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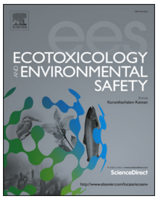

# Lichen as a Biomonitor for Vehicular Emission of Metals: A Risk Assessment of Lichen Consumption by the Sichuan Snub-Nosed Monkey (*Rhinopithecus roxellana*)

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## ABSTRACT

Two lichen species, *Usnea aciculifera* and *Usnea luridorufa*, were used as biomonitors for the deposition of traffic-related metals in China's Shennongjia National Nature Reserve. The suitability of the two lichen species for use as biomonitors was compared. The health threat to the Sichuan snub-nosed (aka golden) monkey (*Rhinopithecus roxellana*) from consuming lichen with elevated metal concentrations due to vehicular traffic was then assessed. Lichens, with large surface areas and neither roots nor stomata, efficiently absorb both particulate and gaseous air pollutants. The resulting data was used to assess the effect of heavy metal accumulation on the lichens as well as the health risk imposed on the monkeys as lichen is a primary food source. Lichen samples were collected in the core area of the reserve at three locations of varying traffic intensity. A forth site in the reserve, with no proximate traffic, was used as the control. Results show: (1) lichen from high traffic sites has significantly higher concentrations of Fe, Cd, Pb Zn, and Cr than lichen collected from the control site; (2) vehicular traffic is the primary source of metals in lichen; (3) *U. luridorufa* collected at high traffic sites displayed decreased photosynthetic efficiency, an indication of stress; (4) intake of Cd and Pb from vehicle emissions in the Shennongjia National Nature Reserve could adversely affect snub-nosed monkey health. This research advances the science of biomonitoring, contributes to environmental protection efforts in China's nature reserves and helps improve food safety for Sichuan snub-nosed monkey, a national treasure of China.

## 1. Introduction

Two lichen species, *Usnea aciculifera* and *Usnea luridorufa*, found in Shennongjia National Nature Reserve were used to assess the environmental effect of trace metals emitted from vehicles. Lichens, composite organisms consisting of fungus and a photosynthetic partner, are widely used in environmental research because they are widespread, easy to sample, and sensitive to various pollutants. Because they lack roots, stomata and cuticle, lichens accumulate elements deposited from air and are widely used as biomonitors for air pollutants (Rola and Osyczka, 2019; Cecconi et al., 2018). Although the response is species specific, lichens are sensitive to heavy metals and have been used in a large scale study comparing natural and anthropogenic sources of metals (Varela et al., 2018).

Heavy metals are known to impact photosynthetic parameters, chlorophyll content (Bajpai and Upreti, 2012), chlorophyll degradation rate (Bajpai et al., 2010), chlorophyll stability index (Yemets et al., 2015) and chlorophyll fluorescence (Álvarez et al., 2012; Branquinho et al., 1997). Heavy metals interfere with chlorophyll synthesis by direct enzyme inhibition or by inducing deficiency of an essential nutrient (Wang et al., 2014). Bajpai and Upreti (2012) found that photosynthetic parameters of the lichen *Pyxine cocoes* changed significantly with varying levels of metal and is suitable for metal biomonitoring. The ratio of variable fluorescence to maximum fluorescence, Fv/Fm, is a measure of photosynthetic efficiency and it decreases with stress. The effects of Pb on *Lobaria pulmonaria* and *Parmelia caperata* include large

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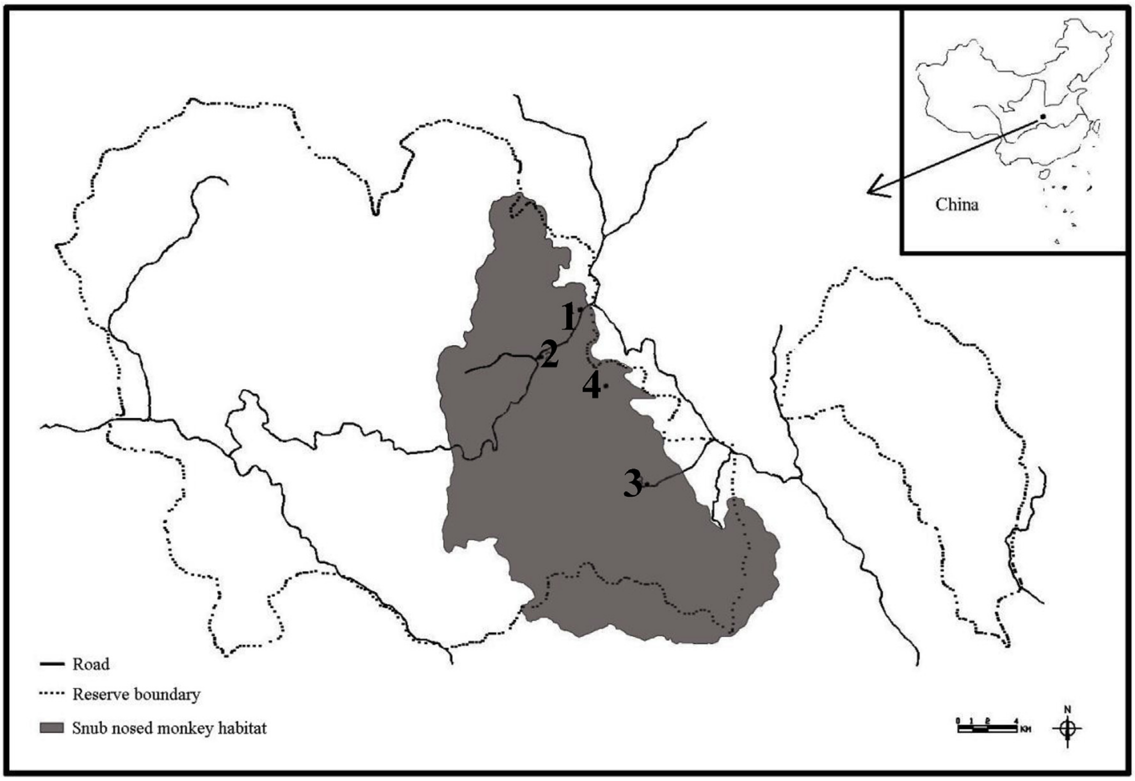

**Fig. 1.** Sampling sites.

reductions in Fv/Fm, showing that chlorophyll fluorescence is a sensitive indicator of damage (Branquinho et al., 1997). Chlorophyll fluorescence is also a non-destructive, non-invasive method that is rapid and convenient and can be used for *in situ* measurements.

Trace element pollution in natural areas within or near urban or industrial areas deserves special attention. Shennongjia National Nature Reserve (**Fig. 1**) is located in central China (31°15′-31°75′N, 109°56′-110°58′E) with an average elevation of 1700 m. The climate is subtropical monsoon, with a mean annual temperature of 12 °C and a mean annual rainfall of 900–1000 mm. The area is covered by primary and secondary temperate, deciduous, broadleaf coniferous forest. Young forest (30–50 years) occupies most of the area (Li et al., 2002) but with numerous patches of primary forest. Shrub forest and grassland account for small proportions of the area, and are found mainly on the plains and some peaks. The Sichuan snub-nosed monkey or golden monkey (*Rhinopithecus roxellana*) is an arboreal forest dweller, inhabiting temperate broadleaf and coniferous forest at elevations between 1500 and 3500 m. The monkey has reddish-gold hair and a bluish face with a small, upturned nose. The species is categorized as Endangered on the IUCN Red List (http://www. iucnredlist.org). Like the panda, the Sichuan snub-nosed monkey is considered a national treasure of China. Lichens (*Usneaceae*) are an important source of food for the golden monkey, accounting for 5.69% of the diet from spring to autumn and 16.51% in the winter (Tie et al., 2010).

Emission of traffic-related elements (TREs) originates from fuel combustion (Pb and Ba), tire, brake, engine, and vehicle deterioration (Cd, Cr, Cu, Fe, Mn, Ni, Sb, V, Zn) and, indirectly, by resuspension of soil (Al, Fe, and associated elements) and street dust (Bargagli, 1998). Lichens have been widely used as biomonitors to measure environmental quality in urban environments influenced by vehicular traffic. However, they have been rarely employed to monitor natural areas and, particularly, the natural reserves in China where the large increase in tourism is leading to heavy vehicular traffic. Metal accumulation in lichen, if sufficiently high, will threaten the health of golden monkeys. It is therefore important to investigate the extent of metal accumulation in lichens to assess both ecosystem health and the health risk imposed on the golden monkey.

This investigation had three interrelated objectives: (1) Evaluate the use of lichen as a biomonitor in the Shennongjia National Nature Reserve. (2) Investigate the stress imposed on lichen from vehicular emissions by correlating metal concentration with photosynthetic fluorescence. (3) Assess the potential health risk to the golden monkey of heavy metal exposure from dietary intake of lichen using a modified THQ (target hazard quotient).

## 2. Material and methods

### 2.1. Study site

Two lichen species, *Usnea aciculifera* and *Usnea luridorufa*, were chosen for study because they are the favorite food of the Sichuan snub-nosed monkey. Samples of the two species were collected from trees along the roadsides of Shennongjia National Nature Reserve during August 2013 (**Fig. 1**). Sample sites with different traffic intensities were selected to give a range of metal deposition (**Table S1**).

### 2.2. In situ determination of chlorophyll fluorescence

Five samples of *U. aciculifera* and *U. luridorufa* (average size of thallus is 5.00 × 3.00 cm diameter) were selected from each site and cleaned *in situ* with a brush to remove dust, leaf debris, and degraded material from the surface. The selected lichens were rehydrated until photosynthesis recovered completely and chlorophyll fluorescence parameters were measured using an open gas exchange system (LI-6400, LI-COR, Inc., Lincoln, Nebraska (NE), USA) with an integrated fluorescence chamber (LI-6400–40 leaf chamber fluorometer, Nebraska (NE), USA). This parameter was measured after a 30 min period of dark adaptation, ensuring that all photosystem II (PSII) reaction centers were open. These processes were carried out *in situ*, i.e., the rehydrated lichen thallus was placed on the tree trunk, covered with the Li-COR leaf chamber, and a live value of PSII Fv/Fm was obtained. The method is non-destructive and non-invasive, crucial for making repeated *in situ* measurements. In the natural environment, water loss is the primary stress on lichen and the ratio, Fv/Fm, is sensitive to the degree of dehydration. The stress of dehydration must be eliminated and measured values of Fv/Fm should be compared to a control. Lichens in the control site (site 4) were re-wetted with a water spray and Fv/Fm values were measured at selected time intervals until Fv/Fm values had reached the normal value.

### *2.3. Metal analysis*

After measuring chlorophyll fluorescence parameters, the samples were carefully removed from the tree and sealed individually in valve bags. A total of 20 samples were collected and transported to the laboratory for metal analysis. Lichen thallus samples were oven dried to a constant weight at 60 °C and ground until powdered. 0.1 g was digested in a mixture of concentrated $HNO_3$: $HClO_4$ (v/v 9:1) for 2 h (Rusu, 2002). The resulting solution was filtered with Whatman filter paper no. 45 and diluted to 50 ml with double distilled water. Metals were determined using Inductively Coupled Mass Spectrometry (ICP-MS; Thermo XseriesII, Germany). Parameters of the instrumentation to carry out the analysis were used as Bressy et al. (2013). For quality assurance, $^{115}In/^{187}Re$ (100 mg/L; Darmstadt, Germany) internal standards were added to all samples and four quality control samples were introduced every eight samples to correct for any signal drift (Bressy et al., 2013). All concentrations were corrected based on the recovery ($C_{measured}/C_{certified}*100$) obtained for each element. Nine selected metals were analyzed, including Fe, Mn, Co, Ni, Cd, Pb, Cu, Zn and Cr. The recoveries were 100 ± 15% for Mn, Co, Ni, Cd, Cu, Zn and Cr, and the recoveries of Fe and Pb were 118% and 124% respectively. For ICP-MS, detection limits range from 1 to 9 μg/L.

### *2.4. Statistical analyses*

The mean value of the five samples from each site was used for statistical analysis. Data analysis was performed using SPSS 15 (IBM, USA) with significance set at 0.05. Two-way ANOVA with “sites” (traffic intensity) and “species” as the main factors was used to compare the metal content of lichens. The Pearson correlation test was used to obtain correlation coefficients between element pairs and between the chlorophyll fluorescence parameters and the elemental content of the lichens. Factor analysis was employed to determine correlations among metals in the lichen and provide information on probable sources of the metals.

### *2.5. Determination of target hazard quotients (THQ)*

The target hazard quotient, THQ, is the ratio of exposure dose to reference (“safe”) dose and is used to express the risk of exposure to non-carcinogenic toxic substances (USEPA, 2000). If THQ < 1, the exposure is less than the reference dose and unlikely to cause adverse effects. The model, developed to assess health risk for humans, was modified and used to assess the health risk of consuming lichen to monkeys, given the metal concentrations found in this study. The assumptions made for the monkey health risk calculations are shown in **Table 1**.

THQ is normally calculated using **Equation (1)** (Chien et al., 2002):

$$THQ = \frac{\mathrm{EFr} \times \mathrm{EDtot} \times \mathrm{FIR} \times \mathrm{C}}{\mathrm{RfDo} \times \mathrm{BWa} \times \mathrm{ATn}} \times 10^{-3} \tag{1}$$

where EFr is exposure frequency (365 days/year); EDtot is the exposure duration (18 years, average lifetime); FIR is the food ingestion rate (g/day); C is the metal concentration of lichen (mg/g); RfDo is the oral reference dose (mg/kg/day) for humans given in **Table S2**; BWa is the average adult body weight (male 16.4 kg, female 8.4 kg); and ATn is the exposure time (365 days/year x 18 years). The food ingestion rate of the golden monkey varies with season (**Table 1**) and the term EFr × FIR was modified according to Equation (2) to give total annual intake of lichen:

$$\mathrm{EFr}{\times}\mathrm{FIR} = \mathrm{FIR}_1{\times}\mathrm{EFr}_1 + \mathrm{FIR}_2{\times}\mathrm{EFr}_2 \tag{2}$$

Where $FIR_1$ is the food ingestion rate from December to March (99 g/day); $EFr_1$ is exposure frequency from December to March (122 days/year); $FIR_2$ is the food ingestion rate from April to November (34 g/day); $EFr_2$ is exposure frequency from April to November (243 days/year). THQ was then calculated using **Equation (3)**:

$$THQ = \frac{\mathrm{EFr}_1 \times \mathrm{EFr}_1 \times \mathrm{FIR}_2 \times \mathrm{C}}{\mathrm{RfDo} \times \mathrm{BWa} \times \mathrm{ATn}} \times 10^{-3} \tag{3}$$

Two or more pollutants may interact to produce either synergistic or antagonistic effects (Hallenbeck, 1993). However, in this study, the total target hazard quotient, TTHQ, is given by the arithmetic sum of the individual THQ values according to.

To date, there are no regulations regarding the maximum allowable residue limits of metals in lichen for monkey consumption. In order to evaluate the potential health risk posed by lichen consumption via Fe, Mn, Co, Ni, Cd, Pb, Cu, Zn and Cr, we defined a maximum allowable concentration of the elements considering non-carcinogenic risk on the basis of the reference dose (RfD) of the elements and the maximum allowable lichen consumption rate considering the non-carcinogenic effect of a contaminant (USEPA, 2000). The maximum allowable concentration (mg/kg, wet weight or dry weight) was calculated as:

$$C_{max} = \frac{\mathrm{RfDo}{\times}\ \mathrm{BWa} \times\ \mathrm{ATn}{\times}\mathrm{THQ}}{(\mathrm{FIR}_1 \times \mathrm{EFr}_1 +\ \mathrm{FIR}_2 \times \mathrm{EFr}_2)\ {\times}\mathrm{EDtot}} \tag{4}$$

where, $C_{max}$ is the maximum allowable concentration of metals in lichen, when THQ is set to 1.

## 3. Results and discussion

### *3.1. Metal content of lichens*

Concentrations of Fe, Mn, Co, Ni, Cd, Pb, Cu, Zn and Cr were estimated for all four sites. Of the nine metals, the level of Fe was the highest in both lichens, followed by Cr and Zn, Mn, Pb, Cu , Cd, Ni, and Co (**Table 2**). Bajpai and Upreti (2012) also found that Fe concentrations in *Lepraria lobificans Nyl.* were significantly higher than those of other metals and that Fe occurs at relatively high levels in the thallus of lichens.

Two-way ANOVA was used to find significant differences in lichen metal concentrations between species and among sites and results are displayed in **Table 3**. Fe, Cd, Pb, Zn, and Cr displayed significant concentration differences both between species and among sites. Mn and Ni displayed significant differences in concentration between species, but not among sites. Co and Cu displayed no significant differences in concentration between species or among sites.

Fe, an essential nutrient of low toxicity, occurs at relatively high concentrations in soil and is weakly associated with vehicle traffic due to wear of iron and steel components. At the other extreme, Cd and Pb are toxic heavy metals with no known biological function and strongly associated with vehicular traffic. Among sampling sites, both Cd and Pb displayed significant variation ($p < 0.01$) attributed to differences in traffic intensity. Nieboer and Richardson (1981) also observed maximum deposition of heavy metals around high traffic areas, with distance from source explaining 90% of the total variance. Other researchers have studied the accumulation of Pb in lichen and found higher concentrations in lichen growing near roads (Zhaoet al., 2019). In addition to automobile exhaust, abrasion of metallic vehicle parts also releases Fe, Pb, and Cr, while Zn is released from abrasion of tires and brake pads (Ward and Sampson, 1989). Zn is another micronutrient that is toxic at high levels and it also varied significantly with both

**Table 1**
Assumptions for THQ calculation.

| Parameter | | Description | Reference |
|---|---|---|---|
| Do | mg/kg/day | Oral dose received by monkeys | USEPA (1989) |
| RfDo | mg/kg/day | Oral reference dose for humans | USEPA (2009) |
| BWa | 16.40 kg | Average adult body weight | Liang et al. (2001) |
| EDtot | 18 years | Average lifetime of golden monkey | Liang et al. (2001) |
| FIR$_1$ | 99 g/day | Lichen ingestion rate from December to March | Liang et al., (2001) & |
| EFr$_1$ | 122 days/year | December–March | Tie et al. (2010) |
| FIR$_2$ | 34 g/day | Lichen ingestion rate from April to November | |
| EFr$_2$ | 243 days/year | April–November | |
| ATn | 365 days/year × 18 years | Exposure time | |

species and site. In *U. aciculifera*, the highest level of Zn was found at site 2 with the highest vehicular flow followed by the samples collected from site 1 (car park) having a higher automobile density. In addition to abrasion, Cr emissions are found in vehicle exhaust and Cr content of lichens was found to vary significantly with traffic intensity. Mn and Ni concentrations varied by species but not with location and do not provide information on the environmental effects of traffic. Cu is an essential trace nutrient that is toxic, especially to algae, at higher concentrations. While no significant difference was observed among sites or between species in this study, Bajpai and Upreti (2012) found that lichen, *Pyxine cocoes*, collected near roadsides had elevated Cu levels. Because Cu and Co concentrations did not vary significantly with species or site, they provide little insight useful for this study.

### *3.2. Factor analysis and correlation of metals in lichen*

The factor analysis (FA) technique is used to reduce the number of variables and is useful for examining the relationships between metals to provide information about metal sources. FA was performed by evaluation of principal components and computing the eigenvalues. The three eigenvalues larger than 1 (Kaiser Criterion) were retained and led to a cumulative variance > 85%. Principle components were then rotated using the Varimax normalization method. The results, presented as factor loadings of the rotated matrix, are shown in **Table 4**.

The three factors adequately account for the total metal content of lichen (**Table 4**). Cd, Pb, Zn, and Cr are associated with Factor 1 (F1); Co, Ni and Cu are associated with Factor 2 (F2), and Fe and Mn are associated with Factor 3 (F3). Metals associated with F1 and F2 had positive loadings, implying a correlation among the metals. Two-way ANOVA (**Table 3**) indicates a significant difference in Cd, Pb, Zn and Cr accumulation among sampling sites; these metals are associated with F1 and are positively correlated. The four metals are released by abrasion of metallic vehicle parts, tires and brake pads and in automobile exhaust. F1 accounts for traffic-related sources of metal. Co, Ni, and Cu, associated with F2, display little concentration variation in lichens collected at different sites, indicating that atmospheric concentration of these metals is not much related to the motor vehicle. In this study, the concentration of Co in lichens ranged from 0.26 ± 0.09 to 0.34 ± 0.05 mg/kg dry weight. This is consistent with the findings of (Pokrovsky et al., 2010), who also reported that the primary source of Co was crustal contributions from local rocks and soils. Thus, F2 may be related to surface deposition from soil-derived dust.

According to Loppi et al. (2000), Fe is an abundant metal in the Earth's crust and local soil concentrations correlate strongly with concentrations in lichen. This is an indication of lichen's potential as a biomonitor. The level of Fe showed significant variation among sites, and the highest concentration was found, as with Zn, at site 2. The primary source of Fe to lichens is soil derived dust and the site with the highest traffic flow has elevated road dust particles due to the turbulence produced by vehicles. Mn is also an abundant trace element in the lithosphere (Bowen et al., 1979). Majumder et al. (2013) suggested that the occurrence of this element in lichen was derived at least partially from wind erosion of the upper soil layer. The negative loadings for Fe in F3 suggest an antagonistic effect between Fe and Mn in the lichen samples from this study. Basile et al. (2008) also confirmed Mn and Fe originated from suspended geogenic particles. The source of Fe and Mn appears to be road dust, but their accumulation antagonistic. Our results show that lichen can be an excellent biomonitor but that the potential varies by species. *U. aciculifera* is a useful biomonitor for airborne metals that records pollutant loading and provides information useful for identifying pollute sources. Results also point to the need for considering antagonism among metals.

**Table 2**
Metal content of *U. aciculifera* and *U. luridorufa* (mg/kg dry weight).

| Sites | Fe | Mn | Co | Ni | Cd | Pb | Cu | Zn | Cr |
|---|---|---|---|---|---|---|---|---|---|
| *U.a* | | | | | | | | | |
| s1 | 170.04 ± 24.12 | 29.70 ± 11.27 | 0.26 ± 0.09 | 0.87 ± 0.25 | 1.40 ± 0.27 | 14.24 ± 6.08 | 4.40 ± 1.32 | 45.05 ± 14.84 | 35.61 ± 13.62 |
| s2 | 193.48 ± 38.59 | 42.22 ± 9.70 | 0.30 ± 0.10 | 0.88 ± 0.48 | 1.04 ± 0.21 | 8.17 ± 0.91 | 4.31 ± 0.61 | 57.39 ± 14.77 | 38.63 ± 8.55 |
| s3 | 141.76 ± 14.91 | 36.70 ± 14.51 | 0.31 ± 0.04 | 0.84 ± 0.19 | 0.79 ± 0.04 | 6.20 ± 0.64 | 3.95 ± 1.38 | 49.84 ± 15.95 | 32.42 ± 10.03 |
| s4 | 130.27 ± 2.26 | 44.46 ± 7.04 | 0.34 ± 0.05 | 0.60 ± 0.31 | 0.39 ± 0.04 | 2.44 ± 0.34 | 4.19 ± 1.37 | 33.28 ± 2.52 | 27.99 ± 4.13 |
| *U.l* | | | | | | | | | |
| s1 | 171.91 ± 22.26 | 13.10 ± 5.30 | 0.30 ± 0.18 | 0.30 ± 0.10 | 0.55 ± 0.16 | 5.98 ± 1.28 | 3.47 ± 0.18 | 25.74 ± 1.33 | 31.86 ± 5.09 |
| s2 | 186.46 ± 21.83 | 15.77 ± 3.92 | 0.31 ± 0.13 | 0.36 ± 0.13 | 0.52 ± 0.14 | 4.06 ± 0.20 | 3.74 ± 0.36 | 25.70 ± 2.65 | 28.16 ± 4.73 |
| s3 | 175.95 ± 10.86 | 16.98 ± 8.60 | 0.30 ± 0.13 | 0.56 ± 0.13 | 0.45 ± 0.05 | 3.51 ± 0.27 | 3.74 ± 0.98 | 25.34 ± 3.45 | 26.49 ± 6.54 |
| s4 | 176.26 ± 8.49 | 17.55 ± 3.58 | 0.28 ± 0.14 | 0.64 ± 0.02 | 0.38 ± 0.13 | 2.47 ± 0.37 | 3.55 ± 0.77 | 27.09 ± 1.61 | 25.55 ± 4.16 |

**Table 3**
Two-way ANOVA with sites (Si) and species (Sp) as factors.

| Metal | | Fe | Mn | Co | Ni | Cd | Pb | Cu | Zn | Cr |
|---|---|---|---|---|---|---|---|---|---|---|
| Si | *F* | 4.918** | 1.009 | 0.499 | 0.350 | 13.082** | 14.315** | 0.070 | 5.881** | 5.217** |
| | *p* | 0.006 | 0.400 | 0.685 | 0.790 | 0.000 | 0.000 | 0.975 | 0.002 | 0.004 |
| Sp | *F* | 6.552* | 90.427** | 0.544 | 8.514** | 40.076** | 19.747** | 3.762 | 79.049** | 61.126** |
| | *p* | 0.015 | 0.000 | 0.466 | 0.006 | 0.000 | 0.000 | 0.061 | 0.000 | 0.000 |
| Si*Sp | *F* | 5.324** | 10.724** | 0.661 | 16.372** | 29.415** | 15.306** | 0.622 | 37.837** | 26.492** |
| | *p* | 0.000 | 0.000 | 0.700 | 0.000 | 0.000 | 0.000 | 0.730 | 0.000 | 0.000 |

**Table 4**
Total variance and component matrices for the heavy metals in lichens.

| Component | Initial Eigenvalues | | | | Extraction Sums of Squared Loadings | | | Rotation Sums of Squared Loadings | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | %of Variance | Cumulative % | | Total | %of Variance | Cumulative % | Total | %of Variance | Cumulative % |
| 1 | 3.916 | 43.516 | 43.516 | | 3.916 | 43.516 | 43.516 | 3.049 | 33.873 | 33.873 |
| 2 | 1.382 | 15.355 | 58.871 | | 1.382 | 15.355 | 58.871 | 2.215 | 24.611 | 58.484 |
| 3 | 1.315 | 14.609 | 73.480 | | 1.315 | 14.609 | 73.480 | 1.350 | 14.996 | 73.480 |
| Elements | | Component matrix | | | | | | | Rotated component matrix | |
| | | F1 | F2 | F3 | | | F1 | | F2 | F3 |
| Fe | | | | −0.831 | | | | | | −0.857 |
| Mn | | | | 0.641 | | | | | | 0.811 |
| Co | | | 0.795 | | | | | | 0.599 | |
| Ni | | | 0.581 | | | | | | 0.740 | |
| Cd | | 0.955 | | | | | 0.921 | | | |
| Pb | | 0.872 | | | | | 0.869 | | | |
| Cu | | | 0.764 | | | | | | 0.646 | |
| Zn | | 0.789 | | | | | 0.686 | | | |
| Cr | | 0.798 | | | | | 0.814 | | | |

**Table 5**
Correlation between Fv/Fm values and metal concentration (n = 20).

| sp | Correlation | Fe | Mn | Co | Ni | Cd | Pb | Cu | Zn | Cr |
|---|---|---|---|---|---|---|---|---|---|---|
| *U. a* | $R^2$ | −0.538* | 0.260 | 0.293 | −0.017 | −0.777** | −0.681** | −0.111 | −0.463* | −0.639** |
| | *P* | 0.014 | 0.269 | 0.209 | 0.944 | 0.000 | 0.001 | 0.641 | 0.040 | 0.002 |
| *U. l* | $R^2$ | −0.352 | −0.053 | −0.008 | −0.048 | 0.088 | 0.160 | −0.315 | 0.184 | 0.051 |
| | *P* | 0.128 | 0.825 | 0.973 | 0.842 | 0.712 | 0.480 | 0.176 | 0.437 | 0.830 |

**Table 6**
Estimated target hazard quotients, THQ, for individual metals and total metals, TTHQ, for lichen in Shennongjia Nature Reserve and Maximum allowable residue limits (mg $kg^{-1}$) for selected toxic trace metals in lichen consumed by the golden monkey.

| Sp | Si | THQ | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Fe | Mn | Co | Ni | Cd | Pb | Cu | Zn | Cr | Cd + Pb | TTHQ |
| *U.a* | 1 | 0.21 | 0.18 | 0.75 | 0.04 | **1.21** | **3.03** | 0.09 | 0.14 | 0.02 | **4.24** | **5.67** |
| | 2 | 0.23 | 0.21 | 0.86 | 0.04 | 0.89 | **1.74** | 0.09 | 0.18 | 0.02 | **2.63** | **4.26** |
| | 3 | 0.17 | 0.19 | 0.90 | 0.04 | 0.68 | **1.32** | 0.08 | 0.13 | 0.02 | **2.00** | **3.52** |
| | 4 | 0.16 | 0.20 | 0.97 | 0.03 | 0.34 | 0.52 | 0.09 | 0.09 | 0.02 | 0.86 | **2.40** |
| *U.l* | 1 | 0.21 | 0.08 | 0.85 | 0.01 | 0.47 | **1.27** | 0.07 | 0.07 | 0.02 | **1.74** | **3.06** |
| | 2 | 0.23 | 0.10 | 0.87 | 0.02 | 0.44 | 0.86 | 0.08 | 0.07 | 0.02 | **1.30** | **2.68** |
| | 3 | 0.21 | 0.10 | 0.85 | 0.02 | 0.38 | 0.75 | 0.08 | 0.07 | 0.02 | **1.13** | **2.49** |
| | 4 | 0.21 | 0.11 | 0.80 | 0.03 | 0.32 | 0.53 | 0.08 | 0.08 | 0.01 | 0.84 | **2.16** |
| $C_{max}$(wet weight) | | 206.00 | 41.20 | 0.09 | 5.89 | 0.29 | 1.18 | 11.76 | 88.20 | 41.00 | / | / |
| $C_{max}$ (dry weight) | | 824.01 | 164.80 | 0.35 | 23.54 | 1.18 | 4.71 | 47.04 | 352.80 | 1764.00 | / | / |

### *3.3. Chlorophyll fluorescence*

Chlorophyll fluorescence has been used to study the effect of pollutants on lichen, via the electron transport chain of PSII (Scheidegger and Schroeter, 1995). The ratio of variable fluorescence to maximum fluorescence (Fv/Fm) is related to the efficiency of PSII in higher plants (Hendrik et al., 2012) and in lichens (Álvarez et al., 2012) and used as an indicator of stress. For example, Álvarez et al. (2012) and Branquinho et al. (1997) demonstrated that Fv/Fm is useful for monitoring the effect of Pb on lichens by showing the ratio was a sensitive indicator of damage. In this study, chlorophyll fluorescence parameters were measured *in situ* and the correlation between Fv/Fm and lichen metal concentration was examined. The stress of dehydration was eliminated and measured values of Fv/Fm were compared to the control. Lichen in the control site (site 4) was re-wetted with a water spray and Fv/Fm values were measured at selected time intervals. After 24 h, Fv/Fm values had reached the normal value (**Fig. S2**).

Lichens samples were re-wetted for at least 24 h, shaded for 20 min for dark adaption, and Fv/Fm values were measured (**Table S3**). The Fv/Fm values of *U. aciculifera* varied significantly among sampling sites, while those of *U. luridorufa* did not. Analysis indicates that *U. aciculifera* accumulates metal more efficiently than *U. luridorufa*. Concentrations of Mn, Ni, Cd and Pb in *U. aciculifera* were more than double those in *U. luridorufa.* Furthermore, variations in the former were more pronounced than those in the latter. Studies have shown that differences in metal accumulation, as well as sensitivity to stress, arise from differences in lichen phycobiont. The results indicate that *U. aciculifera* functions well as a biomonitor, while *U. luridorufa* does not.

The correlation between Fv/Fm values and metals are listed in **Table 5**. In this study, Fv/Fm values of lichen *U. aciculifera* had significantly negative correlation with accumulation ofFe, Cd, Pb, Zn and Cr, but not in *U. luridorufa.* Álvarez et al. (2012) and Branquinho et al. (1997) concluded that lichen PSII photochemical reactions were sensitive to Pb. Beckett and Brown (1984) studied the differential sensitivity of lichens to heavy metals and showed that Cd inhibit photosynthesis in lichens at even lower concentrations. Bajpai and Upreti (2012) found that Fe impacted the chlorophyll parameter in lichens. Our results indicate that vehicular traffic has affected lichen photosynthesis. Lichen *U. aciculifera* and *U. luridorufa* displayed different sensitivities to metal pollution; *U. aciculifera* accumulated more metal and was the more sensitive in terms of PSII photochemical response. Accordingly, in the Shennongjia National Nature Reserve *U. aciculifera* is superior to *U. luridorufa* as a biological indicator for metal pollution. Moreover, *in situ* measurement of Fv/Fm provides prompt and reliable information on the stress on lichen due to elevated metal concentration.

### 3.4. Health threat from consuming lichens

The health threat to golden monkeys from consuming lichen with elevated metal concentrations due to vehicular traffic was assessed using the target hazard quotient, THQ. Because the reference dose includes a margin of safety, the THQ value is conservative (Yi et al., 2011). The total target hazard quotient, TTHQ, given by the arithmetic sum of the individual THQ values, does not account for synergistic or antagonistic effects. While the TTHQ is useful as an index for comparing exposures, a value $> 1$ should not be interpreted as predictive of adverse health effects. **Table 6** displays THQ and TTHQ values by metal, site and lichen species. The TTHQ of all sites exceeded 1 and the THQ summation of non-essential metals (Cd and Pb) exceeded 1 in the sites influenced by traffic (site 1, 2 and 3). The THQ generally followed the order: site 1 > site 2 > site 3 > site 4. With THQs exceeding 1, Pb and Cd could pose a risk to monkey health. Cd and Pb are toxic heavy metals with no known biological function. They are also cumulative and can cause harm, even at low concentrations, when ingested over a long period of time (Somers, 1974). The oral reference dose for Co is low and the THQ for Co was nearly 1. However, Co is an essential nutrient, a component of vitamin B12 and important for blood pressure regulation and proper thyroid function (Blakhima, 1970). Excess Co can be eliminated by excretion and of relatively low toxicity (Young, 1960). The THQ value for all other metals is well below 1. Our results indicate that the health risk of monkeys feeding on lichens is from Pb and Cd and both are strongly associated with vehicular traffic. Thus, the traffic in Shennongjia National Nature Reserve poses a risk to golden monkey health.

Lichen from near roads with TTHQ > 1, is collected to feed captive bred monkeys. By setting THQ to 1 (no adverse effects anticipated), the $C_{max}$ of metals can be calculated and these values are given in **Table 6**. It should be noted that *U. aciculifera* had a higher THQ for each metal than *U. luridorufa*.

## 4. Conclusion

This is the first report on the health risk posed to the Sichuan snub-nosed monkey from metal accumulation in lichen due to vehicular traffic. Cd and Pb from automobile exhaust accumulate in lichens and could pose a health risk to the monkeys. Of the two lichen species used in this study, *U. aciculifera* was the superior biomonitor, showing greater sensitivity as indicated by higher metal accumulation and larger differences in both metal concentration and in chlorophyll fluorescence (Fv/Fm) among the four sampling sites. The sensitivity of chlorophyll fluorescence to varying levels of metal proved to be a suitable marker. From the standpoint of metal toxicity, *U. luridorufa*, with lower concentrations of Cd and Pb, is the safer source of food for the monkeys. These findings will advance the science of biomonitoring, contribute to environmental protection efforts in China's nature reserves and help improve food safety for Sichuan snub-nosed monkey, a national treasure of China.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.05.047.

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