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Temporal and spatial variation in the composition of the lichen *Hypogymnia physodes* from the Niepołomice Forest (Poland)

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The Niepołomice Forest, though relatively natural, is affected by air pollutants transported from nearby urban areas. To assess the impact of air pollution, we analyzed the bioaccumulation of elements (Ca, Cd, Cu, Fe, Hg, Pb, S, Zn) in thalli of *Hypogymnia physodes* (L.) Nyl., together with oxidative stress biomarkers (SOD, TBARS) and thallus condition, at 15 sites during heating and non-heating seasons. Seasonal variability was observed: Cd and TBARS were higher in non-heating season (0.97 µg·g^{-1} and 0.95 mmol·g^{-1} FM respectively), while S increased during heating season (1331 µg·g^{-1}), suggesting emissions from fuel combustion. Spatial differences were most pronounced for Cd and Zn. In the western part of the forest, *H. physodes* was absent at some sites, and lichens showed elevated Pb and Cu concentrations with increased SOD activity, indicating strong traffic-related pollution. In the east, thalli contained a high proportion of degenerated algae, associated with elevated Cd, Hg, and S, as well as other stressors. Overall, element concentrations were comparable to values reported from other regions of Poland. The study highlights that even seemingly natural forests are subject to significant pollution pressure. Combining chemical data with biomarkers offers deeper insight into the effects of toxic elements on lichen bioindicators.

Since the mid-20th century, the Niepołomice Forest has been affected by incoming pollution caused mainly by the development of the urban-industrial agglomeration of Kraków[1] and other heavily polluted cities, such as Tarnów and Nowy Sącz[2]. The location of the pollution sources, together with the prevailing westerly winds carrying pollution from the Kraków agglomeration towards the forest, have created gradient of contamination in the area, intensifying the environmental pressure on this ecosystem[3,4]. Therefore, the forest is a suitable place to study long-term ecological and environmental impacts of air pollution. These impacts have already been monitored using various groups of organisms, including mosses, trees and soil invertebrates[5–11]. One of the key bioindicator groups used to track environmental changes in the Niepołomice Forest has been lichens[3,12–14]. Due to the lack of protective cuticle and roots, they are susceptible to disturbance, especially air pollution[15–18]. Widespread species, such as *Hypogymnia physodes* (L.) Nyl., are excellent long-term indicators of air quality[19], and their use remains central to contemporary biomonitoring approaches[20,21].

Pollutants can disrupt the cellular homeostasis of lichens and induce the formation of reactive oxygen species (ROS), the excessive production of which can lead to cell damage, including damage to cell membranes. These changes may also be visible externally as deformation of the thallus, such as darkening or bleaching of their fragments, and altered structures[22–24]. In the long term, oxidative stress leads to cell death, which is signaled by an increase in the level of Thiobarbituric Acid Reactive Substances (TBARS) in the organism[25,26]. As a consequence, lichen populations decline and species diversity of the polluted habitat decreases[4,15,23,27]. To prevent this, organisms activate defense mechanisms, such as enzymatic antioxidant systems neutralizing ROS. One of the key enzymes involved is Superoxide Dismutase (SOD), whose increased activity converts superoxide anions into less reactive oxygen species, thereby limiting cell damage[28–30]. This enzymatic response plays an important role when lichens are exposed to environmental pollutants, such as chemical compounds and individual elements, particularly metals[31]. Metals, based on function, can be divided into those that are essential for life (e.g., calcium (Ca), copper (Cu), iron (Fe) and zinc (Zn)) and non-essential ones, which have no role in organisms (e.g., cadmium (Cd), lead (Pb) and mercury (Hg))[32–34]. In addition to metals, sulfur and its oxides also exert harmful effects, especially during the heating season, when fuel combustion in households significantly increases their

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emissions into the atmosphere. Nowadays, in the environment concentrations of most of these pollutants stem from anthropogenic sources, such as traffic, industrial activities and domestic heating[35–37], with their levels varying seasonally[31,38]. The heating season is characterized by increased emissions of particulate matter and heavy metals from domestic and industrial heating systems[37]. Differences in the distribution of emission sources may cause site-specific differences in pollution levels, resulting in distinct spatial patterns of lichen pollution. Recognizing these patterns helps to identify the areas most vulnerable to harmful deposition, pinpoint the largest sources, and thus target potential protective measures.

Research on lichens in the Niepołomice Forest began as early as the 1960s, leading to the classification of most of the forest as moderately polluted, except for a more heavily polluted small enclave in the western part of the southern complex[4,39]. Since the 1970s, the extent of the moderately polluted zone has decreased as a result of increasing industrial emissions. Consequently, a decline in many sensitive species and noticeable damage to lichens were recorded[4,13,40–42] Lichen-based surveys conducted in the 1990s showed that the western part of the forest had shifted into the very heavily polluted zone, while the area classified as moderately polluted expanded at the expense of the strongly polluted zone[3,4]. The last lichen studies in the Niepołomice Forest were carried out 20 years ago[14]. Since then, air protection policies, industrial activity, and transportation intensity have undergone significant changes. It remains unclear how these changes have influenced lichen condition and whether the current pollution levels are reflected in their physiological state. Additionally, to date, no measurements of TBARS levels or SOD activity have been performed for lichens in this area, limiting a more complete understanding of their exposure and responses in the Niepołomice Forest.

Despite extensive historical research on lichens in this area, no recent analyses integrating chemical composition with oxidative stress biomarkers have been conducted. Therefore, this study aims to assess the impact of air pollution within the Niepołomice Forest on the monk's-hood lichen (*Hypogymnia physodes* L. Nyl.). We measured concentrations of eight elements: Ca, Cd, Cu, Fe, Hg Pb, S and Zn in thalli sampled three times over a one year period: April 2018, October 2018 and April 2019, covering both heating and non-heating seasons. In addition to elements, we also measured the activity of SOD, TBARS level, as well as the proportion of dead algae in the thallus as markers of lichen condition. The data were analyzed using a multivariate approach and GIS visualization. Based on the collected information, we were able to address the following hypotheses: (1) the concentrations of elements, the activity of SOD and TBARS level depend on the season; (2) the above-mentioned parameters reveal spatial variability; (3) all the parameters are linked to the thallus condition. We expected both spatial and seasonal variability in the measured parameters, with a particular focus on the negative influence of non-essential elements (especially Cd, Hg, and Pb), associated with increased oxidative stress response and, consequently, deterioration of the condition of lichens.

Materials and methods

Study site

The study was conducted in the area of the Niepołomice Forest (49°59′-50°07′N, 20°13′-20°28′E) in southern Poland, Europe (Fig. 1). It is a complex of several forest areas covering 110 km^2, located east of Kraków agglomeration in the western part of the Sandomierz Basin. It is dominated by pine and mixed oak-pine (Pino-Quercetum) as well as oak-hornbeam (Tilio-Carpinetum) forests[14], whose current stands have been shaped by human management[43]. The area is characterized by little variation in relief[44].

The highest temperatures in the area occur in July (average of 18.4 °C) and the lowest (-2.6 °C) are recorded in January[45]. Summer lasts up to 100 days, and winter up to 85 days. Average annual precipitation ranges from 560 to 700 mm, with rainfall recorded on 160–170 days per year. Snowfall occurs on about 45 days, and the average duration of snow cover is 110 to 120 days[44]. The climate of the Niepołomice area is characterized by frequent fog and temperature inversions, which significantly reduce the number of sunny days, especially in autumn and spring[45].

The research was conducted in the southern, largest part of the complex, covering an area of approximately 85 km^2. In this area, 20 evenly distributed survey plots were initially selected, with their final location determined after field verification. In the western part of the Forest, representing about 20% of the planned study area, no *H. physodes* were found. Consequently, the number of study sites was reduced to 15 (Fig. 1).

Sampling

Hypogymnia physodes thalli were sampled after the end of the heating season (April 2018 and 2019), and before the start of the heating season (October 2018). Considering the accumulation time of pollutants, we categorized these samplings as heating and non-heating seasons, respectively. Samples were collected from pine (*Pinus sylvestris* L.) trunks at heights of 50 cm and 2 m above ground. Depending on the site and the collection period, the number of trees sampled ranged from 1 to 15. In total, 45 samples were collected in the whole study. After collection, the samples were dried at room temperature and stored in paper envelopes.

Algal condition

The morpho-anatomical analysis of lichens was performed to determine the degree of thallus damage based on visible changes, according to the following criteria: (1) rosettes without disease damage, i.e., healthy, uniformly colored and morphologically intact; (2) rosettes with moderate damage, i.e., those with blackened or faded patches, excessive shrinkage, affecting no more than 50% of the rosette; (3) rosettes with severe damage, with more than 50% thallus degeneration[46]. At most sites no thalli classified to the moderate damage group were found, therefore, two groups - healthy and severely damaged - were used.

To study internal thallus morphology, microscopic preparations were made from excised fragments of thalli with a specific and fixed diameter. From each site and damage group, three randomly selected thalli were sampled, and three preparations made from the middle part and both margins of each rosette. Using 40x magnification

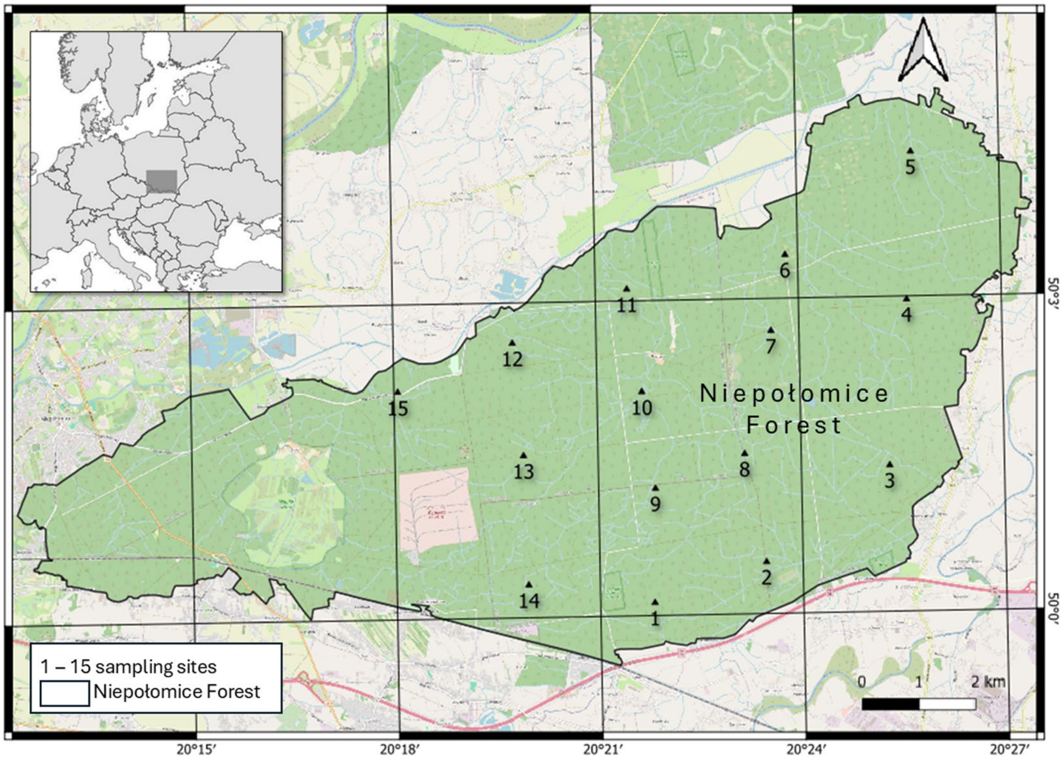

Fig. 1. The Niepołomice Forest (southern Poland, Europe) with sampling sites (1–15) of *H. physodes*.

of the microscope (Delta Optical Genetic Pro), all algal cells were counted according to the following criteria: (1) healthy algae: all cells with uniformly green-colored chloroplast; (2) algal cells with damage, i.e., showing changes in chloroplast coloration (browning), degeneration in the form of shrinking, fragmentation, and with signs of plasmolysis; (3) dead algal cells lacking chloroplast content[46].

The obtained numerical data on the external and internal morphology of the thalli were presented as percentages for each group (damaged and dead algal cells are grouped into one category).

Elemental analysis

Air-dried samples were used in three separate protocols to measure Hg, other metals, and S consecutively. Hg concentrations were determined using a cold vapor atomic absorption spectrometer (MA-2, Nippon, Japan) in sub-samples of ca. 50 mg. Blanks were analyzed at the beginning, middle and end of each set of samples, with their results always below the limit of detection. Three, finally averaged replicates were performed for each lichen sample. During the measurements, quality control was performed using a standard solution of mercury (II) chloride ($HgCl_2$, 100 $ug \cdot ml^{-1}$, Nippon, Japan) diluted to 0.01 $ug \cdot ml^{-1}$. If the RSD between results of triplicates was higher than 15%, the sample was reanalyzed.

For quantification of other metals, air-dried samples were further dried (at 60 °C for 72 h) and aliquots of 2 g were mineralized in an open digestion system (Velp Scientifica, DK-20) using ultrapure nitric acid (Baker Instra, 65%) and ultrapure perchloric acid (Sigma-Aldrich, 70%) in a 4:1 volume ratio, kept first at 140 °C (ca. 2 h) and then at 160 °C (ca. 20 h). The mineralized solution was diluted with ultrapure water (Direct Q-3, Merck Millipore) to a volume of 10 ml, and the concentration was measured in a flame atomic absorption spectrometer (AAnalyst 200, PerkinElmer, USA).

Another part of the sample (approx. 80 mg) was used to measure sulfur concentrations using the modified Butters-Chenery method[47]. Briefly, a sample was mineralized in the presence of an oxidizing agent, leading to the conversion of sulfur compounds into sulfates (SO_4^{2-}), which are then, after reaction with barium chloride ($BaCl_2$), quantified turbidimetrically using a spectrophotometer (Evolution 260 Bio UV-Visible Spectrophotometer, Thermo Scientific, USA). The results were expressed as micrograms per gram of dry weight of the lichen sample. Blanks were used to calibrate the background and zero the instrument before each analysis.

The mean values, number of replicates and quality control monitoring for the elements are provided in the Supplementary materials (Table S1). For Cd, Cu, Hg, Pb and Zn, the accuracy of the method was tested against a Certified Reference Material (CRM; BCR 482, JRC, IRMM). Recoveries ranged from 97.16 for Cu to 104.65 for Zn, confirming the reliability of the analytical protocol. Due to the lack of appropriate CRM available on the market, the accuracy of the method for Ca and Fe was tested using control solutions and spikes only (Table S1). At every control the results were satisfactory (recoveries within 95 and 105%). Blanks were analyzed at the beginning, middle and end of each set of samples, with their results always below the limit of detection.

Oxidative stress biomarkers

Before the analyzes, the lichens were placed into distilled water for 15 min to saturate and acclimatized in a climate chamber for 48 h (10 °C, 60–70% RH, 12 L:12D).

Quantification of thiobarbituric acid-reactive substances (TBARS) as a proxy of the extent of lipid peroxidation was assessed according to Gawrońska et al.[48]. Briefly, lichen powder (0.2 g) was homogenized in 2 mL of 0.1% trichloroacetic acid (TCA, A.C.S., POCH). Homogenates were centrifuged at 10 000×g for 5 min at 4 °C. The obtained supernatant was mixed 1:4 (v/v) with 0.5% barbituric acid solution (A.C.S., POCH) in 20% TCA, incubated at 95 °C for 30 min, cooled on ice and centrifuged at 10 000×g for 5 min. The colored complexes of TBARS (products of lipid peroxidation) were determined at 532 nm, and the non-specific absorption at 600 nm was subtracted (Ultrospec 2100 pro-Classic, GE Healthcare, UK). An extinction coefficient of 1.56 · 105 $M^{-1}cm^{-1}$ was used for TBARS concentration calculations. The results were expressed as mmol TBARS per gram of fresh weight of the lichen sample.

To analyze the superoxide dismutase (SOD) activity, crude protein was extracted in accordance with the procedure described by Egger et al.[49]. Briefly, lichen powder (60 mg) was homogenized in 1 ml of 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA (99+%, Sigma-Aldrich), 1% PVP (99%, Sigma-Aldrich), 0.2% Triton X-100 (99%, Sigma-Aldrich), and 5 mM 2-mercaptoethanol (99%, Sigma-Aldrich). The homogenate was centrifuged at 12 000×g for 5 min at 4 °C, and supernatant was used for measuring antioxidant enzyme activity. According to Bradford[50] protein concentration was determined. According to Laemmli[51] separation of soluble protein fractions was performed using native (without sodium dodecyl sulfate) PAGE at 4 °C and 180 V. According to Beauchamp and Fridovich[52] method, visualization of SOD bands was performed. Briefly, the gels were incubated in staining buffer for 30 min, in darkness, at room temperature and then exposed to white light until SOD activity bands became visible. Densitometric analysis of SODs bands was performed with ImageJ 2 (GPL license) and the results were expressed in activity units [AU].

GIS and statistical analyses

Based on the lichen sampling locations and values of each studied parameter, interpolation maps were created using the Inverse Distance Weighted (IDW) tool. Maps were prepared in QGIS ver. 3.2.2 Bonn. Coordinates were expressed in the EPSG: 2180 – 189 ETRS89/Poland CS92 system. For parameters which showed spatial differences, two maps for each season were created.

Prior to the analysis, we plotted the data to identify the distribution and potential outliers. Since the element concentrations and dead algae fraction (DAF, hereafter) were log-normally distributed, further analysis was conducted on their logged data. Values of SOD and TBARS were normally distributed. The data from the measurements above were accompanied by the following variables: season (non-heating season vs. heating season), and location (15 sampling points).

The main analysis (factorial ANOVA) verified the variability of all element concentrations. For metals, Principal Component Analysis (PCA) was performed to identify patterns of variation and relationships between the studied variables, and to prepare a PCA-derived metal concentration index. For DAF, SOD and TBARS, a set of General Linear Mixed Models (GLMMs) were built to explain their variability using location as a random factor, and explaining variables: season, PCA-derived metal index, S, SOD (for DAF and TBARS models), TBARS (for DAF and SOD models) and DAF (for SOD and TBARS models). The set of models included a null model, single-factor models and a full model. Finally, the AIC value corrected to the small sample size (AICc) was used to compare all the models and choose the best-fitted one. To assess potential multicollinearity effects on the models, correlations between all the numeric variables were examined (correlations were generally weak, with the strongest Pearson coefficient being − 0.330). In order to check the potential spatial dependencies, the spatial autocorrelation in residuals of the best-fitted models was calculated with Moran's I test.

Additionally, the strength and significance of potential correlations between elements were evaluated with r Pearson coefficient. Arithmetic mean, geometric mean, standard deviation, minimum, and maximum were calculated as descriptive statistics. Values were presented with at least three significant digits for the lowest value. In all the analyses, the significance level was set as 0.05. The data were compiled in Microsoft Excel (ver. 16). All the analyses were performed with R Studio (ver. 1.3) in R (ver. 4.0.2)[53] using the packages: factoextra[54], AICcmodavg[55], lme4[56], sf[57], spdep[58], spData[59], effectsize[60] and emmeans[61].

Results

All the measured element concentrations were higher than the quantification limits of the applied method (Table S1). Due to logistical constraints, SOD and TBARS activities could not be measured in the samples from April 2018.

Concentrations of elements

Elemental concentrations built the following order: Hg < Cd < Pb < Cu < Zn < Fe < S < Ca. The lowest concentration was noted for Hg (0.04 $\mu g \cdot g^{-1}$), while the highest was for Ca (7 569 $\mu g \cdot g^{-1}$). The lowest standard deviation was observed for Hg (0.040), and the highest for Ca (1762.054; Table 1).

Concentrations of Cd and S differed between seasons, in contrast to other elements (Table 2). The influence of location was significant only for Cd and Zn concentrations. No interaction was observed between season and location for any of the elements (Table 2). Therefore, according to the principle of parsimony, we used linear models without the interaction in the detailed reporting (Table S4-S12). Cd concentrations were higher in the non-heating season, while S concentrations were higher during the heating season (Table 1). For Cd, the highest concentrations were found in the north-eastern part of the forest (sites 2, 6 and 7), while the lowest concentrations were observed in the central-western part (sites 8, 9, 12 and 15). For Zn, the highest

Models	Non-heating season	Heating season	Pooled
Ca	1852 (1181) ± 1988 312–6112; *n* = 13	1706 (1230) ± 1687 280–7569; *n* = 29	1751 (1214) ± 1762 280–7569; *n* = 42
Cd	0.97 (0.96) ± 0.14 0.81–1.18; *n* = 13	0.87 (0.85) ± 0.17 0.61–1.29; *n* = 29	0.90 (0.88) ± 0.17 0.61–1.29; *n* = 42
Cu	5.72 (5.69) ± 0.67 4.35–6.44; *n* = 13	5.71 (5.65) ± 0.84 4.10–7.34; *n* = 29	5.71 (5.66) ± 0.78 4.10–7.34; *n* = 42
Fe	721 (709) ± 127 463–897; *n* = 13	648 (620) ± 211 343–1412; *n* = 29	671 (647) ± 191 343–1412; *n* = 42
Hg	0.11 (0.11) ± 0.02 0.07–0.14; *n* = 14	0.10 (0.10) ± 0.05 0.04–0.29; *n* = 30	0.11 (0.10) ± 0.04 0.04–0.29; *n* = 44
Pb	9.15 (8.90) ± 2.04 4.72–11.33; *n* = 13	9.13 (8.75) ± 2.46 3.46–13.20; *n* = 29	9.14 (8.80) ± 2.31 3.46–13.20; *n* = 42
S	979 (938) ± 252 372–1237; *n* = 14	1421 (1368) ± 402 837–2463; *n* = 30	1284 (1217) ± 415 373–2471; *n* = 44
Zn	108 (102) ± 39 69–197; *n* = 13	110 (105) ± 35 72–217; *n* = 29	109 (104) ± 36 69–217; *n* = 42
DAF	33.8 (33.4) ± 4.7 26.9–41.5; *n* = 15	34.0 (33.7) ± 5.0 26.3–46.5; *n* = 30	33.9 (33.6) ± 4.9 26.3–46.5; *n* = 45
SOD	23.231 (22396) ± 6094 12.463–32.728; *n* = 15	19.577 (19395) ± 2615 12.996–22,868; *n* = 15	21.404 (20841) ± 4968 12.463–32.728; *n* = 30
TBARS	0.95 (0.95) ± 0.07 0.89–1.14; *n* = 15	0.88 (0.88) ± 0.04 0.80–0.96; *n* = 15	0.91 (0.91) ± 0.07 0.80–1.14; *n* = 30

Table 1. Descriptive statistics (mean followed by geometric mean (in the bracket) and SD) of elements [$\mu g \cdot g^{-1}$], DAF (dead algae fraction) [%], SOD [AU] and TBARS $mmol \cdot g^{-1}$ FM in *H. physodes* thalli.

Variables	Season	Location	Interaction
Ca	$F_{1, 14} = 0.034$, $p = 0.856$	$F_{14, 14} = 2.212$, $p = 0.075$	$F_{12, 14} = 1.631$, $p = 0.190$
Cd	**$F_{1, 14} = 6.340$, p = 0.025**	**$F_{14, 14} = 2.787$, p = 0.033**	$F_{12, 14} = 0.558$, $p = 0.842$
Cu	$F_{1, 14} = 0.023$, $p = 0.882$	$F_{14, 14} = 1.869$, $p = 0.127$	$F_{12, 14} = 0.675$, $p = 0.750$
Fe	$F_{1, 14} = 1.915$, $p = 0.188$	$F_{14, 14} = 0.969$, $p = 0.523$	$F_{12, 14} = 0.525$, $p = 0.865$
Hg	$F_{1, 15} = 1.151$, $p = 0.300$	$F_{14, 15} = 1.129$, $p = 0.408$	$F_{13, 15} = 0.287$, $p = 0.985$
Pb	$F_{1, 14} = 0.026$, $p = 0.875$	$F_{14, 14} = 1.387$, $p = 0.274$	$F_{12, 14} = 0.343$, $p = 0.965$
Zn	$F_{1, 14} = 0.152$, $p = 0.703$	**$F_{14, 14} = 5.512$, p = 0.002**	$F_{12, 14} = 2.397$, $p = 0.061$
S	**$F_{1, 15} = 18.580$, p = 0.001**	$F_{14, 15} = 1.529$, $p = 0.212$	$F_{13, 15} = 1.121$, $p = 0.412$

Table 2. ANOVA models testing the influence of season, location and their interaction on element concentrations in *H. physodes* thalli collected in the Niepołomice Forest. Bold indicates significant differences ($p < 0.05$).

concentrations were recorded in the northern and western parts of the forest (sites 6 and 15), while the lowest concentrations were in the central part (sites 7, 9, 12 and 14) (Fig. 3).

Relationships between elements were generally weak (r Pearson below 0.6), with the strongest observed between Cu and Pb (r Pearson 0.505), followed by Fe and Pb (r Pearson 0.486), and Zn and Pb (r Pearson 0.436) (Figure S3). Other potential correlations were statistically insignificant. PCA analysis of metals showed that the first two principal components (PC1 and PC2) explain 52.8% of the total variability (PC1: 33.1%, PC2: 19.7%, respectively) (Figure S2).

Oxidative stress biomarkers
The best-fitted GLMM model for SOD was the one including PCA-derived metal index (Table 3).

For TBARS, the final model included season only (Table 3). TBARS mean values were higher in the non-heating season (0.95 $mmol \cdot g^{-1}$ FM and 0.88 $mmol \cdot g^{-1}$ FM respectively. Autospatial correlation for SOD and TBARS was statistically insignificant (Table S3).

Algal condition
The examined preparations showed the presence of healthy algal cells, algal cells with severe damage involving more than 90% of chloroplast content, and dead cells devoid of chloroplast content. At most sites, both healthy and severely damaged thalli were observed; however, no thalli with moderate damage were detected.

In samples collected during the heating season, the proportion of dead algal cells ranged from 26.3% to 46.5%, while in the non-heating season, it ranged from 26.9% to 41.5% of all algal cells and was not influenced by the parameters examined in this study (Table 3).

The best-fitted GLMM for DAF was a null model (Table 3). Further analysis showed a weak, but significant spatial autocorrelation for this variable (Moran I statistic 0.2151, $p = 0.019$, Table S3).

Variables	Null model (AICc)	Full model* (ΔAICc**)	Chosen model	Chosen model (ΔAICc)
DAF	-44.382	28.635	null model	– 44.382
SOD	601.667	-34.708	PCA-derived metal index	558.880
TBARS	-69.666	5.219	Season	-79.682

Table 3. Corrected for small sample size Akaike information criterion (AICc) for GLMMs models explaining the condition parameters of *H. physodes* thalli collected in the Niepołomice Forest. The model with the lowest AICc was considered the best-fitting explanatory model. *Full model included the PCA-derived metal index (Figure S2), S, DAF (dead algae fraction; for SOD and TBARS models), SOD (for DAF and TBARS models) and TBARS (for DAF and SOD models) with localization as a random. ** ΔAICc calculated as the difference between the AICc of a given model and the null model (intercept-only).

Discussion

We found that only Cd and S concentrations in studied lichens showed seasonal variation, while Cd and Zn concentrations varied spatially. TBARS also revealed seasonality, while SOD values were not influenced by the studied factors. The condition of algae indicates significant spatial correlation, and was not strongly related to any parameters analyzed in the study.

Over the past 40 years of research in the area, it has been shown how the number of lichen species (lichenized fungi), site frequency and abundance have changed, as well as the strong response to air pollution in the form of damage or even degeneration of entire thalli. Our research showed that the average concentrations of elements in *H. physodes* thalli were comparable to those reported from other parts of Poland[19,62–64]. According to the classification of Nimis et al.[65], only the average concentrations of Cd and Zn reached values indicating potential negative impacts on the natural environment. This shows fairly significant environmental changes, that could become a serious threat if they continue to intensify.

Seasonal differences

Many studies indicate that the process of metal accumulation by lichens is closely related to seasonality, with higher concentrations of certain elements typically occurring during the heating season[19]. Literature also reports increased emissions of Cd and S during the combustion of coal and wood[66–69]. In our study, however, the higher concentration of Cd was observed outside the heating season (Table 1), suggesting that other sources may contribute to its elevated levels. Cd is released not only in combustion processes, but also through industrial activity, waste disposal[70,71], and fuel combustion[72]. Therefore, we suggest that these sources may be responsible for higher Cd emissions during the non-heating season compared to the heating season.

In the study, we also observed seasonality of S concentrations (Table 2). In the environment, S occurs mainly as sulfur oxides, which are released, particularly by thermal power plants[73–75]. Research conducted in the Świętokrzyski National Park on *H. physodes* pollution showed that the concentration of sulfur (IV) oxides (SO_2) increases significantly during the heating season[72,76], which supports our hypothesis. In Kraków, located in the vicinity of the western part of the forest, the observed concentrations are lower (Fig. 2). This may be related to the anti-smog resolution enacted in 2019, which introduced a legal ban on burning coal and wood for residential heating within the city boundaries. In contrast, sites located in the eastern part of the forest are near Tarnów and smaller towns, where no such ban has been implemented[2,77].

Our results indicate seasonal differences in oxidative stress biomarker - TBARS (Table 3). Besides air pollution, their activity is influenced by environmental conditions. During the non-heating season, lichens are exposed to increased UV radiation and low humidity, which disrupt homeostasis and promote ROS production[78,79], indicating oxidative stress. In relation to TBARS, their higher average concentration was observed during the non-heating season, which suggests the potential influence of microclimatic conditions, including humidity and temperature. However, their spatial distribution (Figure S2) indicates its increased concentration during the heating season in the western part of the forest. Both SOD and TBARS can serve as indicators of environmental pollution, as their levels increase in response to metal pollution[26,80], which is higher during the heating season. Although our study does not show a clear impact of the measured elements, we suggest that pollution from Kraków may contribute to their higher levels at those sites. However, we cannot exclude the influence of other factors, such as the climatic conditions mentioned above.

Spatial differences

The spatial distribution of Cd is diversified, with the highest values recorded in the middle-eastern part of the forest (Fig. 3). According to literature, Cd is released in combustion processes, as a result of industrial activity and waste disposal, as discussed in the previous subsection. It is worth noting, however, that less urbanized areas may also contribute significantly to emission of this metal - for example, through waste burning, agricultural practices, or local transport. This indicates a real threat of metal pollution even in areas considered to be more natural[70,81,82].

Over the years, studies conducted in the Niepołomice Forest have consistently reported higher Zn pollution in its western part[14,83], suggesting a significant impact of the Kraków agglomeration. Our results confirmed this pattern, with the highest concentrations observed in the western part (site 15; Fig. 3), where Zn coincided with Pb and Cu (Figure S1), supporting the hypothesis that road transport is a major source[84–86]. A similar distribution was observed in the eastern part of the forest for SOD values, which may be linked to increased

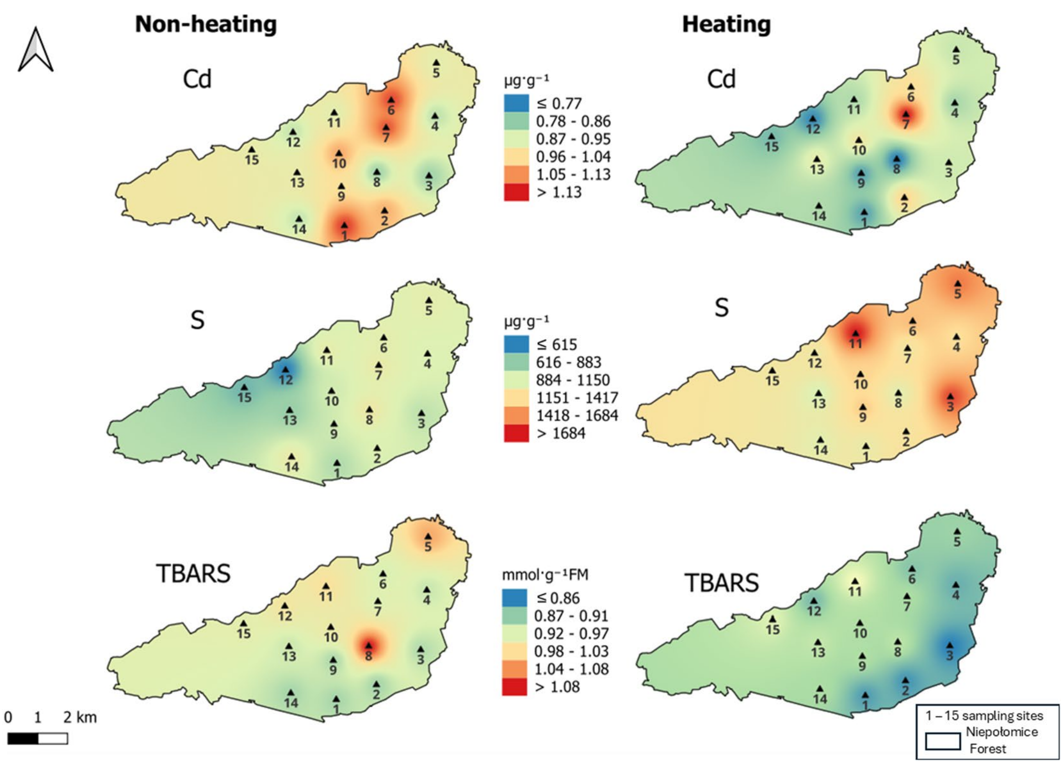

Fig. 2. Spatial distribution of Cd and S in *H. physodes* showing statistically significant seasonal differences in the Niepołomice Forest with sampling sites (1–15).

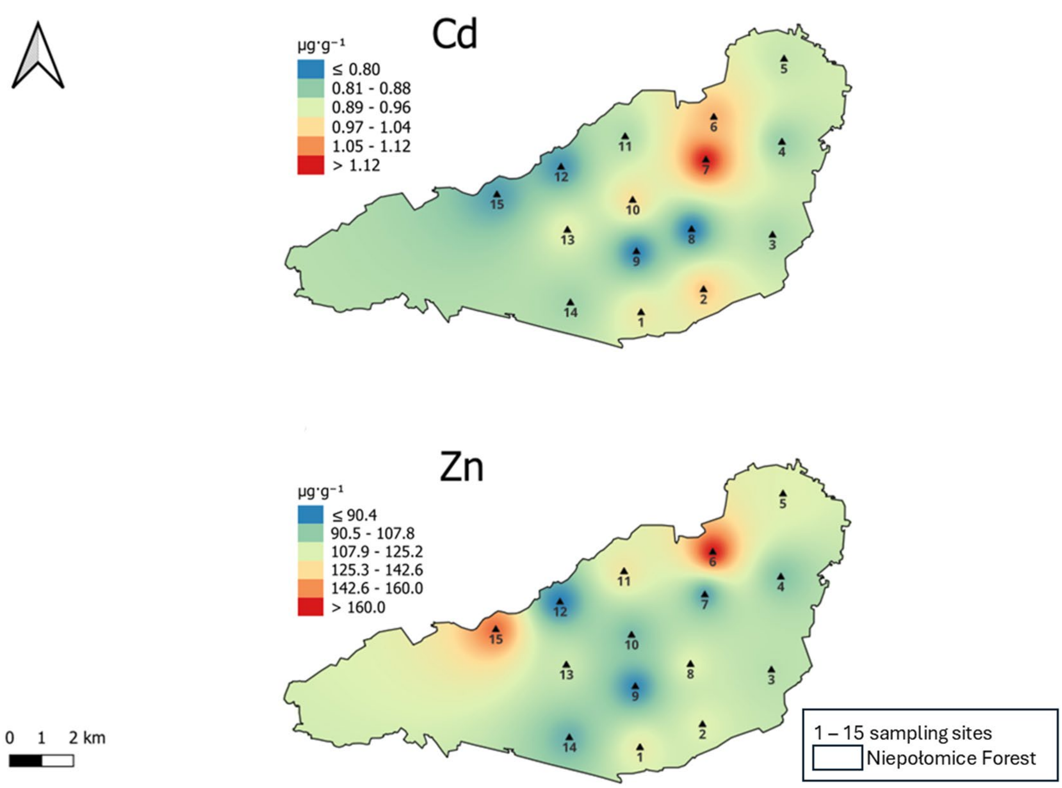

Fig. 3. Spatial distribution of Cd and Zn concentrations in *H. physodes*, the only elements showing statistically significant spatial variation in the Niepołomice Forest with sampling sites (1–15).

concentrations of the mentioned metals. In contrast, an additional Zn maximum was recorded at a northern site located near the village of Olszyny, adjacent to agricultural fields. This pattern cannot be explained by traffic. Combined with elevated Cd and Ca concentrations and the highest percentage of damaged thalli, it may indicate a contribution of agricultural activities. On the other hand, Pb pollution can also be linked to incomplete fuel and oil combustion as well as the legacy of leaded gasoline use[87]. We also observed an antagonistic spatial relationship between Pb and Cu versus Ca. The highest Ca concentrations were found at eastern sites, where Pb and Cu levels were relatively low, compared to the western part where the opposite trend occurred. We suggest that this may be related to the protective role of Ca as a competitor for binding sites with toxic metals such as Cd[88,89]. In contrast, in the eastern part, higher concentrations of S, Cd, Hg, and Ca, were related to greater damage to lichens. This may indicate a stronger impact of local emission sources, such as the energy industry, municipal and domestic combustion, and emissions related to agricultural activities[90]. Furthermore, for Hg and S, long-range transport from small towns and surrounding agricultural areas located in the immediate vicinity of this region cannot be ruled out[91,92]. Unlike most metals, which tend to deposit relatively close to their emission sources, elemental Hg and sulfur oxides can remain airborne for extended periods, allowing them to disperse and deposit far from their origin[93–96]. Concentration of elements in the air is mainly influenced by local sources of their release, as well as meteorological conditions in a given area, such as wind speed, temperature, and precipitation. In addition, rainfall favors pollutant leaching, while strong winds and low temperatures promote their spread[97]. According to the emission balance for 2018 and 2019 in Poland[90,98], industrial processes were the main source of Cd in the environment, with emissions related to energy production also playing a significant role. Therefore, the high concentrations recorded in our study may have been caused by weather conditions, as - according to the thermal classification made by the Institute of Meteorology and Water Management[99] - years 2018 and 2019 were classified as extremely warm, characterized by low rainfall, which might have favored accumulation of pollutants in *H. physodes* thalli.

Although, our study did not indicate a significant connection between lichen damage and oxidative stress biomarkers, we observed that increased TBARS levels and SOD activity were noted in the western part of the forest. ROS are mainly produced in the mitochondria of living cells[100], so their low concentrations in the eastern part of the forest may be related to reduced metabolic activity caused by higher cell degeneration that indicates lower activity of SOD and TBARS levels. Thus, stressors present in the eastern part of the forest may be responsible for the condition of lichens.

Environmental significance

The study shows that effective biomonitoring with lichens requires consideration of both seasonal and spatial dynamics. Since different emission sources dominate at different times of the year, seasonal sampling helps to distinguish between sources of pollution. Ignoring this aspect can mask important signals or lead to misinterpretation of pollution sources. The inclusion of spatial mapping further enhances the diagnostic value of lichens. Presenting biomonitoring data in a spatial context helps identify pollution hotspots, source regions, and potential pathways for the spread of pollutants in the forest environment. This is particularly important in semi-natural areas that appear intact but are exposed to diffuse pollution from outside sources.

By combining data on elemental bioaccumulation with biomarkers of oxidative stress (SOD, TBARS) and lichens condition the study highlights records the biological consequences of exposure, allowing for a more sensitive assessment of pressure on the ecosystem, especially in areas where absolute concentrations may appear moderate.

Conclusions

The concentrations of elements in *H. physoides* thalli from the Niepołomice Forest were generally comparable to those reported from other parts of Poland. Seasonal differences were observed, with higher S levels during the heating season, likely due to domestic heating and higher Cd and TBARS values in the non-heating season, suggesting influences from industrial, agricultural and environmental stress factors.

Spatial patterns indicated elevated Cd concentrations in the central-eastern forest, and higher Cu, Pb and Zn levels in the western and northern areas, suggesting the impact of transport, industrial activities, and historical pollution. Antagonistic distributions between Pb/Cu and Ca may indicate a protective role of Ca in lichens. Overall, eastern sites exhibited higher S, Cd and Hg concentrations along with greater lichen damage, likely linked to local emissions from energy production and domestic combustion. Future research should focus on long-term monitoring of metal accumulation and oxidative stress in lichens, and on distinguishing between anthropogenic and natural sources of these pollutants.

Data availability

The data are available in Open Research Data Repository of Krakow Universities RODBUK: https://doi.org/10.24917/VYJKFU.

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Declarations

Competing interests
The authors declare no competing interests.

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