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Short-term effects of very low dose cadmium feeding on copper, manganese and iron homeostasis: a gastropod perspective

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Highlights

- Low-dose Cd feeding to land snails induced an increase of hepatopancreas Cu and Mn concentrations
- No significant effect was triggered by Cd intake on hepatopancreas iron values
- Low levels of Cd feeding have no long-term effects on the Cu, Mn and Fe homeostasis

Abstract

The available information on the interplay between low-dose cadmium intake and copper, manganese, and iron homeostasis in invertebrates is limited. We have currently studied the accumulation of these trace metals in the hepatopancreas of adult snails, *Cantareus aspersus*, following 14 and 28 days of exposure to low doses of dietary cadmium, up to 1 mg/kg dw

(dry weight). The cadmium dose, but not the duration of exposure, had a significant effect on hepatopancreas copper deposition, the values being significantly elevated compared to controls. A significant peak in manganese levels at 14 days was found in snails administered the lowest cadmium dose. These increases occurred even in the absence of cadmium increase in the hepatopancreas. Our data suggest that low dose cadmium feeding can produce a transient disturbance in hepatopancreas copper and manganese homeostasis. Such responses may serve as early biomarkers of physiological changes occurring during the initial stages of cadmium intoxication.

Keywords: cadmium, copper, manganese, iron, dietary exposure, land snails

1. Introduction

Cadmium (Cd) is one of the most well-studied environmentally hazardous trace metals (TMs) due to its extreme toxicity to humans and animals, long biological half-life, and high mobility along food webs (ATSDR, 2012). The typical natural abundance of Cd in soils ranges from 0.1 to 0.5 mg/kg dw, with the values measured in plants growing on non-contaminated soils varying between 0.01 and 1 mg/kg dw. Dietary intake is the main route of Cd exposure in humans and most terrestrial fauna (Landner and Reuther, 2006; Peralta-Videa *et al.*, 2009; ATSDR, 2012), and hence there is an extensive body of legislation worldwide dealing with the hazard associated with dietary cadmium. For example, the European Union (EU) has set restriction limits for concentrations (expressed as milligrams per kilogram on a dry weight basis, mg/kg dw) allowed in vegetal foods for human consumption, such as fruits (0.05 mg/kg dw), stem vegetables (0.1 mg/kg dw), and leafy vegetables (0.2 mg/kg dw) (EC Regulation no. 629/2008). Similarly low, but environmentally-relevant dietary Cd levels, are commonly used in toxicological studies with mammal study systems, but rarely with invertebrate model organisms.

Once accumulated, cadmium interferes with normal physiological functions at multiple levels, including the delicate equilibrium existing between endogenous TM concentrations (ATSDR, 2012). Such interactions have been demonstrated in mammals for several key essential TMs, such as copper (Cu), manganese (Mn), and iron (Fe) (Davies and Campbell, 1977; Goering and Klaassen, 1985; Sarhan *et al.*, 1986; Gruden and Matausic, 1989; Kotyzova *et al*., 1990; Hook and Lucier, 1998; Ryu *et al.*, 2004; Eybl and Kotyzova, 2010). These metals serve as important prosthetic groups for many metalloenzymes, including

DNA polymerase, carbonic anhydrase, superoxide dismutase, and ribonucleotide dismutase (Kessissouglou, 2012). However, little is known about similar interactions in land snails, despite their importance as major herbivores in terrestrial ecosystems and pertinent bio-indicators of environmental contamination (Gerlach *et al.*, 2013). These invertebrates serve as excellent macro-concentrators for cadmium (Dallinger and Rainbow, 1993), with the hepatopancreas being the main retention endpoint (Russel *et al.*, 1981; Rabitsch, 1996; Dallinger *et al.*, 2004; Fritsch *et al.*, 2011; Pauget *et al.*, 2013). Therefore, the concentration of Cd in this organ is a reliable tool for exposure assessment and provides accurate estimates of its bioavailability for these mollusks (Rabitsch, 1996; Nica *et al.*, 2015).

We have currently investigated the short-term effects of dietary Cd uptake (as cadmium sulfate) on the concentration of Cd, Cu, Mn, and Fe in the hepatopancreas of land snails. *Cantareus aspersus* (Müller, 1774) was used as a study system because its physiology is well understood and because it is easily reared, both under field, as well as laboratory conditions (Garcia *et al.*, 2013). Mature specimens were given low, but environmentally-relevant dietary Cd doses for 28 days and TM concentrations were determined at 14 and 28 days. Copper plays a central role for molluscan metabolism (Nica *et al.*, 2013) and the currently available information on cadmium-iron and cadmium-manganese interactions in invertebrates is relatively limited. The understanding of these interactions provides a new perspective on the metalomic implications of low-level cadmium exposure and may reveal new insights into cadmium toxicokinetics.

2. Materials and Methods

2.1. Breeding methods

Newly matured specimens of *Cantareus aspersus* (mean height 25.9 mm; mean weight 8.25 g) were obtained in February 2017 from the "Mokry Dwór" snail farm (Krzymów, Wielkopolska, Poland). Gastropods were transferred to a climate-controlled room (18-20°C, 12 h L: 12 h D) and placed, in groups of 15, in 30-liter aerated polypropylene terrariums (70.5 x 39.5 x 18.5 cm) with a perforated lid. After being held for four days without food to acclimatize to laboratory conditions (Itziou*et al.*, 2011), the live snails were fed Cd-spiked diets for 28 days. Five experimental doses, with three replicate jars per dose, were used; the nominal concentrations were: 0, 0.02, 0.05, 0.2, and 1 mg/kg dw. These doses were selected based on the reference values for maximum Cd concentrations allowed in vegetal foods on which the snails routinely feed, such as fruits (0.05 mg/kg dw), stem vegetables (0.1 mg/kg

dw), and leafy vegetables (0.2 mg/kg dw) (MWFEP 2002; EC Regulation no. 629/2008). The treatments were abbreviated as 0Cd (controls), 0.02Cd, 0.05Cd, 0.2Cd, and 1Cd. We used cadmium sulfate ($CdSO_4$, 99.99% trace metal basis, Sigma-Aldrich) as a source of cadmium. Cadmium sulfate will extend our knowledge on the bioavailability of different chemical forms of Cd in land snails, which is currently limited to cadmium chloride (Cœurdassier *et al.*, 2002; Itziou and Dimitriadis, 2011; Nica *et al.*, 2015; Nica *et al.*, 2017a, 2017b) and cadmium nitrate (Laskowski and Hopkin, 1996).

An artificial fodder was used to achieve an even distribution of cadmium in the snail feeding medium. The feeding medium was prepared by mixing 15 g agar powder (A-1296, Sigma), 20 g carrot baby food (HiPP, UK), 50 g fortified infant cereals (Nestle Nestum5 – Five Cereals), and 3 mL fungicide (1% methyl paraben solution) with solutions of cadmium sulfate in double distilled water to give 1L of medium containing the desired concentration of cadmium. The feeding medium was divided in forty Petri plates (containing 25 mL medium/plate). After cooling, the Petri plates were maintained in a refrigerator for a maximum period of one week. To provide a moist micro-environment for snail breeding, a layer of ash-free filter paper was placed on the bottom of each terrarium and was wetted twice daily with double distilled water by using a pressure sprayer. The daily activity schedule involved fresh fodder supply (two Petri dishes per each terrarium), monitoring snail fitness, removing feces and remaining food, replacing the paper sheets and collecting the dead specimens. The terrariums were cleaned three times weekly with double distilled water. For each treatment group, eight snails were randomly collected at 14 and 28 days, with at least two animals being sampled for each replicate jar. The snails were kept for two days without food to empty their gut (Notten *et al.*, 2006) and were then sacrificed. The hepatopancreas was detached from the visceral mass and was washed in double distilled water. The samples were dried on cellulose tissue and stored at -80°C until further analyses.

2.2. Chemical analyses

Hepatopancreas samples to be analyzed for Cd, Cu, Mn and Fe were thawed, oven dried (105°C, 24 hours), and then weighed to the nearest 0.01 mg, using an analytical balance (TP-214, Denver Instrument Gmbh), in order to obtain the dry weight. After calcination in a muffle furnace (Nabertherm B150, Lilienthal; 550°C, 6 hours), the resulting ash was submitted to wet acid digestion. Briefly, the ash was treated with 0.5 mL of 65% HNO_3 (Merck, suprapure), heated to dryness and dissolved in 20 mL of 0.5 N HNO_3. After filtration

through ash-free filter paper, the volume of each sample was brought to 30 mL with 10 mL HNO_3 0.5 N. A similar approach was used for assessing Cd levels in food samples.

The metal ion content in the filtrates was determined by flame (air-acetylene) atomic absorption spectrophotometry (VARIAN AA240FS), fitted with a metal-specific hollow-cathode lamp as a source of radiation. Standard solutions (1000 mg/L) of Cd, Cu, Mn, and Fe - ICP Multielement Standard solution IV CertiPUR, were obtained from Merck (Merck KGaA, Darmstadt, Germany). The reagents and standard solutions were prepared with double distilled water (spectroscopic pure). All glassware was treated with Pierce solution 20% (v/v), rinsed with cold tap water, washed with 20% (v/v) nitric acid, and then rinsed again with double distilled water. Blank samples were also analyzed during the procedure to assure the accuracy and homogeneity of the results, while samples were analyzed in triplicate. For quality assurance, the NCS Certified Reference Materials DC 85104a and DC 85105a (China National Analysis Centre for Iron&Steel) were used. The percent recovery means were: Cd (104%), Cu (103%), Mn (98%), Fe (93%). The variation coefficients were below 8%. All detection limits (expressed in mg/kg) were assessed by using the calibration curve method: Cd (0.01), Cu (0.08), Mn (0.13), and Fe (0.04). Blank samples were run every 10 samples, and after analyzing 30 samples one standard was measured to verify consistency in measurements.

2.3. Statistical analysis

Hepatopancreas TM levels were first log-transformed (decimal logarithmation). At each time point, the data sets were evaluated for normality using Anderson-Darling tests and for homoscedasticity with Levene's tests, respectively. Prior to conducting the MANOVA analysis, a series of Pearson correlations were performed between all the dependent variables (DVs) in order to test whether these variables correlate with each other in the moderate range (Meyers *et al.*, 2006). If this condition was not met, separate two-way between-subjects ANOVAs of dietary dose and exposure duration were conducted on each DV (Meyers *et al.*, 2006). Whenever necessary, post hoc analysis was performed using the Bonferroni approach. In case of a significant main effect, all pairwise comparisons were conducted against the earliest time point. For a significant interaction effect between the two predictors, these comparisons were conducted at each time point using the corresponding controls as reference groups.

3. Results

Average levels of cadmium in food were: (***i***) for the 0Cd treatment (controls), below the detection limit (0.010 mg/kg dw); (***ii***) for the 0.02Cd treatment, 0.02 ± 0.01 mg/kg dw; (***iii***) for the 0.05Cd treatment, 0.04 ± 0.02 mg/kg dw; (***iv***) for the 0.2Cd treatment, 0.19 ± 0.03 mg/kg dw; (***v***) for the 1Cd treatment, 0.98 ± 0.13 mg/kg dw. Hepatopancreas Cd levels tended to increase with exposure dose and duration (Table 1). In contrast, no dose-dependent response to low dose Cd feeding was observed for the hepatopancreas copper, manganese, and iron (Table 1). However, copper levels were higher in the hepatopancreas of Cd-exposed specimens than in controls during the 0-14 days period and the 15-28 days period (Table 1). We also noted elevated concentrations of manganese relative to those found in controls during the first period, whereas no clear trend was evident for hepatopancreas iron (Table 1). Despite all these changes in the levels of TMs analyzed, the mortalities recorded were very low during both the 0-14 days period and the 15-28 days period (up to 4.65%); these mortalities are similar to that observed within the acclimatization period (2.44%).

Because no significant correlations were observed between most DVs, (Table 2), separate two-way between-subjects ANOVAs of dietary dose and exposure duration were conducted on each DV instead of MANOVA analysis (Meyers *et al.*, 2006). The $\log_{10}$-transformed data for Cd, Cu, Mn, and Fe concentrations in the hepatopancreas of adult *C. aspersus* were found to be statistically normal (Anderson-Darling test, $p \geq 0.075$) and homoscedastic (Levene's test, $p \geq 0.081$). This shows that the conditions necessary for the application of the two-way ANOVA were met for all DVs.

We identified a significant main effect of dietary dose on cadmium content of the hepatopancreas ($F(4, 36) = 128.98$, $p < 0.001$; Fig. 1A). Post hoc analyses with the Bonferroni procedure showed a significant dose-dependent increase of the measured values from the second highest dose onward (Table 1). There was also a consistent main effect of exposure duration ($F(1, 39) = 58.54$, $p < 0.001$; Fig. 1B), with hepatopancreas cadmium being significantly elevated for the 15-28 days period when compared to the 0-14 days period (Fig. 1B). However, these effects were qualified by a significant interaction between these variables ($F(4, 34) = 9.89$, $p < 0.001$), which implies that the main effect of exposure duration is dependent on the dose of cadmium. More precisely, at 14 days of exposure (Table 1), hepatopancreas cadmium was significantly higher for the 1Cd treatment compared to controls, whereas at 28 days (Table 1), this response was observed for both the 0.2Cd treatment and the 1Cd treatment.

The dietary cadmium exerted a significant main effect on the hepatopancreas copper content ($F(4, 36) = 17.77$, $p < 0.001$; Fig. 1A). For all treatment groups, the measured values at 14 days were significantly increased, but the magnitude of response was quite homogeneous among different dose groups (Table 1). However, no consistent main effect was found for exposure duration ($F(1, 39) = 0.087$, $p = 0.770$; Fig. 1B), as well as no significant interaction between dietary Cd dose and exposure duration ($F(4, 34) = 2.126$, $p = 0.102$).

Although no significant main effect on hepatopancreas manganese was identified for either food Cd level ($F(4, 36) = 2.59$, $p = 0.058$; Fig. 1A) or exposure time ($F(1, 39) = 0.08$, $p = 0.778$; Fig. 1B), statistical analysis revealed a significant interaction between these two variables ($F(4, 34) = 3.89$, $p = 0.011$). Posthoc analysis with the Bonferroni procedure indicated a significant increase in manganese concentrations in gastropods exposed for 14 days to the lowest dietary dose, but no consistent effect was detected for the other paired comparisons (Table 1). Moreover, there were no main effects of cadmium dose ($F(4, 36) = 1.410$, $p = 0.254$; Fig. 1A) or exposure duration ($F(1, 39) = 2.917$, $p = 0.097$; Fig. 1B) on iron hepatopancreas content, nor a significant interaction between these two variables ($F(4, 34) = 1.871$, $p = 0.125$).

4. Discussion

Numerous studies have examined the retention of dietary cadmium in the soft tissues of land snails (Russell *et al.*, 1981; Berger and Dallinger, 1989; Laskowski and Hopkin, 1996; Gomot, 1997; Notten *et al.*, 2006; Gimbert *et al.*, 2008; Itziou and Dimitriadis, 2011), but not at very low, but environmentally-relevant dietary levels below 0.1mg/kg dw (Nica *et al.*, 2017b). In the current study, a 28-day exposure to doses as low as 0.2 mg/kg dw Cd (as cadmium sulfate) yielded significantly elevated hepatopancreas cadmium levels. This is similar to the trend we observed for diets contaminated with Cd in the form of cadmium chloride (Nica *et al.*, 2017a). In contrast, no evident effect of dietary dose on Cd levels in the soft tissues was found following a 56-day exposure to 0.28 mg/kg Cd in the form of cadmium nitrate (Laskowski and Hopkin, 1996). The different bioavailability of the various chemical forms of cadmium — as has been already described in the case of copper (Gomot and Pihan, 1997) — as well as methodological issues, may contribute towards the discrepancies mentioned above.

The results of the present study support the existence of a threshold (approximately 10 mg/kg dw), below which *C. aspersus* adults are able to maintain stable Cd levels in this organ. This threshold is in accordance with our previous findings, when using cadmium chloride as a source of cadmium (Nica *et al.*, 2017a). Two physiological mechanisms, relocation into the foot and excretion via faeces, have been proposed for the control of Cd organ contents (Gimbert*et al.*, 2006). It is, however, likely that similar to the grove snail *Cepaea nemoralis* (Linnaeus, 1758), excretion via mucus also contributes to cadmium regulation in *C. aspersus* (Notten *et al.*, 2006).

With regard to metal-metal interactions, we provide evidence, for the first time, that low-level dietary cadmium intake can temporarily perturb copper and manganese homeostasis in terrestrial gastropods. This finding contributes significantly towards the understanding of the interplay between toxic and essential TMs in invertebrate study systems. Absolute levels of Cu, Mn, and Fe in controls were comparable to those measured for *C. aspersus* adults collected from areas with normal concentrations of Cd in soil/vegetation (De Vaufleury and Pihan, 2000; Menta and Parisi, 2001; Beeby and Richmond, 2002; Amaral *et al.*, 2004; Scheifler *et al.*, 2006), or used as a reference group for dietary Cd exposure (Hispard *et al.*, 2008; Hockner *et al.*, 2011).

Although exposure duration had no influence on hepatopancreas copper, we identified a significant, but homogeneous effect of Cd dose, irrespective of the treatment. This trend is consistent with the results of previous studies showing that any increase of Cu concentration in the soft tissues of terrestrial gastropods is generally transient (Moser and Wieser, 1979; Swaileh and Ezzughayyar, 2000) and this TM is homeostatically regulated in the hepatopancreas of *C. aspersus* (Hockner *et al.*, 2011). In contrast to our findings, no effect was observed in adult specimens fed a high-dose Cd diet (17.2 mg/kg dw Cd) for 15 days (Hispard*et al.*, 2008). In vertebrates, cadmium exposure can increase hepatic copper accumulation (Davies and Campbell, 1977; Honda and Nogawa, 1987; Hollis *et al.*, 2001; Satarug *et al.*, 2001), although the opposite responsehas also been reported (Mills and Dalgarno, 1972; Doyle and Pfander, 1975; Nomiyama *et al.*, 1982, 1983; Brzoska *et al.*, 2002). This effect is mediated by changes in the gastrointestinal absorption of Cu, related to Cd-induced enteropathy (Murata *et al.*,1970), with the latter case being observed in advanced stages of cadmium intoxication (Foulkes, 2000). Therefore, it is reasonable to assume that this temporary disruption of Cu homeostasis reflects changes in gastrointestinal copper absorption which occur during the initial stages of Cd intoxication.

According to our findings, low-dose cadmium feeding had no significant influence on hepatopancreas iron. However, this exposure (the lowest dietary dose for 14 days) was associated with a significant increase of the manganese content in the hepatopancreas of *C. aspersus* adults, despite the lack of a dose- or time-dependent effect. In mammalian systems, elevated Mn concentrations can inhibit/reduce gastrointestinal Cd absorption (Goering and Klaassen, 1985; Sarhan *et al.*, 1986; Kotyzova *et al*., 1990; Ryu *et al.*, 2004; Eybl and Kotyzova, 2010) by competing with cadmium uptake via shared transport mechanisms (Himeno *et al.*, 2009). Our results may be indicative, like in the case of Cu, of a transient disturbance of Mn gastrointestinal absorption during the early stages of cadmium intoxication.

According to our findings, these significant increases in copper and manganese levels occurred even in the lack of observable cadmium elevation in the hepatopancreas. This response is interesting from an (eco)toxicological point of view, especially with respect to copper. Based on the homogeneity of response, Cu levels in the hepatopancreas seem to be more sensitive to short-term exposure to very low, but environmentally-relevant dietary Cd doses than cadmium accumulation itself. This may be related to the special needs of these invertebrates for copper (Nica *et al.*, 2013).

In conclusion, cadmium feeding at low, but environmentally-relevant levels (within the maximum Cd concentrations allowed in vegetal foods) have no long-term effects on the homeostasis of Cu, Mn and Fe in the hepatopancreas of *C. aspersus* snails. The interactions Cd-Cu and Cd-Mn observed here may also play a role in cadmium kinetics and toxicity. As a result, the present data could be used as a benchmark for future studies aiming at developing more accurate, physiologically-based, pharmacokinetic (PBPK) models for short-term Cd exposure, hence facilitating the extrapolation of animal data to support risk assessment in humans.

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Figures

Figure 1. Effect of low dose cadmium feeding on hepatopancreas cadmium, copper, manganese, and iron in adult snails, *Cantareus aspersus*.(**A**) Estimated marginal means for dietary dose (as the main effect). (**B**) Estimated marginal means for time (as the main effect).Eight snails were sampled for each treatment group at each time point. Post hoc tests were applied in case of significant main effects of cadmium dose or exposure duration. Marked columns (*) indicate significant differences as compared to the reference group (Bonferroni test, *—$p < 0.05$).

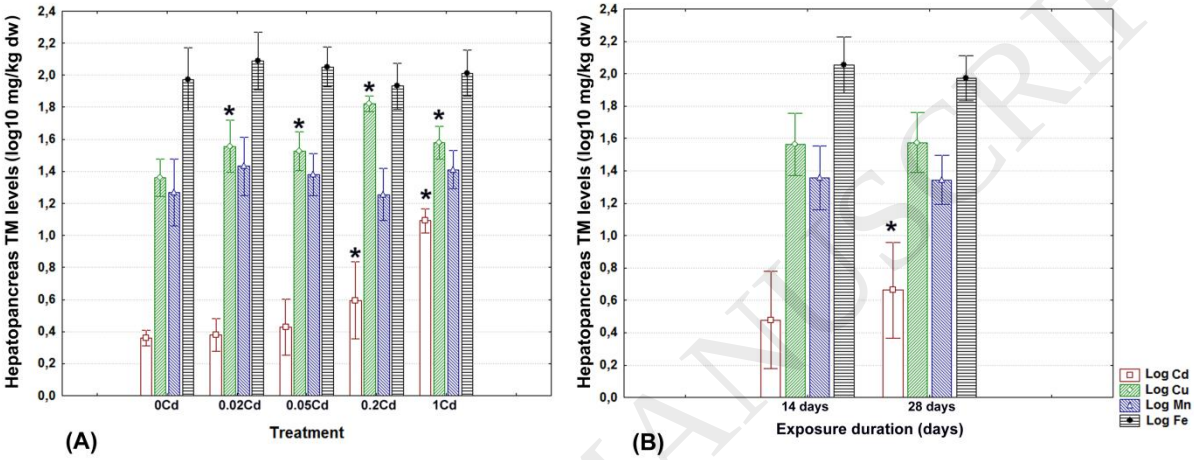

Table 1. Levels of Cd, Cu, Mn, and Fe in the hepatopancreas of adult *C. aspersus* at different time points.

		0Cd	**0.02Cd**	**0.05Cd**	**0.2Cd**	**1Cd**
Cd	14 days	2.23 ± 0.32	2.16 ± 0.44	2.01 ± 0.05	2.37 ± 0.29	11.30 ± 1.49 *
	28 days	2.38 ± 0.22	2.73 ± 0.55	3.71 ± 0.93	6.54 ± 0.27 *	13.73 ± 2.57 *
Cu	14 days	20.07 ± 5.00	43.77 ± 15.51	37.53 ± 6.15	63.78 ± 5.91	34.57 ± 7.43
	28 days	27.26 ± 5.17	32.79 ± 12.80	31.70 ± 9.88	69.65 ± 9.25	42.99 ± 9.98
Mn	14 days	14.59 ± 3.31	35.91 ± 6.09 *	26.45 ± 6.28	15.55 ± 5.15	31.54 ± 9.18
	28 days	26.72 ± 8.73	21.81 ± 8.15	23.40 ± 8.52	22.70 ± 6.72	21.93 ± 4.02
Fe	14 days	88.17 ± 38.66	166.25 ± 57.38	136.60 ± 27.64	86.61 ± 25.39	129.63 ± 47.95
	28 days	117.60 ± 53.69	99.78 ± 39.18	96.26 ± 23.29	92.26 ± 34.01	87.55 ± 19.68

Data measured at 14 days and 28 days are shown as mean ± standard deviation. Eight snails were sampled for each treatment group at each time point. The values are expressed as mg/kg dry weight. Post hoc tests were applied for DVs for which significant interactions between cadmium dose and exposure duration were found. Marked columns (*) indicate significant differences as compared to the reference group (Bonferroni test, *—$p <$ 0.05).

Table 2. Pearson correlations among log_{10}-transformed Cd, Cu, Mn, and Fe levels in the hepatopancreas.

	Log_{10} Cd	**Log_{10} Cu**	**Log_{10}Mn**	**Log_{10} Fe**
Log_{10} Cd				
Log_{10} Cu	0.190			
	(*p*=0.195)			
Log_{10}Mn	0.170	-0.010		
	(*p*=0.246)	(*p*=0.946)		
Log_{10} Fe	0.018	0.751	0.724*	
	(*p*=0.903)	(*p*=0.612)	(***p*=0.000**)	

For all metals, correlational analysis was conducted using data measured at both time points (n =16). Marked values (*) indicate significant correlations ($p< 0.05$)