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Title	Lead concentrations and isotope ratios in blood, breastmilk and feces : contribution of both lactation and soil/dust exposure to infants in a lead
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Citation	Environmental pollution, 286, 117456 https://doi.org/10.1016/j.envpol.2021.117456
Issue Date	2021-10-01
Doc URL	https://hdl.handle.net/2115/90474
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Manuscript 20210518 Without Tracking.pdf

1 **Lead concentrations and isotope ratios in blood, breastmilk and feces:**

2 **contribution of both lactation and soil/dust exposure to infants in a lead mining**

3 **area, Kabwe, Zambia**

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Abstract

41

42   Lead (Pb) poses a serious public health concern. Breastmilk may be a possible

43   source of Pb exposure in infants, as Pb can be transferred from the maternal blood to

44   breastmilk. The present study was undertaken to determine the Pb exposure and the

45   contribution of lactation as one of the exposure pathways to infants in a Pb mining area,

46   Kabwe, Zambia. Blood, breastmilk and infants' feces were collected from 418 pairs of

47   infants and mothers. The Pb concentrations, isotope ratios in the samples, and

48   biochemistry in mothers' plasma were analyzed. The overall mean of blood lead levels

49   (BLLs) in infants and mothers were 18.0 and 11.3 μg/dL, respectively. High Pb

50   concentration in breastmilk (range: 0.4–51.9, mean: 5.3 μg/L) above the WHO

51   acceptable level between 2 and 5 μg/L were found and could be one of the sources of Pb

52   exposure in infants. The Pb isotope ratios in infants' feces were the most similar to Pb

53   ratios in the soil samples. The results suggest that infants are also exposed to Pb from

54   the environment. Pb exposure in infants through breastfeeding and soil ingestion could

55   potentially exceed daily intake of Pb which causes neurodevelopmental toxicity. In

56   contrast to the high BLLs in mothers, the plasma biochemical profiles of most analyzed

57   parameters were interestingly within, or close to, the standard reference values. Our data

58   suggest that environmental remediation is urgently needed to reduce the Pb exposure in

59   infants and mothers from the environment in Kabwe in parallel with chelation therapy.

60

61   **Keywords:** Lead poisoning, Infant, Mother, Breastmilk, Lead stable isotope

62

1. Introduction

Lead (Pb) poses a serious public health concern, accounting for 0.6% of the global burden of disease (WHO, 2010). Serious cases of Pb exposure have been reported in both developed and developing countries (Ajumobi et al., 2014; Haefliger et al., 2009; Ruckart et al., 2019). Lead poisoning causes various symptoms, including anemia, nephropathy, and death (Meyer et al., 2008). Children, especially infants are more vulnerable to Pb, compared to adults. To measure exposure to Pb, blood lead level (BLL) has been widely used. In the blood, Pb has a short half-life of 30–40 days (Barbosa et al., 2005). Even at low levels, Pb exposure can cause pediatric neurodevelopmental impairments, such as a reduction in intelligence quotient (IQ) (Canfield et al., 2003). Due to this, the blood Pb reference value for Pb exposure has been set to 5 µg/dL (CDC, 2019, 2012). A BLL above 45 µg/dL is considered the level where treatment is required (CDC, 2012, 2002; Needleman, 2004), and a BLL above 100 µg/dL is considered a fatal level in children, which causes serious clinical symptoms such as encephalopathy, even in adults (Meyer et al., 2008; NAS, 1972). The European Food Safety Authority (EFSA) observed that Pb dietary intake of 0.5 µg/kg body weight (bw)/day would be associated with developmental neurotoxicity in young children (EFSA, 2010).

Breastmilk is vital for infants, to ensure normal development and to prevent infectious diseases. However, breastmilk may be a possible source of Pb exposure in infants, as Pb can be transferred from the maternal blood to breastmilk. As a result, BLLs in mothers should be monitored to prevent Pb exposure in infants via breastfeeding. Therefore, to minimize Pb exposure in infants through breastmilk, WHO (1989) has set the acceptable level of breastmilk Pb concentration to be between 2 and 5

μg/L. Mothers with confirmed BLLs above 40 μg/dL should pump and discard their breastmilk (CDC, 2010).

Lead exposure primarily occurs via ingestion and inhalation, and can be traced to numerous sources, including battery recycling, gasoline, paint, as well as mining (Calabrese and Stanek, 1995; Meyer et al., 2008; Schoning et al., 1996; Yabe et al., 2010). Identifying the source of Pb exposure is important to prevent exposure. One such method for identifying sources is the use of Pb isotopic tracing (Komarek et al., 2008; Gulson, 2008). Lead is present in the environment as four main isotopes: ^{208}Pb (52%), ^{207}Pb (23%), ^{206}Pb (24%), and ^{204}Pb (1%) (Komárek et al., 2008). The compositions of these isotopes are not affected to a measurable extent by physicochemical fractionation processes (Bollhöfer and Rosman, 2001; Veysseyre et al., 2001).

The Zambian town of Kabwe accommodates a Pb-zinc (Zn) mining area which, up until its closure in 1994, was operated without adequate pollution laws to regulate mining emissions. Elevated BLLs and Pb concentrations in the feces and urine of children near the mine have been reported, all of which exceeded the 5 μg/dL blood Pb reference value (Yabe et al., 2018, 2015). Yabe et al. (2015) found that BLLs in children between the ages of one and two years old were higher compared with those in children between the ages of four and seven years old in Kabwe, as has been observed in many other studies. It is necessary to reduce and prevent Pb exposure in children. This is especially important in infants, who are more vulnerable to Pb poisoning. Moreover, a more recent study has revealed a high Pb exposure also in mothers, where approximately 5% of mothers were found to have a BLL above 45 μg/dL which indicated that treatment was required (Yabe et al., 2020, Nakata et al., 2021). The breastfeeding practices of mothers with high BLLs are a possible source of Pb exposure

111 for infants in Kabwe. However, the precise sources and routes of Pb exposure in infants

112 have not yet been determined. Furthermore, no clinical studies of Pb poisoning have

113 been done in Kabwe, despite high BLLs being reported in the local people. Some

114 previous studies have reported that metallothionein concentrations, which is a cysteine-

115 rich protein that binds and detoxifies toxic metals, increase as metal concentrations in

116 the blood increase (Bizoń and Milnerowicz, 2014; Kowalska et al., 2015).

117 Metallothionein may therefore play an important role in reducing Pb toxicity in the

118 people of Kabwe.

119   The current study aimed to determine the Pb exposure and the contribution of

120 lactation as one of the exposure pathways to infants in a lead mining area, Kabwe,

121 Zambia. Pb concentrations in mothers' breastmilk, infants' feces, and both infants' and

122 mothers' blood, were analyzed. Daily intake of Pb in infants through breastfeeding and

123 soil ingestion was calculated to estimate the burden of routes of Pb exposure. The Pb

124 isotope ratios in samples were analyzed in a limited number of samples to determine the

125 source of Pb exposure. Furthermore, a plasma biochemical analysis including

126 metallothionein concentrations was conducted in the mothers to evaluate the health

127 impact of Pb exposure in this population.

128

129 **2. Materials and Methods**

130 **2.1 Sampling Sites**

131   The town of Kabwe is located at about 28°26′E and 14°27′S, and is the

132 provincial capital of Zambia's Central Province. It is the fourth largest town in Zambia,

133 with a population of about 230,000 inhabitants and an area of 1547 km². In Kabwe,

134 metallic residues from abandoned tailings and waste stored in the mine have continued

135 to serve as potential sources of metal pollution even after the closure of the mine. Dust

136 emanates from the mine dumps, and residents in townships close to the mine may be

137 exposed to high levels of Pb in contaminated dust and soil.

138 　　The present study was conducted at health centers in four sites near the mine

139 (Kasanda, Makululu, Chowa and Katondo) and one site far from the mine (Bwacha) in

140 the rainy season from January to March of 2017 (Fig. 1). Kasanda, Makululu, Chowa,

141 Katondo, and Bwacha health centers were located about 0.9, 2.6, 1.4, 4.5, and 6.1 km

142 from the mine, respectively. Kasanda and Makululu are located on the western side of

143 the mine and in the direction of prevailing winds.
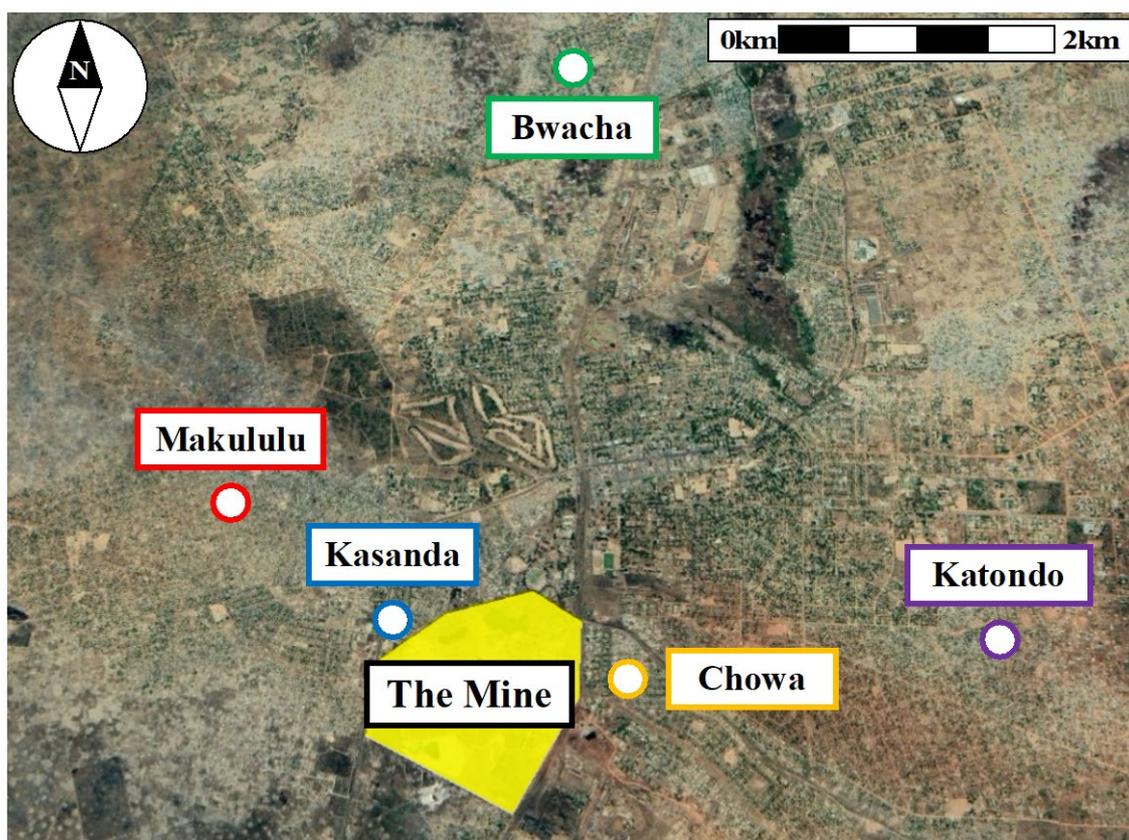
144

145 Fig. 1. Map of the sampling sites in Kabwe, Zambia (image modified from Google

146 Earth).

147

148 **2.2 Sampling**

149   This study was approved by the University of Zambia Research Ethics

150 Committee (UNZAREC; REF. No. 012-04-16). Further approvals were granted by the

151 Ministry of Health through the Zambia National Health Research Ethics Board and the

152 Kabwe District Health Office.

153   A sensitization campaign about the research activities was conducted by

154 community health workers before sampling in their catchment areas around the health

155 centers. Mothers and guardians were encouraged to participate in the study, and were

156 asked to take their breastfed infants under the age of 1 year and 6 months to the selected

157 health centers for sample collection. Only infants with mothers/guardians that willingly

158 agreed to participate and signed the informed consent were included in the present study.

159 After informed and written consent were obtained from the mothers/guardians, blood

160 samples were collected as described by Yabe et al. (2015). The mothers/guardians were

161 also interviewed to obtain necessary personal details about themselves and their infants,

162 such as age and sex. Sample collection and questionnaire administration were

163 undertaken by certified local nurses. In accordance with ethical requirements,

164 confidentiality was upheld in the study.

165   Blood samples up to 2 mL and 5 mL were collected from the cephalic veins of

166 each infant and mother, respectively, and were placed into heparinized blood collection

167 tubes. Breastmilk samples from mothers were collected in clean sample cups by gentle

168 compression of the breast and transferred to 2 mL sample tubes for storage and

169 transportation. To avoid contamination, the hands as well as venipuncture and breast

170 sites were cleaned and sanitized with an ethanol swab before the sample collection.

171 Plasma samples were separated only from the mothers' blood after centrifugation. For

172   infants' fecal samples, mothers/guardians were handed 30 mL stool containers equipped

173   with scoops and were instructed to scoop feces into the container from a used diaper in

174   the morning of the following day. Household soil samples were collected in June 2016

175   from Kasanda (n = 12) and Makululu (n = 20) as a reference of environmental samples

176   for Pb stable isotope analysis.

177   The processed samples were transported to the laboratory of The University of

178   Zambia, School of Veterinary Medicine, Zambia, and stored at −20 °C. The material

179   transfer agreement (MTA) for human samples from the Zambia National Health

180   Research Ethics Committee (approval No. E00417) was obtained before transportation.

181   Similarly, the phytosanitary certificate from plant quarantine and phytosanitary service,

182   Zambia Ministry of Agriculture, and import permission by plant protection station,

183   Japanese Ministry of Agriculture, Forestry and Fisheries (approval No. 28–313) was

184   also granted for soil samples. The human samples were transported in temperature-

185   controlled boxes with ice packs, and the soil samples in temperature-controlled boxes,

186   for further analysis at Hokkaido University, Japan.

187

188   **2.3 Pb and Metal Concentration Analysis**

189   Pb and other metals (iron (Fe), copper (Cu), Zn, and silver (Ag)) were extracted

190   from the samples. Thawed fecal and bulk soil samples were weighed on heat-resistant

191   tissue drying plates and dried for 48 h in a tissue drying oven at 60 °C, whereas whole

192   blood and breastmilk samples were only thawed. Each blood and breastmilk sample

193   (1mL) and 50 mg of each dried fecal and soil sample were analyzed. Detailed method

194   was described in the supplementary materials.

195

196 **2.4 Calculation of Daily Intake of Pb in Infants through Breastfeeding and Soil**

197 **Ingestion**

198   Daily intake of Pb in infants through breastfeeding and soil ingestion was

199 calculated. The calculation was conducted using the maximum, mean, and minimum Pb

200 concentrations in breastmilk and soil samples in the present study using the formulas

201 below. The amounts of daily breastmilk intake and soil ingestion in infants were set as

202 0.78 L/day and 30 mg/day reported by Costa et al. (2010) and United States

203 Environmental Protection Agency (US EPA, 2011), respectively.

204

205 *Daily intake of Pb = (Pb concentrations in breastmilk × Amount of daily breastmilk*

206 *intake) + (Pb concentrations in soil × amount of daily soil ingestion)*

207

208   In the present study, 0.5 μg/kg bw/day was used as the reference value as the

209 limit of daily intake of Pb for infants (EFSA, 2010).

210

211 **2.5 Stable Pb Isotope Analysis**

212   Only 26 sample sets with high Pb concentrations of infants' and mothers' blood,

213 breastmilk, and infants' feces from Kasanda and Makululu, were chosen and analyzed

214 during Pb isotope analysis as well as 32 soil samples. As mentioned above, these soil

215 samples were collected at the preliminary survey in 2016. The sampling area between

216 blood and soil are same, but not all the locations were exactly from the same household.

217 The sample dissolution procedure was similar to previously described methods

218 (Kuritani and Nakamura, 2002; Nakayama et al., 2019). The detailed methods of the

219 high precision isotope analysis were described in the supplementary materials. We have

220 measured duplicates of breastmilk samples and the results of Pb isotope ratios had the

221 similar trends. As sample amounts decrease, the uncertainty of ion beam intensity

222 measurements for the minor isotope ^{204}Pb tends to increase, and the accuracy and

223 precision of the isotopic ratios involving ^{204}Pb decrease (^{204}Pb error; Hamelin et al.,

224 1985). Therefore, the isotopic composition of Pb is commonly expressed as ratios of

225 ^{208}Pb, ^{207}Pb, and ^{206}Pb. However, since normalization to ^{204}Pb yields the largest

226 variability between reservoirs (Komárek et al., 2008), we included ^{204}Pb data with

227 correction (Kuritani et al., 2003, 2002). This was done in order to observe the detail

228 variability of isotope ratio among the breastmilk, blood, feces and soil samples in this

229 study.

230

231 **2.6 Plasma Biochemical Analysis and Metallothionein ELISA**

232 A conventional blood biochemical analyzer (FUJI DRICHEM 7000V;

233 FUJIFILM corporation, Tokyo, Japan) was used to analyze the concentrations of

234 alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate

235 aminotransferase (AST), gamma glutamyl transpeptidase (GGT), lactase dehydrogenase

236 (LDH), total bilirubin (T-Bil), total protein (TP), albumin (Alb), blood urea nitrogen

237 (BUN), creatinine (Cre), and urea acid (UA) in mothers' plasma samples.

238 Metallothionein in mothers' plasma samples was measured by ELISA using an antibody

239 against iso-Metallothionein I and II (Metallothionein ELISA kit; Frontier Institute Co.,

240 Ltd., Hokkaido, Japan). The standard reference ranges for each parameter in humans

241 were provided by the kit manufacturers.

242

243   **2.7 Statistical Analysis**

244   All data from the experiments and questionnaires were combined into a single

245   electronic database and checked for accuracy and outliers. All statistical analyses were

246   performed at a significance level of $p < 0.05$ using JMP 13.1.0 (SAS Institute, USA).

247   Mean values were indicated in addition to standard deviation (SD) values. The

248   collinearity between factors was analyzed using Spearman's rank correlation test. A

249   Steel–Dwass multiple comparisons test was used to compare the differences between

250   the factors among areas and samples.

251

3. Results

3.1 Characteristics of the Infants and Mothers

A total of 418 pairs of infants and mothers participated in this study. Of these, 333 participants came from four sites near the mine, and 85 came from Bwacha, which is located about 6 km from the mine (Table 1; Fig. 1). None of the infants and mothers had overt signs of Pb poisoning. Infants from Chowa were younger than those from other areas, except for Bwacha ($p < 0.05$). In regard to height, infants from Chowa were significantly shorter and infants from Katondo were significantly taller than infants from other areas ($p < 0.05$). Body weight of the infants from Chowa was significantly lower than that of the infants from other areas ($p < 0.05$).

The overall height of boys was greater than that of girls overall ($p < 0.01$), with boys found to be significantly taller in the four areas near the mine considered together ($p < 0.01$) and in Makululu alone ($p < 0.01$, Supplementary Table S3). In Katondo, the height of boys tended to be greater than that of girls, but no significant difference was recorded ($p = 0.09$). The weight of boys was significantly higher than that of girls when considering all infants ($p < 0.01$), and for infants from near the mine ($p < 0.01$), and infants in Kasanda ($p < 0.05$), Makululu ($p < 0.01$), Katondo ($p < 0.01$), and Bwacha ($p < 0.045$) alone.

270   Table 1. General characteristics of infants and mothers, as well as Pb concentrations in blood, breastmilk and fecal samples in Kabwe,

271   Zambia; mean ± SD values (sample size, minimum–maximum).

Area (N)	Sex of infants, boy:girl	Age of infants, months	Height, Cm	Weight, kg	Age of mothers, years	BLLs in infants, μg/dL	BLLs in mothers, μg/dL	Pb in breastmilk, μg/L	Pb in infants' feces, mg/kg dry weight
Overall (418)	221:197	7.1 ± 3.8 (417, 0.1–16.8)	64.2 ± 7.7 (361, 45.0–95.0)	7.3 ± 1.7 (411, 1.9–12.1)	26.1 ± 6.5 (412, 16.3–46.1)	18.0 ± 18.1 [A] (406, 0.8–93.4)	11.3 ± 9.2 [B] (417, 1.5–82.6)	5.3 ± 7.0 [C] (407, 0.4–51.9)	39.2 ± 217.7 [D] (212, 0.08–3002.7)
Near the mine (333)	171:162	7.2 ± 4.0 (332, 0.1–16.8)	64.5 ± 8.0 (280, 45.0–95.0)	7.2 ± 1.7 (329, 1.9–12.1)	26.1 ± 6.6 (329, 16.3–46.1)	21.4 ± 18.9 [**, A] (321, 1.6–93.4)	13.0 ± 9.5 [**, B] (333, 1.9–82.6)	6.1 ± 7.5 [**, C] (324, 0.4–51.9)	49.2 ± 248.0 [**, D] (162, 0.09–3002.7)
Kasanda (82)	43:39	7.3 ± 3.8 [a] (82, 0.6–16.7)	64.5 ± 8.4 [a] (74, 49.0–95.0)	7.6 ± 1.5 [a] (79, 4.0–12.1)	27.1 ± 6.7 (82, 17.4–45.4)	24.8 ± 20.9 [a, A] (80, 3.7–93.4)	15.8 ± 10.6 [a, B] (82, 2.3–82.6)	9.2 ± 8.9 [a, C] (79, 1.9–50.4)	38.8 ± 88.9 [ab, D] (28, 0.6–451.0)
Makululu (102)	44:58	7.7 ± 3.7 [a] (102, 1.5–16.7)	63.0 ± 7.0 [a] (102, 45.0–85.0)	7.4 ± 1.4 [a] (101, 4.1–11.9)	26.2 ± 7.0 (102, 16.6–45.9)	30.8 ± 19.4 [b, A] (102, 5.6–82.7)	17.6 ± 10.1 [a, B] (102, 3.3–67.3)	7.1 ± 7.1 [ab, C] (100, 1.1–40.2)	82.4 ± 362.7 [a, D] (69, 1.1–3002.7)
Chowa (58)	35:23	5.3 ± 3.9 [b] (58, 0.1–15.2)	56.2 ± 7.1 [b] (13, 49.0–67.0)	5.6 ± 1.4 [b] (58, 3.5–8.7)	26.9 ± 6.3 (58, 17.6–41.8)	15.7 ± 15.5 [c, A] (49, 3.2–62.3)	11.2 ± 6.1 [b, A] (58, 3.0–36.2)	5.6 ± 6.5 [b, B] (57, 1.4–42.1)	33.7 ± 139.4 [bc, C] (31, 0.09–780.4)
Katondo (91)	49:42	7.7 ± 4.3 [a] (90, 0.9–16.8)	67.5 ± 7.6 [c] (91, 48.0–94.0)	7.8 ± 1.7 [a] (91, 1.9–11.1)	24.6 ± 5.9 (87, 16.3–46.1)	10.9 ± 9.9 [c, A] (90, 1.6–51.0)	6.3 ± 3.5 [c, B] (91, 1.9–21.6)	2.3 ± 5.5 [c, C] (88, 0.4–51.9)	4.4 ± 5.1 [c, D] (34, 0.1–19.4)
Bwacha (85)	50:35	6.9 ± 3.2 [ab] (85, 1.6–13.5)	63.0 ± 6.6 [a] (81, 51.0–86.0)	7.6 ± 1.6 [a] (82, 4.6–11.4)	26.2 ± 5.9 (83, 16.6–43.3)	5.2 ± 4.1 [d, A] (85, 0.8–22.0)	4.7 ± 3.4 [d, A] (84, 1.5–23.1)	2.3 ± 2.9 [c, B] (83, 0.5–17.9)	6.9 ± 27.6 [d, C] (50, 0.08–184.0)

272   Note: ** indicates a significant difference ($p < 0.01$) between sites near the mine and Bwacha. Various small letters indicate a significant

273   difference among areas ($p < 0.05$). Various capital letters indicate a significant difference among infants' and mothers' blood, breastmilk,

274   and infants' feces ($p < 0.05$).

3.2 Pb and Other Metals Concentrations in Blood, Breastmilk, Infants' Feces, and Soil.

The overall mean of Pb concentrations in the soil samples (n = 32) was 1048 ± 1470 mg/kg (dry weight) and ranged from 346 to 6327 mg/kg.

The overall mean values of BLLs in infants and mothers were 18.0 ± 18.1 and 11.3 ± 9.2 µg/dL, respectively (Table 1 and Fig. 2a). We found 76.8% of infants (312/406) and 73.6% of mothers (307/417) had BLLs above the reference value for Pb exposure (5 µg/dL; CDC, 2019, 2012). Moreover, BLLs in 8.9% of infants (36/406) and 1.2% of mothers (5/417) were above 45 µg/dL, the recommended threshold BLL for chelation therapy (Meyer, 2008). No infants or mothers had BLLs above the lethal level for Pb exposure (100 µg/dL), however, the highest BLL in an infant in the present study was 93.4 µg/dL. The overall mean of Pb concentrations in breastmilk and infants' feces were 5.3 ± 7.0 µg/L and 39.2 ± 217.7 mg/kg (dry weight), respectively (Fig. 2b and 2c). Overall, 30.0% of breastmilk samples (122/407) had Pb concentrations of more than 5 µg/L, which is above the accepted level for breastfeeding (WHO 1989).

There were significant differences in Pb concentrations among sample types in the samples from all sites: infants' feces > infants' blood > mothers' blood > breastmilk ($p < 0.05$). Among the samples from Chowa and Bwacha, there were no significant differences in Pb concentrations between infants' and mothers' blood. Pb concentrations in infants' blood were 1.8 ± 1.5 and 60.2 ± 67.6 times higher than those in the mothers' blood and Pb concentrations in breastmilk, respectively (Supplementary Table S4). On the other hand, Pb concentrations in infants' blood were 5.3 ± 7.4% of the Pb concentrations in infants' feces.
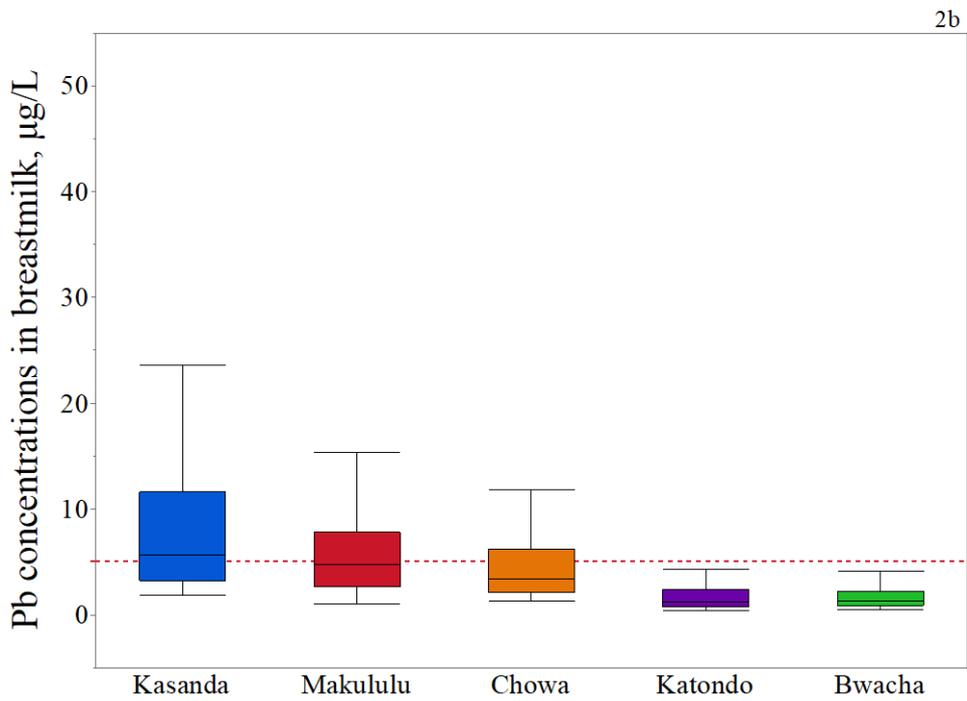
298   Pb concentrations in infants' and mothers' blood, breastmilk, and infants' feces

299   from sites near the mine (Kasanda, Makululu, Chowa, and Katondo) were significantly

300   higher than the concentrations in samples from Bwacha. Among sites, the mean of Pb

301   concentrations in each sample type from Makululu were the highest, except in

302   breastmilk. Pb concentrations in samples from Bwacha were significantly lower than

303   those from other sites ($p < 0.05$), except in breastmilk.

304   BLLs in boys were significantly higher than those in girls ($p = 0.04$) in

305   Makululu, and higher in Bwacha ($p = 0.06$, Supplementary Table S5). Pb concentrations

306   in infants' feces of boys were significantly higher than those of girls in Chowa ($p <$

307   0.01). The same trend was found in all infants, although the association was not

308   statistically significant ($p = 0.08$).

309   Supplementary Tables S6, S7, S8, and S9 show Fe, Cu, Zn, and Ag

310   concentrations in the samples, respectively. Fe concentrations in infants' blood and Fe,

311   Cu, Zn, and Ag concentrations in mothers' blood from sites near the mine were

312   significantly lower than the concentrations in samples from Bwacha. On the other hand,

313   Fe concentrations in breastmilk from sites near the mine were significantly higher than

314   the concentrations in samples from Bwacha.

315

316

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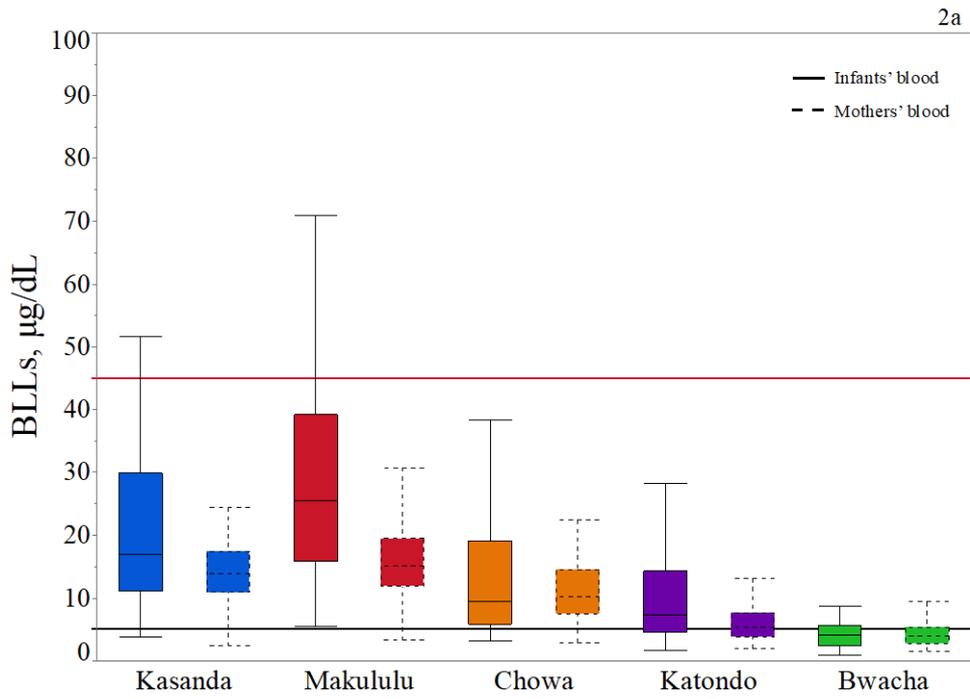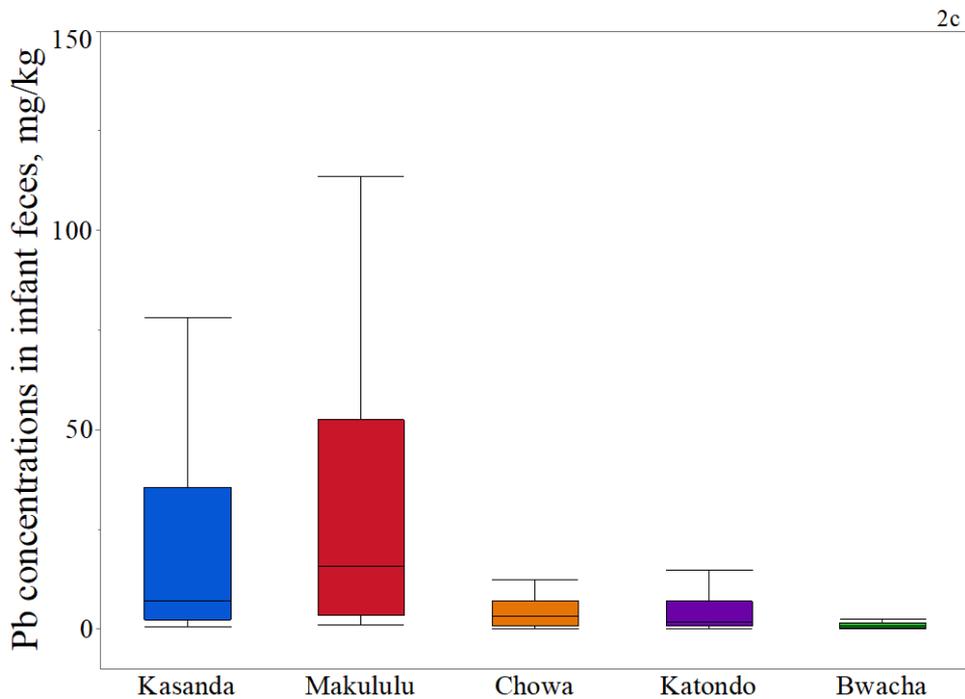
Fig. 2. BLLs in infants and mothers (2a), Pb concentrations in breastmilk (2b), and infants' feces (2c) among areas. The blue, red, orange, violet, and green colors indicate samples from Kasanda, Makululu, Chowa, Katondo, and Bwacha, respectively. Solid and dot box plots in Fig. 2a indicate BLLs in infants and mothers, respectively. Red and black lines in Fig. 2a indicate 45 and 5 μg/dL, respectively. The red dotted line in Fig 2b indicates the 5 μg/L level.

3.3 Relationships among Samples and Factors

In the samples from all areas, significant positive correlations existed among infants in the factors of age, height, and weight (Table 2, $p < 0.001$). There were significant positive correlations of Pb concentrations among all sample types ($p < 0.001$, Supplementary Fig. S2). BLLs in infants and Pb concentrations in infants' feces had a

331 significant positive correlation with the age (Supplementary Fig. S3), height, and weight

332 of infants ($p < 0.001$).

333   In all samples from sites near the mine, the same trend was found

334 (Supplementary Table S10). Moreover, the height of infants had a significant negative

335 correlation with BLLs in mothers ($p < 0.01$, $\rho = -0.19$) and Pb concentrations in

336 breastmilk ($p < 0.05$, $\rho = -0.13$). In the samples from Bwacha, the same trend was

337 found as in the samples from all other sites, except for the relationship between Pb

338 concentrations in breastmilk and other samples, and the BLLs in mothers and Pb

339 concentrations in infants' feces (Supplementary Table S11). Supplementary Tables S12,

340 13, and 14 show the relationships among Pb and other metals in the samples from all

341 areas, from sites near the mine, and Bwacha, respectively. In the samples from all areas,

342 Pb concentrations in infants' blood significantly increased as Cu ($p < 0.001$, $\rho = 0.34$)

343 and Ag ($p < 0.001$, $\rho = 0.28$) concentrations in infants' blood. There were significant

344 negative correlations between Pb and Fe concentrations in infants' blood as well as in

345 mothers' blood.

346 Table 2. Correlation coefficients (R^2) among factors and Pb concentrations in samples in all infants and mothers in the present study.

	Age of infants	Height	Weight	Age of mothers	BLLs in infants	BLLs in mothers	Pb in breastmilk	Pb in infants' feces
Age of infants		0.64***	0.59***	NS	0.46***	NS	NS	0.44***
Height			0.76***	NS	0.36***	NS	NS	0.27***
Weight				NS	0.32***	NS	NS	0.28***
Age of mothers					NS	NS	NS	NS
BLLs in infants						0.68***	0.52***	0.83***
BLLs in mothers							0.58***	0.57***
Pb in breastmilk								0.46***
Pb in infants' feces								

347 Note: *** indicates $p < 0.001$. BLL, blood lead level; NS, not significant.

3.4 Daily Intake of Pb in Infants through Breastfeeding and Soil Ingestion

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349   Table 3 shows the results of daily intake of Pb in infants through breastfeeding

350   and soil ingestion. The results ranged from 0.3 to 40.5 µg/day through breastfeeding and

351   from 10.4 to 189.8 µg/day through soil ingestion depending on Pb concentrations in the

352   samples. Daily intake of Pb through combined breastfeeding and soil ingestion ranged

353   from 10.7 to 230.3 µg/day. The reference value of daily intake of Pb which would be

354   associated with developmental neurotoxicity in infants in the present study was 3.7

355   µg/day (the mean bw: 7.3 kg) and ranged from 1.0 µg/day (the minimum bw: 1.9 kg) to

356   6.1 µg/day (the maximum bw: 12.1) based on the EFSA's reference value (0.5 µg/kg

357   bw/day) (EFSA, 2010). The minimum of calculated daily intake of Pb even only

358   through soil ingestion exceeded the maximum of the limit.

359

360

361   Table 3. Sum of possible daily intake of Pb (µg/day) through breastfeeding and soil ingestion.

			Daily intake of Pb through soil ingestion (30 mg/day)		
			Maximum Pb (6326.7 mg/kg) 189.8 µg/day	Mean Pb (1047.6 mg/kg) 31.4 µg/day	Minimum Pb (345.7 mg/kg) 10.4 µg/day
Daily intake of Pb through breastmilk (0.78 L/day)	Maximum Pb (51.9 µg/L)	40.5 µg/day	230.3	71.9	50.8
	Mean Pb (5.3 µg/L)	4.1 µg/day	193.9	35.5	14.5
	Minimum Pb (0.4 µg//L)	0.3 µg/day	190.1	31.8	10.7

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371   Note: The reference value of daily intake Pb in the present study was 3.7 µg (the mean bw: 7.3 kg) ranged from 1.0 µg (the minimum

372   bw: 1.9 kg) to 6.1 µg (the maximum bw: 12.1) based on the EFSA's reference value (0.5 µg/kg bw/day) (EFSA, 2010).

373

374 **3.5 Pb Isotope Ratio Analysis**

375   Table 4 shows the mean values of the $^{208}Pb/^{206}Pb$, $^{207}Pb/^{206}Pb$, $^{208}Pb/^{204}Pb$, and

376 $^{207}Pb/^{204}Pb$ ratios in the samples. The $^{208}Pb/^{206}Pb$ Pb isotope ratios in mothers' blood

377 (2.127 ± 0.006) were significantly different from those in infants' blood and feces and

378 the soil $(2.129 \pm 0.006, 2.130 \pm 0.004,$ and $2.131 \pm 0.002,$ respectively, $p < 0.05$). On

379 the other hand, there was no significant difference in $^{207}Pb/^{206}Pb$ ratios between infants'

380 and mothers' blood. Both $^{208}Pb/^{204}Pb$, and $^{207}Pb/^{204}Pb$ ratios in mothers' blood were

381 significantly different from those in other samples ($p < 0.05$), except for those in

382 breastmilk. Pb isotope ratios in soil samples were similar to those reported for Kabwe

383 galena (Kamona et al., 1999).

384   Pb isotope ratios in infants' and mothers' blood, and infants' feces were closer

385 to those reported for Kabwe galena (Kamona et al., 1999) as the reciprocal of Pb

386 concentrations decreased (Supplementary Fig. S4).

387   The $^{208}Pb/^{204}Pb$ and $^{207}Pb/^{204}Pb$ ratios (Fig. 3b) clearly show trends of samples

388 compared to $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ ratios (Fig. 3a and Supplementary Fig. S5).

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391 Table 4. Mean $\pm$ SD values of the Pb isotope ratios in different samples from Kasanda
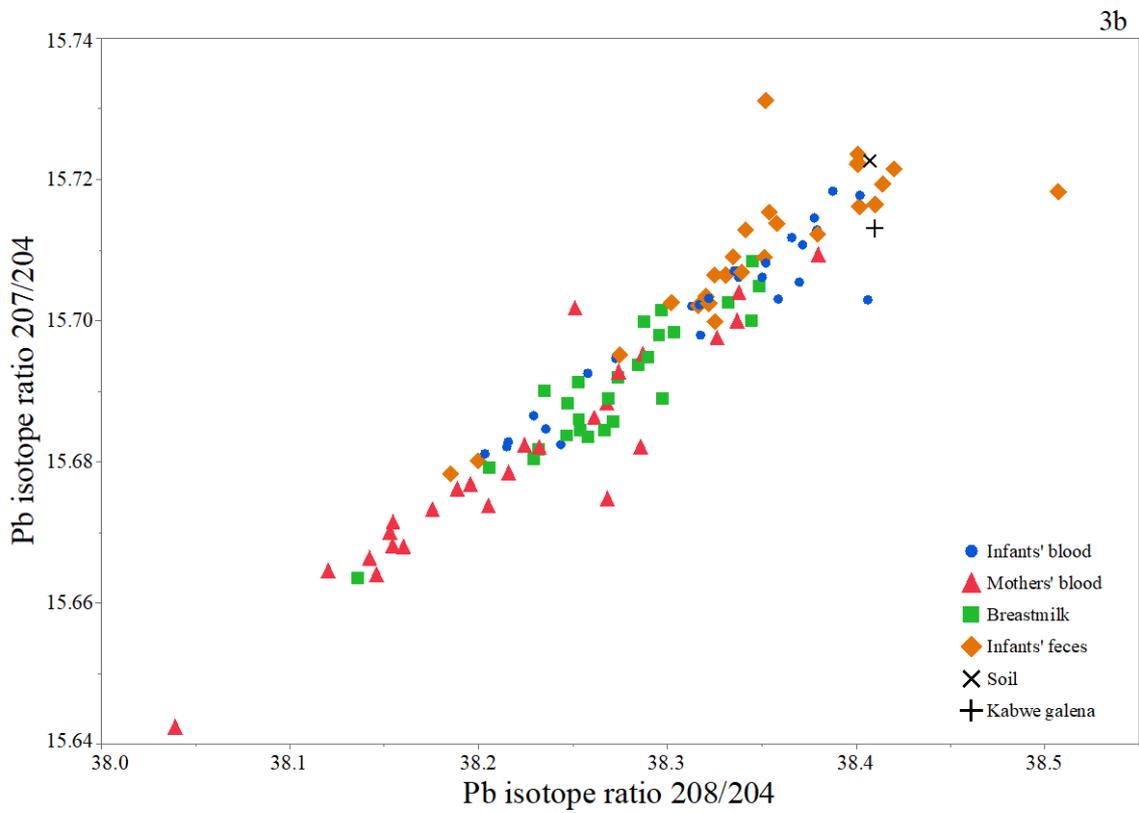392 and Makululu.

Samples (N)	$^{208}Pb/^{206}Pb$	$^{207}Pb/^{206}Pb$	$^{208}Pb/^{204}Pb$	$^{207}Pb/^{204}Pb$	$^{206}Pb/^{204}Pb$
Infants' blood (26)	2.129 ± 0.006 [ac]	0.8724 ± 0.003 [ac]	38.271 ± 0.063 [acd]	15.691 ± 0.012 [a]	17.997 ± 0.062 [ab]
Mothers' blood (26)	2.127 ± 0.006 [b]	0.8726 ± 0.002 [a]	38.223 ± 0.080 [bc]	15.680 ± 0.015 [b]	17.970 ± 0.059 [a]
Breastmilk (26)	2.129 ± 0.001 [bc]	0.8729 ± 0.0005 [b]	38.271 ± 0.046 [c]	15.691 ± 0.010 [b]	17.972 ± 0.018 [a]
Infants' feces (26)	2.130 ± 0.004 [ac]	0.8725 ± 0.002 [c]	38.349 ± 0.067 [d]	15.709 ± 0.012 [a]	18.005 ± 0.048 [b]
Soil (32)	2.131 ± 0.002 [a]	0.8724 ± 0.001 [d]	38.407 ± 0.036 [e]	15.723 ± 0.005 [c]	18.022 ± 0.027 [c]
Kabwe galena (Kamona et al., 1999)	2.134 ± 0.0009	0.8731 ± 0.0003	38.410 ± 0.033	15.713 ± 0.010	17.997 ± 0.007

393 Note: Various small letters indicate a significant difference among areas ($p < 0.05$).

394   Note: The number of significant digits in each ratio value were adjusted with those of

395   the reference (Kamona et al., 1999)

3a

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3b

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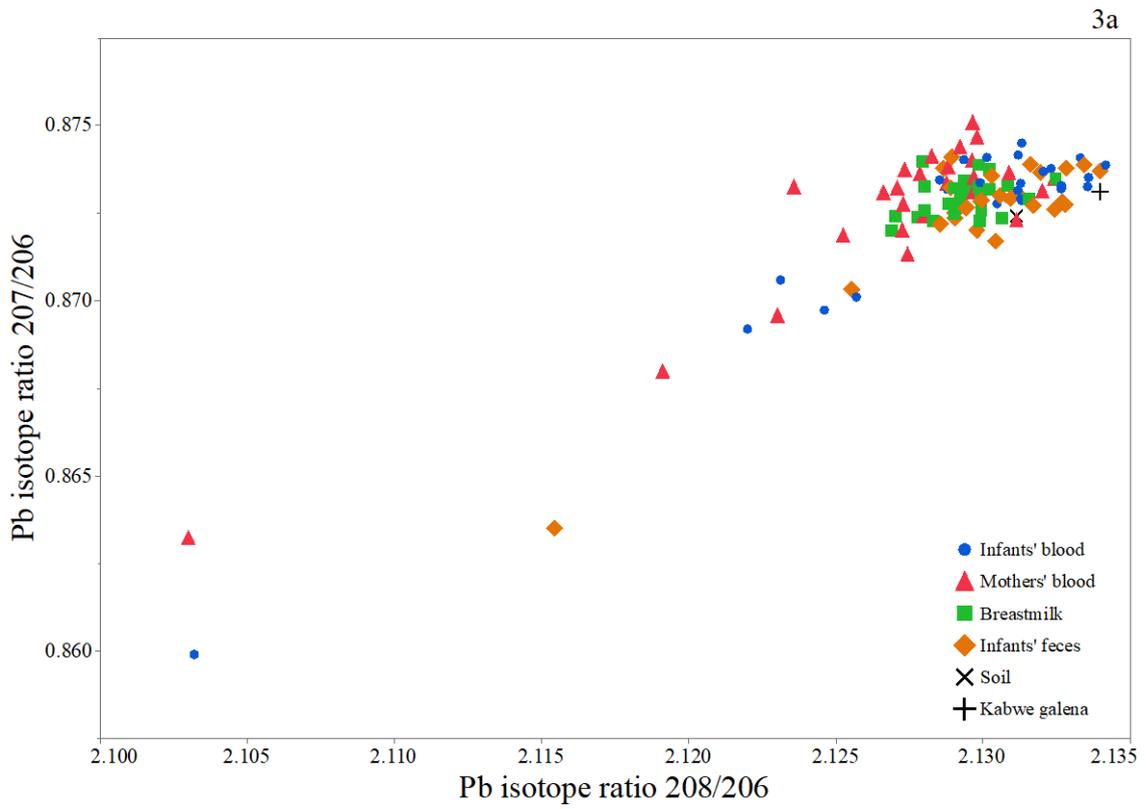
Fig. 3. Pb isotope ratios of individual sample (3a: $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ and 3b:

$^{208}Pb/^{204}Pb$ and $^{207}Pb/^{204}Pb$) in different samples from Kasanda and Makululu. The blue

circle, red triangle, green square, orange rhombus, and black cross markers indicate

infants' blood, mothers' blood, breastmilk, infants' fecal, and soil samples, respectively.

The reference value of Kabwe galena was obtained from a report by Kamona et al.

(1999) and is indicated by a plus sign.

3.6 Plasma Biochemical Analysis

Supplementary Table S15 shows the mean values of the plasma biochemical

analysis on mothers' plasma. The mean values of all parameters in mothers were within

the reference range or slightly higher, except for ALP. The ALT and ALP

concentrations in the mothers from sites near the mine were significantly higher than

those in the mothers from Bwacha ($p < 0.05$). On the other hand, LDH ($p < 0.05$), Alb

($p = 0.049$), and metallothionein ($p < 0.001$) concentrations in the mothers from Bwacha

were significantly higher than those in mothers from sites near the mine. T-Bil

concentrations in the mothers from Bwacha were higher than those in the mothers from

sites near the mine, although this difference was not significant.

In all mothers, there was a significant positive correlation between the Pb and

AST concentrations (Supplementary Table S16, $p < 0.05$, $\rho = 0.16$), and Fe and

metallothionein concentrations (Supplementary Table S17, $p < 0.05$, $\rho = 0.17$). The

ALT concentrations displayed an almost significant increase as Pb concentrations

420   increased (p = 0.08, ρ = 0.13). The Alb (p < 0.05, ρ = −0.14) and metallothionein

421   concentrations (p < 0.001, ρ = −0.53) significantly decreased as the Pb concentrations

422   increased.

423   In the mothers from sites near the mine, the Pb concentrations were significantly

424   associated with increased AST (p < 0.05, ρ = 0.17) and LDH (p < 0.05, ρ = 0.19)

425   concentrations. On the other hand, the BUN (p < 0.05, ρ = −0.26) and metallothionein

426   concentrations (p < 0.001, ρ = −0.43) significantly decreased as Pb concentrations

427   increased. Moreover, the metallothionein concentrations significantly associated with

428   decreased Cu concentrations (p < 0.05, ρ = -0.21). In the mothers from Bwacha, there

429   were no significant correlations between Pb and plasma biochemical factors.

4. Discussion

430

431   To the authors' knowledge, the present study undertook the first ever analysis of

432 Pb in breastmilk of mothers from the sites around the mine in Kabwe. The overall mean

433 Pb concentration in breastmilk was 5.3 μg/L, which was marginally above the

434 acceptable level of 2 to 5 μg/L for breastfeeding (WHO, 1989), and 30.0% of breastmilk

435 samples contained Pb levels above the acceptable level. Compared with previous

436 studies that reported elevated Pb concentrations in breastmilk ranged from 8.8 to 35.4

437 μg/L (Isaac et al., 2012; Turan et al., 2001), Pb concentrations in the breastmilk of

438 mothers from sites near the mine were comparable or even lower. On the other hand, the

439 mean values of Pb concentrations in breastmilk of mothers from Katondo and Bwacha

440 (2.3 μg/L) were within the acceptable level, which agrees with the results obtained in

441 unpolluted areas in other reports (Ettinger et al., 2014; Klein et al., 2017), although the

442 highest individual Pb breastmilk concentration in this study was found in Katondo (51.9

443 μg/L). Pb in breastmilk may be one of the sources of Pb exposure in infants. Pb

444 concentrations in breastmilk in this study were 5.6% of Pb concentrations in maternal

445 blood. This result agreed with previous studies reporting breastmilk/mothers' blood

446 ratios between 1% and 10% (Anastácio et al., 2004; Ettinger et al., 2005; Koyashiki et

447 al., 2010; Koyashiki, et al., 2010; Gulson et al., 1998). In an evaluation of

448 breastmilk/mothers' blood relationships, Gulson et al. (1998) suggested that a ratio of

449 Pb concentration in breast milk to the concentration of Pb in maternal blood greater than

450 15% should be treated with caution, higher values than this arising probably from

451 sampling and/or analytical contamination.

452   High overall mean Pb concentration in infants' feces (39.2 mg/kg) was recorded

453 in the present study. The highest Pb concentration in infants' feces was 3002 mg/kg.

454 These results are in agreement with a previous study conducted in Kabwe (Yabe et al.,

455 2018). Even in Bwacha, which is far from the mine, high Pb concentrations were found

456 in infants' feces. This suggests that infants in Kabwe were exposed to Pb via ingestion.

457   Among sample types, Pb concentrations in infants' feces were significantly

458 higher than in blood and breastmilk. There were significant positive correlations among

459 samples. BLLs in infants significantly increased with BLLs in mothers ($p < 0.001$, $\rho =$

460 0.68), Pb concentrations in breastmilk ($p < 0.001$, $\rho = 0.43$), and infants' feces ($p <$

461 0.001, $\rho = 0.82$). These results suggest that mothers, as well as infants, are exposed to

462 Pb from the environment, as they share the same living conditions. Pb concentrations in

463 infants' feces may be a useful indicator of Pb exposure in infants, in addition to BLLs.

464   The present study found different trends of Pb exposure in infants and mothers

465 among the studied areas in Kabwe. The mean BLL in infants and mothers from

466 Makululu, which is further from the mine than Kasanda, were the highest among the

467 area. Following Makululu, those from Kasanda and Chowa were the second and the

468 third highest, respectively. Yabe et al. (2015) reported a similar trend for BLLs in

469 children under seven years old, but the highest BLL mean was found in Kasanda,

470 among the three sites. Since most areas in Makululu are dusty and unpaved compared to

471 Kasanda, residents could easily come in to contact with polluted soils or dusts in the

472 area. Pb exposure in infants and mothers from Kasanda and Makululu, which are

473 located on the western side of the mine and in the direction of prevailing winds, could

474 be higher than that in Chowa, which lies in the opposite direction. Pb concentrations in

475 breastmilk and infants' feces showed similar trends to BLLs in infants and mothers.

476 These trends agreed with those determined in earlier studies by Tembo et al. (2006) and

477 Nakayama et al. (2011), who reported similar trends in the soils in Kabwe. Toyomaki et

478   al. (2020) reported that Pb concentrations in dog blood decreased with the distance from

479   the mine in Kabwe, and that the exposure to Pb in dogs remarkably decreased about 5

480   km away from the mine. These findings suggested that location of the townships in the

481   relation to the wind direction and distance from the mine influence the extent of the

482   exposure to Pb in infants and mothers in Kabwe, as has been shown in other studies

483   (Soto-Jiménez and Flegal, 2011; Yun et al., 2018).

484   In the present study, Fe, Cu, Zn and Ag were also analyzed. In contrast to Pb,

485   the concentrations of Fe, Cu, Zn and Ag in the blood of infants and mothers tended to

486   be lower in areas surrounding the mine area than in Bwacha. Given that Pb poisoning is

487   known to cause anemia, the negative correlation between Pb and Fe concentrations

488   suggested that the higher Pb concentrations negatively affected Fe metabolism,

489   especially absorption (Hegazy et al.,2010; Chen et al., 2019). Positive correlations were

490   found between Pb and Cu, Zn, as well as Ag. These positive co-exposures could be

491   attributed to the presence of Pb, Zn and Ag in the galena in Kabwe as confirmed in

492   previous studies (Nakayama et al., 2011).

493   Blood lead levels in boys were significantly higher than those in girls in

494   Makululu. The same trend for children under seven years old in the same township was

495   previously reported (Yabe et al., 2015). Moreover, Pb concentrations in infants' feces of

496   boys were significantly higher than those of girls in Chowa. This difference could be

497   attributed to differences in breastmilk consumptions as reported by Costa et al. (2010)

498   were boys consumed breastmilk 0.05 kg/d more than girls. These results suggest that

499   boys are more exposed to Pb via ingestion than girls.

500   Significant positive correlations between BLLs in infants and age of the infants

501   were found. The hand-to-mouth or object-to-mouth (pica) behavior of children, and

502   high absorbance of ingested Pb from the gastrointestinal tract are well known factors

503   attributed to high Pb exposure in children (Wani et al., 2015). However, younger infants

504   who are not ambulatory could display less hand-to-mouth behaviors, as they are under

505   the care of their mothers or guardians although inhalation may be an important pathway

506   for very young children in windy environments (Gulson et al., 2009). Given that BLLs

507   in infants increases from birth to around two years of age in Kabwe, it is important to

508   pay more attention to activities of infants during this period. Moreover, cleaning floors

509   in the house where infants spend most of the time and the kitchen where food could be

510   contaminated by house dust would be important to reduce Pb exposure in infants. On

511   the other hand, only BLLs in mothers from Bwacha significantly decreased as the age of

512   mothers increased. Adults in Bwacha, which is farthest from the mine, could be less

513   exposed to Pb, thus, Pb in the blood of adults may mainly occur from redistribution of

514   endogenous bone-derived Pb (Gulson et al. 1998; Manton et al. 2003). From this point

515   of view, adults even from sites far from the mine in Kabwe could be chronically

516   exposed to Pb via endogenous exposure.

517   The Pb isotope ratios in infants' samples, especially feces, were almost identical

518   to those in the soil samples. The soil samples exhibited Pb isotope ratios similar to those

519   in Kabwe galena (Kamona et al., 1999). Furthermore, a Pb exposure study on rats

520   exposed to lead in the Kabwe soil revealed that the Pb isotope ratios in these biological

521   samples were also similar to those in Kabwe galena (Kamona et al., 1999; Nakayama et

522   al., 2019). These results suggest that contaminated soil or dust from the mine could be

523   one of the important sources of Pb exposure in infants of Kabwe as well as breastmilk.

524   Understanding which infant behaviors and activities are related to their Pb exposure is

525   required to determine the routes and to minimize the exposure. Pb isotope ratios in

526   infants' blood were similar to those in mothers' blood as infants could be exposed to Pb

527   through breastfeeding and through the placenta before birth. On the other hand, Pb

528   isotope ratios in mothers' samples, especially blood were different from those in

529   infants' feces and the soil samples. Pb isotope ratios in chicken, goats, and vegetables in

530   Kabwe reported by Nakata et al. (2016) were similar to the results in the present study,

531   suggesting that consuming contaminated food could be an important route of Pb

532   exposure in mothers. In the present study, both ^{206}Pb and ^{204}Pb ratios were used to

533   compare the differences of Pb isotope ratios among sample types. Both results were

534   similar, but ^{204}Pb-based ratios displayed clear differences among sample types.

535   Therefore, ^{204}Pb ratios could be more useful than ^{206}Pb ratios to elucidate the source of

536   Pb exposure as recommended by Gulson (2008).

537   Daily intake of Pb in infants through breastfeeding and soil ingestion was

538   calculated to estimate the burden of the possible routes of Pb exposure. Given that Pb

539   exposure can cause pediatric neurodevelopmental impairments even at low level

540   (Canfield et al., 2003), the reference value 0.5 μg/kg bw/day was used (US EPA, 2011).

541   From the results obtained in the present study, daily intake of Pb even through soil

542   ingestion alone highly exceeded the reference value. Although the bioavailability of Pb

543   in galena is known to be minimal (Rasmussen et al., 2011), Pb exposure through soil

544   ingestion could be the important source due to the high concentrations. Moreover, the

545   results of daily intake of Pb through breastfeeding using the maximum Pb

546   concentrations were larger than the reference value. These results suggest that both Pb

547   though breastfeeding and soil ingestion are important sources of Pb exposure in infants.

548   In the present study, the daily intake of Pb from the environment was only calculated

549   from soil ingestion. However, US EPA (2011) estimated the same amount (30 mg/day)

550   of dust ingestion in parallel with soil ingestion. This implies that the actual daily intake

551   in the field could be underestimated in the present study. Thus, future studies need to

552   evaluate the detail of Pb exposure in infants as well as mothers in Kabwe including

553   other routes, such as dust ingestion.

554   In contrast to the high BLLs in mothers, the plasma biochemical profiles of most

555   analyzed parameters were interestingly within, or close to, the standard reference values,

556   except in the case of ALP. These results indicate that Pb exposure in Kabwe mothers

557   did not significantly impact their health, as was observed during sampling, where all

558   sampled mothers appeared healthy. More specifically, the ALT and ALP values in the

559   mothers from sites near the mine were significantly higher than those in the mothers

560   from Bwacha although these values were not significantly correlated with BLLs in

561   mothers. Therefore, these results could not only be attributed to Pb exposure, but also

562   other factors. On the other hand, LDH and AST, which are indicators of liver function,

563   significantly increased as BLLs increased. These results suggest that Pb exposure in

564   mothers may have caused some mild liver damage. High Pb exposure is known to cause

565   kidney damage in conjunction with an increase in BUN and a decrease in Alb. However,

566   both biomarkers significantly decreased as BLLs increased in mothers. During a

567   previous study on Pb poisoning in refugee children in the United States, the CDC

568   reported chronic and acute malnutrition as risk factors for Pb poisoning (CDC, 2005).

569   Further studies in Kabwe should therefore focus on the relationship between Pb

570   exposure and nutrition status. In our study, metallothionein concentrations significantly

571   decreased as BLLs increased. A previous study by Mustonen et al. (2014) found

572   constant metallothionein expression in earthworms from a contaminated site, and

573   therefore suggested that the inducibility of the metallothionein response could be lost in

574 earthworms with a history of metal exposure. It is probable that local people in the sites

575 near the mine may be chronically exposed to metals, including Pb, over a long period of

576 time compared to people in sites far from the mine, such as Bwacha. Therefore,

577 metallothionein expression in people residing near the mine was lower than that in

578 people residing far from the mine. Further studies should focus on both metallothionein

579 concentrations and gene expression. In the present study, it is difficult to exclude the

580 possibility of other factors or diseases since a detailed questionnaire survey or medical

581 check-ups were not performed.

582   The findings in the present study suggest that one of the important sources of Pb

583 exposure in infants could be Pb from the environment, especially from soils. Daily

584 intake of Pb in infants through soil ingestion could be enough to exceed the EFSA's

585 reference value of daily intake of Pb. It is important to minimize Pb exposure in

586 infants from soil and dust.. Thus, remediation of the environment in Kabwe is

587 urgently needed to reduce Pb exposure. High BLLs in mothers could also be one of the

588 important sources of Pb exposure in infants via breastfeeding. Also, high BLLs in

589 mothers may cause their fetus to be exposed to Pb during pregnancy. In the current

590 situation, chelation therapy for Pb poisoning is prioritized more in children than in

591 adults, as children are more vulnerable to Pb. However, in utero exposure to

592 environmental lead may be adversely associated with neurodevelopment at two years of

593 age (Lin et al., 2013). Pilsner et al. (2009) reported that the epigenome of the

594 developing fetus can be influenced by the maternal cumulative lead burden, which may

595 influence long-term epigenetic programming and disease susceptibility throughout a

596 child's life. Reducing the Pb exposure in mothers is important to reduce Pb exposure in

597 fetuses via the placenta, as well as Pb exposure in infants, via breastfeeding. Thus, it is

598   necessary that mothers with high BLLs are treated with chelation therapy, as well as

599   their children.

600

601 **5. Conclusions**

602   High Pb concentrations in breastmilk, which were above the WHO acceptable

603 level for breastfeeding, could be one of the important sources of Pb exposure in infants.

604 The results of the isotope ratio analysis suggest that Pb from the environment, such as

605 contaminated soil is one of the important sources of Pb exposure in infants. Moreover,

606 Pb exposure in infants through breastfeeding and soil ingestion potentially exceeded the

607 EFSA's reference value of daily intake of Pb. Therefore, environmental remediation, in

608 parallel with chelation therapy, is urgently needed to reduce the Pb exposure in infants

609 and mothers in Kabwe. Moreover, mothers with high BLLs in Kabwe should be treated

610 with chelation therapy to reduce the Pb exposure of their infants via breastfeeding.

611

Conflict of interest
The authors declare no conflict of interest.

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