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$\delta^{15}N$ in Birch and Pine Leaves in the Vicinity of a Large Copper Smelter Indicating a Change in the Conditions of Their Soil Nutrition

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Abstract: $\delta^{13}C$ and $\delta^{15}N$ were analyzed in the leaves of *Pinus sylvestris* L. and *Betula* spp. under the conditions of severe heavy metal (Zn, Cu, Cd, and Pb) contamination. Twenty-seven plots located near the Karabash copper smelter (Russia) were studied. No reliable correlation of ^{13}C in tree leaves with the level of pollution was observed. $\delta^{15}N$, both in *Pinus sylvestris* and *Betula* spp., increased similarly in polluted areas. $\delta^{15}N$ was increased by 2.3‰ in the needles of *Pinus sylvestris* and by 1.6‰ in the leaves of *Betula* spp. in polluted plots compared to the background ones. The probable reasons for the increase in $\delta^{15}N$ were estimated using multiple regression. The regression model, which includes two predictors: $\delta^{15}N$ in the humus horizon and the occurrence of roots in the litter, explains 33% of the total variability of $\delta^{15}N$ in leaves. Thus, in ecosystems polluted with heavy metals, the state of trees is determined not only by the direct toxic effects of heavy metals but also by indirect ones associated with the features of plant mineral nutrition. This fact opens the way to the search for opportunities to control the state of plants in disturbed ecosystems by regulating the content of mineral nutrition elements.

Keywords: anthropogenic pollution; heavy metals; $\delta^{13}C$; $\delta^{15}N$; *Pinus sylvestris*; *Betula* spp.; leaves; soil; litter

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1. Introduction

The stable isotopic ratios of carbon $^{13}C/^{12}C$ and nitrogen $^{15}N/^{14}N$ in living organisms and other components of ecosystems are indicators (tracers) of many physiological and environmental processes [1–5]. The $\delta^{13}C$ in plants is determined by the nature of their photosynthesis, the life span of leaves, the canopy structure of photosynthetic organs, and environmental conditions [2,3,6,7]. The value of $\delta^{15}N$ reflects the ^{15}N content in soil, the diversity of nitrogen sources and the ability of symbiotic nitrogen fixation, as well as the presence of other symbioses [8–10]. The differences in plant species and functional groups in terms of $\delta^{15}N$ can indicate the general level of nitrogen supply of ecosystems, as well as the degree of its availability and the competition for it [11–14]. Since the isotopic composition of nitrogen reflects the changes in edaphic conditions in ecosystems as a whole [4], its analysis is used in the study of ecosystems processes [13,15–17], including successions [18,19].

Under the conditions of anthropogenic impact, $\delta^{13}C$ and $\delta^{15}N$ in plants change in different ways. For example, $\delta^{13}C$ in tree rings might be increased [4,20,21] or decreased [22–24] during pollution. Recently, an increase in $\delta^{13}C$ in plant leaves near a large metallurgical smelter was demonstrated [25]. The $\delta^{15}N$ may also be increased [26–29] and decreased [23,24] during urbanization and under the influence of gaseous pollutants. In forest habitats polluted with heavy metals, $\delta^{15}N$ in plants increases [25]. Despite the majority of published results revealing that $\delta^{13}C$ and $\delta^{15}N$ increase under anthropogenic impacts, it is still difficult to draw unambiguous conclusions. This can be explained by a wide variety of combinations of different types of impacts, geographical and landscape conditions, and taxonomic and functional specifics of plants. This indicates the need for the further accumulation of data on the features of $\delta^{13}C$ and $\delta^{15}N$ distribution in plants under anthropogenic impacts.

Previously, a unidirectional increase in the content of ^{15}N isotope in the plants of several functional groups near a large metallurgical smelter in the taiga zone of Eurasia was established [30,31]. Nevertheless, the changes in $\delta^{13}C$ in plants have not been observed with an increase in heavy metal pollution. The difference in $\delta^{15}N$ values between uncontaminated and polluted forests in the leaves of plants of the same functional groups, ectomycorrhizal, with ericoid and arbuscular mycorrhiza is about 2–3.5‰. This is comparable to the differences in $\delta^{15}N$ between different soil horizons [12,32] or between plants having different methods of soil nutrition [8]. Thus, the changes in $\delta^{15}N$ under heavy metal pollution were found to be significant. This result suggests that pollution is either accompanied by a change in nitrogen sources for plants or nitrogen uptake mechanisms by plants.

However, our past materials [30,31] were only partly reliable due to some methodological features. The $\delta^{13}C$ and $\delta^{15}N$ measurements from 10 plots were available. The plots were unevenly distributed along the heavy metal pollution gradient. Eight sites were located in the zone of severe pollution and only two in forests with a background level of pollution. Previously, no comparison between $\delta^{13}C$ and $\delta^{15}N$ in plants and soils was made. In this paper, the main conclusions obtained earlier in the study of $\delta^{13}C$ and $\delta^{15}N$ across the gradient of strong anthropogenic pollution are verified, and some assumptions about the reasons for the change in $\delta^{15}N$ considered as possible hypotheses are tested. As opposed to our first research cycle [31,32], the present work is limited to the analysis of the reactions of trees only.

The aim of the present work is to analyze the changes in the composition of stable carbon and nitrogen isotopes in trees under the conditions of strong transformation of natural ecosystems by the emissions from a large copper smelter in the Southern Urals. Based on published information and our own early materials [30,31], three working hypotheses are formulated and tested. The first hypothesis is that under the conditions of heavy metal contamination, the content of heavy ^{15}N and, possibly, ^{13}C isotopes increase in tree leaves. The second and third hypotheses are assumptions about the possible mechanisms for changing $\delta^{15}N$. The second hypothesis is that the change in $\delta^{15}N$ in the trees across the pollution gradient reflects the change in $\delta^{15}N$ in soils. The third hypothesis suggests that the change in $\delta^{15}N$ in trees across the pollution gradient is associated with deeper rooting in the habitats with higher concentrations of heavy metals in soil.

2. Materials and Methods

2.1. The Area and Source of Anthropogenic Impact

The study was carried out in the area affected by the industrial emissions from the Karabash copper smelter (KCS) and in the Ilmensky State Reserve (ISR) in the Chelyabinsk region, Southern Urals (Figure 1). The area is situated in the subzone of southern taiga pine-birch forests of the eastern macroslope of the Southern Urals (the Chelyabinsk region in the vicinity of the Karabash and Miass towns). The typical heights of the uplands are 250–600 m a.s.l. Brown mountain-forest and forest, gleyed podzolic, gray mountain-forest, mountain-forest chernozems, and mountain-podzolic shallow soils are represented. The climate is continental and moderately cold. The coldest month is January (average monthly temperature is between −16 and −17 °C), the warmest month is July (+18 °C); the duration of the growing season is 160–170 days from April to September; the rainfall is about 430 mm per year; the snow cover height is up to 40 cm.

Figure 1. Location of the study area: (**a**) in Russia and (**b**) in the world. (https://www.google.ru/maps).

The prevailing types of vegetation are forb pine forests and secondary grass-forb birch forests. Regular ground-level vegetation species in the ISR in the absence of pollution are *Vaccinium vitis-idaea* L., *Vaccinium myrtillus* L., *Calamagrostis arundinacea (*L.) Roth, and *Rubus saxatilis* L. The moss cover is dominated by *Pleurozium schreberi (*Willd. ex Brid.) Mitt., *Rhytidiadelphus triquertus (*Hedw.) Warnst., *Climacium dendroides (*Hedw.) F.Weber & D.Mohr, and *Hylocomium splendens (*Hedw.) Bruch et al. Soil coverage by mosses in the ISR ranges from 50 to 100% of the soil surface with an average of 70–80%.

The ecosystems of the region are strongly anthropogenically transformed due to various contaminating impacts, including industrial pollution. The Karabash copper smelter (KCS, JSC Karabashmed, Karabash) is a major source of anthropogenic emissions. The main emission components are SO_2 and the dust of heavy metals (Cu, Zn, Pb, and Cd). Copper production started in Karabash in 1910, and the largest emissions (up to 140–360 thousand tons per year) were achieved in 1970–1980 [33]. In the period of 1989–1997, copper production stopped, and after the re-opening and modernization of production, the emissions decreased to about 10 thousand tons per year [34,35]. Due to the severe accumulated anthropogenic pollution in the territories closest to the smelter, the zonal ecosystems were completely destroyed: vegetation and the upper parts of the original soils are absent, and a vast anthropogenic wasteland has formed. The levels of accumulation of heavy metals emitted by the KCS in the two-year-old needles of *Pinus sylvestris* L. are: Cu 8–18 µg/g; Zn 70–150 µg/g; and Pb 30–105 µg/g in the impact zone of the KCS; Cu 2–3 µg/g; Zn 40–45 µg/g, and Pb 1.5–3 µg/g in the Ilmensky State Reserve [36,37]. Due to the accumulation of toxicants, plant damage occurs [33,38] and their diversity decreases [39]. The most stable species of ground-level vegetation are *Vaccinium myrtillus* L., *Calamagrostis arundinacea (*L.) Roth, *Adenophora lilifolia (*L.) A. DC., *Lathyrus pisiformis* L., *Orthilia secunda (*L.) House, *Sanguisorba officinalis* L., *Vicia cracca* L., and *Vicia sylvatica* L. The mossy groundcover near the smelter has been fully destroyed, and the ground mosses are missing.

2.2. Sample Plots

The material (leaf samples from five trees, samples of forest litter (A0 horizon) and of the soil mineral part (A1 horizon)) was collected on 27 sample plots. Fifteen areas were located at distances of 5.5–6.5 km in the northeast and south directions from the KCS (impact zone); twelve areas were located 33–50 km south of the KCS (the Ilmensky State Reserve (ISR), background zone) in pine, birch, and mixed pine-birch forests (Table 1). The areas were selected on the middle parts of the slopes on mountain fragmentary and mountain-forest brown incompletely developed soils. The criteria for selecting sample plots were as follows: (1) the forest stands must be of natural origin, i.e., not planted; (2) the age of the main generation of trees must be is close to 100 years or more; and (3) severe or recent anthropogenic disturbances such as logging and soil disturbance must be absent.

2.3. The Determination of Cu, Zn, Pb, and Cd in Litter

The concentrations of Cu, Zn, Pb, and Cd were measured in sample weights taken from the mixed samples of forest litter (A0). Quality control of the analytical procedure was performed by the analysis of the national certified reference materials (CRM). The metals were extracted from soils using nitric acid with a molar concentration of 5 mol/dm^3. The ratio of the soil sample weight and acid was 1:5. The quantitative characterization of metals was carried out by ICP-MS on an Agilent 7700X inductively coupled plasma mass spectrometer according to the certified analytical methodology (GOST R 56219-2014). The measurements were performed at a certified laboratory (accreditation certificate No. AAC.A.00330 at the time of measurements; valid until 07/31/2020).

The degree of pollution of each sample plot was characterized by the "litter pollution index":

$$\text{Litter pollution index} = \frac{1}{4} \times \sum \frac{C_\mathrm{i}}{C_\mathrm{min}} \tag{1}$$

where C_i and C_min are the concentrations of one of the four metals (Cu, Zn, Pb, or Cd) in the litter at a certain sample plot (C_i) and minimal in the entire studied range (C_min). The litter pollution index shows how many times the measured four metals are greater compared to the least contaminated plot. The litter pollution index natural logarithm (litter pollution index (*Ln*)) of this value was used for the calculations.

2.4. Litter Thickness and Root Occurrence in the Litter

In the summer (July–first half of August) of 2017, 20 measurements of the forest litter thickness were performed on each sample plot. The measurements were carried out one by one in 20 pits randomly placed across the sample plot. The thickness of the enzymatic layer of the forest litter was recorded with an accuracy of 0.5 cm. The presence or absence of roots of any plants in the litter was recorded in a plot with a 20 $\times$ 20 cm area at 20 random points.

2.5. The Collection of Leaves, Litter and Soil to Determine δ^{13}C and δ^{15}N

The leaves of 3–5 individuals of two tree taxa, *Pinus sylvestris* or *Betula* spp., were collected for isotopic analysis across each sample plot in the summer of 2017 (July–first half of August). Across the three sample plots, only *Betula* spp. leaves were selected. There were two taxonomically and ecologically similar species of birch in the region that were difficult to distinguish during mass surveys: *Betula pendula* and *Betula pubescens*. We did not differentiate these species during leaf collection. On each plot were separately collected *P. sylvestris* leaves (leaves from five separate *P. sylvestris* individuals growing on the sample plot were combined into one sample) and *Betula* spp. leaves (leaves from five separate *Betula* spp. individuals were combined into one sample). Moreover, for each plot, using the envelope method, one sample of the litter enzymatic horizon and one sample of the upper layer of the soil mineral part were taken, 3–5 cm below the litter boundary.

The samples were dried first in the shade to an air-dry state, then for 48 h at 70 °C in the laboratory's drying cabinet.

In total, 105 samples were analyzed: 51 samples of tree leaves (24 *Pinus sylvestris*—1 sample from each of 24 sample plots; 27 *Betula* spp.—1 sample from each of the 27 sample plots), 27 samples of litter (1 sample from each of the 27 sample plots), and 27 samples of soil mineral parts (1 sample from each of the 27 sample plots).

Table 1. Sample plot characteristics *.

Plot Number	Distance from the KCS, km	Heavy Metal Concentration in Litter, mg/kg				Litter Pollution Index, Times	Forest Stand Composition	Age of Major Tree Generation, Years	Canopy Cover, %	Grass-Shrub Layer Cover, %	Litter Thickness, cm	Root Occurrence in Litter, %
		Cu	Zn	Pb	Cd							
Ilmensky State Reserve												
31	37.2	29.9	244.0	64.3	1.91	3.3	10 *P.s.*	170	10	50–60	4.0	100
37K	37.5	26.5	96.4	45.9	0.86	2.0	10 *P.s.* + *B*.spp.	170	50–60	70–80	5.5	100
221\37	50.0	12.4	121.0	15.2	0.68	1.2	9 *B*.spp. 1 *P.s.*	120	50–60	80–90	2.6	90
221\28	50.0	13.7	78.0	18.6	0.60	1.1	8 *P.s.* 2 *B*.spp.	185	50	30–40	3.6	90
207\18	48.4	14.9	108.0	19.9	0.80	1.3	9 *P.s.* 1 *L.s.*	115	30–40	50–60	5.5	100
204\36	48.5	16.0	118.0	25.1	0.70	1.4	10 *P.s.*	185	50–60	50–60	3.5	90
204\7	48.1	15.9	117.0	31.8	0.88	1.6	8 *P.s.* 2 *B*.spp.	85	50–60	50–60	3.7	90
199\26	47.7	28.4	157.0	20.8	0.59	1.7	9 *B*.spp. 1 *P.s.*	175	70–80	60–70	5.6	100
199\12	47.5	19.8	137.0	29.3	0.82	1.7	5 *P.s.*1 *L.s.*4*B*.sp	105	60–70	50–60	3.9	100
198\22	48.5	18.8	127.0	26.7	0.60	1.5	9 *P.s.* 2 *L.s.*	175	50–60	70–80	6.9	100
78\13	32.8	25.4	201.5	39.7	1.10	2.3	10 *B*.spp.	115	40	60–70	3.1	90
77\20	33.0	33.7	339.0	44.0	1.41	3.1	10 *B*.spp.	115	40	70–80	2.4	100
The vicinity of the Karabash copper smelter												
186\1	6.6	1255.0	1539.0	1141.0	15.5	55.6	7 *P.s.* 3 *B*.spp.	110	50–60	10–20	3.9	90
186\4	7.0	1219.0	1956.0	1482.0	16.2	62.1	8 *P.s.* 2 *B*.spp.	120	50–60	5–10	4.3	60
186\4K	7.0	2175.0	3177.0	2580.0	25.3	107.2	8 *P.s.* 2 *B*.spp.	120	50–60	5–10	3.9	60
186\16	7.1	896.0	2491.0	987.0	22.7	51.9	9 *B*.spp1*P.t.* + *P.s*	110	30–40	<5	2.9	25
186\31	6.4	3436.0	2600.0	1353.0	22.00	109.2	10 *P.s.*	110	40	<10	5.9	40
186\35	6.5	3061.0	1987.0	2528.0	16.80	116.8	10 *P.s.* + *B*.spp.	110	30–40	<5	9.5	40
186\37	6.1	5372.0	2238.0	2274.0	14.70	159.1	10 *P.s.* + *B*.spp.	100	30–40	<5	8.6	25
185\39	5.6	1968.0	4514.0	2489.0	37.70	111.1	10 *B*.spp.	100	40–50	<5	2.7	55
175\57	8.8	640.0	1733.0	594.0	11.30	33.0	9 *B*.spp. 1 *P.s.*	90	40	40–50	3.3	75
175\56	8.6	1210.0	2408.0	1633.0	21.20	68.0	5 *P.s.* 5 *B*.spp.	90	50–60	30–4	5.3	85
175\40	8.6	1090.0	1548.0	1073.0	14.40	50.7	9 *P.s.* 1 *B*.spp.	120	40	30	5.0	95
175\39	8.5	629.0	947.0	807.0	8.98	32.8	8 *P.s.* 2 *B*.spp.	110	40	20–30	3.5	80
175\37	9.1	790.0	2278.0	1176.0	17.30	49.9	8 *B*.spp. 2 *P.s.*	95	50	30–40	2.8	95
166\50	9.1	293.0	666.0	385.0	5.48	16.7	10 *P.s.* + *B*.spp.	120	40	50–60	3.8	100
166\49	9.5	893.0	1632.0	748.0	11.60	40.5	10 *P.s.* + *B*.spp.	140	50–60	30–40	6.2	100

* "Forest stand composition" column demonstrates the number of trees of each species calculated per 10 trees; "+" indicates the tree species with number less than 10% of the total tree amount on a sample plot. Abbreviations: *P.s.*–*Pinus sylvestris*; *B*.spp.–*Betula* spp.; *L.s.*–*Larix sibirica*; *P.t.*–*Populus tremula*.

2.6. Isotopic Analyses

The determination of $\delta^{13}C$ and $\delta^{15}N$ was carried out at the "Geonauka" Center for Collective Use of the Institute of Geology, Komi Research Center, Ural Branch, Russian Academy of Sciences. Measurements were made using a helium continuous flow mass spectrometry (CF-IRMS) on an analytical complex, including a Flash EA 1112 elemental analyzer connected via a ConFlo IV gas interface to a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, USA). The isotopic composition of nitrogen and carbon were reported per mil relative to the international V-PDB and AIR atmospheric nitrogen standards, δ (‰):

$$\delta\ X_{sample} = ((R_{sample}/R_{standard}) - 1) \times 1000, \quad (2)$$

where X is the element (nitrogen or carbon) and R is the molar ratio of the heavy and light isotopes of the corresponding element. The mass spectrometer was calibrated using the USGS-40 (L-Glutamic acid) international standard and the Acetanilide (C_8H_9NO) in-house standard. The measurement error was ±0.15‰. The measured $\delta^{13}C$ and $\delta^{15}N$ values in leaves, litter, and soil are given in the Supplementary Materials.

2.7. Data Analysis

Statistical analysis was performed using the JMP 10.0.0 software (SAS Institute Inc., Cary, NC, USA, 2012). The differences in $\delta^{13}C$ and $\delta^{15}N$ due to the influence of various factors were evaluated by calculating regressions (simple and multiple) and correlations (Pearson's correlation coefficient (r)). Generalized linear models (GLM) with categorical and continuum predictors were used as well. The combinations of predictors that optimally explained $\delta^{15}N$ in plant leaves were selected using the corrected Akaike's information criterion (*AICc*) [40]. The values of $\delta^{13}C$ and $\delta^{15}N$ in each sample of soil, litter, or plant leaves of one taxon on each sample plot or the value of another feature on a sample plot were the units for statistical analysis. The measurement of variability was the standard error (±*SE*).

3. Results

3.1. $\delta^{13}C$ in Tree Leaves

The lowest values of $\delta^{13}C$ were observed in the leaves of *Betula* spp. (Figure 2). In *Betula* spp. Leaves, the average $\delta^{13}C$ was equal to −30.85 ± 0.19‰, and for the needles of *P. sylvestris* $\delta^{13}C$, it was −29.88 ± 0.16‰. These differences were significant in the GLM with the "taxon" and "litter pollution index" factors: F_{taxon} = 12.09; p = 0.0003 (Figure 3a). The level of anthropogenic pollution affected the content of ^{13}C in leaves on the boundary of statistical significance ($F_{pollution}$ = 3.22; p = 0.0479). The average values of $\delta^{13}C$ in the absence of pollution were $\delta^{13}C$ = −30.10 ± 0.16‰, and near the smelter $\delta^{13}C$ = −30.62 ± 0.21‰. Thus, the differences between tree species in $\delta^{13}C$ were noticeably larger (the differences were 0.95–1.02‰) than the differences determined by the level of pollution (0.51–0.58‰). The $\delta^{13}C$ in *Betula* spp. leaves and in *P. sylvestris* needles in different plots did not correlate with each other (Figure 4a): r = 0.09; n = 24; p = 0.6595.

3.2. $\delta^{15}N$ in Tree Leaves

The lowest values of $\delta^{15}N$ were found in some samples of pine needles (Figure 2), but the differences between *Betula* spp. and *P. sylvestris* were not significant. In the GLM with the "taxon" and "litter pollution index" factors F_{taxon} = 0.01; p = 0.9469. Depending on the level of pollution, $\delta^{15}N$ changed significantly ($F_{pollution}$ = 20.56; $p < 0.0001$). The content of the heavy nitrogen isotope in both tree species near the smelter was higher than in the absence of pollution: in *Betula* spp. by 1.60‰, and in *P. sylvestris* by 2.33‰ (Figure 3b). The $\delta^{15}N$ in *Betula* spp. leaves and in *P. sylvestris* needles in different plots significantly correlated with each other (Figure 4b): r = 0.72; n = 24; $p < 0.0001$.

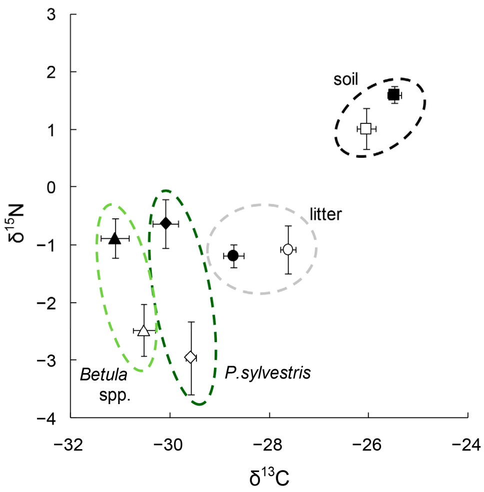

Figure 2. Average $\delta^{13}C$ and $\delta^{15}N$ in *Betula* spp. leaves (△, ▲) and *Pinus sylvestris* needles (◇, ◆), in litter (○, •) and soil humus horizon (□, ■) in the habitats of the Ilmen State Reserve (white symbols) and near the Karabash copper smelter (black symbols). Vertical and horizontal lines correspond to the standard error (SE) bars. The dotted lines show the ranges of $\delta^{13}C$ and $\delta^{15}N$ values in the same objects in the absence and presence of heavy metal pollution.

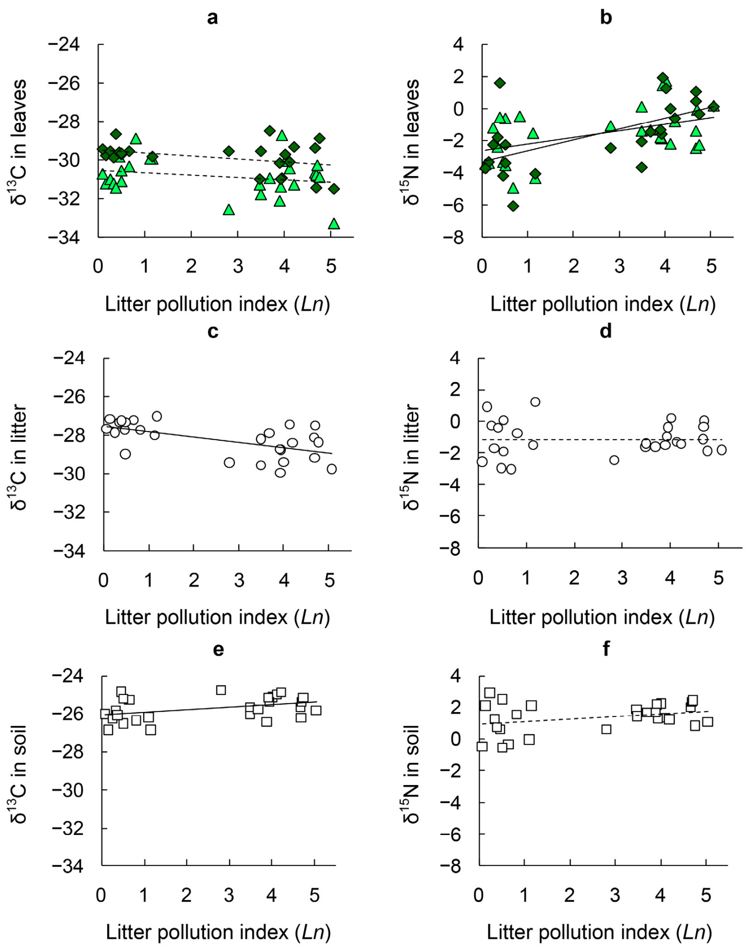

Figure 3. The dependencies of $\delta^{13}C$ (**a**,**c**,**e**) and $\delta^{15}N$ (**b**,**d**,**f**) content in the leaves (**a**,**b**), litter (**c**,**d**) and soil humus horizon (**e**,**f**) on the index of litter pollution with heavy metals. Additional symbols: light-green triangles—*Betula* spp.; dark-green diamonds—*Pinus sylvestris*. Solid lines—statistically significant values; dotted lines—statistically insignificant values.

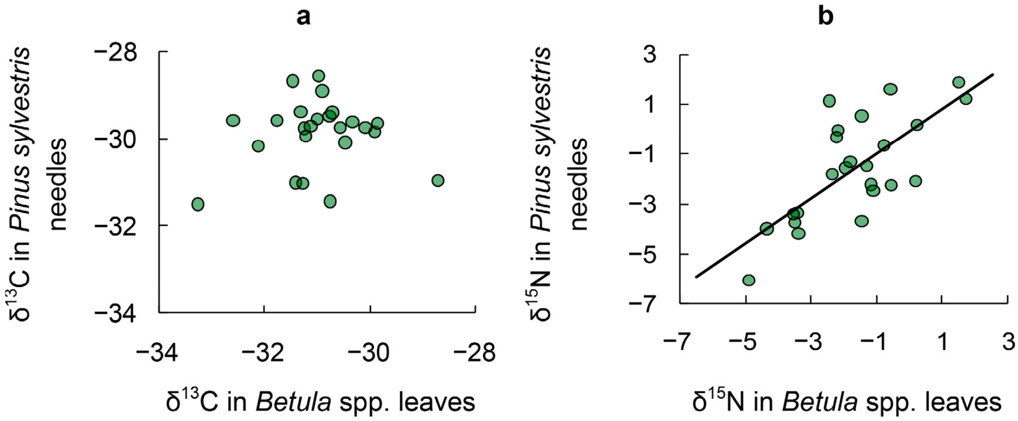

Figure 4. The scatter plots of $\delta^{13}C$ (**a**) and $\delta^{15}N$ (**b**) in the *Pinus sylvestris* needles on the $\delta^{13}C$ and $\delta^{15}N$ in the *Betula* spp. leaves. Solid line in (**b**) is the empirical regression line.

3.3. *δ*^{13}*C in Litter and Soil*

A similar increase in $\delta^{13}C$ was observed in a row "tree leaves–litter–humus soil horizon", both at the background level of pollution and near the smelter. In the ISR forests, the average $\delta^{13}C$ values were: −27.63 ±0.15‰ in litter, and −26.05 ± 0.19‰ in the humus soil horizon. In the forests near the copper smelter, the average $\delta^{13}C$ values were: −28.73 ± 0.21‰ in litter, and −25.48 ± 0.13‰ in the humus soil horizon. The increase in $\delta^{13}C$ during the transition from the leaves to the litter was 2–2.5‰, and 1.5–3‰ during the transition from the litter to soil. Depending on the level of contamination, significant changes in the content of ^{13}C were observed both in litter (Figure 3c) and in soil (Figure 3e). As the pollution increased, the amount of $\delta^{13}C$ in the litter decreased. The correlation between the pollution index and $\delta^{13}C$ in this horizon was r = −0.58 (p = 0.0015). A similar correlation in the humus horizon was opposite in sign: r = 0.40; p = 0.0411. Consequently, $\delta^{13}C$ in the soil increased with increasing pollution.

3.4. *δ*^{15}*N in Litter and Soil*

In uncontaminated ISR forests, an increase in $\delta^{15}N$ was found in a row "leaves (−2.70 ± 0.37‰)—litter (−1.08 ± 0.41‰)—humus soil horizon (1.02 ± 0.35‰)". Near the smelter, the content of ^{15}N in leaves (−0.76 ± 0.26‰) and in the litter (−1.19 ± 0.19‰) did not differ statistically. However, from the litter to the soil near the smelter, an increase in $\delta^{15}N$ was observed up to a value of $\delta^{15}N$ = 1.60 ± 0.14‰, i.e., by almost 3‰. Depending on the level of contamination with heavy metals, the content of ^{15}N (Figure 3d,f) did not change significantly in the litter (r = 0.01; p = 0.9693) or in the humus horizon (r = 0.32; p = 0.0987).

3.5. *Litter Thickness and Occurrence of Roots in the Litter*

We did not observe an increase in the thickness of the litter layer in the vicinity of KCS (Figure 5a). The correlation coefficient between the litter thickness and the index of its contamination was r = 0.25 (p = 0.2126). However, another noticeable consequence of heavy metal contamination was the disappearance of roots from the litter near the KCS (Figure 5b). The occurrence of roots in relation to the degree of litter contamination was better described by a logistic approximation (R^2 = 0.57) rather than by a linear approximation (R^2 = 0.46). Consequently, the occurrence of roots in the litter was non-linearly related to the degree of contamination.

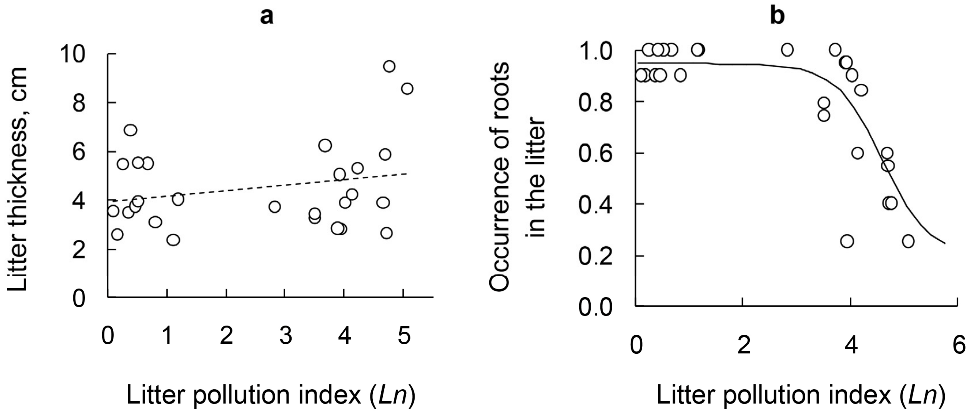

Figure 5. The dependencies of the litter thickness (**a**): linear approximation; statistically insignificant dependency) and the root occurrence in the litter (**b**): logistic approximation; statistically significant dependency) on the index of litter pollution with heavy metals.

3.6. δ15. N in Tree Leaves versus δ^{15}N in Soil and Root Depth

We suggest that the dynamics of δ15N in tree leaves in the pollution gradient are associated with a change in one of two or both of the following characteristics: the content of δ15N in soils and/or the depth of the root distribution. We first tested these assumptions by evaluating three GLM models. In each model, one of the factors described some property of the habitat, and the second factor was the taxon of the tree—*Betula* spp. or *P. sylvestris*. In all three cases, the "taxon" factor and the interaction with its participation were statistically insignificant. The GLM results with the "taxon" and "δ^{15}N in litter" factors: R^2 = 0.09; F_{taxon} = 0.001 (p = 0.9853); $F_{15N\ litter}$ = 4.57 (p = 0.0377); $F_{interaction}$ = 0.14 (p = 0.7073). The GLM results with the "taxon" and "δ^{15}N in soil" factors: R^2 = 0.14; F_{taxon} = 0.001 (p = 0.9780); $F_{15N\ soil}$ = 7.45 (p = 0.0089); $F_{interaction}$ = 0.02 (p = 0.8960). The GLM results with the "taxon" and "occurrence of roots in the litter" factors: R^2 = 0.25; F_{taxon} = 0.001 (p = 0.9761); $F_{occurrence\ of\ roots\ in\ the\ litter}$ = 14.70 (p = 0.0004); $F_{interaction}$ = 1.52 (p = 0.2239). The parameters "δ^{15}N in soil" and "occurrence of roots in the litter" did not correlate with each other (r = –0.11; p = 0.4602). Therefore, they were used as independent predictors in multiple regression. The "taxon" factor did not affect δ^{15}N in leaves. Therefore, to describe δ^{15}N in leaves depending on δ^{15}N in soil and on the depth of the roots, a two-factor regression was used:

$$\delta^{15}N_{leaves} = 0.231 - 3.348 \times \text{occurrence of roots in the litter} + 0.643 \times \delta^{15}N_{soil}. \quad (3)$$

The same expression in a standardized form with *beta* coefficients:

$$\delta^{15}N_{leaves} = -0.444 \times \text{occurrence of roots in the litter} + 0.323 \times \delta^{15}N_{soil}. \quad (4)$$

Thus, δ^{15}N in leaves depended more strongly on the occurrence of roots in the litter than on δ^{15}N in the soil (Figure 6). The significance of the coefficients was as follows: with the predictor of the occurrence of roots in the litter, p = 0.0005; with the predictor of $\delta^{15}N_{soil}$, p = 0.0091. Two predictors explained one third of the variability in δ^{15}N in leaves (R^2 = 0.33).

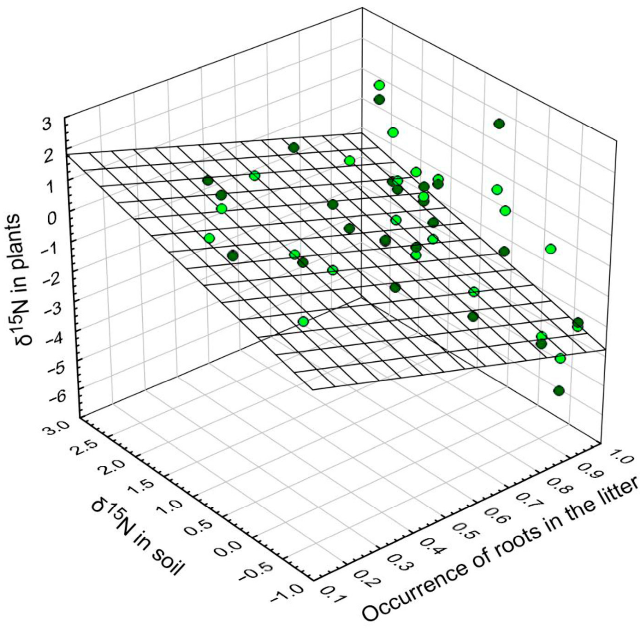

Figure 6. $\delta^{15}N$ in the *Betula* spp. (light-green circles) and *Pinus sylvestris* (dark-green circles) leaves depending on $\delta^{15}N$ content in the soil humus horizon and the root occurrence in the litter.

3.7. Other Possible Explanations for $\delta^{15}N$ in Tree Leaves

Based on the *AICc* values, the combination of "$\delta^{15}N$ in soil" and "occurrence of roots in the litter" predictors was not optimal for explaining $\delta^{15}N$ in tree leaves (Table 2). The best combination of predictors to explain $\delta^{15}N$ in leaves was the combination of $\delta^{13}C$ and $\delta^{15}N$ in litter. The combination of these predictors made it possible to improve the quality of explanation of $\delta^{15}N$ variability in tree leaves by 10% compared to the model considered in the previous section. The multiple two-factor regression equation was:

$$\delta^{15}N_{leaves} = -34.945 - 1.203 \times \delta^{13}C_{litter} + 0.537 \times \delta^{15}N_{litter}. \quad (5)$$

Table 2. The quality of GLM models explaining $\delta^{15}N$ in tree leaves by the combination of different predictors. *AICc* and R^2 estimates are given for the predictor combinations with *dF* equal to 1 or 2.

№	Predictor Combination	*dF*	*AICc*	R^2
1	$\delta^{13}C$ in litter + $\delta^{15}N$ in litter	2	186.03	0.43
2	Occurrence of roots in the litter + $\delta^{13}C$ in litter	2	188.65	0.40
3	$\delta^{13}C$ in litter + $\delta^{15}N$ in soil	2	189.60	0.39
4	Litter pollution index (*Ln*) + $\delta^{15}N$ in litter	2	189.89	0.38
5	$\delta^{13}C$ in litter	1	192.14	0.33
6	Litter pollution index (*Ln*) + $\delta^{15}N$ in soil	2	193.32	0.34
7	Occurrence of roots in the litter + $\delta^{15}N$ in soil	2	194.04	0.33
8	Litter pollution index (*Ln*)	1	194.49	0.29
9	$\delta^{13}C$ in litter + $\delta^{13}C$ in soil	2	194.50	0.33
10	Litter pollution index (*Ln*) + $\delta^{13}C$ in litter	2	194.97	0.32
11	Litter pollution index (*Ln*) + Occurrence of roots in the litter	2	195.05	0.32
12	Occurrence of roots in the litter + $\delta^{15}N$ in litter	2	195.57	0.31
13	Litter pollution index (*Ln*) + $\delta^{13}C$ in soil	2	196.02	0.30
14	Occurrence of roots in the litter	1	198.98	0.23
15	Occurrence of roots in the litter + $\delta^{13}C$ in soil	2	201.33	0.23
16	$\delta^{13}C$ in soil + $\delta^{15}N$ in soil	2	202.46	0.21
17	$\delta^{15}N$ in litter + $\delta^{13}C$ in soil	2	202.95	0.20
18	$\delta^{15}N$ in soil	1	204.71	0.14
19	$\delta^{15}N$ in litter + $\delta^{15}N$ in soil	2	206.95	0.14
20	$\delta^{15}N$ in litter	1	207.51	0.09
21	$\delta^{13}C$ in soil	1	211.46	0.01

The same expression in a standardized form with *beta* coefficients:

$$\delta^{15}N_{leaves} = -0.584 \times \delta^{13}C_{litter} + 0.322 \times \delta^{15}N_{litter}. \quad (6)$$

Based on the *AICc* values, it was also evident that the degree of litter contamination with heavy metals could be a component of models that satisfactorily explained the variability of $\delta^{15}N$ in leaves.

4. Discussion

Higher $\delta^{13}C$ values were found in the evergreen needles of *P. sylvestris* compared to the leaves of deciduous birches. A similar result previously demonstrated by [25] was associated with the peculiarities of stomatal conductance in the leaves of evergreen and deciduous plants. A working hypothesis was formulated about the possible increase in $\delta^{13}C$ under pollution conditions, firstly, taking into consideration the predominance of similar results among published works [4,20,21], and secondly, based on the results of a recent study by [25], which was methodologically very close to our work. An increase in $\delta^{13}C$ in the leaves of boreal plants in the vicinity of the Ni–Cu smelter was demonstrated [25]. However, the hypothesis about $\delta^{13}C$ increase in leaves during the contamination with heavy metals was not confirmed. On the contrary, a slight decrease in $\delta^{13}C$ was observed when approaching the smelter. That fact corresponded neither to most of the published data nor to our earlier result, also obtained in the vicinity of the KCS [31].

The conclusion about the decrease in $\delta^{13}C$ in trees under polluted conditions does not seem reliable to us for two reasons. First, when we analyzed $\delta^{13}C$ separately in the leaves of *P. sylvestris* and *Betula spp.*, we did not observe a statistically significant decrease in $\delta^{13}C$ (which is demonstrated in Figure 3a by dashed lines). Secondly, we did not observe the consistency of the $\delta^{13}C$ content in the leaves of *P. sylvestris* and *Betula spp.* (see Figure 4a). Therefore, it is highly probable that the content of the ^{13}C isotope in the leaves of trees in our study was due not to the level of contamination of the territory but to some other reasons that we did not evaluate.

Among the most often discussed environmental mechanisms of $\delta^{13}C$ variability in plants are the factors regulating the operation of the stomatal apparatus such as the effect of gaseous pollutants [4,23] or moisture level [6]. The emissions from the Karabash smelter during our study were 10–30 times lower than during the highest emissions observed in the mid-1980s [35]. At the same time, even our most polluted areas are 5–7 km away from the source of emissions. There are no forests closer to the smelter that meet the criteria for inclusion in the study. This is due to the influence of high soil toxicity accumulated in the previous period [36,37]. We are not familiar with the data on current levels of air pollution at the studied plots. However, the established slight changes in $\delta^{13}C$ across the pollution gradient in the leaves may indicate the absence of strong effects caused by the influence of gaseous pollutants on trees.

We found that $\delta^{13}C$ in litter as well as $\delta^{13}C$ in leaves decreases across the pollution gradient. However, such a decrease in $\delta^{13}C$ in the litter is more pronounced than in the leaves. As a result, the difference between the carbon isotope signature in leaves and in litter decreases across the pollution gradient. In background forests, this difference is 2.91 ± 0.29‰ for *Betula* spp. and 2.01 ± 0.20% in *P. sylvestris*, while in the vicinity of KCS it is 2.38 ± 0.33 and 1.40 ± 0.31‰, respectively. Usually, an increase in $\delta^{13}C$ from leaves to litter and further to soil is associated with the process of litter microbiological transformation [11,41]. Therefore, a decrease in the difference between $\delta^{13}C$ in leaves and litter near the smelter may be an indicator of a slowdown in the process of litter destruction under the conditions of heavy metal pollution. The decrease in the rate of litter degradation under the influence of heavy metals is well documented [42,43]. The plausible mechanism for this is a strong inhibition of destructor organisms under the influence of heavy metal pollution [44–46].

We observed the same increase in $\delta^{15}N$ in *P. sylvestris* needles and *Betula* spp. leaves with increasing heavy metal pollution. This result confirmed our first working hypothesis and our previous results [30,31]. The conclusion about the increase in the ^{15}N heavy isotope in the tree leaves in contaminated areas was also previously made in the vicinity of another metallurgical plant with a similar emission pattern [25]. After a decrease in atmospheric pollution in the area of the Central Appalachian Mountains, a decrease in δ^{15}N was observed in the needles of *Picea rubens* [47]; this corresponded to the direction of δ^{15}N alteration that we observed. In another case, an increase in δ^{15}N was found in the annual rings of trees during soil acidification in the province of Alberta (Canada) [48], which was also consistent with the facts we have established.

The second working hypothesis assumed the following situation. An increase in δ^{15}N in trees could be due to an increase in δ^{15}N in soil. This hypothesis was partially confirmed. We found that the isotopic signature of nitrogen in the leaves was statistically related to the isotopic signature of nitrogen in the soil mineral part. However, at the same time, the significant increase in δ^{15}N during pollution was found neither in the litter nor in the soil.

The third hypothesis suggested that changes in δ^{15}N in trees across the pollution gradient were associated with deeper root occurrence at high concentrations of heavy metals. An increase in δ^{15}N in the soil with increasing depth is a stable pattern [32,41]. If we assume that near the smelter, the roots of trees, due to the toxicity of the upper soil horizons, are located in deeper soil horizons, the increase in δ^{15}N in the leaves becomes well understood. The deep rooting of coniferous trees is strictly demonstrated under the conditions of another similar polymetallic pollution in the Urals [49]. At the same time, damage to the roots due to increased soil toxicity can be a sufficient explanation for their deep occurrence [50]. In this work, we have shown that roots are rarely found in the litter in polluted ecosystems. However, this does not agree with another estimate obtained, among other things, across the gradient near the KCS [35]. In this work [35], it was shown that the mass of plant roots either in the litter or in the soil significantly changes depending on the level of ecosystems pollution with heavy metals. However, it is important to note that the estimates provided in [35] are not fully comparable with ours, since the studies across the KCS gradient were carried out in birch forests and not in pine forests. We considered the occurrence of roots in the litter as a characteristic associated with the root depth. We assumed that the lower the occurrence of roots in the litter, the deeper the bulk of the roots would be located.

Our results demonstrated that the δ^{15}N variability in tree leaves depended more strongly on the occurrence of roots in the litter rather than on the δ^{15}N in the soil. Out of 33% of the total δ^{15}N variability in leaves explained by Equations (3) and (4), 21% is explained by the influence of the "occurrence of roots in the litter" predictor, and only 12% by the influence of the "δ^{15}N in soil" predictor. In other words, the assumption about the change in the localization of roots in the soil under the influence of pollution explains the variability of δ^{15}N in the leaves better than the assumption about the change in the ^{15}N content in the soil.

Thus, we confirmed the earlier assumptions about the mechanisms of δ^{15}N transformation in plants under pollution conditions [30,31].

At the same time, the explanation of the reasons for the δ^{15}N variability in leaves given in the previous subchapters is neither the only nor the best one. Using *AICc*, we found that δ^{15}N in leaves was best explained by a combination of the "δ^{13}C in litter" and "δ^{15}N in litter" predictors (see Equations (5) and (6); since we have not tested any hypothesis related to that dependence, the figure illustrating it is given in the Supplementary Materials). The values of δ^{15}N in leaves are more strongly related to δ^{13}C in the litter than to δ^{15}N in the litter. The first predictor is associated with 33% of the δ^{15}N variability in leaves out of 44% explained by two predictors. Accordingly, only 10% of the δ^{15}N variability in leaves is associated with the δ^{15}N in the litter. Unfortunately, a statistically reliable description of the dependence of δ^{15}N in leaves on δ^{13}C and δ^{15}N in the litter does not yet have a clear biological explanation. It is difficult to predict how the content of the ^{13}C isotope in the litter affects the content of the ^{15}N isotope in trees. It is possible that δ^{13}C is an indicator of some microbiological processes in the soil, which, in turn, determine nitrogen metabolism and the content of ^{15}N in leaves. For example, it is possible that $\delta^{13}C$ in the litter reflects the degree of its decomposition. This is supported by the negative correlation between the litter pollution index and $\delta^{13}C$ in it ($r = -0.62$; $n= 27$; $p = 0.0006$). However, we have not been able to explain how a low degree of litter decomposition can lead to an increase in the content of $\delta^{15}N$ in trees. For the correct explanation of complex ecosystem processes, a lot of interrelationships based on the great number of measurements have to be taken into account similarly to those given in [48,51,52].

There are two additional arguments about the possible reasons for the $\delta^{15}N$ increase in the tree leaves near the copper smelter.

The first additional reasoning is related to the assumption that ^{15}N enters with gaseous nitrogen-containing pollutants and their direct (via leaves) entry into trees. This mechanism is suggested to be very important in [25]. NO_x nitrogen oxides are formed from nitrogen in air, presumably with the same ratio of $^{15}N/^{14}N$ as in air, during various high-temperature production processes. Nitrogen oxides, according to the official reporting of Rosstat (https://https.rpn.gov.ru/open-service/analytic-data/statistic-reports/air-protect/, accessed data on 24 May 2022), are present in noticeable amounts in the KCS emissions. It is known that plants can assimilate NO_x from the air, including it in the composition of free aminoacids [53,54]. It is known that the proportion of nitrogen absorbed in this way can be appreciable [55,56]. Therefore, it cannot be ruled out that a direct supply of additional doses of ^{15}N from the KCS emissions to trees is possible. However, this is still unlikely. Discussing above the mechanisms of regulation of $\delta^{13}C$ in trees, we stated that strong effects caused by the influence of gaseous pollutants on trees were most likely absent in our study. This conclusion applies equally to SO_2, CO_2, and NO_x atmospheric pollution. In addition, in 2018–2020: (i) the total amount of nitrogen compounds in the KCS emissions was an order of magnitude less than SO_2 emissions; (ii) in our study area, NO_x emissions from factories and vehicles were about equal.

The second additional reasoning, which can explain the increase in the content of ^{15}N in trees under the conditions of heavy metal pollution, is related to the role of mycorrhiza in nitrogen metabolism. Since the highest content of ^{15}N is usually observed in non-mycorrhizal plants compared to plants with different mycorrhizae [16,57], a decrease in $\delta^{15}N$ in the leaves of trees near the KCS could be caused by a weakening of the development of ectomycorrhiza. It is known that arbuscular mycorrhiza in herbaceous plants in anthropogenic habitats can be formed less actively than in the absence of anthropogenic impacts [17]. However, tree ectomycorrhizae near metallurgical smelters are highly resistant [58–60]. Ectomycorrhizal fungi are less susceptible to negative impacts from metallurgical smelters than saprotrophic ones [45,46]. Thus, the assumption of a change in mycorrhizal status cannot be a sufficient explanation for the $\delta^{15}N$ change in trees near the KCS.

5. Conclusions

In ecosystems polluted with heavy metals, the content of the ^{15}N isotope in the leaves of *Pinus sylvestris* and *Betula* spp. increased similarly in the vicinity of a large metallurgical smelter. No reliable correlation of ^{13}C content in tree leaves with the level of anthropogenic pollution was established. The assumption about the change in the root localization in the soil under the influence of pollution explains the variability of $\delta^{15}N$ in the leaves better than the assumption about the change in the ^{15}N content in the soil. At the same time, our data indicate the existence of other mechanisms of changes in nitrogen metabolism in trees and ecosystems under the conditions of heavy metal pollution.

Supplementary Materials: The following supporting information can be downloaded at: Table 1: forests-1764430_supplement_1_data_table [Data set]. Zenodo. https://doi.org/10.5281/zenodo.6962560; Figure 2: forests-1764430_supplement_2_figure. Zenodo. https://doi.org/10.5281/zenodo.6962650.

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