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# Fractionation of Mercury Stable Isotopes in Lichens

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**Key words: Mercury, Stable isotopes, Bio-monitoring, Lichens, Air**

## Highlights:

- 1: The isotopic composition of Hg in lichens changes seasonally, with the highest  $\delta^{202}\text{Hg}$  in the summer.
- 2: At the contaminated sites, the local Hg isotopic fingerprint overrides any other trends.
- 3: Lichens are a suitable bio-monitor for the assessment of spatial and temporal trends of Hg in air in areas with elevated Hg in the environment.
- 4: Natural variability of Hg in lichens prevents their use for spatial and temporal trends at background sites.

## Abstract

Bio-monitoring of mercury (Hg) in air using transplanted and *in-situ* lichens was conducted at three locations in Slovenia: (I) the town of Idrija in the area of the former Hg mine, where Hg contamination is well known; (II) Anhovo, a settlement with a cement production plant, which is a source of Hg pollution, and (III) Pokljuka, a part of a national park. Lichens from Pokljuka were transplanted to different sites and sampled four times—once per season, from January 2020 to February 2021. Lichens were set on tree branches, fences, and under cover, allowing them to be exposed to different environmental conditions (e.g., light and rain). The *in-situ* lichens were sampled at the beginning and the end of the sampling period. The highest concentrations were in the Idrija area, which was consistent with previous research.

33 Significant mass-dependent fractionation has been observed in transplanted lichens during  
34 summer period. The  $\delta^{202}\text{Hg}$  changed from -3.0 ‰ in winter to -1.0 ‰ in summer and dropped  
35 again to the same value in winter the following year. This trend was observed in all samples,  
36 except those from the most polluted Idrija sampling site, which was in the vicinity of the  
37 former Hg ore-smelting plant. This was likely due to large amounts of Hg originating from  
38 polluted soil close to the former smelting plant with a distinct isotopic fingerprint in this local  
39 area. The  $\Delta^{199}\text{Hg}$  in transplanted lichens ranged from -0.1 ‰ to -0.5 ‰ and showed no  
40 seasonal trends. These findings imply that seasonality, particularly in summer months, may  
41 affect the isotopic fractionation of Hg and should be considered in the sampling design and  
42 data interpretation. This trend was thus described in lichens for the first time. The mechanism  
43 behind such change is not yet fully understood.

## 44 **1 Introduction**

45 Mercury (Hg) is one of the most toxic elements. There is a global legally binding agreement  
46 regarding decreasing anthropogenic emissions and releases of Hg and its compounds—the  
47 Minamata Convention on Mercury (United Nations Environment Programme, 2019). An  
48 important aspect of the Minamata Convention is the monitoring of Hg in the atmosphere to  
49 assess the effectiveness of measures aimed at decreasing anthropogenic Hg emissions. As Hg  
50 can be transported over long distances within the atmosphere, it is often difficult to assess the  
51 contribution of local sources of Hg in the overall Hg composition of the atmosphere. This is  
52 additionally complicated by the presence of different forms of Hg in the atmosphere—  
53 gaseous elemental mercury (GEM;  $\text{Hg}^0_{(\text{g})}$ ), gaseous oxidised mercury (GOM;  $\text{Hg}^{2+}_{(\text{g})}$ ), and  
54 particulate-bound mercury (PBM;  $\text{Hg}_{(\text{p})}$ )—all of which have different biogeochemical  
55 properties and cycle differently through environmental compartments (Ariya et al., 2015;  
56 Lyman et al., 2020; Si & Ariya, 2018). Therefore, it is important to improve our  
57 understanding of how to best study and measure Hg in the atmosphere in order to design

58 efficient and effective monitoring programs able to track the effectiveness of the  
59 implementation of the Minamata Convention.

60 There are two groups of methods for measuring Hg in the air—the so-called active and  
61 passive methods. Active methods include instruments that actively measure concentrations  
62 based on the detection of Hg either by atomic absorption or atomic fluorescence  
63 spectrophotometry (Gustin et al., 2015). Passive methods include passive samplers (McLagan  
64 et al., 2016; Naccarato et al., 2021), pre-concentration by sorbent traps, and bio-monitoring  
65 using lichens, mosses, and other vegetation-based matrices. Correlations between such lichen  
66 use and the active methods have been reported (Mlakar et al., 2011). Bio-monitors include  
67 types of vegetation that absorb Hg from the atmosphere (Zhou & Obrist, 2021), such as  
68 mosses (Nickel et al., 2018; Szczepaniak & Biziuk, 2003) and lichens (Abas, 2021; Çobanoğlu  
69 Özyiğitoğlu, 2020). Bio-monitors are notable in the present context for the following reasons:  
70 (I) they can readily be found in nature, (II) they can provide insight into past air  
71 contamination, (III) they are less expensive and (IV) they are less likely to be vandalised than  
72 active methods (Garty, 2002). Bio-monitors are, however, climate dependent. For example,  
73 lichens have been observed as the most metabolically active at temperatures between 0 and 15  
74 °C, somewhat limiting their operating temperature range (Kappen et al., 1996).

75 There are two ways to apply lichens as bio-monitors—the *in-situ* method and the transplanted  
76 method, which are sometimes referred to as passive and active bio-monitoring methods,  
77 respectively (Çobanoğlu Özyiğitoğlu, 2020). In the case of *in-situ* lichens, the lichens are  
78 collected directly from where they are found, and in the case of transplanted lichens, they are  
79 collected at one spot and then exposed in another location. *In-situ* lichens can also record past  
80 contamination, while transplanted lichens can only record contamination after their exposure  
81 at a new location. The transplantation method is flexible in that it allows for the exposure of  
82 lichens under a predetermined set of conditions, such as being covered to avoid rain and/or

83 sun exposure. There are a number of studies where the concentrations of Hg in lichens (*in situ*  
84 and/or transplanted) have been measured (Berdonces et al., 2017; Horvat et al., 2000; Jeran et  
85 al., 2007; Klapstein et al., 2020; Lars Gunnar Sillén, 1967; Mlakar et al., 2011). In some  
86 cases, the connection to pollution sources has been clearly established (Horvat et al., 2000).  
87 However, in some cases, the reality might be more difficult to interpret, especially if the  
88 relative differences in concentrations during exposure of transplanted lichens are low (Mlakar  
89 et al., 2011).

90 Over the last two decades, advanced analytical equipment has opened the door for researchers  
91 to determine stable isotopes of Hg. Ability for isotopic analysis is an important tool used to  
92 improve our understanding of Hg cycling and behaviour in different environmental processes  
93 (Tsui et al., 2020). Hg has seven different stable isotopes, and they can fractionate during  
94 transport through and/or after deposition from the atmosphere (Blum et al., 2014). There are  
95 two types of Hg fractionation—mass dependent and mass independent. Mass-dependent  
96 fractionation (MDF) is represented as  $\delta^{xxx}\text{Hg}$ , and mass-independent fractionation (MIF) as  
97  $\Delta^{xxx}\text{Hg}$  (Bergquist & Blum, 2007). There are a number of processes that affect Hg isotopic  
98 composition, including photo-reduction, evaporation, volatilisation, absorption, microbial  
99 methylation, and microbial and/or chemical reduction (Yin et al., 2010). This means that Hg  
100 isotope ratios could be used to improve our understandings of the sources of Hg in the  
101 environment and assess the processes it has undergone.

102 Hg isotopes in lichens have already been utilised in studies attempting to discriminate  
103 pollution sources (Barre et al., 2018, 2020; Olson et al., 2019; Wang et al., 2020). In general,  
104 Hg isotopes in lichens have negative MDF (-2.5 to -0.5 ‰ for  $\delta^{202}\text{Hg}$ ) and MIF (-0.8 to 0.0 ‰  
105 for  $\Delta^{199}\text{Hg}$ ) (Blum et al., 2014), while atmospheric Hg exhibits mostly positive MDF (0.0 to  
106 1.5 ‰ for  $\delta^{202}\text{Hg}$ ) and negative MIF (-0.4 to 0.0 ‰ for  $\Delta^{199}\text{Hg}$ ) (Demers et al., 2013; Gratz et  
107 al., 2010; Sonke, 2011; Szponar et al., 2020; Wang et al., 2021). Various physicochemical

108 processes can affect the Hg isotope ratios determined in lichen samples to the extent that they  
109 are not representative of the actual Hg isotopic composition in the air in which they were  
110 placed. For example, lower MDF values in foliage could be caused by the uptake and  
111 fractionation of Hg passing into their interior, which preferably takes up the lighter isotopes  
112 (Demers et al., 2013); this might also hold true for lichens, as speculated by Blum et al.  
113 (2014). Negative MIF might be caused by photochemical reduction and loss of some Hg from  
114 the foliage (Blum et al., 2012; Demers et al., 2013). It is presently not understood how these  
115 processes are affected by seasonal changes in temperature, sunlight exposure, or proximity to  
116 sources of Hg pollution. To the best of our knowledge, such processes have not yet been  
117 systematically studied in lichens. It should be noted that there are some MDF processes that  
118 also occur on sorbent traps and active samplers. Moreover, as described by Szponar et al.  
119 (2020), the configuration of a sampling device can alter the  $\delta^{202}\text{Hg}$  by  $\approx 1.0\%$ .

120 In a study by Enrico et al. (2016), the shift in  $\delta^{202}\text{Hg}$  was observed between the samples  
121 collected during the spring–summer and fall–winter seasons. There was no discrimination  
122 among individual seasons, spring and summer values are presented together and the fall and  
123 winter ones together. The spring–summer isotopes showed an average increase of  $0.4\%$   
124 compared to those in the fall–winter ones. Some of the mosses in this study were left covered.  
125 They showed a lesser response of  $\delta^{202}\text{Hg}$  by up to  $0.7\%$  compared to the covered mosses. In  
126 a study by Fu et al. (2019), a similar seasonal trend of  $\delta^{202}\text{Hg}$  increase during the summer in  
127 the forest air was linked to vegetation activity. However, no explanation of the mechanism  
128 was provided in the study, apart from the speculation that higher vegetation activity likely  
129 induces higher  $\delta^{202}\text{Hg}$ .

130 It has been observed that the concentration of Hg in deciduous forest air is cyclical. When  
131 leaves are growing, they take up Hg from the air (Jiskra et al., 2018), leaving the air depleted  
132 in Hg. When they fall of Hg is reemitted it back. Another discovery is that over long periods

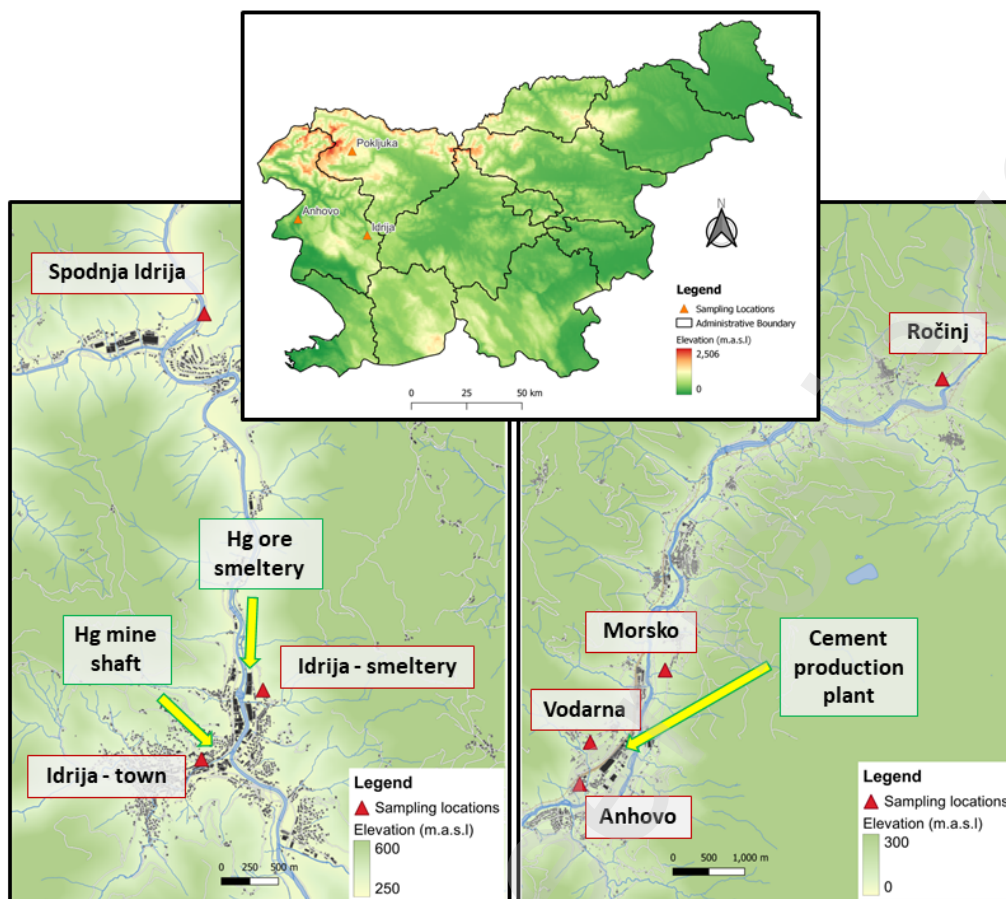
133 of time the average Hg isotopic composition does not change by much (0.2 ‰ for  $\delta^{202}\text{Hg}$ )  
134 despite any other trends in global emission of Hg. This is seen from the work of Yamakawa et  
135 al. (2021), who measured Hg isotopic composition in spruce needles which stay on the tree all  
136 year long.

137 This study aimed to provide unique information on the use of lichens as bio-monitors for Hg  
138 presence in air, including stable isotopes measurements, at sites with a wide range of Hg  
139 concentrations in air. These sites include former mercury mining area Idrija and the second  
140 site is around the cement production plant.

141 These findings are especially relevant due to recently issued monitoring guidelines for the  
142 evaluation of the effectiveness of the Minamata Convention; the guidelines outline options for  
143 measuring Hg in air via a tiered approach, offering several monitoring possibilities. These  
144 include active and passive measurements of elemental Hg, Hg speciation, and methods based  
145 on passive sampling and bio-monitoring. The tiered approach of these guidelines also  
146 envisage the use of stable isotopes for source apportionment (UNEP, 2022).

## 147 **2 Sampling locations**

148 The sampling locations are presented in Figure 1.



149  
 150 **Figure 1: Sampling locations are marked by orange triangles, and individual sampling sites are**  
 151 **marked by red triangles in the zoomed-in view. Data from the Surveying and Mapping Authority**  
 152 **of the Republic of Slovenia (2022).**

153  
 154 **Idrija** is a town with a wealthy history of Hg mining. The metasomatic Hg-rich ore in the  
 155 dolomite is associated with Triassic igneous activity (Drovenik et al., 1990). It has been  
 156 mined for about 500 years. The mine and a nearby smeltery have not operated since the  
 157 1990s; however, even decades after the closure, the concentrations of Hg in the air remain  
 158 high, up to 5000 ng/m<sup>3</sup> (Grönlund et al., 2005; Kocman et al., 2011; Kocman & Horvat, 2011;  
 159 Kotnik et al., 2005). For this study, the in-situ lichens were collected, and transplanted lichens  
 160 were exposed at the following three locations: (I) Spodnja Idrija, a settlement located about 3  
 161 km downstream from the town of Idrija, (II) Idrija, a town located above the mine shafts in  
 162 the town, and (III) the Idrija smeltery, located at the site of former smelting plant. At the same  
 163 sites, in-situ lichens were also collected. The average temperature at the Idrija meteorological



164 station was 10.1 °C in 2020, and the temperatures were below freezing for 8% of the year  
165 (Slovenian Environmental Agency, 2022).

166 **Anhovo** is a settlement in western Slovenia, located in the middle of the Soča Valley. It is the  
167 site of the Salonit–Anhovo cement production plant. The annual average output of Hg from  
168 the plant has been estimated at 10 kg, with two-thirds comprised of GOM, one-third of GEM,  
169 and less than one percent of PBM (Mlakar et al., 2010). Transplanted lichens were exposed at  
170 the following four sites around the plant: (I) Anhovo and (II) Vodarna, which are both to the  
171 south of the Salonit–Anhovo cement plant, (III) Morsko, which is just north of the plant, and  
172 (IV) Ročinj, which is about 3 km up the river. At the same sites, *in-situ* lichens were also  
173 collected. The prevailing wind direction is from the north-east to the south-west along the  
174 Soča river valley (Mlakar et al., 2011). There is no meteorological station in Anhovo, but the  
175 average temperature in 2020 in nearby Tolmin was 9.6 °C. The temperatures were below 0 °C  
176 for 6 % of the year (Slovenian Environmental Agency, 2022).

177 **Pokljuka** is a plateau with an average height of around 1,200 m<sub>asl</sub>. It is part of the Triglav  
178 National Park. It can be considered a clean or reference location with no known local Hg  
179 sources. Lichens were collected at five spots, spread 1 km apart in the spruce woods covering  
180 Pokljuka. The average temperature in 2020 was 8.6 °C, and the temperatures were below  
181 freezing for 10% of the year (Slovenian Environmental Agency, 2022).

## 182 **3 Methodology**

### 183 **3.1 Sampling of lichens and sample preparation**

184 The epiphytic *Hypogymnia physodes* lichens were chosen, as they can be found at all of the  
185 study locations. This enables a direct comparison between the concentrations of Hg  
186 (Bergamaschi et al., 2007). The collection and transplantation processes were in line with the  
187 recommendations for sampling from Garty (2002). The *in-situ* lichens were collected at the  
188 beginning and end of the study period, January 2020 to February 2021. At first sampling,

189 enough lichens were collected at Pokljuka to transplant them to all of the sampling sites. For  
190 each site, four bags of lichens were prepared for exposure. One bag per season was collected  
191 as indicated in Supplementary material S1. The *in-situ* lichens were collected at the same site  
192 as or close to where the transplants were exposed. In the case of two sites—the towns of  
193 Anhovo and Idrija—additional bags with lichens were set under a roof cover (abbreviated  
194 further by C) but were still unsheltered from the sides. This was done to prevent direct  
195 exposure to rain and kept the lichens shaded, protected from direct sun exposure.

### 196 **3.2 Direct measurements of Hg in air**

197 The sampling of atmospheric Hg was performed at the same sites from July to December  
198 2020 (Table 1). The sampling and measurements were performed using a LUMEX Hg  
199 Analyzer RA915M (Lumex Scientific, St. Petersburg, Russia). It measures only GEM using  
200 cold-vapor atomic absorption spectroscopy with the applied Zeeman background correction.  
201 At each location, the detector was active for 30 min. Background correction was done every  
202 10 min. The detection time was set at 1-second intervals. The obtained data was recorded by a  
203 computer using the Lumex software Rapid. The variability of the individual measurements  
204 was calculated as the standard deviation among all the measurements at a given time. The  
205 natural variability is presented as the standard deviation among all the measurements. The  
206 manufacturer specifies the limit of detection (LOD) for the instrument as 0.5 ng/m<sup>3</sup> for air.  
207 During our long term experiments the precision at was 22% (n = 600, k = 1, at 6 ng/m<sup>3</sup>)

### 208 **3.3 Lichen preparation**

209 In the laboratory, lichens were moistened with deionised water, but not washed, in order to  
210 ease their removal from pieces of bark. Care was taken to minimise the removal of the PBM  
211 physically trapped in and/or bound to the cell wall. Lichen thalli were separated from the bark  
212 using polytetrafluoroethylene tweezers. The samples were lyophilised, and after immersion in  
213 liquid nitrogen, they were crushed in a zirconium mortar and ground with a vibration micro-

214 pulveriser. The pulverised lichens were then homogenised. The dry weights of the samples  
215 were determined by weighing  $\approx 0.1$  g of the sample and heating it at 105 °C for 24 h in a  
216 drying oven.

217 Lichens were digested using a microwave digestion system (UltraWave, Milestone, Italy).  
218 About 0.3 g of each sample was weighed in pre-cleaned polytetrafluoroethylene tubes. Then,  
219 3 mL of 65% HNO<sub>3</sub> and 0.4 mL of 30% HCl (both suprapure grade) were added. The samples  
220 were subjected to closed-vessel microwave digestion at a max power of 1,500 W and max  
221 pressure of 100 bar. The solution was filtered (0.45  $\mu$ m), quantitatively transferred into 10-mL  
222 polyethylene graduated tubes, and diluted with Milli-Q water to 10 mL. Before  
223 measurements, 2 ml of the samples was again diluted to 10 mL with 5% HNO<sub>3</sub>.

#### 224 **3.4 Determination of Hg concentration in lichens**

225 The determination of Hg concentrations was performed with the Agilent 8800 (Santa Clara,  
226 CA, USA) Triple-Quadrupole Inductively Coupled Plasma Mass Spectrometer (QQQ-ICP-  
227 MS). Instrumental operating conditions are provided in Table 1. The measuring curve was  
228 derived from the NIST 3133 Standard Reference Material (Hg Standard Solution by National  
229 Institute of Standards and Technology).

230 Quality assurance and control were assessed using two matrix-matched Reference Materials  
231 (RMs)—IAEA 336 (Lichen) and BCR 482 (Lichen)—which were analysed in each batch of  
232 samples. Repeatability was as high as 8%, while reproducibility was as high as 6%. The LOD  
233 was determined by tripling the standard deviation of the procedural blanks and was 0.014  
234  $\mu$ g/g, representing less than 10% of the lowest Hg concentration in a lichen sample.

235

236 **Table 1: Results of the repeated measurements of the two reference materials**

	Reference value (95% confidence interval) in [ $\mu$ g/g], n = 28	0.200 (0.160–0.240)
IAEA 336	Avg. of measured values $\pm$ SD [ $\mu$ g/g], n = 28	0.201 $\pm$ 0.014
	Recovery $\pm$ RSD [%]	100 $\pm$ 8
	Reference value (95% confidence interval) [ $\mu$ g/g], n = 12	0.480 (0.460–0.500)
BCR 482	Avg. of measured values $\pm$ SD [ $\mu$ g/g]	0.510 $\pm$ 0.020
	Recovery $\pm$ RSD [%]	106 $\pm$ 4

237

238 The samples were measured in duplicates. The differences were less than 3% in the majority  
239 of cases. However, in some cases in samples from the Idrija smeltery, the differences were as  
240 high as 16%. This marks the inhomogeneity of the sampled material. The reported results are  
241 the average of the two measurements. To evaluate the natural variability in the transplants, the  
242 standard deviation among the five lichens taken at Pokljuka at 0 M was calculated. The  
243 average concentration was  $0.23 \pm 0.05 \mu\text{g/g}$ , and the range from the lowest to highest was  
244 0.18 to  $0.3 \mu\text{g/g}$ .  
245 Natural variability had to be applied to all the lichens subsequently transplanted from  
246 Pokljuka. Variability due to inhomogeneity and natural variability have been combined into a  
247 one overall variability, used here according to Equation 3.

$$\text{overall variability} = \sqrt{\text{inhomogeneity}^2 + \text{natural variability}^2} \quad \text{Equation 3}$$

### 248 **3.5 Hg isotope ratio analysis**

249 Hg isotopes of the transplanted lichens were analysed with a Nu Plasma II (Nu instruments  
250 Ltd., Ametek, UK) Multi-Collector Inductively Coupled Plasma Mass Spectrometer (MC-  
251 ICP-MS) at the Jožef Stefan Institute (JSI). The instrument and the preparation lab are kept  
252 under clean conditions. Its operating conditions are provided in the Supplementary material.  
253 The in-situ lichens were analysed at the Institut des Sciences Analytiques et de Physicochimie  
254 pour l'Environnement et les Matériaux (IPREM). The procedure was the same as described in  
255 Yamakawa et al. (2021).  
256 An auto-sampler ASX-520 (Teledyne Cetac, NE, USA) was used for sample introduction.  
257 The sample solution was pumped via a peristaltic pump to a temperature section where it was  
258 mixed with  $\text{SnCl}_2$  in a solution (3% w/v in 10% v/v HCl) to reduce Hg. The mix of solutions  
259 was transported to the phase separator. The recovery was as high as 99.6%. It was estimated  
260 using the concentration of Hg in the waste solution pumped out from the separation cell. From  
261 the phase separator, the elemental Hg vapor was carried over to the instrument by Ar sweep

262 gas flow. The measurements were performed at Hg concentrations of 1 ng/mL. Intensities at  
 263 m/z of 202 have been between 0.5 and 1.2 V. Outlier rejection of an individual measurement  
 264 inside the cycle was done for the values above the relative three standard deviations (SD).  
 265 All measurements were done according to the sample–standard–sample bracketing technique  
 266 (Peel et al., 2008). The results were reported using  $\delta$  and  $\Delta$  notations as is shown in Equations  
 267 1 and 2 (Blum et al., 2014; Blum & Johnson, 2017), where  $f$  represents the correction factor,  
 268 which was 0.2520 for  $\Delta^{199}\text{Hg}$ , 0.5024 for  $\Delta^{200}\text{Hg}$ , 0.7520 for  $\Delta^{201}\text{Hg}$ , and 1.4930 for  $\Delta^{204}\text{Hg}$ ,  
 269 and  $^{xxx}$  represents the relative atomic mass of an isotope.

$$\delta^{xxx}\text{Hg} = \left( \frac{\frac{^{xxx}\text{Hg}}{^{198}\text{Hg}}_{\text{sample}}}{\frac{^{xxx}\text{Hg}}{^{198}\text{Hg}}_{\text{SRM3133}}} - 1 \right) \times 1000 \quad \text{Equation 1}$$

$$\Delta^{xxx}\text{Hg} = \delta^{202}\text{Hg} - \delta^{xxx}\text{Hg} \times f \quad \text{Equation 2}$$

270 At IJS and IPREM, the trueness of the measurements were evaluated using the NIST 8610  
 271 (UM-Almaden) (National Institute of Standards & Technology, 2017) while, accuracy was  
 272 reported as the standard deviation of the repeated NIST 8610 measurements (Table 2). All the  
 273 uncertainties of the Hg concentration data in this paper are expressed at  $k = 2$ .

274  
 275 **Table 2: Values of stable isotopes from the NIST 8610 [‰] certificate with the U in parentheses,**  
 276 **as well as the ones measured in this study with the standard deviation of the samples in**  
 277 **parentheses**

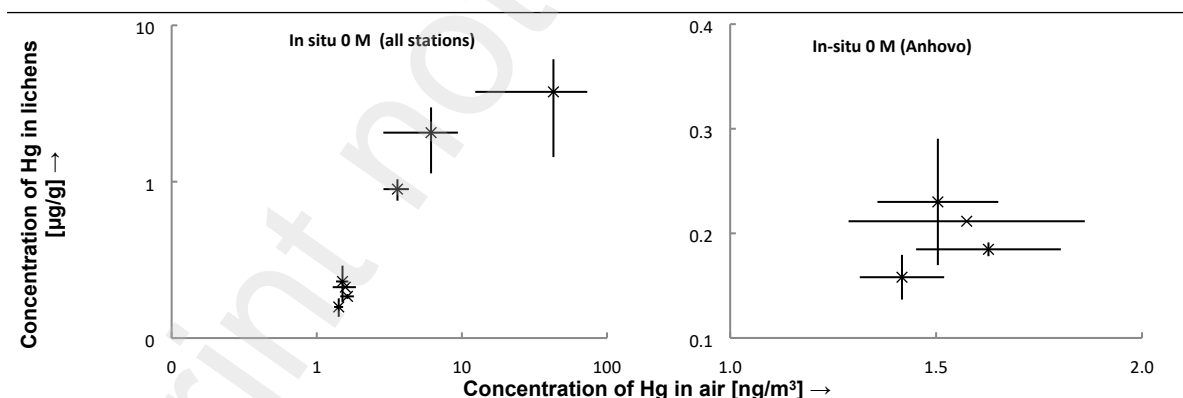
Organisation	n		$\delta^{199}\text{Hg}$	$\delta^{200}\text{Hg}$	$\delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\delta^{204}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{200}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\Delta^{204}\text{Hg}$
NIST	n/a	‰	-0.17	-0.27	-0.46	-0.56	-0.82	-0.03	0.00	-0.04	0.00
		U (k = 2)	(0.01)	(0.01)	(0.02)	(0.03)	(0.07)	(0.02)	(0.01)	(0.01)	(0.02)
IJS	19	‰	-0.17	-0.25	-0.44	-0.49	-0.74	-0.05	0.00	-0.06	-0.02
		2 * S.D.	(0.15)	(0.12)	(0.17)	(0.20)	(0.18)	(0.11)	(0.07)	(0.09)	(0.25)
IPREM	20	‰	-0.15	-0.24	-0.40	-0.51	-0.77	-0.02	0.01	-0.02	0.02
		2 * S.D.	(0.12)	(0.10)	(0.16)	(0.16)	(0.19)	(0.09)	(0.06)	(0.09)	(0.12)

## 278 4 Results and discussion

### 279 4.1 *In-situ* lichens

280 The highest Hg concentration in *in-situ* lichens was found in the Idrija area—in order from  
281 highest to lowest were the Idrija smeltery, the town of Idrija, and Spodnja Idrija. In Figure 2,  
282 the Hg concentrations in the lichens are plotted against Hg concentrations in the air. This was  
283 in agreement with previously published data for Idrija (Horvat et al. 2000; Kocman et al.  
284 2011; Kotnik, Horvat, and Dizdarevič 2005). In the lichens from Anhovo and Vodarna  
285 (downwind from the cement plant), the Hg concentrations were higher than in the lichens  
286 upwind, as was previously observed by Mlakar et al. (2011).

287 There was some observable variability in the Hg concentrations between the first and second  
288 *in-situ* samplings (0 M and 13 M). Relative difference can be up to 400%, as was the case  
289 with the Idrija smeltery ( $1.4 \pm 0.15$  to  $6.0 \pm 0.26$   $\mu\text{g/g}$ ). This could be a consequence of the so-  
290 called ‘nugget effect,’ where a single unit of PBM or a droplet of elemental Hg present on a  
291 single lichen thalli might significantly increase the Hg concentration; this has been observed  
292 by Kocman et al. (2011).

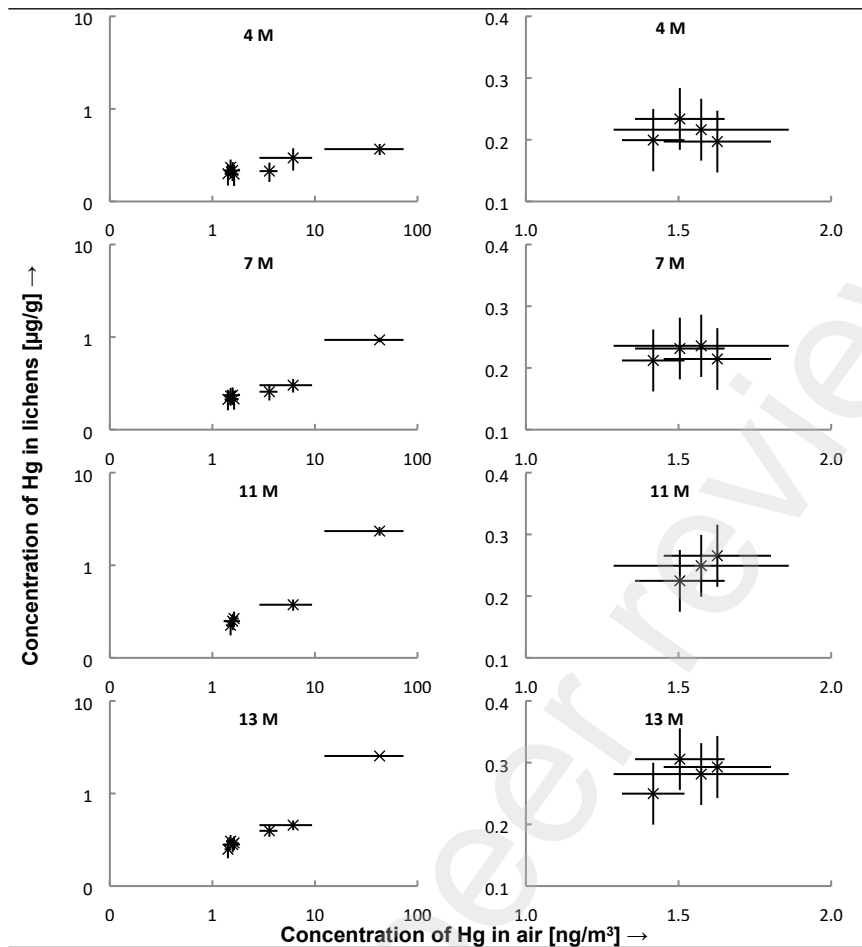


293  
294 **Figure 2: Relationship between the *in-situ* lichens and air concentration of Hg. The Anhovo**  
295 **area sites are expanded on the right. Air bars represent the natural variability, and the lichen**  
296 **bars represent the spread between the two samplings of the *in-situ* lichens (0 and 13 M).**

#### 297 4.1.1 Transplanted lichens

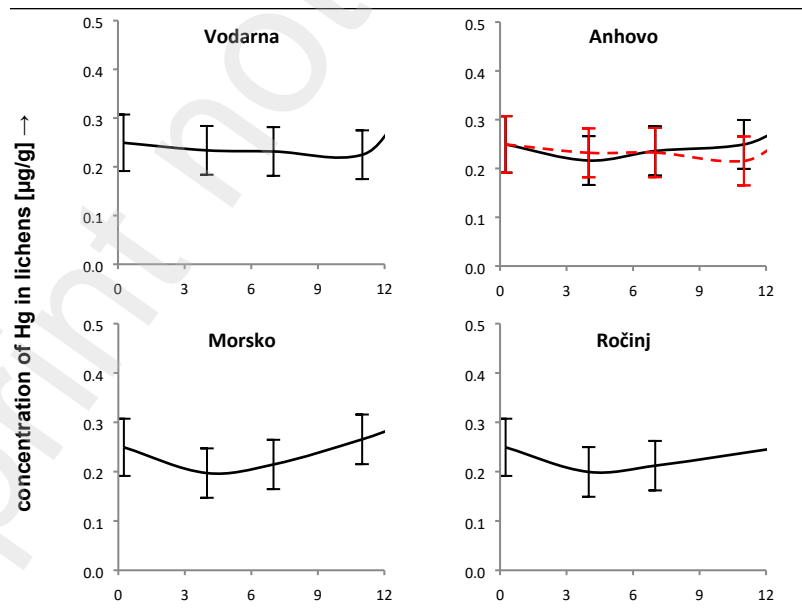
298 In Figure 4 Hg concentrations in the transplanted lichens are plotted against Hg  
299 concentrations in the air and over the exposure time at different sites.

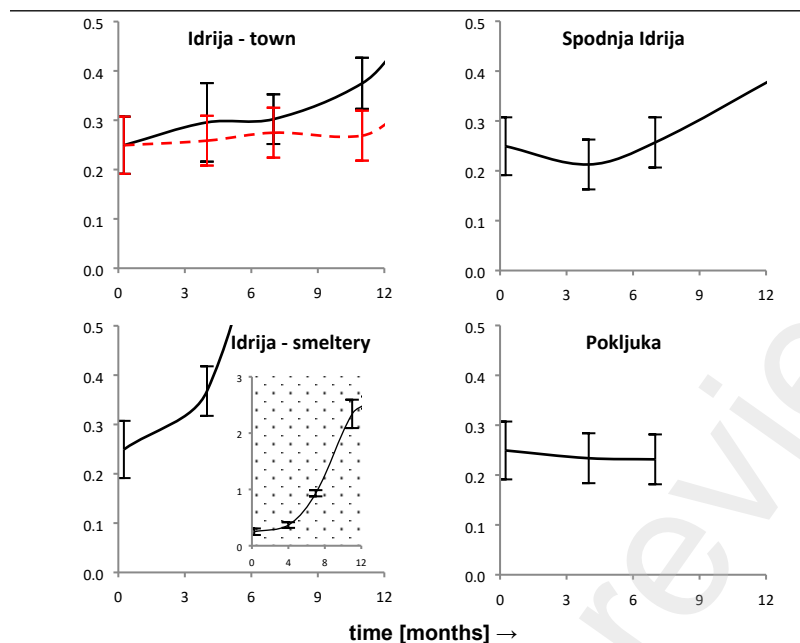
300



301  
302  
303  
304

**Figure 3: Relationship between transplanted lichens and air Hg concentrations for each sampling period. The Anhovo area sites are expanded on the right.**





305 **Figure 4: Graphs representing changes in Hg concentration in the transplanted lichens**  
 306 **(normally exposed indicated in black and covered indicated by dashed red lines). To cover the**  
 307 **whole concentration range an additional zoomed out graph is present at Idrija smeltery region.**  
 308

309 The Hg concentrations in lichens increased in the Idrija area and remained largely the same at  
 310 both Pokljuka and Anhovo. The highest increase was observed at the Idrija smeltery, while  
 311 the increase was lower at the other two sampling locations in Idrija. There seemed to be a  
 312 slight dip in concentration in spring (4 M) at all locations apart from the Idrija smeltery. The  
 313 reason for this is unknown.

314 Lichens protected from direct sunlight and dry and wet deposition gained Hg more slowly  
 315 than the openly exposed lichens in the town of Idrija (red dotted lines in Figure 4 for Idrija  
 316 town and Anhovo). This is also confirmed by the results obtained which are  $0.46 \pm 0.05 \mu\text{g/g}$   
 317 for covered lichens compared to  $0.31 \pm 0.05 \mu\text{g/g}$  for openly exposed lichens at the Anhovo;  
 318 study site. The differences between the shielded and open air-exposed lichens were likely the  
 319 consequence of the cover preventing some of the wet- and dry-deposited Hg from reaching  
 320 the shielded lichens.

321 The concentrations of Hg in air showed the same trend as the one observed in lichens, with  
 322 the highest concentrations at the Idrija smeltery and the lowest in Anhovo and Pokljuka.  
 323 Similar findings were already described by Mlakar et al. (2011). A relationship between the



324 average Hg concentrations in air and lichens can therefore be estimated. At sites with lower  
325 concentrations of Hg (1–2 ng/m<sup>3</sup>), correlation was not evident (Figures 2 and 3).

326 The Hg concentrations in air fluctuated significantly over time. At the Idrija smeltery, the  
327 concentrations ranged from 5.10 to 78.2 ng/m<sup>3</sup>. This could be the consequence of the changes  
328 in wind direction and speed between different measurements. At other locations, the Hg  
329 concentration was relatively similar. There were no discernible changes between the summer  
330 and winter Hg concentrations in Anhovo. In summer (July and August data), the average  
331 concentration of Hg was  $1.58 \pm 0.31$  ng/m<sup>3</sup>, while in the winter (November and December  
332 data), the average concentration of Hg was  $1.55 \pm 0.28$  ng/m<sup>3</sup>.

#### 333 **4.2 Hg isotope ratios**

334 Figure 4 represents the temporally sorted values for the mass-dependent and -independent  
335 fractionation presented by  $\delta^{202}\text{Hg}$  and  $\Delta^{199}\text{Hg}$ , respectively, for in-situ and transplanted  
336 lichens.

337 The  $\delta^{202}\text{Hg}$  in lichens ranged from -0.5 to -3.0 ‰, and the  $\Delta^{199}\text{Hg}$  from -0.0 to -0.5 ‰. This  
338 was in line with the previously published data (Barre et al., 2020; Blum et al., 2012; Carignan  
339 et al., 2009). The in-situ  $\delta^{202}\text{Hg}$  was the lowest in Pokljuka, with  $-3.1 \pm 0.6$  ‰ among the five  
340 samples measured there. In general, the Idrija samples had lower  $\delta^{202}\text{Hg}$  values than those  
341 from the Anhovo area ( $-2.0 \pm 0.3$  vs.  $-1.6 \pm 0.6$  ‰).

342 To evaluate the natural variability, the isotopic composition of the five lichens from Pokljuka  
343 was analysed. The standard deviation among them for  $\delta^{202}\text{Hg}$  was 0.3 ‰, and for  $\Delta^{199}\text{Hg}$ , it  
344 was 0.1 ‰. The variations among the locations that were relatively close to one another were  
345 also high. For example, in Anhovo and Vodarna, which are only a few hundred meters away  
346 from each other, the difference was 0.6 ‰. The highest  $\delta^{202}\text{Hg}$  for Anhovo was  $-2.0 \pm 0.2$  ‰,  
347 while at Vodarna, which is only a few hundred meters away; the highest  $\delta^{202}\text{Hg}$  was  $-1.4 \pm 0.2$   
348 ‰.

349 There was a major change in  $\delta^{202}\text{Hg}$  of the transplanted lichens during the time of their  
350 exposure. In winter, at the beginning and end of the exposure period, the  $\delta^{202}\text{Hg}$  values were  
351 the lowest, while in the summer, the highest values were observed. The average difference  
352 between summer and winter values for  $\delta^{202}\text{Hg}$  was 1.7 ‰. The  $\delta^{202}\text{Hg}$  values returned close to  
353 the starting values of the Pokljuka in-situ values and not to those found in the in-situ lichens  
354 at an individual site. This trend was observed at all sampling sites except the Idrija smeltery.  
355 There, the isotopic composition only continued to increase slightly throughout the whole year,  
356 ending close to the in-situ value. Covered lichens from the location in the town of Idrija  
357 showed a lesser response of  $\delta^{202}\text{Hg}$  than those that were exposed to open air (by 0.3 ‰).  
358 The  $\delta^{202}\text{Hg}$  was the lowest where the temperatures were the lowest and the portion of the year  
359 with below-freezing temperatures was the longest. This correlated well with the in-situ  
360 lichens from all locations. Pokljuka had the lowest temperatures, the most days below 0 °C,  
361 and the lowest  $\delta^{202}\text{Hg}$ , while the opposite held true for Idrija and Anhovo. Temperature,  
362 therefore, had an effect on the  $\delta^{202}\text{Hg}$  in the in-situ lichens.

363 The situation at the Idrija smeltery could be explained through the new rapidly accumulating  
364 Hg, as seen in the Hg concentration data. This was due to the proximity to the former  
365 smeltery. As described above, the concentration of GEM and PBM in air was higher than in  
366 any location. Any previous Hg accumulated on the lichen was overridden by this local Hg.  
367 The proportion of accumulated GEM versus PBM is not known, nor is the  $\delta^{202}\text{Hg}$  of either  
368 species. The world average for PBM is  $\approx -1.0$  ‰ according to Wang et al. (2021). Any new  
369 Hg deposited to the lichen would have to have  $\delta^{202}\text{H}$  higher than  $-3.0$  ‰. The similarity of the  
370 Hg isotopic composition of both the shielded and open air-exposed lichens indicated that the  
371 shade provided by the cover did not significantly influence the isotopic composition.

372 Some correlations can be made between the lichen  $\delta^{202}\text{Hg}$  and other environmental factors.  
373 The first is with temperatures, which followed the same trend of lowest in the winter and

374 highest in the summer. The other is with leaf cover. The leaves start growing in spring and  
375 fall off in autumn. As previously discussed, another correlation is with the concentrations of  
376 Hg in the forest air. When the leaves are growing, they take up Hg from the air (Jiskra et al.,  
377 2018) and deplete the forest air of Hg. Furthermore, the forest  $\delta^{202}\text{Hg}$  fluctuates in the exact  
378 same way as the highest values in summer (Fu et al., 2019). It must be considered that the  
379 range of change of  $\delta^{202}\text{Hg}$  described by Fu et al. (2019) is similar to that in lichens in this  
380 study, albeit from  $\approx 0$  to  $\approx 2\%$ . Thus, there was correlation but no proof for causation among  
381 these factors.

382 There are a few potential mechanisms that could affect the isotopic composition of the  
383 lichens. One is the fractionation during the uptake, as suggested first by Demers et al. (2013).  
384 This uptake is dependent on the biological activity, that is in lichens strongly governed by the  
385 temperature (Kappen et al., 1996). Therefore the process of uptake and fractionation is  
386 determined by the temperature. It is supposed that the lighter isotopes are preferably  
387 incorporated into the leaf surface, and same could be true of lichens. This would mean that  
388 during the time of greater biological activity in the summer, the lichens are fractionating the  
389 isotopes in a way that increases the  $\delta^{202}\text{Hg}$ . However, this is contrary to the observations in  
390 lichens in the present study.

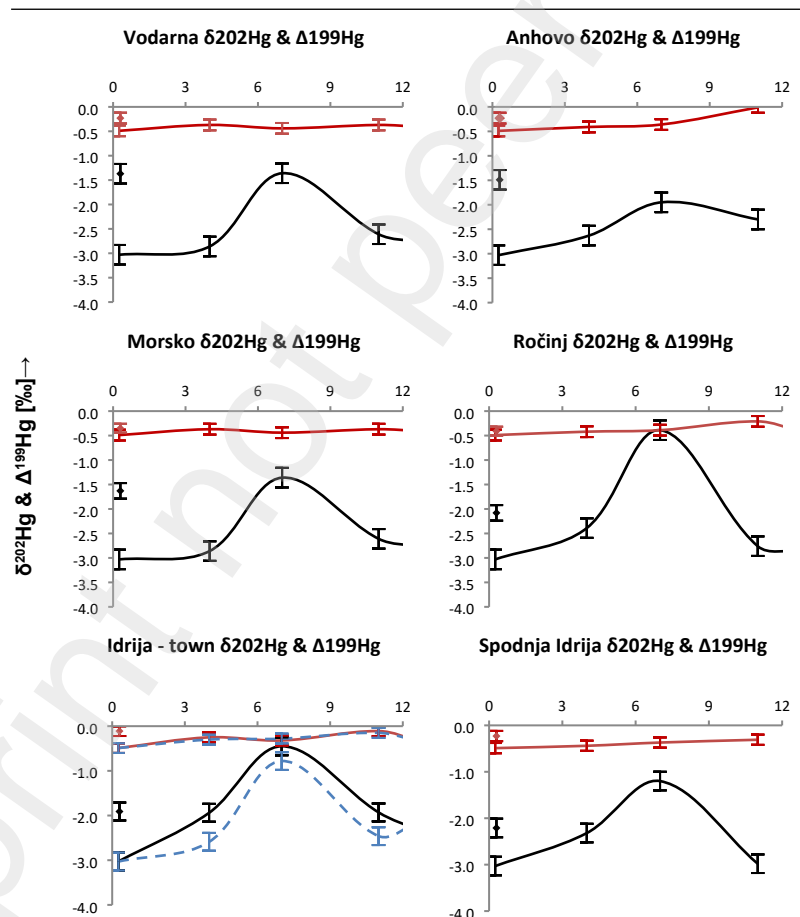
391 Another mechanism that supports some of these observations is the increased evaporation of  
392 lighter isotopes from the lichen thalli during the hotter summer months of the year, leaving  
393 the heavier behind and thereby increasing the  $\delta^{202}\text{Hg}$ . During evaporation experiments, a  
394 positive MDF shift of  $\approx 1.2\%$  for  $\delta^{202}\text{Hg}$  has been observed (Ghosh et al., 2013).

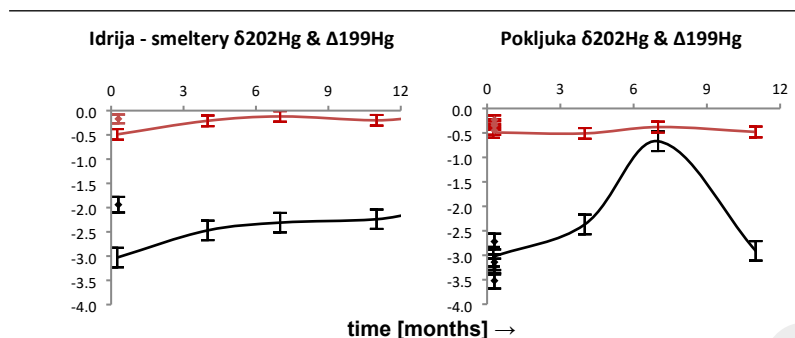
395 The  $\delta^{202}\text{Hg}$  in the lichens was likely a complex mix of these two mechanisms and potentially  
396 others. Since the concentration of Hg did not increase, there should also be some Hg leaving  
397 the lichen. It was peculiar that the  $\delta^{202}\text{Hg}$  value ended closer to the value from Pokljuka and  
398 not the values found in the in-situ lichens.

399 There was a slight trend of increase in  $\Delta^{199}\text{Hg}$  in the transplanted lichens in all locations (from  
400  $-0.5$  to  $-0.0$  ‰), but there were no discernible seasonal trends. The  $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$  ratio in the  
401 lichens was  $\approx 1.1$ . For evaporation, a  $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$  of 1.6 was observed in experiments by  
402 Ghosh et al. (2013). Therefore, the reactions on lichens are less likely to be directly connected  
403 to evaporation.

404 The trend of slight increase in  $\Delta^{199}\text{Hg}$  could be explained by the fact that the lichens in  
405 Pokljuka received higher amounts of light before they were collected and transplanted. After  
406 transplantation, some of the lichens in the bags had less exposure to light and therefore were  
407 not as subject to photo-reduction or inducing MIF and began to reflect  $\Delta^{199}\text{Hg}$  closer to  $0.0$ ‰.

408





409 **Figure 4: Graphs representing the  $\delta^{202}\text{Hg}$  (black) and  $\Delta^{199}\text{Hg}$  (red) in lichens over time. The**  
 410 **dotted blue lines represent the isotopic composition of the covered sample. The  $\blacklozenge$  marks**  
 411 **represent the in-situ lichens collected at the beginning of the sampling period (0 M).**

## 412 5 Conclusions

413 This study showed that lichens can be used as bio-monitors for air Hg concentration and/or  
 414 isotopic composition. There are, however, some constraints as to sensitivity of the approach.

415 It was shown that lichens can be effectively used for Hg monitoring in areas above  
 416 background concentrations, particularly in areas where Hg concentrations are emitted from a  
 417 point source and/or dispersed sources, such as contaminated soils. Both approaches, active  
 418 and passive biomonitoring provide similar results.

419 A trend in the change of  $\delta^{202}\text{Hg}$  throughout the four seasons has been observed. The drivers  
 420 behind the increase and decrease of  $\delta^{202}\text{Hg}$  are not known but are correlated with temperature  
 421 and leaf growth. The potential mechanisms could be linked to fractionation upon the uptake  
 422 of Hg and after its release. The exact mechanisms affecting the Hg isotopic composition of  
 423 the lichens are likely a complex mixture of Hg uptake, evaporation, and photo-reduction.

424 This, in turn, means that lichens cannot be viewed as a one-time probe into atmospheric Hg  
 425 but rather as part of a larger system that includes natural and anthropogenic sources that  
 426 change through time and interact with one another. In the case of heavily contaminated sites,  
 427 these trends are overridden due to the high Hg concentrations with a determined isotopic  
 428 composition.

429 This study affirmed the idea that bio-monitoring using lichens can be successfully applied in  
 430 regional and global monitoring as it offers cost effective approach, but overall, the protocols  
 431 should precisely defined or even better standardized.

432 **Author roles**

433 **Dominik Božič:** Methodology, Investigation, Data Curation, Writing – Original Draft,  
434 Visualization. **Igor Živkovič:** Methodology, Investigation. **Marta Jagodic Hudobivnik:**  
435 Investigation, **Jože Kotnik:** Investigation, **Amouroux David:** Investigation. **Marko Štok:**  
436 Methodology, Supervision, Writing – Review. **Milena Horvat:** Conceptualization, Writing –  
437 Review, Supervision, Project administration, Funding acquisition.

438 **Declaration of interests**

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

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