

1 **Pre-conception serum ferritin concentrations are associated with metal concentrations in blood**  
2 **during pregnancy: A cohort study in Benin**

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42 **Title**

43 **Pre-conception serum ferritin concentrations are associated with metal concentrations in**  
44 **blood during pregnancy: A cohort study in Benin**

45 **Abstract**

46 **Background:** Iron deficiency is a common nutritional deficiency that impacts maternal health and  
47 fetal development and is also associated with increased uptake of toxic metals. Women in sub-  
48 Saharan Africa are highly exposed to both iron deficiency and metals in the environment. As research  
49 on the developmental origins of health and disease increasingly shows impacts of pre-conception  
50 maternal health on pregnancy and fetal health, these environmental exposures are of concern.

51 **Objectives:** This study investigated the association between iron status pre-pregnancy and blood  
52 metal concentrations in the first trimester of pregnancy with potential implications for iron  
53 supplementation.

54 **Methods:** Pre-conception and first trimester blood samples taken from 262 Beninese women were  
55 tested for serum ferritin, inflammation markers, manganese (Mn), cadmium (Cd), lead (Pb), copper,  
56 zinc, selenium, mercury and arsenic. Associations between serum ferritin adjusted for inflammation  
57 and metal concentrations were analyzed using multivariate linear regression.

58 **Results:** Women with iron deficiency before conception (13%) were more likely to remain iron  
59 deficient in the first trimester (4%) (adjusted OR=41.2, 95%CI 6.2; 275.0) even within the context of  
60 routine iron supplementation during pregnancy.

61 Lower pre-pregnancy serum ferritin concentrations were significantly related to higher  
62 concentrations of Mn, Cd and Pb in the first trimester. Every 1% increase in serum ferritin  
63 concentration was associated with a 0.13% decrease in Mn (adjusted  $\beta$ =-0.13, 95%CI -0.18; -0.07), a  
64 0.22% decrease in Cd (adjusted  $\beta$ =-0.22, 95%CI -0.28; -0.15) and a 0.06% decrease in Pb  
65 concentration (adjusted  $\beta$ =-0.06, 95%CI -0.12; -0.006).

66 **Discussion:** These results suggest that increasing iron stores prior to pregnancy may prevent  
67 excessive uptake of toxic concentrations of the metals Mn, Cd and Pb and argue in favour of testing  
68 the effects of iron supplementation prior to pregnancy on metal concentrations.

69 **Keywords:** Iron deficiency, metals, environmental exposure, pregnancy, pre-conception

70

71

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75 **Competing Interests:** The authors declare they have no actual or potential competing financial  
76 interests or personal relationships that could have appeared to influence the work reported in this  
77 paper.

78 **Ethics approval:** The authors confirm that consent from subjects participating in the cohort study  
79 was received prior to conducting the study and that the study was approved by the Ethics Committee  
80 of the Institut des Sciences Biomédicales Appliquées (ISBA) in Benin.

81

## 82 1. Introduction

83 Iron deficiency is the most common nutritional deficiency worldwide and with or without anemia has  
84 important consequences for human health and child development. Globally, about 33% of all women  
85 of reproductive age are estimated to be anemic (WHO, 2016a), with 16-25% estimated to be due to  
86 iron deficiency (Petry et al., 2016). Women's iron requirements increase significantly during  
87 pregnancy, rising during the second trimester and continuing to increase throughout the remainder  
88 of pregnancy (Bothwell, 2000).

89 Iron is essential for brain development, particularly in late fetal and early postnatal life, where  
90 deficits during these critical periods can permanently affect brain function (Georgieff et al., 2018).  
91 Maternal iron deficiency also reduces iron stores in the fetus which further exposes neonates to  
92 neurodevelopmental risks as infants cannot regulate iron absorption until 6-9 months of age  
93 (Radlowski & Johnson, 2013). The WHO recommends oral iron and folic acid supplementation for  
94 pregnant women to prevent maternal anaemia, puerperal sepsis, low birth weight, and preterm birth  
95 (WHO, 2016b).

96 Higher iron levels, however may be detrimental in malaria endemic areas such as Benin, as some  
97 studies in children have found malaria risk increases in relation to serum ferritin concentrations and  
98 iron deficiency seems to act as a protective factor (Moya-Alvarez et al., 2017; Muriuki et al., 2019). A  
99 recent systematic review of malaria risk and iron status in pregnancy found that iron deficiency was  
100 only associated with increased risk of malaria when measured by serum ferritin and not when other  
101 biomarkers of iron deficiency were included. In the light of limited evidence however, iron  
102 supplementation is still recommended to be provided in combination with malaria prevention during  
103 pregnancy (Sangaré et al., 2014).

104 Iron deficiency is also associated with the increased uptake of divalent metals which can adversely  
105 affect neurodevelopment (Kim, 2018). Dietary non-heme iron is transported into intestinal cells by  
106 the divalent metal-ion transporter 1 (DMT1) and then exported into circulation via ferroportin 1  
107 (FPN1) or stored as ferritin. Iron homeostasis is regulated by the hormone hepcidin, which is in low  
108 concentrations when tissue iron stores are low, up-regulating DMT1 and FPN1 (Anderson & Frazer,  
109 2017). DMT1 has been demonstrated to transport divalent metal ions other than iron, notably  
110 manganese, cadmium and lead as well as to a lesser extent copper and zinc (Bressler et al., 2004;  
111 Garrick et al., 2006). There is also evidence that FPN1 can transport manganese and zinc (Loréal et  
112 al., 2014; Madejczyk & Ballatori, 2012).

113 Research conducted within the framework of the Developmental origins of Health and Disease  
114 (DoHAD) continues to provide evidence of the long-term consequences on health of environmental  
115 exposures from conception and including the role of maternal nutrition prior to conception (Mandy  
116 & Nyirenda, 2018; Stephenson et al., 2018). Many studies have examined the effects of iron  
117 deficiency and its relationship to a range of environmental metal exposures during pregnancy and in  
118 relation to fetal and childhood development. This study examines data from a unique cohort where  
119 women were recruited prior to conception, allowing iron status before pregnancy to be examined in  
120 relation to whole blood concentrations in early pregnancy of 8 potentially toxic metals: Manganese,  
121 Copper, Zinc, Selenium, Lead, Cadmium, Mercury and Arsenic.

122 Manganese is an essential nutrient but also a neurotoxicant at high exposure levels. Lead and  
123 cadmium are long-lived, toxic heavy metals that have no known beneficial function in the human  
124 body. The presence of these metals in the environment and the likelihood of increased uptake before  
125 and during pregnancy for women with low iron stores can result in a range of deleterious effects.

126 Cd accumulation over time can damage renal function and affect bone metabolism (ATSDR, 2012a)  
127 as well as some evidence it is toxic to endocrine systems resulting in decreased birth weights  
128 (Rahman et al., 2016). There is some evidence that prenatal Cd exposure may impact cognitive  
129 development in children (Kippler et al., 2012). Although Cd accumulates in the placenta acting as a  
130 partial barrier to foetal exposure with cord blood concentrations significantly lower than maternal  
131 blood (Esteban-Vasallo María D. et al., 2012; Truska et al., 1989), Cd also affects placental transport  
132 of calcium and zinc which are essential for foetal development (Gundacker & Hengstschläger, 2012).

133 The health effects of Pb are diverse, not only is Pb a known developmental neurotoxicant but  
134 exposure to Pb is associated with toxicity to every organ system (ATSDR, 2020). Lead readily crosses  
135 the placenta (Bellinger, 2005; Rossipal et al., 2000) and there is good evidence that maternal lead  
136 exposure during pregnancy can adversely affect both maternal and child health. There is no apparent  
137 threshold below which adverse effects of lead do not occur (Budtz-Jørgensen et al., 2013; CDC,  
138 2010). Bone lead stores persist for decades and are mobilized during periods of increased bone  
139 turnover such as pregnancy and lactation. Iron status, along with intake of dietary calcium and zinc  
140 can affect absorption of Pb, potentially leading to alterations in blood lead concentrations (ATSDR,  
141 2020). In addition, there is some evidence that combined Pb and Mn exposures in-utero and in  
142 childhood could aggravate negative impacts on neurodevelopment (Kim et al., 2009; Lin et al., 2013;  
143 Menezes-Filho et al., 2018).

144 Se is an essential trace element which can also be toxic and has a complex interplay of metabolic  
145 interactions with numerous other nutrients and toxic substances (Arnaud & van Dael, 2018; Sun et  
146 al., 2014). Se intake varies widely dependent on concentrations in soils transferred to crops.

147 The objective of this study was to investigate the association between iron status pre-pregnancy and  
148 during the first trimester with the blood concentrations of eight metals in the first trimester of  
149 pregnancy: manganese, cadmium, lead, copper, zinc, selenium, mercury and arsenic.

150

## 151 **2. Methods**

### 152 *2.1 Study Population*

153 This study includes 262 women from the REtard de Croissance Intra-uterin et PALudisme (RECIPAL)  
154 cohort in southern Benin primarily designed to assess the consequences of malaria in early  
155 pregnancy for the fetus as detailed in Accrombessi et al., 2018 and summarized in Figure 1. Women  
156 in the RECIPAL cohort were recruited prior to conception between June 2014 and December 2016  
157 and followed monthly until pregnancy and through to delivery. At inclusion demographic, household  
158 and socioeconomic characteristics were collected. In addition, blood samples were taken at

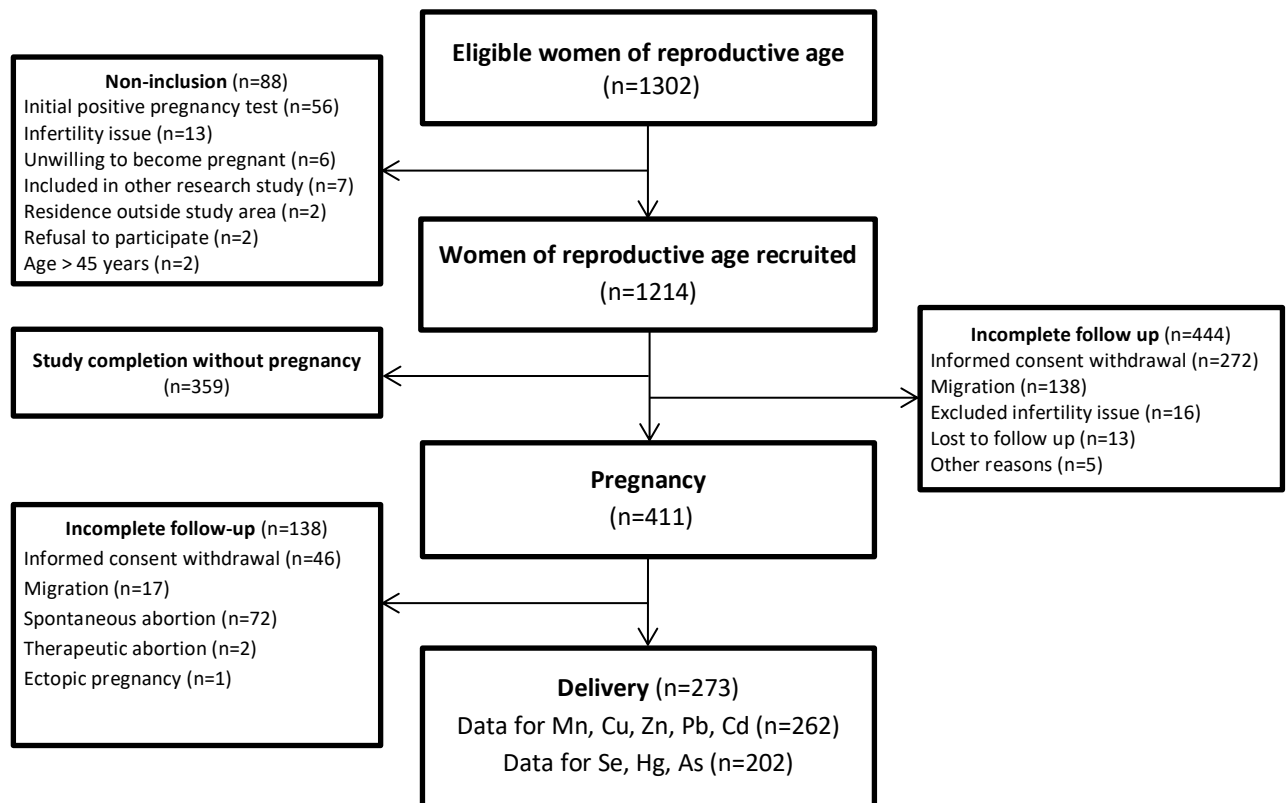
159 enrolment before conception and at the second antenatal care (ANC2) visit which occurred for most  
160 women during the 1st trimester of pregnancy.

## 161 2.2 Ethics Statement

162 The RECIPAL study was approved by the Ethics Committee of the Institut des Sciences Biomédicales  
163 Appliquées (ISBA) in Benin. Women enrolled in the cohort received regular free medical follow-up  
164 treatment as necessary during the life of the study. Blood samples were stored and assessed for  
165 metals at a later date, these results at the population level were presented to health authorities at  
166 local and national levels.

167

168 **Figure 1:** Flowchart diagram of the study



169

## 170 2.3 Gestational Follow-up

171 According to Beninese national recommendations, a kit including iron and folic acid tablets for 1  
172 month was given to each woman at the first ANC visit and maternity staff were encouraged to  
173 administer iron and folic acid supplementation throughout the pregnancy (200 mg oral ferrous  
174 sulfate and 10 mg folic acid daily). As part of the study, the cost of drugs were supported by the  
175 project; however, the iron and folic acid supplementation were not observed by the study staff.

176 *2.4 Blood Measurements*

177 Blood samples were initially tested for parameters related to iron deficiency, inflammation markers  
178 and malaria. Blood serum samples were tested for ferritin, C-reactive protein (CRP) and  $\alpha_1$ -acid  
179 glycoprotein (AGP). Ferritin may be measured in either serum or plasma, with both positively  
180 correlated with the size of total body iron stores (WHO, 2020). As nutrition was one of the core  
181 issues in the RECIPAL cohort, serum ferritin was used as part of the assessment of nutritional status  
182 and the micronutrient intake of women. Hb level was measured with a HemoCue. Before conception  
183 and during pregnancy, malarial screening was performed using both microscopy (thick blood smear)  
184 and PCR. Subsequently two sets of ANC2 whole blood samples of women who completed the study  
185 follow-up were tested for metals. A first group of 60 tested for Manganese (Mn), Copper (Cu), Zinc  
186 (Zn), lead (Pb) and Cadmium (Cd) with results reported previously (Guy et al., 2018). Samples from a  
187 further 202 were later tested for these five metals with the addition of tests for Selenium (Se),  
188 Mercury (Hg) and Arsenic (As).

189 The 262 whole blood samples stored at  $-18^{\circ}\text{C}$ , were analyzed by a plasma torch coupled with tandem  
190 mass spectrometer (ICP-MSMS 8800, Agilent Technologie) after mineralization. Mineralization is  
191 performed by adding nitric acid and heating at  $83^{\circ}\text{C}$  for 4 hours using a graphite block (Hotblock Pro,  
192 model SC-189, Environmental Express). The mineralization diluted at 1:20 was then analyzed, the use  
193 of the tandem mass spectrometer and the oxygen mode made it possible to manage the  
194 interferences in particular for the determination of selenium and arsenic. An online addition of  
195 internal standards (Sc, Ge,  $^{77}\text{Se}$ , Rh, Re and Ir) was performed to correct matrix effects and to verify  
196 the absence of drift (signal variation less than 25%). The quantification of Se was performed by  
197 isotope dilution with  $^{77}\text{Se}$  to resolve persistent effects. At each analysis sequence, consumable  
198 blanks and certified blood controls were systematically analyzed (Utak level 1, Utak level 2). The  
199 results of the blood tests were validated if the concentrations of the blood controls were within the  
200 limits of the control cards. The method of analysis was validated by 2 good inter-laboratory tests over  
201 1 year. This method is accredited by the French accreditation committee (Cofrac). The limits of  
202 quantification (LOQ) of Pb, Mn, Cu, Zn, Cd, As, Se and Hg are respectively 2, 5, 200, 200, 0.25, 1, 20  
203 and  $0.4\ \mu\text{g/L}$ .

204 *2.5 Definitions*

205 *Iron deficiency* was defined as serum ferritin less than  $15\ \mu\text{g/l}$  after adjustment for inflammation.  
206 Serum ferritin is a reliable indicator of total body iron stores when inflammation is not present. In  
207 regions such as the study area in Benin where there is widespread infection or inflammation, defining  
208 iron deficiency using serum ferritin becomes complicated as ferritin is a positive acute phase  
209 response protein. WHO recommends in these areas to assess serum ferritin with concurrent  
210 measurement of CRP and AGP and apply one of four possible methods: raising the cut-off value that  
211 defines iron deficiency to  $70\ \mu\text{g/l}$ ; excluding individuals with elevated CRP or AGP concentrations; use  
212 an arithmetic correction; or use a regression correction (WHO, 2020). A comparison of methods on a  
213 cohort of Kenyan pregnant women concluded that using a regression correction approach led to  
214 better estimates of iron deficiency (Mwangi et al., 2019). This regression correction approach  
215 according to the following equation was also used in this study as it allowed a correction to

216 continuous data and the inclusion of a correction for inflammation present due to malaria infection.  
217 (Namaste et al., 2017; WHO, 2020).

$$218 \text{ Ferritin}_{\text{adjusted}} = \text{Ferritin}_{\text{unadjusted}} - \beta_1(\text{CRP}_{\text{obs}} - \text{CRP}_{\text{ref}}) - \beta_2(\text{AGP}_{\text{obs}} - \text{AGP}_{\text{ref}}) - \beta_3\text{malaria}$$

219 where  $\beta_x$  are the regression coefficients, obs is the observed value and ref is the external reference  
220 value generated to define low inflammation.

221 *Inflammation* was defined as either CRP > 5 mg/l and/or AGP > 1 g/l (Namaste et al., 2017; WHO,  
222 2020)

223 *Anemia* was defined prior to pregnancy as hemoglobin <120 g/l and during pregnancy as <110 g/l  
224 with *moderate anemia* between 70 to 110 g/l and *severe anemia* < 70 g/l (WHO, 2015)

225 *Iron deficiency anemia* was defined as those meeting the criteria both for iron deficiency and anemia.

226 *Insufficient iron stores for pregnancy* was defined as serum ferritin <32  $\mu\text{g/l}$  approximately equivalent  
227 to 300 mg of total body iron stores, the estimated minimum with which a woman must enter  
228 pregnancy to meet her requirements fully assuming an optimal diet.

229 *First trimester* was defined as less than 14 weeks gestation based on both ultrasound scan and LMP

## 230 2.6 Statistical Analysis

231 The association between serum ferritin pre-pregnancy and at ANC2 and blood metal concentrations  
232 for Mn, Cu, Zn, Pb and Cd, Se, Hg and As were modelled separately using multivariate linear  
233 regression. Log transformations of the metal concentrations were made due to non-normal  
234 distributions except for Se which did not have a distribution significantly different from normal when  
235 using a Shapiro-Wilk test. Log transformations of serum ferritin concentrations were also used in the  
236 models for all metals except Se as this improved the linear relationships checked by visual inspection.  
237 Confounders were selected based on literature review, the consideration of directed acyclic graphs  
238 and after testing for collinearity between candidate variables. Gestational age at ANC2, gravidity and  
239 indicators for education level and wealth were included in the models for all metals. As there was  
240 little variability in education level, literacy was used as the variable to represent whether any formal  
241 education had been received. At inclusion in the cohort, detailed questions were asked on the  
242 ownership of assets, the number owned was used as an indicator of wealth. Other potential  
243 confounders were tested such as age, body mass index (BMI), the time between pre-pregnancy and  
244 ANC2 testing, being positive for malaria by microscopy at ANC2 and living lakeside or inland as a  
245 potential indicator of different environmental and dietary exposures. Univariate analysis was first  
246 conducted retaining variables with a p value less than 0.25. These variables were then removed  
247 stepwise from multivariate models if p values were greater than 0.05. Analysis was carried out using  
248 Stata version 15 for Windows.



249 **3. Results**

250 *3.1 Population characteristics*

251 Table 1 presents the general characteristics of the 262 women included in the study population.  
 252 Women were on average 27 years old, primarily of Toffin ethnicity (74.4%) and living within 4 sub-  
 253 districts of Sô-Ava district: Vekky and Houedo-Aguekon on the edge of the polluted Lake Nokoue; Sô-  
 254 Ava along the River So; and Akassato where women lived within 3 to 10 km from Lake Nokoue. The  
 255 majority of women were multigravidae (92.4%), were non-smokers (99.2%) and were within a  
 256 healthy BMI range at the start of pregnancy (67%). Table 2 presents the blood test results related to  
 257 iron deficiency at pre-pregnancy and ANC2 visits and Table 3 gestational age information at the first  
 258 two ANC visits. Before pregnancy, 12.7% of women were iron deficient, 57.9% anemic and 7% tested  
 259 positive for malaria. Most women became pregnant within 6 months of inclusion (51.2%) with a  
 260 further 37.3% conceiving within a year. Pregnancy was confirmed at the first ANC visit which  
 261 occurred at an average gestational age of 6.9 weeks at which time iron supplementation was  
 262 prescribed. Gestational age at ANC2, at which blood was sampled for iron parameters and metals  
 263 analysis, averaged 11.7 weeks with 84.4% considered in the first trimester. By ANC2 only 3.5% of  
 264 women were still considered as iron deficient, 38.9% anemic and 5.4% tested positive for malaria.

265 **Table 1:** Study Population Descriptive Statistics

<b>Maternal Characteristics</b>	<b>No.</b>	<b>Mean ± SD, %</b>
Age	262	26.7 (±4.9)
Subdistrict		
Vekky	92	35.1%
Sô-Ava	91	34.7%
Akassato	65	24.8%
Houedo-Aguekon	14	5.3%
Marital Status		
Married (Monogamy)	173	66.0%
Married (Polygamy)	71	27.1%
Cohabitation	18	6.9%
Ethnicity		
Toffin	195	74.4%
Aizo	32	12.2%
Fon	20	7.6%
Other	13	5.8%
Household density <sup>a</sup>	262	6.1 (±3.9)
Education		
Illiterate	183	69.8%
Gravidity		
Primigravidae	20	7.6%
Multigravidae	242	92.4%
Tobacco during pregnancy		
During 1st trimester	1	0.4%
Throughout pregnancy	1	0.4%
None	254	99.2%
BMI in early pregnancy		
Underweight (<18.5)	24	9.2%
Normal (18.5-25)	175	66.8%

266	Overweight/Obese ( $\geq 25$ )	63	24.0%
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<sup>a</sup>Number of people per household

267 **Table 2:** Maternal blood test results for iron deficiency parameters

	No.	Pre-pregnancy visit	No.	2nd Ante Natal Care (ANC2) visit
Hemoglobin (g/l)				
Mean (±SD)	261	117.6 (±13.0)	260	114.1 (±11.8)
Range (min - max)		84 - 151		84 - 146
Ferritin (µg/l)				
Mean (±SD)	260	58.1 (±34.6)	261	84.0 (±40.7)
Range		4.3 - 208		10.3 - 187
Adjusted Ferritin (µg/l) <sup>a</sup>				
Mean (±SD)	260	42.0 (±23.6)	261	74.1 (±35.8)
Range		4.3 - 105		9.9 - 171
Anemia <sup>b</sup>	261	151 (57.9%)	260	101 (38.9%)
Iron Deficiency <sup>c</sup>	260	33 (12.7%)	261	9 (3.5%)
Iron Deficiency Anemia <sup>d</sup>	260	25 (9.6%)	261	5 (1.9%)
Inflammation present <sup>e</sup>	260	48 (18.5%)	261	63 (24.1%)
Malaria positive by microscopy	259	18 (7.0%)	261	14 (5.4%)
Insufficient iron stores for pregnancy <sup>f</sup>	260	98 (37.7%)		

268 <sup>a</sup>Ferritin results adjusted for inflammation based on C-Reactive Protein (CRP) and α<sub>1</sub>-acid-glycoprotein (AGP) biomarkers  
 269 according to the BRINDA approach

270 <sup>b</sup>Anemia defined pre-pregnancy as hemoglobin <120g/l and during pregnancy as <110g/l

271 <sup>c</sup>Iron deficiency defined as adjusted serum ferritin <15 µg/L

272 <sup>d</sup>Iron deficiency anemia defined as meeting both anemia and iron deficiency criteria

273 <sup>e</sup>Inflammation defined as either CRP>5 µg/l or AGP>1 µg/l

274 <sup>f</sup>Insufficient iron stores for pregnancy defined as adjusted serum ferritin <32 µg/L

275

276 **Table 3:** Gestational age information and maternal blood test timing

	No.	1st Ante Natal Care (ANC1) visit <sup>g</sup>	No.	2nd Ante Natal Care (ANC2) visit
Gestational age (weeks)				
Mean (±SD)	262	6.9 (±2.3)	261	11.7 (±2.8)
Range		3.7 - 19.3		7.0 - 24.0
1 <sup>st</sup> Trimester (≤14 weeks gestation)			262	221 (84.4%)
2 <sup>nd</sup> Trimester (>14 weeks gestation)			262	41 (15.6%)
Time from pre-pregnancy blood test to confirmation of pregnancy (weeks)	260			
Geometric Mean (95%CI)		17.4 (15.6; 19.4)		
Range		2.1 - 106		
n (%) > 26 weeks		97 (37.3%)		
n (%) > 52 weeks		30 (11.5%)		

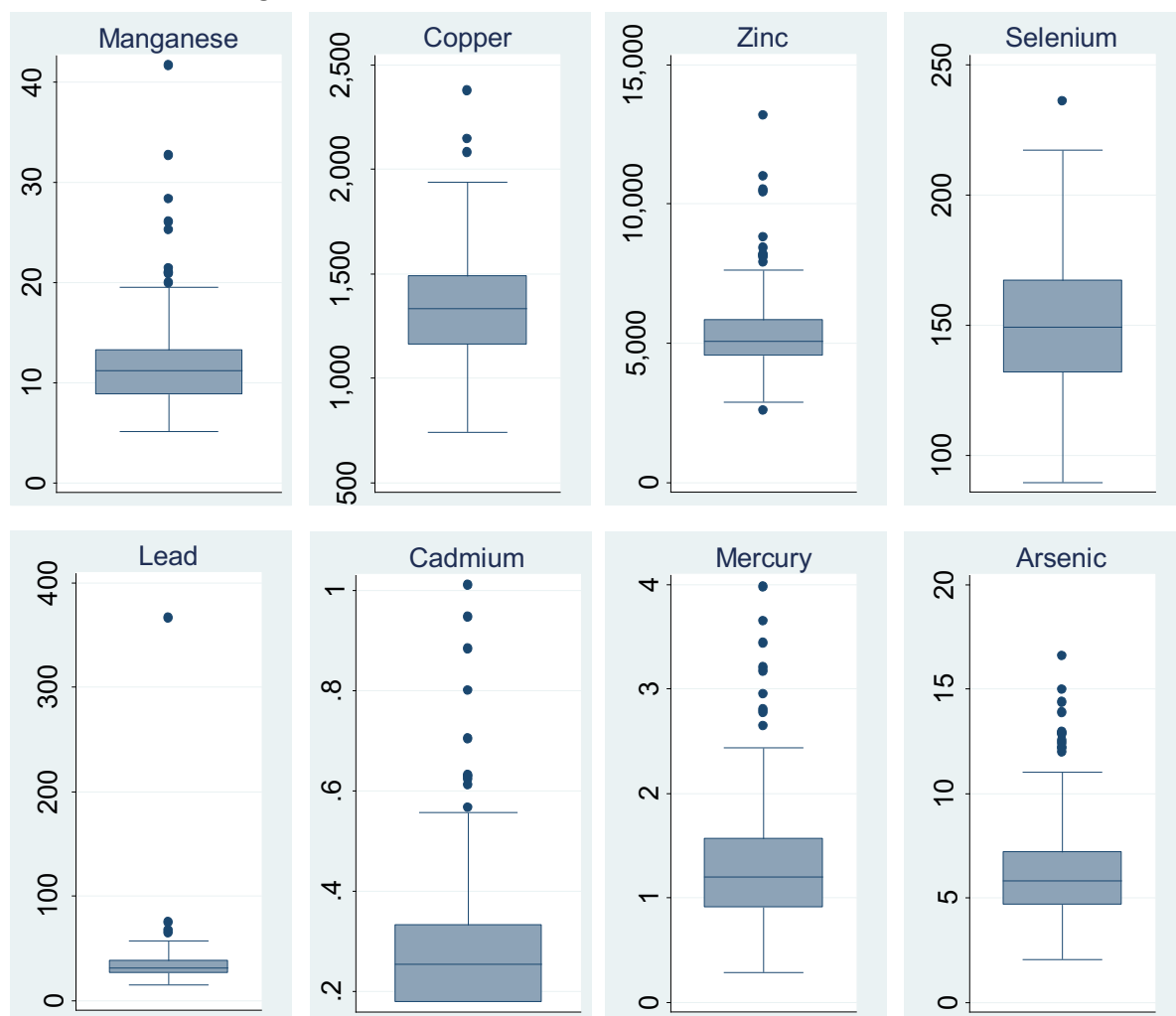
277 <sup>g</sup>Pregnancy confirmed at the first ante-natal visit and iron supplements provided to the mother from this point onwards

278

### 279 3.2 Metal Associations

280 The range of concentrations in maternal blood at the ANC2 visit for the eight metals tested is shown  
 281 in Figure 2. The median and interquartile range concentrations were for Mn 11.2 µg/l (IQR 8.76-  
 282 13.2), Cu 1333 µg/l (IQR 1165-1490), Zn 5074 µg/l (IQR 4541-5830), Se 149.1 µg/l (IQR 132.1-167.3),  
 283 Pb 31.3 µg/l (IQR 25.7-38.9), Cd 0.253 µg/l (IQR 0.177-0.331), and Hg 1.20 µg/l (IQR 0.907-1.56).

284 **Figure 2:** Maternal blood concentrations of elements ( $\mu\text{g/l}$ ) at ANC2 for n=262 for Mn, Cu, Zn, Pb, Cd  
 285 and n=202 for Se, Hg, As\*



286 \*Boxes represent median values with interquartile range (IQR) and whiskers highest and lowest points within 1.5 IQR length  
 287 from the box. Dots represent outliers

288 The relationship between pre-pregnancy iron deficiency and iron deficiency at ANC2 is presented in  
 289 Table 4. Women found to be iron deficient before conception were significantly more likely to remain  
 290 iron deficient when re-tested in the first trimester (adjusted OR=41.2, 95%CI 6.18; 275). However,  
 291 the numbers of iron deficient women, were small with 13% of the sample found to be iron deficient  
 292 before conception and 4% in the first trimester.

293 **Table 4:** Effect of pre-pregnancy iron deficiency on iron deficiency at ANC2

	n	Crude OR (95% CI)	Adjusted OR (95% CI) <sup>a</sup>
Iron deficient <sup>b</sup> at ANC2			
Iron deficient <sup>b</sup> pre-pregnancy	259	16.5 (3.24;106)	41.2 (6.18;275)
Iron stores insufficient <sup>c</sup> at ANC2			
Iron stores insufficient for pregnancy <sup>c</sup> at pre-pregnancy blood test	259	10.3 (4.23; 27.2)	12.5 (5.11;30.6)

294 <sup>a</sup>Adjusted for G2 gestational age, gravidity, education and wealth (based on no. possessions)  
 295 <sup>b</sup>Iron deficiency defined as serum ferritin adjusted for inflammation <15 µg/L  
 296 <sup>c</sup>Insufficient iron stores for pregnancy based on an estimated requirement of at least 300mg which corresponds to a serum  
 297 ferritin level of ≥32 µg/L. As iron requirements are low in the first trimester, the same cut-off was used for pre-pregnancy  
 298 and ANC2 blood test results  
 299

300 The results for blood metal concentrations at ANC2 showed that higher pre-pregnancy serum ferritin  
 301 concentrations and higher ANC2 serum ferritin concentrations were significantly related to lower  
 302 concentrations of Mn, Cd and Pb in the first trimester after adjustment for potential confounders  
 303 (Table 5). Every 1% increase in pre-pregnancy serum ferritin concentration was associated with a  
 304 0.13% decrease in Mn (adjusted  $\beta$ =-0.13, 95%CI -0.18; -0.07), a 0.22% decrease in Cd (adjusted  $\beta$ =-  
 305 0.22, 95%CI -0.28; -0.15) and a 0.06% decrease in Pb concentration (adjusted  $\beta$ =-0.06, 95%CI -0.12; -  
 306 0.006). Similarly, every 1% increase in ANC2 serum ferritin concentration was associated with a  
 307 0.19% decrease in Mn (adjusted  $\beta$ =-0.19, 95%CI -0.25; -0.13), a 0.21% decrease in Cd (adjusted  $\beta$ =-  
 308 0.21, 95%CI -0.29; -0.13) and a 0.11% decrease in Pb concentration (adjusted  $\beta$ =-0.11, 95%CI -0.17; -  
 309 0.04).

310 The only other metal to show a significant positive association was Se where every 1 microgram unit  
 311 increase in pre-pregnancy serum ferritin concentration was associated with a 0.19 microgram  
 312 increase in Se concentration (adjusted  $\beta$ =0.19, 95%CI 0.043; 0.34) and every 1 unit microgram  
 313 increase in ANC2 serum ferritin concentration was associated with a 0.11 microgram increase in Se  
 314 concentration (adjusted  $\beta$ =0.11, 95%CI 0.013; 0.22).

315 After adjusting the models no significant associations were found between serum ferritin  
 316 concentrations and Zn, Cu, As or Hg blood concentrations in the first trimester.

317 **Table 5:** Associations between serum ferritin concentrations pre-pregnancy and ANC2 blood metal  
 318 concentrations

	No.	Crude $\beta$ (95% CI)	Adjusted $\beta$ (95% CI)
Log Mn (µg/l)			
Log Serum ferritin pre-pregnancy (µg/l)	259	<b>-0.13 (-0.18, -0.07)</b>	<b>-0.13 (-0.18, -0.07)<sup>a</sup></b>
Log Serum ferritin at ANC2 (µg/l)	259	<b>-0.19 (-0.25, -0.13)</b>	<b>-0.19 (-0.25, -0.13)<sup>a</sup></b>
Log Cd (µg/l)			
Log Serum ferritin pre-pregnancy (µg/l)	259	<b>-0.21 (-0.28, -0.15)</b>	<b>-0.22 (-0.28, -0.15)<sup>b</sup></b>
Log Serum ferritin at ANC2 (µg/l)	260	<b>-0.20 (-0.28, -0.13)</b>	<b>-0.21 (-0.29, -0.13)<sup>b</sup></b>
Log Zn (µg/l)			
Log Serum ferritin pre-pregnancy (µg/l)	259	-0.03 (-0.07, 0.01)	-0.03 (-0.07, 0.01) <sup>b</sup>
Log Serum ferritin at ANC2 (µg/l)	260	-0.03 (-0.08, 0.01)	-0.04 (-0.09, 0.003) <sup>b</sup>
Log Cu (µg/l)			
Log Serum ferritin pre-pregnancy (µg/l)	258	-0.02 (-0.06, 0.01)	-0.03 (-0.06; 0.004) <sup>c</sup>
Log Serum ferritin at ANC2 (µg/l)	258	-0.03 (-0.07, 0.01)	-0.02 (-0.05; 0.02) <sup>c</sup>
Log Pb (µg/l)			
Log Serum ferritin pre-pregnancy (µg/l)	259	-0.04 (-0.10, 0.02)	<b>-0.06 (-0.12, -0.006)<sup>d</sup></b>
Log Serum ferritin at ANC2 (µg/l)	260	-0.07 (-0.14, 0.001)	<b>-0.11 (-0.17, -0.04)<sup>d</sup></b>
Log As (µg/l)			
Log Serum ferritin pre-pregnancy (µg/l)	201	-0.03 (-0.10, 0.04)	-0.02 (-0.09, 0.05) <sup>a</sup>
Log Serum ferritin at ANC2 (µg/l)	201	-0.07 (-0.15, 0.004)	-0.06 (-0.13, 0.02) <sup>a</sup>
Se (µg/l)			

Serum ferritin pre-pregnancy (µg/l)	201	<b>0.16 (0.004, 0.31)</b>	<b>0.19 (0.043, 0.34)<sup>e</sup></b>
Serum ferritin at ANC2 (µg/l)	201	<b>0.11 (0.010, 0.22)</b>	<b>0.11 (0.013, 0.22)<sup>e</sup></b>
Log Hg (µg/l)			
Log Serum ferritin pre-pregnancy (µg/l)	201	<b>0.003 (0.000, 0.01)</b>	-0.07 (-0.01, 0.16) <sup>f</sup>
Log Serum ferritin at ANC2 (µg/l)	201	0.002 (-0.000, 0.003)	0.03 (-0.06, 0.13) <sup>f</sup>

319 <sup>a</sup>Adjusted for G2 gestational age, gravidity, education, wealth and time from pre-pregnancy blood test to conception

320 <sup>b</sup>Adjusted for G2 gestational age, gravidity, education and wealth

321 <sup>c</sup>Adjusted for G2 gestational age, gravidity, education, wealth, time from pre-pregnancy blood test to conception, BMI and  
322 living lake-side

323 <sup>d</sup>Adjusted for G2 gestational age, gravidity, education, wealth and living lake-side

324 <sup>e</sup>Adjusted for G2 gestational age, gravidity, education, wealth and BMI

325 <sup>f</sup>Adjusted for G2 gestational age, gravidity, education, wealth, BMI and living lake-side

326

## 327 4. Discussion

328 These results show that serum ferritin concentrations prior to conception have an influence on blood  
329 concentrations of iron, Mn, Cd, Pb and Se during pregnancy. Pre-pregnancy serum ferritin  
330 concentrations were inversely associated with higher whole blood concentrations of Mn, Cd and Pb  
331 and positively associated with higher Se concentrations in the first trimester. With iron deficiency  
332 pre-pregnancy strongly related to iron deficiency in the first trimester, similar associations are seen  
333 between ANC2 serum ferritin concentrations and Mn, Cd, Pb and Se, as would be expected.

### 334 4.1 Iron

335 The number of women found to be anemic prior to pregnancy was high (57.9%) with that due to iron  
336 deficiency at 17%. Although iron deficiency is commonly assumed to cause half of all cases of  
337 anemias (WHO, 2015), this figure is in line with a recent meta-analysis of national survey data which  
338 found where anemia prevalence was greater than 40% in rural areas the percentage associated with  
339 iron deficiency was 16% (Petry et al., 2016).

340 All women received iron supplements from their first ANC visit and overall serum ferritin  
341 concentrations increased, and the numbers of anemic and/or iron deficient women decreased  
342 between the pre-pregnancy and ANC2 visit blood tests implying that supplementation was effective.  
343 It was not however possible to know from the data collected the compliance of individual women in  
344 regularly taking iron supplements. Women were asked at each ANC visit if they had taken iron  
345 supplements the previous day but no relationship was found between the number of positive  
346 responses and iron status. Iron status prior to pregnancy had an important effect on iron status in  
347 early pregnancy. The odds of being iron deficient at the ANC2 visit were 41.2 (95%CI 6.2;275.0) times  
348 more likely if iron deficient pre-pregnancy. The wide confidence interval of this result reflects the  
349 small number of women in the sample who remained iron deficient at ANC2. For this reason, a  
350 different cutoff was also tested based on an estimate of iron stores that are needed to be present at  
351 the start of pregnancy to fully meet requirements and was consistent with the previous result with  
352 the odds of a woman remaining without sufficient iron stores for pregnancy at ANC2 being 12.5  
353 (95%CI 5.11;30.6) times higher if low in iron stores prior to pregnancy. This finding suggests that for  
354 women who are not classified as iron deficient but have lower iron stores, the iron supplementation  
355 that they receive is not sufficient to carry them through all the iron requirements of pregnancy.

356 The current recommendations for iron supplementation in pregnancy in Benin is daily oral iron and  
357 folic acid supplementation usually provided through ANC visits and coverage is relatively high with  
358 85% of women who had a live birth in the previous 5 years reporting having taken oral iron  
359 supplement in the most recent national demographic and health survey (INSAE & ICF, 2019). A  
360 comparison of rates of adherence to iron supplementation during pregnancy between 22 sub-  
361 Saharan found Benin had the 4<sup>th</sup> highest reported rate of these countries and varies as may be  
362 expected with the number of antenatal visits, and levels of education and family wealth (Ba et al.,  
363 2019). It appears that iron supplementation is well accepted in Benin but may start later than ideal as  
364 seen in some of the quite late first ANC visits in this cohort, despite regular follow-up. It is also  
365 possible that the same socio-economic factors that contribute to lower adherence to iron  
366 supplementation may also contribute to higher environmental exposures to metals.

#### 367 *4.2 Manganese, Cadmium and Lead*

368 Blood Mn concentrations in the range 4–15 µg/L are considered normal (ATSDR, 2012b). Although  
369 the majority of blood samples taken at ANC2 in this study fell within this range, 15% of women  
370 recorded concentrations above 15 µg/L. As blood manganese concentrations are known to increase  
371 during pregnancy and be significantly higher in cord than in maternal blood samples at delivery  
372 (Gunier et al., 2014; Takser et al., 2004; Tholin et al., 1995; Yamamoto et al., 2019), it is likely that an  
373 even greater percentage of women would have been exposed to higher concentrations of Mn by the  
374 end of pregnancy than that measured at ANC2. Several studies link adverse effects on birth size  
375 parameters with both low and high concentrations of Manganese in maternal and/or cord blood  
376 (Guan et al., 2014; Yamamoto et al., 2019; Zota et al., 2009) and between higher concentrations of  
377 manganese and impaired child neurodevelopment (Chung et al., 2015; Claus Henn et al., 2017; Lin et  
378 al., 2013; Takser et al., 2003). Evidence from other studies also supports a close interaction between  
379 manganese and iron, particularly in an iron deficient state, in terms of their transport and absorption  
380 mechanisms into the body (Bjørklund et al., 2017; Margrete Meltzer et al., 2010). Blood Mn  
381 concentrations have been found to be higher among pregnant women (Tholin et al., 1995). Low iron  
382 status may also increase manganese concentration in certain brain areas as demonstrated in animal  
383 studies (Erikson et al., 2004, 2005).

384 The results for Mn, Cd and Pb appear to be consistent with reported transport affinities of DMT1  
385 whereby Mn and Cd are transported in preference to iron and Pb (Garrick et al., 2006). The slightly  
386 lower increase in Pb concentrations compared to Mn and Cd for the same change in serum ferritin  
387 concentrations for both pre-pregnancy and ANC2 results may indicate the competition between  
388 these metals for absorption through this transport mechanism. A similar pattern was found in a  
389 Norwegian cohort monitored for metals mid-pregnancy where blood concentrations of Mn and Cd  
390 were found to be significantly higher in women who were iron deficient, with a greater difference in  
391 Mn concentrations compared to Cd concentrations. Pb concentrations in this cohort however were  
392 not found to be significantly different between iron deficient and non-iron deficient women  
393 (Caspersen et al., 2019).

394 *4.3 Selenium*

395 For optimal activity of Se dependent proteins, concentrations in whole blood would be expected to  
396 be in the range 125-163 µg/L (Muecke et al., 2018). This is similar to the concentrations measured in  
397 the first trimester for women in this study where the median value was 149.1 and the inter-quartile  
398 range 132.1-167.3 µg/L. Although a significant positive association was found between serum ferritin  
399 concentrations and Se concentrations, the effect was quite small. For example all other parameters  
400 being equal, the difference between Se concentration if increasing from the minimum pre-pregnancy  
401 serum ferritin concentration of 4.3 to the average of 42 µg/l would be an increase of 7 µg/l which is a  
402 relatively small difference on the scale of the Se results measured. Similar results were found in a  
403 mid-pregnancy cohort where a small but significant increase in blood Se concentrations was found in  
404 non iron deficient women compared to iron deficient women (Caspersen et al., 2019). The adjusted R  
405 squared values for the Se models in this study were the lowest of those that found a significant  
406 association explaining only 7% to 8% of the variation in the dataset indicating that other important  
407 factors are certainly influencing Se values. Although there is some evidence that low selenium  
408 concentrations can be associated with anaemia and iron deficiency anaemia, increasing iron stores is  
409 not thought to have a direct effect on selenium uptake (Gürgöze et al., 2004; Semba et al., 2009;  
410 Viita et al., 1989; Yetgin et al., 1992).

411 *4.4 Limitations and Strengths*

412 There are several factors that add to measurement uncertainty in the dataset. The time between the  
413 blood draw before pregnancy and pregnancy confirmation varied widely from just over 2 weeks to  
414 almost 2 years and iron status may have changed over time. There were also differences in the  
415 timing of ANC visits meaning that iron supplementation would have started at different points during  
416 the pregnancy and that what was intended as the first trimester blood draw did not take place until  
417 early in the second trimester for 16% of women in the sample and both of these could have affected  
418 the concentrations of metals measured. In particular Mn has a relatively low half-life in blood of  
419 around 30 days (Finley et al., 2003) so changes in iron status between the pre-pregnancy and ANC2  
420 blood draw may not fully reflect Mn exposure over that time period. Nevertheless, there was a  
421 strong correlation between serum ferritin concentrations at the pre-pregnancy and ANC2 blood  
422 draws ( $r_s=0.65$ ) which implies that the differing time periods did not change relationships too much.  
423 If some women experienced longer periods of iron supplementation than others, this may have  
424 resulted in some low pre-pregnancy serum ferritin concentrations not corresponding to higher  
425 concentrations of Mn at ANC2. This would have reduced the strength of the associations found in the  
426 regression analysis as well as reducing the value of the coefficients. Conversely, for the women who  
427 were not tested for metals until the second trimester of pregnancy, metal concentrations may look  
428 higher than they actually were for a normal serum ferritin concentration, given that the uptake of  
429 some metals increase during pregnancy. This would have had the opposite effect on the regression  
430 coefficients, potentially increasing above their true value while also reducing the strength of the  
431 association.

432 The major strength of this study is that detailed information was collected prior to conception which  
433 is not often available in comparable pregnancy cohorts. The number of metals assessed and the  
434 ability to correct for inflammation in this context adds to understanding of environmental exposures



435 in Benin for which there are few existing studies. It highlights that women with lower iron stores  
436 prior to pregnancy, even if not classified as iron deficient, are at risk of absorbing higher amounts of  
437 metals from their environments that are detrimental to the health of both mother and child. It is  
438 suggested that testing the effects of iron and folic acid supplementation beginning prior to  
439 conception on concentrations of Mn, Cd and Pb during pregnancy would be worthwhile to determine  
440 if fetal exposures can be reduced without any other negative effects.

## 441 **5. Conclusion**

442 This study shows that serum ferritin concentrations are negatively associated with increased uptake  
443 of the toxic metals Mn, Cd and Pb in pregnancy. Importantly, lower serum ferritin concentrations  
444 prior to conception were found to be associated with higher concentrations of Mn, Cd and Pb  
445 suggesting that increasing iron both before and during pregnancy may limit uptake of these metals.  
446 These results argue in favour of testing the effects of iron supplementation prior to pregnancy to  
447 better safeguard foetal development.

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660 **Supplementary Materials**

661 **Table S1:** Maternal blood metal concentrations at ANC2 visit (µg/l)

Metal	n	LOQ (µg/L)	%<LOQ	Geometric Mean (95%CI)	Median	Min	Max	IQR
Mn	262	5	0	11.0 (10.6, 11.4)	11.2	5.12	41.7	8.76-13.2
Cu	262	200	0	1318 (1289, 1348)	1333	743.4	2378	1165-1490
Zn	262	200	0	5157 (5019, 5298)	5074	2596	13167	4541-5830
Se	202	20	0	148.1 (144.4, 151.9)	149.1	89.6	235.7	132.1-167.3
Pb	262	2	0	31.6 (30.3, 32.9)	31.3	14.7	366.5	25.7-38.9
Cd <sup>a</sup>	262	0.25	43	0.254 (0.242, 0.266)	0.253	0.177	1.01	0.177-0.331
Hg <sup>a</sup>	202	0.4	1.5	1.20 (1.13, 1.27)	1.20	0.283	3.98	0.907-1.56
As	202	1.0	0	5.96 (5.67, 6.26)	5.84	2.04	16.6	4.65-7.22

662 <sup>a</sup>Results below the limit of quantification (LOQ) were replaced by the LOQ divided by the square root of 2. For Cd the  
 663 number of results below LOQ was 113 and for Hg 3.

664 **Table S2:** Correlations between metals at ANC2 – Spearman rank correlation test

Metal	Mn	Cu	Zn	Se	Pb	Cd <sup>a</sup>	Hg <sup>a</sup>	As
Mn	1							
Cu	0.03	1						
Zn	0.29	-0.03	1					
Se	-0.01	0.01	0.10	1				
Pb	0.31	0.03	0.14	0.08	1			
Cd <sup>a</sup>	0.37	0.03	0.21	0.008	0.29	1		
Hg <sup>a</sup>	0.11	0.08	0.14	0.65	0.14	0.07	1	
As	-0.003	0.17	-0.04	0.12	0.10	0.04	0.20	1

665 <sup>a</sup>Results below the limit of quantification (LOQ) were replaced by the LOQ divided by the square root of 2. For Cd the  
 666 number of results below LOQ was 113 and for Hg 3