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Hair as a Biomarker of Environmental Manganese Exposure

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Abstract

The absence of well-validated biomarkers of manganese (Mn) exposure in children remains a major obstacle for studies of Mn toxicity. We developed a hair cleaning methodology to establish the utility of hair as an exposure biomarker for Mn and other metals (Pb, Cr, Cu), using ICP-MS, scanning electron microscopy, and laser ablation ICP-MS to evaluate cleaning efficacy. Exogenous metal contamination on hair that was untreated or intentionally contaminated with dust or Mn-contaminated water was effectively removed using a cleaning method of 0.5% Triton X-100 sonication plus 1N nitric acid sonication. This cleaning method was then used on hair samples from children (n=121) in an ongoing study of environmental Mn exposure and related health effects. Mean hair Mn levels were 0.121 μg/g (median = 0.073 μg/g, range = 0.011 – 0.736 μg/g), which are ~4 to 70-fold lower than levels reported in other pediatric Mn studies. Hair Mn levels were also significantly higher in children living in the vicinity of active, but not historic, ferroalloy plant emissions compared to controls (P<0.001). These data show that exogenous metal contamination on hair can be effectively cleaned of exogenous metal contamination, and they substantiate the use of hair Mn levels as a biomarker of environmental Mn exposure in children.

Introduction

Studies in occupationally exposed adults[1–5] and environmentally exposed children[6–10] have reported associations between elevated manganese (Mn) exposure and neurological deficits, though details of the exposure-effect relationship are still being defined[5,11,12]. In part, this may be due to the challenges in accurately characterizing exposure, and to the fact that there are no well-recognized and validated biological markers of Mn exposure like there are for other trace metals such as lead (Pb)[13]. The identification and validation of exposure biomarkers is fundamental to human toxicology and risk assessment, and knowing the dose-response relationship is essential for the demonstration of cause and effect[14].

Biological markers of exposure should reflect an integration of the internalized dose over time. Studies in environmentally-exposed children have reported that hair Mn[6,10,15–18], Mn in the exposure medium (e.g., water)[6,9], but not blood Mn were predictors of exposure and/

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Supporting Information Available

Supplemental Section includes Supplemental Information, Supplemental Table S1 and Supplemental Figures S1 and S2. This information is available free of charge via the Internet at http://pubs.acs.org

or neurotoxic outcomes. The toxicokinetics of Mn suggest that exposure biomarkers such as blood and urine may at best reflect recent exposure (i.e., days - weeks), while hair may integrate or reflect exposures over longer timeframes (e.g., months)[5,19]. This suggests hair may integrate changes in circulating Mn levels and body burden over time better than blood or urine[6,10,16,20].

Analytical challenges associated with the accurate and precise measurement of Mn in hair may confound the assessment of hair Mn levels as a predictor of Mn body burden. Hair is highly susceptible to exogenous contamination, since Mn and other metals of toxicological interest are relatively common constituents of environmental media, such as soil and dust, and Mn can be naturally elevated in well-water[6,9,15]. However, there has been relatively little effort devoted to developing a cleaning/processing method to effectively remove exogenous hair Mn contamination[21–23], in contrast to the significant efforts devoted to developing and validating exposure biomarkers for other toxic metals such as lead[24–25]. The existing literature suggests substantial variability in hair Mn levels in environmentally exposed subjects, though interpreting these disparities is difficult. Studies suggest there are inherent differences in hair metal concentrations between individuals due to differences in the chemical composition of hair (e.g., amino acid and melanin content) and personal habits[26,27].

In addition, reported differences in hair Mn levels of environmentally-exposed subjects may also reflect the fact that studies have often used different methods for cleaning hair prior to analysis[6,10,15,16, 28,29]. For example, Sakai et al.[28] reported mean hair Mn levels of 2.3 μg/g (± 0.5 SD) in Japanese subjects aged 6 mo – 20yrs (n=418), where hair was washed with distilled deionized water followed by repeated washing with an ethanol-acetone mixture. Wright et al.[10] cleaned hair by sonicating in 1% Triton X-100 for 15 minutes, and reported mean hair Mn levels of 0.47 μg/g (range 0.089 – 2.15 μg/g, n=31) for children living in the vicinity of the Tar Creek, Oklahoma Superfund site. Bouchard et al.[15] did not wash hair samples before analyses and reported median hair Mn levels of 5.1 μg/g (range 0.28 – 20.0 μg/g, n=46) for children exposed to well-water Mn (160 or 610 μg Mn/L) in Québec; in a subsequent expanded study they utilized the cleaning method of Wright et al.[10] and reported median hair Mn levels of 0.7 μg/g (range 0.1 – 21 μg/g, n=302) from children exposed to Mn in water containing 0.1 – 2,700 μg Mn/L[6].

Here we developed a hair cleaning methodology to remove exogenous Mn contamination, so as to improve the potential utility of hair Mn as an exposure biomarker. Cleaning methods using Triton X-100, 1N nitric acid, and sonication in different combinations were explored using hair from human subjects that was untreated or intentionally contaminated with dust or Mn-contaminated water. Levels of Mn, Pb, chromium (Cr) and copper (Cu) were measured by high resolution ICP-MS. In addition, we used scanning electron microscopy to evaluate hair surface morphology, and laser ablation ICP-MS to specifically evaluate surface versus endogenous hair Mn under different conditions. We then used the developed cleaning method on hair samples from children (n=121) in an ongoing study of environmental Mn exposure and related health effects. Results show that hair Mn levels were significantly associated with residency in environments containing elevated air and surface soil Mn levels resulting from current or historic ferroalloy pant operations, substantiating the use of hair Mn levels as a biomarker of environmental Mn exposure in children.

Experimental

Study Design, Sample Collection and Treatment

Ten adult volunteers, five male and five female age 22 – 65 yrs, each donated ~2 g of hair to the study. Subsamples of hair from each subject were processed in triplicate (~50 mg each)

using one of five different hair cleaning/processing methodologies described below, and analyzed for Mn, Pb, Cr, Cu concentrations by ICP-MS (see Supplemental for details on hair collection and processing).

In addition, hair samples from a subset of five subjects were intentionally contaminated with house dust, or Mn-contaminated water containing 70 or 700 μg Mn/L, and cleaned using the methodologies described below to evaluate the efficacy of each cleaning procedure on highly contaminated hair. For this, hair from each subject was exposed to house dust (sieved <150 μm) at a ratio of 4:10 (hair:dust, w/w), and thoroughly mixed in a polyethylene bag (house dust typically contains ~160 μg Mn/g, based on our analyses of dust samples similar to that used here). Hair was then removed with stainless steel forceps and shaken vigorously to remove excess dust. For Mn water contamination, hair samples were immersed for 2 hours in tap water solutions containing 70 or 700 μg Mn/L, and then rinsed five-times with ultrapure (18 MOhm-cm^2) Milli-Q (MQ) water before cleaning.

Hair Cleaning and Processing

The untreated hair from each of the 10 subjects was aliquoted in triplicate (~50 mg each) into 5 mL polypropylene syringe bodies, and processed using one of the following five procedures: (1) No cleaning; hair was processed for analyses without cleaning. (2) MQ water rinse; hair was rinsed five-times with ultrapure MQ water. (3) Triton sonication (T); hair was sonicated in 0.5% Triton X-100 for 10 minutes, rinsed five-times with MQ water, rinsed once with 1N trace metal grade (TMG) nitric acid, and rinsed five-times with MQ water. (4) Triton and nitric sonication (TN); hair was sonicated for 20 minutes in 0.5% Triton, rinsed five-times with MQ water, sonicated for 10 minutes in 1N TMG nitric acid, rinsed once with 1N TMG nitric acid, and then rinsed five-times with MQ water. And (5) 3xTN; the Triton and nitric acid sonication (TN) cleaning described above was performed three-times in succession. Hair samples from the subset of five subjects that were intentionally contaminated with dust or Mn-contaminated water were cleaned with the same procedures, except the 'No cleaning' method was omitted. In all cases the final 5x MQ water rinse was performed in a HEPA filtered air cleanroom using trace metal clean procedures. Our selection of cleaning methods was based on several factors, including 1) building on the methods that have been used in prior studies reporting hair Mn levels in Mn-exposed children[6,10,17], and 2) our experience over the past two decades developing methods for the accurate quantitation of Pb in environmental and biological samples[24,30–32].

Hair processing and analysis for metal concentrations

Hair samples were processed for analyses using established trace metal clean techniques in a HEPA filtered-air laboratory following procedures previously described[25]. Briefly, following the final MQ water rinse, hair samples were dried overnight at 65 °C, and ~30 mg of dry hair was digested in 0.5 mL 15.7 N double quartz-distilled nitric acid at 80 °C for 6 h. Rhodium (Rh) and thalium (Tl) were added as internal standards, and samples were analyzed for ^{208}Pb and ^{205}Tl (low resolution), and ^{52}Cr, ^{55}Mn, ^{63}Cu, and ^{103}Rh (medium resolution) by magnetic sector inductively coupled plasma mass spectrometry (Thermo Scientific - Element XR ICP-MS). The analytical detection limits for Mn, Pb, Cr and Cu were 0.051, 0.003, 0.023, and 0.175 ng/mL, respectively. (See Supplemental for processing details).

Scanning electron microscopy analysis of hair

To evaluate the visible morphology of the hair samples, two strands (~2 cm each) of untreated or intentionally contaminated (700 μg Mn/L water) hair per treatment/cleaning scheme per subject were selected from three subjects and analyzed by scanning electron microscopy at 1500X magnification (see Supplemental for details).

Laser ablation ICP-MS

Selected hair strands were analyzed for surface and interior Mn distribution using a Photon Machines Analyte 193 nm ArF excimer laser, interfaced with the XR ICP-MS. Three separate 500 μm sections, separated by ~500 μm were analyzed on each hair strand. Each 500 μm section represented ~2 days of hair growth, based on average adult hair growth rates of ~250 μm/d[33] (see Supplemental for details).

Hair Mn levels in children exposed to environmental Mn

Hair samples were collected from 121 children age 11 – 13 yrs as part of an on-going epidemiologic study of the health effects of environmental Mn exposure from ferroalloy plant operations in northern Italy[29]. Briefly, subjects were lifelong residents in one of three study areas: (1) Bagnolo Mella (n= 44), a town with a currently active ferroalloy plant that has been in operation since approximately 1970; (2) Valcamonica (n=41), a region with several historically-active ferroalloy plants that operated for a century, ceasing operation in 2001; and (3) Garda Lake (n=36), a reference region with no history of ferroalloy plant operations. Recent studies have documented elevated air, deposited dust, and surface soil Mn levels resulting from current or historic ferroalloy pant operations in these regions[34,35]. Subjects were recruited through the public school system according to a community-based participatory approach. Full details of subject recruitment, exposure assessments, and health outcome assessments are reported elsewhere[29]. Hair samples (5 – 30 mg) were processed for analyses using trace metal clean techniques and the 0.5% Triton + 1N nitric acid sonication cleaning process (TN) detailed above.

Data analyses

Summary data are expressed as mean ± standard error (SE), or mean ± standard deviation (SD), as appropriate. Data were analyzed to test specific hypotheses using one-way analysis of variance (ANOVA) using JMP software (Version 10, 2012). Individual comparisons were done using Tukey's post-hoc test. If necessary, data were square root-transformed or log-transformed to achieve normality and variance equality. A P-value ≤0.05 for the various outcomes was considered statistically significant.

Results and Discussion

1) Cleaning hair with 0.5% Triton + 1N nitric acid sonication effectively removes exogenous Mn and other metals from hair

In order to determine how cleaning affected hair Mn, Pb, Cr, and Cu concentrations, we cleaned hair from 10 adult subjects with five different procedures. Results show that there was a significant effect of cleaning procedure on hair Mn levels (ANOVA $F_{(4, 148)} = 38.55$, $p < 0.0001$), which decreased as the apparent strength of cleaning procedure increased in the order: MQ water rinsing (MQ) > Triton sonication + 1N nitric acid rinse (T) > Triton sonication + 1N nitric acid sonication (TN) (Figure 1, Table 1). The latter TN cleaning method reduced hair Mn levels by ~80% compared to uncleaned hair. Similar results were obtained with reductions in Pb, Cu and Cr hair levels (ANOVA $F_{(4, 148)} = 19.77$, $F_{(4, 148)} = 7.87$, and $F_{(4, 148)} = 2.68$, respectively, $P<0.0001$ for Pb and Cu, and $P=0.034$ for Cr), with significant overall reductions of ~70% compared to uncleaned hair (Table 1). Collectively, these results indicate that the Triton sonication + 1N nitric acid sonication cleaning method reduces significantly more exogenous Mn (and Pb, Cu, and Cr) compared to the less-rigorous cleaning methods evaluated (methods MQ or T). Moreover, without any cleaning or with simple MQ water rinsing, hair Mn levels remained elevated by ~4 – 5-fold over levels in Triton +1N nitric acid sonication cleaned hair.

Previous studies that specifically explored cleaning methods to remove exogenous hair Mn were unable to settle on an effective cleaning procedure. Stauber et al.[21], using adult hair intentionally contaminated with synthetic sweat and dust, evaluated 20 different cleaning agents, which did not include those evaluated here, and found at best a 15-minute sonication in 0.1 M HCl or 0.1 M cysteine-HCl reduced exogenous Mn by 71% and 51%, respectively, in addition to the undesirable effect of (presumably) removing endogenous hair Mn. Buckley et al.[22], using adult hair intentionally contaminated with Mn-contaminated water, tested sonication in Triton X-100, sodium lauryl sulfate or sodium EDTA. Sodium lauryl sulfate removed the most Mn (~75%), but none of the methods removed all exogenous Mn. Mikasa et al.[23], using untreated adult hair, tested procedures using various combinations of acetone, ethanol, polyethylene glycol lauryl ether, HCl, and sonication, and concluded that hair Mn contamination was not effectively removed by any of the procedures tested. These studies underscore the need for a validated hair cleaning methodology that effectively removes exogenous Mn without altering metabolically incorporated Mn so as to improve the potential for hair to serve as a biomarker of internal Mn exposure.

As with any potential exposure biomarker that is subject to external contamination, it is important to effectively minimize or remove exogenous contamination without 'over-cleaning', i.e., altering metabolically incorporated metals that may reflect exposure and body burden[5,25]. We evaluated if the Triton sonication + 1N nitric acid sonication (TN) cleaning method, which appeared effective based on our results above, was possibly removing endogenous hair Mn and other metals. For this, hair samples were cleaned sequentially three-times with the TN cleaning method (3xTN); if the TN cleaning method was removing endogenous hair metals, we would expect metal levels in hair cleaned with the 3xTN method to be significantly lower than in hair cleaned only once. Results show that hair levels of Mn, Pb, Cr, or Cu in the TN versus 3xTN-cleaned were not significantly different from each other (Figure 1, Table 1).

Since one cannot definitively distinguish Mn in hair derived exogenously versus metabolically, it is not possible to definitively distinguish removal of exogenous versus metabolically incorporated endogenous hair Mn with cleaning. Nonetheless, these results suggest that the Triton+nitric (TN) cleaning method does not over-clean and remove metabolically incorporated metals from hair. The relative difference in cleaning strength between the Triton vs TN methods may be regarded as relatively small (i.e., the latter used a slightly longer sonication in Triton plus sonication in 1N nitric rather than simply rinsing in 1N nitric as in the Triton method), but these methodological differences resulted in significant additional reductions in hair Mn and Pb (but not Cr and Cu) levels in the Triton vs TN-cleaned hair. In contrast, the relative difference in cleaning strength between the TN vs 3xTN methods may be regarded as quite large, with the 3xTN method being much more aggressive. It is therefore noteworthy that the 3xTN method did not significantly reduce levels of hair Mn, Pb, Cr, or Cu compared to those in the TN-cleaned hair samples (Figure 1, Table 1). If endogenous, metabolically incorporated metals were being inadvertently removed from hair with cleaning, one would expect significant reductions in all metals with repeated TN cleanings, which was not observed.

2) The TN method cleans highly contaminated hair

Since the potential exists for hair to become highly contaminated from prolonged contact with Mn-contaminated water, dust, and airborne particles[5,6,9,15–17,29], we investigated whether the Triton sonication + 1N nitric acid sonication (TN) method would also remove excessive exogenous contamination from hair treated with house dust (hair:dust, 4:10 (w/w)) or Mn-contaminated water at levels of 70 or 700 μg Mn/L. The Mn levels in water were selected to slightly exceed the USEPA secondary standard for Mn in water (50 μg Mn/L)[36], or to be comparable to highly elevated Mn levels in well-water reported in several

epidemiologic studies of Mn exposure[6,9,15]. We immersed hair samples in the Mn-contaminated water for 2 hrs, which is significantly longer than a typical shower or bath, but it is less than the ~4 hrs or more of cumulative water contact estimated from routine bathing over the duration of hair growth (see Supplemental for detailed rationale). These contamination treatments, followed by rinsing with MQ water, increased hair Mn levels by ~7 to 13-fold compared to untreated, uncleaned hair (Table 1), and are comparable to hair Mn levels reported for subjects in Mn-contaminated environments[6,15,16,18,37,38].

Results show that Mn, Pb, Cr, and Cu levels in hair excessively contaminated with house dust were significantly reduced with increasing strength of cleaning (ANOVA $F_{(3, 56)}$ = 58.81, $F_{(3, 56)}$ = 19.34, $F_{(3, 56)}$ = 11.89, and $F_{(3, 56)}$ = 5.58, respectively, with P's<0.0001 for Mn, Pb, and Cr, and P=0.002 for Cu) (Table 1). Similarly, Mn levels in hair contaminated with water containing 70 or 700 μg Mn/L were also significantly reduced by cleaning (ANOVA $F_{(3, 68)}$ = 7.298, P=0.0003, and $F_{(3, 67)}$ = 12.29, P<0.0001, respectively), with the TN method again performing best (Table 1).

In order to specifically test whether exogenously contaminated hair could be sufficiently cleaned using the Triton sonication +1N nitric acid sonication (TN) method, we normalized hair Mn levels from each cleaning method to the Mn levels in the untreated, TN-cleaned (UTN) hair sample within subject. We considered the UTN hair sample to best reflect endogenous hair Mn levels in the absence of external contamination. Levels of Mn in dust-contaminated hair cleaned with the TN or 3xTN method were not significantly different than levels in the UTN sample (P's >0.4) (Figure 2A); similar results were obtained with Pb, Cr, and Cu (P's ≥ 0.9). However, Mn levels in dust-contaminated hair cleaned with the slightly less-rigorous Triton sonication + nitric acid rinse (T) method remained significantly higher than levels in the UTN sample (P<0.0001 for Mn; P's=0.043, 0.11, and 0.013 for Pb, Cr, and Cu, respectively) (Figure 2A), suggesting this slightly less rigorous 'T' cleaning method may not adequately clean highly contaminated hair.

Similarly, hair contaminated with water containing 70 or 700 μg Mn/L and cleaned with the TN or 3xTN method were not measurably different from untreated hair cleaned with the TN method (UTN) (P's >0.1, based on Tukey's post-hoc comparison within subject) (Figure 2B, C). In contrast, however, Mn levels in hair cleaned with the less rigorous Triton sonication + nitric acid rinse (T) method remained significantly elevated compared to Mn levels in the UTN hair sample (P=0.0005 and P=0.0004 for the 70 and 700 μg Mn/L treatments, respectively). Collectively, these data indicate that the TN cleaning method is able to remove even excessive contamination with Mn and other metals without over-cleaning, since the 3xTN -cleaned hair was not statistically different from the UTN hair within subject. Moreover, the TN cleaning method provides significant improvement over the slightly less rigorous Triton sonication + 1N nitric rinse method (T).

3) Individual subjects vary in level of contamination as well as response to cleaning procedures

While only a small number of subjects were utilized in this study, it appeared that hair samples from the different subjects varied in their susceptibility to contamination with Mn-contaminated water, and in their response to cleaning. This is illustrated in hair samples from the five subjects contaminated with 700 μg Mn/L water. Manganese levels in subject 1's hair treated with 700 μg Mn/L water and rinsed with MQ water were actually lower than the levels in his untreated, uncleaned hair. Further cleaning of subject 1's 700 μg Mn/L water-treated hair readily reduced Mn to levels comparable to those in the untreated, Triton + 1N nitric acid sonication-cleaned (UTN) sample (Figure 3). In contrast, the other four subjects were all more susceptible to Mn water contamination when comparing their 700 μg Mn/L water-treated and MQ rinsed sample to their untreated, uncleaned hair sample. Subject

2 shows a ~40 fold increase in hair Mn concentration, while subjects 3, 4, and 5 increase ~5 to 10-fold (Figure 3). The basis for these differences in susceptibility to contamination is not known, but previous studies have reported similar findings with other metals. Buckley et al.[22] examined zinc absorption and removal from hair, and found that hair from different individuals placed in zinc-contaminated water absorbed zinc to varying degrees. Metal contamination is believed to localize to specific parts of the hair shaft, and perhaps differences in the composition of molecular binding sites for Mn (and other metals) between subjects contributes to the individual susceptibility to contamination that we observed[39,40].

Interestingly, subjects also differed in their response to cleaning in a fashion that appeared to be inversely related to individual differences in susceptibility to contamination with the 700 μg Mn/L water. Hair from subject 1 was resistant to contamination and was readily cleaned even with the less rigorous Triton sonication + 1N nitric rinse (T) method used here. In contrast, hair from subjects 2 to 5 was more susceptible to contamination from Mn in water and required more rigorous cleaning to effectively reduce hair Mn to levels comparable with the untreated, Triton sonication + 1N nitric sonication cleaned (UTN) sample within each subject (Figure 3). These data underscore the need to use a hair cleaning method that is sufficiently rigorous to clean hair that may be both susceptible to contamination and resistant to cleaning.

4) Cleaning hair with Triton + nitric acid sonication does not alter the visible morphology of hair

To determine whether the TN cleaning method or the intentional contamination of hair with Mn-contaminated water visibly altered the morphological appearance, and hence possibly the structural integrity of hair, selected hair samples from three subjects were analyzed by scanning electron microscopy (SEM). Results show that there was no discernible difference in the hair surface morphology across any of the treatment conditions within subject (see Supplemental and Figure S1). This substantiates that the Triton sonication + 1N nitric acid sonication cleaning method does not visibly alter the structural morphology of hair, consistent with our assessment above that this cleaning method does not appear to 'over-clean' metals from hair - a result that may have been more likely if the structural integrity of hair was visibly damaged by cleaning.

5) Mn contamination from water localizes to both the surface and interior hair shaft, and contamination is removed with the Triton + 1N nitric acid sonication cleaning procedure

We used laser ablation ICP-MS (LA-ICPMS) to selectively measure surface versus endogenous hair Mn levels, in order to determine whether the pattern of Mn distribution in hair changes following various contamination and cleaning conditions. Analyses were performed on two hair strands each from the same three subjects and treatment/cleaning conditions selected for SEM analyses. Representative data from a hair strand that was contaminated with 700 μg Mn/L water and rinsed with MQ before analysis shows ^{34}S counts remained relatively stable over ablation through the hair shaft (similar results were obtained for hairs from all treatment/cleaning conditions), while ^{55}Mn counts peaked dramatically coinciding with ablation of the hair surface, and rapidly declined to asymptotically lower counts corresponding to the interior hair shaft (Figure 4A).

Subsequently, hair ^{55}Mn counts were normalized to ^{34}S counts (as $^{55}Mn/^{34}S$ ratios), and the relative levels of Mn in surface versus interior hair shaft were evaluated. In untreated, uncleaned hair the $^{55}Mn/^{34}S$ ratios in the surface of the hair shaft were universally higher compared to the interior of the hair (Figure 4B, Figure S2, Table S1). Notably, contamination of hair with 700 μg Mn/L water led to significant increases in both surface and interior hair shaft Mn levels, compared to Mn levels in untreated, uncleaned hair for

both subjects 5 and 4, i.e., the two subjects analyzed that were susceptible to water Mn contamination (Figure 4B, Figure S2a, Table S1), but not for subject 1, whose hair was resistant to contamination by water Mn (Figure S2b, Table S1). Importantly, however, there was no significant difference or increase in surface or interior $^{55}Mn/^{34}S$ ratios in the untreated hair or 700 μg Mn/L water-contaminated hair that was cleaned with the Triton sonication + 1N nitric acid sonication (TN) method for any of the subjects analyzed (Figure 4B, Figure S2, Table S1), indicating that the TN cleaning method removed hair contamination from both the surface and interior of the hair shaft.

The between-subject differences in susceptibility of hair to Mn contamination and cleaning likely reflect differences in the molecular composition of both the surface and interior of the hair shaft. This is supported by evidence suggesting that the trace metal content in hair may be affected by the composition of metal-binding ligands in hair, such as amino acids and melanin pigment, which are known to vary between individuals based on a number of factors, including individual genetics, diet, weathering of hair, and cosmetic treatments[26,27], as noted above. Studies on hair calcium (Ca) and magnesium (Mg), which as partial Mn homologues may provide some clues on Mn binding in hair, have shown that Ca and Mg bind specifically to amino and carboxyl functional groups on the edge of cuticle scales on the surface of hair[39,40], and co-localize with melanin molecules in the interior of the hair shaft[39].

6) Children's hair Mn levels reflect environmental exposures

In order to better validate the use of hair Mn as a biomarker of environmental Mn exposure, we applied the Triton sonication + 1N nitric acid sonication cleaning method to hair samples collected from children age 11– 14 yrs living in the vicinity of active ferroalloy plant emissions (Bagnolo Mella, n=44), a historic but currently inactive ferroalloy plant (Valcamonica, n=41), and a reference region with no history of industrial activity (Garda Lake, n=36) in northern Italy. Importantly, recent reports on children living in the Valcamonica versus Garda Lake region have shown significant impairment of motor coordination, hand dexterity, and odor identification associated with environmental Mn levels, while motor tremor intensity was positively associated with hair Mn levels[29].

Here, there was a significant effect of exposure region on hair Mn levels (ANOVA $F_{(2,117)} = 8.28$, $P<0.001$), with children from the active ferroalloy emission site (Bagnolo Mella) having significantly higher hair Mn levels (median = 0.134 μg/g, range 0.011 – 0.736 μg/g) compared to children from the reference Garda Lake region (median = 0.060 μg/g, range 0.023 – 0.344 μg/g) ($P<0.001$) or historically active Valcamonica region (median = 0.070 μg/g, range 0.025 – 0.642 μg/g ($P=0.019$) (Figure 5). Hair Mn levels were no different between children living in the Garda Lake versus Valcamonica regions ($P=0.43$, all based on Tukey's post-hoc on log-transformed data). These data show that Mn levels in children's hair cleaned with the Triton sonication + 1N nitric sonication (TN) method are significantly associated with elevated environmental Mn levels, and they support the utility of hair Mn levels as a biomarker of environmental Mn exposure in children.

Hair Mn levels across the cohort of 121 children averaged 0.121 μg/g (median=0.073 μg/g, range 0.011 – 0.736), which is ~4 – >6-fold lower than levels in referent children and children environmentally exposed to Mn via airborne particles or contaminated well-water reported in other recent studies[6,10,16,17,37,41]. For example, Haynes et al.[41] investigated Mn exposure in children living near a ferromanganese plant in the USA and reported mean hair Mn levels of 0.47 μg/g (0.3 SD, range 0.085 – 1.25 μg/g, n=38). Similarly, Menezes-Filho et al.[17] investigated hair Mn levels in children living in the vicinity of a ferromanganese plant in Brazil, and reported geometric mean hair Mn levels of 1.37 μg/g in reference (unexposed) children (median 1.19, range 0.39 – 8.58 μg/g, n=43), and mean levels of 9.61

μg/g in children living 1–2 km downwind of the ferromanganese plant (median = 9.68, range 1.1 – 46.2 μg/g, n=75). Wright et al.[10] reported mean hair Mn levels of 0.47 μg/g (range 0.089 – 2.15 μg/g, n=31) in children living in the vicinity of the Oklahoma Tar Creek Superfund site, while Bouchard et al.[6] reported mean hair Mn levels of 0.7 μg/g (range 0.1 – 21 μg/g, n=302) in children exposed to Mn-contaminated well water in Quebec (range 1 – 2700 μg Mn/L). All of these studies cleaned hair via a 15 minute sonication in 1% Triton X-100 and rinse with MQ or distilled deionized water – a method that is less rigorous than the 0.5% Triton sonication + 1N nitric acid sonication (TN) method used to clean the children's hair in the present study, and likely less aggressive than even the 0.5% Triton sonication + 1N nitric acid rinse (T) method that our data indicated did not reliably clean Mn-contaminated hair (Table 1, Figure 2). Thus, the extent that the differences in hair Mn levels reported in these studies versus the present study reflect differences in hair cleaning and processing versus differences in internalized Mn exposure is not clear.

Several of these previous studies that used a 1% Triton X-100 sonication and water rinse cleaning method did report associations between hair Mn levels and health effects in children as a result of increased environmental exposure to Mn[6,10,16]. Again, however, the extent that reported hair Mn levels reflect the Mn body burden versus external environmental Mn contamination of hair is not clear; certainly both endpoints are of interest in the environmental epidemiology of Mn toxicity, but a biomarker of the internalized Mn dose, integrated over the duration of hair growth may be expected to better predict health risk than measures of exogenous environmental Mn. Notably, our recent study reported an association between hair Mn levels and tremor intensity in children age 11 – 14 yrs exposed to environmental Mn from historic ferroalloy plant emissions[29]. This latter study also used the 0.5% Triton sonication + 1N nitric acid sonication (TN) method in a similar but distinct pediatric group from what we report here, and reported median hair Mn levels of 0.11 μg/g (mean 0.16, range 0.02 – 3.45 μg/g, n=258 children); levels that were quite similar to those we report in the present study.

The utility of hair as a biomarker of Mn (and other metals) exposure and body burden rests to a large extent on the ability to remove exogenously derived Mn contamination without altering levels of metabolically incorporated Mn. However, achievement of this goal cannot be known with certainty, since one cannot definitively distinguish Mn in hair derived exogenously versus metabolically. Nonetheless, we believe the Triton sonication + 1N nitric acid sonication (TN) method developed here may approach this goal (see Supplemental for detailed rationale). Using this cleaning method, hair Mn levels were found to be significantly higher in children living in the vicinity of active, but not historic, ferroalloy plant emissions, substantiating the use of hair Mn levels as a biomarker of environmental Mn exposure in children. Finally, these results underscore the importance of validated hair processing methodologies in studies using hair metal levels as an exposure biomarker[42].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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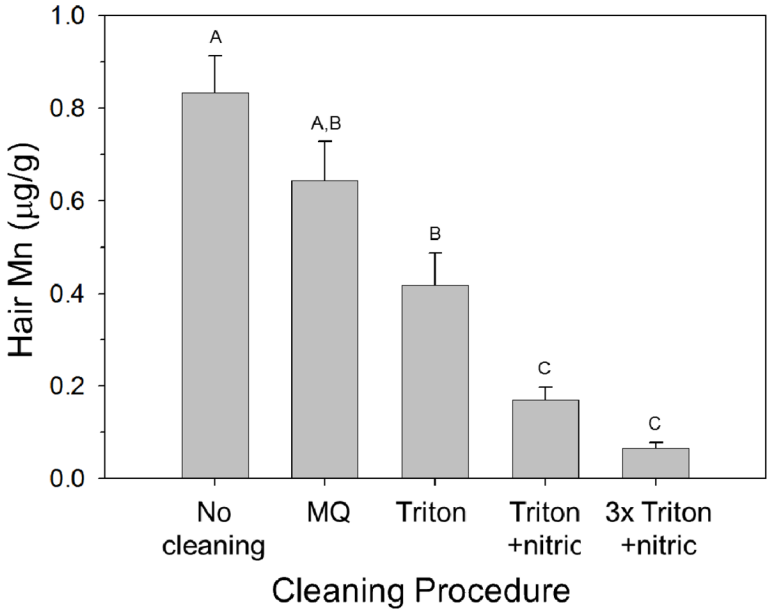

FIGURE 1.

Hair Mn levels were reduced with increasing strength of cleaning. Data reflect hair Mn concentrations after cleaning with each procedure. Values are mean (± SE, n=10 subjects). Superscript letters denote significant differences between cleaning methods; bars that do not share a common superscript are statistically different from one another ($p < 0.05$), based on Tukey's post-hoc analyses performed on square root-transformed data.

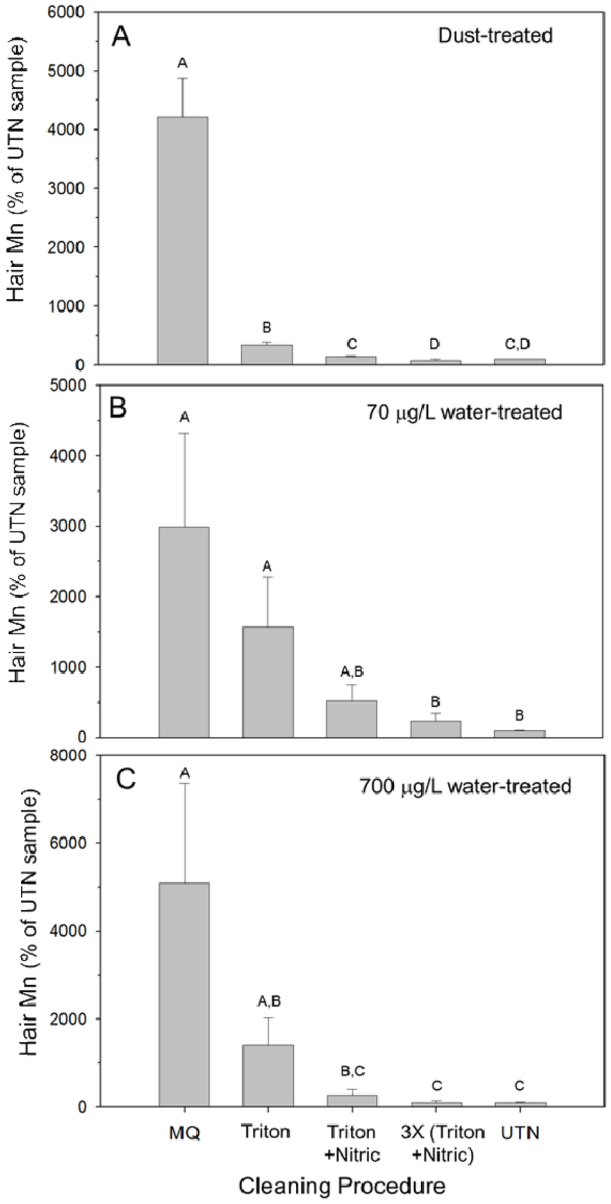

FIGURE 2.

Manganese levels in hair that was intentionally contaminated with (A) house dust, or water containing either (B) 70 μg Mn/L, or (C) 700 μg Mn/L, and then cleaned with either MQ water rinsing (MQ), Triton sonication + 1N nitric acid rinsing (Triton), Triton sonication + 1 N nitric acid sonication (Triton + nitric), or the latter Triton + nitric cleaning performed three times. Manganese levels are expressed as a percent of the untreated hair cleaned with the Triton + nitric method (UTN), calculated within each subject. Values are mean (± SE; n=5 subjects). Superscript letters denote significant differences between cleaning methods; bars that do not share a common superscript are statistically different from one another ($p < 0.05$), based on Tukey's post-hoc analyses performed on log-transformed data.

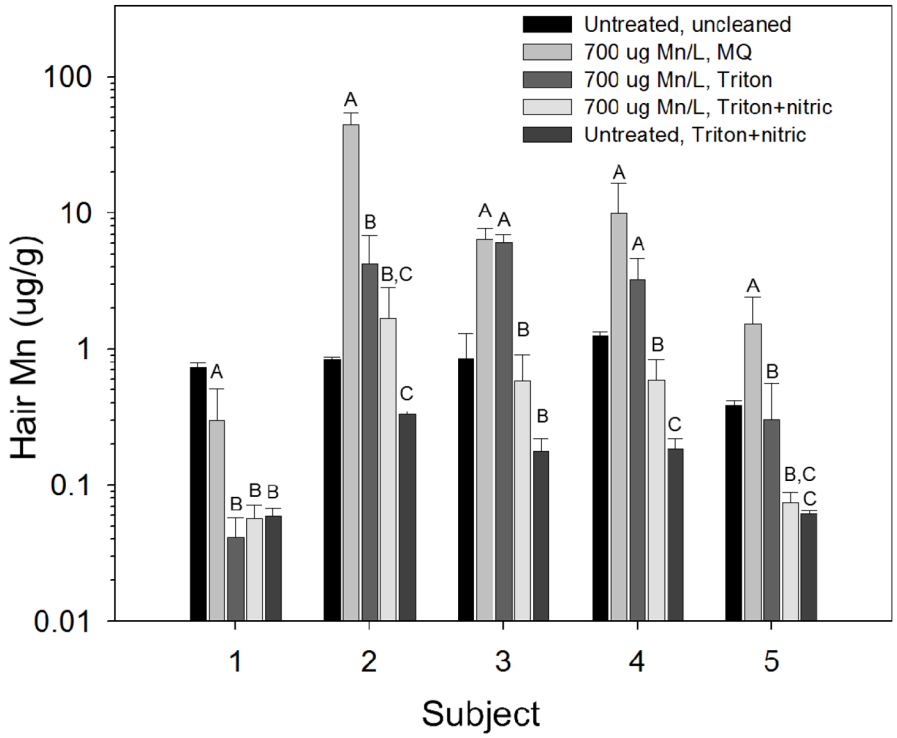

FIGURE 3.

Hair from individual subjects varies in susceptibility to contamination and response to cleaning. Hair Mn levels (mean ± SD, n=3 hair samples per bar) in samples from each subject that were untreated and uncleaned, contaminated with 700 μg Mn/L water and rinsed with MQ water, contaminated with 700 μg Mn/L water and cleaned with Triton sonication + 1N nitric acid rinsing (Triton), contaminated with 700 μg Mn/L water and cleaned with Triton sonication + 1N nitric acid sonication (Triton + nitric), or untreated and cleaned with Triton sonication + 1N nitric acid sonication. Superscript letters denote significant differences among the latter four bars (cleaning methods) per subject; bars that do not share a common superscript are statistically different from one another ($p < 0.05$), based on Tukey's post-hoc analyses ($P < 0.05$) (ANOVA $F_{(4,21)} = 15.99$, $F_{(4, 10)} = 35.46$, $F_{(4, 10)} = 33.48$, $F_{(4, 10)} = 34.21$, $F_{(4, 10)} = 29.98$ on log-transformed data for subjects 1 – 5, respectively; $p < 0.0001$ for all). Data from the 'untreated and uncleaned' samples are included for reference only and were not used in the statistical analyses.

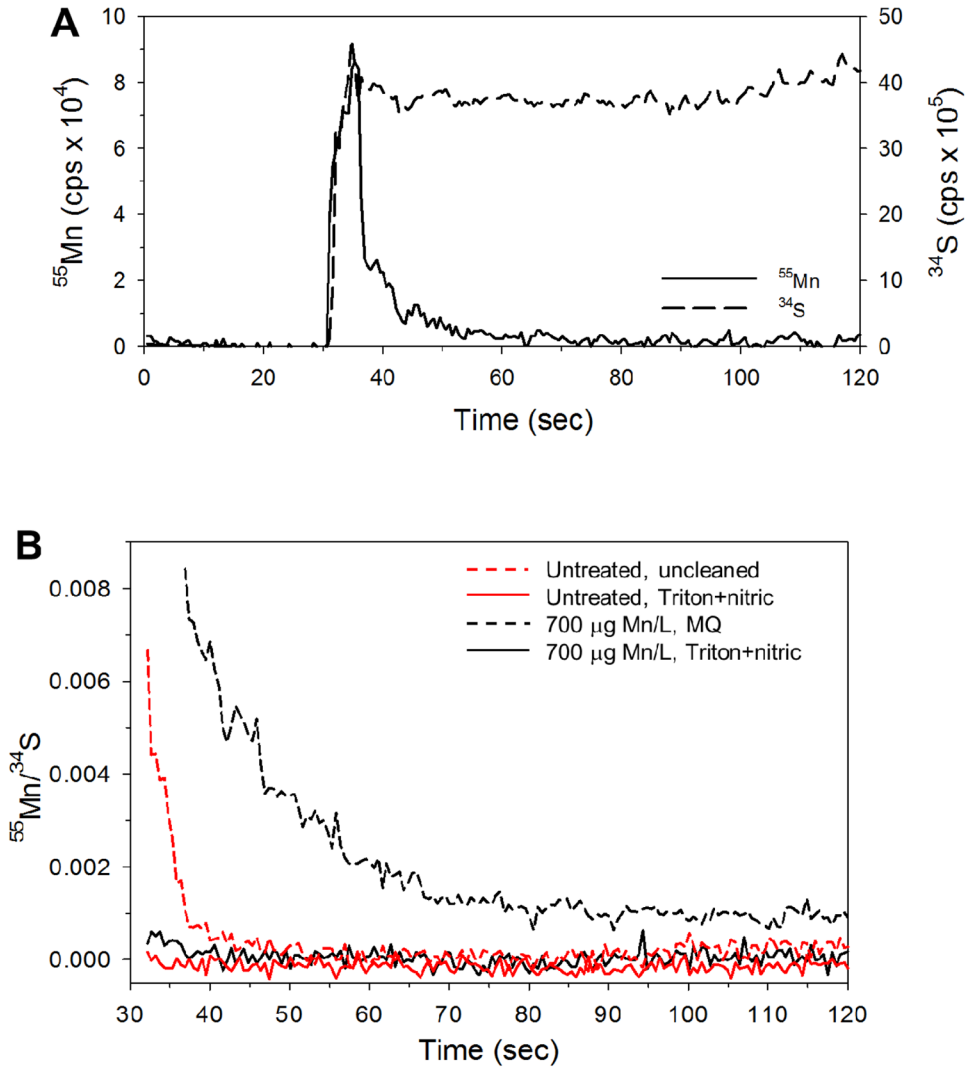

FIGURE 4.

A) ^{55}Mn and ^{34}S counts per second (cps) in a hair sample from subject 5 contaminated with 700 μg Mn/L and cleaned with MQ water, measured using laser ablation (LA) ICP-MS (hair diameter was 60 μm). B) ^{55}Mn/^{34}S cps ratios measured in hair by LA ICP-MS following four different treatment/cleaning schemes, as follows: Untreated and uncleaned, untreated cleaned with Triton sonication + 1N nitric acid sonication, contaminated with 700 μg Mn/L water and MQ rinsed, and contaminated with 700 μg Mn/L water and cleaned with Triton sonication + 1N nitric acid sonication (hair diameters ranged from ~60–100 μm).

The ^{55}Mn/^{34}S cps ratio is plotted versus time starting when the laser was turned on. The first ~5 seconds corresponds to the surface layer of each hair. Data for each hair in 'B' reflect the average ^{55}Mn/^{34}S cps ratio from analysis of three 500 μm sections/hair (see Supplemental for details).

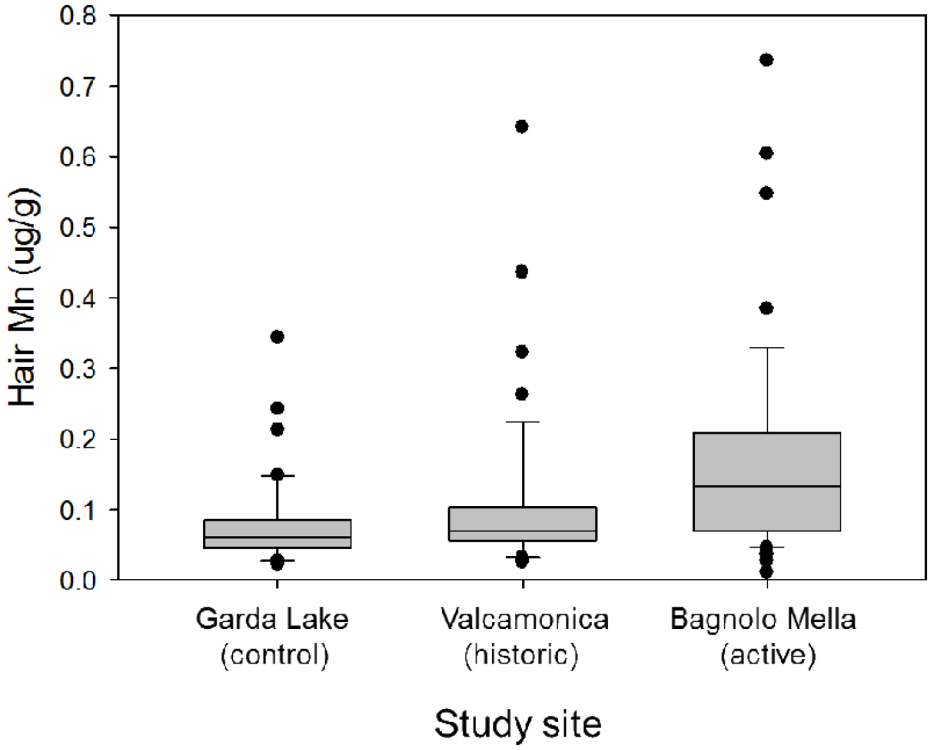

FIGURE 5.

Hair Mn concentrations in children living in the vicinity of active ferroalloy plant emissions (Bagnolo Mella, n=44), a historic but currently inactive ferroalloy plant (Valcamonica, n=41), and a reference region with no history of industrial activity (Garda Lake, n=36). There is a significant effect of exposure region on hair Mn levels ($F_{(2,117)} = 8.28$, $P<0.001$), with levels in children from the active ferroalloy emission site (Bagnolo Mella) having significantly higher hair Mn levels compared to children from the reference Garda Lake region ($P<0.001$) and historically active Valcamonica region ($P=0.019$). Hair Mn levels are not different between Garda Lake and Valcamonica ($P=0.43$, all based on Tukey's post-hoc on log-transformed data).

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TABLE 1

Metal concentrations in untreated and intentionally contaminated hair processed with different cleaning methods (see text).

	Cleaning Method	Mn*	Pb	Cr	Cu
Untreated	No cleaning	0.833 (0.080)^{a}	0.796 (0.123)^{a}	0.273 (0.064)^{a}	73.3(15.2)^{a}
	MQ	0.644 (0.085)^{a,b}	0.813 (0.134)^{a}	0.241 (0.077)^{a,b}	68.2(18.5)^{a}
	Triton	0.419 (0.068)^{b}	0.696 (0.137)^{a}	0.194 (0.068)^{a,b}	45.0(10.4)^{a,b}
	Triton+nitric	0.170 (0.027)^{c}	0.250 (0.043)^{b}	0.181 (0.070)^{a,b}	24.4 (5.20)^{b}
	3x Triton+nitric	0.066(0.012)^{c}	0.084 (0.012)^{b}	0.134(0.047)^{b}	17.5 (3.45)^{b}
Contaminated	*Dust-treated*				
	MQ	5.73 (0.632)^{a}	1.62 (0.367)^{a}	1.07 (0.161)^{a}	120.0 (36.4)^{a}
	Triton	0.593 (0.108)^{b}	0.972 (0.268)^{a,b}	0.491 (0.153)^{b}	75.7 (20.2)^{a,b}
	Triton+nitric	0.238 (0.042)^{b}	0.315(0.068)^{b}	0.342 (0.146)^{b,c}	36.0 (7.75)^{b}
	3x Triton+nitric	0.154 (0.055)^{c}	0.0885 (0.013)^{c}	0.264 (0.116)^{c}	29.7 (7.42)^{b}
	70 μg/L water-treated				
	MQ	5.51(1.55)^{a}	0.960 (0.241)^{a}	0.313 (0.120)^{a}	95.2 (30.4)^{a}
	Triton	2.68 (0.667)^{a}	0.898 (0.221)^{a}	0.246 (0.113)^{a}	71.6(19.6)^{a,b}
	Triton+nitric	0.89 (0.258)^{a,b}	0.304 (0.056)^{b}	0.190 (0.095)^{a}	33.0 (7.28)^{a,b}
	3x Triton+nitric	0.414 (0.158)^{b}	0.098 (0.011)^{b}	0.292 (0.112)^{a}	29.4 (9.08)^{b}
	700 μg/L water-treated				
	MQ	10.5(3.90)^{a}	1.17 (0.315)^{a}	0.372 (0.162)^{a}	98.6 (25.0)^{a}
	Triton	2.31 (0.620)^{a,b}	1.09 (0.235)^{a}	0.233 (0.111)^{a}	66.5 (18.2)^{a,b}
	Triton+nitric	0.506 (0.170)^{b,c}	0.306 (0.045)^{b}	0.246 (0.105)^{a}	32.0 (7.14)^{b}
	3x Triton+nitric	0.181 (0.066)^{c}	0.044 (0.009)^{b}	0.231 (0.099)^{a}	22.0 (5.48)^{b}

*Mn, Pb, Cr, Cu concentrations are μg/g hair; Data are mean (standard error); n = 10 and n = 5 subjects for the 'Untreated and 'Contaminated' values, respectively.

*a, b, c*Values with different superscripts are significantly different from one another based on Tukey's post-hoc analysis ($P < 0.05$) performed on square root-transformed 'Untreated' data and log-transformed 'Contaminated' data within metal and contamination category.