EVALUATION OF TRACE METAL RUNOFF FROM CHICKEN LITTER APPLICATION IN THE BUGABOO WATERSHED

A Thesis
By
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ABSTRACT

EVAULATION OF TRACE METAL RUNOFF FROM CHICKEN LITTER APPLICATION IN THE BUGABOO WATERSHED
(December 2009)

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Trace elements present in chicken litter are both naturally occurring and the result of anti-coccidial treatments and growth supplements. Specifically, arsenic, copper, manganese, and zinc are added to the feed. The impacts of elevated concentrations of toxic trace elements present in poultry litter (PL) have been a recent environmental concern, especially for plant and aquatic toxicity. If trace elements from poultry litter application are leaching into rivers and streams, aquatic organisms (particularly fish and aquatic invertebrate species) are at risk from elevated trace element exposure. The following three hypotheses were developed based on this notion: H_1- PL application increases level of metals in soil, water, and sediment; H_2- Fish in streams adjacent to PL application sites will have higher trace element concentrations than those in reference streams; H_3- Fish will express elevated levels of metallothionein in sites with elevated trace elements. Soil, sediment, water, and fish tissues were analyzed for As, Cu, Mn, and Zn using ICP-OES. Elevated arsenic concentrations were not found in soil, sediment, water, or fish. Copper and zinc concentrations were found at levels known to induce phytotoxicity; soil values ranged from 8.6 to 56 mg/kg and from 103 to 172 mg/kg,

respectively. Copper and zinc concentrations in streams were found at levels of concern to aquatic organisms with Cu concentrations of 4 to 20 ug/L and Zn concentrations of 20 and 100 ug/L. The results from this study indicate that the current practices of supplementing feed with trace elements and applying this poultry litter on agricultural fields is not a sustainable practice.

DEDICATION

To all of the women scientists who have laid the path before me and to those who will continue to succeed after me, thank you.

"The "control of nature" is a phrase conceived in arrogance, born of the Neanderthal age of biology and philosophy, when it was supposed that nature exists for the convenience of man"

-Rachel Carson, *Silent Spring*

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INTRODUCTION

Annual chicken consumption in the United States has risen from 23.1 to 81.2 pounds per person in the time between 1966 and 2000 [1]. To meet the increasing demand, U.S. broiler meat production is expected to rise to 36.1 billion pounds by 2010 [2], with the majority of this chicken being produced within the southeastern region of the U.S. The southeastern states within the U.S. Environmental Protection Agency's Region 4 (Alabama, Florida, Georgia, Kentucky, Mississippi, and North Carolina) are the largest producers of poultry in the US with 56% of the U.S. broiler market, which equates to 4.9 billion birds per year and over $10 billion in annual sales [3]. Among the costs of doing business is the management of 5 million tons per year of poultry litter [3]. Looking at North Carolina in particular, roughly 749 million broiler chickens were produced in 2006; nearly 10% of the total produced in the U.S. [2]. Much of the broiler production is confined to the rural northwestern corner of the state where Wilkes County alone produced over 91.7 million broiler chickens in 2006, as well as an estimated 250,000 tons of chicken litter.

Chicken litter is defined as a biomass waste consisting of a mixture of wood shavings/other bedding materials, chicken urine and feces, feathers, and dead birds. The current method of spreading the litter on fields as a cheap source of fertilizer (because it consists of up to 4% total nitrogen) as well as a convenient means of disposal has several well-documented environmental impacts including nitrogen and phosphorus related water quality issues [4]. The impacts of elevated levels of toxic trace elements present in poultry litter have been a recent environmental concern, especially regarding plant and aquatic toxicity. If trace elements from poultry litter application are leaching into rivers and streams,

aquatic organisms (particularly fish and aquatic invertebrate species) are at risk from elevated metal exposure.

Trace elements present in chicken litter are both naturally occurring and the result of anti-coccidial treatments and growth supplements [5]. Roxarsone (4-hydroxy-3-nitrophenyl arsonic acid), an organoarsenical compound, is added to control/treat coccidial intestinal parasites which indirectly increases weight gain and promotes growth, thus ultimately increasing yield and profits. As much as 25-50 mg of roxarsone per kg of feed has been traditionally added [6]. Copper (Cu) and Zinc (Zn) are also added to poultry feed to act as fungicides and nutrient supplements [7]. Cu and Iron (Fe), furthermore, help to prevent anemia and Zn and Manganese (Mn) are added to guarantee sufficient eggshell deposition and feather growth [8]. Although aluminum is not a supplement given to the birds, it is present in the litter. Aluminum sulfate (formula) is added to poultry houses to reduce the amount of ammonia present within the air of the buildings and has been shown to reduce the amount of soluble phosphorus and metal content of litter [9]. Soluble phosphorus can be reduced by 67%, arsenic (As) by 63%, Cu by 37%, and Zn by 48% when aluminum sulfate is added to poultry litter [10].

Since a number of trace elements are added to either feed or litter (Al, As, Cu, Fe, Mn) and subsequently accumulated in litter, it is important to understand the interactions amongst the metals. Many of the trace elements are added in excess of requirements, which can contribute to the bioavailability and antagonism between metals and other nutrients [11]. When litter is applied to soils these interactions can be of importance to the mobility and bioavailability of the metals to plants and animals.

The question of the fate of arsenic from poultry litter application is one that has been studied by many researchers in the past. Arsenic (e.g. roxarsone) present in feed is not easily absorbed by the gut and therefore is mostly excreted [7], thus most of it is found in poultry litter. Roxarsone changes to inorganic As – a more toxic form- in composted litter [12]. One study found that regardless of the degradation process (composting, microbial, or addition of water) roxarsone was primarily converted to arsenate As(V) [7]. Concentrations of As have been found in the range of 1 to 39 mg/kg dry weight in poultry litter [13]. With concentrations of this magnitude, continuous applications could introduce a significant amount of As onto agricultural soils. Garbarino et al. [7] estimated that from 60 to 250g per hectare of arsenic could be introduced with each application. Several studies have shown that the As found in poultry litter is highly soluble [7,13,14]. The mobility of As by water has been shown to occur readily from poultry litter in laboratory studies, however, the leach rate from amended soils in the environment seems to be slow [14]. Arani et al. [15] found no evidence of arsenic accumulation in agricultural soils, and suggested that the soluble As in litter is either leaching into surface water or is being up taken by crops– though noted that no significant accumulation of As in crops had been found. Although unclear, from these past studies it seems quite possible that As is readily leached from the litter and could end up in adjacent streams where poultry litter application is occurring.

Understanding the chemistry and occurrence of As is important in assessing its risk of toxicity to various organisms. Arsenic can be found in soils both from natural and anthropogenic sources (Figure 1). The average naturally occurring concentration of As in soils is usually from 5 to 6 ppm; however, ranges from 0.2 to 40 ppm have been found [16]; concentrations vary depending on the geological history of the region. Inorganic As can be

found in four oxidation states (-3, 0, +3, and +5). Arsenite (As^{+3}) and arsenate (As^{+5}) are the most common forms found in soils. Arsenate is typically found in oxic soil conditions where the activity of the electrons (Eh) is >200mV and pH ranges from 5 to 8 and arsenite is predominately found under reducing conditions [17]. Speciation of As can occur in soils and many factors influence this, including other elements such as free Fe oxide, MgO, Al_2O_3, clay content [16], pH, redox potential, organic matter, and fungal and bacterial presence [18].

Microbes present in the soil can encourage oxidation or reduction and demethylation and methylation of arsenic. Monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), and trimethylarsine oxide can form from biomethylazation of inorganic As by microbial species [18]. Microbes can also demethylate organic forms of As to inorganic As [19]. If As is methylated, then it can be transformed by microorganisms into volatile forms of arsenic [20], which can be lost from soils to the atmosphere. The toxicity of As to organisms is highly dependent on its oxidation state. According to the World Health Organization, inorganic As is more toxic than organic As, and between arsenite and arsenate (the two most commonly found forms of As), arsenite is 60 times more toxic than arsenate which is 70 times more toxic than methylated forms (MMAA and DMAA) [21]. Due to its highly toxic nature, the US EPA limits safe drinking water levels of arsenic to less than 10 ppb and soil levels to about 25 ppm. Arsenic can also be highly toxic to aquatic organisms. North Carolina's current freshwater standards for aquatic life for As is 50 ppb [22].

Many factors affect the toxicity of arsenic to aquatic organisms, for example at higher temperatures fish become more tolerant of arsenic [23]. Arsenite and arsenate are also the predominate forms of arsenic found in aquatic environments [24]. Arsenite binds to protein sulfhydryl groups [25] and arsenate interferes with phosphorylation reactions [26]. Fish have

the ability to biotransform arsenic using either of the oxidation states into organic arsenic compounds which vary in toxicity; including methylated species, arseno-sugars, and arsenolipids [27].

Copper is a naturally abundant metal and is found in soil, water, sediments, and organisms throughout the world. Weathering of rock is the primary source of naturally occurring Cu in the environment. Cu is an essential micronutrient to many organisms and Cu is a necessary element to plants and helps in photosynthesis, respiration, perception of ethylene, reactive oxygen metabolism and cell wall remodeling. Cu in the environment can be in three phases: aqueous, solid, and biological [28]. Background levels of naturally occurring Cu in freshwater have been reported to be from 0.20 to 30 ug/L [29]. However, at elevated levels Cu can be toxic to many aquatic organisms ranging from plants to animals. North Carolina's current freshwater standard for aquatic life for Cu is 7 ug/L (at 25 mg/L hardness) [22].

The bioavailability of Cu depends on several factors, including: pH, redox potential, soil and sediment type, water hardness, organic content, and the species that is being exposed [28]. Increased water harness reduces the toxicity of Cu to aquatic species [30]. The toxicity of Cu is also affected by the presence of other metals, particularly Mn and Fe which can increase the toxicity of Cu to aquatic species [31]. According to the EPA [32], fresh water plant growth can be inhibited from Cu concentrations from 1 to 8,000 ug/L. According to Handy [33], similarly reduced growth in both the freshwater aquatic midge and rainbow trout occurred (63%) when water Cu concentrations were 600 ug/L.

Fish are particularly sensitive to high levels of Cu in the water and there are many ways in which Cu can affect fish. Copper shows affinity for the liver, however, it can also

accumulate in the gill, kidney and sometimes in the muscle [34]. High levels of copper acquired through dietary uptake can cause the production of free radicals in tissues where it accumulates. Dietary copper uptake can also inhibit digestive enzymes and reduce gut motility [35]. When copper is taken up by the gills in high concentrations, branchial function is affected through perturbation of sodium homeostasis [36] and can lead to osmoregulation failure [37]. Copper exposure has also led to increased glycolytic activity in fish [38], altered cellularity including cell type and turnover in the gut and gill epithelium [34] and has been shown to have genotoxic effects on fish [39].

Copper concentrations found in poultry litter have been directly correlated to Cu content in chicken feed, with concentrations being 5 to 6 times higher in litter where birds received Cu supplements [4]. Cu has also been found in high concentrations in surface soils amended with poultry litter [40]. Although Cu is one of the least mobile metals in soils, runoff from poultry litter has been found with Cu concentrations up to 450 ug/L that are potentially toxic to aquatic life [41].

Zinc is an essential trace element for all living organisms. It is a naturally occurring element and ranges in soils can be anywhere from 30 to 150 mg/kg [42]. Under acidic conditions Zn can be quite mobile [42]. The bioavailability of Zn is influenced by the total Zn content, pH, organic matter, microbial activity, moisture, and interactions with other nutrients [43]. In excess amounts Zn can be toxic to plants and animals, especially aquatic organisms. North Carolina's current fresh water standard for aquatic life for Zn is 50 ug/L [22]. Zinc can have acute toxic effects on freshwater fish at water levels as low as 15 mg/L [44]. Levels of Zn can be especially high compared to other metal levels with background levels of Zn reported in fish up to 300 mg/kg d.w. [45]. Zinc normally concentrates in the

gonads but is also usually found in gills and sometimes it may be found in the liver and kidney [45]. Zinc does not form free radicals in fish because it is itself beneficial as an antioxidant, however high levels of zinc do interfere with calcium homeostasis, causing toxicity to fish [46].

Metals can have numerous affects on fish species and fish are commonly studied for metal concentrations because of their high position in aquatic food chains and humans and other predators readily consume them. Metals and other contaminants that are found in fish have been shown to accumulate in their predators, including humans. Metals can be taken up by fish either through the digestive tract (from their diet) or through the gill surface (from water). The metals are then transferred through the blood to other organs, particularly liver and kidney organs [47]. Metal accumulation in fish is dependent upon many factors, including environmental factors such as water pH, temperature, and salinity, as well as fish physiology, morphology, and the duration of exposure and fish age [47]. Metals can affect the fish through synergistic, antagonistic, or through additive effects [48]. Fatality is one effect of heavy metals on fish; however other studies have discovered that low levels of metals may reduce the fecundity of fish populations either by directly affecting the reproductive organs or the free gametes that are released into the water [49]. An increase in the levels of antioxidant enzymes (catalase, glutathione S-transferase, superoxide dismutase, glutathione) in liver, kidney, and gills [50] has been reported to be a biomarker of metal and oxidative damage to cell membranes. Metals can also act as endocrine disrupters, either through the disturbance of the thyroid gland [51], or inhibit estrogen receptors [52] or by generally inhibiting protein production and hormone synthesis and release.

When fish are exposed to high levels of metals, they regulate these metals through the induction of metallothionein (MT). Metallothioneins are proteins that are capable of binding metals, and thereby bind the metals during an excess of metal pollution, and reduce the amount of metal toxicity at undesirable cellular sites [53]. While the primary role of MT is unknown, it is known that it plays an important role in the homeostasis of essential metals, including copper and zinc and the seizure of nonessential metals, including cadmium, mercury [54] and arsenic [55]. Induction of MT in fish varies amongst different species and tissues [56] and varies in different environments due to the numerous variables affecting metal uptake.

The purpose of this study was to assess the levels of trace metals in riparian zone soils, sediments, water, and fish in streams near fields receiving poultry litter (PL) application and compare the levels to reference streams where no poultry litter application has occurred. My null hypothesis is: PL application does not result in increased trace element levels in soil, water, sediment, or fish. My two hypotheses are: H_1- PL application increases the level of trace elements in soil, water, and sediment; H_2- Fish in streams adjacent to PL application sites will have higher levels of trace elements than those in reference streams; H_3- Fish will express elevated levels of MT in sites with elevated trace element content.

MATERIALS AND METHODS

Collection Sites

Ten sites were chosen along the Big and Little Bugaboo Creeks in east central Wilkes County, North Carolina (Figure 2) in cooperation with the Wilkes County Soil and Water Conservation District. Poultry litter has been applied to the fields adjacent to these sites from 10 to almost 30 years, receiving between 3 and 6 tons/acre/year [57]. The fields typically contain either corn or fescue crops. Two nearby sites to the northwest were chosen as reference points, one on the East Fork Roaring River just outside of Stone Mountain State Park and another on Basin Creek in Doughton Park (a segment of the Blue Ridge Parkway). Collections were taken in spring (March), summer (June), and fall (September) of 2008.

Manure Samples

Chicken litter samples (traditional (3 week) composted and forced air (3 week) composted) were collected from a farm near the study sites and were sent to the North Carolina Department of Agriculture (Raleigh, NC) for analysis. Chicken litter samples were tested for As, Al, Cu, Mn, Mo, Ni, Pb, and Zn and a reference standard of cow manure was analyzed for comparison.

Soil and Sediment Samples

Soil samples were taken (0 to 20.32 cm in depth) from both sides of the streams 10 and 20 meters from the streambank using a soil corer. Fine sediments were collected from the stream sites and placed in Whirlpack® bags. All samples were kept on ice until they were transportedt to the ecophysiology and aquatic toxicology lab at Appalachian State University. Sediments and soils were frozen at -20°C and freeze-dried using a LabConco lyophilizer (Model number 117, Kansas City, MO). A sample mass of 0.5g was weighed and 10 mL of

70% nitric acid (Omni Trace EDC) was added to 60 mL MARSXpress Teflon vessels. Samples were digested using a MARS 5 System (CEM Matthews, NC) following EPA method 3051. Samples were then cooled and 10 mL of deionized water was added. Samples were filtered through 9 cm qualitative filter paper using a glass funnel into a 50 mL volumetric flask. Deionized water was used to bring the sample up volume and were then transferred to 50 mL centrifuge tubes for trace element analysis using ICP-OES (see section below).

Water Samples

Stream pH, dissolved oxygen, temperature, and conductivity were measured using an Orion 5-Star Portable multi meter (Thermo Scientific Inc., Waltham, MA) at all sites. Water samples were collected by dipping acid washed plastic containers (250 mL) below the surface of the water at each site; samples were then preserved by adding 2.5 mL (1%v/v) of 70% Omni Trace Nano pure nitric acid (EDC). Water samples were kept cool in the refrigerator at 4°C until digested using a MARS 5 System (CEM Matthews, NC) following EPA protocol 3015 for total recoverable metals. Samples were cooled and filtered through 9 cm qualitative filter paper using a glass funnel and transferred to 50 mL centrifuge tubes for trace element analysis using ICP-OES (see section below). Following ICP-OES analysis water hardness was calculated using the following formula: $CaCO_3 = 2.5\,[Ca] + 4.1\,[Mg]$.

Fish Samples

Fish [blue head chub (*Nocomis leptocephalus*), creek chubs (*Semolitus atromaculatus*), and white suckers (*Catostomus commersonii*)] were collected through backpack electroshocking for determination of trace element body burdens from sites with habitat suitable for sustaining fish populations. Fish were tagged and maintained with an

aerator until they were anesthetized in the lab using MS 222. Blood (ranging from 50 to 200 uL, depending on the species and fish size) was drawn by cardiac puncture using a syringe filled with 200 uL phosphate buffered saline (PBS) (all chemicals purchased from Sigma Aldrich) with anticoagulant. The anticoagulant solution was made by adding 0.4 g Glycine Ethyl Ester (GEE) and 0.006 g of Ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA) to 20 mL of PBS and then extracting 10 mL of this mixture and adding 1 protease inhibitor tablet (Complete mini (Roche)). The blood was then centrifuged at 2000 x g for 10 minutes and the supernatant was stored at -80°C for metallothionein analysis. Fish species, wet weight, total length and sex were recorded. Gills, liver, muscle, and gonad samples were dissected and placed in polypropylene cryovials and frozen at -80°C until further analysis. Fish tissues to be analyzed for metal content were lyophilized and then 0.5- 1.0g were digested using EPA protocol 3050b. Samples were then cooled and 10 mL of deionized water was added. Samples were filtered through 9-cm qualitative filter paper using a glass funnel into a 50 mL volumetric flask. Deionized water was used to bring the sample up to volume and transferred to 50 mL centrifuge tubes for trace element analysis using ICP-OES (see section below).

Two Creek Chubs (*Semolitus atromaculatus*) and one White Sucker (*Catostomus commersonii*) were collected via backpack electroshocking from the South Fork New River in Watauga County, NC to be used as positive controls for metallothionein induction. Fish were brought back to the lab and placed in a 10 gallon tank with creek water and were allowed to acclimate for one week in the tank. Fish were then injected with a dose of 100ug/kg body weight $CdCl_2$. Four days later the fish were anesthetized in the lab using MS

222 and blood was drawn using the same procedure aforementioned. Plasma was stored at -80°C until further analysis.

Metallothionein analysis

Metallothionein (MT) protein analysis was done via western blotting [58]. Blood plasma from the positive controls and from the study sites were thawed on ice. The samples were loaded onto a precast 10% SDS-polyacrylamide gels (NuSep) and run at 40 mA for ~1.5 hr to separate plasma proteins. The gel proteins were then transferred by Western blotting overnight at 40 mA at 4°C onto PVDF membranes (VWR). The blot was then probed for MT using Rabbit anti-cod MT polyclonal antibody (KH-1, Cayman Chemical) as the primary antibody followed by the secondary antibody (goat anti-rabbit POD, IgG (H + L) Jackson ImmunoResearch). Color development was achieved by the addition of 4-Cl-1-Naphthol substrate and the reaction was terminated with the addition of several changes of diH_2O. Blots were then dried and digitally photographed for analysis.

ICP-OES

After digestion all samples were analyzed for As, Cu, Mn, and Zn using inductively coupled plasma optical emission spectroscopy (ICP-OES Varian 710 ES) with CCD detector. Water samples were also analyzed for Ca and Mg for calculation of water hardness. Stock solutions (1000 ppm) were used to prepare standards of As, Cu, Mn, and Zn ranging from 10ppb to 2ppm and were prepared in a matrix of 1% (v/v) HNO_3. A Cetac autosampler with 15-mL polypropylene sample tubes was connected to the ICP-OES by a peristaltic pump. A concentric glass nebulizer (Seaspray, Varian) and glass cyclonic spray chamber were used for sample introduction. Yttrium (2 mg/L) was used as an internal standard and was added to all standards. Plasma power was set at 1.00 kW, the argon flow was set at a rate of 15.0

L/min with an auxillary flow of 1.50 mL/min and the nebulizer pressure was set at 300 psi. Sample read time was 60 seconds with 1 replicate/sample.

Statistical Analysis

All data were analyzed using SAS® (SAS Institute). Reference sites 12 and 11 were combined into one site to increase statistical power. For soil, sediment, and water trace element analysis a one-way ANOVA was performed using Scheffe to test one site (reference) against others, and a Bonferoni test was also run to ensure a more conservative approach of analysis. The experimental design for fish tissue metal analysis was a two-way factorial, an ANOVA and mean separation was performed using the Duncan Multiple Range Test. Water, sediment, and soil transformed log data was normal. Trace element concentration data of fish tissues at each site were log transformed and most of the data was normal; confidence in normality was accepted for analysis of variance and was quite robust with respect to normality. Differences between data were considered significant if $p < 0.05$.

RESULTS

Manure Analysis

Aluminum, arsenic, copper, manganese, and zinc concentrations from the poultry litter samples were between 6 to 80 times higher than the cow manure samples (Table 1). Forced air compost decreased the amount of Al, but no other elements, compared to the traditional composting.

Soil Samples

Soil samples from the riparian zones of sites 1 to 10 had slightly to moderately acidic pH in a range from 4.7 to 6.3 (Table 2). Arsenic concentrations in soils ranged from 0.77 to 2.93 mg/kg, Cu concentrations varied from 8.6 to 56 mg/kg, Zn concentrations were from 103 to 172 mg/kg, and Mn concentrations values ranged from 70.7 to 299 mg/kg in soils (Figure 3). Sites 2, 3, 4, 5, and 6 were significantly higher in As content than the combined reference sites (0.77 mg/kg). Sites 5 and 8 were significantly higher in Cu than the combined reference sites (12.7 mg/kg). There were no significant differences in Zn soil concentrations between the reference and test sites. Site 5 was the only site that showed a significant difference in Mn concentrations from the reference sites (130 mg/kg).

Water

Stream pH ranged from 6.0 to7.3 (Table 3). The creek temperatures from September 2008 collections were from 15.8 to 16.6 °C and dissolved oxygen ranged from 5.9 to 9.3 mg/L. Water hardness ranged from 7.7 to 12.7 mg/L, indicating that all sites have very soft water. Water conductivity ranged from 21.47 to 221.3 uS/cm. Mean As water concentrations for total recoverable trace elements ranged from not detected to 4.9 ug/L, Cu values ranged from 4.5 to 24.7 ug/L, Mn ranged from 8 to 50 ug/L, and water Zn concentrations ranged

from 25 and 117 ug/L (Figure 4). Site 1 (4.9 ug/L) was the only site that was significantly higher in As than the reference sites (non detectable). None of the PL applied sites were significantly different in Cu or Mn concentrations from the reference sites. Sites 5 (117 ug/L), 9 (100 ug/L), and 10 (101 ug/L) were significantly higher in Zinc concentrations than the reference sites (25 ug/L).

Sediment

Sediment As concentrations from the Bugaboo Creeks had a range from 0.047 to 1.99 mg/kg, Cu concentrations varied from 4 to 11 mg/kg, Zn concentrations were found from 32 to 63 mg/kg, and Mn concentrations varied from 47 to 95 mg/kg (Figure 5). There was significantly more As in sediments in sites 4 (1.83 mg/kg) and 5 (1.99 mg/kg) than the reference areas (0.36 mg/kg). There were no significant differences between the PL applied sites for Cu and Mn concentrations in sediments. Site 1 (62.7 mg/kg) was the only site that was significantly higher from the reference sites (37.3 mg/kg) for Zn.

Fish

Sites 2,4,5,9 and the reference sites were the only sites where fish habitat was suitable and fish were collected. From site 2, *S. atromaculus, N. leptocephalus,* and *C. commersonii*, were collected, from site 4 *N. leptocephalus* was the only species collected, from site 5 *N. leptocephalus,* and *C. commersonii* were collected, from site 9 *N. leptocephalus* was the only species collected and from the reference sites all of the above species were found. Since there were no significant differences between the tissues, the concentrations from all of the tissues were averaged together for comparision.

Arsenic in tissues of *Nocomis leptocephalus* ranged from 0.11 to 0.20 mg/kg, Cu from 0.31 to 0.65 mg/kg, Mn from 0.3 to 0.5 mg/kg and Zn from 2.8 to 5.2 mg/kg (Figure 6).

There were no significant differences in arsenic between tissues. Sites 2, 4, 5, had significantly higher Cu and Zn concentrations compared to the reference sites. Sites 2 and 4 had significantly higher Mn concentrations in tissues than the reference sites.

In *Semolitus atromaculus* tissue concentrations of As were 0.40 mg/kg, Cu concentrations were 0.94 mg/kg, Mn was 0.34 mg/kg, and Zn was 5.1 mg/kg in the study sites (Figure 7). Site 2 fish tissues were significantly higher in As, Cu, Mn, and Zn compared to the reference sites.

In Catostomus commersonii arsenic tissues ranged from 0.21 to 0.28 mg/kg, Cu concentrations were from 0.37 to 0.51 mg/kg, Mn tissue concentrations were from 0.57 to 0.80 mg/kg, and Zn tissues were from 2.2 to 3.4 mg/kg (Figure 8). For this species copper was the only element that was significantly higher at sites 2 and 5 compared to the reference sites.

Metallothionein

Following electrophoretic separation of fish plasma samples, there was a positive transfer of the proteins from the gel to the blot as indicated by staining of the blot with Red Alert western blot stain (Novagen) (Figure 9). After completion of the primary and secondary antibody recognition procedures no bands were present not even in the positive control (Figure 10).

DISCUSSION

The data found in this study support the hypothesis that Cu, Mn, and Zn accumulate in PL amended soils. Trace element levels found in the soils would be higher in the fields where PL application is occurring compared to the sites where the soil was collected in this study; thus, phytotoxicity in the fields where agricultural crops are grown should be addressed. Concentrations of trace elements in water samples indicate that Cu and Zn are of a toxic concern for aquatic organisms in the Bugaboo Creek.

The manure samples from the Wilkes county area that were tested for toxic trace elements were consistent with what has been reported in the literature. Arsenic levels were lower in our samples than some reports of the past, up to 39 mg/kg [13]; the values in the Bugaboo litter samples were from 6 to 7 mg/kg. By comparing the trace element concentrations in cow manure to chicken litter, it was clear that the higher levels of Al, As, Cu, Mn, and Zn were due to the addition of these specific trace elements to poultry feed (Table 1). Testing of the feeds would strengthen this analysis- but was proven to be difficult due to previously arranged farm agreements with integrators.

Soil

Excess trace metal concentrations in soils can induce phytotoxicity in many plant species and can be toxic to microbes and invertebrates that live in soils. In this assessment only phytotoxicity will be addressed primarily because PL is applied to cropslands- thus phytotoxicity is of importance from an agricultural perspective. In this study riparian zone soils were tested and assuming farmers are following best management practices, there would be no spreading of PL up to the creek; thus, resulting in lower levels of trace elements in the riparian zones than in the fields.

Arsenic levels in the study sites 2 through 6 were significantly higher than the reference sites, however, when determining the risk of toxicity it is important to compare these levels with the normal ranges and those of known toxic thresholds and removal action levels specific to a particular area. All of the sites ranged from 0.7 to 2.9 mg/kg, which are all well below the national naturally occurring levels of arsenic (ranging from 5 to 6 mg/kg); thus, the levels of As in the soil are not of concern at this time.

Copper concentrations ranged from 8 to 56 mg/kg in the study sites. Even though sites 5 and 8 were higher than the reference they are unlikely to induce phytotoxicity because the concentrations are below NC's critical concentration for phytotoxicity (60mg/kg). However, the copper concentration at site 5 (56 mg/kg) is reaching North Carolina's critical concentration for phytotoxcity (60 mg/kg). The soil riparian zone samples that were taken in this study were 10 and 20 feet from the stream and it is to be expected that the levels of copper in the fields are probably higher than those found on the banks of the streams due to receiving significantly more PL application. Since PL is directly applied on the fields Cu induced phytotoxicity could occur if Cu concentrations are higher in the field than in adjacent stream banks. Previous work has found Cu concentrations in fields amended with PL vary from 211 to 840 mg/kg [59], thus it is possible that the Cu from PL could be phytotoxic to sensitive agricultural crops, such as peanuts (which are in fact no longer grown in Wilkes County).

Soil manganese concentrations in the study sites ranged from 70 to 300 mg/kg. Past research has found critical values for Mn toxicity ranging from 100 to 5,000 mg/kg [60]. Manganese phytotoxicity is species dependent [61]. Mn accumulates in the leaves and at high

levels can cause foliar damage such as chlorosis [61]. This would indicate that the levels found at the Bugaboo sites could induce Mn phytotoxicity in the most sensitive species.

The soil Zn concentrations found at the study sites (except sites 8 and 10) exceeded NC critical concentrations for potential phytotoxicity (120 mg/kg). Zn toxicity can decrease leaf chlorophyll content as well as decrease the rate of photosynthesis [62] – resulting in energy loss for the plants. From this study Zn concentrations are high enough to be a toxic threat to agricultural crops in the Wilkes county area.

Sediment

All of the sediment levels for arsenic, copper, and zinc were quite low. The threshold effect concentration (dry weight) for As is 5.9 mg/kg, Cu 35.7 mg/kg, and Zn 123 mg/kg [63]. The highest reported elemental levels in this study: arsenic concentrations were <2 mg/kg, copper <12 mg/kg, and zinc <6 mg/kg. Sediments with these concentrations do not pose a serious health risk to aquatic organisms and therefore are not of concern for aquatic toxicity.

Water

Arsenic concentrations in the water samples were relatively low at all sites, in fact, concentrations were on average 50 times lower (0 to 4.9 ug/L) than what is considered to be a threat to NC aquatic life in fresh water streams (50 ug/L) (Table 4). Typical background concentrations of arsenic in streams are approximately 1 ug/L [64] and all of the sites (except site 1) were at these levels. From these findings arsenic is not approaching the toxic threshold in streams receiving runoff from the PL amended fields. Since arsenic is fairly mobile in soils, if high levels were found in PL, it is expected that there should be high levels of arsenic in the water. This is especially true since PL contains relatively high levels of

inorganic phosphorus and this can compete for arsenic binding sites in soils [65] – thus making arsenic more available and mobile. The levels of arsenic in the PL samples and soil samples were relatively low, so it is not surprising that the water concentrations are also low. One possible explanation is that the local integrator (Tyson) at the farms in the study announced in 2004 that they would no longer use roxarsone in their feeds; this would provide support for the low levels of arsenic in the soil, sediment, and water samples. However, arsenic was still detectable in PL samples, but not at high levels.

Even though there were no significant differences between the study sites and the reference sites, there was a detectable trend for higher Cu concentrations in the study sites compared to the reference sites. Copper concentrations in the watershed exceeded action levels for NC (7 ug/L) in five of the sites (Table 4); indicating that these concentrations could cause toxicity to aquatic organisms. Researchers have found toxic affects on aquatic organisms at seemingly low levels. Hodson et al. [30] found altered growth, reproduction, and behavior in salmonids when exposed to 5 ug/L of Cu. According to Buhl and Hamilton [66], the LC 50 for rainbow trout during a 96-hr test was 13.8 ug/L. Dorgelo et al. [67] reported that inhibition of growth rates in snails occurred at a Cu concentration of 10ug/L and the inhibition of reproduction at 30 ug/L. The highest level of Cu in the Bugaboo drainage was 25ug/L indicating that Cu levels there are a risk to sensitive species of aquatic organisms.

All of the sites except for the references were above the NC Aquatic Life Standards for Zn (50 ug/L), with three (5,9,&10) sites being twice as high. The data found here for Cu and Zn supports the conclusions of Hann et al. [68] and Kingery et al. [69] who reported that long term PL application could potentially increase loadings of trace elements in soils and

leachates and these levels could be toxic to aquatic organisms. Another study found that survival was reduced by 42% after crayfish were exposed to 63.3 ug/L (water exposure) of zinc sulfate and at 130 ug/L survival was reduced by 61% [70]. The highest level found in the Bugaboo streams was 100 ug/L, indicating that these levels could be toxic to local crayfish and/or fish. Spehar [71] found that when the freshwater flagfish were exposed to 85 ug/L of zinc sulfate through the water, survival was reduced by 80%. Although other studies have found no effect of Zn on survival, growth, or reproduction at levels as high as 534 ug/L in brook trout [72].

Fish

Arsenic concentrations in the Bugaboo Creek (water and sediment samples) were not at elevated levels and therefore we did not expect to find high levels arsenic in the fish tissues, which was supported by the data. Biomagnification of arsenic does not occur in aquatic food chains [73] nor does As bioaccumulate, so for toxicity to occur fish would need to be continually exposed to high levels of arsenic. Another factor that may have affected the findings of arsenic in tissues in this study was the lack of diversity of trophic levels in the sites, which can affect the arsenic concentrations found- depending on their food source. For example, fish that consume plankton are more likely to have higher concentrations of arsenic than those who are omnivores or piscivores [74]. One study showed that when rainbow trout were exposed to arsenic, growth was reduced and the corresponding muscle concentrations were 3 ug/g [23]. Sorensen [75] found survival was reduced and death occurred in the Green sunfish when exposed to arsenic and muscle levels were at 6 ug/g. These values are more than 10 fold higher than the highest tissue concentration found in fish from this study (0.4

mg/kg), indicating that the fish observed do not have elevated body burdens of arsenic which does not support my 3rd hypothesis.

Looking at copper, however, the body burdens found at Bugaboo are relevant to toxic concentrations found in lab studies where there was an effect on survival and growth. When muscle tissue levels in rainbow trout, *Oncorhynchus mykiss* were 0.50 ug/g survival was reduced by 63% [33]. Murai et al., [76] found that when channel catfish were exposed to Cu through diet, muscle concentrations of 0.30 ug/g were concurrent with reduced growth. The highest Cu levels in the tissues in the fish in this study were 2 and 3 times as high (1 mg/kg) as those aforementioned. Fish in the study sites have elevated levels of Cu in their tissues; thus, there could be toxic effects in these fish such as reduced survival and growth.

The Zn tissue levels in fish at the sample sites were relatively low compared to values found in tissues where toxic endpoints have been measured. Holcombe et al. [71] found when tissue values between 36 to 66 ug/g (tissue dependent) were reached hatchability was reduced in brook trout eggs. Another study found no effect in the Atlantic salmon when whole body residue was 60 ug/g [77]. The highest Zn tissue levels found in this study were 5 mg/kg. Assessment of the fish tissues in this study indicates that fish were not exposed to high enough levels of Zn to pose a health risk-even to highly sensitive species such as trout and salmonids.

Results from the MT western blotting would indicate that there was no MT present, however, the MT antibody used was from cod and MT's are very species specific [30]; thus they did not cross react with the blotted plasma proteins. A more sucessful assessment for this study would involve creating antibodies specific to the species collected to ensure

specific MT identification in our fish species. This was not done in this study due to time and resource constraints.

CONCLUSION

From this study arsenic is not of concern in terms of soil, sediment, water, and fish toxicity. Many of these sites potentially have been receiving arsenic for thirty years and there is no evidence of accumulation or aquatic toxicity, suggesting that arsenic from poultry litter application does not have detectable long-term effects on surrounding aquatic ecosystems. Copper toxicity is of concern for plants, and exposure via water but not sediments to aquatic organisms. Copper concentrations in agricultural soils should be monitored and more aquatic studies need to be addressed in this area. Manganese values in fields receiving PL need to be monitored. Zinc induced phytotoxicity could occur in this study area and further monitoring is recommended, however, there is no evidence of a threat for aquatic toxicity. In the time period of this study there were no high rainfall events; thus, there were no collections taken where high volume runoff events could increase the load of metals that the streams were receiving. Further studies on phytotoxicity in fields receiving PL is something that should be addressed, especially since the crops grown in these fields are of economic importance. More aquatic toxicity studies would be beneficial in addressing the full toxic potential of metal runoff from PL. This includes increased sample size, fish of different trophic levels, and caging experiments.

The results from this study indicate that the current practices of supplementing feed with trace metals and applying this poultry litter on agricultural fields is not a sustainable practice. Spreading has occurred for approximately 30 years in this region and the toxic threshold for Cu and Zn in soils and streams has been reached. If spreading continues, further accumulation of these toxic trace elements will continue; thus, growing crops in this region may no longer be a viable option and the streams may be severely impacted. Removal of

these elements from the diet would be a sustainable option to allow the continuation of poultry litter application.

Table 1. Comparison of chicken litter (composted) to cow manure (NC Department of Agriculture). All values in ppm.

Element	Al	As	Cu	Mn	Mo	Ni	Pb	Zn
Cow Manure	846	0	9	32	0	2	1	43
Chicken litter 3 week Compost	4998	7	731	593	8	14	2	498
Forced Air Compost	2862	6	721	631	6	10	2	478

Table 2. pH of soils at each site from September, 2008. Reference sites (11 & 12 combined).

Site	1	2	3	4	5	6	7	8	9	10	Reference
pH	5	5	5.3	5	5.8	5	5.9	6.3	5.9	5.3	4.7

Table 3. Water chemistry: average pH, temperature, dissolved oxygen, and water hardness from September, 2008. Reference sites (11 & 12 combined).

Site	1	2	3	4	5	6	7	8	9	10	Ref
pH	6.6	6.5	6.7	6.8	6.6	6.5	6.6	6.3	7.3	6.7	6.4
Avg Temp °C	16.7	16.6	16.6	16.6	16.6	16.6	16.4	16.6	16.8	17.4	15.8
DO	8.4	7.8	9.07	8.15	7.61	7.4	7.98	8.87	8.87	5.9	9.3
Cond (uS/cm)	N/A	34.2	35.5	37.3	34.9	40.4	28.46	37.8	40.2	221.3	21.47
Hardness	10.4	11.9	10.5	11.4	12.5	11.4	15.8	8.7	12.7	11.5	7.7

Table 4. Comparison of North Carolina freshwater standards (NC DWQ 2009) for aquatic life and values found in the study area. All values in ppb. Shaded values are higher than the NC standards.

Metal	NC Freshwater Stds	1	2	3	4	5	6	7	8	9	10	Ref
Arsenic	50	4.9	0.57	0.77	0	0.13	0.57	0.43	0	0.17	0	0
Copper	7	5.3	24.7	6.6	5.9	7.3	6.5	10.2	5.7	8.7	7.3	4.5
Zinc	50	77	72	74	66	117	77	76	88	100	101	25

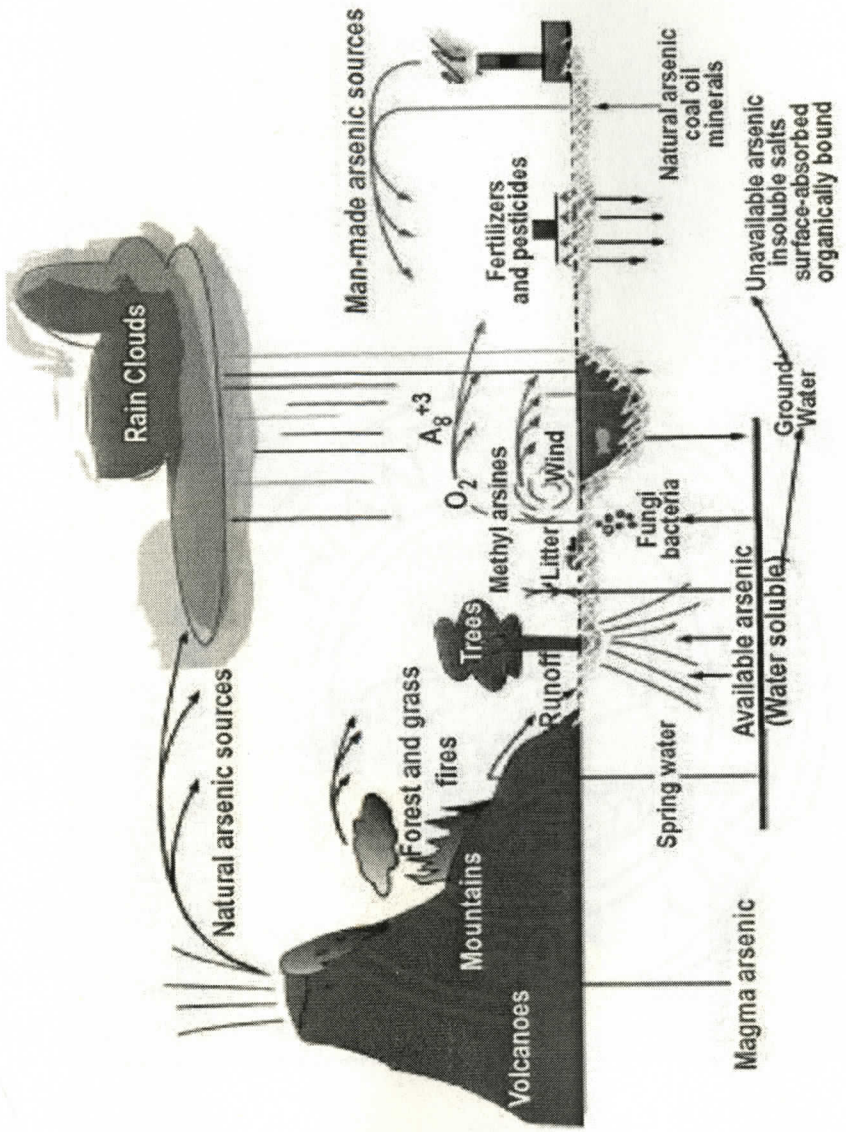

Figure 1. Natural Cycle of Arsenic (Jones 2007)

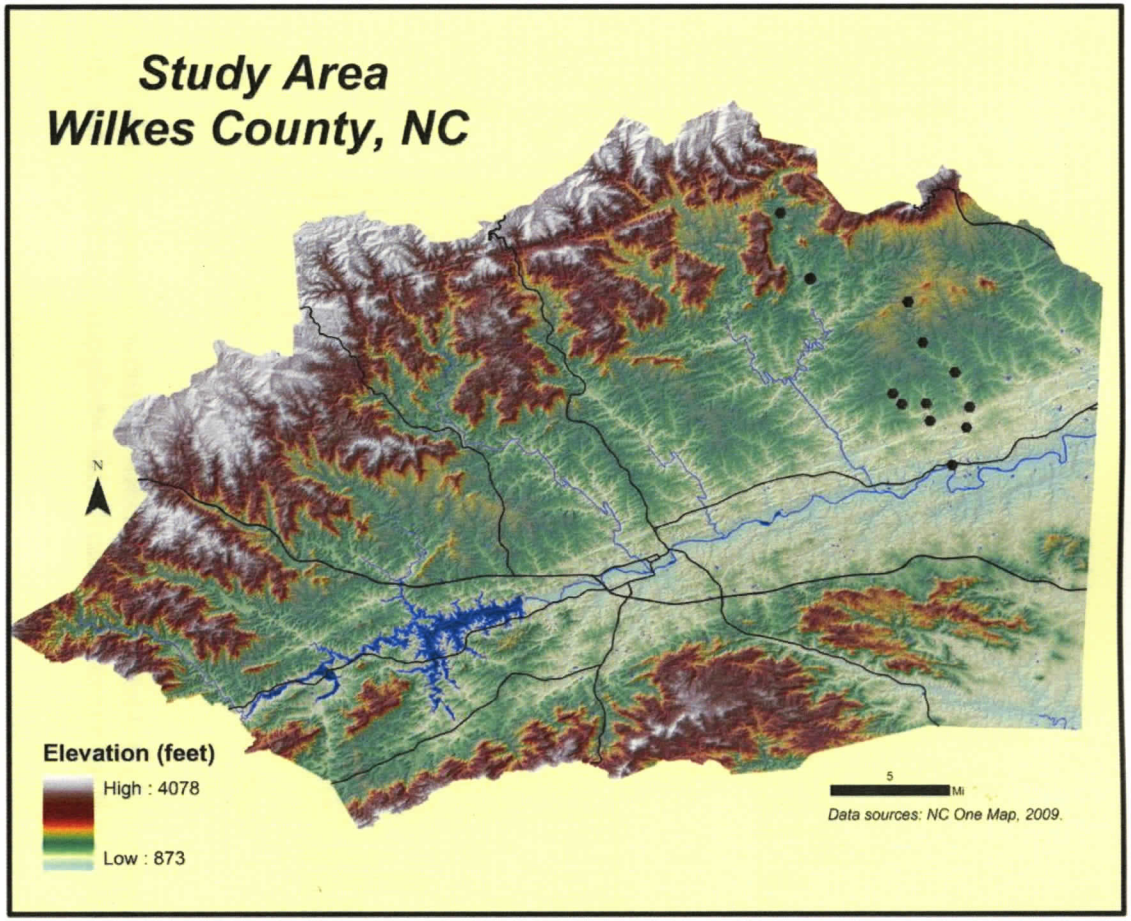

Figure 2: Study Sites in Wilkes County, NC. Black dots indicate sample sites

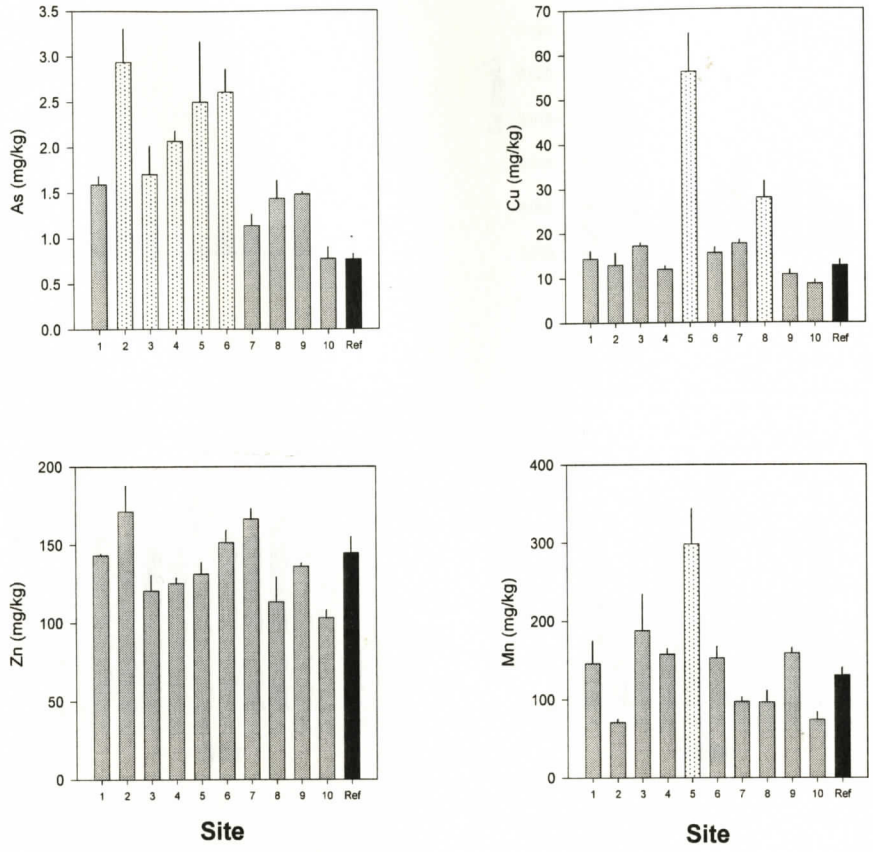

Figure 3. Soil trace element analysis. Black Bars= Combined Reference Streams Gray= No Significant Difference Stippled= Significant Difference ($p<0.05$). Error bars= Standard Error.

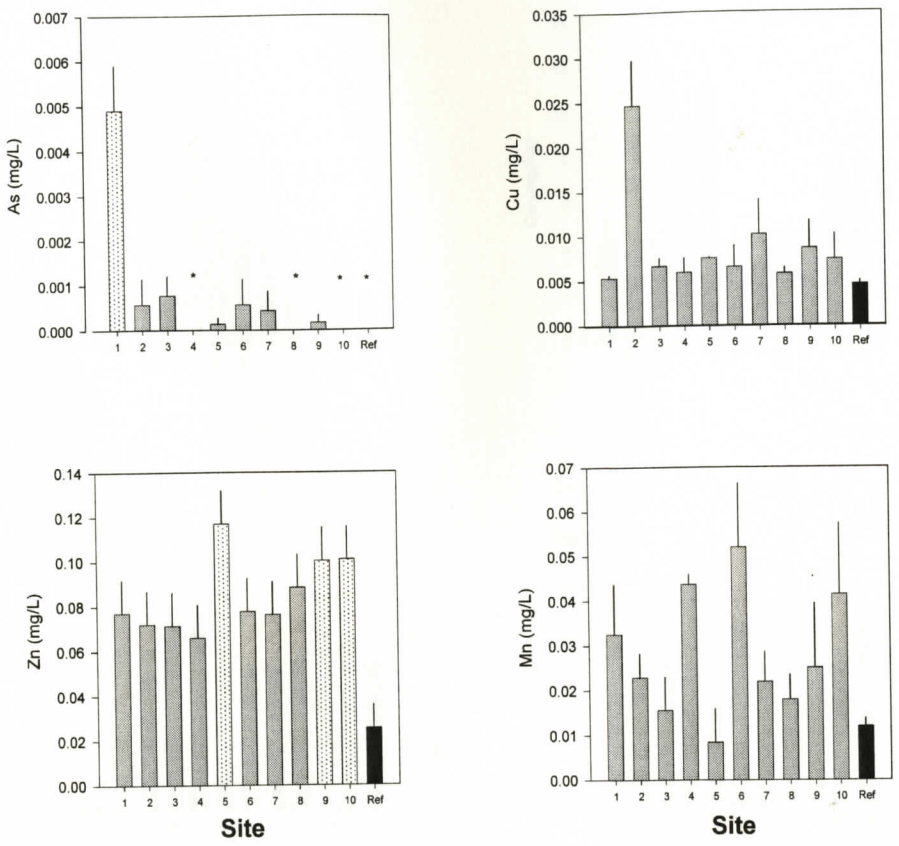

Figure 4. Water Trace element analysis. Black Bars= Combined Reference Streams Gray= No Significant Difference Stippled= Significant Difference ($p<0.05$). * Values below detection limit. Error bars= Standard Error.

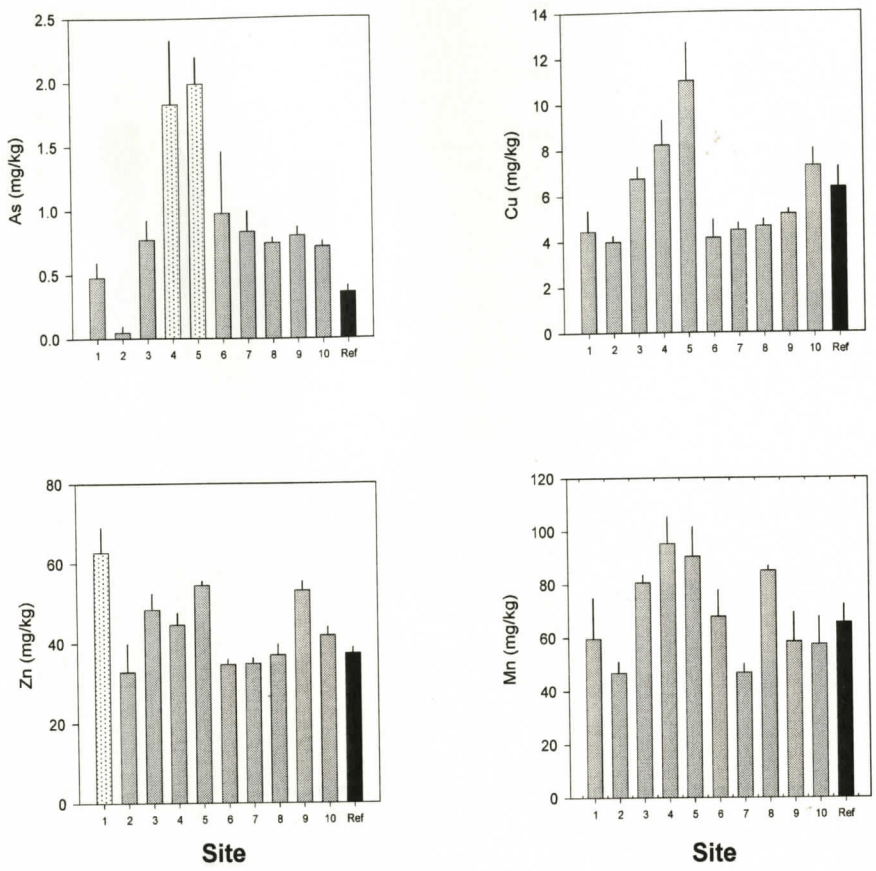

Figure 5. Sediment trace element analysis. Black Bars= Combined Reference Streams Gray= No Significant Difference Stippled= Significant Difference ($p<0.05$). Error bars= Standard Error.

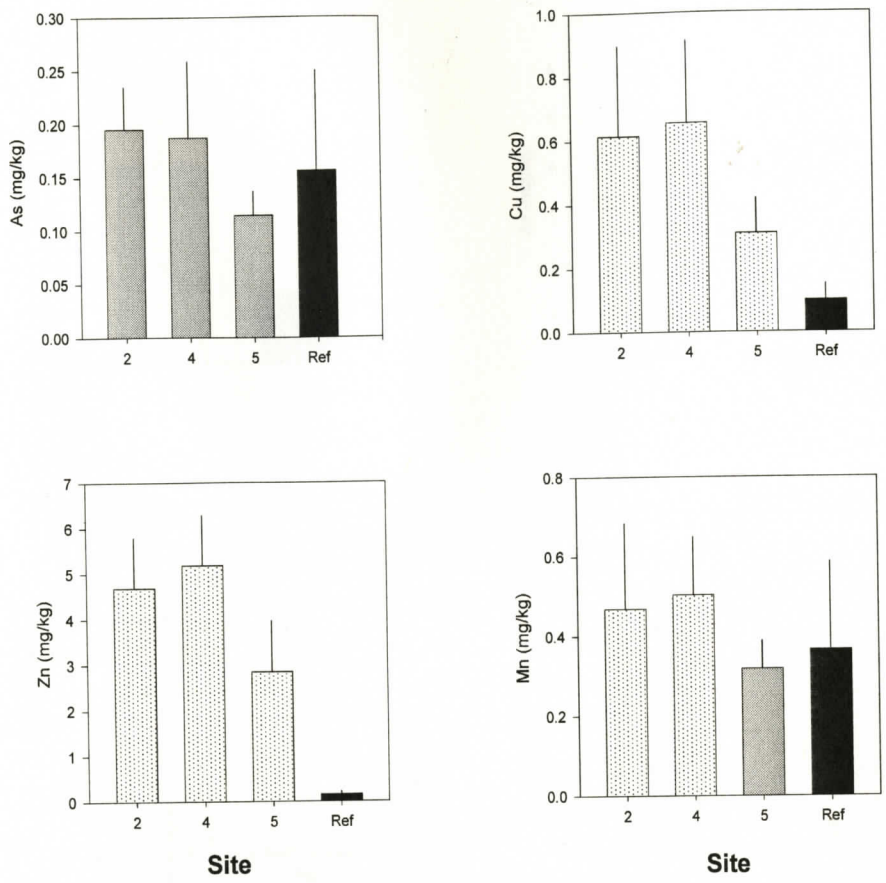

Figure 6. ***Nocomis leptocephalus*** **tissue trace element analysis. Black Bars= Combined Reference Streams Gray= No Significant Difference Stippled= Significant Difference ($p<0.05$). Error bars= Standard Error.**

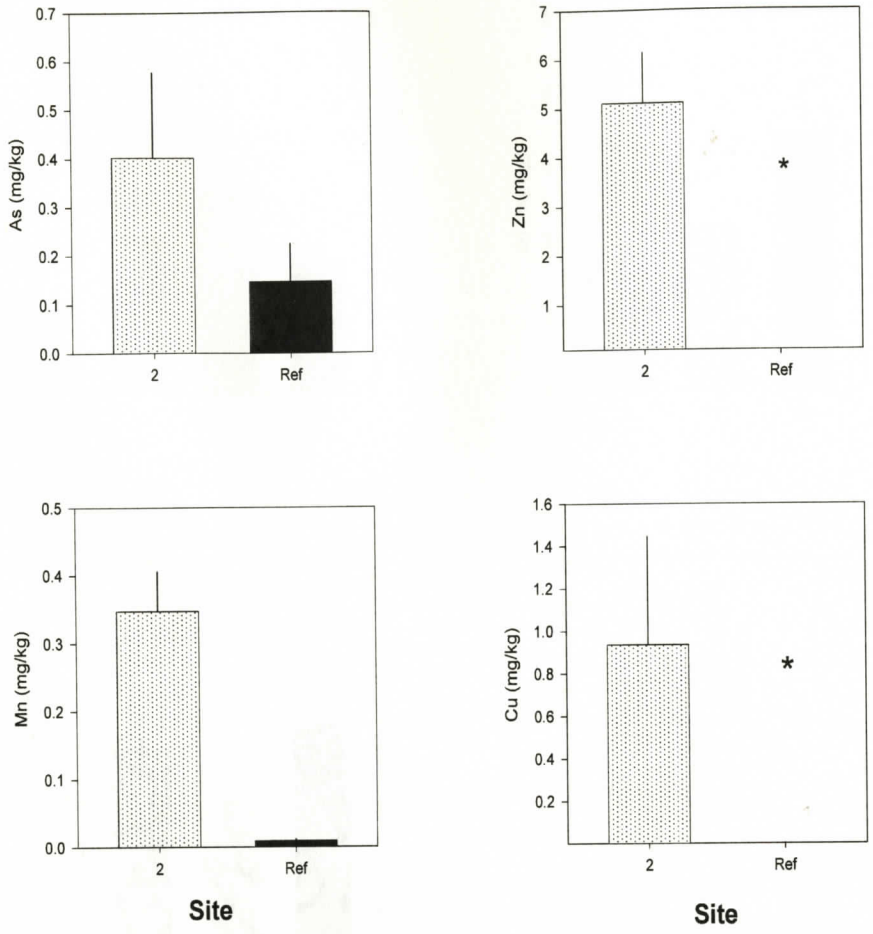

Figure 7. *Semolitus atromaculus* tissue trace element analysis. Black Bars= Combined Reference Streams Gray= No Significant Difference Stippled= Significant Difference ($p<0.05$). * Values below detection level. Error bars= Standard Error

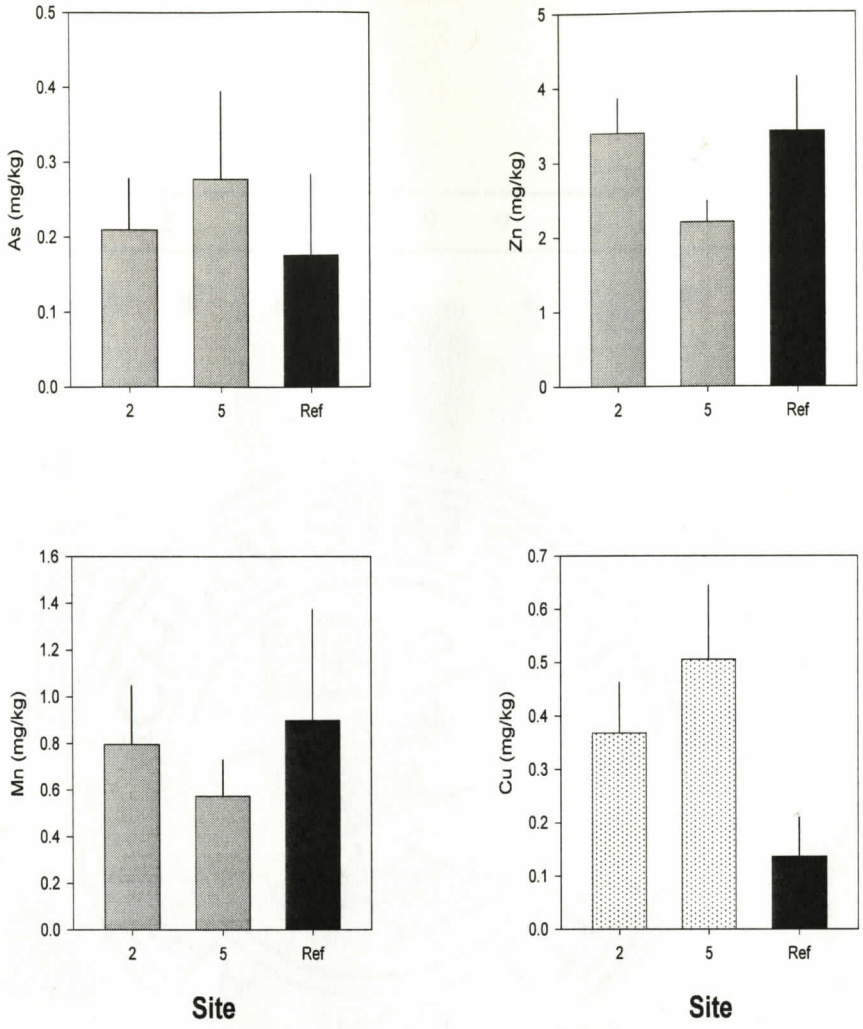

Figure 8. ***Catostomus commersonii*** **tissue trace element analysis. Black Bars= Combined Reference Streams Gray= No Significant Difference Stippled= Significant Difference ($p<0.05$). Error bars= Standard Error.**

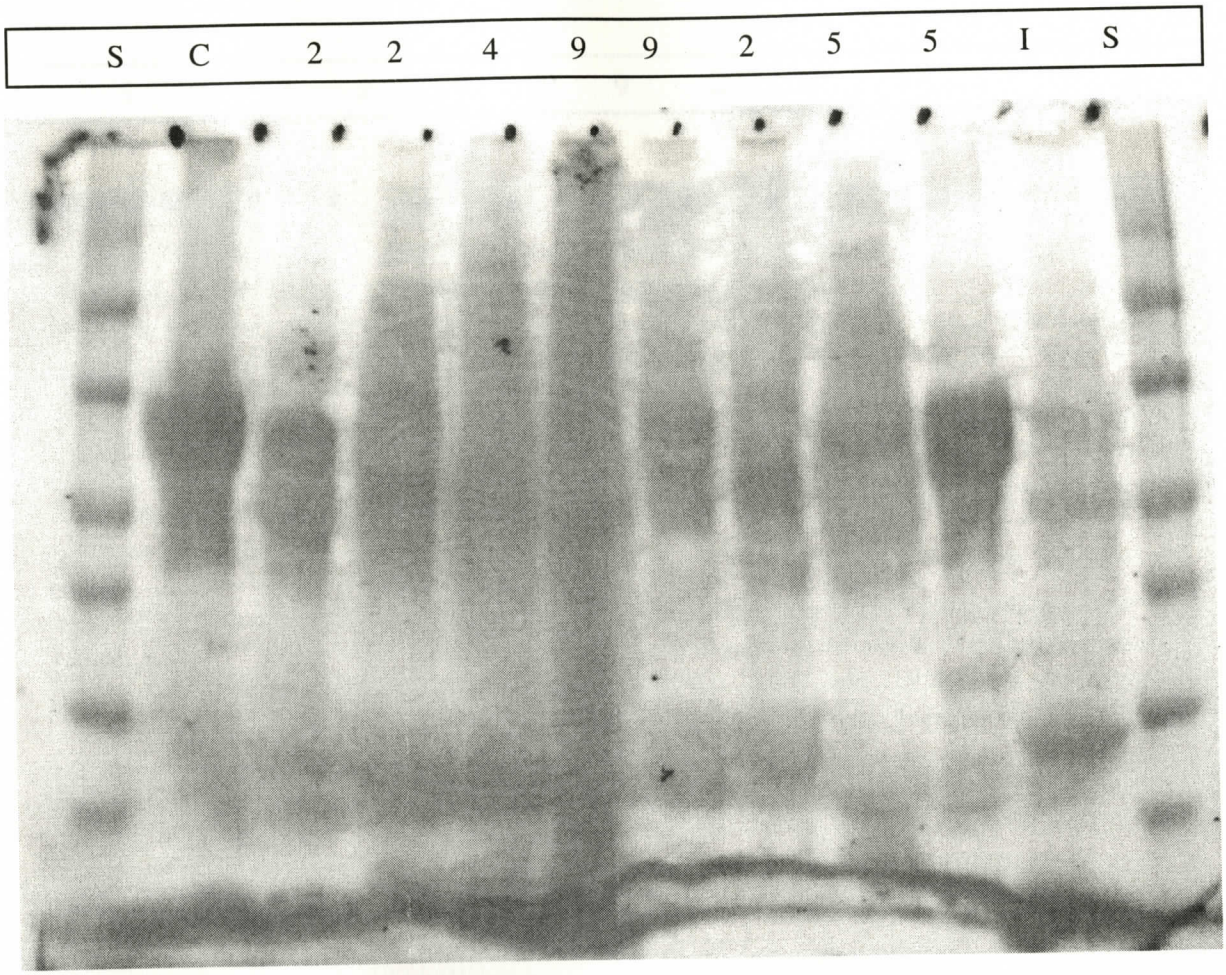

Figure 9. Red Alert stained bands after electrophoresis protein separation. S= Molecular weight standards, C= Reference Site, 2,4,5,9= Fish Serum from Bugaboo sites, I= MT induced controls ($CdCl_2$) injected.

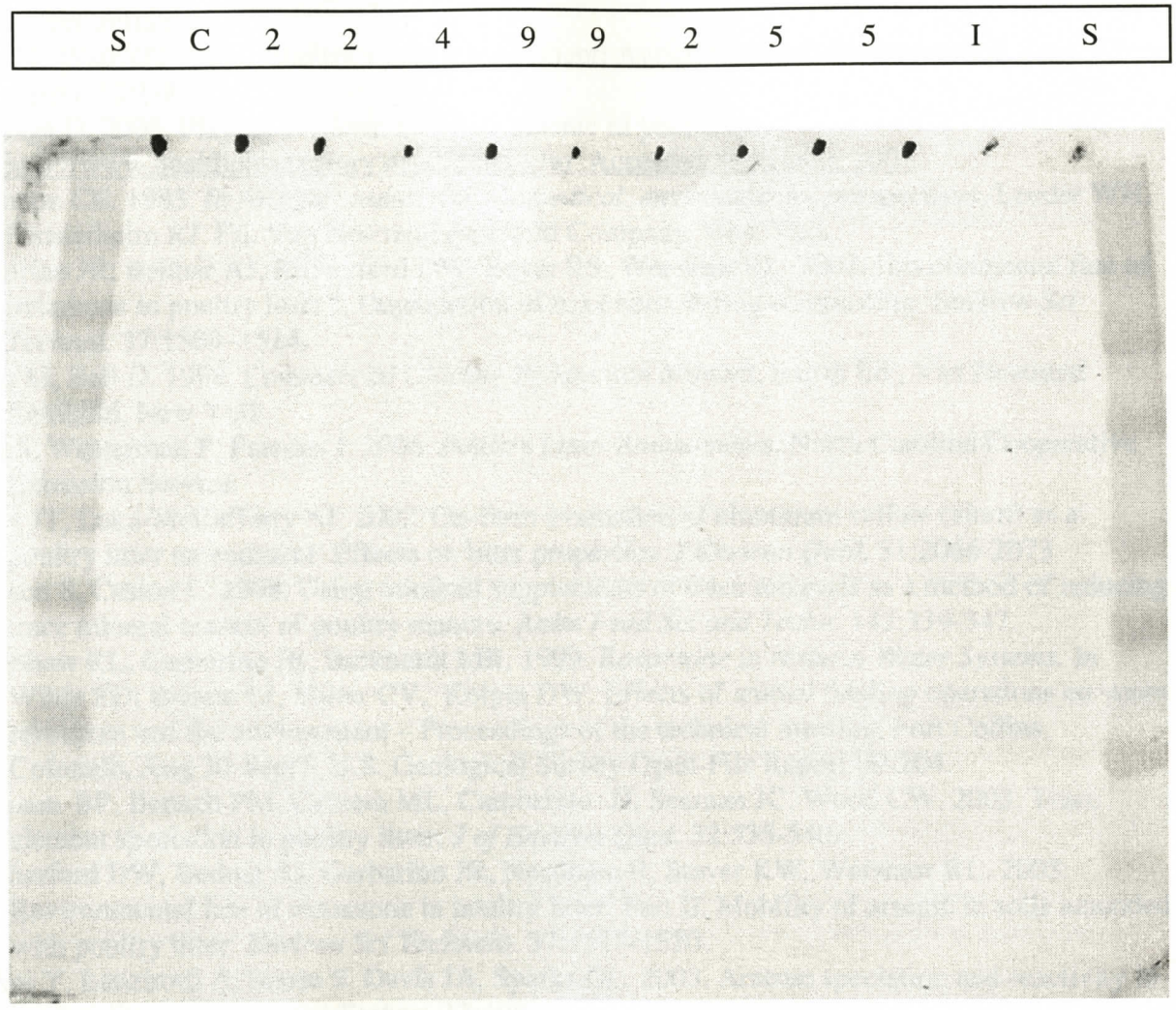

Figure 10. Metallothionein Western Blotting, after reaction with primary and secondary antibody. S= Molecular weight standards, C= Reference Site, 2,4,5,9= Fish Serum from Bugaboo sites, I= MT induced controls ($CdCl_2$) injected.

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BIOGRAPHICAL SKETCH

Jessica Elaine Pack was born in Greenville, North Carolina, on February 20, 1983 and she remained in eastern North Carolina until college. She received a B.S. in biology at Appalachian State University in Boone North Carolina, graduating cum laude in 2006. She then attended Appalachian State University for a M.S. in biology, specializing in the field of toxicology. A graduate certificate in women's studies was also completed at Appalachian State University. She plans to continue her education in toxicology and obtain a PhD in the field. Research interests include reproductive toxins and effects of toxins on women and children. She is interested in bridging the gap between women's studies and science and in environmental outreach and education.