leaves". Plant roots were separated from substrate and carefully washed to remove any adhered soil particles. All parts were weighed and individually dried in an oven at 60 °C for at least 72 h, after which dry biomass was measured.

Dried plant tissues were used for chemical analysis. Tissues were ground with an IKA A11 basic analytical mill (IKA-Werke, Staufen, Germany) to a fine powder. A 1-gram sample was weighed, dry-ashed in concentrated HNO_3 vapors, and dissolved in HCl:water (3:100, v/v). The amounts of Cu, Zn, and Mn were measured with an AAnalyst 700 atomic absorption spectrophotometer (PerkinElmer, Waltham, MA, USA), using acetylene–air flames. Three individual samples per treatment were analyzed.

The results were analyzed with KaleidaGraph (v. 5.0, Synergy Software, Reading, PA, USA). The statistical significance of differences was evaluated by one-way ANOVA using post-hoc analysis with minimum significant difference. Significant differences were indicated at $p < 0.05$.

5. Conclusions

The accession of *H. maximum* used in the present study was found growing in a drift line habitat on a coast of the Baltic Sea with relatively high substrate salinity, indicating the presence of putative tolerance mechanisms against osmotic stress and ion toxicity. As these basic mechanisms operate also in the case of resistance against heavy metals, it was hypothesized that *H. maximum* plants could tolerate high substrate heavy metal concentrations. In conditions of vegetation pot experiments in a greenhouse, these plants indeed showed a high level of tolerance against the biogenous heavy metals Cu, Zn, and Mn. A high accumulation potential for Cu and a very high potential for accumulation of Zn and Mn were found, suggesting that this particular accession of *H. maximum* has very good potential for use in practical phytoremediation. However, more studies are necessary to understand the particular physiological mechanisms of metal tolerance and accumulation potential in this species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/stresses2040031/s1, Figure S1: Typical *Hylotelephium maximum* plants 2 (above) and 4 (below) weeks after full treatment; Figure S2: Typical *Hylotelephium maximum* plants 5 (above), 6 (middle), and 8 (below) weeks after full treatment.

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