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DNA Double-Strand Breakage as an Endpoint to Examine Metal and Radionuclide Exposure Effects to Water Snakes on a Nuclear Industrial Site

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ABSTRACT

This study examined metal levels (especially U and Ni) in the tail tissues of water snakes from contaminated (Tim's Branch) and reference areas on the Department of Energy's Savannah River Site (SRS). Home ranges of snakes were quantified to determine the ratio of the habitat that they use in relation to the contaminated areas to better estimate exposure. Compared to conventional methods that do not, the exposure assessment indicated that water snakes in the contaminated areas could expect U exposure at 3–4 orders of magnitude greater than the Agency for Toxic Substances and Disease Registry's Minimum Risk Level (MRL) from ingestion of amphibians and fish. Ni and U, in addition to Se, Mn, and Cu, were related to increased DNA double-strand breakage (DDSB) in water snakes. We report burdens for each metal individually, but the results of the DDSB indicated that these metals did not behave independently, but as a suite. If we did not have a secondary endpoint (DDSB), we might have assumed from the exposure predictions and tissue burden analyses that U was the sole metal of concern to water snakes in Tim's Branch. These data also imply that these toxicants do not biomagnify at the spatial and temporal scale of this study.

Key Words: DNA double-strand breaks, exposure, uranium, *Nerodia*, nickel, water snakes.

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INTRODUCTION

No risk to a biological system exists unless there is the potential for exposure, and accumulation that might cause adverse effects (Sparling *et al.* 2000). Risk assessment must be preceded by an understanding of the behavior of the toxicant in a particular environment. There are only a limited number of studies examining bioaccumulation and the effects of metal and radionuclide contamination in snakes and subsequently how these toxicants may pose adverse effects (Campbell and Campbell 2001). Snakes are model receptor species for risk assessment in relation to the extent of contamination through an ecosystem via trophic transfer because of their life history and ability to bioaccumulate contaminants (Hopkins 2000). For example, studies have found correlations between blood and tail snips with tissue burdens for metals that were elevated at their sample site (Hopkins *et al.* 2001).

On the U.S. Department of Energy's (USDOE) Savannah River Site (SRS), the effects of contamination can still be detected in the environment from disturbances over the past 50 years from production facilities. Specifically, the biogeochemical cycling of heavy metals in a riparian ecosystem (Steed's Pond–Tim's Branch) contaminated with U, Cr, Cu, Al, Mn, and particularly Ni, were investigated in relation to their bioavailability to carnivorous aquatic snakes: the banded water snake (*Nerodia fasciata*), the brown water snake (*Nerodia taxispilota*), and the cottonmouth (*Aghistrodon piscivorus*)—which is not taxonomically considered a water snake, but occupies a similar ecological niche.

Presently, predicting risk to reptile populations is difficult because of insufficient data for the estimation of individual responses as a function of heavy metal concentrations (Hopkins 2000). However, numerous effects of heavy metals on small mammal DNA have been summarized by Hartwig (1995), where specific metals such as Ni, Cr, As, and Cd have been associated with DNA damaging effects. Similarly, Lamb *et al.* (1991) illustrated DNA damage associated with gamma and strong beta radiation exposure from radiocesium and radiostrontium in slider turtles (*Trachemys* spp.). Because reptiles exhibit DNA damage as a result of a toxicant stress, the use of this metric as an endpoint of ecological risk in reptiles may be ideal in some systems (Lamb *et al.* 1991). Since reptiles have nucleated erythrocytes, a small blood sample can be used to quantify DNA double-strand breakage (DDSB) thus providing a non-destructive endpoint in modeling ecological risk.

This research examines the relationship between metal tissue concentration of water snakes to DDSB with a specific focus on U and Ni. Both the USDOE and the U.S. Environmental Protection Agency (USEPA) are focused on these contaminants in the efforts to remediate the disturbed watershed associated with Tim's Branch (Punshon *et al.* 2003b). DDSB was chosen as a specific endpoint because of the documented relationship of this metric to these and other toxicants. Specifically, Ni interferes with the repair of UV (ultra-violet) and X-ray-induced DNA damage during the recognition/incision and polymerization/ligation stages (Christie 1989). In fact, Christie (1989) has shown that DNA damage that has been affected by X-rays, and ultraviolet light may be more susceptible to cellular processing in the presence of Ni. Further, U gamma-emitting daughter products present in the Steed's Pond–Tim's Branch corridor may potentially contribute to DNA damage in water

snakes since similarities exist between the behavior of gamma rays and X-rays in DNA function.

Using Sample and Suter's model (1994) for estimating exposure of wildlife to toxicants, another aim was to produce a model for Ni and U exposure, and relate the estimated exposure values to biological effects. This model is used by both the USEPA and the USDOE in the risk assessment process, and is based on all exposure pathways. An exposure model for a semi-aquatic organism with an aquatic food base, such as *N. fasciata* (used in this study), has not been previously attempted. Although exposure from all pathways (E_{total}) is the sum of oral, dermal, and inhalation exposure, the estimates calculated here were only for oral exposure that included food and incidentally ingested soils. Several studies have indicated diet as the major exposure pathway for snakes (Fontenot *et al.* 2000; Hopkins *et al.* 2002). Inhalation and dermal exposure are considered minimal since the moisture level in this system is extremely high thus reducing air-borne particles and the aquatic nature of the snakes would preclude dermal exposure since most emissions from the beta and gamma-emitting daughter products of U as well as U itself will not penetrate far enough into the water (contamination is within the stream sediments) to cause exposure. Specifically, *Nerodia* spend much of their time in trees to seek prey and then dive into the water to obtain the prey at which most of their time is spent in the upper portions of the water column. Further, diffusion of metals across the skin is negligible (as compared to susceptible taxa such as anurans).

The specific objectives of this study were to: determine the home range of *N. fasciata* to estimate Ni and U exposure; determine the Ni, U and other associated metal concentrations in blood and tail snips of water snakes; compare the degree of DDSB found in the blood of water snakes collected from contaminated and reference sites; model the relationship between DDSB in water snakes with blood and tail snips metal concentrations. Using a field-based approach to examine ecological risk is unfortunately still relatively novel in the field of ecotoxicology, where the majority of studies are laboratory-based to avoid the burdens associated with natural variation. Although performing such studies in the field inherently adds more uncertainty and thus increases the need for sample sizes that can rarely be achieved (especially when focusing on a top predator), the ecological risk assessment field will not move forward until we examine the relationships between exposure estimates and endpoint effects in contaminated systems.

MATERIALS AND METHODS

Study Site

The Steed Pond–Tim's Branch depositional system (Figure 1) is located in the northwestern portion of the SRS, a 777 km² USDOE facility in the upper coastal plain of South Carolina (Pickett 1990). This stream system was impacted during the 1950s and the 1980s by discharges of depleted and natural U, Al, Ni, Cu, Cr, Zn, Pb, and other metals associated with nuclear production (Punshon *et al.* 2003a). Steed's Pond was used as a settling basin for contaminated sediments as a result of fuel and target processing activities. It is estimated that 44,000 kg of depleted U was released into Steed's Pond (Pickett 1990; Punshon *et al.* 2003a,b). The breach of a

Ni and U in Water Snakes

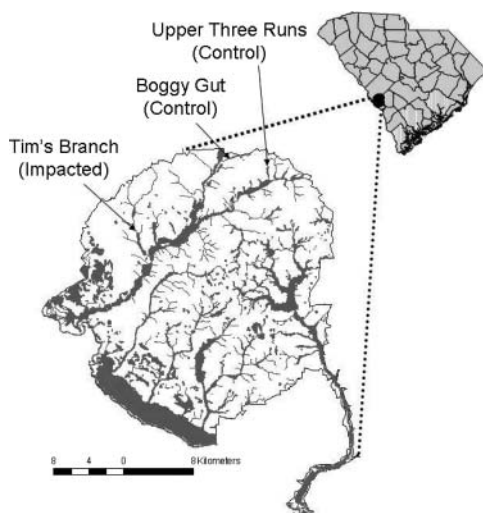


Figure 1. Map of the U.S. Department of Energy's Savannah River Site (SRS) located in west-central South Carolina, USA. Impacted and reference areas are shown in relationship to the wetlands and drainage system of the SRS (in gray).

wooden spillway in 1984, caused U and Ni contaminated sediment to flow into Tim's Branch. Downstream from Steeds Pond, along the Tim's Branch riparian area, is a beaver pond, hereafter referred to as Beaver Pond. The last wetland type along this watershed is a farm pond (Pond 25), which has also reverted to a stream after the failure of its dam from the influx of water from the Steed's Pond failure. Punshon *et al.* (2003a) indicated that U in Steed's Pond did not accumulate in high levels in the tissues of either cotton rats (*Sigmodon hispidus*) or marsh rice rats (*Oryzomys palustris*). However, Ni was shown to be bioavailable and accumulated in leaf tissues of black willow (*Salix nigra*) greater than published "background" concentrations. Similarly, Ni concentrations were elevated in liver and muscle tissues in both cotton rats and marsh rice rats from this site (Punshon *et al.* 2003b).

Field Collection and Home Range

Snakes (*N. fasciata*, *N. taxispilota*, and *A. piscivorous*) were collected during the months of April 2001 through July 2002 to account for seasonal differences in population abundance and heavy metal loads. Minnow traps were set at three locations within the impacted water shed (Steed's Pond, Beaver Pond, Pond 25) and at two reference areas (Boggy Gut and Upper Three Runs, Figure 1). The two reference sites are not a part of the Steed's Pond watershed. These sites have been commonly used as reference areas for SRS-based contaminant studies since there have been no reported disturbances to these sites from operations, and therefore are presumed to be representative of southeastern U.S. watersheds. To assess toxicant body burdens in prey, fishes and amphibians that are considered common prey items of *N. fasciata* (Gibbons and Dorcas 2004), were collected from the minnow traps set at each of the study sites for use in exposure estimation. Snakes captured in the field were brought

to the lab for no more than 2 days to obtain blood and muscle tissue samples and for morphometric measurements (snout-vent length, body weight, sex). A 8–10 mm of the tail tip (considered a muscle sample) was snipped as a representative for whole body tissue metal burden. At that time the tail blood was allowed to drip into two centrifuge tubes where 1–3 cc of blood was collected. Samples were immediately put on ice and transported to a –80°C freezer to await analysis. These activities were conducted under the Rutgers University (97–017) and University of Georgia's (A960205) animal care and use protocols.

N. fasciata with a mass greater than 100 g, caught during field collection (except for one-month prior to end of the activity season to allow for a tracking period) were fitted with a 5.0 g radio-transmitter manufactured by Holohil®. Transmitters were implanted by subcutaneous placement following the use of Ketamine to anaesthetize the snake at rate of 0.77 ml/kg (Reinert and Cundall 1982). When the snakes were fully anaesthetized, an incision was made at a pre-sterilized site in the latter 1/3 of the snout-vent length. The transmitter was inserted ventrally and placed so that the ribs “comfortably” surround it with the antenna threaded subcutaneously and anteriorly to allow for maximum reception. After transmitter insertion, the incision was closed using 4-0 gauge absorbable suture. Snakes were housed for one night with no water (to prevent drowning), in aquaria with dark shelter boxes and a heat strip. After the first night, water was placed back into the aquaria and the snakes monitored daily until normal activity appeared and no complications were observed. They were then released at the point of capture.

After release, each snake was located using radio-telemetry. Locations were determined by either triangulation or visual location. A global positioning system (GPS) was used to mark the animal's location or to mark radio-telemetry triangulation stations. Compass bearings were taken at a minimum of three stations and recorded onto a field map with a protractor. The bearings were then translated into ArcView© and x-y-coordinates were calculated to quantify home range. A minimum of 30 location points was determined to adequately minimize variance for the home range estimate (Worton 1987). A minimum period of 12 h was used between tracking sessions to prevent temporal autocorrelation between locations (Swihart and Slade 1985). Data points were collected at least once for each hour of the day as the maximal activity period (nocturnal v. diurnal) for *N. fasciata* is not known with certainty but also varies with season (Mills *et al.* 1995). Tracking was only performed weekly during the period of November through February (the typical hibernating period for snakes in the Southeast) once it was determined that individuals were indeed hibernating. Home range was quantified using CALHOME (Kie *et al.* 1996), utilizing the adaptive kernel method (Worton 1989).

Exposure Estimates for Ingestion

Oral exposure was estimated using the model described by Sample and Suter (1994) that utilizes the home range of an animal proportional to the size and utilization of the contaminated area expressed as body-weight normalized daily dose (mg/kg/day; Eq. (1)). Mean exposure estimates from food ingestion for the Tim's Branch watershed and the reference areas, Boggy Gut and Upper Three Runs, were determined using the data gathered from this study as well as previous

Ni and U in Water Snakes

studies on and off the SRS. Specifically, fishes and amphibians that are considered common prey items of *N. fasciata* (as cited in Gibbons and Dorcas 2004) were collected from the minnow traps set at each of the study sites for use in exposure estimation. These species were: bluegill and dollar sunfish (*Lepomis marginatus*), golden shiners (*Notemigonus crysoleucas*), red-fin pickerel (*Esox americanus*), pirate perch (*Aphredoderus sayanus*), green frog (*Rana clamitans*), and the southern toad (*Bufo terrestris*). Exposure was estimated using the concentration of Ni and U in each food type (amphibians, fishes), ingestion rate (0.26 g/g-day; Brown 1958; USEPA 1992), body weight (mass), waste site area (ha; from GIS-based contaminant distribution data layers), and home range size (ha) of endpoint species (*N. fasciata*) as determined from the home range portion of the study. The water in the Tim's Branch system was not contaminated (unpublished data) and soil ingestion rates for piscivores (and those animals that consume amphibians) are considered negligible (Sample and Suter 1994). Therefore exposure was estimated as:

$$E_j = \left(\frac{A}{HR} \left[\sum_{i=1}^m \left(\frac{FIR_i * C_{ij}}{BW} \right) \right] \right) \quad (1)$$

where: E_j = total exposure to contaminant (j) (mg/kg/d), m = total number of ingested media (*e.g.*, food, water, or soil), FIR_i = food ingestion rate (i) (kg/d or liter/d); see Eq. (2), C_{ij} = concentration contaminant (j) in medium (i) (mg/kg or mg/l), BW = body weight of endpoint species (kg), A = area (ha) of the waste site, HR = home range size (ha) of endpoint species.

Food ingestion rates (FIR_{dry}) were calculated based on models of the northern water snake (*N. sipedon*) presented in the Wildlife Exposures Factors Handbook (USEPA 1993), which were calculated using data from Brown (1958). Differences in ingestion rates between the two species, previously classified taxonomically as the same species, are likely to be insignificant. This ingestion rate was converted to fresh weight to reflect what the animal is ingesting in the field by considering the water content of the food, by using the following formula:

$$FIR_{fresh} = \sum_{i=1}^f \left(P_i \times \frac{FIR_{dry}}{1 - WC_i} \right) \quad (2)$$

where: FIR_{fresh} = total food ingestion rate (kg food_{fresh weight} / individual / day), P_i = proportion of the i th food type in the diet, WC_i = proportional water content (by weight) of the i th food type (as determined from drying the potential food items prior to metal analysis).

Four diet composition scenarios were used to estimate exposure based on Brown (1958): (1) 100% amphibians/0% fish; (2) 79% amphibians/21% fish; (3) 21% amphibians/79% fish; (4) 0% amphibian/100% fish. Amphibian species were pooled for analyses because preliminary Analysis of Variance (ANOVA) results showed no differences in toxicant load between the two species. Loads differed significantly between fish species, however, and subsequently were not combined. Since fish dietary preference for *N. fasciata* has not been recorded, each of the four fish species assumed equal contribution to exposure at 25%.

Calculated home range for *N. fasciata* (HR) was overlaid on top of waste site boundaries within a GIS (ArcView ©) and it was determined that the area of the

home range was smaller than the contaminated area (A). Therefore, the waste site area-to-home range ratio equaled one (1) and dropped from the model. Exposure for *N. fasciata* was divided into two age classes: juvenile and adult based on the minimum snout-vent length observed at sexual maturity (52 cm for males and 58 cm for females; Tinkle 1959). However, preliminary ANOVA models indicated that no differences existed based on sex; therefore, exposures were only calculated separately by age group.

Metal Analyses

After collection, blood and muscle tissue samples were freeze-dried and digested with 5 ml 5 M HNO₃ (trace metal grade) in pure Teflon(tm) PFA vessels using microwave digestion apparatus (MDS-2000, CEM Corporation, Matthews, NC). A HNO₃ blank and a U.S. National Institute of Standards and Technology (NIST) standard reference material (TORT) were included with each carousel after which samples were analyzed using inductively coupled plasma-mass spectrometry (ICP-MS). Ni concentrations in prey items were determined in the same way as the blood and tail snips. Animals were freeze-dried, digested using the whole body to reflect what the snake ingests, and analyzed using ICP-MS. Digested tissue samples followed a modified methodology outlined in USEPA method 3052 and quality control procedures based on USEPA method 6020.

DNA Double-Strand Breakage

Blood samples for DNA double-strand breakage (DDSB) were taken from field-captured snakes (*N. fasciata*, *N. taxispilota*, *A. piscivorous*). After snipping, the tail blood was allowed to drip into two centrifuge tubes. Tubes contained a mixture of heparin and high salt SDE buffer to prevent clotting and stabilize the mixture to keep cell disruption to as low a level as possible. Samples were immediately put on ice and thereafter into a freezer at -20°C until lab analyses were performed.

DNA samples were prepared for agarose-based Pulsed Field Gel Electrophoresis (PFGE). Blood samples were first embedded in agarose plugs and then incubated with proteinase K for a minimum of 12 h. After this treatment, the samples were run on a BIORAD CHEF-DR® III (*Clamped Homogeneous Electrical Field*) PFGE unit. Methods for the running gel were as follows: 16-h run, 4.5 volts/cm, 120° angle of current, and a ramp of 40–120 s between “switching” angles. Each gel contains 45 lanes consisting of ten samples replicated once and three size markers and a blank lane between all sample lanes. A second gel containing the same samples but in a random order was run for a second replication between gels. The size markers are Hi-Lo (50 base pairs to 10 kilobase pairs), 10–200 (0.1 kilobases to 194 kilobases), and yeast chromosomes (*Saccharomyces cerevisiae*; 225 kilobases to 2,200 kilobases). After the gel was run, it was allowed to soak in SyberGold® stain solution on a shaker table for 1 h for later UV visualization. The gel was then photographed under UV light, and the images were quantified as presence or absence of double-strand breakage. Longer, unbroken pieces of DNA (approx. 3M bases and larger) are expected to remain in the well. Each sample was replicated four times (twice within gels and twice between gels) to quantify and remove handling artifacts. Presence of breakage was determined using all replicates of an individual.

Statistical Analyses

A one-way ANOVA (with Bonferroni corrections), revealed no statistical differences between toxicant concentrations in blood or muscle tissues within the contaminated site types and reference site types; therefore, the two reference sites, Boggy Gut and Upper Three Runs, were combined in addition to the three contaminated sites, Tim's Branch, Beaver Pond, and Steed's Pond (PROC GLM, SAS© Institute 2003). Individual one-way ANOVA's of blood and muscle for both species of water snake and *A. piscivorous*, were used to examine differences in toxicant body burdens for each species between sampling locations (PROC GLM; SAS© Institute 2003). Stem-and-leaf and normality plots suggested log-transformation prior to analysis (PROC UNIVARIATE; SAS© Institute 2003). After transformation, all variables were normally distributed. Statistical significance was tested at the $\alpha = 0.05$ level.

The relationship between DDSB versus toxicant concentration in blood and muscle was established based on assigning each individual a score of either broken or unbroken by assessing all the replicates for each individual. A log-likelihood ratio test (G) was used to determine the extent of damage between the reference site and Tim's Branch for all snakes (including *A. piscivorous*) and for *N. fasciata* alone, based on the expected number of strand breaks from the reference site (Zar 1999). For DDSB, no statistical difference existed within the contaminated site types or reference sites types at the $\alpha = 0.05$ level, therefore, the two reference sites, Boggy Gut and Upper Three Runs, were combined, as well as the three contaminated sites; Tim's Branch, Beaver Pond, and Steed's Pond.

A logistic regression model was used to determine how the metal body burdens were related to the probability of breakage (PROC LOGISTIC, SAS© Institute 2003). We used an information-theoretic model fitting procedure (Burnham and Anderson 2002) to fit the most parsimonious model. Specifically, the corrected Akaike Information Criterion (AICc) was utilized as the information-theoretic statistic for the procedure (Akaike 1974). All variables in all models were tested for collinearity utilizing a principal components analysis of matrix structure. Metals were eliminated individually one by one from the model based on their correlation to the dependent variable (the independent variable with the lowest correlation was removed first) as long as the AICc continued to decrease. A final logistic regression model was determined when the AICc stabilized or increased. Individual logistic regressions were run for the log-transformed values of the eight metals (Al, Ni, Cr, Cu, Se, Mn, U, and Hg). We chose these particular metals because of their prevalence in the soil and plant tissues of the Tim's Branch watershed (Table 1), and to further examine trophic differences in concentrations (Punshon *et al.* 2003a,b).

A correlation analysis was used to determine the association of metals between the blood and muscle samples (PROC CORR, SAS© Institute 2003). This was done to estimate the relative independence of metal concentrations between the tissue types.

RESULTS

Home Range

Twelve *N. fasciata* were tracked from the three experimental sites (Steed's Pond, Beaver Pond, and Pond 25; Figure 1). The mean home range area for the 12 snakes

Table 1. Comparison of U and Ni concentrations in reference areas and Steed's Pond media (located on the Department of Energy's Savannah River Site (South Carolina, USA)) as reported from a simultaneous study (Punshon *et al* 2003b).

Compartment	Mean U concentration (mg/kg) (SE)	Mean Ni concentration (mg/kg) (SE)
Soils (reference) (n = 3)	8.15 (0.850)	26.2 (1.20)
Steed Pond Soil (n = 3)	1675.5 (382.1)	876.5 (197.7)
<i>Salix nigra</i> (reference) (n = 5)	<BDL	4.7 (5.8)
<i>Salix nigra</i> (leaf tissue) (n = 5)	0.03 (0.002)	75.5 (3.6)

<BDL: Below detection limits.

was 1.77 ha (SE = 0.24). Individual home ranges overlapped in almost all cases between and among the sexes, except at Pond 25. During the tracking period, June 2001 to August 2002, the southeastern U.S. experienced the tail end of a 5-year drought. Regardless of wetland hydrology, it was found that each snake remained within the geographical boundaries of each wetland. No differences in home range as a function of gender were found, and there was no linear association between home range and snout-vent length or body weight for this species ($p > .05$ for all tests).

Metal Concentrations

U and Ni concentrations in water snake prey items from Tim's Branch used for exposure estimates were elevated compared to the control areas (Tables 2–3). Both blood and muscle metal concentrations tended to be higher in the contaminated areas versus the control areas (Table 4). Blood Ni levels were significantly greater in snakes from Tim's Branch than the reference area ($p < .0001$) for *N. fasciata*. No other metal in *N. fasciata* blood was significantly different between Tim's Branch and the reference area.

In the muscle tissue, U was the only metal found to be significantly greater in the Tim's Branch *N. fasciata* compared to the reference area ($p < .0001$). U was also found to be significantly greater ($p = .0002$) in muscle tissues of *A. piscivorous* from the Tim's Branch area. There was no difference between metal loads in *N. taxispilota* blood or muscle tissue. No relationship existed between body burden and sex, mass, or snout-vent length.

Cu exhibited a strong negative correlation while Se exhibited a strong positive correlation between blood and muscle when all snakes were pooled and when *N. fasciata* was evaluated alone (Table 5; p 's $< .05$). U had a weak positive correlation with muscle and blood with the relationship similar whether all species were pooled or if *N. fasciata* was evaluated alone. All other metals exhibited no correlation between muscle and blood.

fasciata Exposure Estimates

U exposure from the Tim's Branch watershed is 2–3 orders of magnitude greater than that which is predicted for the reference site (Table 3). For Ni, the exposure

Ni and U in Water Snakes

Table 2. Mean metal concentrations (fresh weight mg/kg (standard error)) of potential *Nerodia fasciata* food items (n = # of samples) collected from contaminated areas on the Tim's Branch watershed and a reference area located on the Department of Energy's Savannah River Site (South Carolina, USA). (a.) U concentrations in prey items. (b.) Ni concentrations in prey items.

(a)		
Food item	U contaminated	U reference
Amphibians	0.485 (0.101); n = 36	0.004 (0.002); n = 14
Minnows	0.399 (.067); n = 25	0.005 (0.001); n = 14
Perch	0.213 (.023); n = 41	0.004 (0.001); n = 15
Pike	0.100 (0.020); n = 24	0.001 (0.0001); n = 29
Sunfishes	0.203 (0.020); n = 42	0.009 (.002); n = 2
(b)		
Food item	Ni contaminated	Ni reference
Amphibians	0.501 (0.096); n = 36	0.455 (0.137); n = 14
Minnows	0.476 (.065); n = 25	0.134 (0.052); n = 14
Perch	0.764 (.077); n = 41	0.489 (.098); n = 15
Pike	0.619 (0.191); n = 24	0.248 (0.074); n = 29
Sunfishes	0.319 (0.062); n = 42	0.197 (0.115); n = 2

from Tim's Branch is nearly double that of the reference site. The majority of U exposure from Tim's Branch is attributed to ingestion of amphibians. Also, as a function of the exposure equation, exposure decreased from juvenile to adult due to body mass for both metals.

DNA Double-Strand Breakage

The log-likelihood ratio test indicated significantly greater breakage in all species, as well as *N. fasciata* alone, from the Tim's Branch area compared to individuals from the reference area (Figure 2, $G = 18.27$, $p < .0001$). In both instances, breakage from Tim's Branch was approximately three times more likely. Slope estimates for the metals that were included in the logistic regression model relating them to the probability of strand breakage for all species and *N. fasciata*-blood and muscle tissues are shown in Table 6. No meaningful model could be fitted for blood pooled for all species. The strongest model, yielding a max-rescaled r -square value of 0.53, was for *N. fasciata*-blood indicating that Cr, Hg, Ni, and Se positively related to strand-breakage, while Al, Cu, and U were negatively related.

DISCUSSION

Bioaccumulation of toxicants in snakes has been demonstrated in several studies (Burger and Gibbons 1998; Hopkins 2000; Hopkins *et al.* 1999; Ohlendorf *et al.* 1988). Specifically, Hopkins (2000) has shown blood sampling and tail clips in *N. fasciata* as a possible means to demonstrate heavy metal burden within the organism

Table 3. Predictions of maximum (3a) and mean (3b) U and Ni exposure (mg/kg/day) for *Nerodia fasciata* residing in contaminated areas of the Tim's Branch watershed and a reference area located on the Department of Energy's Savannah River Site (South Carolina, USA). Predictions are presented separately by age classes and by proportion of amphibians and fishes in the diet (Brown 1958).

Age	Weight (kg) (contaminated)	Weight (kg) (reference)	U exposure (contaminated)	U exposure (reference)	Ni exposure (contaminated)	Ni exposure (reference)	% Amphib in diet	% Fish in diet
3a: Maximum contaminant exposure								
Juveniles	0.05	0.08	94.17	0.06	82.39	42.73	100	0
Adults	0.22	0.23	23.14	0.02	20.24	14.54	100	0
Juveniles	0.05	0.08	32.18	0.12	56.21	19.76	21	79
Adults	0.22	0.23	7.91	0.04	13.81	6.72	21	79
Juveniles	0.05	0.08	77.96	0.08	75.43	36.63	79	21
Adults	0.22	0.23	19.09	0.03	15.54	12.46	79	21
Juveniles	0.05	0.08	15.70	0.13	49.25	13.66	0	100
Adults	0.22	0.23	3.86	0.05	12.10	4.65	0	100
3b: Mean contaminant exposure								
Juveniles	0.05	0.08	15.82	0.10	16.61	9.98	100	0
Adults	0.22	0.23	3.89	0.03	4.08	3.39	100	0
Juveniles	0.05	0.08	7.01	0.07	11.80	4.98	21	79
Adults	0.22	0.23	1.72	0.03	2.90	1.69	21	79
Juveniles	0.05	0.08	13.48	0.09	15.33	8.65	79	21
Adults	0.22	0.23	3.31	0.03	3.77	2.94	79	21
Juveniles	0.05	0.08	4.67	0.07	10.52	3.65	0	100
Adults	0.22	0.23	1.15	0.02	2.58	1.24	0	100

Ni and U in Water Snakes

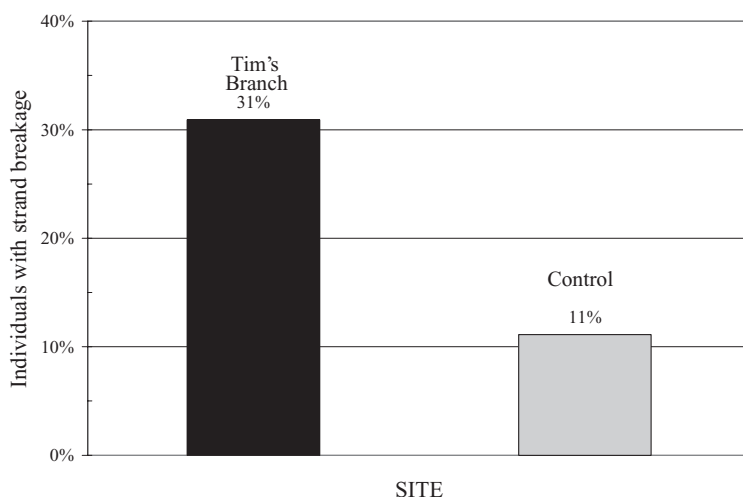


Figure 2. DNA double strand breakage (DDSB) expressed for all species of water snakes (*Nerodia fasciata*, *N. taxispilota*, and *Akistrodon piscivorou*; $G = 18.27$, $df = 1$, $p < .0001$). The black bar represents percentage of DDSB in blood of snakes from Tim's Branch ($n = 55$) and the gray bar represents percent breakage from the control sites ($n = 20$).

and reported tail snips and blood samples as good predictors of arsenic exposure. However, in this study, data did not indicate a correlation between the tissue types (blood and tail) for the particular metals analyzed, and blood concentrations did not reflect whole body exposure (Tables 3–5). These contrasting results are likely due to sample size differences, magnitude of contamination, and chemical behavior of the different metals. Specifically, since so many toxicants are present in the contaminated watershed, the permutations of their chemical interactions in the environment are extremely complex to model. The logistic regression showed a relationship between DDSB and metals (Table 6). However, these models only explain a portion of the variation due to environmental stochasticity. Bioavailability of metals may increase or decrease with the presence or absence of other specific metals depending on the mixture and other abiotic factors surrounding the matrix (Pyle *et al.* 2002; Stewart 1999). Our study showed a repeated association between Se and U, which prompts further investigation. The influence of Se on metal bioavailability is not surprising as the protective effects of Se on Hg uptake have been documented by several reports (see Burger *et al.* 2001). In this study, Se had an opposite directionality to U in each of the four logistic regression models (Table 6).

U and Ni Exposure

Previous studies in the Tim's Branch watershed have shown that levels of both metals residing in the soil, and plant tissues, are heterogeneously distributed (Punshon *et al.* 2003a,b). The home ranges of *N. fasciata* showed that this animal resided exclusively within the contaminated area during the entire study period. Exposure within the contaminated area of Tim's Branch was greater than the reference area,

Table 4. Comparison of water snake (*Aghistron piscivorous* (apisc), *Nerodia fasciata* (nfasc), *N. taxispilota* (ntaxi)) metal concentrations in muscle and blood collected from reference and contaminated areas located in the Upper Three Runs and Tim's Branch watersheds, respectively, on the Department of Energy's Savannah River Site (South Carolina, USA).

Site	Tissue	Species	Element																
			Al	Cr	Cu	Hg	Mn	Ni	Se	U									
Reference	Blood	apisc	Mean	0.082	0.671	0.145	0.061	0.102	0.496	0.050									
		n = 3	SE	0.053	0.169	0.050	0.027	0.045	0.258	0.038									
		nfasc	Mean	1.202	0.670	0.460	0.149	0.090	0.296	0.012									
		n = 9	SE	0.269	0.063	0.084	0.029	0.043	0.017	0.000									
		ntaxi	Mean	0.643	0.020	0.339	0.134	0.096	1.024	0.126									
		n = 2	SE	0.296	0.006	0.021	0.005	0.078	0.355	0.000									
		All	Mean	1.645	0.132	0.623	0.459	0.093	0.443	0.036									
	n = 14	SE	0.637	0.043	0.060	0.083	0.030	0.092	0.013										
	Muscle	apisc	Mean	392.951	0.464	3.282	1.103	4.619	2.827	1.493	0.071								
		n = 6	SE	102.977	0.131	0.488	0.167	0.411	1.141	0.215	0.020								
		nfasc	Mean	45.162	0.594	1.486	0.860	34.138	3.257	1.003	0.045								
		n = 12	SE	9.453	0.075	0.276	0.081	7.227	0.088	0.081	0.024								
		ntaxi	Mean	119.290	0.553	1.729	0.971	49.331	6.108	1.770	0.423								
		n = 3	SE	66.534	0.142	0.343	0.179	14.501	2.650	0.735	0.312								
All		Mean	155.120	0.551	2.034	0.945	27.874	3.541	1.253	0.107									
Contaminated	Blood	n = 21	SE	44.849	0.059	0.272	0.071	5.618	0.502	0.133	0.049								
		apisc	Mean	4.475	0.139	0.457	0.117	0.199	0.275	0.710	0.070								
		n = 2	SE	3.289	0.047	0.007	0.090	0.160	0.062	0.301	0.057								
		nfasc	Mean	2.131	0.155	0.705	0.379	0.177	0.220	0.408	0.048								
		n = 25	SE	0.310	0.023	0.173	0.057	0.019	0.032	0.038	0.011								
		ntaxi	Mean	2.209	0.137	0.303	0.611	0.192	0.132	0.730	0.053								
		n = 7	SE	0.594	0.022	0.054	0.180	0.039	0.016	0.078	0.020								
	Muscle	All	Mean	2.285	0.151	0.607	0.411	0.182	0.205	0.492	0.050								
		n = 34	SE	0.305	0.018	0.130	0.058	0.017	0.025	0.042	0.009								
		apisc	Mean	551.396	1.600	4.214	1.204	31.706	7.883	1.206	3.667								
		n = 7	SE	142.106	0.506	0.948	0.475	5.910	1.752	1.184	1.489								
		nfasc	Mean	109.882	0.935	1.895	0.538	76.847	6.521	1.220	1.291								
		n = 35	SE	26.697	0.268	0.218	0.047	7.415	0.872	0.115	0.244								
		ntaxi	Mean	225.072	1.181	4.064	0.644	100.007	5.154	1.637	0.672								
n = 15	SE	58.915	0.492	1.074	0.100	10.498	1.239	0.434	0.152										
All	Mean	194.416	1.081	2.751	0.648	77.398	6.328	1.328	1.420										
n = 57	SE	33.497	0.217	0.355	0.073	5.932	0.661	0.136	0.258										

Ni and U in Water Snakes

Table 5. Pearson correlation coefficients for blood and muscle tissues in pooled individuals of all snake species (n = 57) as well as only *Nerodia fasciata* (n = 38) individuals from the Department Energy's Savannah River Site located in South Carolina, USA.

Metal	All species		<i>Nerodia fasciata</i>	
	r-value	p-value	r-value	p-value
Al	-0.0110	0.9407	-0.2489	0.1557
Ni	-0.0002	0.9988	-0.0631	0.7229
Cr	0.0457	0.7576	-0.1487	0.4012
Cu	-0.5903	< 0.0001	-0.6160	0.0001
Se	0.5288	0.0001	0.4187	0.0137
Mn	0.0433	0.7702	-0.1777	0.3148
U	0.2552	0.0800	0.3025	0.0821
Hg	0.1007	0.4960	0.1950	0.2691

especially for U, which was 2–3 orders of magnitude greater than the reference area. Interestingly, the majority of U exposure from Tim's Branch was due to the predicted consumption of amphibians. Amphibians collected from Tim's Branch had higher U burdens than fish collected from the same site (Table 2). Notably, the relationship between exposure and uptake was demonstrated with *N. taxispilota* residing in contaminated areas that had markedly lower concentrations of Ni and U in blood or tissue samples compared to other snake species. Although *N. taxispilota* were excluded from the exposure analysis due to sample size limitations, their diet, which consists almost exclusively of fishes (Mills *et al.* 1995), bolsters the inference that fish may not be the vector for U or Ni exposure in Tim's Branch. This parallels exposure assessments conducted for wild hogs (*Sus scrofa*) for Tim's branch showing that both Ni and U exposure was related to diet (Gaines *et al.* 2005).

The Agency for Toxic Substances and Disease Registry (ATSDR) has derived a minimal risk level (MRL) for soluble compounds of U of 0.002 mg/kg/day based on the lowest observed adverse effect level (LOAEL) of 0.05 mg/kg/day for renal effects in rabbits (ATSDR 1999; Gilman *et al.* 1998). Mean U exposure from reference areas ranged from 0.02–0.1 mg/kg/day level (Table 2), exceeding the MRL. However, it is difficult to extrapolate what the MRL should be for reptiles since their physiologies differ from mammals. Since exposure estimates for both juvenile and adults from the impacted Tim's Branch study were up to three orders of magnitude greater than the reference area the potential for risk of an adverse effect for this population from oral exposure may be very real.

The mammalian oral reference dose (RfD) for Ni is 0.02 mg/kg/day (Ambrose and Paillet 1980; IRIS 1996). Exposure estimates to Ni for the control snakes exceeded the mammalian RfD by several orders of magnitude and snakes from the impacted areas had exposure estimates roughly double over the control snakes. Most likely due to depuration, concentrations of Ni in muscle tissue for this species did not significantly exceed those from the reference area, although the Ni concentration in the blood of *N. fasciata* was significantly greater from Tim's Branch than the reference area.

Table 6. Slope estimates and maximum-rescaled r -square values for the logistic regression modeling DNA double-strand breakage as a function of metal concentration in tissue from snakes collected on the Department of Energy's Savannah River Site located in South Carolina, USA.

Snake and tissue (n)	$R^2/C.I.$	Al	Cu	Cr	Hg	Mn	Ni	Se	U
All species, muscle (95)	0.24/3.9	N/A	1.1981	N/A	N/A	-0.4447	N/A	-1.4447	0.3465
<i>N. fasciata</i> , muscle (53)	0.16/6.5	N/A	1.0729	N/A	N/A	N/A	N/A	-1.8852	N/A
All species, blood (57)				No meaningful model fitted					
<i>N. fasciata</i> , blood (38)	0.53/4.39	-2.0282	-5.2966	1.828	1.8283	N/A	1.2614	3.8688	-1.3614

N/A indicates the metal did not fit the model. Maximum intercept adjusted condition index (C.I.) is reported with the r -square.

DNA Double-Strand Breakage (DDSB)

Our findings showed that the snakes from the impacted site had higher DDSB compared to snakes from control locations, indicating that DDSB has the potential to be used as an endpoint to identify ecological risk. Hartwig (1995) suggested that the mechanism for metal-induced breakage is the inhibition of repair processes while Sugg *et al.* (1995) showed that adducted nucleotides produced by radiation may prevent the repair of naturally occurring strand breaks due to interference. This latter process occurs in addition to direct breakage from ionizing radiation or from induction of free radicals (Sugg *et al.* 1995). Heavy metals may also cause direct breakage through the induction of oxygen radicals (Hoffman and Heinz 1998; Snow 1991; Tsuzuki *et al.* 1994). The presence of U in muscle tissue and the correlation to the observed DDSB (Table 6) allows speculation that gamma-emitting daughter products of U in Tim's Branch might have had some effect on the integrity of the DNA of these organisms. Since U is a metal, an alpha emitting radionuclide with strong beta and gamma daughter products, the totality of its effect is combination of all of these components.

It was hypothesized that compounds of Ni, which have been known to cause strand breakage, were possibly acting as agents to increase the background level of DNA strand breaks in water snakes. The logistic regression for the strongest model using *N. fasciata* blood showed this positive relationship of Ni as well as Cr, Hg, and Se with DDSB (Table 6). This finding is consistent with previous studies that have shown that the induction of DNA breaks by both Se and Hg, which is mediated by the formation of active oxygen species (Hoffman and Heinz 1998, Tsuzuki *et al.* 1994). Hopkins *et al.* (2002) found that Se accumulation in *N. fasciata* fed food from a contaminated ecosystem on the SRS exceeded toxic thresholds. However, other studies have demonstrated a protective effect of Se on Hg exposure at the intracellular level (Lourdes 1991). Moreover, Cr is well known to inhibit DNA repair and can form intermolecular DNA cross-links (Snow 1991; Tsuzuki *et al.* 1994).

Interestingly, U, Al, and Cu in the blood exhibited a negative relationship with DDSB (Table 6). This may be due to the residence time of the metals in the respective tissues, with blood in general having faster metal depuration rates than muscle in reptiles (Sparling *et al.* 2000). Therefore, metals that tend to directly cause DDSB (rather than inhibit repair) might not be present in the blood long enough to cause DDSB. Conversely, if the toxicant inhibits repair, residence time is not as an important factor since this is a more continuous process. This can best be seen in the fitted model using muscle tissue for the three snake species combined that showed an inverse relationship with Se and Mn, no relationship between DDSB and Ni, but a direct relationship with U and Cu (Table 6). Cu has also shown a direct relationship to DDSB (Lloyd and Phillips 1999), and U can break DNA due to its alpha emissions.

A suite of contaminants were deposited in the Tim's Branch system over the last 50 years and how they behave as a mixture warrants further consideration. The logistic regression analysis (Table 6) demonstrated the relative association of these contaminants acting as a mixture. The biogeochemistry of these metals may change and not behave in the field as independent agents. The mixtures may be additive, compensatory, minimizing, or all three, thereby complicating predicted transfer

pathways of the metals in the environment. More research to demonstrate relationships between genetic damage and combined contaminant exposure, uptake, and physiological response, is needed.

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