Landuse and Water Quality Threats to Current and Historical *Cryptobranchus Alleganiensis* Streams Across Multiple Ecoregions

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Abstract

The hellbender (*Cryptobranchus alleganiensis*) is a large, long-lived, and fully aquatic salamander species endemic to streams and rivers across 15 states in the Midwest and Eastern United States. Hellbender populations have experienced drastic declines due to habitat degradation, accelerated sedimentation, aquatic contaminants, and infectious diseases. Although declining water quality is often suggested as a major contributing factor to hellbender population declines, few studies have evaluated the presence of aquatic contaminants at sites with current and historical *C. alleganiensis* populations. We used a novel passive sampling technique to assess the presence and concentration of two herbicides (glyphosate and atrazine) in the water and analyzed heavy metal (cadmium (Cd), mercury (Hg), and lead (Pb)) concentrations in stream sediment samples at 30 sites across a watershed development gradient throughout the *C. alleganiensis* range in Tennessee (TN) and North Carolina (NC). Our results indicated the presence of atrazine in 23% of our sites. All 30 sites contained Cd and Pb, and 26 sites contained Hg. Levels of glyphosate were too low to detect using our methods. Although percent watershed development was not significantly different among ecoregions, Blue Ridge watersheds were overall less developed than watersheds in the Ridge and Valley and Interior Plateau ecoregions. The model with greatest support suggested that percent watershed development and specific conductivity were negatively correlated with hellbender population status. Because this research focused on assessing the prevalence of commonly encountered aquatic contaminants, our results and study design are broadly applicable to *C. alleganiensis* populations across the species range.

Introduction

Anthropogenic disturbances have degraded the integrity of lotic ecosystems, and these impacts tend to be greatest in landscapes with high levels of urban and agricultural development (Masterson and Bannerman 1994; Booth and Jackson 1997; May et al. 1997). Urban and agricultural development across watersheds often cause declines in water quality and aquatic species diversity through significant alterations in channel morphology and hydrological patterns, increased runoff, and increased erosion and subsequent sedimentation (Hammer 1972; Klein 1979; Wang et al. 2001; Paul and Meyer 2001). Characteristics of degraded streams include a lack of, or reduced, riparian buffers, fine sediment loading, elevated conductivity, and high levels of bacteria, nutrient, and agricultural chemical input through runoff (Farrell-Poe 1995; Ritter and Shirmohammad 2000; Muenz et al. 2006). Agricultural nonpoint pollution (i.e., sediment, manure, and chemicals) and altered hydrologic regimes represent one of the greatest threats to aquatic biodiversity in North America and globally (Richter et al. 1997; Vörösmarty et al. 2010). As land altering practices and associated pollution of aquatic systems are likely to continue, greater research is needed to understand the environmental legacy of pollutants and how they are distributed throughout ecosystems.

Herbicides and heavy metals are common pollutants that are associated with urban and agricultural development (Farrell-Poe 1995, Ritter et al. 2000; Naimo 1995). The pesticide amounts used in both 2000 and 2001 in the United States exceeded 1.2 billion pounds, which accounted for 24% of global pesticide use (Kiely 2004). Glyphosate and atrazine are the most common active ingredients in herbicides (i.e., a form of pesticide) used worldwide. In 2004, more than 85 million pounds of glyphosate and more than 60 million pounds of atrazine were used annually in the United States (Kiely 2004). Most water bodies in the Northern Hemisphere contain heavy metals such as cadmium (Cd), mercury (Hg), lead (Pb), and copper (Cu) (Naimo 1995), which is due primarily to anthropogenic activities (e.g., mining, application of agricultural fertilizer, and coal fired power generation). Herbicides and heavy metals can harm wildlife through acute and chronic exposures (Hayes et al. 2002a, b; Boone and James 2003; Naimo 1995; Clements 2000). Notably, pollution and habitat destruction are considered major contributors to the decline of aquatic biodiversity, including fish, amphibians, mussels, and macroinvertebrates (Vitt et al. 1990, Wilcove et al. 1998). Despite extensive research on the impact of common pollutants on wildlife, little is known about the presence and concentration of these pollutants across ecosystems and especially at larger spatial scales. Such data are essential for the creation of effective and efficient management plans that can potentially mitigate the negative effects of pollution on aquatic biodiversity.

The hellbender salamander (*Cryptobranchus* alleganiensis) is a fully-aquatic, large-bodied salamander species that has experienced drastic population declines across its historical range since the 1980s (Wheeler et al. 2003; Nickerson and Briggler 2007; Burgmeier et al. 2011a; Freake and DePerno 2017). Impoundments and dams, accelerated sedimentation, emerging pathogens, and compromised water quality are suggested to be major contributors to these declines (Wheeler et al. 2003; Foster et al. 2009). Hellbenders are habitat specialists that are restricted to streams and rivers with cool, well-oxygenated, flowing water with a heterogeneous substrate composed of unembedded rocks of diverse shapes and size (Humphries and Pauley 2005; Da Silva Neto 2019). Individuals of all age classes use the interstitial spaces between substrate rocks as shelter and breeding site (Nickerson and Mays 1973). Because hellbenders are fully aquatic, benthic, long-lived, have cutaneous respiration, and are thus sensitive to pollutants and degraded aquatic habitats, they can be reliable indicators of stream health where they occur (Nickerson and Mays 1973; Nickerson et al. 2002). Despite extensive research completed on the species' habitat requirements and demographics of several populations across numerous states (Taber et al. 1975; Nickerson et al. 2003; Humphries and Pauley 2005; Burgmeier et al. 2011b; Hecht-Kardasz et al. 2012)little is known about the presence and concentration of herbicides and heavy metals throughout the geographic range of *C.alleganiensis* (Solis et al. 2007). Assessment of the presence and concentration of herbicides and heavy metals across large landscapes may help researchers identify factors that contribute to hellbender population declines. Additionally, based on literature reviews, few methods are available for testing herbicide presence and concentration are relevant to benthic aquatic amphibians. Therefore, the purpose of our study was to 1) create a sampling protocol that quantifies in-site herbicide concentrations in which hellbenders and other aquatic organisms are potentially exposed, 2) assess the level of agricultural and urban development within HUC12 watersheds across a large landscape, 3) acquire baseline data on presence and concentration of two herbicides and three heavy metals at sites with current and historical *C. alleganiensis* populations across a watershed development gradient, and 4) assess relationships among watershed development, herbicides and heavy metals, water quality, and hellbender population status. We expected that herbicide (glyphosate and atrazine) and heavy metal (Cd, Hg, Pb) concentrations would be either absent or in relatively low concentrations at sites in watersheds with low anthropogenic development and would increase in watersheds with

greater anthropogenic development. Further, we expected that hellbender population status would mirror patterns of aquatic impairment, with stable and recruiting *C. alleganiensis* populations in streams with the lowest amounts of anthropogenic impacts.

Materials And Methods

Study Area: We conducted this study in the summer of 2017 at 30 sites distributed throughout the geographic range of *C. alleganiensis* in Tennessee and North Carolina. Although the range of *C. alleganiensis* extends from northern Alabama and Georgia and north through Pennsylvania and into southern New York (Petranka 1998), portions of Tennessee and North Carolina provide some of the best remaining habitat for *C. alleganiensis* across the geographic range (Freake and DePerno 2017; USFWS 2018). As state boundaries are often not biologically relevant, we performed our analysis considering the study area to be the joint range of the species between both states (Figure 1). The range across both states is composed of 1,174 HUC12 (i.e., smallest hydrological unit identified by a unique12-digit hydrological unit code) watersheds located in their entirety within both state boundaries (i.e., watersheds that are not shared with adjacent states), with 208 watersheds located within North Carolina, 876 watersheds located within Tennessee, and 90 watersheds shared between eastern Tennessee and western North Carolina. In addition, the joint range encompasses the following six EPA Level III ecoregions: Southeastern Plains, Ridge and Valley, Southwestern Appalachians, Central Appalachians, Interior Plateau, and Blue Ridge. The species range in Tennessee includes all ecoregions, whereas the species range in North Carolina only includes the Blue Ridge ecoregion. It is important to note that the range of *C. alleganiensis* in Tennessee includes several large metropolitan areas (e.g., Nashville, Knoxville, Chattanooga), compared to the species range in North Carolina, which lacks urban areas of comparable size.

Watershed Development Analysis and Site selection: We merged HUC12 watershed layers (USGS; National Hydrography Dataset [NHDPlus]) from Tennessee and North Carolina using the Unite tool in ArcGIS 10.3 (ESRI Inc. 2017) to create a contiguous watershed layer. We used the Dissolve and Editor tools to ensure that polygons were united correctly and gaps between polygons were not present. We used the National Land Cover Database (NLCD 2011) to identify and quantify percent of land cover dedicated to agricultural practices and urban development within each HUC12 watershed (USGS 2011; Seaber et al. 1987). We used the Reclassify tool to merge categories 21 (Developed, Open Space), 22 (Developed, Low Intensity), 23 (Developed, Medium Intensity), 24 (Developed, High Intensity), 81 (Pasture/Hay), and 82 (Cultivated Crops) of the NLCD layer into one category that represented the total amount of land dedicated to urban and agricultural land uses, respectively. Then, we used the Geospatial Modeling Environment software (Spatial Ecology LLC) to extract the NLCD data to each HUC12 watershed and quantify the percent of each land use category per watershed. Watersheds were divided into three groups based on the percent of watershed surface area dedicated to development: low watershed development (agricultural and urban development = 0 – 25% of watershed surface area), moderate watershed development (agricultural and urban development = 25 – 50% of watershed surface area), and high watershed development (agricultural and urban development = 50% and above of watershed surface area; Lu and Weng 2006). We stratified sites according to level of watershed development across the range of *C. alleganiensis* in Tennessee and North Carolina. We selected 30 sampling sites (15 locations per state; 5 sites per watershed development category) that historically had (i.e., populations were extirpated) or currently have hellbender populations that are healthy, stable, or declining, which were assessed based on recent *C. alleganiensis* survey data and data from the recent United States Fish and Wildlife Species Status Assessment (USFWS 2018). Specifically, we classified hellbender population status via the following categories: Recruiting (RE), Declining (DE), Functionally Extirpated (FE), and Extirpated (EX). We focused site allocation on the three largest Level III ecoregions in Tennessee and North Carolina, which included the Blue Ridge, Ridge and Valley, and Interior Plateau ecoregions, which in total span over 550 km from east to west and include a considerable portion of the geographic range for *C. alleganiensis*.

Passive Sampling Protocol Development: We developed a novel passive sampling technique that relied on diffusion to collect contaminants diluted in water that are neutral and lipophilic and can be absorbed into a medium through non-facilitated diffusion. The principle of passive sampling is based on the ability of analyte molecules to flow freely (i.e., non-assisted) from the sampled medium into a receiving phase in a sampling device due to the difference between analyte concentrations of the two media (Vrana et al. 2005). Analytes flow into the receiving phase (i.e., deionized water inside the passive sampler) until the analyte concentration reaches equilibrium between the receiving phase and the sampled medium (i.e., stream water; Vrana et al. 2005). Similarly, active compounds in pesticides are absorbed through plant and animal cell membrane via simple diffusion (Sterling 1994). Amphibians are especially susceptible to environmental pollutants due to their permeable skin responsible for gas exchange and water update (Brühl et al. 2011), Therefore, we assumed that the concentration of analytes in the receiving phase of the passive sampler potentially represented the concentration that an individual hellbender would be exposed to in-situ. Our goal was to develop a biologically-relevant sampling technique that would expose the sampling units to the same environmental factors that an individual hellbender would experience in natural conditions and to replicate the diffusion of analytes across the skin surface. Although, we acknowledge that absorption of herbicides through animal skin is a much more complex process.

The passive samplers consisted of a 2.54 cm (1 inch) x 15.24 m (50 foot) dialysis tube made of seamless regenerated cellulose with a molecular weight cutoff of 12,000 – 14,000 Daltons (Carolina Scientific, Burlington, NC, catalog #684214). We measured and cut the dialysis tubes into 43.1 cm sections and placed them into a soaking beaker with deionized water to soften the membrane, which facilitated handling. After five minutes, we removed each section from the deionized water, and tied a knot on one end. We used a syringe to fill the tube with 60 mL of deionized water and knotted the other end of the dialysis tube. We wore disposable nitrile gloves while handling the dialysis tubes.

Prior to field deployment, we conducted a pilot study to evaluate the effectiveness of the passive samplers for glyphosate and atrazine detection in a water medium. We acknowledge that streams are likely impacted by multiple herbicides and pesticides, but we selected glyphosate and atrazine because they are the most utilized herbicides in agriculture. We used the PESTANAL analytical standards for both atrazine (Molar mass = 215.68 g/Mole) and glyphosate (Molar mass = 169.07 g/Mole; Sigma-Aldrich, St. Louis, Missouri) to evaluate our sampling method. We diluted 100 mg of glyphosate in 100

mL of deionized water and 20.4 mg of atrazine in 2.0 mL of 95% ethanol to acquire a 1.0 mg/mL master solution for both herbicides. We pre-washed 10 glass mason jars and filled each jar with 1 L of deionized water. We placed 0.1 mL, 0.5 mL, 1 mL, 3 mL, 5 mL of each herbicide's master solution into separate jars which resulted into 10 jars (five jars for atrazine and five jars for glyphosate) with the following concentrations of herbicides in each jar: 0.1 mg/L, 0.5 mg/L, 1 mg/L, 3 mg/L, and 5 mg/L. We submerged two passive samplers in each jar for 48 hours and shook each jar twice a day to ensure equal distribution of analytes in the water medium. After the 48-hour sampling period, we removed the passive samplers from the sample jars, and used a scalpel blade to make a small incision at the end of each sampler. We poured the contents of the receiving phase into 50 mL centrifuge tubes (Fisher Scientific, catalog # 14-432-22). We immediately placed tubes on ice and stored them at -20° C until subsequent processing at the Biological Small Molecule Mass Spectrometry Core at the University of Tennessee, Knoxville, Tennessee.

Field Survey Methods

Passive Sampler Deployment: After assuring that passive samplers were able to detect aquatic contaminants (presented in Results), we prepared all passive samplers in the lab and transported them in a clean container to deployment sites. At each site, we deployed two passive samplers placed in a protective plastic mesh tube (ALS Global, Brisbane, Australia). We attached a 3 oz (85.05 g) weight at each end of the mesh tubing and closed both ends with zip ties. To mimic the benthic behavior of *C. alleganiensis*, we deployed passive samplers under or next to large rocks (>25 cm in length) and possible cover rocks, and as close as possible to the substrate. We secured each protective tube to a 0.91 m piece of rebar sunk 0.61 m into the substrate with 70-pound braided fishing line. We recorded water quality parameters (i.e., pH, specific conductivity [µS/cm], temperature [°C], and dissolved oxygen [%] at each site with a YSI ProDSS Multiparameter water quality unit with four-port cable assembly and probes (YSI Inc., Yellow Springs, OH) during deployment and recovery (June, July, and August) at all sites.

To account for seasonal variation in herbicide concentration, we deployed sampling units every 30 days in June, July, and August. During each sampling event, we deployed a total of 30 samplers with two sampling units per site at 15 different sites. Each group of sampling units was deployed for a 48-hour exposure period to allow the concentration of analytes between the sampled medium (i.e., river water and substrate) and the receiving phase (i.e., deionized water inside passive sampler) to reach equilibrium with low effect from environmental variables, such as heavy rain, which can dilute analytes and alter analyte exchange dynamics (Vroblesky 2001). We recovered sampling units in the order they were placed in respective streams (i.e., first units placed were the first units recovered). Immediately after recovering the sampling units, we made an incision in at the end of the passive sampler and stored the contents of the receiving phase in 50 mL centrifuge tubes (Fisher Scientific, catalog # 14-432-22). The tubes were stored on ice and later frozen at -20° C until subsequent processing at the Biological Small Molecule Mass Spectrometry Core at the University of Tennessee, Knoxville, Tennessee following the protocols described below.

Soil collection: We used a modified sampling method described by the United States Geological Survey to collect stream sediment samples in each survey stream (Nelson et al. 2014). At each site, we selected areas with slow-moving water with accumulated fine sediment and collected 50 mL of sediment into 50 mL centrifuge tubes (Fisher Scientific, catalog # 14-432-22). If sediment was composed of mud or silt, we collected samples by pressing a 50 mL centrifuge tube 2 cm into the sediment surface. If sediment was composed mainly of sand or loose material, we used a small plastic scoop to collect the top 2 cm of sediment and placed it into 50 mL centrifuge tubes. In areas with larger substrate structure (i.e., pebble, cobble, and boulders), we moved those larger structures until we reached fine sediment (< 2 cm). We stored each sample on ice during transport and then froze and shipped samples overnight for heavy metal analysis at the Center of Environmental Science and Engineering at the University of Connecticut, Storrs, Connecticut.

Lab Analysis

Herbicide Lab Analysis**:** Herbicides were analyzed using reverse-phase Ultra Performance Liquid Chromatography coupled to High-Resolution Mass Spectrometry (UPLC-HRMS) on an Orbitrap Exactive Plus (ThermoFisher Scientific, Waltham, MA). 10 µL of each sample were injected onto an Accucore Phenyl-X column which measured 100 x 2.1 mm and contained 2.6 µm particles (ThermoFisher Scientific, Waltham, MA). Analytes were eluted using a two-solvent system (A= 0.1 % formic acid in water; B= Methanol) with the following gradient: 5% B at 0 min, 50% B at 2.5 min, 90% B at 3 min, 90% B at 7 min, 5% B at 10 minutes, and 5% B at 12 minutes. Samples were ionized using heated electrospray with a sheath gas flow of 35, aux gas flow of 10, spray voltage of 3.6 kV, capillary temp of 300° C, S-lens RF level of 50.0, and aux gas heat of 305° C. Mass spectrometry was performed using a polarity-switching method which acquired in negative mode from 0 to 3.6 minutes and in positive mode from 3.6 to 12 minutes. All other parameters were constant for the duration of the run (resolution = 140,000, AGC target = 1e6, maximum inject time = 250). Glyphosate was detected as the $[M-H]^-$ with a retention time of 1.5 min, and atrazine was detected as the $[M+H]^+$ with a retention time of 5.0 min. After the data was collected, .RAW files were converted to .mzml and the peaks were integrated using Metabolomics Analysis and Visualization EngiNe ([MAVEN], Melamud et al. 2010, Clasquin et al. 2012).

Heavy Metal Lab Analysis**:**

*ICP/MS Analysis for Cd and Pb***:** Stream soil samples were brought to room temperature and 0.35 g of each sample was placed into a separate hot block tube. We added 5 mL of concentrated trace metal grade nitric acid to each tube, placed each sample on a hot block, and refluxed each sample for four hours at 95° C. We cooled each sample and added 2 mL of deionized water and 3 mL of trace metal grade hydrogen peroxide. We then heated the sample in the hot block until effervescence subsided. We cooled samples and filled them to a final volume of 25 mL with deionized water (DIW).

We analyzed undiluted samples on a Perkin Elmer DRCe Inductively Coupled Plasma Mass Spectrometer (ICP/MS) using standard protocols. We analyzed interference check solutions (ICS A and ICS A+B; High Purity Standards, Charleston, SC) with all sample runs to compensate for any matrix effects that would interfere with sample analysis.

CVAAS Analysis for Hg: We brought samples to room temperature and placed approximately 0.25 g of each sample in a separate hot block tube. We added a total of 2.5 mL of DIW and aqua regia (3:1 HCl: HNO_3) to each tube and placed each sample in the hot block at 95º C for 5 minutes. We removed samples from the hot block, cooled them to room temperature, and added 5 mL of DIW and 7.5 mL of potassium permanganate (KMnO4) solution. We heated each sample to 95º C for 30 minutes. We added 1.5 mL of hydroxylamine hydrochloride (NH2OH-HCl) to each sample and filled samples to a final volume of 50 mL with DIW. We analyzed samples on a Perkin Elmer FIMS Cold Vapor Atomic Absorption Spectrometer using standard protocols.

We used standard quality assurance procedures, which included analysis of method blanks (Blank), post digestion spiked samples, and laboratory control samples (LCS). Sample mass was too small to conduct duplicate analysis. We evaluated instrument response initially, every 10 samples, and at the end of an analytical run using a calibration verification standard and blank.

Statistical Analysis:

Pilot Study Evaluation: We used general linear models (GLMs) in R v. 4.0.2 to evaluate differences among the main effects of herbicide concentration (0.1 mg/L, 0.5 mg/L, 1 mg/L, 3 mg/L, and 5 mg/L) and sample type (dilution standard or passive sampler), along with their interactions on the relative amount of herbicide (atrazine and glyphosate) detected in the sample. Prior to analysis, we evaluated frequency distributions of the response variables (relative amount of atrazine and glyphosate detected) and used a square root transformation to achieve statistical normality. We used a Poisson data distribution with a log-link function when statistical normality was not achieved following transformation. We evaluated confidence intervals of fixed effects and report biological significance (especially when confidence intervals did not overlap zero).

Ecoregion Level Differences: For field collected samples, we evaluated differences in watershed size, urban and agricultural development, water quality parameters, and herbicide/heavy metal concentrations among ecoregions. We first evaluated ecoregion level (Blue Ridge, Ridge and Valley, and Interior Plateau ecoregion) differences in stream response variables. We used GLMs in the R statistical package to evaluate ecoregion-level differences in watershed size (ha); relative amount of anthropogenic watershed development (based on percent of watershed development); water quality variables (pH, dissolved oxygen, specific conductivity); and heavy metal/herbicide concentrations (Cd, Hg, Pb, atrazine) within stream variables among ecoregions (Blue Ridge, Ridge and Valley, and Interior Plateau). Prior to analysis, we assessed whether stream predictor variables were normally distributed via frequency distributions, quantile-quantile plots, and the Shapiro-Wilk's test. When response data were not distributed normally, we used log10, natural log, and square root transformations to achieve statistical normality. In instances when a predictor variable was not normally distributed (even after transformation), we used an alternative data distribution (e.g., Inverse Gaussian distribution) in the GLM structure to model the response. We evaluated confidence intervals of fixed effects and plotted the response when predictor variables had 95% confidence intervals that did not overlap zero.

Hellbender Population Responses: We used a multiple hypothesis approach to evaluate the effects of watershed size (ha); percent watershed development; water quality variables (pH, dissolved oxygen, specific conductance); and heavy metal/herbicide concentrations (Cd, Hg, Pb, atrazine) on hellbender population status. Prior to analysis, we developed a series of 26 candidate models (Table 2) to evaluate the singular and additive effects of landscape condition and water quality variables on hellbender population status. We used Cumulative Link Mixed Models (CLMMs) via the Ordinal package (Christensen and Christensen 2015) in R v. 4.0.2 to evaluate the effects of watershed size, percent watershed development, water quality, and heavy metal/herbicide concentrations (fixed effects) on hellbender population status (response variable), while controlling for non-independence of sample stream selection within the ecoregions (Blue Ridge, Ridge and Valley, and Interior Plateau) of Tennessee and North Carolina (random effect). Briefly, CLMMs are used when response data are ordinal (i.e., data have natural ordered categories, but the distance among categories are not known) and there is non-independence within predictor variables that necessitates the use of a mixed structure. We evaluated correlations among fixed effects and maintained the variable with greatest biological relevance when correlations were > 0.75.

We used Akaike's Information Criterion adjusted for small sample sizes (AIC_c) to evaluate relative support of individual models. We considered models competitive when ΔAIC_c estimates were $\leq$ than 2.0 and used model averaging when model terms were contained in multiple top models. We reported model-averaged beta coefficients, standard errors, and 95% confidence intervals for all fixed effects for models with confidence intervals that did not overlap zero.

Results

Laboratory Assessment of Herbicide Sampling Protocol: In our pilot evaluation of passive sampler efficacy, we did not detect a significant difference in the amount of atrazine detected between passive samplers and known standards (β = -1242 ± 2526, C.I.: -3709, 3709; (Figure 2). We observed a significant, non-linear increase in total atrazine detected in each subsequent dilution (Standard Dilution: $r^2 = 0.94$, $y = 1.0 \text{ X } 10^9 e^{0.7821x}$; Passive Sampler: $r^2 = 0.95$, $y = 9.0 \text{ X } 10^8 e^{0.8021x}$), and even at the lowest dilutions, we observed relatively low variance among replicates (Figure 2). In contrast to our detection results for atrazine, we did not detect a significant trend in glyphosate detection across the serial dilutions (β = -8.30 ± 22.24, C.I.: -51.90, 35.30), which suggest that we are unable to use the dilution curve to estimate glyphosate concentrations from biological samples. We were unable to reliably detect glyphosate (either with the passive samplers or from known standards) when dilutions were less than 300 ml/l (Figure 2). As our analytical method was not adequate to estimate glyphosate concentrations from the standard dilutions and passive samplers, we did not include biological estimates of glyphosate in further analyses. We calculated atrazine concentrations from passive sampling using a log-transformed linear standard curve ($R^2 = 0.999$) injected in triplicate and analyzed the same day as the environmental samples to reduce day-to-day variance in instrument signal.

Assessment of Agricultural and Urban Development within HUC12 Watersheds: We found that HUC12 watershed size (total hectares) of sample sites in the Interior Plateau were greater (mean = 10,839.94 ha ± 3,197.34 SD; β = 0.14 ± 0.06, C.I.: 0.02, 0.26) compared to sites within the Blue Ridge ecoregion (mean size = 7,756.23 ha ± 2,525.35; Table 1). Anthropogenic land use (i.e., percent watershed development) within the HUC12 watersheds was not significantly different among ecoregions (Interior Plateau: β = 0.03 ± 0.02, C.I.: −0.01, 0.07; Ridge and Valley: β = −0.03, 0.15). Our analysis indicates that 78% (340/473) of the watersheds within the Blue Ridge ecoregion had 0 – 25% of their land surface developed, and 8% (38/473) had more than 50% of their land surface developed. Approximately 24% (90/375) of the watersheds in the Ridge and Valley ecoregion had 0 – 25% of their land surface developed, and 37% (140/375) had more than 50% of their land surface developed. Finally, approximately 34% (148/440) of the watersheds in the Interior Plateau ecoregion had 0 – 25% of their land surface developed, and 34% (147/440) had more than 50% of their land surface developed.

Assessment of Atrazine, Cd, Pb, Hg, and Water Quality Field Data: Our field results indicated the presence of atrazine in 23% of our sites; six sites in Tennessee (min=0.005 ppm, max= 0.020 ppm) and one site in North Carolina (0.002 ppm). All 30 sites contained Cd (0.09 – 0.83 ppm) and Pb (1.22 – 10.4 ppm), and 26 sites contained Hg (15 in Tennessee and 11 in North Carolina; 0.005 – 0.032 ppm). Atrazine and heavy metal concentrations differed among ecoregions. Atrazine concentrations were greater in Interior Plateau ecoregion streams (β = 19.8 ± 5.87, C.I.: 8.3, 31.3; mean = 0.01 ± 0.02 ppm), compared to Ridge and Valley and Blue Ridge ecoregion streams (Figure 3). As per heavy metal concentrations, Cadmium was greater in Interior Plateau streams (β = 0.37 ± 0.07, C.I.: 0.23, 0.51; mean = 0.50 ± 0.22 ppm) compared to Blue Ridge ecoregion streams (0.20 ± 0.07 ppm), whereas Hg concentrations were greater in Interior Plateau (β = 0.003 ± 0.001, C.I.: 0.001, 0.005; mean = 0.20 ± 0.008 ppm) and Ridge and Valley (β = 0.006 ± 0.003, C.I.: 0.000, 0.006; mean = 0.02 ± 0.01 ppm) streams compared to Blue Ridge ecoregion streams (mean = 0.009 ± 0.007 ppm; Figure 3). We detected differences in water quality measurements among ecoregions. Notably, pH was greater in the Interior Plateau streams (β = 0.38 ± 0.09, C.I.: 0.20, 0.56; mean = 7.82 ± 0.15) compared to Blue Ridge ecoregion streams (mean = 7.44 ± 0.27), whereas specific conductivity was greater in Interior Plateau (β = 0.57 ± 0.08, C.I.:0.41, 0.73; mean = 204.53 ± 97.75 mS/cm) and Ridge and Valley (β = 0.55 ± 0.16, C.I: 0.24, 0.86; mean = 176.22 ± 48.46 mS/cm) ecoregion streams, compared to Blue Ridge ecoregion streams (mean = 54.11 ± 27.15 mS/cm; Figure 4).

Assessment of Hellbender Population Responses: We used a multiple-hypothesis approach to evaluate the influence of 26 a priori models that included landscape/land-use, water quality, and aquatic contaminant variables on hellbender population status. The model with greatest support (AIC_c = 63.04; k = 5; ω_i = 0.55) included percent watershed development and specific conductivity variables. A second model (AIC_c = 64.45; k = 6; ω_i = 0.27) also included percent watershed development and conductivity variables, along with the HUC12 watershed size variable. These models suggest that percent watershed development (−1.90 ± 0.63; C.I.: −3.13, −0.67) and specific conductivity (−1.59 ± 0.66; C.I.: −2.87, −0.32) were negatively correlated with hellbender population status (Figure 5). The effect of watershed size was negligible as indicated by confidence intervals that overlapped zero (−0.56 ± 0.42; −1.38, 0.26).

Overall, we found considerable decline in hellbender population status (i.e., recruiting versus declining, functionally extirpated, and extirpated) when the percent watershed development in the HUC12 watershed exceeded 25% and when specific conductivity at the study site exceeded 50 µS/cm (Figure 5).

Discussion

Our results indicate that anthropogenic watershed development in the form of urbanization and agriculture are negatively impacting hellbender populations through degradation of water quality. Loss of physical and chemical function in aquatic systems are often associated with channelization, dredging, loss of riparian buffers, flow alteration, and excess sediment input derived from development. Conductivity is a parameter often used as a general measure of water quality. Low levels of conductivity occur naturally (below 70-140 mS/cm; Dow and Zampella 2000), but high levels of conductivity (above 140 mS/cm) are often associated with detrimental impacts to water quality associated with watershed development (Likens et al. 1970; Paul and Meyer 2001). The mean conductivity in sample sites with stable and recruitment hellbender populations was 42.9 mS/cm, whereas mean conductivity at sampled sites with declining (100.7 mS/cm), functionally extirpated (120.6 mS/cm), and extirpated (213.4 mS/cm) hellbender populations was above 100 mS/cm. Our results support the concept that watershed development often degrades stream systems and poses a threat to aquatic wildlife (Lenat and Crawford 1994, Paul and Meyer 2001). Notably, high levels of conductivity are likely associated with anthropogenic practices that promote deforestation, loss of riparian buffers, and elevated erosion and runoff. Our results are congruent with other studies indicating that loss of forest and natural landuse at the catchment level contribute to decline in water quality, which is negatively impacting hellbender populations. (Keitzer et al. 2013; Pitt et al. 2017; Bodinof Jachowski and Hopkins 2018; Wineland et al. 2018). It is important to note that riverine systems in Tennessee and North Carolina have been directly or indirectly affected by landscape alteration and development since the early 1800s. Although conductivity can be an indicative of the current effect land use on water quality, historical land use changes may also contribute to hellbender population declines through delayed negative impacts on stream habitat quality and availability (i.e., legacy sediment transport and dam controlled rivers).

Although we did not find a difference in percent watershed development among ecoregions, it is important to discuss possible ecoregion specific effects related to ecoregion size. According to our analysis, the Blue Ridge ecoregion had more HUC12 watersheds (473) than the Ridge and Valley (375) and Interior Plateau (440). However, the Interior Plateau has significantly larger watersheds compared to sites within the Blue Ridge ecoregion. In general, larger watersheds have greater surface area available for development which increases the possibility of land use alteration and associated negative impacts to water resources. In our study area, 147 (34%) HUC12 watersheds within the Interior Plateau had more than 50% of their surface area developed, compared to 140 (37%) watersheds in the Ridge and Valley and 38 (8%) in the Blue Ridge ecoregion. Similarly, 340 (78%) watersheds in the Blue Ridge ecoregion had less than 25% of their surface developed compared to 148 (34%) watersheds in the Interior Plateau. Although not significant, our results indicate that hellbender populations that are recruiting seem to be located within smaller watersheds with lower percent watershed development like those present within the Blue Ridge ecoregion. Atrazine, cadmium, and pH were significantly greater in the Interior Plateau than the Blue

Ridge, whereas Hg and conductivity were significantly greater in the Interior Plateau and Ridge and Valley compared to the Blue Ridge. Notably, Da Silva Neto et al. (2020) suggested that the greatest area of suitable hellbender habitat available in Tennessee is located within the Blue Ridge ecoregion. It is important to highlight that our analysis considered the combined impact of agricultural and urban development on a landscape level. However, urban, and agricultural development may have separate impacts on water quality that function on a smaller scale that cannot be assessed through our methods. Finally, our landscape-scale analysis does not take into consideration microhabitat quality and availability which are affected by complex biological and chemical processes and may affect hellbender population status.

Our pilot and field study results demonstrated that we successfully used a novel passive sampling approach based on diffusion to quantify the concentration of atrazine at sampled sites. We did not detect a significant difference in the concentration of atrazine between passive samplers and known standards, which suggests that passive samplers are an effective tool to assess concentration of atrazine in an aquatic environment. In addition, there was low variance among replicates and a significant non-linear increase in atrazine detected in each dilution, which suggests that this approach is precise, and the dilution curve can be used to estimate atrazine concentration from field samples. In contrast, we were unable to effectively use our passive sampling approach to detect glyphosate. The pilot study demonstrated that our analysis method did not detect glyphosate dilutions that were less than 300 ml/l. Glyphosate has previously been detected at lower concentrations in freshwater streams using other analytical methods; however, our current method was designed to screen for multiple herbicides that ionized in both positive and negative mode and was not optimized for detection of glyphosate alone (Coupe et al. 2012; Battaglin et al. 2014). Fate and concentration of glyphosate in the environment can vary greatly depending on the characteristics of the chemical compound (e.g., half-life and surfactants), soil composition and microorganism community, rate of precipitation and runoff, presence and quality of riparian buffer, and compliance with herbicide label recommendations (Coupe et al. 2012, Wagner et al. 2013; Annett et al. 2014: Battaglin et al. 2014). Our lack of ability to identify glyphosate in samples sites does not mean that glyphosate was not present. It does, however, highlight the limit of sensitivity of our analytical methods and the necessity for a modified and improved protocol.

Our results indicate the presence of atrazine in seven sites (six in Tennessee and one in North Carolina), including sites where we considered hellbender populations to be stable and recruiting. The mean concentration of atrazine (0.001 ± 0.002 ppm) we detected in sites with stable and recruiting populations is 10 times greater than concentrations that have been shown to cause developmental abnormalities in frogs (0.0001 ppm; Hayes et al. 2002). The concentration of atrazine we found in sites where hellbender populations were extirpated, functionally extirpated, or declining were several times higher than concentrations shown to have negative effects on other amphibian species (Boone and James 2003). However, the mean concentration of atrazine in the Interior Plateau ecoregion was orders of magnitude lower than the 96 h LC50 (i.e., concentration at which 50% of individuals die after 96 h of exposure) for rainbow trout (4.5 ppm). It is important to note that atrazine is highly mobile through soil (i.e., it has a high probably of leaching), it is stable in water, and it will sink in water after 24 h of entering an aquatic environment (Atrazine SDS sheet). The long life-span of *C. alleganiensis* in combination with atrazine's characteristics may increase the potential for chronic exposure of individuals to atrazine. In addition, atrazine may indirectly impact hellbender populations and other aquatic organisms by impacting aquatic food webs. For example, atrazine is highly toxic to aquatic plant communities, which are food source to invertebrates, and moderately toxic to invertebrates (Atrazine SDS sheet), which in turn are a food source for larval hellbenders (Unger et al. 2020). For example, atrazine reduced food supply to frog tadpoles through the reduction of algae available to tadpoles exposed to 200 mg/L of Atrazine in several 1480L cattle tank ponds (James and Bonne 2003). In addition, Cusaac et al. (2021) suggested that chronic exposure to Atrazine can increase susceptibility to emerging infectious diseases that are known to cause high mortality in several amphibian species. Although our study does not establish causation regarding direct impacts of atrazine on hellbender populations, it is likely that presence of atrazine at the observed concentrations has the potential to impact overall hellbender population health and long-term persistence through acute and chronic impacts to individuals and community trophic levels. In addition, the occurrence of elevated levels of atrazine, the elevated conductivity measurements from Interior Plateau ecoregion streams suggests that this ecoregion is experiencing exceptionally high levels of stream impairment, which is likely due to the relatively high amount of agricultural landuse specific to this ecoregion.

We also detected Cd, Hg, and Pb in soil samples at several of our sample sites. The presence of Cd is often associated with mining and industrial waste and it has been shown to cause negative effects on amphibians that are chronically exposed to higher concentrations (0.1ppm and above; Friberg et al. 2019; James and Little 2003). The average Cd concentration detected in the Interior Plateau ecoregion (0.52 ± 0.06 ppm) has been found to affect the development of *Bufo arenarum* embryos (Pérez-Coll et al. 1985). Little is known about the toxicity of Hg to amphibians, although one study reported the 96h LC50 of HgCL to *Microhyla arnata* tadpoles to be 2.04ppm. It is important to note that we measured the concentration of inorganic Hg, and not organic methylHg (MeHg), which is the relevant form of Hg for wildlife exposure. Organic Hg can occur naturally in the environment, while methylmercury (MeHg) is formed once inorganic Hg is methylated into organic Hg by microorganisms. Therefore, our results demonstrate that Hg is indeed present in several sampled sites, but it does not allow us to discuss exposure potential. Finally, Pb is a common heavy metal that can be found in concentrations of on average 0.004 ppm in water and 0.0018 ppm in the sediment of lakes and river across the United States (Berzins et al. 2002). The Pb concentration we detected in the Interior Plateau are approximately 1000 times the average concentration of Pb in found in water and 200 times the average concentration of Pb found in sediments of lakes and rivers of the United States. *Xenopus laevis* tadpoles exposed to Pb concentrations of 0.04 ppm, which is 150 times lower than the concentration we detected on average in the Interior Plateau, experienced significant decrease in body weight (Berzins et al. 2002). Several other studies have shown that Pb can be acutely and chronically toxic to humans and several wildlife species (Berkins and Bundy 2002; Wang and Jia 2009; Tranel and Kimmel 2009, Pain et al. 2019). The half-life of Pb in the environment is approximately 20 years, and therefore it may pose a threat to hellbender populations through chronic exposure (Berzins et al. 2002). Our results provide basic baseline data regarding the presence of Cd, Hg, and Pb at several historical and current hellbender sites. Our study does not permit direct causation among Cd, Hg, and Pb concentrations and hellbender population declines across the study area, however; the potential negative effects of these pollutants in combination with other environmental stressors on hellbender populations cannot be discounted.

There are several limitations to our approach that must be acknowledged. First, it is important to note the limitation of our results. Our goal was not to quantify total absorption and absorption rates to herbicides and heavy metals. Exposure potential and total absorption rates are difficult to assess because they are a product of a complex interaction between physiological characteristics (e.g., skin thickness and cell membrane biochemistry), species natural history (e.g., foraging behavior), and environmental factors (e.g., water velocity, precipitation rate, soil texture; Willens et al. 2006). Absorption rates are also dependent on taxa and life-stage. Therefore, assessing true absorption potential falls outside the scope of our study. However, we acknowledge the importance of those parameters and we recommend that future studies investigate this further. A key component of our study was our ability to estimate herbicide concentrations that wild hellbender individuals may be exposed to in situ. Studies using both in-vitro and in-vivo models have indicated that cutaneous absorption can be a major pathway for herbicide absorption in amphibians (Willens et al. 2006, Brühl et al. 2011). Therefore, we believe that although our passive sampler is not a morphologically identical representation of hellbender skin, our approach is an effective way to estimate potential herbicide exposure concentration of hellbenders located within sampled stream reaches. In addition, our approach allows us to investigate potential herbicide exposure of individual hellbenders without the need to euthanize wild animals. We recommend that future research assessing herbicide presence in hellbender streams using this approach incorporate a greater number of samples and increase the frequency of passive sampler deployment to account for seasonal variation in herbicide application and delivery. Finally, we recommend a long-term assessment of water quality and herbicide presence at known hellbender locations to allow for long-term, quantitative, assessment of the impact of land use changes on water quality and potential chronic effects on hellbender populations.

Declarations

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Tables

Table 1: Mean (± SD) for landscape, heavy metal and atrazine concentrations, and water quality variables within Level III ecoregions and *Cryptobranchus alleganiensis* population status categories. Average values were calculated from data acquired from 30 stream sites within Tennessee and North Carolina.

	HUC12 Watershed Size (ha)	Percent Watershed Development (x100)	Cd Concentration (ppm)	Hg Concentration (ppm)	Pb Concentration (ppm)	Atrazine Concentration (ppm)	Dissolved Oxygen (%)	Specific Conductivity (µS/cm)	pH
Level III Ecoregion									
Blue Ridge	7756.23 ± 2525.35	0.32 ± 0.20	0.20 ± 0.07	0.009 ± 0.007	4.46 ± 2.52	0.0003 ± 0.001	94.44 ± 4.06	54.11 ± 27.15	7.44 ± 0.27
Interior Plateau	10839.94 ± 3197.34	0.42 ± 0.20	0.50 ± 0.22	0.02 ± 0.008	5.98 ± 3.04	0.01 ± 0.02	95.06 ± 4.93	204.53 ± 97.75	7.82 ± 0.15
Ridge and Valley	9030.91 ± 4754.92	0.52 ± 0.06	0.37 ± 0.12	0.02 ± 0.01	7.88 ± 2.14	0.00 ± 0.00	88.36 ± 0.95	176.22 ± 48.46	7.74 ± 0.07
Population Status									
Extirpated	9130.96 ± 3375.83	0.58 ± 0.09	0.35 ± 0.21	0.01 ± 0.009	6.92 ± 2.88	0.01 ± 0.02	92.05 ± 5.80	213.43 ± 122.68	7.66 ± 0.41
Functionally Extirpated	9871.77 ± 2942.56	0.47 ± 0.18	0.46 ± 0.27	0.02 ± 0.008	6.87 ± 3.05	0.001 ± 0.002	93.13 ± 6.36	129.27 ± 75.53	7.61 ± 0.27
Declining	10725.06 ± 3124.92	0.29 ± 0.14	0.34 ± 0.15	0.01 ± 0.009	3.66 ± 1.35	0.006 ± 0.009	94.93 ± 1.38	102.15 ± 38.23	7.65 ± 0.23
Recruiting	7143.24 ± 2525.42	0.22 ± 0.14	0.19 ± 0.07	0.008 ± 0.008	4.02 ± 2.01	0.0006 ± 0.001	95.86 ± 3.00	44.47 ± 20.92	7.49 ± 0.25

Table 2: Description of 26 candidate models to evaluate the singular and additive effects of landscape condition and water quality variables on *Cryptobranchus alleganiensis* population status.

AIC

Model	log-likelihood	k	AIC_c	ΔAIC_c	ω_i
Development + Conductivity	-25.27	5	63.04	0.00	0.55
Watershed Size + Development + Conductivity	-24.40	6	64.45	1.41	0.27
Development	-29.62	4	68.84	5.80	0.03
Watershed Size + Development + Atrazine	-26.99	6	69.63	6.59	0.02
Watershed Size + Development + Cadmium	-27.27	6	70.19	7.15	0.02
Watershed Size + Development + pH	-27.33	6	70.31	7.27	0.01
Development + Cadmium	-28.97	5	70.44	7.40	0.01
Watershed Size + Development + Mercury	-27.58	6	70.81	7.77	0.01
Watershed Size + Development + Lead	-27.62	6	70.89	7.85	0.01
Watershed Size + Development + Dissolved Oxygen	-27.63	6	70.91	7.87	0.01
Conductivity + pH + Dissolved Oxygen	-27.68	6	71.01	7.97	0.01
Development + pH	-29.48	5	71.46	8.42	0.01
Development + Lead	-29.50	5	71.50	8.46	0.01
Development + Mercury	-29.60	5	71.70	8.66	0.01
Conductivity	-31.29	4	72.18	9.14	0.01
Development + Atrazine	-30.10	5	72.70	9.66	0.00
Development + Dissolved Oxygen	-30.24	5	72.98	9.94	0.00
Lead	-37.61	4	84.82	21.78	0.00
Dissolved Oxygen	-38.25	4	86.10	23.06	0.00
Cadmium	-38.92	4	87.44	24.40	0.00
Null	-40.26	3	87.44	24.40	0.00
Atrazine	-39.40	4	88.40	25.36	0.00
Mercury	-39.64	4	88.88	25.84	0.00
Watershed Size	-39.94	4	89.48	26.44	0.00
pH	-40.15	4	89.90	26.86	0.00
Cadmium + Lead + Mercury	-37.38	6	90.41	27.37	0.00

Figures

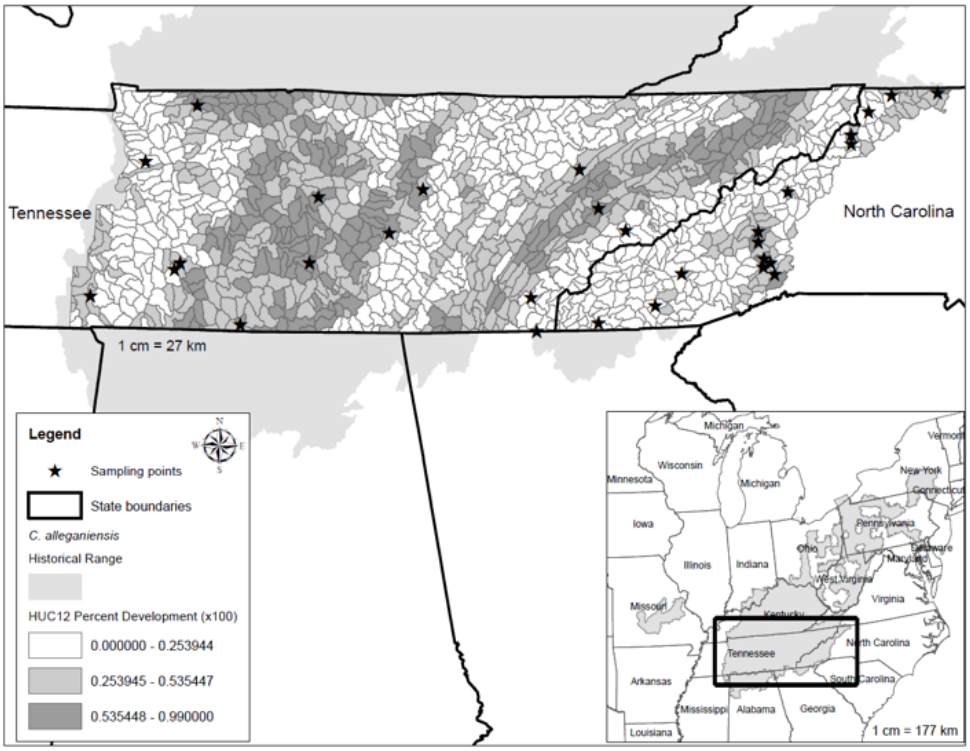

Figure 1

Map representing percent development within HUC12 watersheds within the Cryptobranchus alleganiensis range in Tennessee and North Carolina. The map also includes our sampling sites and Level III ecoregions within the study area. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

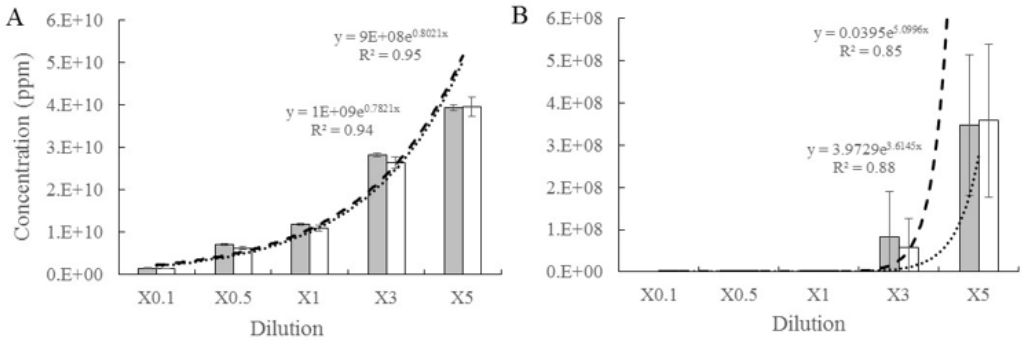

Figure 2

Dilution curves representing the results of the pilot study evaluating passive samplers versus dilution standards to detect atrazine (A) and glyphosate (B) in a water medium. The gray bars and dark dotted lines represent the known concentration of herbicides in the water medium and the white bars and light dotted lines represent the concentration within the receiving phase (i.e., deionized water) of the passive samplers.

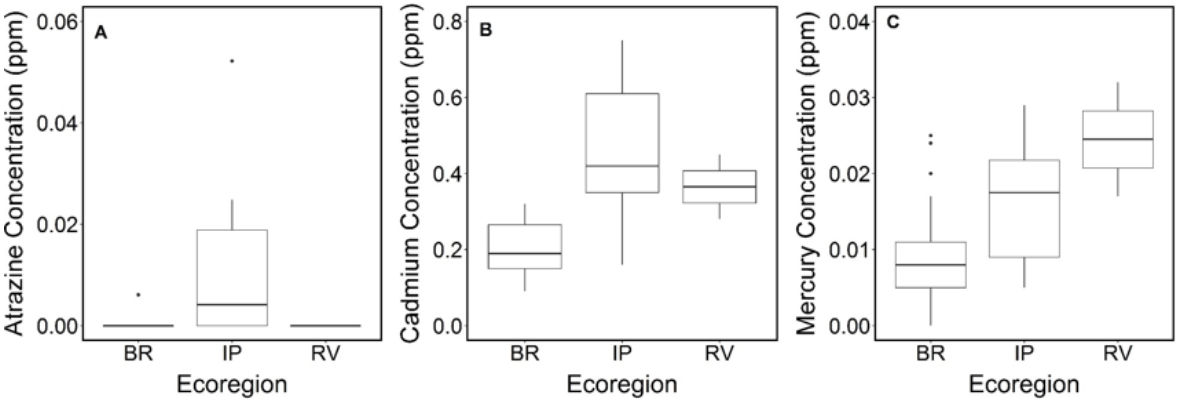

Figure 3

Mean atrazine (ppm), Cd (ppm), and Hg (ppm) in 30 stream sites within three Level III ecoregions (Blue Ridge [BR], Interior Plateau [IP], and Ridge and Valley [RV]) in Tennessee and North Carolina, USA.

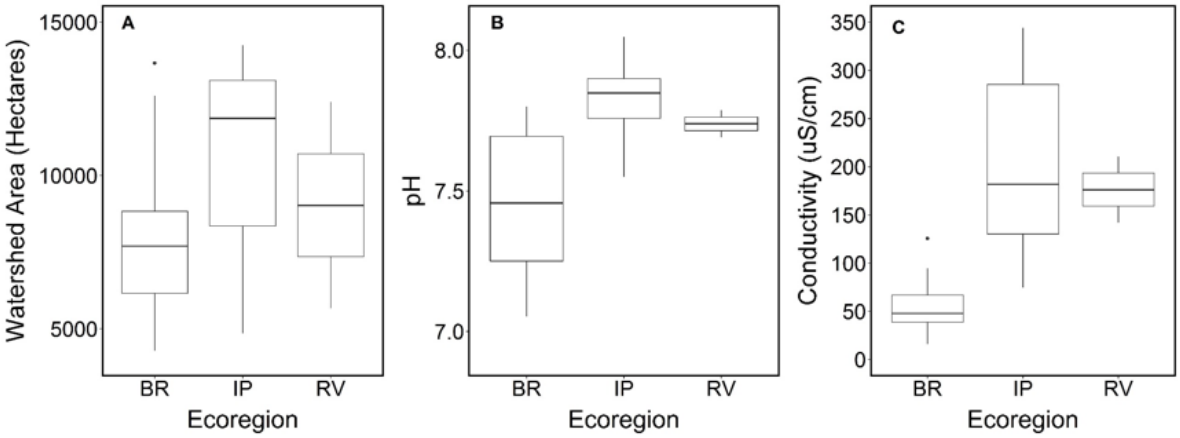

Figure 4

Mean watershed area (ha), pH, and conductivity (µS/cm) at 30 stream sites within three Level III ecoregions (Blue Ridge [BR], Interior Plateau [IP], and Ridge and Valley [RV]) in Tennessee and North Carolina, USA.

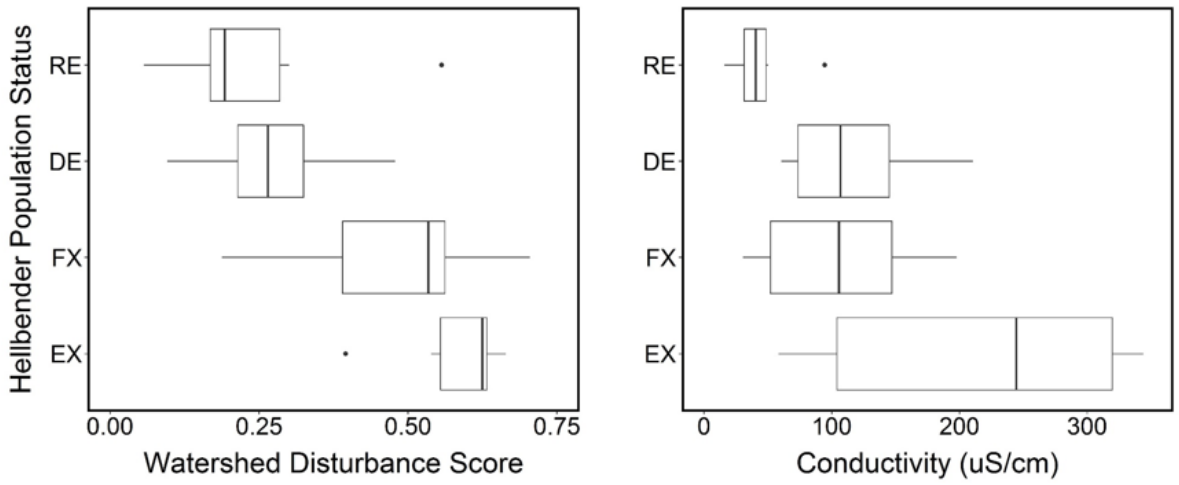

Figure 5

Mean percent watershed development (x100) and conductivity versus Cryptobranchus alleganiensis population status (RE – recruiting, DE - declining, FX – functionally extirpated, EX – extirpated) in 30 stream sites within three Level III ecoregions in Tennessee and North Carolina, USA. These variables were included in a single top model that explained the effects of land use, aquatic contaminants, and water quality parameters on Cryptobranchus alleganiensis population status.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DaSilvaNetoetal.2021AppendixA.docx
- DaSilvaNetoetal.2021AppendixB.docx