

**Susceptibility of Appalachian Mountain Streams to Non-Point Source
Contamination**

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ABSTRACT

Often thought of as pristine, the susceptibility of Tennessee's Appalachian Mountains has remained largely uninvestigated likely due to their government protection and a lack of known point-source contamination. However, a growing body of scientific evidence suggests that depositional inputs can lead to concerning levels of contamination, even in isolated areas. To investigate potential concerns, concentrations of metals and organic pollutants were measured in eastern brook trout (*Salvelinus fontinalis*) in four Appalachian Mountain streams. Additionally, water quality parameters were used to model bioavailability of metals to aquatic organisms. This work is among the first to establish baselines of polychlorinated biphenyls, organochlorine pesticides, dioxins, furans, and phthalates in this area.

TABLE OF CONTENTS

LIST OF TABLES	v
LIST OF FIGURES	vi
BACKGROUND	1
Aluminum Toxicity.....	1
Biotic Ligand Model.....	2
Water Quality Criteria.....	5
Organic Contaminants	6
Polychlorinated biphenyls (PCBs).....	6
Organochlorine Pesticides (OC pesticides)	7
Dioxins and Furans	8
Phthalate Esters	8
INTRODUCTION	8
METHODS	11
Site Selection and Description	11
Sampling Schedule.....	12
Water Sampling and Analysis.....	12
Fish Collection and Analysis	13
Total Metals	13
PCBs	14
Phthalate Esters	15
Organochlorine Pesticides	16
Dioxins/Furans.....	18
Lipid Analysis.....	20
Water Quality Criteria Calculation	20
Statistical Analysis.....	21
RESULTS	22
DISCUSSION	25
REFERENCES	30
APPENDICES	34
APPENDIX A – TABLES.....	35
APPENDIX B – SUPPLEMENTAL TABLES	36
APPENDIX C – FIGURES	40
APPENDIX D – IACUC APPROVAL	43

LIST OF TABLES

TABLE 1 – Mean \pm SE values for water quality parameters.....	35
TABLE 2 – Mean \pm SE values for metal concentrations in <i>Salvelinus fontinalis</i>	35
SUPPLEMENTAL TABLE 1 – Number of replicates sampled.....	36
SUPPLEMENTAL TABLE 2 – Mean \pm SE , minimum and maximum values.....	37
SUPPLEMENTAL TABLE 3 – Detection limits of tested phthalate esters.....	37
SUPPLEMENTAL TABLE 4 – Concentrations of OC pesticides.....	38
SUPPLEMENTAL TABLE 5 – Concentrations of Dioxins/Furans.....	39

LIST OF FIGURES

FIGURE 1 - Map of TEARS sites.....	40
FIGURE 2 – Predicted acute (left) and chronic (right) criteria.....	41
FIGURE 3 - Comparison of field-collected versus EPA-calculated water quality criteria.....	42

BACKGROUND

Aluminum Toxicity

Aluminum comprises approximately 8% of the Earth's crust, making it the most abundant metal and the third most abundant element, after oxygen and silicon (Hamblin 1992). Aluminum is often of no toxicological concern to aquatic life, as it is normally complexed and, consequently, unavailable to cause toxic effects around neutral pH (~ 7). However, in chronically acidified surface water, aluminum availability is of much higher concern and has been implicated in fish die-offs in the northeastern United States and Scandinavia (Henriksen and Rosseland 1984, Baldigo and Murdoch 1997).

In the northeastern United States, many surface waters experience increased acidification (Driscoll *et al.* 2001). Surface water acidification is often attributed to the atmospheric deposition of sulfate and nitrous oxides (i.e. acid rain) that, upon entry into a body of water, cause a disproportionately high concentration of anions, which many systems can neutralize with naturally occurring hardness cations (mainly calcium and magnesium). In systems containing insufficient levels of cations, this influx of anions initiates the release of hydrogen and aluminum cations to maintain electroneutrality. Consequently, high acid deposition rates can cause prolonged surface water acidification as well as high rates of bioavailable aluminum. Unsurprisingly, many studies note an increase in bioavailable aluminum during rain events corresponding with pH drops (Neff *et al.* 2008).

The mechanism for aluminum toxicity mirrors that of other gill-binding metals, which is the interruption of osmoregulation. However, an additional mechanism for

aluminum toxicity has been described by Poleo (1995). Following the liberation of aluminum during toxic events, aluminum re-polymerization occurs as the pH rises back to its base conditions. If re-polymerization occurs in the vicinity of a gill, it can cause irritation and respiratory difficulty for fish, stimulating mucosal release, which can further exacerbate fish respiration and lead to death.

Biotic Ligand Model

When determining the toxicological effect of a metal on an organism, it is important to note that not every metal species or phase will necessarily elicit an equally toxic response. This quality is referred to as a metal's bioavailability and can be affected by several physiochemical factors (e.g. temperature, pH, competing cations, etc.).

For example, metals that produce effects from binding with gill sites must be in a dissolved form to do so; that is, the dissolved form represents the bioavailable fraction. More specifically, the Free-Ion Activity Model (FIAM) states that the aqueous ion form of a metal is directly proportional to the toxic effect seen. The FIAM was among the earliest bioavailability models for metals and was the basis for future models. For a review of the history, derivation, and exceptions to the FIAM, see Campbell (1995).

Early metal criteria for surface waters failed to take metal speciation into account and relied on measurements of total recoverable metal (USEPA 1980a, 1980b, 1980c). Consequently, metal criteria were often 'too rigorous' and did not correspond to expected toxicity in natural waters. It was not until 1993 that the EPA Office of Water released a memo officially suggesting the dissolved fraction of metal be considered when determining

criteria (USEPA 1993). Interestingly, criteria for aluminum are still based on total aluminum.

In addition to speciation, metal bioavailability decreases in the presence of hardness cations (i.e. Ca^+ , Mg^+) due to competitive inhibition for gill sites. The ability of water hardness to ‘buffer’ organisms from metal toxicity has been recognized since at least the 1980s and was considered in early metal criteria calculations (USEPA 1980a, 1980b, 1980c). The Gill-Surface Interaction Model (GSIM) combined this concept with metal speciation models to produce a more sophisticated metal bioavailability model (Pagenkopf 1983).

The next major advancement came with the incorporation of dissolved organic carbon (DOC). DOC can chelate metal, thereby reducing its ability to bind to an organism’s gills and lowering the effective toxicity of an organism; however, DOC can also bind to other cations and other constituents in the water column, making its integration into existing models difficult. The development of the Windemere Humic Aqueous Model (WHAM; Tipping 1994) provided a solution to DOC modeling – the Biotic Ligand Model (BLM; Di Toro *et al.* 2001). The BLM incorporates metal speciation, competing hardness cations, and organic carbon complexation to produce more accurate metal criteria. Additionally, the BLM functions for any organism whose toxic response to a metal is a result of external complexation, expanding the model’s application.

While the BLM was initially created for copper and calculated site-specific toxicity to a single taxon (e.g. rainbow trout), there are now BLMs for several metals (e.g. Al, Ag, Cd, Zn, Ni) and various taxa. Additionally, BLM software can now produce water quality

criteria (WQC). Several metal-mixing BLMs have been produced, and current projects aim to expand the catalog of metals for the BLM and design more sophisticated mixing models.

Water Quality Criteria

The Clean Water Act (CWA) of 1977 requires that criteria be established for water by the Environmental Protection Agency (EPA) for any chemicals or compounds of concern to human or wildlife health. These criteria can manifest as numerical thresholds or as narrative statements and are expected to reflect the current state of scientific knowledge. Consequently, means to derive these criteria have shifted numerous times since the CWA's enactment. The 1985 *Guidelines to Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses*, often referred to as "the guidelines," presents a comprehensive guide for the calculation of numerical criteria. A brief summarization of the guidelines' methodologies is presented below.

A 'criterion,' as defined by the guidelines, consists of two numerical criteria, representing acute and chronic toxicity. Though these values have changed in name and calculation, some iteration of them can be found throughout criteria since the 1980s (USEPA 1980(a), USEPA 1980(b), USEPA 2007). Criteria are intended to be protective to a majority of wildlife present in any given body of water; however, it is not practical to try to establish criteria that are 100% protective in all water bodies to all organisms, so a value of 95% was deemed an appropriate threshold.

The 95% threshold is calculated by compiling toxicological laboratory data on the concentration of a chemical of interest that results in the death of 50% of a species, a metric known as the lethal concentration for 50% of a population (LC₅₀). The average LC₅₀ is calculated for species from available laboratory studies, producing species mean acute values (SMAVs), which are then averaged into genus mean acute values (GMAVs). These values are ranked from lowest to highest and the bottom 5th percentile is calculated

mathematically from the resulting cumulative distribution. The resulting value is the final acute value (FAV). To be sufficiently protective, this value is divided by two to produce the criterion maximum concentration (CMC). The CMC represents the acute criterion set by the EPA. For greater detail of FAV calculation see EPA (1985).

The same process can be completed with chronic data to produce the criterion continuous concentration (CCC), which represents the EPA's chronic criterion. There are much fewer chronic toxicological data, however, because the length of experiments are longer and demand greater resources to complete. This has prompted the use of acute-chronic ratios (ACRs) for estimating chronic values. ACRs are simply the ratio of known acute and chronic values, which, once established empirically for a particular taxon, can be applied to other similar groups that lack chronic data.

In order to incorporate BLM calculations for relevant criteria, calculated LC_{50s} are normalized to reference conditions, allowing normalized SMAVs to be produced. Using these values, the normal CMC and CCC derivation is completed as described above.

Organic Contaminants

Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a class of lipophilic organic compounds that gained commercial popularity due to their extreme stability. Often utilized as varying congener mixtures, PCBs were used in numerous applications such as industrial lubricants, dielectric insulators, and heating system insulators in the United States (Newman 1998). Although their inert nature makes them useful in industrial settings, it also makes them extremely

environmentally persistent, with some models estimating half-lives over 25 years in soils and sediments (Sinkkonen and Paasivirta 2000). Upon entering the food chain, these lipophilic compounds become stored in lipids, building up in organism (bioaccumulating) and increasing up levels of a food chain (biomagnification) (Walters *et al.* 2008; Borga, Gabrielsen and Skaare 2001). Evidence has shown that PCBs can have a range of toxic effects on animal health (see Safe 1994 for review) and are considered a probable carcinogen by the EPA (USEPA 1996). The evidence of adverse health effects caused by PCBs prompted the 1979 ban on the manufacturing of these compounds; however, their persistence and global transport make them a continued concern.

Organochlorine Pesticides (OC pesticides)

Organochlorine Pesticides (OC pesticides) are a group of chlorinated compounds that were widely used in the United States as insecticides, until their eventual ban. OC pesticides can have varying chemical properties that affect their persistence in the environment and their mode of toxicity. The most well-known of these compounds is dichlorodiphenyltrichloroethane (DDT), which first found use during the second world war and later was used as a commercial insecticide both domestically and internationally (Walker *et al.* 2016). DDT and its metabolites were found to cause irregularities in breeding cycles and eggshell thinning in birds, due to their inhibition of calcium incorporation (Peakall 1970). In addition to DDT and its metabolites, there are numerous other OC pesticides that have been used and subsequently banned, due to a range of toxic effects to non-target organisms; most notably are Aldrin, Eldrin, Toxaphene and Lindane.

Dioxins and Furans

Polychlorinated dibenzo-p-dioxins (PCDD or dioxins) and polychlorinated dibenzofurans (PCDF or furans) are a class of environmentally persistent, chlorinated compounds. Dioxins and furans have no commercial value and are by-products of waste incineration and other industrial processes, including the production of chlorinated compounds (Newman 1998). There exist multiple congeners of dioxins and furans that differ based on the level of chlorination and toxicity. To help simplify the interpretation of dioxin and furan data, toxic equivalency factors (TEF) were developed to normalize all congeners to the most toxic congener, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, TEF of 1; Van den Berg *et al.* 1998). Like PCBs, dioxins and furans are relatively stable, can persist in the environment and can travel far distances via atmospheric deposition.

Phthalate Esters

Phthalate esters, referred to hereon simply as phthalates, are a group of chemical plasticizers used most notably in polyvinyl chlorides (PVC). Phthalates are readily biodegradable and show little environmental persistence or bioaccumulation. Phthalate exposure to the general public is widespread (CDC 2017) and has been linked with potential developmental and reproductive issues (USEPA 2012).

INTRODUCTION

In 2017, a report released by the *Lancet* commission on pollution and health identified pollution as the “largest environmental cause of disease and premature death in the world

today” (Landrigan *et al.* 2017). The report goes on to highlight several recommendations regarding pollution research and management – notably, the need for established monitoring systems and multi-sectoral approaches towards pollution control. These recommendations emphasize that the critical first step to managing environmental pollution is to determine current levels of environmental contaminants.

Traditionally, investigations into environmental contamination have focused on areas with known point sources or in close proximity to anthropogenic activity; however, there is now substantial evidence that even isolated areas are susceptible to high contaminant exposure through atmospheric deposition. For example, studies have found polychlorinated biphenyls (PCBs), organochlorinated (OC) pesticides, polychlorinated dibenzo-p-dioxins (dioxins) and dibenzofurans (furans) in arctic mammals (Muir, Norstrom and Simon 1988, Oehme *et al.* 1995, Addison and Smith 1974). As Dewailly *et al.* (1989) showed, many of these organisms serve as significant dietary contributions to the Inuit diet, leading to elevated levels of chlorinated compounds in Inuit breast milk, despite the far distance from where these compounds were manufactured or used. Additionally, mercury has been shown to atmospherically deposit in remote areas, where it can be methylated and magnified to levels of ecological concern (Walters *et al.* 2015, Driscoll *et al.* 2007). Walters *et al.* 2015 found mercury concentrations in fish in Grand Canyon, Arizona to exceed wildlife thresholds for human consumption (0.3 ppm). Growing evidence casts doubt on the existence of truly “pristine” environments.

The Blue Ridge Mountain ecoregion (66) is among the most biologically rich areas in the southeast and houses much of Tennessee’s Appalachian Mountains, including the most visited national park in the United States, the Great Smoky Mountain National Park

(GRSM; NPS 2017). Despite the area's ecological and social importance, relatively little is known outside of GRSM regarding concentrations of persistent contaminants. Although these mountains are typically thought of as pristine and stable, largely due to the afforded protection at both state and federal levels, the geographical features of the Southern Appalachian Mountains can cause relatively high levels of contaminant deposition (Lovett and Kinsman 1990). Historically, these features have led to high levels of inorganic acid deposition (NO_x , SO_4), causing surface water acidification and concerns of potential aluminum toxicity (Neff *et al.* 2008). These areas have also been shown to receive elevated levels of mercury deposition (Risch *et al.* 2012), though there have been little to no investigations into the biological uptake of mercury once deposited. Levels of other depositional contaminants (e.g. PCBs, OC pesticides, dioxins, furans, etc.) have gone largely uninvestigated.

Once established, interpretation of contaminant concentrations requires some threshold or reference to determine whether levels exceed those acceptable for stream health, most often taking the form of numerical criteria. Calculating criteria for metals in surface waters can be especially challenging, as there is now substantial evidence that factors affecting metal bioavailability, which vary by site, must be taken into account when setting criteria (Pagenkopf 1983, Di Toro *et al.* 2001, Campbell 1995, Erickson *et al.* 1996). One approach to this problem was the creation of the Biotic Ligand Model (BLM), which can produce site-specific criteria based on a stream's physiochemical properties (e.g. dissolved organic carbon, hardness). Because of the potential difficulties in collecting all of the data necessary for the BLM, the EPA has released a technical support document that provides protective, estimated BLM parameters for each ecoregion, based on geostatistical

modeling (USEPA 2016). The estimated EPA parameters for low-order streams in ecoregion 66 have relatively low concentrations of organic carbon and hardness cations, so following the principles of the BLM, it can be hypothesized that Tennessee's Appalachian Mountains will afford aquatic life little protection from metal toxicity, though this has not yet been tested.

Investigations into the presence of persistent contaminants in isolated areas can be both logistically difficult and costly. Consequently, a methodology for practically investigating areas where toxicological data is generally absent is needed. The Tennessee's Ecologically At-Risk Streams (TEARS) project is a multiagency effort to establish current contaminant levels and susceptibility of four headwater streams in Tennessee's Appalachian Mountains within ecoregion 66. The TEARS framework uses a broad screening approach to establish contaminant baselines, identify current concerns for stream health, and determine stream susceptibility to future concerns. The aims of the present study were to (1) characterize water quality parameters for four headwater streams; (2) establish current concentrations of common organic and inorganic contaminants, specifically nutrients (nitrate + nitrite, total phosphorous), PCBs, OC pesticides, dioxins, furans, phthalates, and metals; and (3) identify current concerns for stream health.

METHODS

Site Selection and Description

Four sites were selected in headwater streams across Tennessee's Appalachian Mountains: Bald River (35° 16.278'N, 84° 8.295'W), Rock Creek (35° 45.239'N, 83° 12.845'W), Left

Prong Hampton Creek (36° 8.382'N, 82° 2.787'W), and Gentry Creek (36° 33.568'N, 81° 42.669'W) (Fig. 1). All sites were 2nd or 3rd order streams in the Blue Ridge level III ecoregion (ecoregion 66), which is characterized by having well-drained, loamy soils. Despite high channel gradients (~10-20%), flow was maintained year-round, sustaining populations of commercially-important eastern brook trout (*Salvelinus fontinalis*). Although little to no historical toxicological data exists for these sites, all are afforded some level of state or federal protection; Bald River, Left Prong Hampton Creek and Gentry Creek are all in Cherokee National Forest, while Rock Creek is in Great Smoky Mountain National Park (GRSM).

Sampling Schedule

Water and fish samples were collected from August 2015 through August 2017. For a more detailed sampling timeline see Supplemental Table 1.

Water Sampling and Analysis

Water sampling occurred during base flow conditions, (no rainfall within at least 24 hours of a storm event). To minimize variability in measurements caused by diel fluctuations, samples were collected as close to 9:30 a.m. (CST) as possible throughout the entire study.

Water sampling of temperature, dissolved oxygen, conductivity, and pH were measured using a YSI® Pro 2030 conductivity meter and an Orion Star™ A321 pH hand meter. Surface grab samples were collected in high density polyethylene (HDPE) bottles and analyzed for nitrate and nitrite (EPA 353.2), total phosphorous (EPA 365.1), total

metals (acid digestion [EPA 200.2]; Al, Cd, Cu, Pb, Ni, Zn, ICP-MS [EPA 200.8]; Fe, K, Na, ICP [EPA 200.7]), total hardness and calcium hardness (Standard Methods 2340B), organic carbon (total, Standard Methods 5310C; dissolved, syringe-filtered [0.45 µm], Standard Methods 5310C), and alkalinity (Standard Methods 2320B @ pH 4.5). If metal concentrations were below detection at all sites, they were not collected during the following sampling event. Subsurface grab samples were collected in either high density polyethylene (HDPE) or polypropylene (PP) bottles and analyzed for total suspended solids (TSS) (Stand Method 2540D). All sample collections were taken from mid-stream at each site. After collection, all samples were transported to the laboratory on wet ice where they were refrigerated at 4°C until analysis.

Fish Collection and Analysis

Eastern brook trout (*S. fontinalis*) were collected via electrofishing during the summers of 2015, 2016, and 2017. Fish were verified to species and measured for total length and weight before being euthanized. Fish were then wrapped in aluminum foil, placed into individually labeled plastic bags, stored on wet ice, and transported to the laboratory to await processing and analysis.

Total Metals

Ten fish from each of the four sites were analyzed for total metals (Ag, Al, Ar, Cd, Cr, Cu, Pb, Ni, Hg, Se, Zn). Following the removal of the gastrointestinal tract, larger fish were paired with smaller fish to create five homogenates of roughly equal mass from each site.

Homogenized tissue was prepared for analysis via extraction in aqua regia in accordance with EPA Method 200.2. Sample extracts were analyzed via ICP-MS in accordance with EPA Method 200.8. Samples below detection limits are deemed undetectable. All method blanks were below detection.

PCBs

Ten fish from each of the four sites were analyzed for specific PCB Aroclor mixtures (1016, 1221, 1232, 1242, 1248, 1254, 1260) and total PCBs (sum of the 19 congeners listed in SW-846 Method 8082A). Following the removal of the gastrointestinal tract, larger fish were paired with smaller fish to create five homogenates of roughly equal mass from each site.

Approximately 10 grams of each homogenized sample was dried with anhydrous sodium sulfate and spiked with tetrachloro-m-xylene (TCMX) and decachlorobiphenyl (DCB). Samples were solvent extracted in dichloromethane via automated soxhlet extractor in accordance with SW-846 Method 3541 and concentrated to approximately 5 ml in a Kuderna-Danish concentrator. A Hewlett Packard 5890 equipped with dual electron capture detectors (ECDs) and two 30m x 0.32mm capillary columns were used to analyze sample extracts in accordance with SW-846 Method 8082A.

All samples, blanks, and controls were spiked with TCMX and DCB prior to extraction. Mean surrogate recovery was 86% (± 6 RSD) and 92% (± 6 RSD) for TCMX and DCB, respectively. The Practical Quantitation Limit (PQL) was 25.0 $\mu\text{g}/\text{kg}$ and the Minimum Detection limit (MDL) was 12.5 $\mu\text{g}/\text{kg}$. Samples below the MDL are deemed

undetected and samples below the PQL are listed as estimated. A method blank, laboratory control sample (LCS), and a matrix spike/matrix spike duplicate (MS/MSD) were analyzed after every 10 samples. All method blanks were below the PQL. The LCS recovery rate was 78.5% (<1% RSD). MS/MSD recoveries were 84% (<1% RSD) and 88% (<1% RSD), respectively.

Phthalate Esters

Four fish were selected from Gentry Creek, Left Prong Hampton Creek, and Rock Creek for analysis of six phthalate esters: dimethyl phthalate, diethyl phthalate, di-n-butyl phthalate, butyl benzyl phthalate, bis(2-ethylhexyl)phthalate, and di-n-octyl phthalate. Following the removal of the gastrointestinal tract, two grams of muscle tissue, the caudal fin, and the left pectoral fin (tissues used for other assays) fish were paired to create two homogenates of roughly equal mass from each site.

Each homogenized sample was dried with anhydrous sodium sulfate and spiked with six surrogates (2-Fluorobiphenyl, Phenol-d₅, Nitrobenzene-d₅, 2-Fluorophenol, 2,4,6-Tribromophenol, Terphenyl-d₁₄). Samples were then solvent-extracted with dichloromethane using a stainless steel Tissuemizer. After this process was repeated three times, the sample was concentrated to 5-10 ml using a Kuderna-Danish Concentrator. Concentrated sample extracts were analyzed using a gas chromatograph/mass spectrometer (GC/MS) equipped with a narrow-bore capillary column in accordance SW-846 Method 8270D.

All samples, blanks, and controls were spiked with the following surrogates prior to extraction: 2-Fluorobiphenyl, Phenol-d₅, Nitrobenzene-d₅, 2-Fluorophenol, 2,4,6-

Tribromophenol, Terphenyl-d₁₄ with recoveries of 61% (± 19 %RSD), 54% (± 27 %RSD), 56% (± 25 %RSD), 44% (± 28 %RSD), 67% (± 19 %RSD), and 71% (± 17 %RSD), respectively. MDLs varied for each sample due to differences in mass and can be found in Supplemental Table 3. Samples below the MDL are deemed undetectable. A method blank, laboratory controls sample (LCS), and laboratory control sample duplicate (LCSD) were also analyzed. All method blanks were below their respective PQLs (400 $\mu\text{g}/\text{kg}$ for di-n-octyl phthalate, 200 $\mu\text{g}/\text{kg}$ for all other congeners). LCS and LCSD recoveries were as follows: 69% (± 12 RSD) and 71% (± 17 %RSD) for dimethyl phthalate, 74% (± 7 %RSD) and 73% (± 13 %RSD) for diethyl phthalate, 81% (± 7 %RSD) and 88% (± 9 %RSD) for di-n-butyl phthalate, 85% (± 7 %RSD) and 91% (± 10 %RSD) for butyl benzyl phthalate, 87% (± 8 %RSD) and 93% (± 11 %RSD) for bis(2-ethylhexyl)phthalate, and 88% (± 8 %RSD) and 93% (± 10 %RSD) for di-n-octyl phthalate.

Organochlorine Pesticides

Four fish from each of the four sites were analyzed for the following organochlorine pesticides: 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, Aldrin, Chlordane, Dieldrin, Endosulfan I, Endosulfan II, Endosulfan sulfate, Endrin, Endrin aldehyde, Endrin ketone, Heptachlor, Heptachlor epoxide, Methoxychlor, Toxaphene, alpha-BHC, alpha-Chlordane, beta-BHC, delta-BHC, gamma-BHC, gamma-Chlordane. Following the removal of the gastrointestinal tract, two grams of muscle tissue, the caudal fin, and the left pectoral fin (tissues used for other assays) fish were paired to create two homogenates of roughly equal mass from each site.

Each homogenized sample was dried with anhydrous sodium sulfate and spiked with DCB and TCMX. Samples were solvent extracted in dichloromethane via automated soxhlet extractor in accordance with SW-846 Method 3540C and concentrated to approximately 10 ml in a Kuderna-Danish concentrator. A Hewlett Packard 5890 equipped with dual electron capture detectors (ECDs) and two 30m x 0.32mm capillary columns was used to analyze sample extracts in accordance with SW-846 Method 8081A.

All samples, blanks, and controls were spiked with TCMX and DCB prior to extraction. Mean surrogate recovery was 57% (± 13 RSD) and 56% (± 11 RSD) for TCMX and DCB, respectively. MDLs varied for each sample due to differences in mass and can be found in Supplemental Table 4. All method blanks were below the PQL. Mean LCS recovery rates were 73% (± 5 %RSD) for 4,4'-DDD, 75% (± 6 %RSD) for 4,4'-DDE, 87% (± 7 %RSD) for 4,4'-DDT, 67% (± 10 %RSD) for Aldrin, 66% (± 6 %RSD) for Dieldrin, 65 (± 5 %RSD) for Endosulfan I, 72% (± 3 %RSD) for Endosulfan II, 71% (± 3 %RSD) for Endosulfan sulfate, 82% (± 6 %RSD) for Endrin, 48% (± 4 %RSD) for Endrin aldehyde, 75% (± 4 %RSD) for Endrin ketone, 74% (± 8 %RSD) for Heptachlor, 71% (± 7 %RSD) for Heptachlor epoxide, 81% (± 8 %RSD) for Methoxychlor, 67% (± 12 %RSD) for alpha-BHC, 69% (± 6 %RSD) for alpha-Chlordane, 68% (± 9 %RSD) for beta-BHC, 75% (± 9 %RSD) for delta-BHC, 74% (± 8 %RSD) for gamma-BHC, and 75% (± 1 %RSD) for gamma-Chlordane. Relative percent difference (RPD) between the LCS and LCSD was between 0-18% with a mean value of 4%. Matrix spike recovery rates were 80% (± 4 %RSD) for 4,4'-DDD, 76% (± 8 %RSD) for 4,4'-DDE, 96% (± 3 %RSD) for 4,4'-DDT, 81% (± 10 %RSD) for Aldrin, 78% (± 2 %RSD) for Dieldrin, 71 (± 3 %RSD) for

Endosulfan I, 75% (± 3 %RSD) for Endosulfan II, 76% (± 7 %RSD) for Endosulfan sulfate, 84% (± 3 %RSD) for Endrin, 55% (± 4 %RSD) for Endrin aldehyde, 85% (± 3 %RSD) for Endrin ketone, 80% (± 1 %RSD) for Heptachlor, 73% (± 1 %RSD) for Heptachlor epoxide, 84% (± 3 %RSD) for Methoxychlor, 74% (± 1 %RSD) for alpha-BHC, 72% (± 1 %RSD) for alpha-Chlordane, 76% (± 5 %RSD) for beta-BHC, 83% (± 3 %RSD) for delta-BHC, 71% (± 4 %RSD) for gamma-BHC, and 81% (± 4 %RSD) for gamma-Chlordane. MS/MSD RPDs were between 1-13% with a mean value of 5%.

Dioxins/Furans

Four fish were selected from Gentry Creek, Left Prong Hampton Creek, and Rock Creek for analysis of 15 polychlorinated dibenzo-p-dioxin and polychlorinated dibenzofuran homologues (specific compounds listed in Supplemental Table 5). Following the removal of the gastrointestinal tract, two grams of muscle tissue, the caudal fin, and the left pectoral fin (tissues used for other assays) fish were paired to create two homogenates of roughly equal mass from each site.

Each homogenized sample was dried with anhydrous sodium sulfate and spiked with isotopically labeled analogs (for full list see EPA Method 8290A). Samples were solvent extracted via soxhlet extractor in dichloromethane/hexane (1:1 v/v) and concentrated to 1ml via Kuderna-Danish concentrator. Following extractions, ^{37}Cl -2,3,7,8-TCDD was added to sample extracts as a clean-up standard. Sample extracts were analyzed using a high-resolution gas chromatography/high resolution mass spectrometry in accordance with USEPA Method 1613B.

All samples, blanks and controls were spiked with the following isotopically labeled surrogates (mean % recovery \pm %RSD) prior to extraction: 2,3,7,8-TCDF- ^{13}C (74 ± 7), 2,3,7,8-TCDD- ^{13}C (86 ± 6), 1,2,3,7,8-PeCDF- ^{13}C (74 ± 8), 2,3,4,7,8-PeCDF- ^{13}C (78 ± 10), 1,2,3,7,8-PeCDD- ^{13}C (89 ± 10), 1,2,3,4,7,8-HxCDF- ^{13}C (62 ± 10), 1,2,3,6,7,8-HxCDF- ^{13}C (73 ± 9), 2,3,4,6,7,8-HxCDF- ^{13}C (70 ± 8), 1,2,3,7,8,9-HxCDF- ^{13}C (70 ± 6), 1,2,3,4,7,8-HxCDD- ^{13}C (64 ± 9), 1,2,3,6,7,8-HxCDD- ^{13}C (66 ± 8), 1,2,3,4,6,7,8-HpCDF- ^{13}C (65 ± 4), 1,2,3,4,7,8,9-HpCDF- ^{13}C (61 ± 13), 1,2,3,4,6,7,8-HpCDD- ^{13}C (72 ± 7), and OCDD- ^{13}C (70 ± 9). Average recovery of the clean-up standard, ^{37}Cl -2,3,7,8-TCDD, was 79% (± 7 %RSD). MDLs varied for each sample due to differences in mass and can be found in Supplemental Table 5. Samples below the MDL are deemed undetectable. A method blank, laboratory controls sample (LCS), and laboratory control sample duplicate (LCSD) were also analyzed. No detectable concentrations were observed in the method blank for any analyte. LCS and LCSD recoveries were as follows: 102% and 99% for 2,3,7,8-TCDD, 96% and 91% for 2,3,7,8-TCDD, 100% and 104% for 1,2,3,7,8-PeCDF, 93% and 96% for 2,3,4,7,8-PeCDF, 103% and 99% for 1,2,3,7,8-PeCDD, 106% and 98% for 1,2,3,4,7-HxCDF, 102% and 99% for 1,2,3,6,7,-HxCDF, 95% and 91% for 2,3,4,6,7,8-HxCDF, 100% and 94% for 1,2,3,7,8,9-HxCDF, 109% and 90% 1,2,3,4,7,8-HxCDD, 106% and 116% for 1,2,3,6,7,8-HxCDD, 109% and 112% for 1,2,3,4,6,7,8-HpCDF, 101% and 99% for 1,2,3,4,7,8,9-HpCDF, 97% and 93% for 1,2,3,4,7,8,9-HpCDF, 98% and 97% for 1,2,3,4,6,7,8-HpCDD, 110% and 103% for OCDF, and 107% and 102% for OCDD.

Lipid Analysis

Lipid analysis was performed on all organic extracts following solvent-extraction of fish for PCBs, dioxins/furans, organochlorine pesticides and phthalate esters. Sample extracts were allowed to evaporate in pre-weighed dishes. The remaining mass represents the lipid fraction of the sample and could be used to calculate percent lipid.

Water Quality Criteria Calculation

Water quality criteria (WQC) for dissolved copper, zinc, cadmium, and nickel were calculated using the Biotic Ligand Model (BLM) approach (Di Toro et al. 2001), as those metals were regularly detectable in fish tissue across all sites. Acute and chronic criteria were calculated for copper and zinc; only chronic criteria were produced for cadmium and nickel.

All necessary inputs for BLM calculation (Temperature, pH, total organic carbon (TOC), Ca, Mg, Na, K, SO₄, Cl, Alkalinity) were collected across all sites during June and August 2016. While the BLM calls for dissolved organic carbon (DOC) for its calculations, TOC was collected instead, based on the low levels of suspended solids measured and the assumption of low primary production. Since this substitution could lead to under-protective criteria, DOC/TOC ratios were calculated to justify TOC use for current and future investigations. Once calculated, dissolved criteria were compared to total metal concentrations to determine if concentrations exceeded acute or chronic criteria.

In order to evaluate how conservative EPA estimated inputs are for low-order streams in the Blue Ridge ecoregion (III), criteria were calculated using the above methods;

however, all inputs, excluding temperature and pH, were replaced with EPA suggested inputs (USEPA 2016). The produced criteria were compared directly to the field-collected criteria to evaluate to what extent criteria shifted.

Statistical Analysis

Metal concentrations in water, sediment and fish were analyzed for normality using shapiro-wilk tests. Because the assumption of normality was not met in these data sets, differences between sites were tested using the nonparametric Kruskal-Wallis test. Select water quality parameters were also investigated for differences between sites using Kruskal-Wallis tests. All statistical analyses were performed in R 3.2.3 (R Core Team, Vienna, Austria). All tests used a significance levels of $\alpha < 0.05$.

RESULTS

Water quality parameters varied depending on site (Table 1). Significant differences were found in concentrations of nitrogen ($H = 29.05$, $p < 0.05$), TSS ($H = 13.011$, $p < 0.05$), conductivity ($H = 21.278$, $p < 0.05$) and pH ($H = 17.321$, $p < 0.05$) between sites. No difference in TOC ($H = 5.217$, $p = 0.16$) between sites was found. Regardless of variation, all pH measurements were within the 6.0-9.0 range required for wadeable streams in Tennessee and dissolved oxygen was always above the 8.0 mg/L criteria required for naturally reproducing trout streams (TWQCB 2007). Rock Creek exceeded the TDEC numerical interpretation of Tennessee's narrative nitrogen (nitrate + nitrite) criterion for ecoregion 66g (0.31 mg/L); all other sites were below their respective criteria (0.31 mg/L for Bald and Gentry; 0.50 mg/L for LPHC). Average TSS, TOC, and conductivity concentrations across all sites were below TDEC reported averages for eastern Tennessee (7.8 mg/L, 1.41 mg/L, and 289 $\mu\text{S}/\text{cm}$ respectively).

All samples analyzed for PCBs, phthalates, OC pesticides, and dioxins/furans were below their respective quantitation limits, although some analytes were detectable (For details, see supplemental tables 3-5). At every site, at least one OC pesticide was detectable; alpha-chlordane was the only pesticide present at all sites (maximum recorded value (MRV) 1.1 ng/g). Dioxins/furans were detectable at every site with the average 2,3,7,8-TCDD equivalents being 0.015 ng/Kg.

Preliminary water samples of cadmium were never detectable above 0.26 $\mu\text{g}/\text{L}$. Copper was only above quantitation in one sample (11 $\mu\text{g}/\text{L}$). Nickel and lead were detectable only once (MDL 0.39 and 0.29, respectively), but never above quantitation.

Consequently, these metals were not resampled. Zinc was consistently above detection, but only once above quantitation. No differences were found in total mercury in water across sites ($H = 4.3724$, $p = 0.224$). Methyl-mercury was only above quantitation during one sampling event, and detectable in half the samples collected. No difference was found in total aluminum samples between sites ($H = 2.6763$, $p = 0.4443$). Dissolved aluminum was on average $65\% \pm 7\%$ of corresponding total aluminum concentrations; the highest measured dissolved aluminum value was $106 \mu\text{g/L}$.

For select metals, concentrations in whole body fish tissue varied between sites (Table 2). Significant differences were found in concentrations of cadmium ($H = 15.128$, $p < 0.05$), copper ($H = 10.261$, $p < 0.05$), selenium ($H = 12.253$, $p < 0.05$), and zinc ($H = 12.237$, $p < 0.05$) between sites; no differences were found for aluminum ($H = 3.642$, $p = 0.3028$) or nickel ($H = 4.2499$, $p = 0.2357$) concentrations between sites. Though detectable, arsenic, chromium and lead were never above quantitation. Selenium concentrations at all sites were below the EPA's 8.5 mg/kg criteria for whole body fish tissue (EPA 2016). No difference was found for total mercury in fish tissue across sites ($H = 4.8713$, $p = 0.1815$); however, a significant difference was found in methyl-mercury concentrations in fish tissue across sites ($H = 8.7208$, $p < 0.05$). Methyl-mercury was on average $75\% \pm 2\%$ of total mercury. Methyl-mercury concentrations across all sites were on average $0.037 \pm 0.003 \text{ ppm}$, well below the 0.3 ppm human consumption threshold (USEPA 2001).

Calculated metal WQC varied between sites, metals and months (Fig. 2). Because total copper, nickel and cadmium measurements were almost always below detection, the MDL was chosen as a reference point for site-specific concentrations. Acute copper criteria

exceeded the MDL in all but two cases, while the chronic criteria exceeded it thrice. Acute zinc criteria calculated were above the maximum recorded value (MRV, 13 $\mu\text{g/L}$); one chronic value was below the MRV. Acute nickel criteria were in all cases above the MDL. Chronic cadmium values were in all cases below the MDL.

All criteria calculated using field-collected data were equal to or greater than those calculated using EPA-estimated parameters (Fig. 3). Field-collected copper and nickel both produced criteria that were over three times greater than their respective EPA criteria. Chronic cadmium and zinc produced criteria were double their respective EPA criteria. Field-collected acute zinc criteria were consistently within a 2x range of EPA-estimated criteria, and in one instance, was exactly the same.

DISCUSSION

Tennessee's Appalachian Mountains are among the most biologically diverse and protected natural areas in the state. This area, mainly absent of known anthropogenic point sources, has little to no previous data on the presence of persistent contaminants.

In the present study, no samples analyzed for PCBs, OC pesticides, dioxins, furans, or phthalates were above quantitation limits and, when detectable, were below EPA human screening thresholds (USEPA 2009). Consequently, these contaminants were not deemed current concerns for these streams. Regardless, detectable concentrations of pesticides and dioxins/furans were found at all sites, suggesting that these areas are not exempt from anthropogenic contamination, despite their relative isolation. It is worth noting that among the detectable pesticides, 4,4-DDE was present at three sites, which, given DDT's 1972 ban, suggests current contaminant concentrations could come from legacy contamination. This study is the first to investigate levels of PCBs, OC pesticides, dioxins, furans, and phthalates in this area and have established baseline levels for future use.

No sites in the present study displayed signs of declining stream health due to nutrients (nitrate + nitrite, total phosphorous), despite Rock Creek's exceedance ($0.50 \text{ mg/L} \pm 0.02$) of TDEC's numerical interpretation for Tennessee's nutrient criteria (0.31 mg/L). The optimal concentration of nutrients for a waterbody can vary greatly depending on several factors, making a single, representative numerical criterion difficult to establish, even for small regions. This could explain Rock Creek's exceedance and lack of excess nutrient impacts (e.g. low dissolved oxygen, reduced clarity). Macroinvertebrate

assemblages from all sites are currently being analyzed and will likely give further endpoints for determining overall stream health and potential nutrient impacts.

Few studies, if any, have investigated depositional mercury in Appalachian Mountain streams, likely due to the high stream gradients, low water temperatures, and small sediment beds. Although methyl-mercury concentrations in fish were below risk thresholds for humans (0.3 mg/L ww; USEPA 2001), concentrations were not negligible and suggest that deposited mercury is, to some extent, becoming bioavailable. In the present study, total/methyl mercury ratios ($75\% \pm 2\%$) were lower than reported in the literature, which often report a ratio near $\sim 95\%$ (USEPA 2001; Huckabee, Feldman, and Talmi 1974). Huckabee, Feldman, and Talmi (1974) reported average total/methyl mercury fractions for fish in GRSM at $93\% \pm 2.6$, which could reflect higher historic rates of mercury deposition and shows that methylation rates are liable to change. Given that the amount of depositional mercury in this area is not minor and that relatively low concentrations are found in fish, this may imply that the limiting factor in mercury uptake in fish could be due to mercury bioavailability, which is unsurprising given that high methylation rates are normally associated with warm, anoxic, slow-moving waters. If methylation is indeed the limiting step in these systems, climactic changes (e.g. increased water temperatures, reduced flow, less dissolved oxygen) could lead to increased rates of methylation and greater impacts on stream health.

Bioaccumulation Factors (BAF) for methyl-mercury were not calculated in the present study due to methyl-mercury concentrations in the water frequently being below the detection limit. If calculated using detection limits, the resulting BAF's would likely underestimate the extent to which mercury is accumulating in fish across sites. Elucidation

of mercury dynamics at these sites will likely require more sensitive analytical techniques or investigation into food-chain dynamics and the establishment of biomagnification factors (BMFs), which is being investigated in other TEARS projects.

Although the BLM is often used for waters with anthropogenically-added metals, this study took a BLM approach to determine the bioavailability of naturally-occurring metals across sites. Criteria calculated for zinc and nickel are regularly above average measured concentrations and, therefore, are not of current concern. Copper and cadmium criteria were, in several instances, above detection limits for total copper and cadmium. Because these metals were not detectable, it is unclear whether exceedances occurred, suggesting more sensitive analytical techniques must be used in future investigations.

It was hypothesized that there would be relatively little to “buffer” aquatic life from metal toxicity in low-order ecoregion 66 streams, because parameters like DOC and hardness were relatively low across all sites (see supplemental table 2); however, the metals for which criteria were produced (Ni, Zn, Cd, Cu) were naturally in such low concentrations that their bioavailability was irrelevant. As it is unlikely for these metals to greatly increase without anthropogenic supplementation, the sites from this study were not deemed susceptible to copper, zinc, and nickel. Conversely, aluminum concentrations were measured at levels shown to cause toxicity to brook trout during acidic rain events. For example, Baldigo and Murdoch (1997) witnessed trout mortality at concentrations of available aluminum as low as 15 $\mu\text{g/L}$ and calculated a toxicity threshold for trout of 200 $\mu\text{g/L}$ over a 2-hour exposure. While in the present study aluminum concentrations were not measured during storm events, Neff *et al.* (2008) showed drastic drops in pH during storm events near Rock Creek and implicated aluminum in fish die-offs. Consequently,

aluminum is considered a concern for aquatic health in the streams of the present study and requires further investigation, especially considering the potential effect of climate change on the frequency and intensity of rain events. Unfortunately, no models are currently available to estimate the bioavailability of aluminum to trout during these storm events.

BLM parameters showed little inter- and intra-site variation throughout the present study, which may provide useful information for future BLM calculation in this ecoregion. Dissolved organic carbon was the entirety of total organic carbon measured in almost all samples, suggesting that in cases of limited data availability these parameters could be used interchangeably during base flow conditions in low-order, ecoregion 66 streams without greatly affecting criteria calculation. Hardness cations showed the greatest amount of variation, though this is likely due to the small sample sizes collected. While the EPA has released conservative 10th percentile values for ecoregion 66 for use when parameters cannot be measured, the averages presented in supplemental table 2 may provide more representative values for low-order streams in this ecoregion. When these values were compared, EPA values were always more conservative, as expected, but sometimes up to three times more conservative depending on the metal and model being used. Although these comparisons represent an extreme case, as using all EPA-estimated criteria would likely be unrealistic and unrepresentative of any actual stream, the similarity of criteria produced supports use of the EPA's suggested values in ecoregion 66 when needed.

The abundance of persistent and far-travelling contaminants in the environment make investigations into areas with no historic toxicological data both logistically difficult and costly. This work is the first to establish baseline concentrations of PCBs, OC pesticides, dioxins, furans, and phthalates in this area. The present study has identified

mercury and aluminum as potential concerns for future stream health in the ecoregion. It is our hope that the methodologies developed in this study will help lay groundwork for future investigations.

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APPENDICES

APPENDIX A – TABLES

Table 1 - Mean \pm SE values for water quality parameters across all sites. All parameters are reported in mg/L, with the exception of pH and conductivity (μ S/cm). Analytes are listed as less than the detection limit if no samples were detectable. pH (n=11 at Rock, LPHC, and Gentry; n = 10 at Bald) Nitrogen (n=11 at Rock, LPHC and Gentry; n = 10 at Bald), Hardness (n=2/site) ,TOC (n=11 at Rock, LPHC, and Gentry; n=10 at Bald), DOC (n=4 at Rock, LPHC, and Gentry; n=3 at Bald) TSS (n=11 at Bald and LPHC; n=12 at Rock and Gentry), Conductivity (n=12 at Rock, LPHC, and Gentry; 11 at Bald).

	Bald	Rock	LPHC	Gentry
pH	6.71 \pm 0.10	6.42 \pm 0.08	7.02 \pm 0.08	6.51 \pm 0.07
Nitrogen	0.05 \pm 0.01	0.50 \pm 0.02	0.40 \pm 0.03	0.19 \pm 0.06
Hardness	2.62 \pm 0.05	3.40 \pm 0.07	6.12 \pm 2.45	5.80 \pm 2.30
TOC	0.74 \pm 0.14	0.97 \pm 0.10	0.68 \pm 0.11	0.87 \pm 0.15
DOC	1.0 \pm 0.1	1.6 \pm 0.2	1.14 \pm 0.08	2.2 \pm 0.4
TSS	3.23 \pm 0.87	1.49 \pm 0.20	3.29 \pm 0.81	1.53 \pm 0.16
Conductivity	7.97 \pm 0.57	9.52 \pm 0.62	16.13 \pm 1.50	9.98 \pm 0.61

Alkalinity was never detected above 10 mg/L (n=5/site)

Total phosphorous was never detectable above 0.0095 mg/L (n=6/site)

Ammonia was never detectable above 0.03 mg/L (n=6/site)

Table 2 - Mean \pm SE values for metal concentrations in *Salvelinus fontinalis* across all sites (2016). Analytes are listed as less than the detection limit if no samples were detectable. All parameters are reported in mg/kg ww (n = 5/site).

	Bald	Rock	LPHC	Gentry
Aluminum	3.1 \pm 1.2	2.1 \pm 0.1	3.3 \pm 0.8	1.9 \pm 0.1
Arsenic	0.09 \pm 0.01	0.09 \pm 0.00	0.11 \pm 0.01	0.11 \pm 0.00
Cadmium	0.059 \pm 0.007	0.09 \pm 0.01	0.015 \pm 0.002*	0.12 \pm 0.02
Chromium	0.16 \pm 0.01	0.15 \pm 0.01	0.17 \pm 0.01	0.15 \pm 0.01
Copper	0.71 \pm 0.05	1.7 \pm 0.2	0.78 \pm 0.05	1.0 \pm 0.2
Lead	0.016 \pm 0.002*	0.018 \pm 0.002*	< 0.014	0.016 \pm 0.002*
Nickel	0.36 \pm 0.07	0.45 \pm 0.07	0.53 \pm 0.03	0.45 \pm 0.06
Mercury	0.047 \pm 0.002	0.043 \pm 0.001	0.063 \pm 0.010	0.056 \pm 0.006
Selenium	0.55 \pm 0.02	0.55 \pm 0.01	0.39 \pm 0.01	0.51 \pm 0.02
Silver	< 0.04	< 0.04	< 0.04	< 0.04
Zinc	23 \pm 2	30 \pm 2	23 \pm 1	20 \pm 1

* One or more samples were undetectable. Means were calculated using detection limit values for these data.

Supplemental Table 2 - Mean \pm SE, minimum and maximum values for all BLM input parameters from level III ecoregion 66. Alkalinity was consistently below the detection limit (10 mg/L) and therefore includes no minimum or maximum. Temperature is listed in C^o and all other parameters, excluding pH, are in mg/L. N = sample size.

	Mean	Min	Max	N	10th Percentile 66 Values
Total Organic Carbon	1.0 \pm 0.0	0.5	1.9	40	0.5
Calcium	2.9 \pm 0.6	1.5	5.9	8	0.5
Magnesium	1.6 \pm 0.2	1.0	2.6	8	0.3
Sodium	0.8 \pm 0.1	0.4	1.3	8	0.7
Potassium	0.42 \pm 0.02	0.32	0.55	8	0.3
Sulfate	2.7 \pm 0.1	1.9	3.4	40	1.1
Chloride	1.3 \pm 0.1	1.1	1.3	28	0.7
Temperature	11.1 \pm 0.8	0.8	19.6	40	---
Alkalinity	<10	---	---	28	3
pH	6.72 \pm 0.06	6.14	7.58	36	---

Supplemental Table 3 - Detection limits of tested phthalate esters. All analogs are in μ g/Kg. LPHC = Left Prong Hampton Creek.

	Rock 1	Rock 2	LPHC 1	LPHC 2	Gentry 1	Gentry 2
Dimethylphthalate	< 155	< 156	< 147	< 149	< 136	< 152
Diethylphthalate	< 203	< 204	< 193	< 196	< 178	< 200
Di-n-butylphthalate	< 189	< 190	< 180	< 182	< 166	< 186
Butylbenzylphthalate	< 402	< 403	< 382	< 387	< 352	< 394
bis(2-Ethylhexyl)phthalate	< 521	< 523	< 495	< 502	< 456	< 512
Di-n-octylphthalate	< 823	< 826	< 783	< 793	< 721	< 808

Supplemental Table 5 - Concentrations of Dioxins/Furans in two-fish homogenates by site. All analogs are listed in ng/Kg. If concentrations were undetectable, samples are listed below as less than the minimum detection limit. LPHC = Left Prong Hampton Creek.

	Rock 1	Rock 2	LPHC 1	LPHC 2	Gentry 1	Gentry 2
2,3,7,8 - TCDF	< 0.12	<0.17	< 0.20	< 0.13	< 0.24	< 0.15
Total TCDF	< 0.12	<0.17	< 0.20	< 0.13	< 0.24	< 0.15
2,3,7,8 - TCDD	< 0.082	< 0.076	< 0.099	< 0.095	< 0.061	< 0.11
Total TCDD	< 0.082	< 0.076	0.12	0.12	< 0.061	< 0.11
1,2,3,7,8 - PeCDF	< 0.049	< 0.12	< 0.094	< 0.10	< 0.12	< 0.21
2,3,4,7,8 - PeCDF	< 0.026	< 0.11	< 0.039	< 0.062	< 0.080	< 0.14
Total PeCDF	< 0.038	< 0.11	< 0.067	< 0.083	0.31	< 0.18
1,2,3,7,8 - PeCDD	< 0.13	< 0.063	< 0.077	< 0.11	< 0.071	< 0.084
Total PeCDD	< 0.13	< 0.063	< 0.077	< 0.11	< 0.071	< 0.084
1,2,3,4,7,8 - HxCDF	< 0.095	< 0.12	< 0.12	< 0.10	< 0.094	< 0.23
1,2,3,6,7,8 - HxCDF	< 0.066	< 0.066	< 0.065	< 0.097	< 0.084	< 0.11
2,3,4,6,7,8 - HxCDF	< 0.052	< 0.068	< 0.14	< 0.096	< 0.073	< 0.11
1,2,3,7,8,9 - HxCDF	< 0.10	< 0.13	0.21	< 0.14	< 0.12	< 0.25
Total HxCDF	< 0.079	< 0.096	< 0.11	< 0.11	< 0.094	< 0.17
1,2,3,4,7 - HxCDD	< 0.054	< 0.051	< 0.078	< 0.048	< 0.052	< 0.077
1,2,3,6,7,8 - HxCDD	< 0.065	< 0.062	< 0.089	< 0.054	< 0.062	< 0.070
1,2,3,7,8,9 - HxCDD	< 0.061	< 0.069	< 0.12	< 0.065	< 0.080	< 0.068
Total HxCDD	< 0.060	< 0.061	< 0.096	< 0.056	< 0.065	< 0.072
1,2,3,4,6,7,8 - HpCDF	< 0.092	< 0.072	< 0.13	< 0.085	< 0.13	< 0.11
1,2,3,4,7,8,9 - HpCDF	< 0.21	< 0.14	< 0.27	< 0.12	< 0.20	< 0.16
Total HpCDF	< 0.15	< 0.11	< 0.20	< 0.10	< 0.17	< 0.13
1,2,3,6,7,8 - HpCDD	0.17	< 0.086	< 0.10	0.12	< 0.098	< 0.10
Total HpCDD	0.17	< 0.086	< 0.10	0.25	0.14	0.12
OCDF	< 0.11	< 0.10	< 0.13	< 0.091	< 0.13	< 0.13
OCDD	0.28	< 0.16	< 0.23	0.38	< 0.19	< 0.25

APPENDIX C – FIGURES

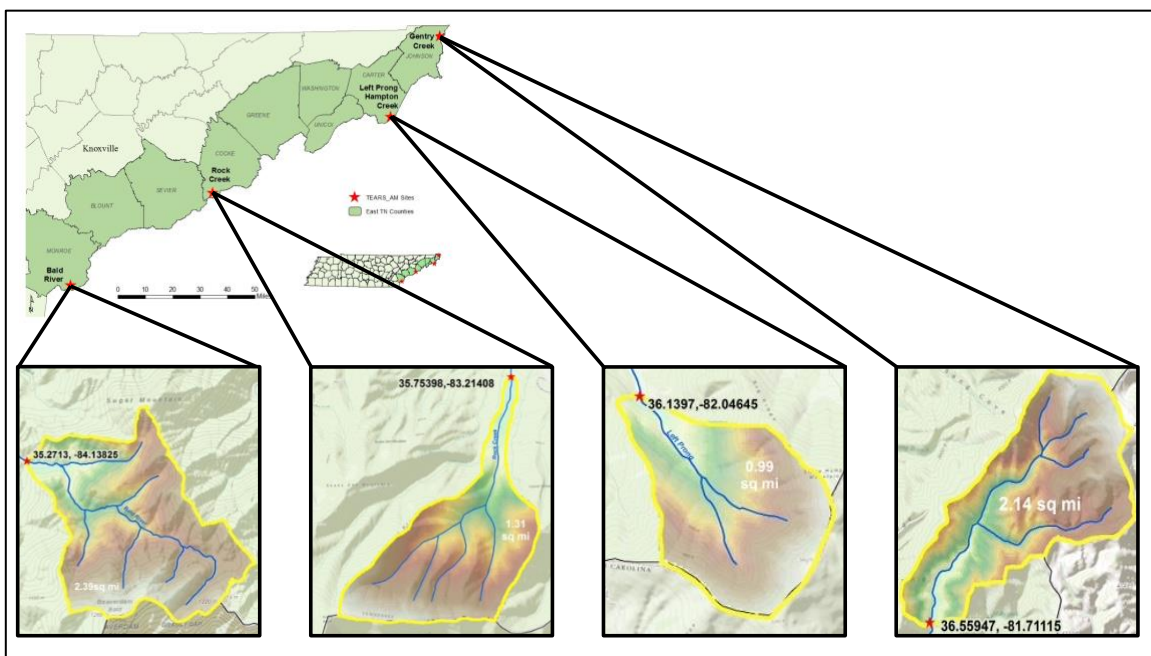


Fig. 1- Map of TEARS sites. From south to north: Bald River, Rock Creek, Left Prong Hampton Creek (LPHC), and Gentry Creek. Rock Creek is located within Great Smoky Mountain National Park. Bald, LPHC and Gentry are located with Cherokee National Forest.

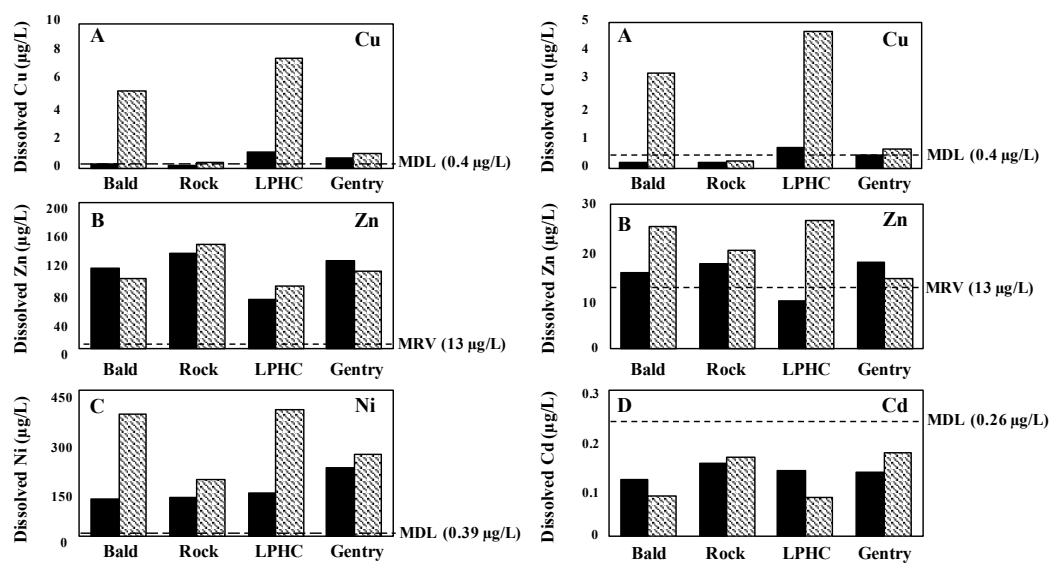


Fig. 2 - Predicted acute (left) and chronic (right) criteria: (A) copper, (B) zinc, (C) nickel and (D) cadmium. All data is from June (black) and August (grey).

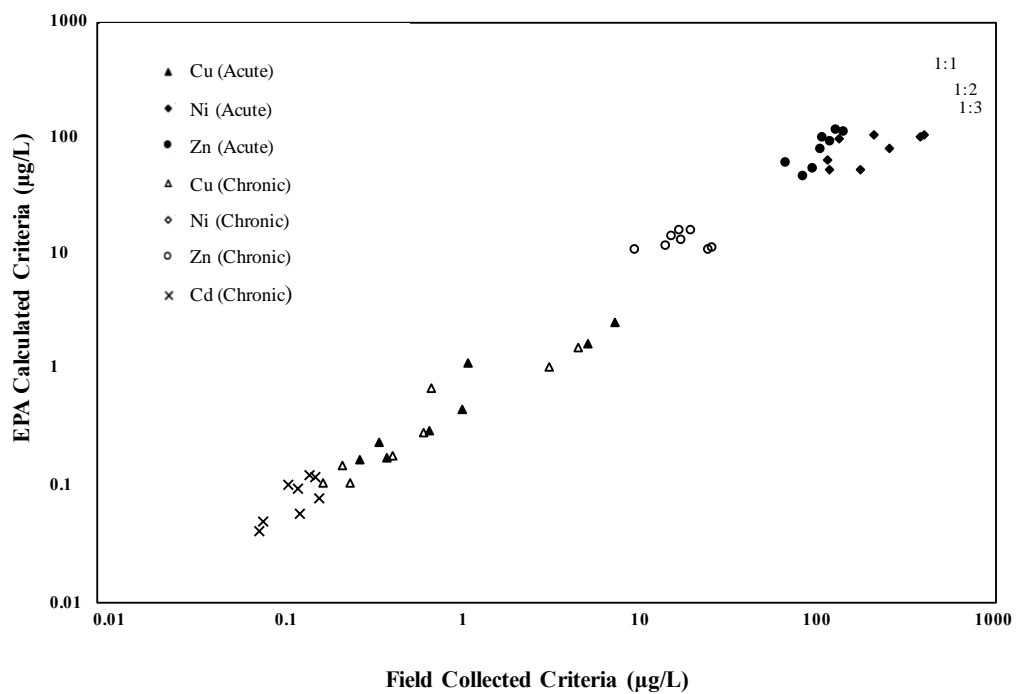


Fig. 3 - Comparison of field-collected versus EPA-calculated water quality criteria for copper, cadmium, nickel, and zinc. Dashed lines represent a 1:1 relationship between criteria, 2:1 represents a two-fold increase in criteria, and 3:1 represents a three-fold increase in field-collected versus EPA-calculated criteria. (N = 8)/ metal.

APPENDIX D – IACUC APPROVAL

IACUC

INSTITUTIONAL ANIMAL CARE and USE COMMITTEE

Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129



PROTOCOL APPROVAL NOTICE

Monday, September 14, 2015

Investigator Name(s): Ryan Otter
Investigator Email(s): ryan.otter@mtsu.edu
Department/Unit: Biology

Protocol ID: **15-013**
Protocol Title: ***Tennessee ecologically at-risk streams (TEARS)***

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above under the ***Full Member Review mechanism*** and has approved your protocol in accordance with PHS policy. This approval is effective for three (3) years from the date of this notice. Your study **expires 9/14/2018**. Investigator(s) MUST file a Progress Report annually regarding the status of the study and submit an end-of-project report.

MTSU Policy defines an investigator as someone who has contact with animals for research or teaching purposes. Anyone meeting this definition needs to be listed on your protocol and needs to complete IACUC training through the CITI program. Addition of investigators requires submission of an Addendum Approval to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918.

Also, all research materials must be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. **Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections** at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University
Tel: 615 494 8918
Email: compliance@mtsu.edu