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Enzyme Activities Transforming Sulphur in Brunic Arenosols in Forest Exposure to the Nitrogen Plants

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Abstract: This study has been conducted to assess the anthropogenic impact of the ANWIL S.A. nitrogen plants, one of the largest producers of nitrogen fertilizers in Poland, on the activity of arylsulphatase (AR), rhodanese (RDN) and catalase (CAT) related to sulphur transformation in forest soils. Samples of rusty brown soils were collected in the area adjacent to the ANWIL S.A. nitrogen plants in Włocławek. The profiles were located in the vicinity of the following production plants: No. W1—approximately 2.5 km away, No. W2—approximately 2 km away, and No. W3—approximately 0.8 km away. The control profile was established in the Tuchola Forest, which is part of the Biosphere Reserve. In order to recognize the impact caused by pollutants and the capacity of soil for re-generation, the resistance index (RS) and resilience index (RL) were determined. The highest RS value (0.964) was noted for rhodanese activity in layer Bv in W2, where the lowest RS for CAT (0.019) was observed. This study demonstrated the resilience of CAT activity in layer Bv in all the soil profiles adjacent to ANWIL. The highest resilience indicator was soil catalase activity. Enzymatic activity can be used to indicate the anthropogenic impact and the transformation of nutrients in forest soil.

Keywords: arylsulphatase; rhodanese; catalase; total sulphur; sulphates; forest soil; resistance index; resilience index

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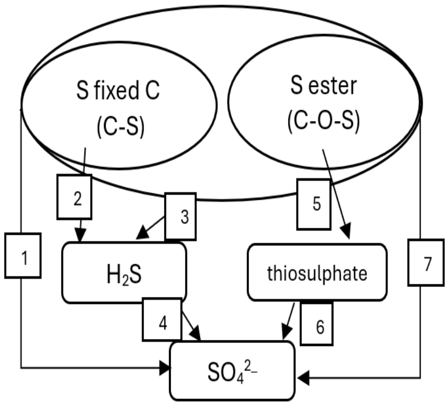
1. Introduction

Sulphur is an essential nutrient for plants due to playing an important role in the formation of chlorophyll and S-containing amino acids viz. cysteine, cystine and methionine, which are the building protein, vitamins and flavour-enhancing compounds, as well as due to activating several enzymes. Deficiency of sulphur influences nitrogen (N_2) fixation as S and N are basic element of proteins [1,2]. Sulphur occurring in soils in quantities depends on the content of organic matter, mineral and organic fertilization and the emissions of sulphur compounds from the polluted atmosphere [3–6]. Excessive sulphur content in soil can be no less harmful to plant growth and development than sulphur deficiency [7,8]. The negative effects of sulphur contamination in soils exposed to human impact include mostly chemical degradation of soils due to acidification [9–11]. Sulphates, after chlorides and nitrates, are the main inorganic anions (Cl^-, $SO_4{}^{2-}$ and $NO_3{}^-$) introduced into the environment mainly through dry and wet deposition. These anions have a significant impact on the chemical and biological processes in soil, the health status of forests and the quality of surface waters [10–12]. The main source of SO_4^{2-} and NO_3 in the atmosphere are anthropogenic emissions of SO_2 and NO_x [10,13–15]. Since the 1990s, a significant decrease in SO_2 emissions has been observed; for example, a 67% decrease in Poland during 1990–2005 [16]. The adverse effects of ambient concentration on forests are directly seen in deteriorating leaves, acidifying soils and reducing the availability of nutrients [17]. In vascular plants, SO_2 causes tissue damage and affects the root system and leaves [17,18]. Dust from industrial emissions settles on leaves, clogging plant stomata and thus disturbing the processes of photosynthesis and transpiration [18]. As for trees, it reduces the number of leaves, deforms the shape of the crown and limits growth [19]. In soil, a high concentration of SO_4^{2-} increases the leaching of nutrients into the soil profile and the displacement of base cations Ca^{2+} and Mg^{2+} from the sorption complex, which ultimately leads to soil acidification. This process increases the mobility of trace elements and disrupts microbiological and enzymatic processes [17,20]. As part of the campaign to reduce SO_2 pollution, the EU introduced emission limits and published directives setting permissible sulphur contents in certain liquid fuels. This has prompted energy-related sectors to shift from high-sulphur solid and liquid fuels to low sulphur fuels such as natural gas and prompted industrial plants to implement technologies that reduce exhaust gas emissions. In Europe, between 1990 and 2011, sulphur oxide emissions decreased by 74% [20]. An alternative to pollution monitoring methods that involve technical equipment, which can be expensive, can be provided by enzymatic indicators. Nutrients cycling in soil is associated with biochemical, chemical and physicochemical reactions. All biochemical reactions are catalysed by enzymes that increase their rate and which are specific to the substrate and reaction type [21–23]. Enzymatic activity in soil is the result of the activity of accumulated enzymes and the activity resulting from the development and reproduction of microorganisms. The enzymes accumulated are those that occur and which are active in soil; however, they are not related to the development and reproduction of microorganisms [23]. The source of enzymes in soil is primarily microbial biomass; however, enzymes can also come from plants and animal residue [24]. Environmental implications of sulphur transformation in soil are multidimensional; e.g., microbial sulphur mineralization releasing sulphate form (SO_4^{2-}) available for uptake by plants [25] as well as taking part in detoxification processes of metals, such as cadmium (Cd) and lead (Pb) to form insoluble sulphides [26]. The balance of microbial sulphur oxidation and reduction is very important. Sulphur-reducing bacteria transform sulphate into hydrogen sulphide (H_2S), preventing sulphur from being washed into waterways. Increased microbial sulphur oxidation converts sulphur compounds into sulphuric acid (H_2SO_4), contributing to soil acidification, thus damaging vegetation and leaching of essential nutrients (Ca^{2+}, Mg^{2+}) [27,28].

The research covered the activities of enzymes involved in sulphur metabolism in soil, i.e., arylsulfatase, rhodanese and catalase. Arylsulfatase (AR) (EC 3.1.6.1.) plays an important role in the processes of organic sulphur mineralization, making it available to plants [29] (Figure 1).

Rhodanese (ROD) (EC 2.8.1.1.) is an enzyme widespread in nature, occurring in humans and animals, as well as in plants and microorganisms [30], that transfers sulphur from thiosulphates to cyanides to produce sulphates (SO_3^{2-}) and thiocyanides (SCN^-) (Figure 1). Catalase (EC 1.11.1.6.) is an enzyme that catalyses a decomposition of H_2O_2 to water and molecular oxygen. Therefore, catalase is one of the key enzymes defending organisms from negative effects of oxidative stress. Oxidoreductase, along with peroxidases and superoxide dismutase, serves as an efficient scavenger of reactive oxygen species [31–33]. Forests and woodlands cover about one-third of the Earth's surface and play an important role in global nutrient cycling. Forests can influence the course of climate change by regulating the water regime, air quality, carbon sequestration and even reduce climate extremes [13,34]. Biochemical indicators of soil quality show a high degree of variability in response to paedogenic factors, geographical location, climate and season. It is therefore important to conduct studies in different soil conditions, and their conclusion can support describing the soil system complexity. The aim of this study was to recognize biochemical properties of the forest soil to determine a complexity of the soil system in close vicinity of the nitrogen plant.



1. Biological mineralization
2. Hydrolysis of cysteine and cystine by desulphydratase
3. Anaerobic oxidation of organic matter
4. Biological oxidation of hydrogen sulphide
5. Incomplete oxidation of sulphur
6. Oxidation of thiosulphate to sulphates by sulphites (the enzyme rhodanase is involved).
7. Biochemical mineralization of sulphate esters by sulfatases (arylsulfatase).

Figure 1. Diagram showing organic sulphur mineralization in soil.

2. Materials and Methods

2.1. Subject of Study and Experimental Site

The nitrogen plant, ANWIL S.A., operating since 1966, is one of the largest producers of nitrogen fertilizers in Poland. Additionally, it has produced ammonium nitrate (NH_4NO_3) since 1971 and saltpeter ($NH_4NO_3^+$ $CaCO_3$*$MgCO_3$) since 2000. It has also produced plastics such as PVC since 1992, including suspension polyvinyl chloride, which is also used for the production of granulates, building profiles, water and sewage pipes. ANWIL S.A. is the only manufacturer of suspension polychloride in Poland. ANWIL S.A. is located in Włocławek (52°41′55″ N, 18°58′09″ E) in the Kuyavian–Pomeranian Province (Figure 2).

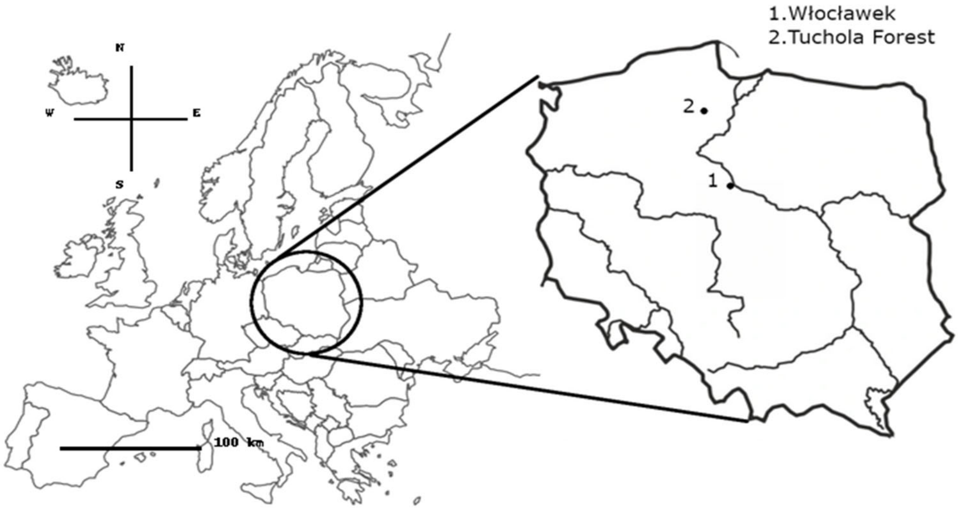


Figure 2. Research site.

In order to determine the effect of natural and anthropogenic environmental factors, the research was carried out as part of the analysis of forest soils exposed to the operation of ANWIL S.A. in 2001, and after 18 years, in 2019, in the area exposed to such impact. The soils were sampled from three profiles of forest Brunic Arenosols in the area adjacent to the Włocławek nitrogen plant, ANWIL S.A., in the zone of the prevailing wind directions of the range affected by the pollution emitted by ANWIL S.A. The profiles were located adjacent to the production plant as follows: No. W1—approximately 2.5 km away from the right bank of the Vistula eastward, where the individual layers of the profile were AEes (0 ÷ 4 cm), ABv (4 ÷ 18 cm), Bv (18 ÷ 60 cm), C (60 ÷ 150 cm); No. W2—about 2 km away to the west, where the individual layers of the profile were AEes (0 ÷ 15 cm), ABv (15 ÷ 39 cm), C1 (39 ÷ 87 cm), C2 (87 ÷ 100 cm); and No. W3—about 0.8 km away to the north-west from the nitrogen plant, where the individual layers of the profile were AEes (0 ÷ 15 cm), Bv (15 ÷ 72 cm), C (72 ÷ 100 cm). The forests in close vicinity of the plant are dominated by Scots pine (*Pinus sylvestris* L.) with American bird cherry (*Prunus serotina*) growing in a fresh mixed forest habitat. To exclude a possible natural and anthropogenic influence from the investigations, it was necessary to find a good control soil. The control was made up by Brunic Arenosols sampled in Szumiąca (53°31′05″ N 17°57′09″ E), which is outside the impact of emissions, in the Tuchola Forest. The Tuchola Forest is one of the largest complexes of pine forests in Poland, recognized due to its biodiversity. Since 2010, the region has been the largest Polish biosphere reserve, established by the UNESCO. It occupies approximately 3000 km^2 of outwash in the Brda and Wda basin. The layers of the profile are as follows: AEes (0 ÷ 20 cm), ABv (20 ÷ 50 cm), Bv (50 ÷ 100 cm), C (100 ÷ 130 cm). All the soils, according to the "Systematics of Soils of Poland" [35], were classified as autogenic soils, in the following order: podzolic soils, type: podzolic soils, subtype: podzolic soils, genus: sand alluvial, species: sand.

2.2. Soil Sampling and Chemical Analysis

Soil was sampled from each layer of soil profiles. The air-dried soil samples were sieved through a 2 mm screen; visible roots and plant residue were removed. Total organic carbon (TOC) and total nitrogen (TN) were determined using a Skalar TOC Primacs analyser (Skalar, Breda, The Netherlands). Selected physicochemical properties were assayed: pH in 1 M KCl (PN-ISO 10390) [36] and grain size composition—with the laser diffraction method, with a Masterssizer MS 2000 analyser (Malvern Panalytical, Malvern, UK) [37]. The sulphate content was determined using turbidimetric methods, following the Bardsley–Lancaster method [38]. Soil samples were extracted (CH_3COONH_4 dissolved in CH_3COOH) by shaking. The filtrate was mixed with HCl, $BaCl_2$ was added and the solution was stirred. The samples were left at room temperature for 15 min. After 15 min, the turbidity was measured at 440 nm with a spectrophotometer. The maximum reading was considered the proper turbidity and the value was compared with a standard curve to determine the sulphate ion concentration. The sulphate sulphur was measured turbidimetrically, applying the Bardsley–Lancaster method, in COMN-IUNG modification [38].

2.3. Biochemical Analysis

The activity of arylsulphatase (AR) (EC 3.1.6.1.) was assayed according to the Tabatabai and Bremner method [29]. The procedure involved 1 h of soil incubation at 37 °C in acetate buffer and potassium-p-nitrophenyl sulphate salt as a substrate. After that, the reaction was stopped by adding 1 mL of $CaCl_2$ and 4 mL of NaOH. The measurement was performed spectrophotometrically (400 nm). One unit of arylsulphatase activity was defined as the number of µmoles of p-nitrophenol released by 1 g of soil at 37 °C per hour (µM pNP$\cdot g^{-1} \cdot h^{-1}$). The activity of rhodanese (RDN) (EC 2.8.1.1) was assayed, incubating the soil with buffered substrates $S_2O_3{}^{2-}$ and CN^- solutions (0.05 M THAM, pH 6.0) at 37 °C for 1 h, applying the Tabatabai and Singh method [30], SCN; production in soil is based on the reaction of SCN^- with Fe^{3+} in an acidic medium forming a Fe–SCN complex. The formation of Fe–$S_2O_3{}^-$ complex SCN was determined calorimetrically with the spectrophotometer (460 nm). One unit of RDN activity was defined as the number of nmoles of SCN^- released by 1 g of soil at 37 °C per hour (nM $SCN^- \cdot g^{-1} \cdot h^{-1}$). Catalase activity (CAT) (EC.1.11.1.6) was assayed according to Johnson and Temple [31]. A mixture of soil, distilled water and 0.3% hydrogen peroxide solution was shaken for 20 min and, to stop the enzymatic reaction, H_2SO_4 was added. After filtration, the residual H_2O_2 was determined by titration with $KMnO_4$. One unit of catalase activity was defined as mg decomposed H_2O_2 of 1 g of soil per minute (mg $H_2O_2 \cdot g^{-1} \cdot min^{-1}$).

2.4. Data Analysis

The indices for resistance (RS) and resilience (RL) for three genetic layers present in all the profiles were calculated according to Orwin and Wardle [39]:

$$\mathrm{RS}(t_0) = 1 - \frac{2|\mathrm{Do}|}{(\mathrm{Co} + |\mathrm{Do}|)} \tag{1}$$

where Co is the value of the parameter analysed in control soil and Do is the difference between the control and disturbed soil (P_0) sampled in 2001 (t_0).

$$\mathrm{RL}(t_x) = \frac{2|\mathrm{Do}|}{\mathrm{Co} + |D_x|} - 1 \tag{2}$$

where Co is the difference between the control and disturbed soil (P_0) sampled in 2001 (t_0) and D_x means the difference between the control and polluted soil sampled in 2019 (t_x).

The enzyme activity results and chemical analysis were subjected to analysis of variance of Tukey's test with the aid of the 5% using a statistical software program analysis of variance for orthogonal experiments of the Bydgoszcz University of Science and Technology, Poland. Principal component analysis (PCA) was performed between the tested parameters. All the analyses were made using "Statistica 12.0 for Windows Pl" package (StatSoft Inc., Tulsa, OK, USA) [40].

3. Results and Discussion

3.1. Chemical Properties

The soils included in this study represent podzolic soils of the rusty soil type. The parent rocks were formed on alluvial sands. The characteristic feature of those sandy soils is their evenness. The analysis of grain size composition demonstrated that those soils of mechanical composition are sand (Table 1).

The soils of the forest adjacent to the Włocławek nitrogen plant, ANWIL S.A., showed an acidic and slightly acidic reaction. The pH value varied significantly across the sites. The soils sampled from the Włocławek area ranged from 4.39 to 5.41 in 2001 (Table 1), which was higher than in the soil from the control site (3.73–4.90). While, after 18 years, in 2019, the pH value in the soils exposed to the impact of ANWIL ranged from 4.29 to 5.13 and it was similar to that in the Szumiąca soil where it ranged from 4.25 to 5.13 (Table 1). The soils under study demonstrated a very acidic and acidic soil reaction. Soils found in Poland in the Silesian Province were exposed to a many-year impact of pollutions from the nearby emitters, especially the "Kędzierzyn" Nitrogen Plant (since the 1950s) and the "Rybnik" Power Plant (since the 1970s), as well as from the nitrogen plant and the power plant located in Blachownia, in close vicinity of Kędzierzyn and, located further away, from the south and from the east, pollutions emitted by the industrial plants of the Upper

Silesian Industrial District and from the Rybnik Coal District; the soil reaction was lower and it ranged from 3.00 to 3.64 [41]. Soils adjacent to the emitter sometimes showed a higher pH than the control soil, which could have been due to the fact that sulphur compounds from coal combustion in the power plant and heating boiler house (SO_2) emitted at 100–300 m enter the higher layers of the atmosphere and, in the atmosphere, they are oxidized to SO_3 and, with sufficient moisture, converted to sulphuric acids (IV and VI) [42]. Total organic carbon (TOC) in the soil profiles in 2001, affected by the nitrogen factory, ranged from 0.343 to 19.23 g·kg^{-1}, while in the soil sampled in the Tuchola Forest—from 0.392 to 17.20 g·kg^{-1} kg. In 2016, TOC was slightly higher at 0.437–25.89 g·kg^{-1} and 0.485–18.83 g·kg^{-1}, respectively, while TOC in the soils sampled in 2019, in the area adjacent to ANWIL, was 17% to 89% higher than in 2001. The highest amount was observed in W2 at bedrock C1 (89%) as well as W3—Bv and C (81% and 86%, respectively). Only in the Szumiąca soil, in ABv, a 7% lower content of TOC was assayed. In all the profiles, more TOC was identified in the surface layers. Total nitrogen content tended to be higher in the soil sampled in 2019. In the samples from the vicinity of Włoclawek, it ranged from 0.040 (C2 in W2) to 1.510 g·kg^{-1} (AEes in W1 and W2) in 2001. TN ranged from 0.051 g·kg^{-1} (C in W1) to 1.82 g·kg^{-1} (AEes in W2) in 2019.

Table 1. Selected properties of soil.

Sampling Location #		**pH 1M KCl**		**TOC g·kg^{-1}**		**TN g·kg^{-1}**		**C:N**		**Grain Size Composition [%]**			**Texture Class USDA ****
		2001	**2019**	**2001**	**2019**	**2001**	**2019**	**2001**	**2019**	**Sand 2.0–0.05 mm**	**Silt 0.05–0.002 mm**	**Clay <0.002 mm**	
W1	AEes	4.39	4.50	19.23	25.89	1.510	1.820	13	14	87	6	7	S
	ABv	4.93	4.78	6.070	8.791	0.672	0.761	9	12	97	2	1	S
	Bv	5.17	4.87	2.120	3.201	0.231	0.270	9	12	95	3	2	S
	C	5.41	5.13	0.362	0.437	0.051	0.051	7	9	97	2	1	S
W2	AEes	4.72	4.74	15.12	25.32	1.510	1.791	10	14	86	11	3	S
	Bv	4.98	4.95	4.045	12.63	0.510	0.892	8	14	93	6	1	S
	C_1	4.98	4.29	0.745	6.851	0.061	0.640	12	11	84	12	4	S
	C_2	4.97	4.35	0.363	0.577	0.043	0.040	9	14	91	6	3	S
W3	AEes	4.46	4.67	8.601	12.38	0.981	1.283	9	10	87	11	2	S
	Bv	4.99	4.46	1.530	8.050	0.210	0.785	7	10	90	9	1	S
	C	5.09	4.59	0.343	2.531	0.041	0.262	9	10	98	2	0	S
Sz	AEes	3.73	5.11	17.20	18.83	1.432	1.271	12	15	88	3	9	S
	Abv	4.90	5.13	4.320	4.043	0.390	0.387	11	10	95	0	5	S
	Bv	4.41	4.25	0.540	1.067	0.051	0.110	11	10	94	1	5	S
	C	4.32	4.31	0.392	0.485	0.040	0.051	10	10	98	1	1	S
Mean value		4.763	4.675	5.399	8.739	0.516	0.695	9.733	11.67				
Standard deviation		0.4265	0.3144	6.6096	8.6908	0.5708	0.6131	1.7925	2.0227				
LSD for													
Year				**		**							
Location				***		***							
Layer				***		***							
Interactions													
Year × Location				0.381 ^		0.627							
Year × Layer				0.900		0.076							
Location × Layers				**		***							

W1—Włocławek 0.8 km away from the nitrogen plant; W2—2 km away from the nitrogen plant; W3—2.5 km away from the nitrogen plant; Sz—Control in Szumiąca; TOC—total organic carbon in soil; TN—total nitrogen; S—sandy # USDA [37], ** the difference is significant for a 99% confidence level $p \leq 0.01$, *** the difference is significant for a 99.9% confidence significant $p \leq 0.001$, ^ the difference is not significant, it is a value of p.

Higher TN values were found in the soil sampled in close vicinity of ANWIL S.A., most likely due to the human impact (Table 1). The C:N ratio in soils sampled in 2001 was wider and it ranged from 7 in bedrock C in W1 and Bv in W2 to 13 in AEes in the soils adjacent to ANWIL and from 10 to 13 in the Szumiąca soils. In 2019, the soils showed a higher C:N ratio than 18 years earlier, which could have been due to N accumulation and complexation with SOM (Table 1). Pollution from atmospheric deposition affected the broader biogeochemical cycling of forest ecosystems, including the carbon (C) balance. Evans et al. [43] and Monteith et al. [44] showed that the decrease in S deposition was a factor leading to the change in the terrestrial C cycle, not only S. Waldrop et al. [45] also indicated that the addition of atmospheric N can stimulate carbon sequestration in the ecosystem due to the reduced decomposition of forest litter, which may contribute to the accumulation of organic matter in mineral layers of spruce forest soils.

3.2. Total Sulphur and Sulphate

Total sulphur (TS), like TOC and TN, tended to be higher in the soil sampled in 2019. In the samples from the close vicinity of Włoclawek, it ranged from 0.030 (in layer 2 in W3) to 1.155 $g \cdot kg^{-1}$ (in AEes in W1) in 2001. After 18 years, TN ranged from 0.068 (AEes in W2) to 0.917 (bedrock C in W1) in 2019. Higher concentrations of TS in Abv and Bv in W1 as well as C2 in W2 and AEes in W3 were identified in 2001, as compared with 2019 (Table 1).

In the soil sampled in Szumiąca a similar trend was noted and in BvC and C, the content of TS was higher in 2001. ST increased considerably for 18 years, and the content of sulphate sulphur decreased (Table 2), which points to soil having accumulated organic sulphur compounds that form a sulphur reservoir in soil. With the total sulphur criterium for Poland's soils and climate conditions developed, the total sulphur in the soil sampled in 2001 was qualified as low content (0.15 $g \cdot kg^{-1}$) and in 2019, an average content (0.16–0.50 $g \cdot kg^{-1}$) [46]. Sulphate sulphur is the most labile sulphur form and it is the form that the plants are most capable of using for their immediate and efficient soil uptake use. And, at the same time, an excess of sulphates in soil increases soil and surface waters acidification and it is harmful to flora and fauna [47]. The content of sulphate sulphur in the ANWIL area in 2001 ranged from 12.33 to 27.61 $mg \cdot kg^{-1}$, and after 18 years, from 6.593 $mg \cdot kg^{-1}$ to 17.64 C. In 2001, the highest SO_4^{2-} contents were found in soil C1 (26.61 $mg \cdot kg^{-1}$) in W2 and C soil (27.32 $mg \cdot kg^{-1}$) in W1; it was 20% higher than the average content calculated for all the samples at that time. A specific distribution of sulphur compactness in the soil profile was noted. Only W3 recorded the highest accumulation of that sulphur fraction, which was found in AEes (19.44 $mg \cdot kg^{-1}$) and a gradual decrease down the soil profile. At the control site, the lowest sulphate content was found in AEes and the highest in the ABv layer, and gradually decreasing. In 2019, the content of SO_4^{2-} was, on average, almost 50% lower. The highest content (18.99 $mg \cdot kg^{-1}$) was assayed in W3 in bedrock C. A displacement of sulphate in soil profiles was also identified. In W1, there was an accumulation of sulphur in Abv, while in W2, the highest amount of sulphate was found in AEes and it gradually decreased, while in W3, the opposite was observed and the lowest amount of sulphate was found in AEes, and it gradually increased down the soil profile. In the profile sampled in Szumiąca, sulphate accumulation decreased from ABv (a higher content in 2001) to BvC, where the content was 37% higher than the average for all the profiles and layers. In the soils studied, the content of sulphate sulphur was not higher due to human impact in both the soils sampled in 2001 (<15 $g \cdot SO_4^{2-} \cdot kg^{-1}$; a low content) and in 2019 (16–25 g $SO_4^{2-} \cdot kg^{-1}$—an average content). Such an amount of sulphur should be sufficient for plants growing in the forest. It is considered that over 80% of S in tree tissue is used for protein synthesis [48]. Sulphur deficit can result in no such tree growth limitation as it is in the case of crops; however, it results in an accumulation of amino acids not containing sulphur, especially arginine, in leaves. The non-containing-sulphur amino acids are used by fungal leaf pathogens (e.g., *Dilodia pinea* and *Dothistroma septospora*) as sources of food and thus facilitating a very fast growth of those organisms [49].

Table 2. Content of total sulphur, sulphate sulphur and the enzymes activity in soils in 2001 and in 2019.

Sampling Location #		**TS** g·kg^{-1}	**SO_4^{2-}** mg·kg^{-1}	**Arylsulphatase** µg pNP·g^{-1}·h^{-1}	**Rhodanese** ng SCN^{-}·g^{-1}·h^{-1}	**Catalase** mg H_2O_2 g^{-1} min^{-1}
2001						
W1	AEes	0.155	26.56	0.192	1.482	0.155
	Abv	0.107	22.15	0.128	1.112	0.107
	Bv	0.091	21.41	0.117	1.079	0.091
	C	0.124	27.32	0.140	1.474	0.093
W2	AEes	0.097	21.58	0.130	1.327	0.760
	Bv	0.082	26.65	0.116	1.137	0.081
	C_1	0.093	27.61	0.202	1.673	0.124
	C_2	0.081	22.17	0.111	1.191	0.097
W3	AEes	0.072	19.44	0.112	1.309	0.082
	Bv	0.069	13.82	0.213	1.979	0.070
	C	0.030	12.33	0.119	1.303	0.029
Sz	AEes	0.029	16.40	0.123	1.339	0.024
	Abv	0.155	26.56	0.192	1.482	0.155
	BvC	0.107	22.15	0.128	1.112	0.107
	C	0.091	21.41	0.117	1.079	0.091
Mean value		0.092	21.84	0.143	1.339	0.138
Standard deviation		0.0363	4.795	0.0367	0.2517	0.176
2019						
W1	AEes	0.177	7.381	0.175	0.396	0.151
	Abv	0.089	17.635	0.037	0.295	0.032
	Bv	0.069	6.254	0.043	0.340	0.037
	C	0.917	8.170	1.032	0.567	1.359
W2	AEes	0.185	14.71	0.089	0.706	0.077
	Bv	0.110	13.02	0.042	0.322	0.036
	C_1	0.836	8.126	0.596	0.449	0.051
	C_2	0.078	6.593	0.042	0.295	0.036
W3	AEes	0.068	9.580	0.034	0.154	0.029
	Bv	0.354	13.54	0.317	0.690	0.273
	C	0.085	18.99	0.049	0.159	0.051
Sz	AEes	0.090	11.61	0.099	0.223	0.085
	Abv	0.177	7.381	0.175	0.396	0.151
	BvC	0.089	17.64	0.037	0.295	0.032
	C	0.069	6.254	0.043	0.340	0.037
Mean value		0.226	11.13	0.187	0.375	0.162
Standard deviation		0.275	4.514	0.279	0.168	0.338
LSD for						
Year		*	***	0.326	***	0.323
Location		0.766 ^	0.876	0.310	0.062	0.364
layer		0.524	0.996	0.617	0.536	0.526
Irrigation						
Year × Location		0.154	0.166	0.271	0.403	0.227
Year × Layer		0.729	0.982	0.707	0.868	0.213
Location × Layers		0.529	0.887	0.729	0.529	0.854

W1—Włocławek 0.8 km away from the nitrogen plant; W2—2 km away from the nitrogen plant; W3—2.5 km away from the nitrogen plant; Sz—Control in Szumiąca; * the difference is significant for a 95% confidence level $p \leq 0.05$, *** the difference is significant for a 99.9% confidence significant $p \leq 0.001$; ^ the difference is not significant, it is a value of p.

3.3. Enzymes Activity

Sulphur enzymes activity transforms sulphur organic bonds into available sulphate (SO_4^{2-}), an essential plant nutrient [25]. The optimal activity improved sulphur availability, supporting protein synthesis in plants. More excessive activity increased soil acidification, harming plant and microbial communities and leaching of essential nutrients (e.g., Ca^{2+}, Mg^{2+}) [26]. The activity of the enzymes studied varied over the years analysed. Arylsulphatase hydrolyses aromatic sulphate esters and release SO_4^{2-} [50–53]. That hydrolase is an indicator of S mineralization in soil and it is important for S cycling [54,55]. The activities of arylsulphatase and catalase were, on average, 23% and 15% higher, respectively, in the soil sampled in 2019, as compared with the activity in 2001. This is in contrast to rhodanese, the activity of which was, on average, 72% higher in 2001. It was observed for the control, both in the soil sampled in 2001 and after 18 years, that the highest activity of all the enzymes tested was found in Abv. The highest content of TS and SO_4^{2-} in 2001 was also recorded in that layer. In contrast, no common rules for enzyme activity could be found in ANWIL's adjacent profiles. In W1, the highest activity of all the enzymes under study was determined in the first mineral layer of the profile (AEes), while after 18 years, the highest activity was identified in the deepest layer, i.e., bedrock (C). In W2, the highest AR activity was determined in both cases in Bv and CAT—in AEes. In the profile closest to ANWIL, AR and RDN showed the highest activity in Bv in both study years. Changes in the activity of constitutive enzymes to much extent depend on the content of nutrients. Nutrients deficits in soil often activate soil enzymes. According to Kertesz [52] and Kunito et al. [53], a production of arylsulphatase by soil microorganisms is probably a mechanism of reactions to ensure the adaption to a lower sulphate availability in the soil ecosystem. In our results, the lower activity of AR in soil in 2001 coincide with Li et al. [54] in terms of the accumulation of SO_4^{2-} being capable of inhibiting the excretion of sulfohydrolases. In the soils in the vicinity of ANWIL, some factors probably occurred to modify the activity of those enzymes. Soil enzymes are very sensitive to nutrient additions, e.g., with nitrogen addition increasing carbon and phosphorus acquisition by enzymes by 9.1% and 9.9%, respectively [55]. The responses of soil enzymes activity to nutrients are commonly explained by the "resource allocation theory" [55,56]. According to this theory, soil microorganisms modulate enzyme activity based on the availability of resources. The microbial caught of enzymes changed as a survival plan, increasing enzymes activity when the resources appeared and decreasing when basic nutrients such as mineral nitrogen or carbon, phosphorus and sulphur are available [55,56]. According to Adamczyk et al. [57], the activity of enzymes is also considerably affected by the tree species: the AR activity was highest under birch; however, the difference was significant only when compared with pine. The activity of beta-glucosidase, on the other hand, was twice as high in the humus layer under pine than under birch and spruce. The activity of beta-glucosaminidase and acid phosphatase was similar under all three tree species. The proteolytic activity was highest under birch, lower under pine and hardly detectable under spruce. In the environment of forest ecosystems overgrown with deep tree roots, hyphae and infested with microorganisms, there are many factors determining the activity of enzymes. According to Chabot et al. [58], the communities of microorganisms exposed to higher levels of native oxidative stress show higher primary intracellular CAT concentrations. All that coincides with the results for the soils in the vicinity of ZAW, where a higher activity was recorded for the soil sampled in 2001 for all the layers of the profiles except for bedrock (C). Rhodanese is a sulphur transferase that mostly catalyses a formation of thiocyanate from cyanide and thiosulphate or other adequate sulphur donors. The oxidation of thiosulphate to sulphate involves the action of rhodanese $S_2O_3^{2-} + CN^- \; SCN^- + SO_3^{2-}$ [59]. The application of extracellular rhodanese obtained from *Aureobasidium pullulans* in a free or immobilized form for a biodegradation of cyanide present in sewage discharged from the mill processing cassava points to a considerable participation of that enzyme in detoxication of various environments from toxic sulphur compounds and a possibility of use in biotechnology [60]. Higher values of rhodanese activity were noted in rhizosphere soils, as compared with non-rhizosphere soils [61]. In addition, the activity of rhodanese was much higher in the soils exposed to S atmospheric deposition [62]. According to Deng and Dick [63], the activity of rhodanese in soils is suppressed by the presence of $SO_4{}^{2-}$, which is the final product of oxidation of elemental S. The activity of that transferase in soils in close vicinity of ZAW, sampled in 2001, was about 72% higher than in the soils sampled in 2019, and it points to sulphur compounds oxidation when exposed to emissions of various sulphur compounds. An accumulation of various toxic substances in the soils sampled in 2001 can also be seen from the activity of catalase, the activity of which is very stable in soil. Therefore, their low activity can suggest the occurrence of potentially toxic substances, e.g., heavy metals in soil [64,65]. The transformation of organic sulphur into available sulphur is primarily regulated by mineralization and immobilization processes, in which microorganisms participate, significantly influencing $SO_4{}^{2-}$ changes [66,67]. In addition, the dynamics of available sulphur is also strongly regulated by reduction and oxidation processes driven by water availability, pH and soil microbial communities [67,68]. The studies showed a slight decrease in soil pH and an increase in TOC, TN and TS contents in soils from the vicinity of ANIWIL over 18 years due to changes in enzymatic activity. Several important mechanisms can be involved in this soil homeostatic effect. First, there was an accumulation of organic compounds, mainly carbon, as well as sulphur (Tables 1 and 2). According to Oulehle et al. [69], past anthropogenic factors contributing to soil acidification inhibited decomposition as well as decreased the solubility of organic matter, which led to the accumulation of a large pool of organic matter on the forest floor. Secondly, it was creating available carbon, which became a universal source of C for microorganisms, which increased the secretion of enzymes, including oxidoreductases and hydrolases, thus increasing the activity of catalase and arylsulfatase. The increase in sulphohydrolase activity was also associated with a decrease in the content of sulphate sulphur.

3.4. Statistical Analysis

Using Ward's method, the layers of the soil profiles were divided into groups with similar parameters analysed. On the dendrogram, standardized distances are marked on the vertical axis. On the dendrogram for the sites in 2001, at Euclidean distances, four groups can be distinguished, with similar parameters studied (Figure 3A). The first of the groups includes AEes of all the three points adjacent to ANWIL (W1, W2, W3); the second largest group covers the remaining layers (ABv, Bv, C) of the furthest profile (W1) and the control (Sz), as well as C2 and Bv in W2 and C and Bv in W3. A separate group is C1 in W2. The AEes layer from the control has differed most in terms of the parameters studied. A hierarchical clustering procedure revealed changes in terms of similar characteristics of the profile layers in 2019. Analysing the order of individual layers, those that are most similar to one another after 18 years, due to the structure of the features at the height of three Euclidean distances, seem to be AEes of W1, Bv of W2, ABv of Sz, C of W1 and C1 of W2. A separate group is formed by ABv of W1, AEes of W2, W1 of Bv, C of Sz, C2 of W2, Bv of Sz and AEes of W3, and the third group by Bv and C of W3 and AEes of Sz (Figure 3). Using Ward's method, the analysis of soil profiles presents some differences and characteristics of layered soils. The analysis shows a pattern of changes over 18 years of soil profiles near ANWIL, which is important for understanding their dynamic response to the anthropogenic impact. The layer that was the most different in 2001 and after

18 years is AEes in Szumiąca. The results present that soil profiles are usually heterogeneous, consisting of various layers due to geological processes [70] and the anthropogenic impact.

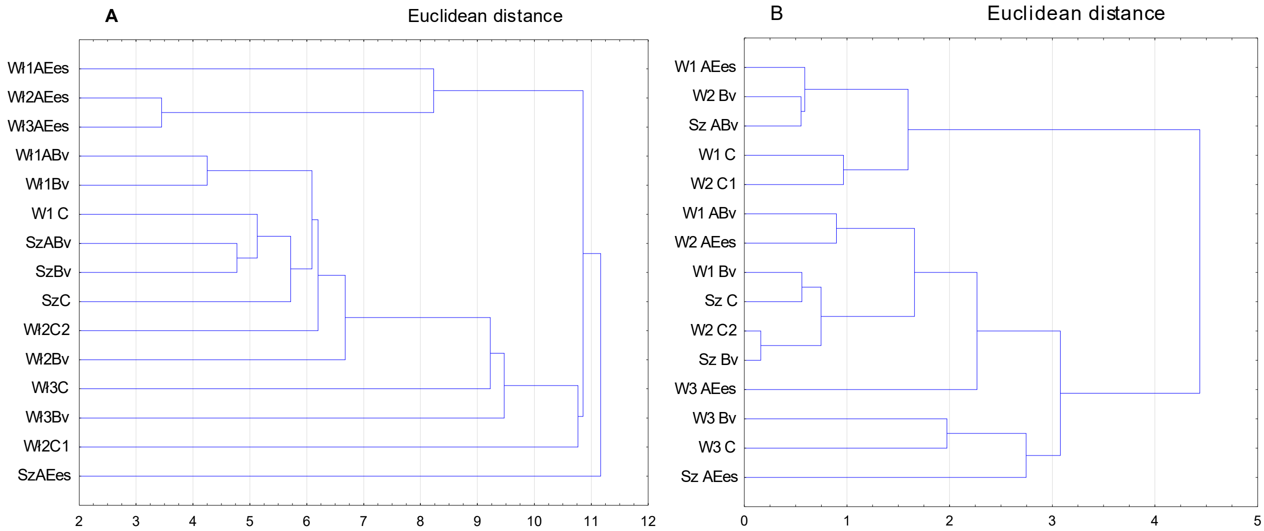


Figure 3. Ward's method cluster analysis for the soil layers in Włocławek (W1, W2, W3) and Szumiąca (Sz) in 2001 (**A**) and 2019 (**B**).

The resistance of soil and its ability to recover the equilibrium, which is soil resilience, are essential quality components and key indicators of the homeostasis between pressure caused by pollutants and the capacity of soil for regeneration [71,72]. Between the most considerable factors affecting soil resistance and resilience, researchers mention (10 of 15) the type of soil and plants, climate, land use and the extent of external interference. The interactions between the RS, RL and duration of the contamination are complex and case specific [73]. The RS and RL values showed that the activities of enzymes and the content of TS and $SO_4{}^{2-}$ varied in their sensitivity to the soil next to ANWIL. According the Orwin and Wardle [39], the index of resistance (RS) and the index of resilience (RL) are fixed by −1 and +1, with a value of +1 showing no effect of disturbance (maximal resistance). When RS equals 1, soil is fully tolerant to a given contaminant, which shows zero effect of contamination [39]. The highest RS value was noted for rhodanese activity (RS = 0.964) (W2) (Table 3). A higher value of RS for RDN was also noted in Bv in W1 (0.708) and W3 (0.828) as well as in bedrock in W3 (0.956). Also, the value of RS for AR activity was high in Bv in all the three profiles (0.793, 0.626, 0.358, respectively) and in AEes in W1 (0.457). The lowest RS was observed for CAT activity (RS = 0.019) in Bv inW2. For $SO_4{}^{2-}$, the highest values of RS were recorded for bedrock (0.620, 0.444 and 0.729, respectively). RS for TS was high in AEes (0.286, 0.358 and 0.590, respectively). The RS index values recorded in our experiment implied that the soil material in the Bv layer was distinguished by the highest resilience. According to Orwin and Wardle [39], the RL value of 1 and at the time of measurement (tx) indicates full recovery (maximal resilience). This study demonstrated the recovery of CAT activity (RL = 0.240, 0.860 and 0.220, respectively) in Bv in all the soil profiles adjacent to ANWIL. A recovery of the TS content in Bv in W1 (RL = 0.393) and in W3 (RL = 0.162) was also observed, as well as in AEes in W1 (RL = 0.286) and in bedrock C in W1 (RL = 0.111). The RL value lower than 1 indicates a slower rate of recovery, and an index value of 0 indicates that the disturbed soil has not recovered [39]. In the present study, RL = 0.044 in W2 in C, which suggests that the TS content in that soil layer has recovered slightly. In all the layer samples, especially for AR, RDN and sulphates content, the soil resilience (RL) index value was negative, thus implying a long-term effect of the nitrogen plant in forest soil.

Table 3. Index of resistance (RS) and index of resilience (RS) for TS and enzymes.

Sampling Location *		TS		SO_4^{2-}		Arylsulphatase		Rhodanese		Catalase	
		RS (to)	**RL (tx)**	**RS (to)**	**RL (tx)**	**RS (to)**	**RL (tx)**	**RS (to)**	**RL (tx)**	**RS (to)**	**RL (tx)**
W1	AEes	0.286	−0.482	0.352	−0.221	0.457	−0.653	0.498	−0.440	0.289	−0.383
	Bv	0.162	0.393	0.386	−0.165	0.793	−0.841	0.708	−0.695	0.156	0.240
	C	0.189	0.111	0.620	−0.625	0.477	−0.535	0.612	−0.566	0.152	−0.038
W2	AEes	0.385	−0.840	0.339	−0.174	0.166	−0.779	0.489	−0.366	0.597	−0.960
	Bv	0.182	−0.316	0.400	−0.285	0.626	−0.558	0.964	−0.974	0.019	0.860
	C_1	0.214	0.044	0.444	−0.269	0.331	−0.368	0.698	−0.673	0.174	−0.125
W3	AEes	0.590	−0.917	0.334	−0.165	−0.126	−0.285	0.691	−0.680	0.389	−0.685
	Bv	0.226	0.162	0.385	−0.431	0.358	−0.385	0.828	−0.831	0.175	0.220
	C	0.251	−0.082	0.729	−0.716	0.169	−0.203	0.956	−0.957	0.171	−0.159

* W1—Włocławek 0.8 km away from the nitrogen plant; W2—2 km away from the nitrogen plant; W3—2.5 km away from the nitrogen plant.

The activity of enzymes in the soil environment depends on many factors, both abiotic and biotic, mostly water–air properties, soil pH, mineral and organic colloid content, temperature, biogenic element content and the number and species status of microorganisms [73,74]. The principal component analysis (PCA) facilitates identifying a consistency in the relationships between variables and the description and classification of element parameters by variables. In that way, two principal components (PC1 and PC2) were established, which revealed 50.30% of the variance changes (Table 4). Component 1 (PC1) accounted for 25.85% of all the differential components and it was highly correlated with TOC values in 2001 (0.851), TOC in 2016 (0.811), TN in 2001 (0.756) and TN in 2001 (0.807). Component 2 (PC2), which accounted for 24.45% of the total variance, demonstrated the AR activity in 2001 (0.731) and 2016 (0.714), RDN in 2001 (−0.747) and RDN in 2016 (−0.779) and TS content in 2019 (−0.750). According to Brejda [75] and Shukla et al. [76], organic carbon content in soil was also recognized as the most powerful soil feature for central and southern high plains and for northern Mississippi loess hills and Palouse prairie in the USA.

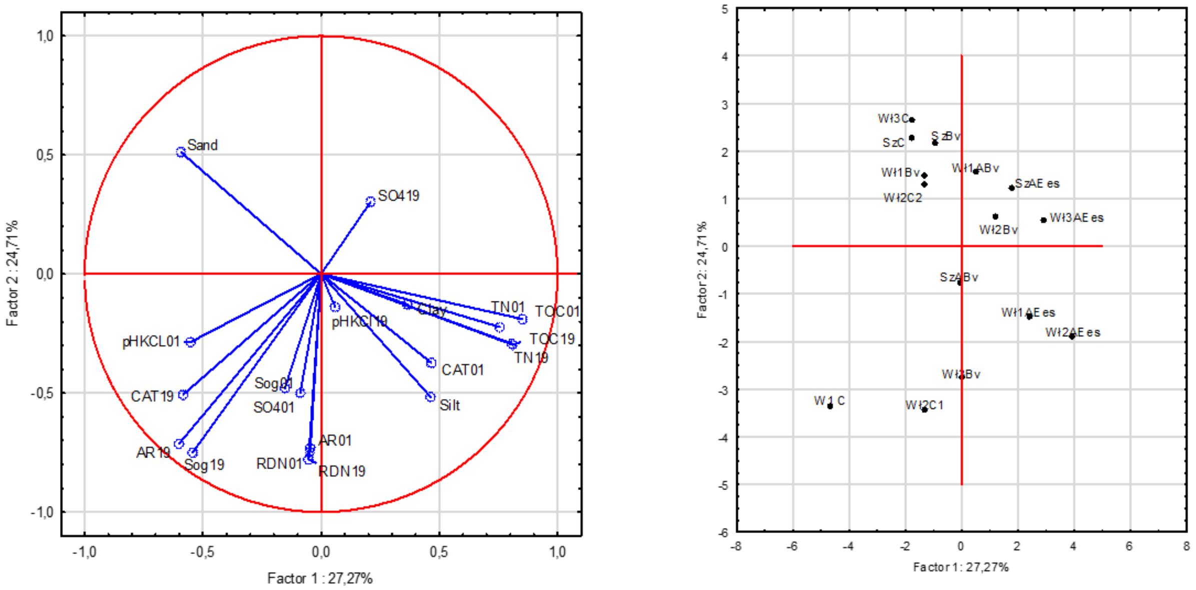
Table 4. Values of the two extracted factor loadings.

Parameters	Factor 1	Factor 2
TS 01	−0.08781	−0.49928
SO_4^{2-} 01	−0.15201	−0.47804
AR 01	−0.04523	**−0.73135**
RDN 01	−0.04733	**−0.74703**
CAT 01	0.466616	−0.37338
TS 19	−0.54285	**−0.75031**
SO_4^{2-} 19	0.209614	0.303077
AR 19	−0.60162	**−0.71386**
RDN 19	−0.0535	**−0.77875**
CAT 19	−0.58311	−0.5067
pH KCL 01	−0.55225	−0.28629
pH KC 19	0.059007	−0.13843
TOC 01	**0.851332**	−0.18937
TOC 19	**0.811415**	−0.29763
TN 01	**0.756057**	−0.22331

Table 4. *Cont.*

Parameters	Factor 1	Factor 2
TN 19	**0.807453**	−0.29308
Sand	−0.59355	0.511994
Silt	0.462774	−0.51666
Clay	0.365151	−0.12844

A comparison of the soils adjacent to ANWIL has identified groups of layers that can be distinguished in terms of AR activity in 2019 and TS content, which include C in W1 and C1 W2 (Figure 4). Layer Bv in W3 and ABv in Sz have indicated a high RDN activity. On the contrary, another group for the layer AEes in W1 and W2 represented similar activity of catalase in 2019 and content of TN and TOC. The results show a strong relationship between the organic carbon, total nitrogen content as well as total sulphur and enzymatic activity in the soil studied. The results of Yuan and Yue [77] for Chinese pine (*Pinus tabulaeformis*) plantations indicated that the plantation age and season significantly affected soil microbial and enzymatic parameters. In addition, soil organic C, total N and soil enzymes were highest on the 21-year-old plantation. In our study, the impact of anthropogenic impact caused significant changes in enzymatic activity but did not eliminate the impact of carbon and nitrogen on their activity.



(**A**) (**B**)

Figure 4. Configuration of variables in the system of the first two axes of principal components (**A**) and scores and loadings of the enzymes' activity data in relation to soil physical and chemical properties (**B**).

4. Conclusions

Our results suggest that anthropogenic impact has altered the forest in close vicinity of the nitrogen plant ecosystem C and S cycles. In Poland, Brunic Arenosols are airy soils poor in nutrients used in forests, and are the core of the country's forest management. Studies that were performed after 18 years of changes in soils around the ZAW showed an increased growth of TOC, TN and TS, which indicated a limited threat of negative human impact. Between the year 2001 and 2019, the mean value of higher 19% of C pool, and 22% of N pool as well as 60% of S pool were recorded, intensely modifying the forest ecosystem biogeochemistry mainly due to suppressed decomposition, and consequent organic matter accumulation in forest pool and later translocated from the organic to the mineral layers of soil. In addition, our findings indicate that the activity of soil enzymes in pine forests is a complex marker of natural occurrence of living cells of microorganisms, fungi and protozoa as well as anthropogenic factors found in the ecosystem. The activity of rhodanese close to the emitter was the highest at the beginning of this study and determined by the highest value of the resistance index, which indicates a transfer of this sub-stance resulting in detoxification of the forest environment. A higher activity of arylsulfatase was recorded after the accumulation of organic sulphur in soil. For the soil sampled in close vicinity of ZAW, our study shows that a mineral transition layer AEes can be used as the diagnostic layer. The enzyme that had the highest resilience index value was catalase. Enzymatic activity can be used to indicate the anthropogenic impact and transformation of nutrients in the forest soil. The emission of harmful compounds from production plants to the environment also poses a major challenge for industry and ecosystem sustainability. It is necessary to apply expert knowledge on the processes in the biological S cycle. Regarding the soil's sulphate content, which is an available form of S for the development of organisms, an excess can accelerate the acidification process, which can damage vegetation and leach nutrients. In general, this study calls for a need for monitoring and modelling studies of composite and interacting actions of different environmental factors on the biogeochemistry of forest ecosystems.

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