A New Method of Environmental Assessment and Monitoring of Cu, Zn, As, and Pb Pollution in Surface Soil Using Terricolous Fruticose Lichens

Yuri Sueoka [1],*, Masayuki Sakakibara [1,2], Sakae Sano [3], and Yoshikazu Yamamoto [4]

[1] Graduate School of Science and Engineering, Ehime University, Bunkyo-cho 2-5, Matsuyama, Ehime 790-8577, Japan; sueoka@sci.ehime-u.ac.jp
[2] Faculty of Collaborative Regional Innovation, Ehime University, Bunkyo-cho 2-5, Matsuyama, Ehime 790-8577, Japan; sakakibara.masayuki.mb@ehime-u.ac.jp
[3] Faculty of Education, Ehime University, Bunkyo-cho 2-5, Matsuyama, Ehime 790-8577, Japan; sano@ehime-u.ac.jp
[4] Osaka Museum of Natural History, Ikeda-Kitamachi 24-1, Neyagawa, Osaka, 572-0073, Japan; yosyamam@gmail.com
* Correspondence: sueoka@sci.ehime-u.ac.jp; Tel.: +81-089-927-9649

Abstract: Levels of trace element pollution in surface soil can be estimated using soil analyses and leaching tests. These methods may reveal different results due to the effect of soil properties such as grain size and mineral composition on elemental availability. Therefore, this study advocates an alternative method for monitoring and assessment of trace element pollution in surface soil using terricolous 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 Cu, Zn, As, and Pb concentrations in lichens and soils, including their absorption properties. Concentrations of these elements in the lichens were positively correlated with those in the soils regardless of lichen species, location, habitat, or conditions of soils. The analyzed lichens had neither competitive nor antagonistic properties in their elemental absorption, which made them good biomonitors of trace element pollution in surface soil. The distribution maps of average Cu, Zn, As, and Pb concentrations at each sampling region detected almost all of the Cu, Zn, and As pollution of the soils. Therefore, lichens could be used in practical applications to monitor Cu, Zn, and As pollution in soils.

Keywords: biomonitor; environmental assessment; elemental competition; bioconcentration factor

1. Introduction

Soil pollution has been estimated using soil analyses and leaching tests. 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 tests 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. Results differing from the actual condition may be obtained by the leaching test if an inappropriate method is chosen. Accordingly, the leaching test methods may lead to misuse and misinterpretation of the results [1].

Many organisms have been investigated as potential biomonitors as a means to assess the level of soil pollution [2]. 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 [2–13]. However, trace element absorption by plants is affected by antagonisms and interactions among elements [14]. For instance, Cd, Co, Cr, Pb, Ni, Mn, and Zn accumulation causes Fe deficiency in plants [15]. The concentrations of these elements are, therefore, affected by Fe

concentrations in soil and/or soil solution. Accordingly, these antagonisms and interactions are a problem in using organisms for biomonitoring and environmental assessment.

In contrast, lichens absorb mineral nutrients and trace elements, including metals, from dry and wet atmospheric deposition and surface water due to a lack of a vascular root system and protective cuticles [13,16,17]. Wet depositions, including precipitation and occult precipitation, such as fog and dew, and dry depositions, including sedimentation, impaction, and gaseous absorption, are important sources of nutrients for lichens [18]. Lichens exchange gases and trap aerosols across their entire surface because of the high surface areas and their lack of stomata [19]. Lichens also uptake elements from trapped soil dust [20] and surface water containing minerals dissolved from their substrata [21]. Accordingly, concentrations of trace elements in lichen thalli may reflect those of surface soil which are unstable and affects environmental pollution.

Moreover, some characteristics of lichens that could be used as biomonitors for pollution of their substrata have been revealed in recent studies. Several lichens absorb heavy metals from the corresponding substrata [22–24]. Osyczka and Rola [25] 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 model. These characters of lichens are an important advantage for practical applications as an alternative to the leaching test.

Terricolous lichens cover more than 6% of the land surface of the earth [23], and are found in areas such as tropical forests, desert, alpine regions, polar regions, urban areas, as well as highly polluted areas [26,27]. Lichens found in naturally heavy metal-enriched sites, such as serpentinite and other ultramafic rocks [28–30] and metal mines [31,32], may be good model systems for biomonitoring. Accordingly, lichens may have the potential to be broadly used as biomonitors in various terrestrial habitats worldwide.

To evaluate a practical application of *Stereocaulon commixtum* and several *Cladonia* spp. as biomonitors, this study investigated the correlation between concentrations of Cu, Zn, As, and Pb in the lichen thalli and those in the corresponding substrata. In addition, distribution maps of the concentrations at each sampling site, including contaminated abandoned mine sites and unpolluted areas in Southwest Japan, were created using the average concentrations of Cu, Zn, As, and Pb in the surface soils and lichen thalli. Finally, the practical application of lichens as biomonitors for the assessment and monitoring of Cu, Zn, As, and Pb pollution in surface 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.
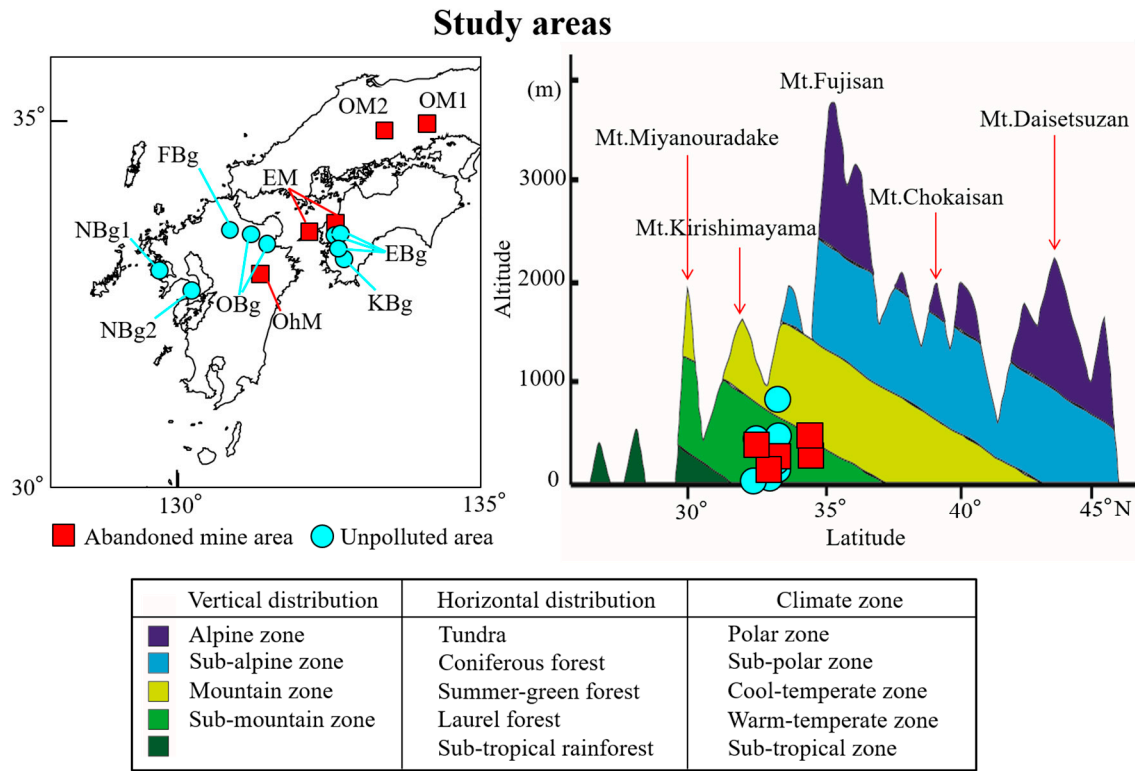
Figure 1. Locations of this study areas and their climate zone.

2.2. Sampling and Pulverizing Methods

A total of 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 stored in dark place at Ehime University. 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 were washed with ultrapure water (Milli-Q; Merck Millipore Corporation, Darmstadt, Germany). The washed samples were oven dried at 80 °C for 24 hours. The dried samples were pulverized using an agate mortar. The soil samples were stored in a desiccator after being oven dried at 120 °C for at least 48 hours. 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. 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) [33].

2.4. Determining Concentrations of Trace Elements in Lichen Materials and Soils

The concentrations of trace elements 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 concentrations of trace elements 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. Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS)

The 20 mg pulverized powder was digested with several acids by the 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; and (8) cooled down to room temperature for 1 h. The digested solution was diluted by 3% HNO_3 as the solution for analysis.

The analytical accuracy and precision were verified using the NIES CRM #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. X-Ray Fluorescence (XRF)

Pressed powder pellets were used for the XRF analysis. The pulverized samples were homogenized by shaking for six hours. Approximately 3.3 g of powdered soil samples were oven dried at 120 °C for two 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 3 g powdered soil samples by shaking for one 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) [34,35] were analyzed to confirm analytical precision.

2.5. Statistical analysis

The normality of the concentrations of trace elements in lichens and soils were assessed using the Shapiro-Wilk test and QQ plots, both exhibiting significantly non-normal distributions. Therefore, correlations between the concentrations of trace elements in lichens and soils 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 [36].

2.6. Bioconcentration Factor

The bioconcentration factor (BCF) has been used to evaluate the bioaccumulation property of plants [28]. The BCF values of lichens were calculated using the following equation [37]:

$$BCF = C_{shoot}/C_{soil} \qquad (1)$$

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.; *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 (Figure 2).

The *S. commixtum*, *Cladia aggregata*, *Cladonia rangiferina*, *C. crispata*, *C. macilenta*, *C. humilis*, *C. ramulosa*, *C. krempelhuberi*, and *C. trassii* grew in both unpolluted and polluted areas. Distributions of these lichens showed several dense areas on the waste dumps (Figure 3). 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 [38–41]. *Cladonia* spp. were often associated with other *Cladonia* lichens.
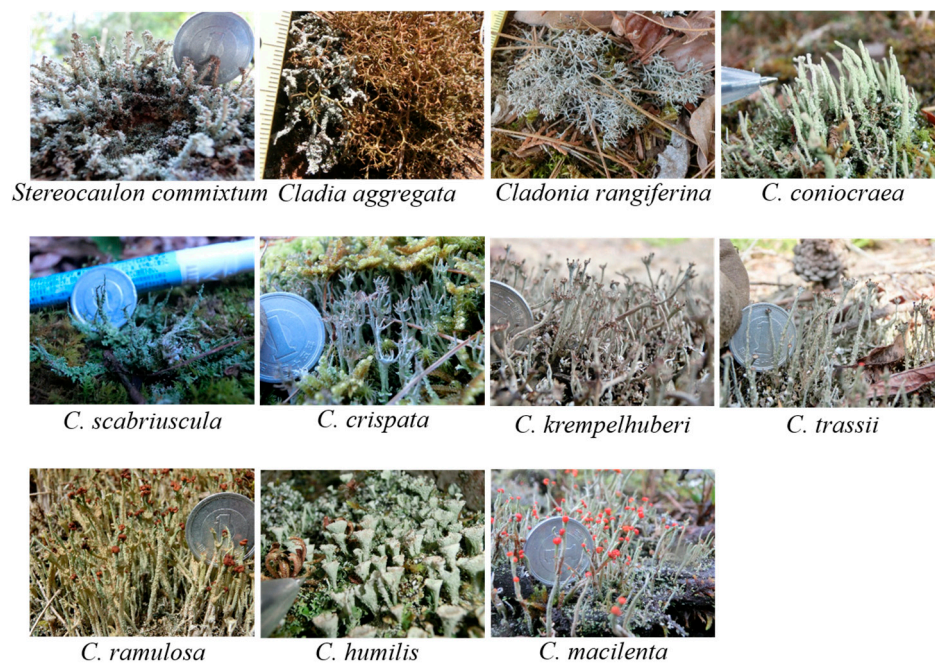
Figure 2. Several lichens growing on soil, rock, and/or mine wastes in the study areas.
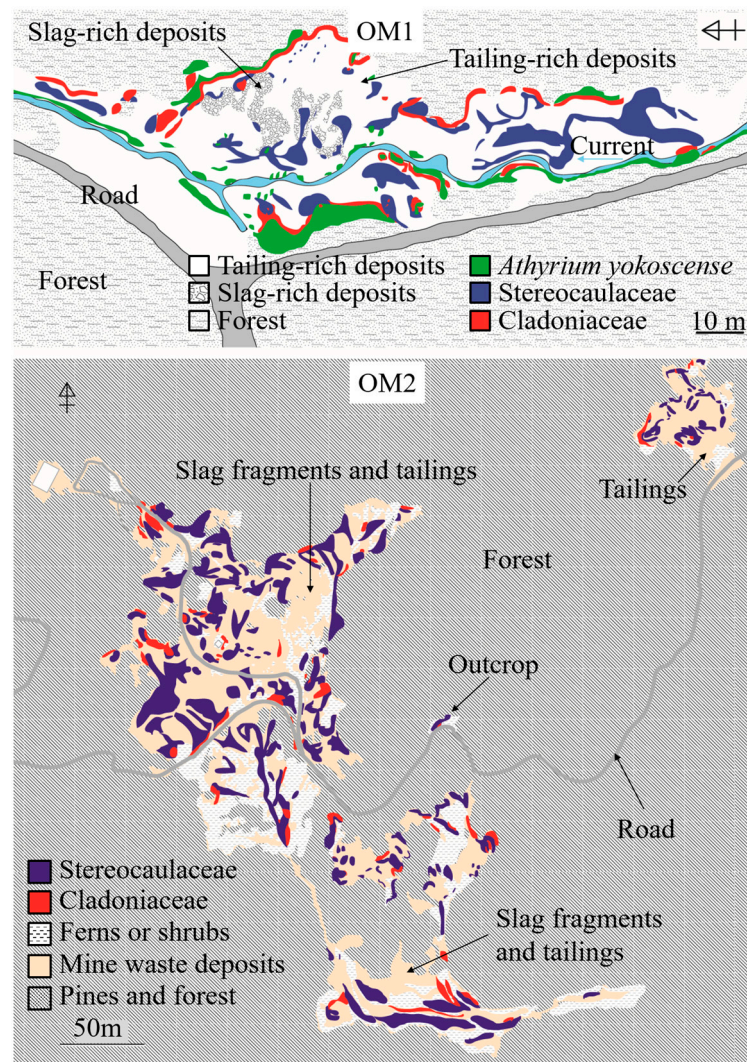
Figure 3. Distribution maps of lichens on mine waste dumps in the study areas OM1 and OM2.

3.2. Relationships between Concentrations of Trace Elements in Lichens and the Corresponding Substata

Scatterplots were used to determine the relationships between concentrations of trace elements in lichens and the corresponding substrata. The scatterplots showed that concentrations of Cu, Zn, As, and Pb in all lichens, including *S. commixtum* and eight *Cladonia* spp., were positively correlated with those in the corresponding substrata regardless of lichen species, location, habitat, types of substrata, constituents of substrata, or particle sizes of soils (Figure 4, 5). The scatterplots showed no local maximum points, but a linear distribution under a logarithmic scale.

Scatter matrices were used to estimate the competitive and antagonistic properties in the element absorption by lichens. All of the scatterplots in the scatter matrices showed positive correlations between concentrations of Cu, Zn, As, and Pb in lichens and soils after logarithmic transformation (Figure 6). The BCF data also have important implications for potential biomarkers. The BCF values decrease with increasing elemental concentrations in soils regardless of lichen species, location, habitat, types of substrata, constituents of the substrata, or particle sizes of substrata, except Pb (Figure 7).
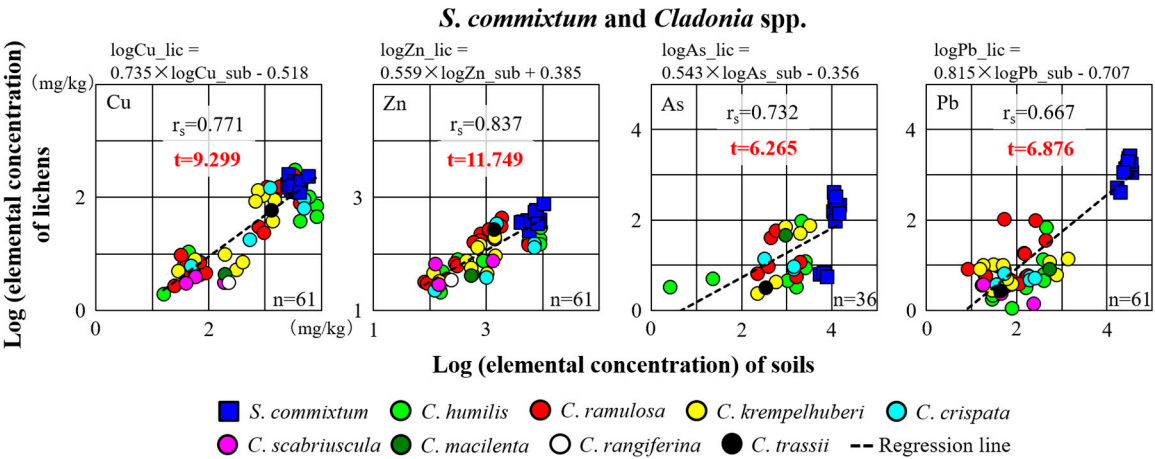
Figure 4. Scatterplots of Cu, Zn, As, and Pb concentrations in lichens and the corresponding substrata, with regression lines.
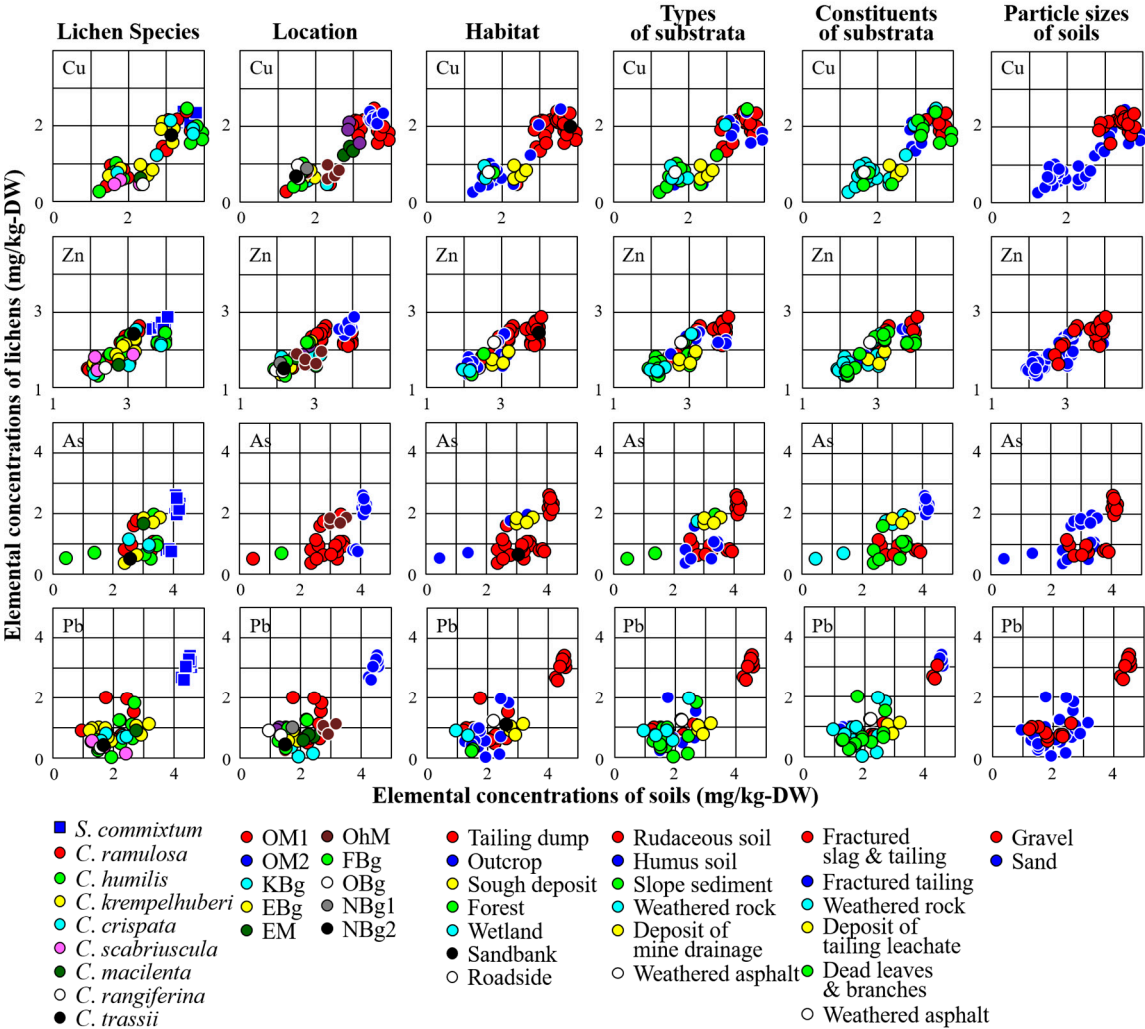
Figure 5. Scatterplots of Cu, Zn, As, and Pb concentrations in lichens and the corresponding substrata, separated by lichen species, location, habitat, types of substrata, constituents of substrata, and particle sizes of soils using different colors.
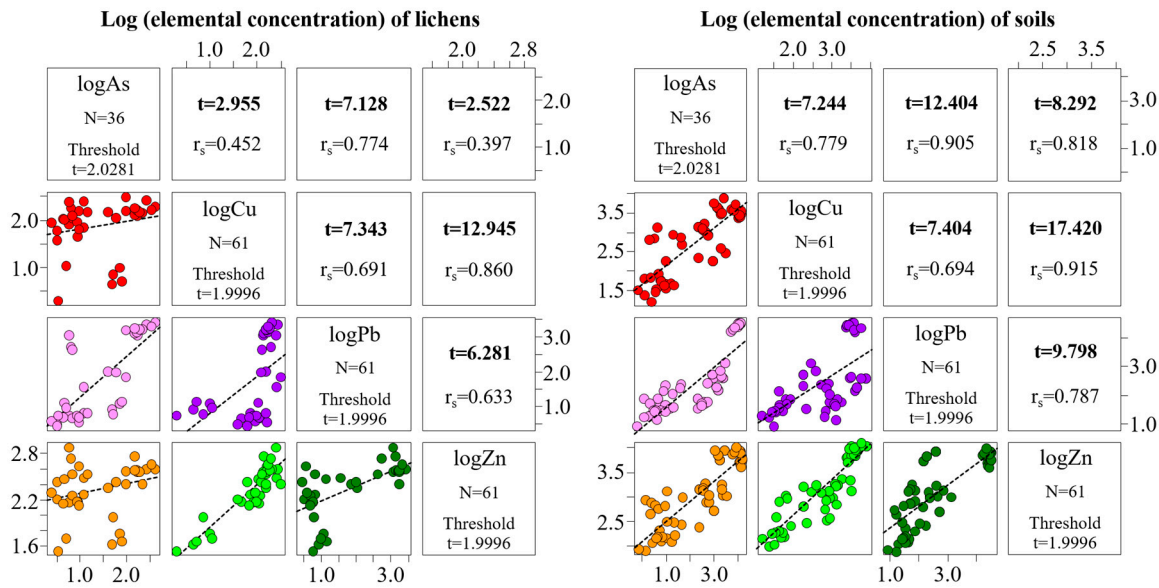
Figure 6. Scatter matrices showing the relationships among each Cu, Zn, As, and Pb concentration in lichens and soils after logarithmic transformation, with regression lines.
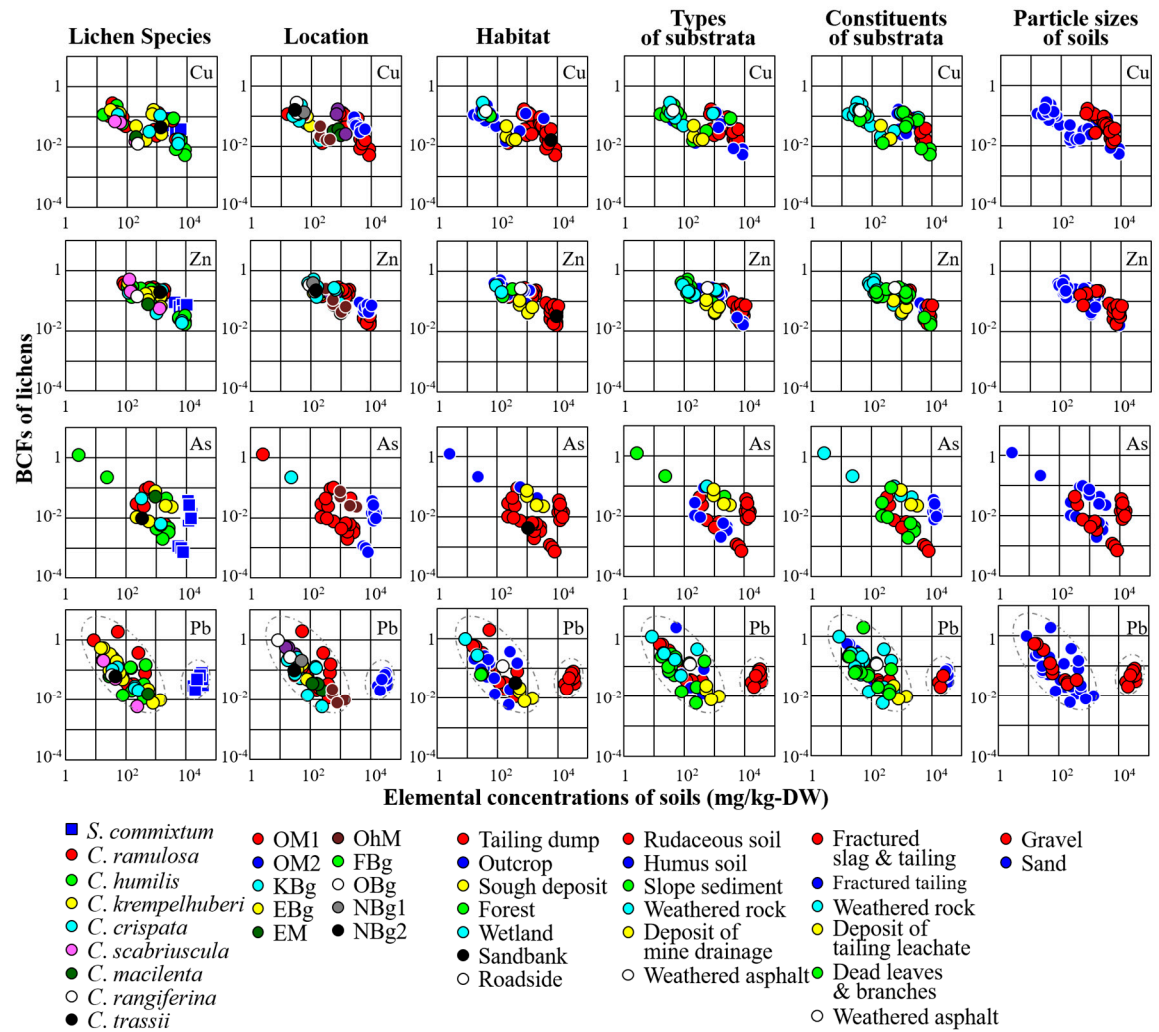
Figure 7. Scatterplots showing relationships between the BCFs and the elemental concentrations in soils, separated by lichen species, location, habitat, types of substrata, constituents of substrata, and particle sizes of soils using different colors.

4. Discussion

4.1. Trace Element Absorption Properties of Lichens

The scatterplots of Pb in Figures 3 and 6 showed two groups related with lichen species and/or locations. Nieboer and Rechardson (1980) [42] demonstrated that the affinity of ions for exchange sites varied in the sequence monovalent Class A < divalent Class A < borderline divalent < divalent Class B ions. The borderline ions include Cu, Zn, As, and divalent Pb ions, while the class B ions include tetravalent Pb. Accordingly, the Pb concentration in lichen thalli may be affected by physiological properties of lichens and/or chemical forms of Pb in surface soil.

Previous studies have demonstrated the heavy metal uptake ability and the accumulation capacity of lichens [25,30,43–45]. Competitive effects in heavy metal absorption of lichens was also clarified in previous studies [46,47]. According to Sueoka et al. [24], Fe and As could be affected by precipitation and adsorption on hyphae of lichen thalli as Fe-hydroxides, but not selective elemental absorption by lichens. In this study, the positive correlations among each Cu, Zn, As, and Pb concentration in lichens were detected. The correlation indicated that these trace elements were not absorbed selectively into the lichen thalli and, therefore, the lichens had neither competitive nor antagonistic properties in their Cu, Zn, As, and Pb absorption. Moreover, the relationship between elemental concentrations in lichens and soils were described by linear regression models in a wide range of the concentrations (Figure 4), even for low BCF values. This property of the lichens imply possible practical applications of the lichens as biomonitors for a wide range of trace element pollution in surface soil.

Ions absorbed by lichens were not evenly distributed throughout the thalli [19]. 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 [19]. The lichens contained dust and soil particles in their thalli [19,48]. Several *Cladonia* lichens showed different concentrations of heavy metals in different parts of their thalli [44]. Our previous study clarified the distribution of trace elements in *S. exutum* thalli as follows: (1) Fe, Cu, Zn, and As were contained between cortex and medulla; (2) the Fe occurred on the surface of hyphae as Fe-hydroxides with As; (3) the trapped soil particulates in the thalli which were the upper 1–5 mm portion from their substrata were mainly quartz and plagioclase [24]. Accordingly, the most effective factor contributing to trace element concentrations in *S. commixtum* thalli may be the precipitation of the elements on the hyphae of the medulla and/or scavenging ions from surface water. Our studies, however, have not revealed the elemental distribution in *Cladonia* spp. thalli. More detail investigations on the chemical forms and allocation patterns of trace elements in the lichen thalli, therefore, can reveal the accumulation potential and suitability of the lichens as biomonitors.

4.2. Practical Application of Lichens as Biomarkers

The relationships between trace element concentrations in lichens and the corresponding substrata have been demonstrated in previous studies [25,30]. Osyczka and Rola [25] 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 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 [25]. The data of this study included different lichen species, locations, habitats, and conditions of soils in contrast to the previous study. This study, therefore, may be able to provide versatile data for practical application of lichens compared with other previous studies.

The statistically positive correlations indicated that the lichens have potential as biomonitors of Cu, Zn, As, and Pb pollution in the soil. However, the practical applications of lichens require more than statistical correlations. Accordingly, this study evaluated the practical application of lichens using distribution maps of concentrations, created using the average concentrations of Cu, Zn, As, and Pb in lichens and soils at each sampling region (Figure 8).

The elemental concentrations were divided into five 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 trace elements in soils 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 Cu, Zn, As, and Pb in lichens were calculated using Equation (2), which is calculated from the linear regression lines in the scatterplot of lichens in Figure 4:

$$\log Y = \log(aX^b) = \log(a) + b \times \log X, \tag{2}$$

$$Y = aX^b, \tag{3}$$

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

As shown in Figure 8, the plots of Cu, Zn, and As in the lichens showed similar distributions as those in the soils. The distribution of Pb plots, however, varied widely. The variation in the Pb distribution may be affected by differences of bioavailability and/or dissolution rate of Pb in metal minerals from those of the other elements. Consequently, the maps created using lichens detected almost all of the Cu, Zn, and As pollution in the soil.
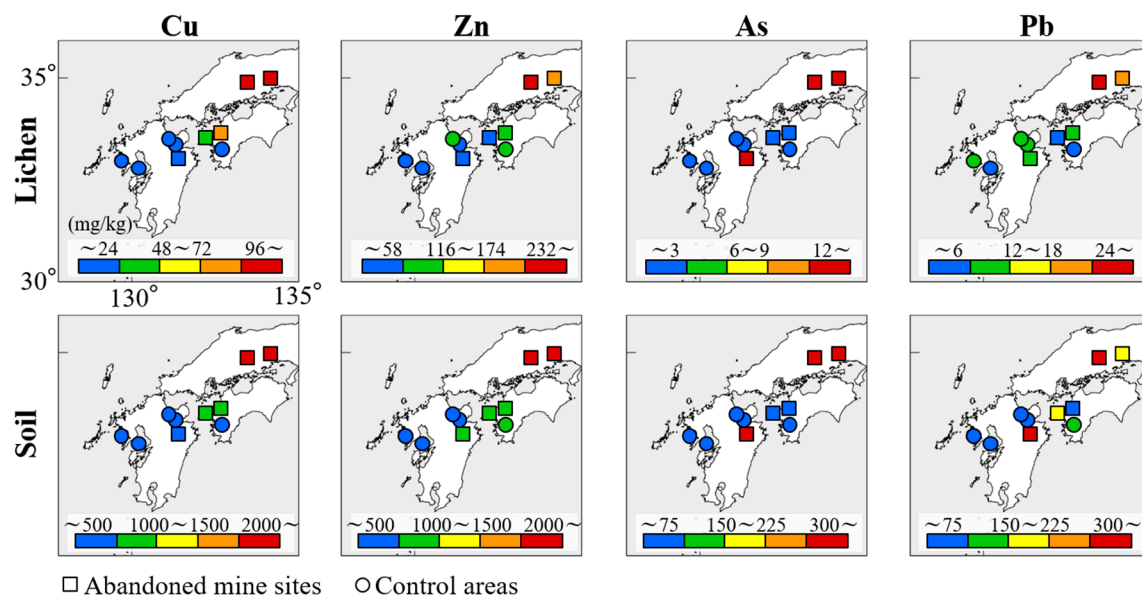
Figure 8. Distribution maps of the average concentrations of Cu, Zn, As, and Pb in lichens and soils at each sampling region in Southwest Japan. The yellow dots indicate the environmental standards in Japan.

To increase the sensitivity of pollution detection, a large number of samples should always be tested. Larger volumes of soil samples are required for analysis compared with lichen samples to meet this condition. Leaching tests may include artificial errors depending on the method chosen. Lichens absorb elements dissolved by surface water from the substrata, as well as wet and dry atmospheric deposition. Accordingly, analysis of lichens is not directly affected by mineral composition and grain size, but is affected by the solubility of elements in metal-rich minerals affecting environmental pollution. Moreover, the analysis of lichens can prevent the artificial contamination of analytical samples by W and the other elements contained in a mill during

pulverizing because lichens are easily pulverized by a mortar. As a result, this study advocated that analysis of lichens may be an alternative method for soil analyses or leaching tests to decrease artificial error, time, cost, and secondary environmental impact for assessment and monitoring of soil pollution, which are important advantages for practical applications. The analysis of lichens may be 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. 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 in surface soil. Lichens occur in a broad range of environments and, therefore, have broad utility as biomonitors of potentially toxic trace elements worldwide.

Acknowledgments: The authors are grateful to Kaoru Kinoshita (Meiji Pharmaceutical University, Japan) for help with LC/MS analysis and Akinori Kawamata (Ehime prefectural science museum, Japan) for help with morphological identifications of lichens. We thank the anonymous referees for their perceptive comments and recommendations. This study was partially supported by grants from Fukada Geological Institute (Fukada Grant-in-Aid, 2013) and JSPS KAKENHI Grant Number 15J04570.

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. 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.

4. 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.

5. 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.

6. 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.

7. 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.

8. 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.

9. 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.

10. 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.

11. 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.

12. 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.

13. 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.

14. Gerloff, G.C.; Stout, P.R.; Jones, L.H.P. Molybdenum-Manganese-Iron antagonisms in the nutrition of tomato plants. *Plant Physiol*. **1959,** *34* (6), 608-613. PMCID: PMC541266.

15. Siedlecka, A. Some aspects of interactions between heavy metals and plant mineral nutrients. *Acta Soc. Bot. Pol.* **1995,** *64* (3), 265-272. DOI: 10.5586/asbp.1995.035.

16. 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.

17. Goyal, R.; Seaward, M.R.D. Metal uptake in terricolous lichens. III. Translocation in the thallus of *Peltigera canina*. *New Phytol*. **1982,** *90*, 58-98. Stable URL: http://www.jstor.org/stable/2433916.

18. Knops, J.M.H.; Nash III, T.H.; Boucher, V.L.; Schlesinger, W.L. Mineral cycling and epiphytic lichens: implications at the ecosystem level. *Lichenologist*. **1991,** *23*, 309–321. DOI: 10.1017/S0024282991000452.

19. 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.

20. Nieboer, E.; Richardson, D.H.S.; Tomassini, F.D. Mineral uptake and release by lichens: an overview. *Bryologist*. **1978,** *81*, 226–246. DOI: 10.2307/3242185.

21. Crittenden, P.D. Nutrient exchange in an Antarctic macrolichen during summer snowfall snow melt events. *New Phytol*. **1998,** *139*, 697–707. DOI: 10.1046/j.1469-8137.1998.00236.x.

22. 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.

23. 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.

24. Sueoka, Y.; Sakakibara, M.; Sera, K. Heavy Metal Behavior in Lichen-Mine Waste Interactions at an Abandoned Mine Site in Southwest Japan. *Metals* **2015,** *5* (3), 1591-1608. DOI: 10.3390/met5031591.

25. 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.

26. 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.

27. Bačkor, M.; Loppi, S. Interactions of lichens with heavy metals. *Biol. Plantarum* **2009,** *53* (2), 214-222. DOI: 10.1007/s10535-009-0042-y.

28. Favero-Longo, S.E.; Isocrono, D.; Piervittori, R. Lichens and ultramafic rocks: a review. *The Lichenologist*. **2004,** *36* (6), 391-404. DOI: 10.1017/S0024282904014215.

29. Rajakaruna, N.; Knudsen, K.; Fryday, A.M.; O'Dell, R.E.; Pore, N.; Olday, F.C.; Woolhouse, S. Investigation of the importance of rock chemistry for saxicolous lichen communities of the New Idria serpentinite mass, San Benito County, California, USA. *The Lichenologist*. **2012,** *44* (5), 695-714. DOI: 10.1017/S0024282912000205.

30. 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.

31. Rajakaruna, N.; Harris, T.B.; Clayden, S.R.; Dibble, A.C.; Olday, F.C. Lichens of the Callahan Mine, a copper- and zinc-enriched superfund site in Brooksville, Maine, U.S.A. *Rhodora*, **2011,** *113* (953), 1-31. DOI: 10.3119/10-03.1.

32. Medeiros, I.D.; Fryday, A.M.; Rajakaruna, N. Additional lichen records and mineralogical data from metal-contaminated sites in Maine. *Rhodora*, **2014,** *116* (967), 323-347. DOI: 10.3119/13-26.

33. Huneck, S.; Yoshimura, I. *Identification of lichen substances*; Springer: Berlin, Germany, 1996.

34. 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

35. 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

36. 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.

37. 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.

38. 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.

39. 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.

40. 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.

41. 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.

42. Nieboer, E.; Richardson, D.H.S. The replacement of the nondescript term 'heavy metals' byabiologically and chemically significant classification of metal ions. *Environ. Pollut.*, *Ser. B*, **1980,** *1* (1), 3–26. DOI: 10.1016/0143-148X(80)90017-8.

43. 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.

44. 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.

45. 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.

46. Beckett, R.P.; Brown, D.H. The control of cadmium uptake in the lichen genus *Peltigera*. *J. Exp. Bot.* **1984,** *35*, 1071–1082. DOI: 10.1093/jxb/35.7.1071.

47. Beckett, R.P.; Brown, D.H. The relationship between cadmium uptake and heavy metal uptake tolerance in the lichen genus *Peltigera*. *New Phytol.* **1984,** *97*, 301–311. DOI: 10.1111/j.1469-8137.1984.tb04134.x.

48. 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.