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Membrane lipid peroxidation in lichens determined by the TBARS assay as a suitable biomarker for the prediction of elevated level of potentially toxic trace elements in soil

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ABSTRACT

The use of biomarkers defined as measurable biological parameters at the sub-organism level in which structural or functional changes indicate the presence of pollutants is a promising approach to identify affected sites. The aim of the study was to test the suitability of the level of membrane lipid peroxidation in the widespread epigeic lichen *Cladonia rei* for the assessment of soil pollution and to develop an effective method to recognise elevated concentrations of potentially toxic trace elements in soil. We collected lichen and soil substrate samples from variously polluted sites and determined the level of membrane lipid peroxidation in the thalli using the thiobarbituric acid-reactive substances (TBARS) assay. Based on the concentrations of Zn, Pb, Cd and As in the soil samples, we calculated the Pollution Load Index (PLI) for particular sampling sites to consider the combined effect of several toxic elements. The model of relationships between TBARS concentrations and PLI revealed that membrane lipid peroxidation does not increase linearly with PLI value, which suggests that above a certain threshold, oxidative stress induced activation of defence aimed at counteracting deleterious effects. We observed that if PLI exceeded the value of 100, the concentrations of TBARS were markedly increased and did not drop below 30 nmol g^{-1} DW. On the other hand, in lichen samples collected at sites with PLI < 10, TBARS concentrations were lower and as a rule did not exceed 25 nmol g^{-1} DW. Therefore, we concluded that the effect of soil pollution on membrane lipid peroxidation in the thalli of *C. rei* can be used in passive soil quality biomonitoring, according to the principle that an increased level of membrane lipid peroxidation is predictive for increased concentrations of elements in the host substrate. Given that this species has not been analysed in detail for the use of biomarkers, this study establishes a baseline and supports practicable use of TBARS concentrations in lichens as biomarkers.

1. Introduction

Bioindication and biomonitoring are commonly used methods for assessing the effect of external factors on ecosystems that help to distinguish affected from not affected sites (e.g., Chuquimarca et al., 2019). Environmental chemicals, including potentially toxic trace elements, influence biological systems at various levels, from a single biochemical substance, through cells, organs, organisms, populations, and communities to ecosystems as a whole. Bioindication comprises all kinds of indicative parameters and involves different components: sub-organism biomarkers, bioindicator and biomonitor species, as well as ecological indicators (Gerhardt, 2002). Various effective methods have been proposed and introduced as bioindication tools. They also include the use of biomarkers to assess the impact of pollutants. Biomarkers are defined as measurable biological parameters at the sub-organism level (molecular, physiological, enzymatic, anatomical) in which structural or functional changes indicate the presence of pollutants in either qualitative or quantitative terms (Markert et al., 2003; Chaudhary, 2022). Pollution stress typically triggers a cascade of biological responses, each of which could theoretically serve as a biomarker (Lemos, 2021). Suitable biomarkers should be easy to measure and provide characteristic symptoms that are not confused with those induced by other environmental factors. Biomarkers are also known as diagnostic and early warning indicators characterized by a quick and sensitive response to changes in the level of pollution. By using biomarkers, we gain the opportunity to use biochemical/physiological response parameters that are

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rapid and cost-effective in the assay for the early recognition of pollutants present in the environment.

Lichens constitute symbiotic associations central to which are a fungus and a phototroph, which is typically an alga and/or cyanobacterium. Both symbiotic partners form a well-defined structural and functional unit; however, they differ in their metabolism, and the details of these heterotroph-phototroph metabolic interactions are still poorly understood (Spribille et al., 2022). They are particularly responsive to various pollutants and agents present in the surrounding environment, which is related to direct uptake of nutrients and toxic compounds throughout the entire surface of the thallus (Nimis et al., 2002). The term biomarker is still not often used in lichenology. Nevertheless, several biomarkers of lichens have already been involved in the assessment of the pressure of pollutants on their condition (e.g., Dhaouadi et al., 2022). They include, for example, the level of cell membrane damage, photosynthetic pigment concentrations, photosynthetic efficiency, membrane lipid peroxidation, ergosterol content, dehydrogenase activity, antioxidant and free radical-scavenging enzyme concentrations (e.g., Cuny et al., 2004a; Munzi et al., 2009; Kováčik et al., 2018; Osyczka and Rola, 2019). The physiological responses to stress were usually tested by means of laboratory experiments or after lichen thalli transplantation into polluted areas and frequently in relation to a specific adverse factor. On the other hand, specific proposals for the use of lichen biomarkers in the biomonitoring of pollutants have been very rarely presented (e.g., Gonzalez and Pignata, 1994; Levin and Pignata, 1995), although such an approach seems worth considering.

Various types of environmental stress can trigger the formation of reactive oxygen species (ROS) that leads to oxidative stress (Kranner et al., 2008). ROS formation has not been quantified in lichens, but the damage and defence mechanisms (i.e., antioxidants and enzymes that scavenge ROS) they cause can be successfully used as biomarkers (Cuny et al., 2002). ROS can separate hydrogen radicals from membrane lipids that initiate their peroxidation and thiobarbituric acid reactive substances (TBARS), which are products of polyunsaturated fatty acid decomposition, are formed (Mittler, 2002). TBARS concentrations were studied in lichens affected by various harmful factors, including potentially toxic trace elements (e.g., Bačkor, et al., 2011; Pisani et al., 2011; Rola et al., 2022), salt stress (Chowaniec et al., 2022), nitrogen excess (e.g., Maslaňáková et al., 2015; Paoli et al., 2015) or air pollutants (e.g., Paoli et al., 2011; Mateos and Gonzales, 2016).

Although in developed countries, emissions of most toxic elements have decreased in recent years (EEA, 2021), environmental pollution still remains a concern in many regions. Potentially toxic trace elements pose a threat to living organisms and whole ecosystems owing to their non-biodegradability, persistence in the environment, bioaccumulative nature, and biotoxicity (Bradl, 2005). The epigeic lichen *Cladonia rei* Schaer. (for detailed characteristics, see Syrek and Kukwa, 2008; Dolnik et al., 2010; Pino-Bodas et al., 2010) demonstrates great adaptability to a wide range of environmental conditions, and it occurs both in natural and semi-natural habitats as well as strongly affected anthropogenic sites, even those extremely enriched with toxic trace elements (e.g., Cuny et al. 2004b; Rola and Osyczka, 2018). Despite the evident physiological symptoms of stress, this lichen tolerates it well and spreads easily as a sturdy pioneer through highly polluted environments (Rola et al., 2014; Rola et al., 2022). During our previous studies on the impact of soil pollution on lichen physiology, we noticed that the level of membrane lipid peroxidation in *C. rei* shows high dependence and regularity with respect to the pollution and accumulation of trace elements in the thalli (see Rola et al., 2022). Since this parameter turned out to be very responsive, we decided to check whether it can be used as a biomarker to assess the degree of soil pollution in sites where this lichen grows.

The aim of the study was to test the suitability of determining the level of membrane lipid peroxidation in widespread lichen *C. rei* for the assessment of soil pollution and to develop a rapid, cheap and effective method to identify elevated trace element concentrations in soil and monitor potentially polluted sites in post-industrial areas. Our general assumption was that individuals from unpolluted or slightly polluted sites demonstrate a membrane lipid peroxidation level always much lower than those inhabiting sites heavily affected by soil pollution.

2. Materials and methods

2.1. Study area and sampling

Fieldwork was conducted in the Silesia-Cracow Upland (S Poland), a large part of which is closely associated with the mining, processing of Zn-Pb ores, and metallurgical industry. A total of 17 sampling sites were examined. The sampling sites were designated both within semi-natural habitats, such as psammophilous grasslands, and various anthropogenic habitats, i.e., post-smelting dumps, post-flotation dumps, and post-industrial wastes (Table 1; Fig. S1). The selection of sampling sites was aimed at considering the widest possible spectrum of soil pollution level. The sampling sites constituted homogenous patch of vegetation with a clear dominance of *C. rei* with an area ranging from 400 m^2 to 900 m^2. Ten sampling plots (1 m^2) were randomly selected at each sampling site and lichen materials were collected from their central parts. Simultaneously, soil substrate sub-samples were collected from the plots at each sampling site to a depth of 5 cm. The soil sub-samples were combined into three to ten independent composite samples (with one composite sample consisting of 10 sub-samples). The number of composite samples per a sampling site depended on the size of the area represented by a given site and its structural complexity. Composite soil sampling was applied to obtain information about the average level of

Table 1
The list of sampling sites included in the study together with their location and habitat type.

Sampling site (locality)	Coordinates (midpoint)	Habitat type
Podlesice	50°34′00.0″N 19°32′27.7″E	Semi-natural psammophilous grassland
Pazurek	50°20′41.1″N 19°37′33.8″E	Semi-natural psammophilous grassland
Pustynia Błędowska	50°20′13.9″N 19°32′41.1″E	Semi-natural psammophilous grassland
Ujków Stary	50°17′33.6″N 19°29′08.1″E	Semi-natural psammophilous grassland
Pustynia Starczynowska (1)	50°15′30.4″N 19°31′18.5″E	Semi-natural psammophilous grassland
Pustynia Starczynowska (2)	50°15′21.6″N 19°30′15.9″E	Semi-natural psammophilous grassland
Bukowno (grassland)	50°16′07.6″N 19°29′36.5″E	Psammophilous grassland within industrial area
Piekary Śląskie - Brzeziny Śląskie	50°21′09.4″N 18°57′52.9″E	Post-smelting dump – Pb smelting
Chorzów	50°20′23.0″N 18°56′30.1″E	Post-flotation dump
Bukowno (Zn smelter)	50°16′28.0″N 19°28′11.5″E	Psammophilous grassland adjacent to the smelter (post-industrial wastes)
Trzebinia	50°09′13.9″N 19°27′48.6″E	Post-smelting dump – Zn smelting
Piekary Śląskie - Brzozowice Kamień (2)	50°21′52.5″N 18°57′58.3″E	Post-smelting dump – Pb smelting
Piekary Śląskie - Brzozowice Kamień (1)	50°22′04.1″N 18°58′05.3″E	Post-smelting dump – Pb smelting
Ruda Śląska - Wirek (3)	50°16′06.3″N 18°52′01.5″E	Post-smelting dump – Zn/Pb smelting
Ruda Śląska - Wirek (2)	50°16′42.8″N 18°51′54.0″E	Post-smelting dump – Zn/Pb smelting
Ruda Śląska - Wirek (1)	50°15′59.3″N 18°52′10.8″E	Post-smelting dump – Zn/Pb smelting
Piekary Śląskie (Dołki)	50°21′11.6″N 19°00′10.0″E	Post-flotation dump

soil pollution for particular sites inhabited by *C. rei* and from which the lichen thalli intended for the TBARS assay were collected. In addition, ten specimens of four other *Cladonia* lichens, i.e., *C. furcata* (Huds.) Schrad., *C. macilenta* Hoffm., *C. merochlorophaea* Asahina, and *C. subulata* (L.) Weber ex F.H. Wigg., were collected at Pustynia Starczynowska (2) locality that corresponds to a habitat not directly affected by metal pollution. This was done to recognize the natural production of TBARS in common *Cladonia* lichens and to relate the TBARS level determined in *C. rei* not exposed to pollution to the levels characteristic for other species of the same genus. After being transported to the laboratory, lichen thalli were carefully cleaned of soil particles and other macroscopic foreign materials adhering to their surfaces.

2.2. Metal concentrations in soil samples

Soil samples were dried and sieved (mesh diameter 2 mm). The samples (5 g of DW) were digested with 70 % $HClO_4$ (Merck, Suprapur) using a digester (FOSS, Sweden). The concentrations of elements (Zn, Pb, Cd, As) were determined by means of flame or graphite furnace atomic absorption spectrometry (AA280FS, AA280Z with a GTA 120; Varian, Australia). Certified reference material CRM048 (Sand 1, Sigma-Aldrich) were used for quality assurance. The recovery values for CRM ranged from 89.00 % to 99.86 % of the certified value. The choice of these elements was due to the fact that they primarily pose a serious environmental threat in the study area. The extraction and processing of zinc and lead ores led to a significant enrichment of soils with these elements.

2.3. Determination of membrane lipid peroxidation

The level of membrane lipid peroxidation in lichen samples was assessed using the thiobarbituric acid-reactive substances (TBARS) assay in accordance with Heath and Packer (1968) with modifications of Politycka (1996). Ten independent samples from each study site were analysed. Ca 40 mg of air-dried lichen thalli (1–2 podetia), were weighed per one sample and homogenised in a rough mortar with 1.5 ml of ice-cold 0.25 % (w/v) thiobarbituric acid (TBA) in 10 % trichloroacetic acid (TCA). The mixtures were then heated in a water bath (JWE 357, Elpin-Plus, Poland) at 95 °C for 30 min. After the samples had cooled down, they were centrifuged for 15 min at 12000 × g (Centrifuge 5424, Eppendorf, Poland). The absorbance of the supernatant was measured at 532 nm and corrected for nonspecific absorption at 600 nm (UV–vis spectrophotometer Genesys 180, Thermo Fisher Scientific, USA). The extinction coefficient (155 $mM^{-1}cm^{-1}$) specific for thiobarbituric acid-malondialdehyde (TBA-MDA) complex was used for calculation of concentration of lipid peroxidation products (TBARS). The level of membrane lipid peroxidation was expressed as nmol TBARS per gram of DW of lichen thallus.

2.4. Calculations and statistical analyses

In order to take account of the combined effect of several toxic elements on membrane lipid peroxidation in lichens, we decided to use indices reflecting the overall level of soil pollution. This approach seems justified since we studied lichens that naturally inhabited polluted sites and their metabolic response did not result from the influence of a single element. We applied Pollution Load Index (PLI; Varol, 2011); it was calculated as a geometric average of PI based on the following formula:

$$PLI = \sqrt[4]{PIsoilZn \times PIsoilPb \times PIsoilCd \times PIsoilAs}$$

where PI_{soil} is a calculated value for a single Pollution Index calculated according to the formula:

$$PIsoil = \frac{Cn_{soil}}{Bg_{soil}}$$

where Cn_{soil} is the concentration of element in soil sample and Bg_{soil} is the value of a geochemical background. The values of Bg_{soil} were adopted from Kabata-Pendias (2011), which represent the worldwide geochemical background of the average element contents in surface horizons. Additionally, local background values were also used for comparative purposes; they were determined based on element concentrations in the soil of psammophilous grassland located far from pollution sources within the study area (Osyczka et al., 2021; see Table S1).

The values of TBARS concentration in the samples collected from the sites with PLI values close to 1 (which indicates no soil pollution; see Varol, 2011; Kowalska et al., 2018) were averaged and treated as a reference point. In this way, we were able to estimate by how many percent the mean TBARS concentration in samples collected from other sites changed compared to the reference (non-affected lichen thalli).

The relationship between TBARS concentrations in lichen thalli and PLI was tested with various regression models to find the best fitted one. To this end, the variables were accordingly transformed to convert curvilinear models into linear models, in which parameters could be determined by least squares estimation (Sen and Srivastava, 1990). The best-fitted model was selected based on the coefficient of determination (R^2) (Motulsky and Christopoulos, 2004). Once the model has been fit, residual analysis was performed in order to validate the model, obtain reliable regression coefficients and detect potential outliers.

The significance of differences in terms of TBARS concentrations between particular sampling sites was tested with one-way analyses of variance ($p < 0.05$) followed by Tukey's HSD post-hoc test. The normality distribution in particular groups was checked with the Kolmogorov-Smirnov test, while the Brown–Forsythe test was used to verify the homogeneity of variances. In the same way, the significance of differences in TBARS concentrations between species of the genus *Cladonia* was analysed.

Principal component analysis (PCA) was performed to show distribution of sampling sites based on mean pollution indices (PI) for particular elements. Pollution indices were normalized prior to analysis. Double normalized (to the range 0 to 1) TBARS concentrations in lichen samples were visualised in a form of bubbles indicating their mean values for sampling sites.

The statistical analyses were performed using Statgraphics Centurion 19 (Statgraphics Technologies, Inc., The Plains, VA, USA), STATISTICA 13 (TIBCO Software Inc., Palo Alto, CA, USA) and PAST 4.06 (Hammer et al., 2001).

3. Results

3.1. Pollution Load Index and membrane lipid peroxidation in lichens

The values of PLI calculated for studied sampling sites based on worldwide and local background values ranged from 0.64 to 1353 and from 0.99 to as much as 2087, respectively (Table S2). PCA showed the distribution of sampling sites according to PI of particular elements (Fig. 1). The main gradient can be observed along the first axis. Generally, moving from the left to the right side of the diagram, the values of PI increase. The level of membrane lipid peroxidation also readily reflects this gradient, since the highest mean TBARS concentrations were recorded in lichens collected from sampling sites that were grouped on the right side of the diagram.

Mean TBARS concentrations in lichen thalli ranged from 18.45 to 44.15 nmol g^{-1} DW and differ significantly between sampling sites (Fig. 2).

3.2. Relationships between TBARS concentrations and Pollution Load Index

The visualisations and regression equations of the relationship

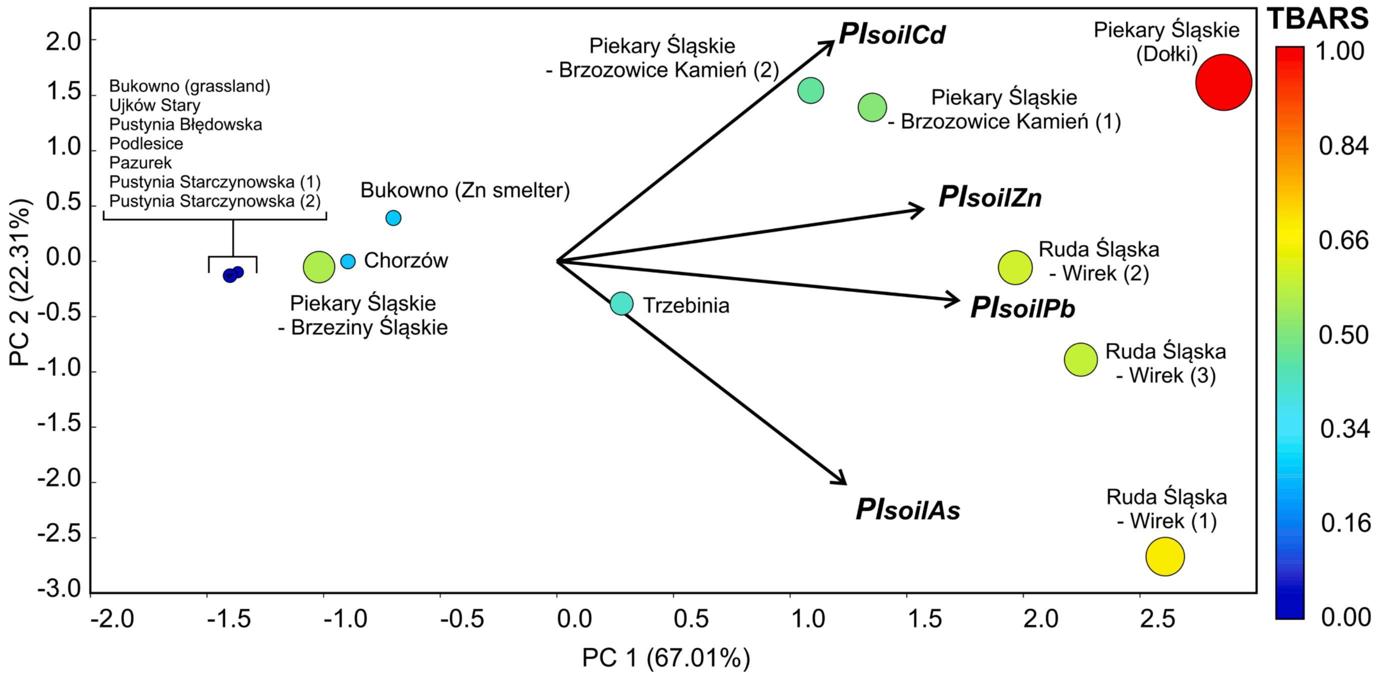

Fig. 1. Principal component analysis (PCA) bubble biplot based on mean pollution indices (PI) of particular elements (worldwide background values considered) calculated for sampling sites. The size and colour of the bubbles indicate the mean concentrations of TBARS measured in lichen samples. TBARS concentration values were double normalized to the range 0 to 1.

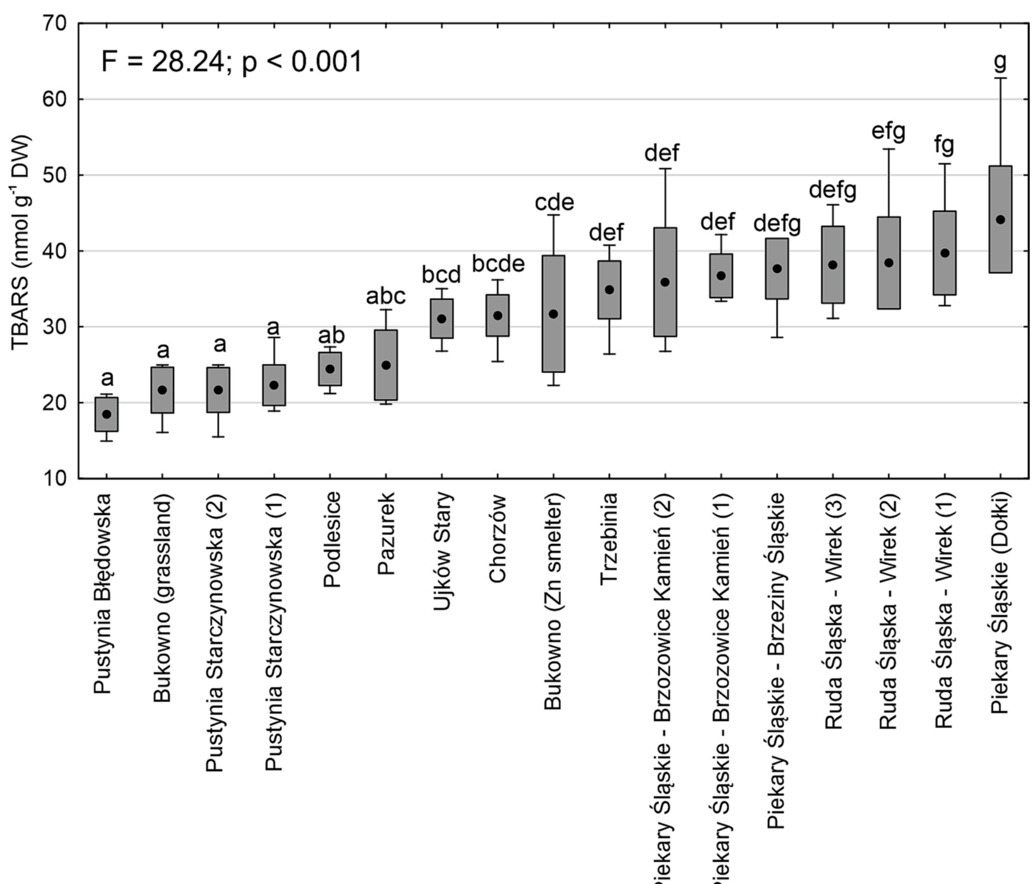

Fig. 2. TBARS concentrations determined in *Cladonia rei* samples collected at particular sampling sites. Boxes indicate SD, whiskers indicate minimum and maximum values (n = 10). The result of one-way ANOVA with Tukey's HSD post-hoc test is provided; the different letters indicate statistically significant differences ($p < 0.05$).

between TBARS concentrations and PLI calculated based on worldwide and local background values are provided in Fig. 3 and Fig. S2; the same pattern reflects this relationship regardless of the background levels. Among all the tested models, the non-linear relationship described by the squared-Y square root-X function turned out to be the best-fitted; the results of analysis of variance indicated significance of the model ($p < 0.001$) and around 83 % of the variability in TBARS was explained (Table S3). Following the course of the curves that illustrate the function, it can be generalized that with the increase of PLI values, the rate of increase of TBARS gradually decreases approximately in line with the given functions (Fig. 3 and Fig. S2).

3.3. Differences in the level of membrane lipid peroxidation between different Cladonia species

The concentrations of TBARS in *Cladonia* representatives collected from semi-natural habitat varied between 14.8 and 21.7 nmol g^{-1} DW. One-way ANOVA revealed significant differences between the species (Fig. 4). Significantly the lowest TBARS concentrations were recorded in *C. subulata*, while *C. rei* was characterized by the highest TBARS concentrations that differ significantly from *C. merochlorophaea* and *C. subulata*.

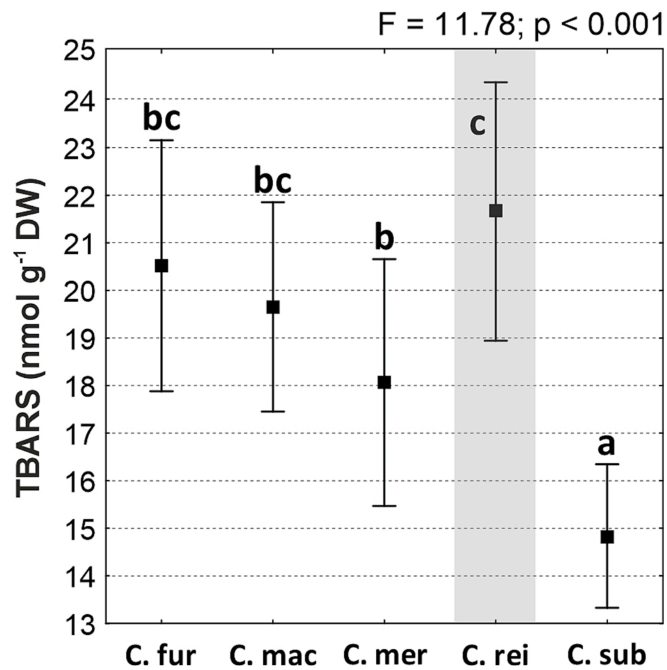

Fig. 4. Comparison of TBARS concentrations (mean ± SD) between different species of *Cladonia* genus; all specimens were collected from the same semi-natural grassland site (denoted as Pustynia Starczynowska 2). The result of one-way ANOVA with Tukey's HSD post-hoc test is provided; the different letters indicate statistically significant differences ($p < 0.05$). Species abbreviations: C. fur – *C. furcata*, C. mac – *C. macilenta*, C. mer – *C. merochlorophaea*, C. sub – *C. subulata*.

4. Discussion

Lichens are among the most commonly used biomonitors in both terrestrial and aquatic ecosystems (Nimis et al., 2002; Krzewicka et al., 2020). Most biomonitoring studies on lichens concerning physiological biomarkers involve an active approach under planned field exposures (Chaudhary, 2022) in which lichens are transplanted in the research area for a particular period of time (e.g., Oztetik and Cicek, 2011; Malaspina et al., 2014). The assessment of pollution impact by physiological response of indigenous lichens in their natural site where they grow (passive biomonitoring) has been used much less frequently (Cuny et al., 2004a). Various studies suggested that indicators of oxidative stress (e.g., lipid peroxidation products) in indigenous or transplanted lichens could serve as a qualitative or quantitative assessment of pollution (e.g., Paoli et al., 2011; Lucadamo et al., 2015; Sujetovienė et al., 2020). As for potentially toxic trace elements, Cuny et al. (2004a) found a significant correlation between intracellular Zn concentrations in lichen *Diploschistes muscorum* and MDA concentrations, which suggests that Zn enhance the peroxidation of membrane lipids. However, all these studies were related to the effect of bioaccumulated elements on lichen metabolism. Our results showed that the physiological response of *C. rei* may result directly from pollution of host soil substrate. We believe that *C. rei* can serve as a model for soil pollution biomonitoring by analysing the response of membrane lipid peroxidation level.

Particular trace elements may affect membrane lipid peroxidation in different ways depending on the doses delivered and the specific lichen species. For example, several studies showed that lichens are sensitive to high doses of Cu, which caused a significant increase in the content of TBARS in both short- and long-term experiments (Bačkor et al., 2011; Bačkorová et al., 2015). Similarly, membrane lipid peroxidation was positively correlated with increased accumulation of Pb and Zn in *Xanthoria parietina* thalli (Vavilin et al., 1998; Dzubaj et al., 2008). A more complicated situation concerns the effect of Cd on lichens since this element is highly intercepted at the cell wall level protecting cell membranes against the toxic effect (Traina, 1999). Nevertheless, intense exposure to Cd stress also increased TBARS levels (Sanità di Toppi et al., 2005). Moreover, our previous study (Rola et al., 2022) provided clear evidence that increased accumulation of trace elements (especially xenobiotics) in *C. rei* results in a marked increase in membrane lipid

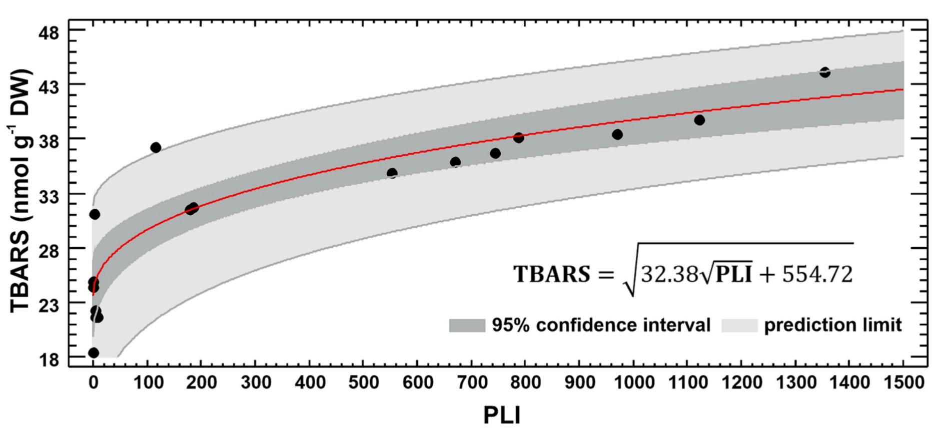

Fig. 3. Regression plot with the best fitted function presenting the relationships between TBARS concentrations in the thalli of *Cladonia rei* and Pollution Load Index (PLI) calculated based on the worldwide background values (see also Table S1 and Table S2).

peroxidation. This supports the idea that this is a sensitive and useful parameter for assessing physiological state of lichens. Consequently, *C. rei* seems to be a perfect candidate for biomonitoring research, as TBARS values in the thalli collected from unpolluted sites were always considerably lower than in those from metal-rich areas (Fig. 2).

The advisability of the proposal to use TBARS concentration in lichens as a biomarker is also in line with high responsiveness of a mycobiont to pollution stress. Cell membranes constitute the first site of biological interaction with external substances (Honegger 1993). Paoli et al. (2018) found that recovery of physiological parameters typical for the mycobiont (including membrane lipid peroxidation level) was much faster compared to those related to the photobiont in lichens transplanted to a new habitat at the unpolluted site. Moreover, our previous study showed that photosynthetic efficiency in tolerant lichens collected from polluted sites, including *C. rei*, remained at a very high level (Rola et al., 2019). The high responsiveness of TBARS concentrations in lichens to environmental stress is especially important for two different reasons. First, the biomarker will fulfil its function in the case of environmental conditions improvement, such as a decrease in the content of potentially toxic trace element in the soil, for example when emissions will cease in a given area or when reclamation or phytoremediation treatments will be implemented. This was demonstrated by a decrease in TBARS concentrations in lichens transplanted to remote unpolluted area from landfill sites affected by metal pollution (Paoli et al., 2018). Second, TBARS levels are likely to be very effective in indicating environmental deterioration in the case of newly created sources of emissions. Paoli et al. (2015) found that membrane lipid peroxidation was significantly higher in lichens growing in metal polluted area around the landfill compared to those inhabiting remote locations. Carreras et al. (2005) also confirmed an increased concentration of MDA in lichens transplanted to the polluted urban site, which suggests that air pollutants increase the level of oxidative stress and directly affect the cell membranes in lichens.

Lichens in their natural environment are influenced by a mixture of substances rather than by a single substance. An environmental study based on lichens that grow in their natural habitat readily reflects the general degree of soil pollution instead of soil enrichment by a single element. It has been shown that a risk assessment based solely on the level of one toxic substance in the environment is not considered reliable due to the ability of different pollutants to interact and enhance their toxic effects (Van der Oost et al., 2005). Therefore, environmental assessment should focus on the effects of the entire mixture of pollutants expressed by complex indices (e.g., Gong et al., 2008; Varol, 2011). The fact that biomarkers generally are more effective in determining the overall toxicity of complex mixtures (Lam and Gray, 2003) supports the approach we used.

The process of membrane lipid peroxidation, which occurs as a result of the influence of factors not directly related to the soil pollution pressure, reaches different levels depending on the species (Paoli et al., 2015; Chowaniec et al., 2022). However, the general range of TBARS concentration that was determined in various *Cladonia* lichens collected from the same site and time within the semi-natural psammophilous grassland was relatively narrow (Fig. 4). On the other hand, it can be observed that *C. rei* specifically tends to achieve higher levels of TBARS concentration in the thalli, especially when compared to the values recorded in the samples of *C. subulata* (Fig. 4). The gradual replacement of certain sensitive species (including *C. subulata* and *C. merochlorophaea*) by resistant species (including *C. rei*) has been observed along the soil pollution gradient (Rola and Osyczka, 2014). Perhaps this is partly related to the peculiar physiological tolerance of *C. rei* to the increased membrane lipid peroxidation that normally occurs in their thalli. The model of relationships between TBARS concentrations in *C. rei* samples and PLI indicates that the increase in membrane lipid peroxidation does not increase linearly with PLI (Fig. 3 and Fig. S2). This suggests that above a certain threshold, oxidative stress induced activation of defence mechanisms including enzymatic and non-enzymatic antioxidants scavenging ROS (Kranner et al., 2003), which may counteract deleterious effects. Nevertheless, it can be observed that if PLI exceeded the value of 100, the concentrations of TBARS increased markedly (at a level of at least 25 % compared to unpolluted sites with PLI close to the value of 1) and did not drop below 30 nmol g^{-1} DW. On the other hand, lichens collected at sampling sites with PLI $<$ 10, TBARS concentrations were always lower and as a rule did not exceed 25 nmol g^{-1} DW. Therefore, an increase in TBARS concentration in *C. rei* by at least 25 % successfully predicts elevated levels of Zn, Pb, Cd and, As in soil substrate. Nevertheless, it should be borne in mind that many external factors may have an additional impact on the level of oxidative stress in lichens. The confounding factors include both biotic and abiotic variables. Therefore, for example, when comparing sites with different air pollution levels, care must be taken when interpreting the results. Moreover, many non-pollution-related factors may have an additional impact on various biochemical or physiological biomarkers (Forbes et al., 2006). Consequently, to utilize our proposal, it is necessary to conduct the study under the same conditions, to use the same protocol for analytical method, and to include the examination of samples collected from unpolluted site (in order to relate the results to a reference value).

5. Conclusions

Our proposal constitutes a passive biomonitoring of soil pollution with toxic trace elements based on a physiological biomarker that is the level of membrane lipid peroxidation (expressed by TBARS concentrations) in samples of *C. rei*, which is a widespread and common lichen species both in anthropogenic and semi-natural habitats throughout Europe (Paus, 1997; Rola et al., 2014). The increase in TBARS concentration in *C. rei* by at least 25 % clearly reflects the elevated level of trace element concentrations in the soil substrate. Although the method does not provide specific quantitative information on soil pollution with individual elements, it is relatively simple in terms of implementation procedure and is incomparably cheaper than specialized analyses of soil samples for trace element determinations. This assessment could be applied to a wide landscape scale in various post-industrial habitats and can serve as an early warning indicator for the detection of elevated element concentrations in the soil. Given that *C. rei* has not been analysed in detail for the use of biomarkers associated with it, this study constitutes a baseline and supports the use of TBARS concentrations in lichens as a biomarker of soil pollution with potentially toxic trace elements.

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CRediT authorship contribution statement

Piotr Osyczka: Conceptualization, Formal analysis, Methodology, Investigation, Resources, Visualization, Writing – review & editing, Project administration. **Karolina Chowaniec:** Formal analysis, Investigation, Resources, Visualization, Writing – review & editing. **Kaja Skubała:** Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Resources, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.109910.

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