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Toxic Effects of Thallium on Biological Indicators of Haplic Chernozem Health: A Case Study

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Abstract: Thallium (Tl) was introduced into Haplic Chernozem in the amounts of 3, 30, and 300 mg/kg, and biological indicators were observed at 10, 30, and 90 days after incubation in the laboratory experiment. An increase in biological activities; i.e., the total number of bacteria, *Azotobacter* spp. abundance, enzymes (catalase, dehydrogenases), and phytotoxic indicators (germination rate of radish) after 30 days of Tl exposure were noted. The total number of bacteria and *Azotobacter* spp. abundance, enzyme activity, and phytotoxicity were more sensitive (16–76%) and informative (12–65%) indicators compared to the control, respectively. Integral biological indicators of soil state (IIBS) noted at 10, 30, and 90 days decreased at a dose of 30 and 300 mg/kg by 13–43% in relation to the control. An increase in Tl concentration and duration of exposure (up to 90 days) inhibited biological properties and caused ecotoxicological effects, respectively. We concluded that the use of individual indicators served as an indicator of the state of the soil.

Keywords: thallium; pollution; soil; ecotoxicity; biological activity

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1. Introduction

Chemical contamination of the soil is most often represented by a mixture of substances in inorganic and organic forms (heavy metals, petroleum hydrocarbons and polycyclic aromatic hydrocarbons) [1–3]. Numerous works are devoted to the analysis of the detrimental impacts of coal stations on the biological conditions of soils and ecosystems [4,5]. However, not all heavy metals have received comparable attention, and thallium (Tl) is the least explored one. The United States Environmental Protection Agency (US EPA) considers Tl as a priority role pollutant [6]. The investigations of Tl have been mainly focused on its content in soils. Priority sources of soil pollution with Tl include: coal combustion at thermal power plants, the operation of cement plants, ferrous and nonferrous metallurgy enterprises, the use of rodenticides, and hydrocarbon oil products [7–9]. The toxic form of Tl relies on its oxidation state: Tl^{3+} is more toxic than Tl^{2+} [8,10,11].

Tl is present in the soil in very small amounts; i.e., less than 1 mg/kg [10,12,13], but it is reported to be highly toxic [14–16] even more toxic than Hg, Cd, Pb, Cu, and Zn [17,18]. The range of Tl detection in areas of pyrite deposits was from 5 to 15 mg/kg, and near sulfide deposits was from 40 to 124 mg/kg [19–21]. An excess of the background content of Tl in the soil by hundreds of thousands of times was recorded near coal mines, and

reached up to 20,000 mg/kg [22]. Some studies revealed that the content of Tl can be up to 7–19 mg/kg in polluted soils [11,19,23]. As a result of the accumulation of Tl in crops, the risk of introducing the element into the food chain is quite significant [24,25]. Horticultural crops can store up to 1100 mg/kg Tl [9].

The assessment of the ecotoxicity of potential toxic elements (PTE) can use microbial properties such as active number of bacteria, the activity of enzymes, and phytotoxicity indicators. The total number of bacteria in the soil reflects the state of reducers in the ecosystem [26]. It can be determined by the luminescence microscopy method while considering the number of soil bacteria after staining with acridine orange dye [27]. Acridine orange is a fluorochromatic dye that binds to nucleic acids in bacteria and other cells. Under the influence of ultraviolet radiation, acridine orange stains ribonucleic acid (RNA) and single-stranded DNA in orange (as soil particles), and double-stranded DNA in green (as bacterial cells). *Azotobacter* spp. abundance traditionally has been used to indicate chemical pollution of soils [28]. Oxidoreductases (as catalase, dehydrogenases, peroxidases, and polyphenoloxidases) are functionally necessary for the decomposition of pollutants, the transformation of organic matter, and the maintenance of the metabolism of microorganisms [29,30]. Catalase activity decreases with soil contamination by petroleum hydrocarbons and heavy metals [1,31–33]. The activity of catalase is related to the metabolic activity of aerobic organisms, and is often used as one of the indicators of soil fertility. The phytotoxic properties of soil were analyzed in terms of the intensity of initial seed growth (germination rate) and length of the roots of radish (*Raphanus sativus* L.) seedlings. Compared to other plant test objects, radish showed a fast response to soil nutrients and moisture [34]. The germination rate and root length of radish were the most informative of the many indicators of soil phytotoxicity [31,35–37]. The use of these biological indicators will open up many opportunities for both cleaning and restoring soil fertility after contamination with various PTEs.

Assessing the effect of Tl on the state of soils and terrestrial ecosystems, establishing the limits of soil resistance to Tl pollution, and standardizing the Tl content in soil are quite urgent tasks. When assessing soil resistance to Tl pollution, it is advisable to use biological indicators, as in the case of other chemical soil pollutants [26,38]. Thus, the objective of the current work was to evaluate the ecotoxicity of Tl by using biological indicators of the condition of Haplic Chernozem health as a case study.

2. Materials and Methods

2.1. Soil Sampling and Site Description

Soil samples were collected from the top layer of Haplic Chernozem from the territory of the Persianovskaya steppe reserve (Rostov region, Russia) [39,40]. The Persianovskaya Steppe Nature Reserve is virgin soil, and the site is listed in the *Red Book of Russian Soils* [41]. In addition, on the territory of the steppe, many rare plants grow (more than 17 species), such as *Túlipa biebersteiniána* L. and *Bellevalia sarmatica* L. Haplic Chernozem was characterized by high soil organic matter (4.0%), the neutral reaction of the medium (pH = 7.6), heavy–loamy granulometric composition with high biological activity: total number of soil bacteria (3.5×10^9 per gram of soil dry weight), catalase activity (8.4 mL O_2 in a gram of soil dry weight for 1 min), dehydrogenases activity (14.5 mg TPF in a gram of soil dry weight for 24 h), and *Azotobacter* spp. abundance (100% of the fouling lumps).

2.2. Spiking of Thallium into Haplic Chernozem and Experimental Conditions

The collected soil was contaminated with Tl in the form of Tl_2O_3 in laboratory conditions. The form of Tl^{3+} was considered due to it being more toxic than Tl^{2+} [42,43]. Thallium was introduced in the amounts of 3, 30, and 300 mg/kg in a mortar with a small volume of soil and then thoroughly mixed with the entire volume of soil. After that, water was added to laboratory vessels with Tl and soil (Figure 1). The use of oxides excluded the influence of accompanying anions on the properties of the soil, which is characteristic of salts of macro- and microelements. The Tl background content in Haplic Chernozem was

defined by an ELAN-DRC-e inductively coupled plasma mass spectrometry instrument (Perkin Elmer, Inc., Waltham, MA, USA; range 10^{-5} to 0.1%).

Figure 1. Scheme of experiments under the laboratory conditions.

The content of Tl in Haplic Chernozem before pollution was 0.98 mg/kg. Soil (1 kg) was incubated in plastic vessels in triplicate at room temperature (20–22 °C) and optimal moistening (25% of water field capacity) and soil density (1.2 g/cm^3). The frequency of assessment of biological activity of Haplic Chernozem was 10, 30 and 90 days after contamination.

2.3. Determination of Biological Indicators

The total number of bacteria, *Azotobacter* spp. abundance, activity of catalase and dehydrogenases, and phytotoxic properties (the germination rate of radish seeds) of soil were determined. The total number of bacteria in the soil was measured using luminescent microscopy and *Azotobacter* spp. abundance by the number of lumps fouling in the Ashby medium. The number of soil bacteria was determined by the luminescence microscopy method while considering the number of soil bacteria after staining with acridine orange [44]. The results were expressed in 10^9 bacteria in 1 g of soil (Equation (1)):

$$M = \frac{4 \times A \times H \times 10^{10}}{P} \quad (1)$$

where M—number of cells per 1 g of soil; A—the average number of cells within one field of vision; H—dilution index; and P—the area of the field of vision in μm^2.

The enzyme activities such as catalase were determined by the decomposition rate of H_2O_2, and dehydrogenases by the rate of conversion of triphenyltetrazolium chloride (TPC) into triphenylformazan (TPF). The activities of catalase and dehydrogenases, total number of bacteria, length of roots, and germination rate of the radish seeds were summed [45,46]. Thereafter, the average assessment point of studied indices was calculated for each variant using Equation (2):

$$B = \frac{B_1 + B_2 + \ldots + B_n}{N} \quad (2)$$

where B—average estimated score of indicators; $B_1 \ldots .B_n$—the relative score of the indicator; and N—the number of indicators.

The integral index of the soil biological state (IIBS) was calculated using Equation (3):

$$IIBS = \frac{B}{B_{max}} \times 100\% \quad (3)$$

where B—the average estimated score of all indicators; and B_{max}—the maximum estimated score of all indicators.

Soil phytotoxicity was investigated by the germination rate of radish in moisture conditions of 25% and temperature conditions of 24–25 °C in the growth chamber after 7 days (Binder KBW 240).

The indices of the intensity of the initial growth of the radish seeds (length of radish shoots and roots) were calculated as the average of the triplicates [44]. The indicator of seed germination rate was calculated using Equation (4):

$$G = \frac{n_1 + n_{2...}}{2} \quad (4)$$

where G—seed germination; n_1—number of the seed of 1st replicate; n_2—number of the seed of 2nd replicate and so on.

For the calculation of IIBS, the value of each of the above indicators on the control was taken as 100%. The percentages in other experimental variants (in polluted soil) were expressed as a percentage relative to the control. For the IIBS condition, a maximum value of each index (100%) was chosen from the array data, and approximately this value of the index was expressed for other variants of experiments using Equation (5):

$$B_1 = \frac{B_x}{B_{max}} \times 100\% \quad (5)$$

where B_1—the relative score of the indicator; B_x—the actual value of the indicator; and B_{max}—the maximum value of the indicator.

The activities of catalase and dehydrogenases indicated the potential biological activity of the soil. Based on the results of determining the biological parameters, the IIBS of the soil was calculated [47]. During diagnostics of contamination value of each index in non-contaminated soil, it was taken as 100%. With reference to its value, the same index in the contaminated soil was expressed in percent. The obtained IIBS value was expressed as a percentage with respect to the control (to 100%). The methodology used allowed the integration of the relative values of different indicators, which ordinarily cannot be integrated since they have different units of measurement.

2.4. Statistical Analyses

An analysis of variance (ANOVA) was carried out to check the reliability of the results, followed by the determination of the least significant difference (LSD) at $p \leq 0.05$. Data are means of triplicate biological samples. Statistical data processing was carried out using Statistica 12.0 and Python 3.6.5 Matpolib package.

3. Results

3.1. Influence of Thallium on Microbiological Indices

The results of the influence of Tl at different concentrations (3, 30 and 300 mg/kg) on the total number of bacteria in Haplic Chernozem after 10, 30 and 90 days of pollution are shown in Figure 2A. Doses of 3 mg/kg caused a significant ($p < 0.05$) decrease in the total number of bacteria by 46, 40 and 39% compared to the control for 10, 30 and 90 days of pollution, respectively. The inhibition of the total number of bacteria was observed at 61, 63 and 56% relative to the control when Tl was introduced into the soil in the amount of 30 mg/kg ($p < 0.05$). Similarly, the total number of bacteria decreased by 68–76% relative to the control values at 300 mg/kg ($p < 0.05$). The results of the influence of the Tl in the studied concentrations (3, 30 and 300 mg/kg) on the *Azotobacter* spp. abundance are shown in Figure 2B. The statistically reliable decrease in the *Azotobacter* spp. abundance was observed in the soil contaminated by Tl in the amount of 3 mg/kg ($p < 0.05$). The percent decline caused by the 3 mg/kg of Tl was 16, 18 and 19% after 10, 30 and 90 days of pollution, respectively. The inhibition of *Azotobacter* spp. abundance was observed at 27, 21 and 26% relative to the control when Tl was introduced into the soil in the amount of

30 mg/kg ($p < 0.05$). The *Azotobacter* spp. abundance decreased by 36–40% relative to the control values at 300 mg/kg ($p < 0.05$).

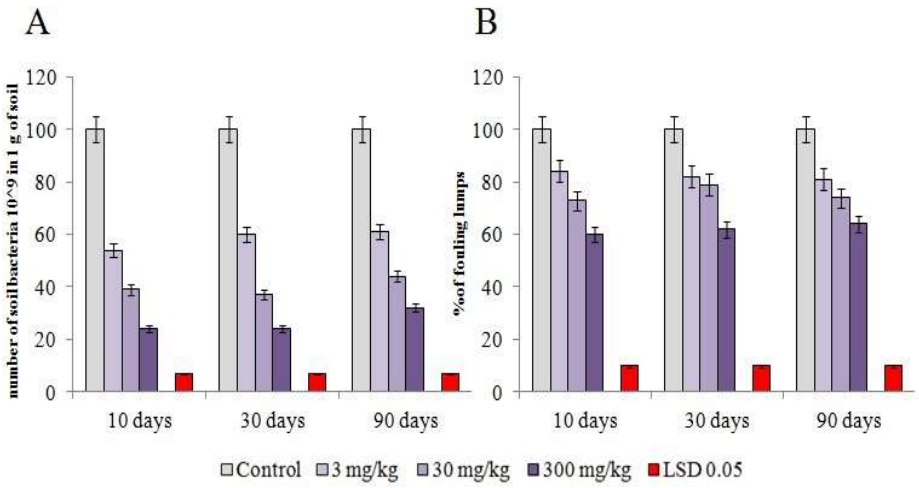

Figure 2. Effects of thallium on microbiological properties of Haplic Chernozem after 10, 30, and 90 days (% of control): (**A**) the total number of bacteria; (**B**) *Azotobacter* spp. abundance.

3.2. Influence of Thallium on the Activity of Enzymes of Soil

Enzymatic activities (catalase and dehydrogenases) of Haplic Chernozem after Tl pollution were altered (Figure 3). The results of the influence of the Tl at the studied concentrations (3, 30 and 300 mg/kg) on the activity of catalase of Haplic Chernozem are shown in Figure 3A. Thallium in the amount of 30 mg/kg caused a decrease in the catalase activity by 19, 12 and 16% ($p < 0.05$) with respect to the control for 10, 30 and 90 days of exposure. Likewise, the decline in the activity of catalase ranged from 61–65% relative to the control at 300 mg/kg.

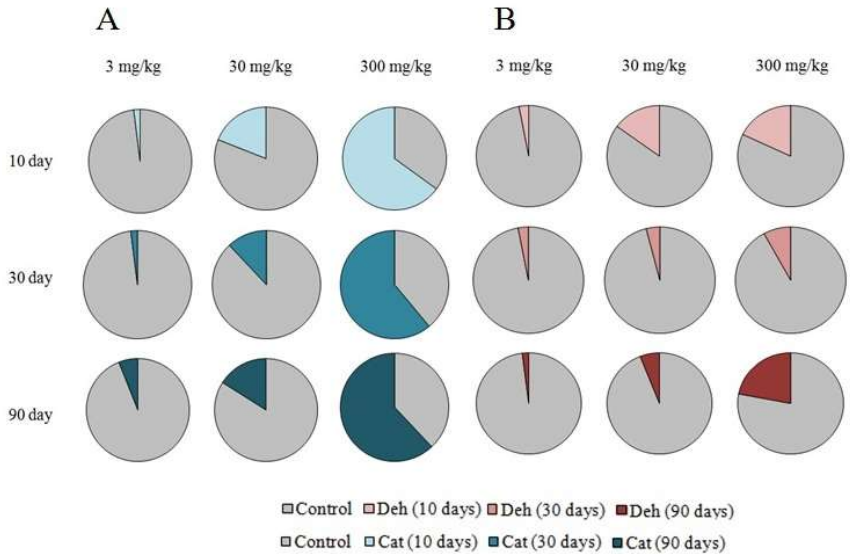

Figure 3. Effects of thallium on the enzymatic activity of Haplic Chernozem after 10, 30 and 90 days (% of control): (**A**) activity of catalase (Cat in legend); (**B**) activity of dehydrogenases (Deh in legend).

Figure 3B shows the results of the influence of Tl at concentrations of 3, 30 and 300 mg/kg on the activity of dehydrogenases in the Haplic Chernozem. The Tl at 30 mg/kg for 10 days of exposure caused a decrease in dehydrogenase activity by 15% ($p < 0.05$) relative to the control. The activity of dehydrogenases at 300 mg/kg decreased by 18 and 22% ($p < 0.05$) in comparison to the control after 10 and 90 days of exposure, respectively.

3.3. Influence of Thallium on the Germination Rate of Radish

The results of the influence of the Tl at the studied concentrations (3, 30 and 300 mg/kg) on the radish germination rate are shown in Figure 4. A statistically unreliable stimulating effect of Tl at the concentrations of 3 and 30 mg/kg on the radish germination rate was recorded. At such doses of pollution by Tl, disturbances in the growth and development of radish were not found. A dose of 300 mg/kg of Tl inhibited seed germination by 17 and 19% as compared to the control for 10 and 30 days of exposure, respectively.

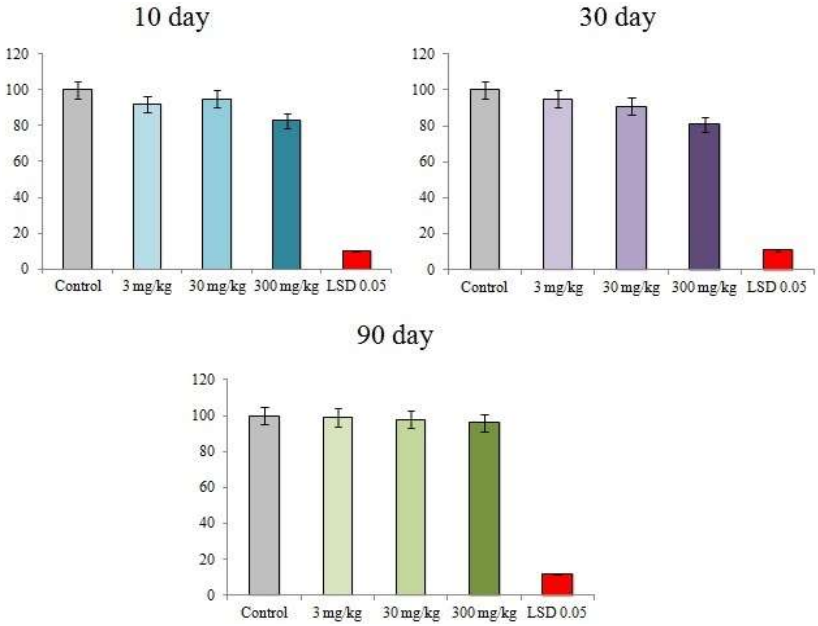

Figure 4. Effects of thallium on germination rate of radish in Haplic Chernozem after 10, 30 and 90 days (% of control).

3.4. Integrated Index of the Biological State of Soil Contaminated with Thallium

The enzymes of the class of oxidoreductases (activity of catalase and dehydrogenases) were more sensitive to chemical pollution than other classes of enzymes. Figure 5 shows the results of the influence of Tl at the concentrations of 3, 30 and 300 mg/kg on IIBS in Haplic Chernozem after 10, 30 and 90 days of contamination. Tl in the concentration of 3 mg/kg caused a significant decrease in the indicator by 15, 14 and 13% ($p < 0.05$) after 10, 30 and 90 days relative to the control. The Tl decreased IIBS at the 30 mg/kg concentration by 21, 22, and 25% ($p < 0.05$) after 10, 30 and 90 days of treatment relative to the control. A dose of 300 mg/kg of Tl reduced IIBS by 43, 40, and 38% after 10, 30 and 90 days, relative to control values. Thus, the degree of reduction was observed to be dose-dependent, and also relied on the exposure period of Tl contamination. When Haplic Chernozem was polluted with Tl, the degree of decrease in the biological properties of the soil directly depended on its concentration in the soil. This pattern was noted in most cases. A month after the pollution of Haplic Chernozem with Tl, the biological properties of the soil were subjected to the strongest negative influence. The biological indicators of Tl-contaminated Haplic Chernozem showed a tendency to recovery three months after incubation. Nevertheless, the biological activity of Haplic Chernozem, even after 90 days, did not reach the value of uncontaminated soil (Table 1).

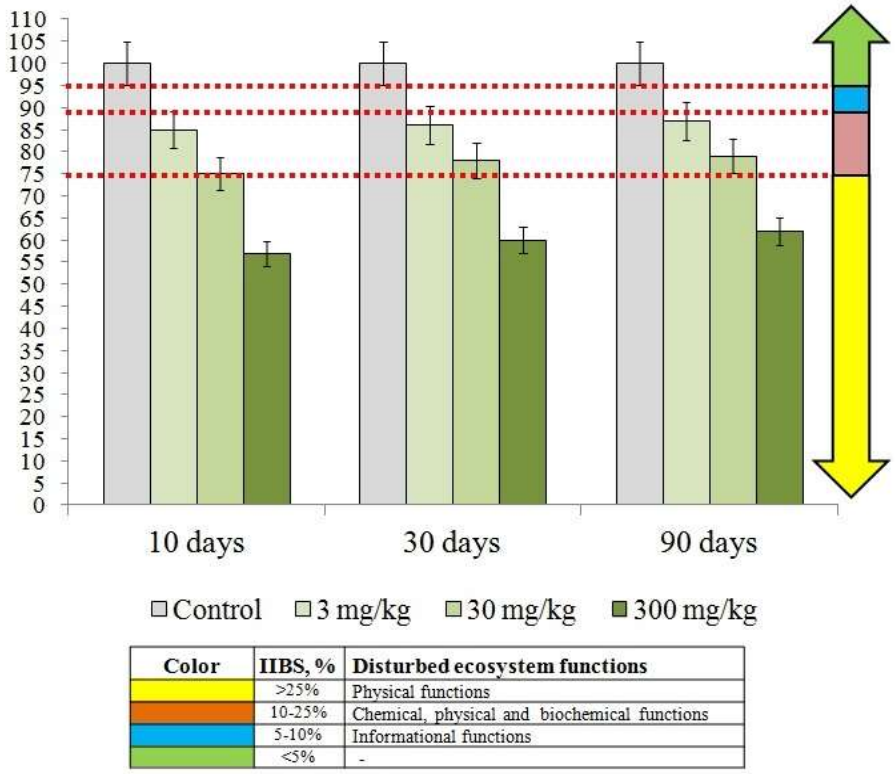

Figure 5. Effects of thallium pollution on the Integrated Index of Biological State (IIBS) of Haplic Chernozem after 10, 30, and 90 days (% of control).

Table 1. Assessment of biological indicators of Haplic Chernozem conditions contaminated with thallium by sensitivity and informativeness.

Indicator	Degree of Sensitivity [1]	Informative Value [2]
Number of soil bacteria	57	−0.70
Azotobacter spp. abundance	59	−0.83
Activity of catalase	88	−0.90
Activity of dehydrogenases	79	−0.88
Germination rate of radish	93	−0.94
IIBS	75	−0.87

[1] Sensitivity of the biological indicator as the intensity of the decrease in the biological indicator of Haplic Chernozem contaminated by Tl, % of uncontaminated soil (average values are indicated for doses and incubation periods). [2] The informative value of the indicator is the correlation coefficient (r) between the amount of thallium in Haplic Chernozem and the biological indicators ($p = 0.05$).

In order to use biological indicators for monitoring and diagnostics of soils contaminated by Tl, as well as for standardizing the Tl content in soils and terrestrial ecosystems, it was necessary to assess the degree of sensitivity and information content of these indicators. The degree of sensitivity of biological indicators was assessed by the intensity of their decrease when polluted with various concentrations of Tl relative to values in uncontaminated soil. The degree of information content of biological indicators in the contamination of Haplic Chernozem with Tl was assessed by the tightness of the correlation between the indicator and the amount of the polluting element in the soil.

Several biological indicators reflecting the degree of their sensitivity (Table 1) by Tl contamination had the following sequence (the % changes in the indicator relative to the control are in parentheses):

Number of soil bacteria (57) ≥ Azotobacter spp. abundance (59) ≥ activity of dehydrogenases (79) > activity of catalase (88) > germination rate (93).

Biological indicators for pollution of Haplic Chernozem with Tl in terms of the degree of information content were arranged in the following order, as described in Table 1:

Germination rate (−0.94) > activity of catalase (−0.90) > activity of dehydrogenases (−0.88) > Azotobacter spp. abundance (−0.83) > number of soil bacteria (−0.70).

The greatest sensitivity to Tl pollution was shown by the total number of soil bacteria and the *Azotobacter* spp. abundance belonging to the group of microbiological indicators. The most informative indicators for pollution of Haplic Chernozem with Tl are enzymes belonging to the class of oxidoreductases (catalase and dehydrogenases) and phytotoxicity indicators (radish seed germination). In addition to Tl, similar patterns apply to most heavy metals and metalloids. The choice of biological indicators for assessing the state of soil when contaminated with Tl was due to the following reasons. The total number of bacteria in the soil characterizes the state of the destructors in the ecosystem. *Azotobacter* spp. abundance is traditionally used as an indicator for assessing the ecotoxicity of chemical elements for the soil. The activity of catalase and dehydrogenases reflects the intensity of mineralization processes in the soil. Among the enzymes, oxidoreductases are the most sensitive to chemical contamination.

4. Discussion

At a low level of soil pollution with Tl, in isolated cases, statistically insignificant stimulating effects of biological activity were noted [6,9,18,48,49]. Statistically insignificant stimulation of the *Azotobacter* spp. abundance trended toward increase in significance with an increasing dose up to 300 mg/kg and duration of Tl exposure up to 90 days. When the soil was contaminated with Tl, the nitrogen balance in the soil did not change, and therefore, *Azotobacter* spp. abundance in the soil depended little on the concentration and duration of Tl exposure [50]. For radish germination, recovery was noted with an increase in exposure to 90 days. This was probably due to the adaptation and transition of Tl to the vegetative parts of the plant [51,52]. A decrease in the permeability of biological membranes, inhibition of enzymes, and, as a result, metabolic disorders, were consequences of the toxic effect of Tl on the biological activity of soils, as well as plants [15,53]. The concentration of the pollutant in the soil determines the sequence of violations of the ecological functions of the soil: first of all, informational, then biochemical, physicochemical, chemical, integral, and then physical.

The soil IIBS is a well-recognized indicator that depicts a disturbance in ecosystem functions. A decrease in IIBS by less than 5% reflects no disturbance in the ecosystem functions. However, a decrease in the value of IIBS by more than 5% shows disturbed functioning in an ecosystem; e.g., a decrease of 5–10% means a violation of informational functions; of 10–25% leads to biochemical, physicochemical, chemical, and integral dysfunction; and of more than 25% is associated with physical properties [47]. Serious disturbances in the functioning of soils caused by Tl contamination were diagnosed when the integral indicator of the IIBS decreased by more than 10%. The concentration of Tl causing such a decrease can be considered the maximum permissible concentration (MPC) of this element in the soil. Based on the regression equations, it is possible to calculate the dependence of the degree of decrease in IIBS values on the concentration of Tl in the soil. The level of decrease in IIBS values, depending on the Tl content, determines the order of the disturbance of the ecosystem functions of the soil that occurred [47]. At a minimum concentration, a violation of information functions occurs, and further with an increase in the content of a pollutant of biochemical, physicochemical, chemical, and integral dysfunction; and at maximum, physical functions. Thallium concentrations calculated using the regression equations causing a violation of certain ecological functions are presented in Table 2.

Table 2. Environmental regulation (for MPC) of thallium pollution of Haplic Chernozem by the degree of violation of ecosystem functions.

Properties	Degree of Violation of Ecosystem Functions			
	Minimal	**Slight**	**Moderate**	**Maximal**
Degree of soil IIBS reduction [1]	<5%	5–10%	10–25%	>25%
Violated ecosystem functions [2]	-	Informational	Chemical, physico-chemical, biochemical, holistic	Physics
Thallium content, mg/kg	<1.4	1.4–2.5	2.5–20	>20
Soil remediation methods	Not required	Phytoremediation	Chemical reclamation	Removing contaminated soil

Note: [1] determination of IIBS of soils according to Kolesnikov et al. [47]. [2] Classification of ecosystem functions of soil.

According to Table 2, rationing of soils contaminated with Tl is directly related to the violation of the ecological functions of the soil. If the concentration of Tl does not exceed 0.5 mg/kg, there is no disruption of the soil's ecological activities. The information functions of soil contaminated with Tl were documented to be disturbed when its concentration ranged from 1.5 to 4.4 mg/kg. If the concentration of Tl in the soil is from 4.4 to 106.0 mg/kg, then the chemical, physico-chemical, and integral functions of the soil will be violated. The physical functions of the soil will be impaired if the Tl concentration exceeds 106.0 mg/kg. Thus, violation of integral functions in soil contamination with Tl has serious consequences for soil fertility.

Thallium concentration of 4.4 mg/kg in Haplic Chernozem causing a violation of integral functions can be considered the MPC. When carrying out a wide range of environmental activities aimed at assessing the impact of Tl on the environment, monitoring soils and terrestrial ecosystems contaminated with Tl, and choosing methods for restoring Tl-contaminated soils. The MPCs for Tl developed in this work can be used to normalize soil pollution. Soils of different regions of the world, similar to Haplic Chernozem in terms of resistance to heavy metal pollution [54,55], can be normalized using the MPC of Tl in the soil established as per this work, which was 4.4 mg/kg. Soils were similar in the terms of stability, and had a similar grain size composition, pH, and soil organic matter content [31]. Methods for the restoration of Tl-contaminated soils, depending on the concentration that had the highest efficiency, are presented in Table 2. Reorganization of Haplic Chernozem is not required if the concentration of Tl in the soil does not exceed 0.5 mg/kg.

It seems promising to continue studies of the toxic effects of Tl on the ecological state of soils in the following directions: an intensive assessment of the ecotoxicity of different doses of Tl in soils from the background concentration up to 20,000 mg/kg; to determine the toxicity of different chemical forms of Tl for soil; to study changes in soil properties under Tl pollution with dynamics (from 10 to 360 days); to study a wider range of biological indicators of soil contamination with Tl; and to investigate the joint effect of Tl polluted with complicated other pollutants in order to identify synergistic and antagonistic effects in relation to soil properties. The relationships between Tl and microbial communities in soil with different Tl-contamination levels were noted [56]. The toxic effects of Tl on soil biological properties and its accumulation in plant tissues were also observed by several researchers [16,57,58].

5. Conclusions

According to the results of the present study, it was found that when Haplic Chernozem was contaminated with Tl, for all biological indicators, the maximal significant impact was observed on the total number of soil bacteria; i.e., *Azotobacter* spp. abundance and catalase activity, while a less obvious toxic effect of Tl was noticeable in relation to the activity of dehydrogenases and a phytotoxical indicator (the germination rate of radish seeds). The highest sensitivity to Tl contamination was established by the number of soil bacteria. However, at low Tl concentrations, there was no significant difference from the control in terms of the activity of dehydrogenases and the germination rate of radish seeds. The IIBS, which characterized the state of the soil biota, varied from 13 to 43% depending on the Tl concentration. We concluded that the results showed toxic effects of Tl on the compositions of soil bacterial communities and enzyme activity.

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Conflicts of Interest: The authors declare that they have no competing interests.

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