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Nondestructive indices of mercury exposure in three species of turtles occupying different trophic niches downstream from a former chloralkali facility

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Abstract Turtles are useful for studying bioaccumulative pollutants such as mercury (Hg) because they have long life spans and feed at trophic levels that result in high exposure to anthropogenic chemicals. We compared total Hg concentrations in blood and toenails of three species of turtles (*Chelydra serpentina*, *Sternotherus odoratus*, and *Graptemys geographica*) with different feeding ecologies from locations up- and downstream of a superfund site in Virginia, USA. Mercury concentrations in turtle tissues were low at the reference site (average ± 1SE: blood = 48 ± 6 ng g^{-1}; nail = 2,464 ± 339 ng g^{-1} FW) but rose near the contamination source to concentrations among the highest ever reported in turtles [up to 1,800 ng g^{-1} (blood) and 42,250 ng g^{-1} (nail) FW]. Tissue concentrations remained elevated ~130 km downstream from the source compared to reference concentrations. Tissue Hg concentrations were higher for *C. serpentina* and *S. odoratus* than *G. geographica*, consistent with the feeding ecology and our stable isotope (δ^{13}C and δ^{15}N) analyses of these species. In addition, we suggest that toenails were a better indication of Hg exposure than blood, probably because this keratinized tissue represents integrated exposure over time. Our results demonstrate that downstream transport of Hg from point sources can persist over vast expanses of river thereby posing potential exposure risks to turtles, but relative exposure varies with trophic level. In addition, our study identifies turtle toenails as a simple, cost-efficient, and minimally invasive tissue for conservation-minded sampling of these long-lived vertebrates.

Keywords *Chelydra serpentina* · *Graptemys geographica* · *Sternotherus odoratus* · Stable isotopes · Reptile

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Introduction

Among inorganic contaminants, mercury (Hg) is one of the greatest threats to the health of fish and wildlife around the globe. Its propensity to affect vertebrates partly stems from its tendency to bioaccumulate, particularly in its highly bioavailable methylated form (Watras and Bloom 1992; Hill et al. 1996). Once accumulated in tissues, reproductive females and early lifestages are at greatest risk of adverse effects from Hg exposure, which include behavioral abnormalities, neurotoxicity, endocrine disruption, and reproductive impairment (Barr 1986; Heinz 1996; Hammerschmidt et al. 2002; Drevnick and Sandheinrich 2003; Scheuhammer et al. 2007; Tan et al. 2009; Wada et al. 2009). Despite the fact that Hg has been studied for decades and its effects on wildlife are well documented, many important knowledge gaps remain unfilled. For example, little is known about the effects of Hg on amphibians and reptiles, particularly in lotic systems, despite

their importance to ecological function (reviewed in Hopkins 2006, 2007; Hopkins and Rowe 2010).

Although much research on Hg has focused on atmospheric deposition in wetlands and other lentic systems, many lotic systems are heavily polluted by point sources of Hg, which pose significant risks to humans and wildlife. For example, mining activities, fiber manufacturing, and chloralkali processing have resulted in considerable loading of Hg into river systems around the world including sites in the U.S., Europe, and South America (Bonzongo et al. 2002; Southworth et al. 2004; Bergeron et al. 2007, 2010a, b, 2011; Cristol et al. 2008; Hallinger et al. 2011). Studies of Hg dynamics and associated ecological effects in flowing water systems have raised important new questions about floodplain fauna and associated pathways of exposure in species that were not previously considered vulnerable (e.g., migratory songbirds; Cristol et al. 2008). In addition, studies in lotic systems allow investigators to quantify Hg exposure, tissue residues, and associated effects on the same species over large concentration gradients. Such approaches are particularly needed for poorly studied taxonomic groups such as reptiles.

In the current study we quantified total Hg concentrations in tissues of three species of turtles across a broad contamination gradient in the North Fork of the Holston River (hereafter NFHR) in southwest Virginia, USA. The river was polluted with Hg by a former chloralkali plant, Olin Corporation's Saltville facility, which was in operation from 1950 to 1972. The Saltville facility, which is now designated as an EPA superfund site along the border of Smyth and Washington counties, contains a disposal pond approximately 30-ha in area. The disposal pond is filled with Hg-laden wastes approximately 24 m deep, and has been the primary source of Hg to the river. We focused on turtles because they possess a suite of life history and ecological characteristics that make them useful for studying bioaccumulative pollutants such as Hg; many turtles are locally abundant and tend to have relatively small home ranges, long life spans, and feed at trophic levels that put them at high risk of exposure (Iverson 1982; Ernst et al. 1994; Meyers-Schöne and Walton 1994; Mitchell 1994; Golet and Haines 2001; Bergeron et al. 2007; Congdon et al. 2008). For example, snapping turtles are top predators that feed upon a wide array of prey items including fish, which are important trophic vectors of Hg (Golet and Haines 2001). Despite their useful traits, little is known about Hg in turtles compared to other vertebrates such as birds, fish, and mammals (Wolfe et al. 1998; Eisler 2006; Bergeron et al. 2007; Turnquist et al. 2011).

We specifically sought to determine whether differences in feeding ecology influenced Hg concentrations in turtle tissues and whether Hg exposure varied over spatial scales. Building upon recent work in a different river system (Bergeron et al. 2007), we used stable isotopes to infer how local feeding ecology might influence relative exposure among species. In addition, we determined whether toenail clippings could be used as an additional or alternative nondestructive index of Hg exposure to blood sampling. We hypothesized that toenails would be an excellent tissue for biomonitoring efforts because they are simple to collect, should contain high levels of Hg due to their high keratin content, and should represent cumulative exposure to Hg that occurred during the months prior to sampling (Bearhop et al. 2003; Hopkins et al. 2007).

Materials and methods

Study species

Seven species of semiaquatic turtles were collected along the NFHR including spiny softshell turtles (*Apalone spinifera*), painted turtles (*Chrysemys picta*), snapping turtles (*Chelydra serpentina*), common map turtles (*Graptemys geographica*), stripe-necked musk turtles (*Sternotherus minor peltier*), stinkpots (*Sternotherus odoratus*), and slider turtles (*Trachemys scripta*). However, only three of these species, *C. serpentina*, *S. odoratus*, and *G. geographica* were available in sufficient numbers to study at the four sampling regions described below. These species have very different foraging ecologies, enabling us to determine whether feeding preferences influenced Hg exposure in this turtle assemblage. *C. serpentina* is a top-level predator that can live longer than 55 years and attain very large body sizes (up to 16 kg in Virginia; Mitchell 1994). Although *C. serpentina* is well known for its piscivory, it is an opportunist and will also feed on items ranging from plant material to invertebrates and other vertebrates. *Sternotherus odoratus* is a small-bodied turtle (max size in Virginia = 318 g) that scavenges the benthos opportunistically, primarily feeding on benthic invertebrates, carrion, and plant material (Mitchell 1994). Although this species is not thought to feed at trophic levels comparable to snapping turtles, recent work in the South River (Virginia, USA) showed that its benthic scavenging habits place it at considerable risk of Hg exposure (Bergeron et al. 2007). Finally, *G. geographica* is a large (max size in Virginia = 1.5 kg) basking turtle that feeds primarily on mollusks, especially snails (Mitchell 1994). Based on the known feeding ecologies of these species and the recent work on *C. serpentina* and *S. odoratus* (Bergeron et al. 2007), we predicted that Hg

concentrations in tissues would follow this pattern among species: *C. serpentina* ≥ *S. odoratus* > *G. geographica*.

Collection of turtles

Turtles were collected from four sites oriented at varying distances upstream and downstream from the source of Hg contamination. Each collection site represented a 1.6–4.0 km reach of river. The superfund site is located at river km 131.2. Our reference site was located upstream from the source of contamination, between river km 149.7–153.7, hereafter referred to as RKM 150. Downstream from the Hg source, turtles were sampled at three locations spread over a 128.1 km contamination gradient. The first downstream site was located ~1.6–4.8 km downstream from the contamination source at river km 125.9–129.6; hereafter RKM 126). The next was 68.1–71.9 km downstream from the source (river km 59.2–63.1; hereafter RKM 60). Our final site was across the state border with Tennessee at river km 3.1–4.7 (hereafter RKM 4), just above the confluence with the South Fork of the Holston River. This furthest downstream site was 126.5–128.1 km below the superfund site. Because these four sites were separated by a minimum of 20.1 km, we treated them as separate sites in our statistical comparisons. While it is possible for individual turtles to move considerable distances for activities such as nesting migrations (>11 km; Obbard 1980), it is highly improbable that a sizeable proportion of the population regularly moves between these distant sites.

Turtles were captured during the summer (June–July) of 2007 by hand, in basking traps, and in baited hoop nets (Memphis Net and Twine, Memphis, TN, USA). Traps were placed in areas that matched the microhabitat requirements of target species (e.g., slow-moving water, presence of coarse/woody debris, and structured bank) and then left for one to three nights. Traps were checked daily, and individual traps were moved if not successful after 2–3 nights. After the third night, traps were removed from the site or rebaited and often moved within a site. Sample sizes of the three species used for Hg analysis varied by site, but were as follows (RKM 150, 126, 60, and 4, respectively): *C. serpentina* n = 13, 23, 17, and 14; *S. odoratus* n = 16, 19, 16, and 16; *G. geographica* n = 14, 22, 12, and 4.

On capture, turtles were measured for carapace length, carapace width, and plastron length and for mass to the nearest 0.5 kg for snapping turtles or the nearest 0.005 kg for the other two species. A ~0.5–1-ml blood sample was drawn from the cervical sinus or caudal vein of each turtle using a 1-ml heparinized syringe for Hg analysis. A second ~0.3-ml blood sample was collected for stable isotope analysis from a subset of individuals using non-heparinized syringes. We also removed 1–2 mm of the tip of 3–4 hind toenails using a pair of fingernail clippers (for smaller turtles) or canine nail grooming clippers (for larger turtles). Care was taken not to penetrate the blood supply to the nail. Samples were immediately placed on ice, returned to the field house, and stored frozen until analyses. Turtles were each given permanent individual marks by notching three marginal scutes of the shell. A handheld Global Positioning System unit (Garmin International, Olathe, KS, USA) was used to obtain geospatial coordinates for each captured turtle. Turtles were then released at their point of capture.

Through the course of the first few weeks of our study, we opportunistically collected eggs from 11 gravid females. After confirmation of gravidity using palpation, these females were returned to the field house where they were injected with oxytocin to induce egg laying. At oviposition, eggs were enumerated, measured, and weighed. One egg from each clutch was immediately frozen for Hg analysis. Remaining eggs were incubated and hatchlings were later released at the site where the female was originally collected. Although the sample sizes were small and reproductive assessments were beyond the scope of this study, these Hg concentrations are included for descriptive purposes because so little is known about maternal transfer of Hg in turtles.

Mercury analysis

All samples were analyzed for total Hg content. We did not analyze blood or nails for methylHg because of small sample masses (for nails) and because it was determined in previous work that most (70–100 %) Hg in turtle blood is methylated (Bergeron et al. 2007). Likewise, it is known that Hg in keratinized tissues such as feather is predominately methylated (Thompson and Furness 1989; Hopkins et al. 2007). Because nails are also keratinized, it is likely that most Hg in this tissue is methylated.

Frozen samples were shipped on ice to the University of Connecticut for analysis. All samples were analyzed on a fresh weight (FW) basis. Blood and egg samples were analyzed for total mercury by EPA method 245.6 (USEPA 1991). Each sample was digested with nitric and sulfuric acids, samples were allowed to cool and potassium permanganate was then added, followed by the addition of potassium persulfate. After the samples were allowed to stand overnight, hydroxylamine hydrochloride was added to each tube and then analyzed using cold vapor atomic absorption (CVAA). Sample mass for blood analysis ranged from 2.0 to 221.5 mg and the egg sample mass was approximately 0.5 g. Nail samples were analyzed for total Hg by EPA method 1631 (USEPA 2002). Each sample was digested with nitric and sulfuric acids, oxidized with bromine monochloride, purged onto a gold amalgamation trap, and desorbed into a cold vapor atomic fluorescence

(CVAFS) for analysis. Sample mass for the nails ranged from 0.2 to 93.5 mg. The calibration curve consisted of five standards for CVAA analysis and six standards for CVAFS, with a correlation coefficient greater than 0.999 for all analytical runs. Standard quality assurance procedures were employed, including analysis of duplicate samples, method blanks, spiked samples, laboratory control samples, and standard reference materials (DOLT-3 and DORM-2, NRC Canada; SRM 966, NIST). Instrument response was evaluated initially, every 20 samples, and at the end of an analytical run using a calibration verification standard and blank.

Stable isotope analysis

Blood samples from a subset of the turtles used in the study were analyzed for their isotopic composition of nitrogen (N) and carbon (C). A total of 109 samples were analyzed, composed of blood from 12 to 13 individuals of each species from RKM 150 (reference site), 126, and 60. We did not analyze any samples from the most downstream site (RKM 4) for N and C because of insufficient sample sizes for one species.

At the Virginia Institute of Marine Sciences, whole-blood samples were lyophilized, weighed to the nearest microgram, and placed into pre-cleaned tin capsules. Samples were then shipped to the Stable Isotope Facility at UC Davis where they were analyzed using an elemental analyzer (PDZ Europa ANCA-GSL) coupled to a continuous-flow isotope ratio mass spectrometer (PDZ Europa 20-20 isotope ratio mass spectrometer; Sercon Ltd., Cheshire, UK). Stable isotope ratios are reported in per mill units (‰) using δ notation ($\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$), where $X = {}^{13}\text{C}$ or ${}^{15}\text{N}$ and R = the ratio of ${}^{13}\text{C}/{}^{12}\text{C}$ or ${}^{15}\text{N}/{}^{14}\text{N}$ in a sample or standard reference material. Values were calibrated to atmospheric nitrogen and Vienna-PeeDee Belemnite and two laboratory standards were run with every 12 samples.

Statistical analyses

Prior to any statistical analyses, we verified whether assumptions of parametric models (homoscedasticity and normality) were met. Neither blood Hg nor nail Hg concentrations were normally distributed so in several cases non-parametric tests were used. All analyses were performed with SAS 9.1 (SAS Institute, Cary, NC, USA). A Bonferroni-corrected alpha value of 0.025 was used to assess statistical significance because blood Hg and nail Hg concentrations were not independent of one another. Our dataset contained one obvious outlier; one *S. odoratus* from RKM 60 had a nail Hg concentration of 74,250 ng g^{-1}, which was 4.8 times the IQR from the third quartile value, and was removed from the dataset. However, this individual's blood Hg concentration (376 ng g^{-1}) fell within the normal range (14–826 ng g^{-1}) so was included in comparisons of blood Hg.

We first determined whether sex or size affected blood Hg or nail Hg concentrations because these factors are known to influence Hg accumulation in some fish and wildlife species (Wiener and Spry 1996; Evers et al. 1998; Rimmer et al. 2005). We conducted Wilcoxon two-sample tests to examine sex differences in Hg concentrations within each of the three turtle species. Next, we examined whether body size affected blood Hg or nail Hg concentrations by regressing log Hg concentration (ng g^{-1} FW) by log body mass (g) at each site for each species. There was no effect of sex on Hg concentrations and the effect of mass on Hg was significant for only one species for one tissue at one site (see "Results"). Therefore, neither sex nor mass was included in subsequent analyses.

To examine the effects of site and species on Hg concentrations, we used the nonparametric equivalent of a 2-way analysis of variance (ANOVA), the Scheirer–Ray–Hare extension of the Kruskal–Wallis test (Sokal and Rohlf 1995). We calculated Spearman correlation coefficients (r_s) to examine the relationship between blood Hg and nail Hg concentrations for each species individually and again with all species combined. The equation describing the linear relationship between both variables was also determined.

We calculated Spearman correlation coefficients to examine the relationship between egg Hg and maternal blood Hg and nail Hg concentrations. Due to small sample sizes, we combined *G. geographica* (n = 8) and *S. odoratus* (n = 3) data for both analyses. The equations describing linear relationships between egg and maternal Hg concentrations were also determined.

The fraction of ${}^{13}\text{C}$ isotopes to ${}^{12}\text{C}$ isotopes, $\delta^{13}\text{C}$, were normally distributed, as were $\delta^{15}\text{N}$ values for all three species. We examined the correlation (Pearson correlation coefficient) between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, pooled across species, and calculated the equation describing this linear relationship. A 2-way ANOVA was used to examine the effects of site, species, and their interaction on $\delta^{15}\text{N}$ values. Tukey's multiple comparisons tests were then conducted to compare $\delta^{15}\text{N}$ values among species. These tests were repeated for $\delta^{13}\text{C}$ values. We used Spearman correlation coefficients to describe the relationships between blood and nail Hg concentrations and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for each species and all species combined. The equations describing the linear relationships among variables were also determined.

Results

Site, species, and tissue differences

Neither sex nor mass significantly affected either blood Hg or nail Hg concentrations (in all cases $p > 0.25$) with one exception: mass was positively correlated with the Hg

concentration in nails of *C. serpentina* at one site (RKM 126; $r^2 = 0.26$, $p = 0.013$). However, Hg concentrations in both tissues varied among species and sites (Fig. 1). There were significant effects of species ($\chi^2 = 92.9$, df = 2, $p < 0.0001$) and site ($\chi^2 = 7.81$, df = 3, $p = 0.020$) on blood Hg concentrations, but their interaction was not significant ($p = 0.21$) suggesting that species differences scaled similarly across sites. Similarly, both species ($\chi^2 = 78.2$, df = 2, $p < 0.0001$) and site ($\chi^2 = 44.4$, df = 3, $p < 0.0001$) significantly affected nail Hg concentrations, but their interaction was not significant ($p = 0.36$). Mercury concentrations in turtle tissues were low at the reference site (RKM 150) but rose by as much as 10 fold at RKM 126, the site nearest the contamination source. Tissue concentrations peaked at RKM 126 and 60, and tended to decline at the most downstream site (RKM 4). However, tissue concentrations remained elevated at the most downstream site compared to the reference site, even though the site was nearly 130 km downstream from the contamination source. In fact, all three species at the most downstream site had nail concentrations of Hg that were ~3–4 times the concentrations found in conspecifics at the reference site.

In general, nail Hg concentrations were more than an order of magnitude higher than blood Hg concentrations. Regardless of site, *C. serpentina* and *S. odoratus* consistently had higher nail and blood Hg concentrations than *G. geographica* downstream from the superfund site, but this effect was most evident for the nail tissue (Fig. 1). There was a strong positive correlation between concentrations of Hg in blood and nail for each turtle species (Fig. 2a–c, all $r_s \geq 0.69$; p values < 0.0001). When data were pooled for all three turtle species, the relationship between nail Hg and blood Hg remained strong (Fig. 2d, $r_s = 0.73$, $p < 0.0001$).

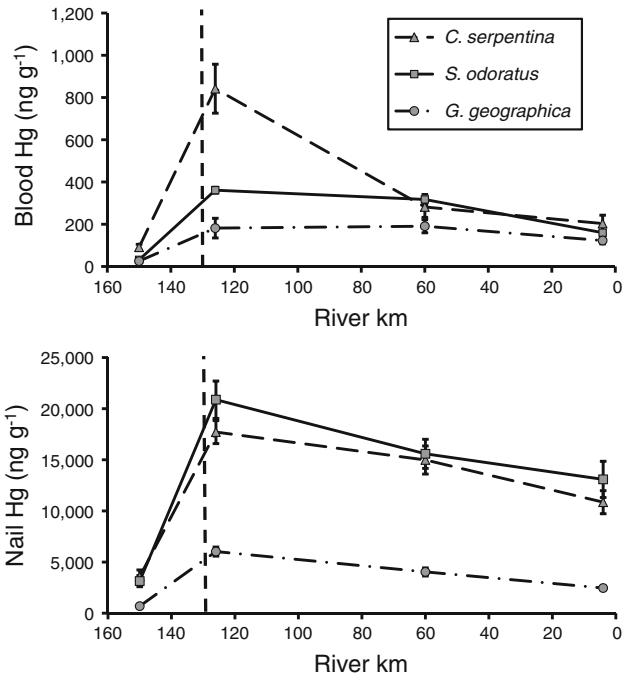

Fig. 1 Blood and nail Hg (ng g^{-1} fresh wt: mean ± standard error) concentrations in three species of turtles (*Chelydra serpentina*, *Sternotherus odoratus*, and *Graptemys geographica*) collected at four sites along the North Fork Holston River (river km 150-4). Mercury contamination occurred at river km 131.2, indicated by a *vertical dashed line*

Stable isotopes

Nitrogen isotope ratios in blood varied among sites and turtle species (site: $F = 122$, $p < 0.0001$; species: $F = 124$, $p < 0.0001$; site * species: $F = 5.0$, $p = 0.001$). Pairwise comparisons showed that mean δ^{15}N values differed for all three species, but *C. serpentina* consistently had higher δ^{15}N levels than *S. odoratus* and *G. geographica* (Fig. 3). The significant interaction term in the model was primarily driven by *S. odoratus* and *G. geographica* differing in δ^{15}N values at the ref site (RKM 150) and RKM 60, but not differing at RKM 126. Similarly, δ^{13}C values were significantly affected by site ($F = 34.2$, $p < 0.0001$), species ($F = 20.2$, $p < 0.0001$) and their interaction ($F = 7.3$, $p < 0.0001$). As was observed for δ^{15}N values, δ^{13}C values for *C. serpentina* were significantly higher than those of *S. odoratus* and *G. geographica*. The significant interaction term in the model was primarily driven by δ^{13}C values remaining consistent across sites for *C. serpentina*, but varying across sites for *S. odoratus* and *G. geographica*. There was a significant linear relationship between δ^{13}C and δ^{15}N values when data were pooled across species ($r = 0.48$, $p < 0.0001$).

Blood Hg and nail Hg concentrations for all three species were positively correlated with blood δ^{15}N values (Fig. 4; $r_s = 0.68$–0.82, p values < 0.0001). The relationship between blood δ^{15}N and blood Hg and nail Hg remained strong when data were pooled across species (Fig. 4; $r_s = 0.70$ and 0.71, p values < 0.0001). In contrast, the results for δ^{13}C were not as consistent. Blood δ^{13}C values in *S. odoratus* were highly correlated with concentrations of Hg in blood ($r_s = 0.71$, $p < 0.0001$) and nail ($r_s = 0.59$, $p < 0.0002$) (Fig. 4). Blood δ^{13}C values in *G. geographica* were also significantly correlated with blood Hg ($r_s = 0.40$, $p = 0.015$) and nail Hg concentrations ($r_s = 0.48$, $p < 0.003$). However, *C. serpentina* δ^{13}C values were not significantly correlated with blood Hg ($r_s = 0.20$, $p = 0.24$) and nail Hg levels ($r_s = 0.33$, $p = 0.045$). When pooled across species, δ^{13}C values were less strongly correlated with blood Hg ($r_s = 0.44$, $p < 0.0001$) and nail Hg ($r_s = 0.42$, $p < 0.0001$) values than δ^{15}N values (Fig. 4).

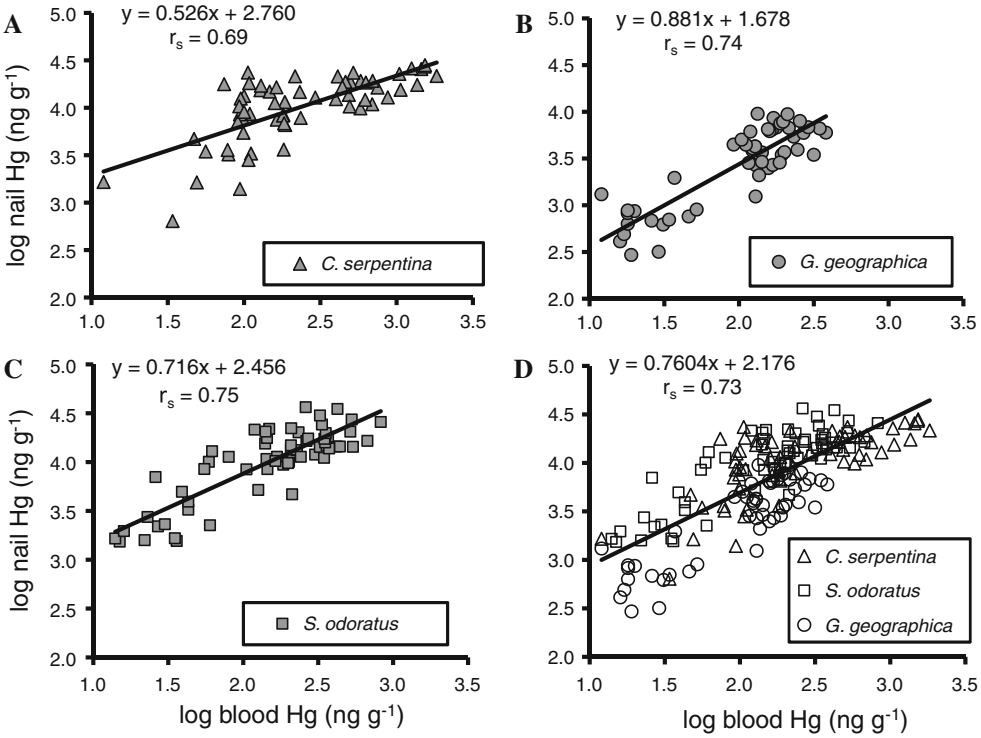

Fig. 2 Spearman's correlations between log blood and log nail Hg concentrations (fresh wt) in three species of turtles (**a** *Chelydra serpentina*, **b** *Sternotherus odoratus*, and **c** *Graptemys geographica*) collected at the North Fork Holston River. The species are shown together (**d**) with individuals as *open symbols*

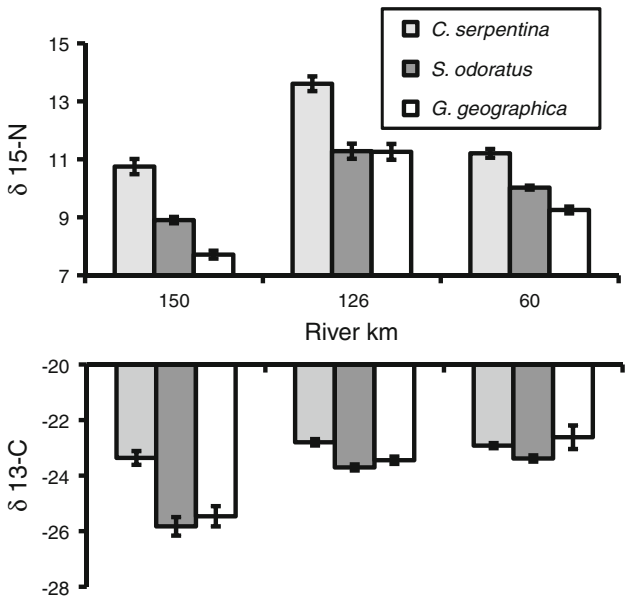

Fig. 3 Blood nitrogen and carbon isotopic ratios (±1 SE) in three species of turtles (*Chelydra serpentina*, *Sternotherus odoratus*, and *Graptemys geographica*) collected at three sites along the North Fork Holston River

Mercury in eggs

Although our sample size for the opportunistic sampling of eggs was small, the results indicate that turtles downstream from the superfund site maternally transfer Hg to their eggs. Mercury concentrations in *S. odoratus* and *G. geographica* eggs from the reference site (RKM 150) averaged 11 ng g^{-1} (FW), but eggs from female conspecifics collected downstream from the superfund site averaged 53 ng g^{-1}. The two *S. odoratus* sampled downstream had much higher Hg concentrations in their eggs (mean = 92 ng g^{-1}) than *G. geographica* (mean = 42 ng g^{-1}), which is consistent with their feeding ecologies and observed Hg concentrations in nails and blood. Egg Hg concentrations were positively correlated with concentrations in nail ($r_s = 0.92$, $p < 0.0001$), but were not significantly correlated with blood Hg concentrations ($r_s = 0.51$, $p = 0.11$; Fig. 5).

Discussion

Spatial and tissue differences

Our study confirmed that turtles inhabiting areas downstream of the source of Hg pollution on the NFHR are at significant risk of Hg exposure. Tissue concentrations of Hg rose quickly after the point source and remained elevated for considerable distances, in most cases ~130 km downstream in Tennessee. The extent of the downstream exposure for turtles was consistent with previous findings for other organisms in the NFHR. For example, previous studies have shown extirpations of mussel populations as far as 112 km downstream of the superfund site (Young-Morgan & Associates 1990) and elevated Hg in fish tissues 133 km (Hildebrand et al. 1980) and >160 km downstream

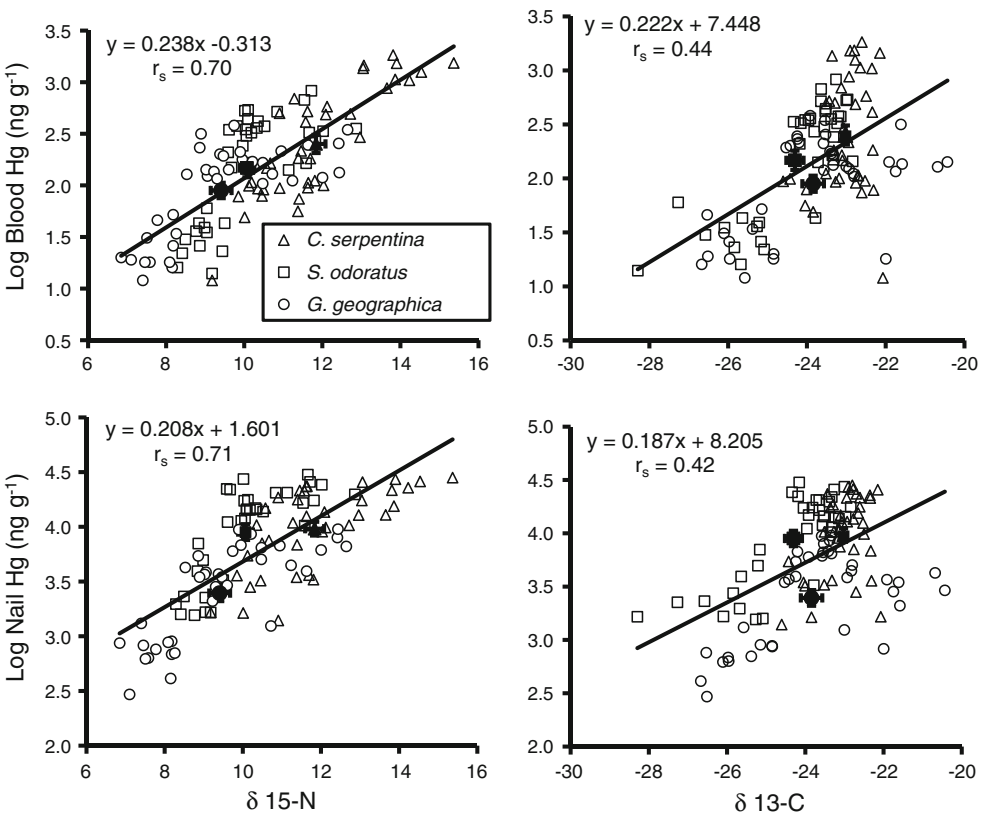

Fig. 4 Relationship between nitrogen and carbon isotopic ratios and tissue Hg concentrations (ng g^{-1} fresh wt) in three species of turtles (*Chelydra serpentina*, *Sternotherus odoratus*, and *Graptemys geographica*) collected at three sites along the North Fork Holston River. *Open symbols* indicate individual values and *solid symbols* indicate species means (±1 SE)

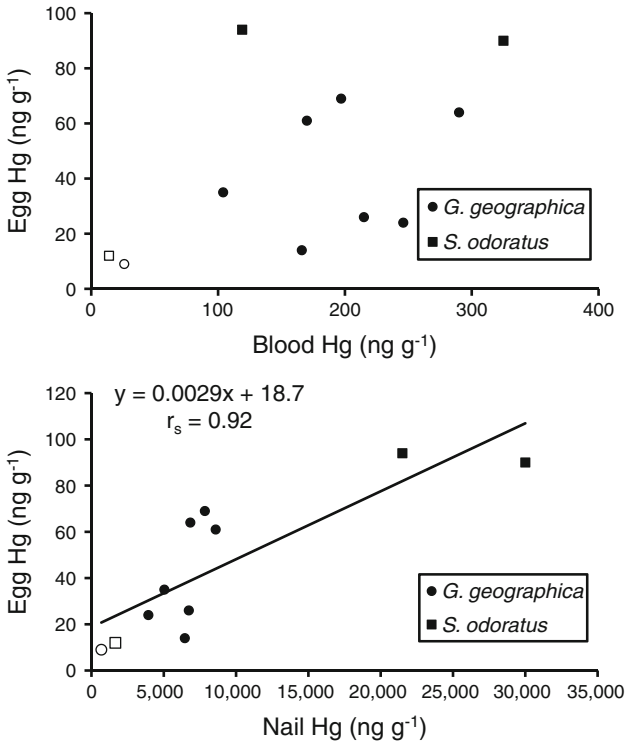

Fig. 5 Correlations between maternal blood Hg ($p = 0.11$) and nail Hg ($p < 0.0001$) with egg Hg concentrations (all fresh wt) from two species of turtle collected at a reference site (*open symbols*) and Hg contaminated site (*solid symbols*) along the Holston River, VA

(Carter 1977). Our results verify that downstream transport of Hg from the superfund site continues to be a significant health concern for certain species of wildlife over vast expanses of river.

Although nail concentrations of Hg in turtles have not been well studied, several published reports of Hg concentrations in turtle blood allow comparisons with our results. Blood Hg concentrations in turtles from the contaminated area of the NFHR (up to 1,800 ng g^{-1} just downstream from the superfund site) were among the highest ever documented in turtles, surpassed only by turtles on the aforementioned South River, VA (up to 3,600 ng g^{-1}; Bergeron et al. 2007) which was also polluted by a point source. Other studies that did not focus on well-defined point sources recorded much lower blood Hg concentrations: *C. serpentina* between 50 and 500 ng g^{-1} (Golet and Haines 2001), *Caretta caretta* (loggerhead sea turtle) between 57 and 141 ng g^{-1} (Day et al. 2005), and *Lepidochelys kempii* (Kemp's ridley sea turtle) between 0.50 and 67.3 ng g^{-1} (Kenyon et al. 2001). Blood Hg concentrations in turtles from our reference site, ranging between 12 and 183 ng g^{-1}, agreed well with these other studies.

One of the most important findings from our study was that nail clippings provided useful information about the Hg exposure history of turtles. Nail concentrations of Hg were generally an order of magnitude higher than blood

concentrations, but Hg concentrations in these two tissues were strongly correlated with one another. The fact that nail concentrations were so high was not surprising given the affinity of keratinized tissue such as nail and feathers for Hg (Thompson and Furness 1989; Hopkins et al. 2007). However, the advantage of using nails over blood was evident when comparing Hg concentrations in these tissues across sites. Site differences were more obvious and consistent with nails than with blood, partly because nail concentrations of Hg were less variable among turtles than blood concentrations. Based on coefficients of variation (CV) calculated for each species at each site, the variance for Hg concentrations in blood was considerably higher than that in nails in 8 out of 12 cases (mean of 8 cases = 70 % higher CV in blood than in nails). In addition, it is important to note that blood concentrations of Hg decreased significantly at the most downstream study area, but declines in nail concentrations of Hg were modest in comparison. Clearly, different conclusions regarding the exposure of turtles might be drawn if only blood was sampled.

Nondestructive tissues such as blood and nail provide different types of information and should be interpreted appropriately with these constraints in mind (Hopkins et al. 2001, 2005, 2007). Blood Hg concentrations primarily represent recent dietary uptake (Hobson and Clark 1993, 1994; Bearhop et al. 2000; Evers et al. 2005) and thus provide information about Hg recently encountered by turtles. In contrast, nail tissue grows continuously (Bearhop et al. 2003; Hopkins et al. 2007) and is more representative of the recent body pool of Hg within several months of sampling. Thus, nail tissue concentrations represent the integration of exposure over previous months, whereas blood Hg concentrations can be influenced greatly by what was ingested in days immediately prior to sampling. Because nails should not be susceptible to variations in dietary Hg that occurred over small timescales, our observation that nail Hg concentrations were less variable than blood Hg concentrations from the same turtles is consistent with the physiology of these tissues.

We hypothesize that nails will be a better predictor than blood of accumulation in target organs such as brain, liver, and kidney. We base this on recent work with piscivorous raptors (osprey) that demonstrated that Hg concentrations in talon were a better predictor of soft tissue Hg concentrations than feathers, the keratinized tissue usually used by scientists studying Hg in birds (Hopkins et al. 2007). Likewise, Day et al. (2005) concluded that scutes from turtles (another keratinized tissue) were more reliable than blood concentrations for predicting liver Hg concentrations. In addition, our pilot work on turtle eggs during this study revealed a strong positive relationship between Hg in nails and eggs despite small sample sizes, but we were unable to detect a statistically significant relationship between Hg in turtle blood and eggs from the same individuals. Our findings, in conjunction with recent work with birds, suggest that future studies on turtles and other clawed vertebrates should consider analyzing this keratinized tissue for Hg as a nondestructive complement to blood. If our predictions about the value of this tissue prove true, future investigations could be improved because nails can be sampled much faster and with less expertise than blood (saving staff time), and lower variance in Hg concentrations might permit smaller sample sizes to meet study objectives (saving analytical costs). The primary drawback that we have encountered with nails is that only a small amount of tissue can be sampled from smaller turtles (e.g., stinkpots), which can complicate laboratory analyses.

Species, sex, and size differences

The exposure of turtles to Hg along the NFHR varied among species. As predicted, the high trophic level predator, *C. serpentina*, and the benthic scavenger, *S. odoratus*, had the highest concentrations of Hg in their tissues. In all of the sites downstream from the Hg source, *G. geographica* had lower Hg concentrations in its tissues compared to the other two species. *C. serpentina* and *S. odoratus* were statistically indistinguishable in most cases, with the exception of blood samples from these two species immediately below the superfund site. This finding is similar to what we recently documented on the South River, VA (Bergeron et al. 2007) and suggests that small benthic turtles may face Hg exposure comparable to that encountered by their large predatory counterparts. Future work is needed to determine whether similar Hg exposure in these two species translates to comparable risks of adverse effects, particularly in terms of reproductive and behavioral outcomes.

Stable isotope analyses supported the known dietary preferences of these three species and were useful for drawing inferences about their relative trophic positions. Assuming a 2–5 ‰ increment between each successive trophic level (Vander Zanden and Rasmussen 2001; Post 2002), the range in individual δ^{15}N values (6.9–15.4 ‰) and mean species δ^{15}N values (9.4–11.8 ‰) suggested that individuals were feeding at more than one trophic level within the NFHR. In general, individual *G. geographica* fed at the lowest trophic level and *C. serpentina* fed at the highest level, but there was significant overlap among species consistent with opportunistic feeding patterns. These observations were remarkably similar to what we documented in recent work (Bergeron et al. 2007) in a different river with a different turtle assemblage. In fact, the δ^{15}N values of the two species that were included in that previous study and the current work, *C. serpentina*

(12.6 and 11.8 ‰, respectively) and *S. odoratus* (10.9 and 10.1 ‰, respectively), were quite consistent despite significant ecological differences between these systems.

In contrast to nitrogen, which displays differential fractionation at successive trophic levels, carbon exhibits little trophic fractionation (<1 ‰). This characteristic of $\delta^{13}C$ makes it useful for drawing inferences about differences in dietary carbon sources among species (DeNiro and Epstein 1978). We found significant differences in $\delta^{13}C$ values in blood among species, with individual turtles ranging from −20.4 to −28.3 ‰. Moreover, Hg concentrations in tissues were positively correlated with $\delta^{13}C$ values in this turtle assemblage. In light of this, and the fact that $\delta^{15}N$ and $\delta^{13}C$ values were positively correlated with each other, our results suggest that turtles were also feeding on multiple carbon sources in the NFHR. Specifically, *C. serpentina* was ingesting carbon sources with higher $\delta^{13}C$ values than *S. odoratus* and *G. geographica*. In addition, the range of $\delta^{13}C$ signatures in blood suggest that *C. serpentina* (range: −22.1 to −24.6 ‰) was feeding more narrowly on carbon sources than the other two species (*S. odoratus* range: −22.8 to −28.3 ‰; *G. geographica* range: −20.4 to −26.7 ‰). In future studies, it may prove useful to analyze stable isotopes in nail tissue to determine if this dietary variability is integrated over time or is an artifact of using blood which is more sensitive to recent dietary composition.

We did not detect any consistent effect of sex or size on tissue Hg concentrations in our study population. Some metal concentrations in tissues are known to differ with sex, presumably due to sex-specific differences in feeding ecology, growth rates, body size, and/or to the elimination of contaminants in eggs by females (Meyers-Schöne and Walton 1994; Wiener and Spry 1996). However, the literature on Hg in turtles is far from comprehensive and currently provides inconsistent findings on the effects of sex and size on Hg exposure. The lack of a sex effect in the current study is consistent with some previous work (Albers et al. 1986; Bergeron et al. 2007), but contradicts the sex differences documented by Kenyon et al. (2001; females higher than males) and Meyers-Schöne et al. (1993; males higher than females). In the case of body size in our study, there was only one instance (nails of *C. serpentina* at one site) where body mass influenced Hg concentrations in tissues, and this effect was fairly weak statistically. In previous work *C. serpentina* was the only turtle species with a detectable correlation between blood Hg concentration and body mass, perhaps because *C. serpentina* had the largest range in body size of all species studied (Bergeron et al. 2007). The literature on this subject for turtles is again inconsistent; Kenyon et al. (2001), Turnquist et al. (2011) and Meyers-Schöne et al. (1993) found a positive correlation between tissue Hg concentration and body size in turtles, but others have not found these relationships (Helwig and Hora 1983; Golet and Haines 2001; Turnquist et al. 2011).

Conclusion

Our study clearly demonstrated that turtles in the NFHR are exposed to elevated levels of Hg for considerable distances downstream from the source of pollution. In all cases, tissue concentrations of Hg rose rapidly immediately below the superfund site and remained significantly elevated 70 km downstream. In most cases, tissue Hg concentrations remained significantly elevated even at the most downstream site (∼130 km) in Tennessee, sometimes ∼3–4 times the concentrations found in conspecifics at the reference site. At least two of the species we studied, *C. serpentina* and *S. odoratus*, appear particularly at risk of exposure to Hg, probably because of their feeding ecologies. We also determined that both blood and nail tissue are useful indices of Hg exposure in turtles, but that nails may be superior because they provide a signal of Hg exposure that is integrated over time. Because nondestructive tissue sampling is often preferred over lethal sampling, especially in long-lived vertebrates (e.g., turtles) and species of conservation concern (Hopkins et al. 2001, 2007; Jackson et al. 2003), we believe both of these techniques show great promise for ecological monitoring of turtles. Future studies should consider using both metrics until the use of nails is further refined and validated.

The major question that remains is whether the observed Hg concentrations are sufficient to elicit adverse effects in turtles. Unfortunately, relationships between tissue concentrations of Hg and adverse effects do not currently exist for turtles or other reptiles, and drawing conclusions based on tissue criteria for other species involves too much uncertainty to be of value (Hopkins 2006). In fact, even within a taxonomic group, sensitivity can vary by more than an order of magnitude. For example, among 23 species of birds LC_{50} values for eggs injected with methylHg ranged from 0.12 to 4.33 μg g^{-1} wet mass (Heinz et al. 2009). Thus, the best approach for determining whether Hg contamination is affecting turtles in the NFHR will be to assess their reproductive status using controlled incubation of eggs collected from females upstream and downstream from the superfund site, and to relate the females' blood, nail, and egg Hg concentrations to these reproductive outcomes. Given the critical status of turtle populations around the world (Gibbons et al. 2000) and the ubiquity of contaminants such as Hg, such tissue residue-response relationships will be critical to future conservation efforts on the NFHR and other sites. We suggest that lotic systems such as the NFHR may be ideal situations for developing these mathematical relationships because large contamination gradients exist within a single ecosystem.

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Conflict of interest The authors declare that they have no conflicts of interest.

References

Albers PH, Sileo L, Mulhern BM (1986) Effects of environmental contamination on snapping turtles of a tidal wetland. Arch Environ Contam Toxicol 15:39–109

Barr JF (1986) Population dynamics of the common loon (*Gavia immer*) associated with mercury-contaminated waters in northwestern Ontario. Canadian Wildlife Service Occasional paper no. 56

Bearhop S, Waldron S, Thompson D, Furness R (2000) Bioamplification of mercury in great skua *Catharacta skua* chicks: The influence of trophic status as determined by stable isotope signatures of blood and feathers. Mar Pollut Bull 40:181–185

Bearhop S, Furness R, Hilton GM, Votier SC, Waldron S (2003) A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. Funct Ecol 17:270–275

Bergeron CM, Husak JF, Unrine JM, Romanek CS, Hopkins WA (2007) Influence of feeding ecology on blood mercury concentrations in four species of turtles. Environ Toxicol Chem 26:1733–1741

Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA (2010a) Mercury accumulation along a contamination gradient and nondestructive indices of exposure in amphibians. Environ Toxicol Chem 29:980–988

Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA (2010b) Bioaccumulation and maternal transfer of mercury and selenium in amphibians. Environ Toxicol Chem 29:989–997

Bergeron CM, Hopkins WA, Todd BD, Hepner MJ, Unrine JM (2011) Interactive effects of maternal and dietary mercury exposure have latent and lethal consequences for amphibian larvae. Environ Sci Technol 45:3781–3787

Bonzongo JC, Lyons WB, Hines ME, Warwick JJ, Faganeli J, Horvat M, Lechler PJ, Miller JR (2002) Mercury in surface waters of three mine-dominated river systems: Idrija River, Slovenia; Carson River, Nevada; and Madeira River, Brazilian Amazon. Geochem Explor Environ Anal 2:111–119

Carter LJ (1977) Chemical-plants leave unexpected legacy for two Virginia rivers. Science 198:1015–1020

Congdon JD, Greene JL, Brooks RJ (2008) Reproductive and nesting ecology of female snapping turtles. In: Steyermark AC, Finkler MS, Brooks RJ (eds) Biology of the Snapping Turtle (*Chelydra serpentina*). Johns Hopkins University Press, Baltimore, pp 123–134

Cristol DA, Brasso RL, Condon AM, Fovargue RE, Friedman SL, Hallinger KK, Monroe AP, White AE (2008) The movement of aquatic mercury through terrestrial food webs. Science 320:335

Day RD, Christopher SJ, Becker PR, Whitaker DW (2005) Monitoring mercury in the loggerhead sea turtle, *Caretta caretta*. Environ Sci Technol 39:437–446

DeNiro MJ, Epstein S (1978) Influence of diet on the distribution of carbon isotopes in animals. Geochim Cosmochim Acta 42:495–506

Drevnick PE, Sandheinrich MB (2003) Effects of dietary methylmercury on reproductive endocrinology of fathead minnows. Environ Sci Technol 37:4390–4396

Eisler R (2006) Mercury hazards to living organisms. CRC Press, Boca Raton

Ernst CH, Lovich JE, Barbour RW (1994) Turtles of the United States and Canada. Smithsonian Institute Press, Washington, DC

Evers DC, Kaplan JD, Meyer MW, Reaman PS, Braselton WE, Major A, Burgess N, Scheuhammer AM (1998) Geographical trend in mercury measured in common loon feathers and blood. Environ Toxicol Chem 17:173–183

Evers DC, Burgess NM, Champoux L, Hoskins B, Major A, Goodale WM, Taylor RJ, Poppenga R, Daigle T (2005) Patterns and interpretation of mercury exposure in freshwater avian communities in northeastern North America. Ecotoxicology 14:193–221

Gibbons JW, Scott DE, Ryan TJ, Buhlmann KA, Tuberville TD, Metts BS, Greene JL, Mills T, Leiden Y, Poppy S, Winne CT (2000) The global decline of reptiles, déjà vu amphibians. Bioscience 50:653–666

Golet WJ, Haines TA (2001) Snapping turtles (*Chelydra serpentina*) as monitors for mercury contamination of aquatic environments. Environ Monit Assess 71:211–220

Hallinger KK, Cornell KL, Brasso RL, Cristol DA (2011) Mercury exposure and survival in free-living swallows (*Tachycineta bicolor*). Ecotoxicology 20:39–46

Hammerschmidt CR, Sandheinrich MB, Wiener JG, Rada RG (2002) Effects of dietary methylmercury on reproduction of fathead minnows. Environ Sci Technol 36:877–883

Heinz GH (1996) Mercury poisoning in wildlife. In: Fairbrother AL, Locke LN, Hoff GL (eds) Noninfectious diseases of wildlife, 2nd edn. Iowa State University Press, Ames, pp 118–127

Heinz GH, Hoffman DJ, Klimstra JD, Stebbins KR, Kondrad SL, Erwin CA (2009) Species differences in the sensitivity of avian embryos to methylmercury. Arch Environ Contam Toxicol 56:129–138

Helwig DD, Hora ME (1983) Polychlorinated biphenyl, mercury, and cadmium concentrations in Minnesota snapping turtles. Bull Environ Contam Toxicol 30:186–190

Hildebrand SG, Strand RH, Huckabee JW (1980) Mercury accumulation in fish and invertebrates of the North Fork Holston River, Virginia and Tennessee. J Environ Qual 9:393–400

Hill WR, Stewart AJ, Napolitano GE (1996) Mercury speciation and bioaccumulation in lotic primary producers and primary consumers. Can J Fish Aquat Sci 53:812–819

Hobson KA, Clark RG (1993) Turnover of 13C in cellular and plasma fractions of blood: implications for nondestructive sampling in avian dietary studies. Auk 110:638–641

Hobson KA, Clark RG (1994) Assessing avian diets using stable isotopes I: turnover of 13C in tissues. Condor 94:181–188

Hopkins WA (2006) Use of tissue residues in reptile ecotoxicology: a call for integration and experimentalism. In: Gardner S, Oberdorster E (eds) New perspectives: toxicology and the environment, vol 3., Reptile toxicologyTaylor and Francis Publishers, London, pp 35–62

Hopkins WA (2007) Amphibians as models for studying environmental change. Inst Lab Anim Res J 48:270–277

Hopkins WA, Rowe CL (2010) Interdisciplinary and hierarchical approaches for studying the effects of metals and metalloids on amphibians. In: Sparling D, Linder G, Bishop CA (eds) Ecotoxicology of amphibians and reptiles, 2nd edn. SETAC Press, Pensacola, pp 325–336

Hopkins WA, Roe JH, Snodgrass JW, Jackson BP, Kling DE, Rowe CL, Congdon JD (2001) Nondestructive indices of trace element exposure in squamate reptiles. Environ Pollut 115:1–7

Hopkins WA, Snodgrass JW, Baionno JA, Roe JH, Staub BP, Jackson BP (2005) Functional relationships among selenium concentrations in the diet, target tissues, and nondestructive tissue samples of two species of snakes. Environ Toxicol Chem 24:344–351

Hopkins WA, Hopkins LB, Unrine J, Snodgrass J, Elliot J (2007) Mercury concentrations in tissues of osprey from the Carolinas, USA. J Wildl Manage 71:1819–1829

Iverson JB (1982) Biomass in turtle populations—a neglected subject. Oecologia 55:69–76

Jackson BP, Hopkins WA, Baionno JA (2003) Laser ablation-ICP-MS analysis of micro-dissected tissue: a conservation-minded approach to assessing contaminant exposure. Environ Sci Technol 37:2511–2515

Kenyon LO, Landry AM, Gill GA (2001) Trace metal concentrations in blood of the Kemp's Ridley sea turtle (*Lepidochelys kempii*). Chelonian Conserv Biol 4:128–135

Meyers-Schöne L, Walton BT (1994) Turtles as monitors of chemical contaminants in the environment. Rev Environ Contam Toxicol 135:93–153

Meyers-Schöne L, Shugart LR, Beauchamp JJ, Walton BT (1993) Comparison of two fresh-water turtle species as monitors of radionuclide and chemical contamination—DNA-damage and residue analysis. Environ Toxicol Chem 12:1487–1496

Mitchell JC (1994) The reptiles of Virginia. Smithsonian Institute Press, Washington, DC

Obbard ME (1980) Nesting migrations of the snapping turtle (*Chelydra serpentina*). Herpetologica 36:158–162

Post DM (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83:703–718

Rimmer CC, McFarland KP, Evers DC, Miller EK, Aubry Y, Busby D, Taylor RJ (2005) Mercury concentrations in Bicknell's thrush and other insectivorous passerines in montane forests of northeastern North America. Ecotoxicology 14:223–240

Scheuhammer AM, Meyer MW, Sandheinrich MB, Murray MW (2007) Effects of environmental methylmercury on the health of wild birds, mammals, and fish. Ambio 36:12–18

Sokal RR, Rohlf FJ (1995) Biometry: principles and practice of statistics in biological research. W.H. Freeman, New York

Southworth GR, Peterson MJ, Bogle MA (2004) Bioaccumulation factors for mercury in stream fish. Environ Pract 6:135–143

Tan SW, Meiller JC, Mahaffey KR (2009) The endocrine effects of mercury in humans and wildlife. Crit Rev Toxicol 39:228–269

Thompson DR, Furness RW (1989) The chemical form of mercury stored in South Atlantic seabirds. Environ Pollut 60:305–317

Turnquist MA, Driscoll CT, Schulz KL, Schlaepfer MA (2011) Mercury concentrations in snapping turtles (*Chelydra serpentina*) correlate with environmental and landscape characteristics. Ecotoxicology 20:1599–1608

USEPA (1991) Determination of mercury in tissues by cold vapor atomic absorption spectrometry. EPA/600/4-91/010

USEPA (2002) Method 1631, Revision E: Mercury in water by oxidation, purge and trap, and cold vapor atomic fluorescence spectrometry. EPA-821-R-02-019

Vander Zanden MJ, Rasmussen JB (2001) Variation in δ^{15}N and δ^{13}C trophic fractionation: Implications for aquatic food web studies. Limnol Oceanogr 46:2061–2066

Wada H, Cristol DA, McNabb FMA, Hopkins WA (2009) Suppressed adrenocortical responses and triiodothyronine levels in tree swallow (*Tachycineta bicolor*) nestlings near a Hg-contaminated river. Environ Sci Technol 43:6031–6038

Watras CJ, Bloom NS (1992) Mercury and methylmercury in individual zooplankton—implications for bioaccumulation. Limnol Oceanogr 37:1313–1318

Wiener JG, Spry DJ (1996) Toxicological significance of mercury in freshwater fish. In: Beyer WN, Heinz GH, Redmon-Norwood AW (eds) Environmental contaminants in wildlife: interpreting tissue concentrations. Lewis Publishers, Boca Raton, pp 297–340

Wolfe MF, Schwarzbach S, Sulaiman RA (1998) Effects of mercury on wildlife: a comprehensive review. Environ Toxicol Chem 17:146–160

Young-Morgan & Associates (1990) An assessment of mussel communities in the North Fork Holston River. Prepared for: Olin Corporation, pp 1–22