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Article

A New Method of Environmental Assessment and Monitoring of Heavy Metal Polluted Soil Using Fruticose Lichens

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Abstract: Soil pollution has been estimated using soil analysis and leaching test. These methods could show different data from reality due to effects by soil properties such as grain size and mineral composition. Therefore, this study advocates a new assessment and monitoring method of heavy metal polluted soil using fruticose lichens. Lichens growing at abandoned mine sites and unpolluted areas in southwest Japan and their substrata were analyzed using inductively coupled plasma-mass spectrometry and X-ray fluorescence spectrometry to clarify the relationships between the heavy metal concentrations in lichens and soils, and their heavy metal absorption properties. Concentrations of Cu, Zn, As, and Pb in the lichens were positively correlated with those in the soil. Variability of the relationships did not depend on the lichen species, location, habitat, or the conditions of soils. The analyzed lichens had neither competitive nor antagonistic properties in their heavy metal absorption, which make them good biomarkers of heavy metal pollution of soil. The distribution maps of average heavy metal concentrations at each sampling region detected almost all of the Cu, Zn, and As pollution of soil. Therefore, lichens could be used in practical applications to assess Cu, Zn, and As pollution of soils.

Keywords: biomarker; environmental assessment; elemental competition; bioconcentration factor; heavy metal absorption

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1. Introduction

Soil pollution has been estimated using soil analysis and leaching test. No single laboratory leaching test can evaluate the leaching behavior of a wide variety of material in a broad range of management scenarios [1]. The methods of leaching test should be chosen after consideration of soil properties, such as chemical and physical properties of soil-forming minerals and climatic conditions of the sampling area. Different results from actual environment may be given by the leaching test if an inappropriate method is chosen. Accordingly, limitation of the leaching test methods may lead to misuse and misinterpretation of the results [1].

Many organisms have been investigated as potential environmental indicators, so-called “bioindicators”, as the other way to estimate the soil pollution [2]. Van and Van (1996) [3] defined a “bioindicator” as one of the three types, according to the different levels of biological organization for ecological assessment: (1) “biomarker” shows biochemical, physiological, histological, and morphological responses within an organism at the below-individual level, or its products; (2) “bioindicator” reflects the environmental conditions of a habitat by its behavior and presence in the habitat; (3) "ecological indicator" shows differences at the ecological level, such as species diversity, population dynamics, and nutrient cycling rates.

The use of locally resident biomarkers growing in a polluted area could reduce the limitation shown in leaching tests. Commonly used indicator organisms include fishes, mollusks, and vascular plants [2,4-13]. However, these organisms commonly absorb selected elements because of their competitive and antagonistic properties of heavy metal absorption. Therefore, they can be used as the indicator for limited elements. This is a problem to use organisms for biomonitoring and environmental assessment.

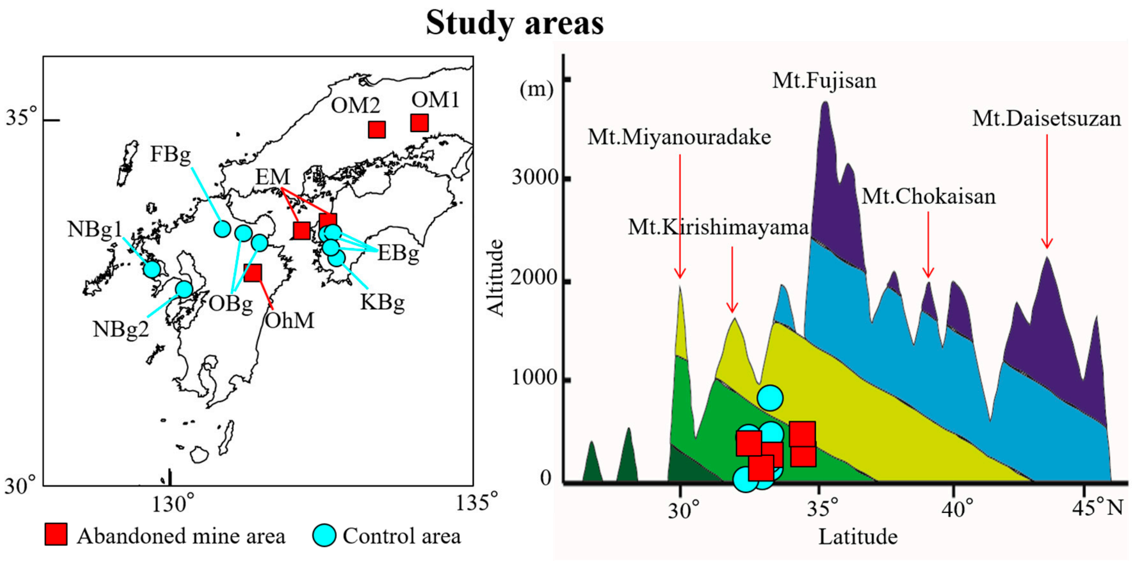
In contrast, lichens absorb mineral nutrients and trace elements, including metals, from dry and wet atmospheric deposition due to lack of a vascular root system [14,15]. Therefore, heavy metal concentrations of lichens may show the mobile heavy metals affecting environmental pollution. Moreover, some characteristics of lichens that could be used as a biomarker for pollution of their substrata have been revealed in recent studies. Several lichens absorb heavy metals from the corresponding substrata [16-18]. Osyczka and Rola [19] determined that the Zn and Cd contents in *Cladonia rei* Schaer. thalli were related to those in the host substrata via a power function, calculated through specific regression models. These characters of lichens are an important advantage for practical applications as an alternative to the leaching test.

Lichens cover more than 6% of the land surface of the earth [17], and are found in areas, such as tropical forests, desert, alpine regions, polar regions, urban areas, as well as highly polluted areas [20,21]. Therefore, lichens may have the potential to be broadly used as biomarkers in various terrestrial habitats worldwide. Although correlations between the accumulation capacity of lichens and the heavy metal concentrations of substrata have been demonstrated, the practical application of lichens as a biomarker for heavy metal pollution of soil has not been examined before. Therefore, the correlation between heavy metal concentrations in lichens and those in the corresponding substrata were investigated in *Stereocaulon commixtum* and several *Cladonia* spp. lichens growing in contaminated abandoned mine sites and unpolluted areas in southwest Japan. In addition, distribution maps of heavy metal concentrations at each sampling site were created using the average concentrations of heavy metals in the soils and lichen thalli. Finally, the practical application of lichens as biomarkers for the assessment and monitoring of heavy metal polluted soil was evaluated.

2. Materials and Methods

2.1. Study Area

This study was conducted at abandoned mine sites and unpolluted areas at altitudes between 10 and 800 m above sea level in warm-temperate and cool-temperate zones in southwest Japan (Figure 1). Metals, such as Cu, Zn, Sn, and Pb had been smelted at the abandoned mine sites, which closed at least 40 years before the experiment. Surfaces of waste dumps at the abandoned mine sites consisted mainly of slag fragments and tailings. Solidified and coherent slag was partially exposed on the dumps. The unpolluted areas had almost no ore deposits, and no heavy metal pollution had been reported in these areas.



	Vertical distribution	Horizontal distribution	Climate zone
🟪	Alpine zone	Tundra	Polar zone
🟦	Sub-alpine zone	Coniferous forest	Sub-polar zone
🟨	Mountain zone	Summer-green forest	Cool-temperate zone
🟩	Sub-mountain zone	Laurel forest	Warm-temperate zone
■		Sub-tropical rainforest	Sub-tropical zone

Figure 1. Locations of this study areas and their climate zone.

2.2. Sampling methods

Totally 61 lichen specimens set with the corresponding substrata were randomly sampled from the slag and tailing dumps, outcrops, sandbanks, slope sediment, and weathered asphalt along a roadside at each of the abandoned mine sites and unpolluted areas from November 3, 2012 to May 18, 2015. The lichen specimens were rinsed with ultrapure water (Milli-Q), were oven dried at 80 °C for 24 hours, and were stored in dark place at Ehime University. The soil samples were stored in a desiccator after being oven dried at 120 °C for at least 48 hours. Powdered samples of lichens and soils were stored in dark place at Ehime University.

2.3. Identification of lichens

The lichen specimens were tested for identification by morphological observation and chemical analyses using a stereomicroscope, solutions for color reactions, thin layer chromatography (TLC), and liquid chromatography-mass spectrometry (LC/MS). Color reactions were carried out using the following three solutions: 10% aqueous potassium hydroxide [KOH], saturated aqueous calcium hypochlorite [$Ca(OCl)_2$], and 5% alcoholic p-phenylenediamine solution. Lichen substances in acetone extracts of specimens were identified using TLC and LC/MS according to Huneck and Yoshimura (1996) [22].

2.4. Determining heavy metal concentrations in lichen materials and soils

The heavy metal concentrations in lichen materials were determined by inductively coupled plasma-mass spectrometry (ICP-MS) using a Varian 820-MS instrument (Agilent Technologies, Santa Clara, USA). The heavy metal concentrations in the substrata were determined by wave dispersive X-ray fluorescence (WD-XRF) spectrometry using a Primus II instrument (Rigaku, Tokyo, Japan).

2.4.1. ICP-MS

Lichen thalli were excised at the upper 1–5 mm portion from their substrata using ceramic scissors to avoid fragments of the substratum trapped by the hyphae and rinsed with ultrapure water

(Milli-Q). The rinsed samples were oven dried at 80 °C for 24 hours. The dried samples were pulverized using an agate mortar. The 20 mg pulverized powder was digested with several acids by following steps: 1. digested with 200 μL of hydrogen peroxide [H_2O_2], 1 mL of 61% nitric acid [HNO_3], and 500 μL of hydrofluoric acid [HF] and heated on a hotplate at 160 ˚C for 30 minutes; 2. cooled down at room temperature for 30 minutes; 3. evaporated to dryness on the hotplate at 80 ˚C; 4. digested with 1 mL of 61% HNO_3 and heated on the hotplate at 160 ˚C for 30 minutes; 5. cooled down at room temperature for 30 minutes; 6. evaporated to dryness on the hotplate at 80 ˚C; 7. digested with 1 mL of 30% HNO_3 and heated on the hotplate at 120 ˚C for 10 minutes; 8. Cooled down at room temperature for 1 h. The digested solution was diluted by 3% HNO_3 as solution for analysis.

The analytical accuracy and precision were verified using the NIES CRM No. 1 environmental sample. The analytical calibration curve was created using Multi-element Calibration Standard 3 (PerkinElmer, Inc., Massachusetts, USA). Rhodium Standard Solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as the internal standard.

2.4.1. XRF

Pressed powder pellets were used for the XRF analysis. Samples were pulverized to a grain size of <1 μm in a tungsten carbide vibrating sample mill (SAMPLE MILL model TI-100, HEIKO, Tokyo, Japan) and an agate mortar. The pulverized samples were homogenized by shaking for 6 hours. Polyethylene series ($C_{13}H_{14}O_6$) was used as a binder (20 wt.% in the pressed powder pellet) and was homogenized with the powdered samples by shaking for 1 hour.

The analysis was conducted at an X-ray tube voltage of 50 kV. Two detectors, the scintillation counter and the proportional counter, were used for the analysis. The X-ray source was a Rh anode. The elemental concentrations were determined using the fundamental parameter method and a matching library. Geological standards, including JSd-1, JSd-2, and JSd-3 (Geological Survey of Japan Reference Materials) [23,24] were analyzed to confirm analytical precision.

2.5. Statistical analysis

The normality of the heavy metal concentrations in lichens and the corresponding substrata were assessed using the Shapiro-Wilk test and QQ plots, and exhibited significantly non-normal distributions. Therefore, correlations between the heavy metal concentrations in lichens and soil were verified by Spearman's rank correlation rho (r_s) or t-values obtained by Spearman's correlation coefficient.

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) that is designed to add statistical functions frequently used in biostatistics [25].

2.6. Bioconcentration factor

The bioconcentration factor (BCF) has been used to evaluate the bioaccumulation property of plants [26]. The BCF values of lichens were calculated using the following equation [27]:

$$\mathrm{BCF} = C_{shoot}/C_{soil} \tag{1}$$

where C_{shoot} is the metal concentration in shoot and C_{soil} is that in soil.

3. Results

3.1. Distribution of lichens

At least 10 Cladoniaceae lichens and a Stereocaulaceae lichen occurred on soil, rock, and/or mine wastes in the study areas. The tested lichens were identified as follows: *Stereocaulon commixtum* (Asah.) Asah.; *Cladia aggregata* (Sw.) Nyl.; *Cladonia rangiferina* (L.) F. H. Wigg. subsp. *rangiferina*; *C. coniocraea* (Flörke) Spreng.; *C. scabriuscula* (Delise ex Duby) Nyl.; *C. crispata* (Ach.) Flot. var. *crispata*;

C. krempelhuberi Vain.; *C. trassii* Ahti; *C. ramulosa* (With.) J. R. Laundon; *C. humilis* (With.) J. R. Laundon; and *C. macilenta* Hoffm. The *S. commixtum*, *Cladia aggregata*, *Cladonia rangiferina*, *C. crispata*, *C. macilenta*, *C. humilis*, *C. ramulosa*, *C. krempelhuberi*, and *C. trassii* grew in the unpolluted and heavy metal polluted areas in all the study areas. Distributions of these lichens showed several dense areas on the waste dumps (Figure 2). The *S. commixtum* occurred mainly on slag fragments, tailings, and rudaceous soil. The *C. crispata*, *C. krempelhuberi*, and *C. trassii* occurred mainly on the rudaceous soil. The remaining *Cladonia* spp. and *Cladia aggregata* occurred mainly on humus soil and dead leaves, e.g., from ferns such as *Athyrium yokoscense* which are known as a resistant, accumulator, and bioindicator for Cu, Zn, Cd, and Pb [28-31]. *Cladonia* spp. are often associated with other *Cladonia* lichens.

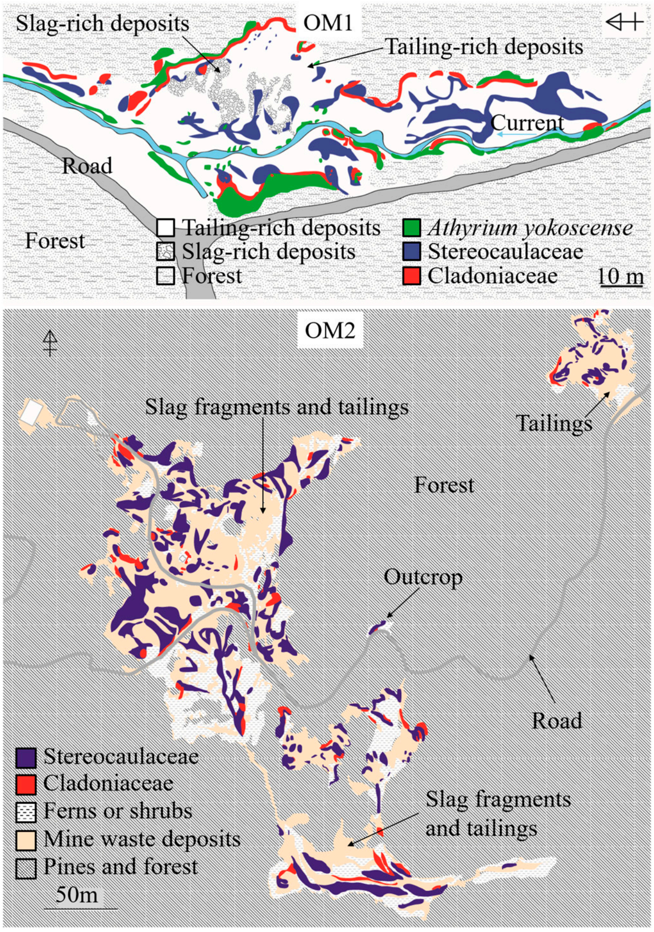


Figure 2. Distribution maps of lichens on mine waste dumps in the study areas OM1 and OM2.

3.2. Relationships between heavy metal concentrations in lichens and the corresponding substata

Scatter plots were used to determine the relationships between heavy metal concentrations in lichens and the corresponding substrata. The scatter plots showed that concentrations of Cu, Zn, As, and Pb in all lichens, including *S. commixtum* and 8 *Cladonia* spp., were positively correlated with those in the corresponding substrata (Figure 3). The scatter plots showed no local maximum points, but a linear distribution under a logarithmic scale. The variability of the scatter plots did not depend on lichen species, location, habitat, types of substrata, constituents of the substrata, or particle sizes of substrata (Figure 4). Accordingly, all lichens absorbed heavy metals in their thalli in unregulated concentrations, irrespective of the species, location, habitat, or conditions of soils in the study areas.

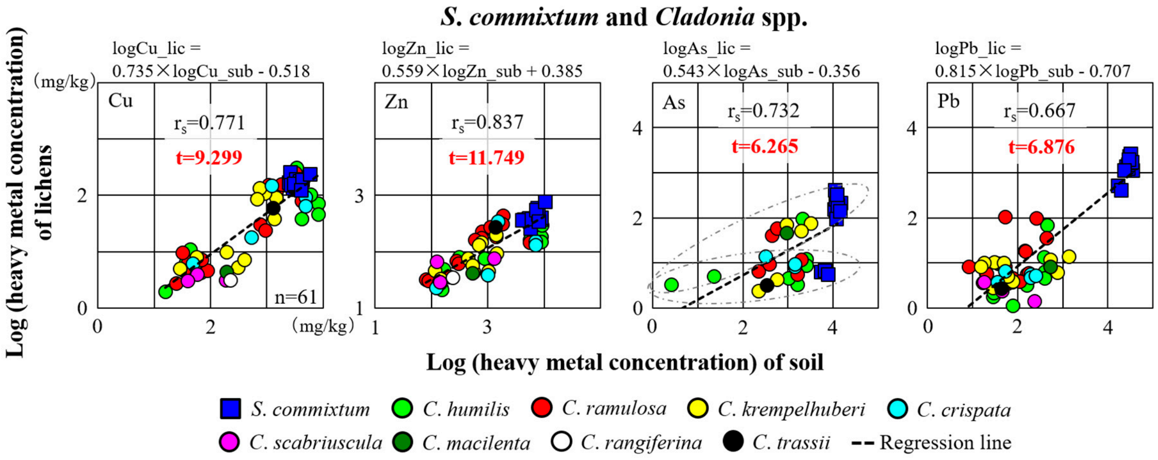


Figure 3. Scatter plots of Cu, Zn, As, and Pb concentrations in lichens and the corresponding substrata, with regression lines.

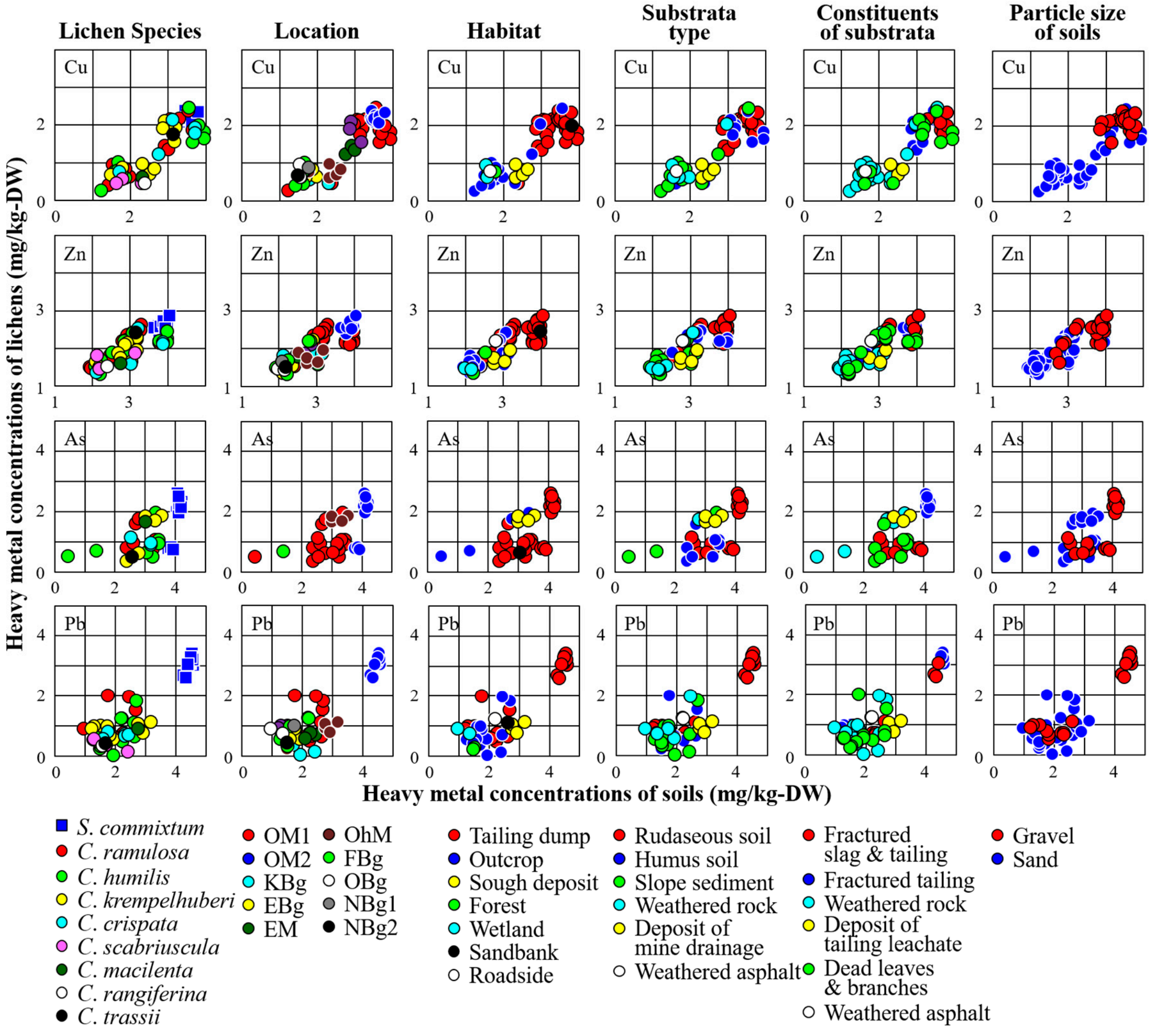


Figure 4. Scatter plots of heavy metal concentrations in lichens and the corresponding substrata separated by lichen species, location, habitat, substrata type, constituents of substrata, and particle size of soils using different colors.

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3.3. Competitive and antagonistic properties of heavy metal absorption

Scatter matrix were used to estimate the competitive and antagonistic properties in the heavy metal absorption by lichens. All of the scatter plots in the scatter matrix showed positive correlations between concentrations of Cu, Zn, As, and Pb in lichens and soil after logarithmic transformation (Figure 5). The positive correlation indicated that these elements were not absorbed selectively into the lichen thalli. Therefore, the lichens had neither competitive nor antagonistic properties in their heavy metal absorption, and the concentrations of heavy metal in lichens were similar to those in soil.

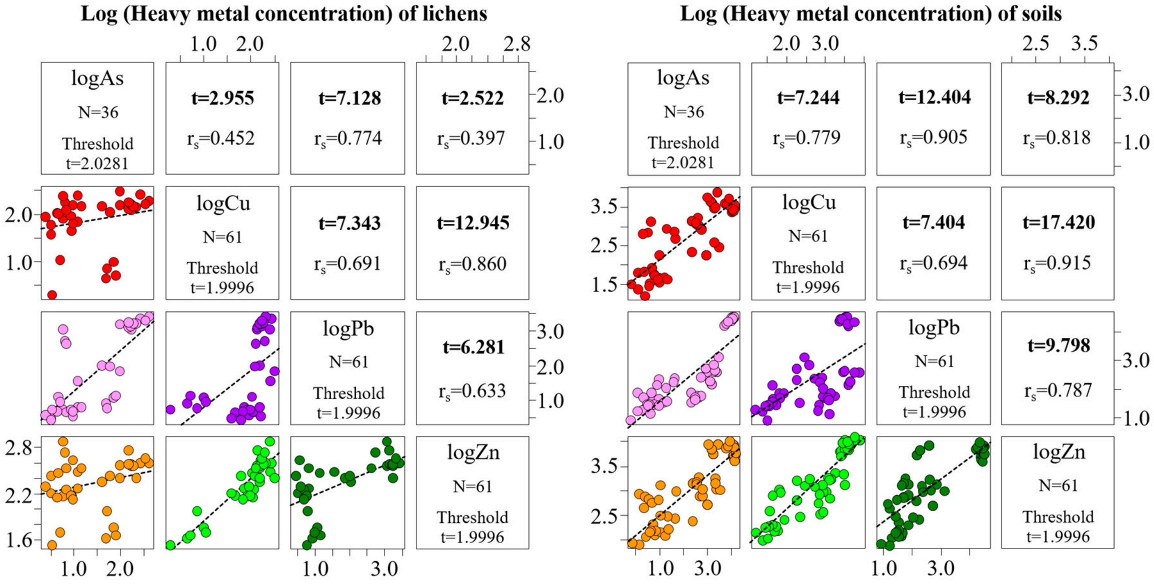

Figure 5. Scatter matrix showing the relationships among each Cu, Zn, As, and Pb concentration in lichens and soils after logarithmic transformation, with regression lines.

3.2. Relationships between bioconcentration factor and heavy metal concentrations.

According to McGrath and Zhao (2003) and Beni *et al*. (2012) [32,33], the lichens growing the study area were not hyperaccumulators, except several samples taken from OM1 (Figure 6). The BCF data also have important implications for potential biomarkers. The BCF values decrease with increasing heavy metal concentrations in soils (Figure 6), so that absorption of heavy metal by lichens is inhibited at higher concentrations. However, the scatter plots of logarithmic values of heavy metal concentrations in lichens and soils showed positive correlations and were described by linear regression models in a wide range of heavy metal concentrations (Figure 3), even for low BCF values. This property of the lichens imply possible practical application of the lichens as biomarkers for a wide range of heavy metal polluted soil. The variability of the scatter plots did not depend on lichen species, location, habitat, or conditions of soils, nor did the variability of heavy metal concentrations (Figure 6).

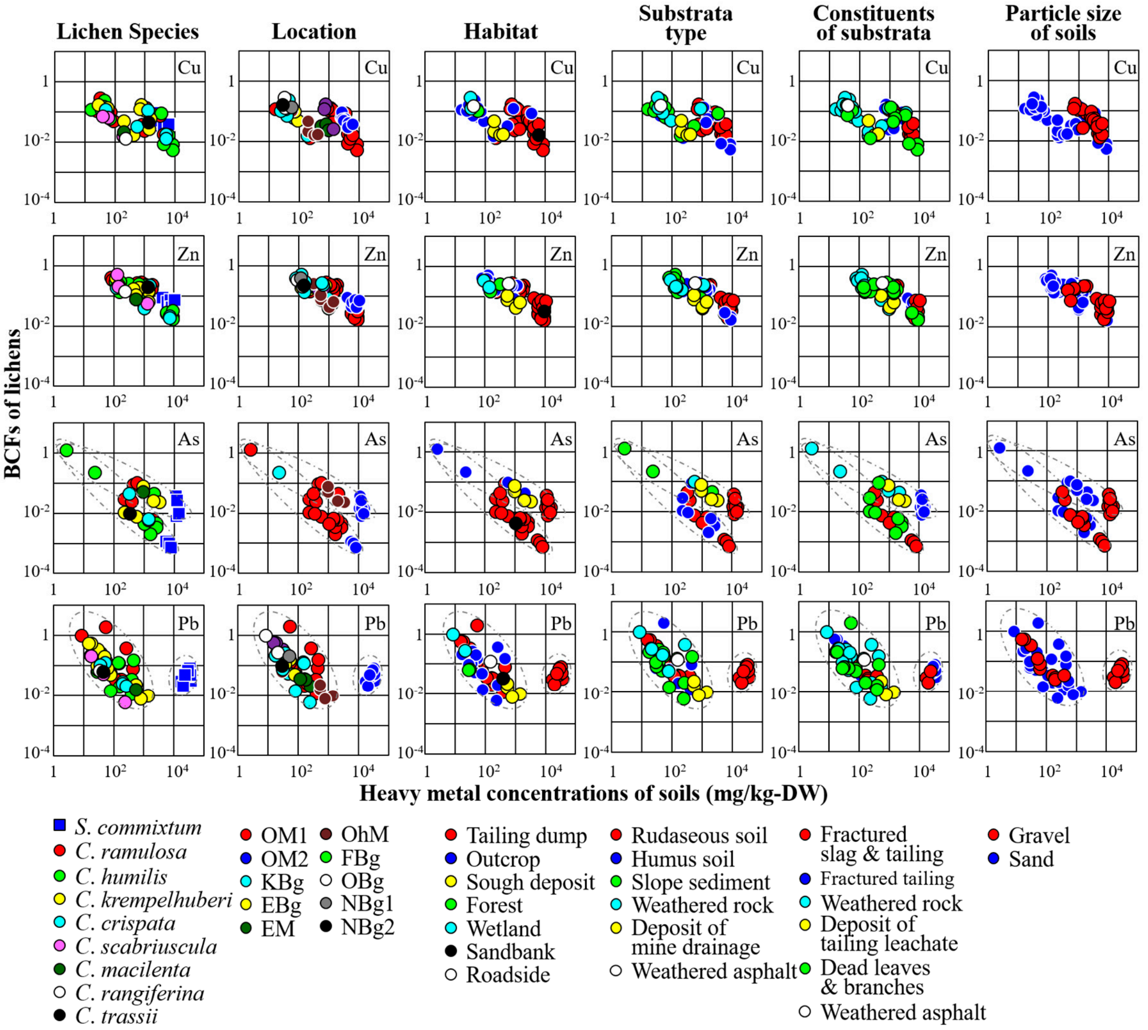

Figure 6. Scatter plots showing relationships between the BCFs and the heavy metal concentrations in soils, separated by lichen species, location, habitat, substrate type, constituents of substrata, and particle size of soils using different colors.

4. Discussion

4.1. Heavy metal absorption properties of lichens

The *Cladonia* spp. and *S. commixtum* showed different absorption properties for heavy metals in this study. The scatter plots of Pb vs the BCF showed two groups, *Cladonia* spp. and *S. commixtum* (Figure 6). Although the plots showed negative correlations with Pb, these two lichen species could have different absorption mechanisms. The plots of As vs BCF also showed two groups (Figure 6), although they showed different patterns to those of Pb. The As groups included both *Cladonia* spp. and *S. commixtum*, especially at higher concentrations (Figures 3,6).

Various previous studies has demonstrated the heavy metal uptake ability and the accumulation capacity of lichens [19,34-37]. However, neither competitive nor antagonistic properties in heavy metal absorption of lichens have been clarified in any previous studies. According to Sueoka *et al.* (2015), Fe and As could be affected by precipitation and adsorption on hyphae of lichen thalli but not selective elemental absorption by lichens. Therefore, the patterns of Pb and As showing in figures 3 and 6 indicated that *Cladonia* spp. and *S. commixtum* had different absorption properties for Pb and As, and were not affected by substratum properties or the habitat, but other possible factors, such as the chemical form of As and its solubility and connectivity.

Ions absorbed by lichens were not evenly distributed throughout the thalli [38]. The distributions of cations were divided into the following fractions: (1) extracellular and surface fraction, (2) ion exchange site fraction, (3) intracellular fraction, and (4) residual fraction [38]. The lichens contained dust and soil particles in their thalli [38,39]. Several *Cladonia* lichens showed different concentrations of heavy metals in different parts of their thalli [36]. Accordingly, chemical forms and accumulation of heavy metals in the lichen thalli should be clarified in future studies to reveal the accumulation capacity and property of the lichens as biomarkers.

4.2. Practical application of lichens as biomarkers

The relationships between heavy metal concentrations in lichens and the corresponding substrata have been demonstrated in previous studies [19,34]. Osyczka and Rola (2013) [19] demonstrated the relationship between Zn and Cd concentrations in *C. rei* thalli and the host substrata using specific non-linear regression models described by a power function. In this study, the heavy metal concentrations in Figure 3 indicate that the lichens were not "hyperaccumulators" or "excluders", but "indicators" as suggested by Ghosh and Singh [40]. In addition, the concentrations of Cu, Zn, As, and Pb in the lichens were positively correlated with those in the corresponding substrata and were analyzed by linear regression models after logarithmic transformation, similar to Osyczka and Rola (2013) [19]. The data of this study included different lichen species, locations, habitats, and conditions of soils in contrast to the previous study. Therefore, this study may be able to provide versatile data for practical application of lichens compared with the other previous studies.

The statistically positive correlations indicated that the lichens have potential as biomarkers of heavy metal pollution in soil. However, the practical applications of lichens require more than statistical correlations. Therefore, this study evaluated the practical application of lichens using heavy metal distribution maps, created using the average heavy metal concentrations in lichens and the corresponding substrata.

Figure 7 shows the distribution maps of the average heavy metal concentrations in the lichens and the corresponding substrata at each sampling region. The heavy metal concentrations were divided into 5 levels, based on the environmental soil standards of Japan. The yellow dots indicate that the concentrations exceeded the environmental standards. The light blue and red dots indicate less than half and more than double levels of the standards, respectively. The standard values of heavy metals in soil were: 1000 mg/kg for Cu and Zn, and 150 mg/kg for As and Pb. The standards for Cu and Zn in soil are not set by the Ministry of the Environment in Japan. Although the standards for Cu and Zn were set as 125 mg/kg and 120 mg/kg, respectively, by the Agricultural Land Soil Pollution Prevention Act in Japan, the standards for these elements were set at 1000 mg/kg in this study as the target was not farmland. The estimated environmental standards of heavy metals in lichens were calculated using the following equation (2), which is calculated from the linear regression lines in the scatter plot of lichens in Figure 3.

$$\mathrm{logY} = \log(\mathrm{a}X^{b}) = \log(\mathrm{a}) + \mathrm{b} \times \mathrm{logX}, \tag{2}$$

$$\mathrm{Y} = \mathrm{a}X^{b}, \tag{3}$$

where Y is heavy metal concentrations in lichens, and X is the environmental standard values of soil. The antilogarithmic transformed equation was the same as the regression model obtained by a power function (3). Therefore, the estimated environmental standards of lichens were obtained using this equation.

As shown in Figure 7, the plots of Cu, Zn, and As in the lichens showed similar distributions as those of the soils. The distribution of Pb plots, however, varied widely. Consequently, the maps created using lichens detected almost all of the Cu, Zn, and As pollution of soil.

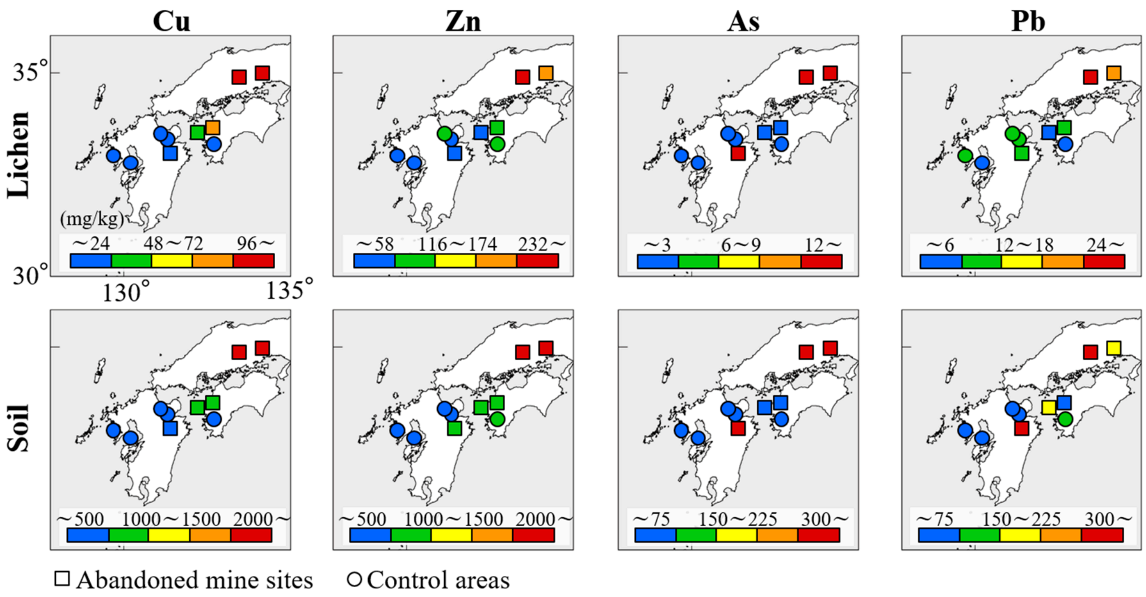

Figure 7. Distribution maps of the average heavy metal concentrations in lichens and soils at each sampling region in southwest Japan. The yellow dots indicate the environmental standards in Japan.

To increase the sensitivity of pollution detection, the error of the data should be reduced by increasing the number of samples. However, larger volumes of soil samples are required for analysis compared with lichen samples to meet this condition. Leaching tests may include artificial error when the methods are chosen. Lichens absorb elements from surface water dissolved from the substrata; therefore, this analysis was not directly affected by mineral composition, grain size, or solubility of elements in metal-rich minerals but showed the results of mobile heavy metals affecting environmental pollution. Moreover, the analysis of lichens is more applicable than that of soils for monitoring short- and long-term changes in soil pollution because lichens grow on-site over the long term. As a result, this study advocates that analysis of lichens may be a new method to decrease artificial error and secondary environmental impacts for assessment and monitoring of soil pollution, which are important advantages for practical applications. In conclusion, *S. commixtum*, *C. humilis*, *C. ramulosa*, *C. krempelhuberi*, *C. crispata*, *C. scabriuscula*, *C. macilenta*, *C. rangiferina*, and *C. trassii* could be used in practical applications for biomonitoring and risk assessment of Cu, Zn, and As pollution of soils. Lichens occur in a broad range of environments and therefore have broad utility as biomarkers of heavy metal pollution worldwide.

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Author Contributions: Yuri Sueoka mainly performed this study and wrote the paper. Masayuki Sakakibara provided advice and recommendations on all analytical methods. Sakae Sano provided advice and recommendations on the ICP-MS analysis. Yoshikazu Yamamoto contributed to the TLC.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviation are used in this manuscript:

TLC	Thin layer chromatography
LC/MS	Liquid chromatography-mass spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
WD-XRF	Wave dispersive X-ray fluorescence spectrometry
BCF	Bioconcentration factor

References

1. Tiwari, M.J.; Bajpai, S.; Dewangan, U.K.; Tamrakar, R.K. Suitability of leaching test methods for fly ash and slag: A review. J. Radiat. *Res. Appl. Sci.* **2015,** *8*, 523-537. DOI: 10.1016/j.jrras.2015.06.003.
2. Niemi, G.J.; McDonald, M.E. Application of ecological indicators. *Annu. Rev. Ecol. Syst*. **2004,** *35*, 89-111. DOI: 10.1146/annurev.ecolsys.35.112202.130132.
3. Van Gestel, C.A.; Van Brummelen, T.C. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology* **1996,** *5* (4), 217-225. DOI: 10.1007/BF00118992.
4. Graney, R.L.Jr.; Cherry, D.S.; Cairns, J.Jr. Heavy metal indicator potential of the Asiatic clam (*Corbicula fluminea*) in artificial stream systems. *Hydrobiologia* **1983,** *102* (2), 81-88. DOI: 10.1007/BF00006071.
5. Suter II, G.W. Applicability of indicator monitoring to ecological risk assessment. *Ecol. Indic.* **2001,** *1* (2), 101-112. DOI: 10.1016/S1470-160X(01)00011-5.
6. Demirezen, D.; Aksoy, A. Common hydrophytes as bioindicators of iron and manganese pollutions. *Ecol. Indic.* **2006,** *6* (2), 388-393. DOI: 10.1016/j.ecolind.2005.04.004.
7. Birungi, Z.; Masola, B.; Zaranyika, M.F.; Naigaga, I.; Marshall, B. Active biomonitoring of trace heavy metals using fish (*Oreochromis niloticus*) as bioindicator species. The case of Nakivubo wetland along Lake Victoria. *Phys. Chem. Earth.* **2007,** *32* (15-18), 1350-1358. DOI: 10.1016/j.pce.2007.07.034.
8. Epelde, L.; Becerril, J.M.; Hernández-Allica, J.; Barrutia, O.; Garbisu, C. Functional diversity as indicator of the recovery of soil health derived from *Thlaspi caerulescens* growth and metal phytoextraction. *Appl. Soil Ecol.* **2008,** *39* (3), 299-310. DOI: 10.1016/j.apsoil.2008.01.005.
9. Hartley, W.; Uffindell, L.; Plumb, A.; Rawlinson, H.A.; Putwain, P.; Dickinson, N.M. Assessing biological indicators for remediated anthropogenic urban soils. *Sci. Total Environ.* **2008,** *405* (1-3), 358-369. DOI: 10.1016/j.scitotenv.2008.06.004.
10. Krizkova, S.; Ryant, P.; Krystofova, O.; Adam, V.; Galiova, M.; Beklova, M.; Babula, P.; Kaiser, J.; Novotny, K.; Novotny, J.; Liska, M.; Malina, R.; Zehnalek, J.; Hubalek, J.; Havel, L.; Kizek, R. Multi-instrumental Analysis of Tissues of Sunflower Plants Treated with Silver (I) Ions - Plants as Bioindicators of Environmental Pollution. *Sensors* **2008,** 8 (1), 445-463. DOI: 10.3390/s8010445.
11. Chang, J.S.; Yoon, I.H.; Kim, K.W. Heavy metal and arsenic accumulating fern species as potential ecological indicators in As-contaminated abandoned mines. *Ecol. Indic.* **2009,** *9* (6), 1275-1279. DOI: 10.1016/j.ecolind.2009.03.011.
12. María-Cervantes, A.; Jiménez-Cárceles, F. J.; Álvarez-Rogel, J. As, Cd, Cu, Mn, Pb, and Zn Contents in Sediments and Mollusks (*Hexaplex trunculus* and *Tapes decussatus*) from Coastal Zones of a Mediterranean Lagoon（Mar Menor, SE Spain）Affected by Mining Wastes. *Water Air Soil Poll*. **2009,** *200* (1), 289-304. DOI: 10.1007/s11270-008-9913-7.
13. Moss, J.C.; Hardaway, C.J.; Richert, J.C.; Sneddon, J. Determination of cadmium copper, iron, nickel, lead and zinc in crawfish [*Procambrus clarkii*] by inductively coupled plasma optical emission spectrometry: a study over the 2009 season in Southwest Louisiana. *Microchem. J.* **2010,** *95* (1), 5-10. DOI: 10.1016/j.microc.2009.07.008.
14. Čeburnis, D.; Steinnes, E. Conifer needles as biomonitors of atmospheric heavy metal deposition: comparison with mosses and precipitation, role of the canopy. *Atmos. Environ.* **2000,** *34* (25), 4265-4271. DOI: 10.1016/S1352-2310(00)00213-2.
15. Loppi, S; Pirintsos, S.A. Epiphytic lichens as sentinels for heavy metal pollution at forest ecosystems (central Italy). *Environ. Pollut.* **2003,** *121* (3), 327-332. DOI: 10.1016/S0269-7491(02)00269-5.
16. Purvis, O.W.; Pawlik-Skowronska, B.; Cressey, G.; Jones, G.C.; Kearsley, A.; Spratt, J. Mineral phases and element composition of the copper hyperaccumulator lichen *Lecanora polytropa*. *Mineral. Mag.* **2008,** *72* (2), 607-616. DOI: 10.1180/minmag.2008.072.2.607.
17. Purvis, O.W.; Bennett, J.P.; Spratt, J. Copper localization, elemental content, and thallus color in the copper hyperaccumulator lichen *Lecanora sierrae* from California. *Lichenologist* **2011,** *43* (2), 165-173. DOI: 10.1017/S0024282910000770.
18. Sueoka, Y.; Sakakibara, M. Primary phases and natural weathering of smelting slag at an abandoned mine site in southwest Japan. *Minerals* **2013,** *3* (4), 412-426. DOI: 10.3390/min3040412.
19. Osyczka, P.; Rola, K. Response of the lichen *Cladonia rei* Schaer. to strong heavy metal contamination of the substrate. *Environ. Sci. Pollut. Res. Int.* **2013,** *20* (7), 5076-5084. DOI: 10.1007/s11356-013-1645-6.

20. Edwards, H.G.M.; Moody, C.D.; Jorge Villar, S.E.; Wynn-Williams, D.D. Raman spectroscopic detection of key biomarkers of cyanobacteria and lichen symbiosis in extreme Antarctic habitats: Evaluation for Mars Lander missions. *Icarus.* **2005,** *174* (2), 560-571. DOI: 10.1016/j.icarus.2004.07.029.
21. Bačkor, M.; Loppi, S. Interactions of lichens with heavy metals. *Biol. Plantarum* **2009,** *53* (2), 214-222. DOI: 10.1007/s10535-009-0042-y.
22. Huneck, S.; Yoshimura, I. *Identification of lichen substances*; Springer: Berlin, Germany, 1996.
23. Imai, N.; Terashima, S.; Itoh, S.; Ando, A. 1996 Compilation of analytical data on nine GSJ geochemical reference samples, "Sedimentary Rock Series". *Geostand. Geoanal. Res.* **1996**, *20*, 165-216. DOI: 10.1111/j.1751-908X.1996.tb00184.x
24. Imai, N.; Terashima, S.; Itoh, S.; Ando, A. 1998 Compilation of analytical data for five GSJ geochemical reference samples: The "Instrumental Analysis Series". *Geostand. Geoanal. Res.* **1998**, *23*, 223-250. DOI: 10.1111/j.1751-908X.1999.tb00576.x
25. Kanda, Y. Investigation of the freely available easy-to-use software "EZR" for medical statistics. *Bone Marrow Transplant.* **2013,** *48* (3), 452-458. DOI: 10.1038/bmt.2012.244.
26. McGeer,J.C.; Brix,K.V.; Skeaff,J.M.; DeForest,D.K.; Brigham,S.I.; Adams,W.J.; Green, A. Inverse relationship between bioconcentration factor and exposure concentration for metals: Implications for hazard assessment of metals in the aquatic environment. *Environ. Toxicol. Chem.* **2003,** *22*, 1017–1037. DOI: 10.1002/etc.5620220509.
27. Epelde, L.; Becerril, J.M.; Hernández-Allica, J.; Barrutia, O.; Garbisu, C. Functional diversity as indicator of the recovery of soil health derived from *Thlaspi caerulescens* growth and metal phytoextraction. *Appl. Soil Ecol.* **2008,** *39* (3), 299-310. DOI: 10.1016/j.apsoil.2008.01.005.
28. Nishizono, H.; Suzuki, S.; Ishii. F. Accumulation of heavy metals in the metal-tolerant fern, *Athyrium yokoscense*, growing on various environments. *Plant Soil* **1987,** *102* (1), 65-70. DOI: 10.1007/BF02370902.
29. Morishita, T.; Boratyski, J.K. Accumulation of Cadmium and Other Metals in Organs of Plants Growing around Metal Smelters in Japan. *Soil Sci. Plant Nutr.* **1992,** *38* (4), 781-785. DOI: 10.1080/00380768.1992.10416712.
30. Yoshihara, T.; Tsunokawa, K.; Miyano, Y.; Arashima, Y.; Hodoshima, H.; Shoji, K.; Shimada, H.; Goto, F. Induction of callus from a metal hypertolerant fern, *Athyrium yokoscense*, and evaluation of its cadmium tolerance and accumulation capacity. *Plant Cell Rep.* **2005,** *23* (8), 579-585. DOI: 10.1007/s00299-004-0877-9.
31. Kamachi, H.; Komori, I.; Tamura, H.; Sawa, Y.; Karahara, I.; Honma, Y.; Wada, N.; Kawabata, T.; Matsuda, K.; Ikeno, S.; Noguchi, M.; Inoue, H. Lead tolerance and accumulation in the gametophytes of the fern *Athyrium yokoscense*. *J. Plant Res.* **2005,** *118* (2), 137-45. DOI: 10.1007/s10265-005-0202-x.
32. McGrath, S.P.; Zhao, F.J. Phytoextraction of metals and metalloids from contaminated soils. *Curr. Opin. Biotechnol.* **2003,** *14*, 277–282. DOI: 10.1016/S0958-1669(03)00060-0.
33. Bini, C.; Wahsha, M.; Fontana, S.; Maleci, L. Effects of heavy metals on morphological characteristics of *Taraxacum officinale* Web growing on mine soils in NE Italy. *J. Geochem. Explor*. **2012,** *123*, 101-108. DOI: 10.1016/j.gexplo.2012.07.009.
34. Nakajima, H.; Fujimoto, K.; Yoshitani, A.; Yamamoto, Y.; Sakurai, H.; Itoh, K. Effect of copper stress on cup lichens *Cladonia. humilis* and *C. subconistea* growing on copper-hyperaccumulating moss *Scopelophila cataractae* at copper-polluted sites in Japan. *Ecotoxicol. Environ. Saf.* **2012,** *84*, 341-346. DOI: 10.1016/j.ecoenv.2012.08.009.
35. Nakajima, H.; Yamamoto, Y.; Yoshitani, A.; Itoh, K. Effect of metal stress on photosynthetic pigments in the Cu-hyperaccumulating lichens *Cladonia humilis* and *Stereocaulon. japonicum* growing in Cu-polluted sites in Japan. *Ecotoxicol. Environ. Saf.* **2013,** *97*, 154-159. DOI: 10.1016/j.ecoenv.2013.07.026.
36. Nakajima, H.; Hara, K.; Yamamoto, Y.; Itoh, K. Effects of Cu on the content of chlorophylls and secondary metabolites in the Cu-hyperaccumulator lichen *Stereocaulon japonicum*. *Ecotoxicol. Environ. Saf.* **2015,** *113*, 447-482. DOI: 10.1016/j.ecoenv.2014.12.038.
37. Osyczka, P.; Rola, K.; Jankowska, K. Vertical concentration gradients of heavy metals in *Cladonia* lichens across different parts of thalli. *Ecol. Indic.* **2016,** *61* (2), 766-776. DOI: 10.1016/j.ecolind.2015.10.028.
38. Nash III, T.H. Nutrients, elemental accumulation, and mineral cycling. In *Lichen Biology*, 2nd ed.; Nash III, T.H. Ed; Cambridge University Press: Cambridge, UK, 2008; pp. 234-251.
39. Banfield, J.F.; Baker, W.W.; Welch, S.A.; Taunton, A. Biological impact on mineral dissolution: Application of the lichen model to understanding mineral weathering in the rhizosphere. *Proc. Natl. Acad. Sci. USA.* **1999,** *96* (7), 3404-3411. DOI 10.1073/pnas.96.7.3404.

40. Ghosh, M.; Singh, S. P. A review on phytoremediation of heavy metals and utilization of its by-products. *Appl. Ecol. Env. Res.* **2005,** *3* (1), 1-18. DOI: 10.15666/aeer/0301_001018.