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Mercury Accumulation in Millipedes (*Narceus* spp.) Living Adjacent to a Southern Appalachian Mountain Stream (USA)

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Abstract

Millipedes are among the most important processors of leaf litter in temperate forests. Through consumption of leaf litter, millipedes may be exposed to mercury that accumulates in leaf tissues prior to senescence. To investigate mercury uptake in millipedes, *Narceus* spp. were collected from a remote site in the southern Appalachian Mountains, an area known to receive high mercury deposition. Additionally, aquatic primary consumers (larval caddisflies and stoneflies), brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) were collected from the same site for comparisons of mercury concentrations and percent methylmercury. Bioaccumulation factors for millipedes were 18.5 and 20.2 for total and methylmercury, respectively. At this site, the mean THg concentration in millipedes was ~ 10 × greater than both brook trout and rainbow trout and ~ 200 × greater than that of aquatic primary consumers. Millipede THg concentrations ranged from 222 to 1620 ng/g ww in an area where EPA fish consumption criteria (300 ng/g MeHg in fish tissue, ww) were not exceeded. The mean percent methylmercury in millipedes was 1.4%, suggesting these animals were accumulating large quantities of inorganic mercury.

Keywords Trace element · *Narceus* spp. · Bioaccumulation · Atmospheric deposition · Methylmercury

Mercury is a global contaminant that can have adverse effects on human health and wildlife (Driscoll et al. 2013). Methylmercury (MeHg), the form of Hg most often of concern for human health and wildlife, bioaccumulates and biomagnifies through food chains and can reach concentrations much greater than those found in the surrounding environment. Mercury methylation is most commonly expected to occur via bacterial processes in water and sediment (Hsu-Kim et al. 2013); consequently, MeHg has traditionally been considered a concern for aquatic organisms (e.g., fish) and predators that consume them (e.g., humans, piscivorous birds).

Mercury is primarily introduced to isolated areas through the deposition of atmospheric mercury onto soils, plant surfaces, and surface waters (Driscoll et al. 2013; Risch et al. 2017). Forested areas, in particular, receive large quantities of atmospheric mercury due to the surface area afforded by foliage and the ability of leaves to uptake mercury through their stomata (Zhang et al. 2009; Rutter et al. 2011). Once associated with plant matter, atmospheric mercury can be re-volatilized to the atmosphere or be transported to soils and surface waters via precipitation (i.e., throughfall) and falling senescent leaves (i.e., litterfall; Gustin et al. 2008). Litterfall has been shown to be a major contributor to overall mercury deposition (Risch et al. 2012, 2017; Wang et al. 2016) and can serve to transfer mercury to those aquatic and terrestrial primary consumers reliant on leaves as dietary items.

Millipedes are terrestrial detritivores and are among the most important processors of plant litter in temperate forests (Edwards 1974). As they consume leaves, millipedes produce fragmented litter that is more easily degraded by other decomposers (e.g., soil bacteria, fungi). This makes millipedes of high ecological importance, but also provides a route of exposure to mercury present in litterfall. This, along

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with their relatively long lifespans, their role as prey items for foraging birds, and their ability to sequester large quantities of metals in intestinal granules (Köhler et al. 1995), warrants investigation into their ability to accumulate Hg. In laboratory trials, Buch et al. (2018) found that millipedes could bioaccumulate inorganic Hg to high levels through consumption of mercury-spiked leaf litter, with bioaccumulation factors (BAFs) ranging from ~ 5 to > 50; however, field studies involving Hg accumulation in millipedes are rare and suggest vastly different BAFs across taxa (Kwon et al. 2015; Vrankovic et al. 2017).

To better understand the ability of millipedes to bioaccumulate mercury, millipedes were collected alongside aquatic primary consumers (caddisflies, stoneflies) and aquatic predators (rainbow trout, brook trout) from an area known to receive substantial levels of mercury deposition via litterfall (Fisher and Wolfe 2012; Risch et al. 2012, 2017). The specific objectives of this study were to (1) compare total mercury (THg) concentrations in terrestrial millipedes (*Narceus* spp.) to aquatic biota (larval stoneflies, larval caddisflies, brook trout, and rainbow trout) from the same area; (2) determine the concentration and percentage of mercury in the methylated form (%MeHg) in millipedes, brook trout, and rainbow trout and compare them to previously reported field values; and (3) calculate millipede BAFs for THg and MeHg.

Materials and Methods

Bald River is a remote site in Tennessee's Appalachian Mountains (USA) located in the Blue Ridge Mountains (EPA Level III; Ecoregion 66) (35° 16.278′N, 84° 8.295′W). All samples in the study were collected in August of 2016 from a 200-meter reach of Bald River and the adjacent riparian area. For a more detailed description of Bald River see Olson et al. (2019).

Adult millipedes (*Narceus* spp.) were collected by hand from riparian vegetation and structures along the shoreline, no further than 2 m inland (n = 6). After capture, each millipede was placed into an individual 50-mL polypropylene tube. Processing of millipedes consisted of measuring total length (cm) and weight (g) of each individual and removing a two-ring portion of the mid-body (for analysis not included in this study). Gut contents were not removed prior to analysis. Mean ± SD total length and weight for millipedes was 9.3 ± 1.8 cm and 7.8 ± 3.0 g, respectively.

Larval caddisflies (*Pycnopsyche* spp.) and larval stoneflies (*Pteronarcys* spp.) collected in the present study are primarily shredders (Morse and Holzenthal 2008). Larval caddisflies were hand-collected from rocks within the stream, removed from their cases, and sorted into six polypropylene tubes (9–13 animals/tube). Larval stoneflies were hand-collected and sorted into six polypropylene tubes (four animals/tube). Gut contents were not removed prior to analysis.

A total of 10 rainbow trout (*Oncorhynchus mykiss*) and 10 brook trout (*Salvelinus fontinalis*) were collected via electrofishing from Bald River. After capture each fish was verified to species and measured for total length (only adult fish within a specified size range [140–165 mm] were collected, all others were released) before being euthanized. Fish were wrapped in aluminum foil, placed into individually labeled plastic bags, stored on wet ice, and transported to the laboratory to await processing and analysis at or below − 20°C. Fish processing consisted of measuring fish total length, weight, and the removing of the gastrointestinal tract. Mean ± SD total length and weight for brook trout was 147.9 ± 6.7 mm and 31.2 ± 5.0 g, respectively. Mean ± SD total length and weight for rainbow brook trout was 150.1 ± 5.4 mm and 29.4 ± 4.0 g, respectively. Species-specific homogenates of roughly equal weight were made by compositing a larger fish with a smaller fish to create five brook trout and five rainbow trout samples for analysis. Sample compositing occurred because fish samples were analyzed for organic contaminants not reported in this study (see Olson et al. 2019).

Millipede, caddisfly, and stonefly samples were analyzed for THg via oxidation, purge and trap, desorption, and cold-vapor atomic fluorescence spectrometry in accordance with USEPA Method 1631 (USEPA 2002). Briefly, composite samples were homogenized and stored frozen in acid-cleaned glass fluoropolymer jars. Samples were then transferred to a digestion vessel, digested with HNO_3 and H_2SO_4 on a 58°C hot block for 1 h. Once cooled, samples were diluted with 0.02 N BrCl and left at room temperature for an additional 4 h. Prior to analysis, an initial calibration verification was tested, and a continuing calibration verification was performed every ten samples. Analysis of THg was conducted utilizing an Analytik Jena automated mercury analyzer. All samples were analyzed alongside two method blanks (all reagents) and at least one paired Laboratory Control Sample (LCS) and Laboratory Control Sample Duplicate (LCSD). In all cases, method blanks were undetectable (< 0.32 ng/g) and below the method reporting limit of 1.1 ng/g ww. Mean ± SD %-recovery for LCS and LCSD were 90 ± 4% and 93 ± 8%, respectively. The mean ± SD relative percent difference (RPD) of the paired LCS and LCSDs was 5 ± 2%, and all were within the acceptable range. Sample quality assurance included the use of Tort-3, a Standard Reference Material (SRM), which was used to make SRM blanks, and Matrix Spikes (MS) and Matrix Spike Duplicates (MSD). In all cases SRM blanks were within the acceptable range. Mean ± SD %-recovery for MS and matrix spike MSD were 101 ± 4% and 101 ± 4%,

respectively. Each paired MS and MSD was within an acceptable range for relative percent recovery.

Brook trout and rainbow trout composites were analyzed for THg via oxidation, purge and trap, desorption, and cold-vapor atomic absorption spectrometry in accordance with USEPA Method 7473 (USEPA 1998a) utilizing a Milestone Direct Mercury Analyzer (DMA-80). All samples were analyzed alongside a method blank (all reagents). In all cases, method blanks were undetectable (< 3.2 ng/g) and below the method reporting limit of 20 ng/g ww. Sample quality assurance included the use of Tort-3, which was used to make SRM blanks, MS, and MSDs. In all cases SRM blanks were within the acceptable range. Mean ± SD %-recovery for MS and matrix spike MSD were 93 ± 1% and 92 ± 1%, respectively. Each paired MS and MSD was within an acceptable range for relative percent recovery.

Subsamples from three of the six individual millipedes collected and from each brook trout and rainbow trout composite were analyzed for MeHg according to EPA 1630 (USEPA 1998b). Briefly, MeHg samples were digested in 10 mL of 25% w/v KOH/MeOH, heated to 65°C for 3–4 h. Once samples were brought to room temperature, the sample was diluted to 25.6 mL with MeOH, inverted, and allowed to sit for a minimum of 1 day. A maximum of a 30-µL aliquot of the digestate was transferred to a 40-mL amber glass vial, and 300 µL of 2 M acetate buffer and 0.04 mL of NaBEt4 were added. Vials were filled (all headspace removed) with the addition of ultra-pure 18 MΩ reagent water. Samples were analyzed via cold vapor atomic fluorescence with a Brooks Rand Model III mercury analyzer. All method blanks were undetectable (< 1.08 ng/g) and below the method reporting limit of 3.16 ng/g ww. The SRM (National Institute of Standards and Technology, Standard Reference Material 1946) was within the acceptable range. Mean ± SD LCS, MS, and MSD were 94 ± 10%, 110 ± 11%, and 107 ± 5%, respectively. Each paired MS and MSD was within an acceptable range for relative percent recovery.

Millipede BAFs were calculated using litterfall THg and MeHg concentrations from a long-standing study site in Great Smoky Mountain National Park (TN11) that is part of the National Atmospheric Deposition Program's Litterfall Mercury Monitoring Initiative (Risch et al. 2017). Site TN11 is located approximately 68.3 km from the Bald River site and is within the same level III Ecoregion. In 2007, mean THg in litterfall was 32.6 ± 12.4 ng/g with 0.8% (0.25 ng/g) being observed as MeHg. Millipede BAFs were calculated as the mean concentration (ng/g ww) of THg or MeHg in millipedes, divided by the mean concentration (ng/g ww) of the respective THg or MeHg concentration in leaf litter. This calculation is based on a modified version of bioaccumulation guidelines for terrestrial oligochaetes (OECD 2010) previously utilized for laboratory bioaccumulation assays in millipedes (Buch et al. 2018).

THg and MeHg concentrations were pooled by species and assessed for normality prior to statistical analysis. THg concentrations were Log_{10}-transformed to meet the assumption of normality and compared across species using a one-way ANOVA, with a Tukey's post hoc test. Millipede THg concentrations were compared to values reported in Kwon et al. (2015) using a Wilcoxon rank-sum test (non-parametric, independent *t* test). All statistical analyses were performed using JMP 11.2.1 software (Cary, NC, USA). Statistical significance was defined as $\alpha = 0.05$.

Results and Discussion

Significant differences in THg concentrations were found between organisms collected at Bald River ($F_{(4,23)} = 251.9$, $p < 0.001$; Fig. 1). As expected, mean THg concentrations in predatory fish species (brook trout [61 ± 2 ng/g ww]; rainbow trout [49 ± 5 ng/g ww]) were found to be significantly higher than in aquatic primary consumers (larval caddisflies [3.47 ± 0.12 ng/g ww]; larval stoneflies [2.72 ± 0.16 ng/g ww]). The larval caddisflies and stoneflies sampled are primarily shredders and would be expected to consume plant matter similar to millipedes; however, millipedes (602 ± 208 ng/g ww) not only had a significantly greater THg concentration than both aquatic primary consumers, but also both species of trout (Fig. 1). Millipede THg concentrations ranged from 222 to 1620 ng/g ww and were ~ 200 × greater than that of aquatic primary consumers and ~ 10 × greater than the top predators in the aquatic food chain.

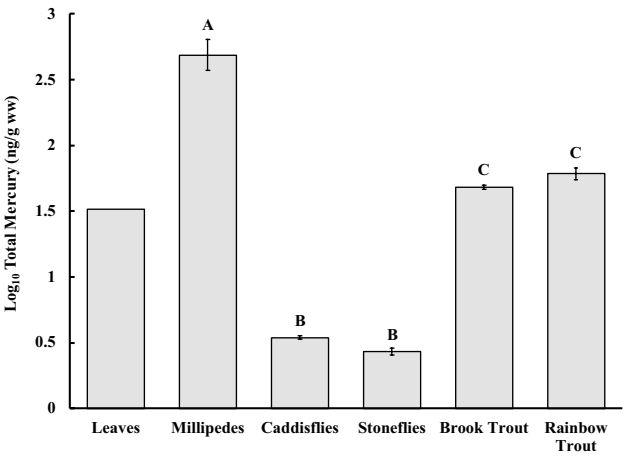

Fig. 1 Log_{10} total mercury concentrations (mean ± SE) in leaves, millipedes (*Narceus* spp.; n = 6), caddisflies (*Pycnopsyche* spp.; n = 6), stoneflies (*Pteronarcys* spp.;n = 6), brook trout (*S. fontinalis*; n = 5), and rainbow trout (*O. mykiss*; n = 5). Leaf data from Great Smoky Mountain National Park (site TN11; Risch et al. 2017). Significant differences are indicated by different letters ($p < 0.05$; ANOVA)

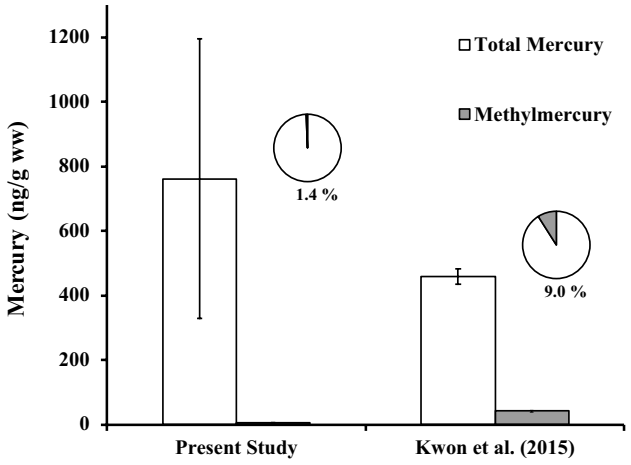

Fig. 2 Total mercury and methylmercury concentrations (mean ± SE) in millipedes from Bald River in the present study (*Narceus* spp.) and Spirobolida from Kwon et al. (2015); pie charts represent the percent methylmercury

Although the concentration of THg in millipedes was higher than those found in both trout species, the mean millipede %MeHg was only 1.4% – significantly lower than the 96.4% and 99.8% observed in brook trout and rainbow trout, respectively ($F_{(2,10)} = 40.75$, $p < 0.001$). These finding match those of Lasorsa and Allen-gil (1995), who found that while %MeHg in fish were generally high (> 90%), %MeHg in terrestrial organisms at lower trophic positions were relatively low. The physiological mechanisms responsible for the high concentration of inorganic Hg and the low %MeHg observed in millipedes is not fully understood and was outside of the scope of this study. When millipedes of the present study were compared to millipedes collected by Kwon et al. (2015) while investigating Hg pathways between Douglas Lake (Michigan, USA) and its surrounding forests, no differences in THg concentrations were found ($H = 0.05$, $p = 0.83$) (Fig. 2). Both the present study and Kwon et al. (2015) found THg concentration in millipedes to be much higher than the other matrices collected and to have < 10% MeHg. These field data along with the high inorganic Hg bioaccumulation reported by Buch et al. (2018) suggest that high levels of inorganic Hg accumulation could be a common phenomenon in some millipedes.

Millipede BAFs were calculated to be 18.5 for THg and 20.2 for MeHg. These values far exceeded field-calculated THg BAFs for earthworms (all < 1; Zhang et al. 2009) and cave millipedes (~ 1; Vrankovic et al. 2017) reported in previous studies. Interestingly, the BAF values from the current study fell within the range (~ 5 to 50) of those calculated in 28-day laboratory trails performed by Buch et al. (2018), even though the laboratory trials involved different millipede species, a short exposure time, and high doses of inorganic mercury. To our knowledge this work is the first to publish millipede THg BAF in the field and the first MeHg BAF published for millipedes in any setting. However, because the collection of leaf litter and millipedes did not occur at the same time, we cannot verify that the leaf litter collected was representative of the millipede diet at the time the millipedes were collected. Shaw (1968) described that *Narceus* adults would preferentially feed on litter from previous years, which could be an important factor for BAF calculations given that Hg concentrations in leaves have been shown to fluctuate as they decay (Demers et al. 2007).

Mercury is typically perceived to be an aquatic concern (Hsu-kim et al. 2013); however, in the present study, an individual millipede was found to be as high as 1600 ng/g ww in an area where EPA fish consumption criteria (300 ng/g MeHg in fish tissue, ww) were not exceeded. These findings may have broader implications for known millipede predators, such as the American and Eurasian woodcock (*Scolopax minor, Scolopax rusticola*; Pettingill 1939; Hoodless and Hirons 2007), and further investigations into the accumulation of inorganic and MeHg in millipedes as well as the associated risk to terrestrial predators are warranted.

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Compliance with Ethical Standards

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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