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The Effects of Total Dissolved Solids on Locomotory Behavior and Body Weight of Streamside Salamanders, and a Baseline Survey of Salamander Diversity and Abundance

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THE EFFECTS OF TOTAL DISSOLVED SOLIDS ON LOCOMOTORY BEHAVIOR AND BODY WEIGHT OF STREAMSIDE SALAMANDERS, AND A BASELINE SURVEY OF SALAMANDER DIVERSITY AND ABUNDANCE

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By

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ABSTRACT

THE EFFECTS OF TOTAL DISSOLVED SOLIDS ON LOCOMOTORY BEHAVIOR AND BODY WEIGHT OF STREAMSIDE SALAMANDERS, AND A BASELINE SURVEY OF SALAMANDER DIVERSITY AND ABUNDANCE

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Thesis supervised by Dr. Sarah Woodley

Increased levels of total dissolved solids (TDS) in stream habitats are of concern due to salinity as well as the presence of potentially toxic ions. Natural gas drilling in the Marcellus shale could increase TDS in nearby streams. This thesis investigated the effects of water with elevated TDS on the locomotory activity and body weight of the streamside salamander *Desmognathus ochrophaeus*. Salamanders were exposed to water collected from streams in southwest Pennsylvania with elevated TDS as well as synthetic ion solutions that mimicked the ionic composition found in streams. Chronic, but not acute exposure to solutions of 1000 ppm TDS caused significant differences in weight loss and locomotory activity, although the effect depended on the exact ion composition of the dissolved solids. Finally, field surveys of salamander abundance were completed on two streams to provide baseline data with which to track population changes should the TDS in the streams increase.

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INTRODUCTION

Amphibians

Amphibian populations have been declining or becoming extinct steadily for the past several decades (Stuart *et al.*, 2004; Houlihan *et al.*, 2000; Dodd, 2010). In 2009 the International Union for the Conservation of Nature listed 30% of world wide amphibian species as threatened, which included vulnerable, endangered and critically endangered species (Dodd, 2010; IUCN, Red List). Amphibian declines can be attributed to several different factors that include introduction of invasive species, over-exploitation, habitat destruction, global climate change, increased usage of pesticides and toxic chemicals, and infectious disease (Collins and Storfer, 2003). Studying amphibian response to environmental changes may lead to a better understanding of the reasons for the population decline.

Amphibians are also studied because their health is thought to be a useful indicator of the overall environmental quality of an area, particularly water quality (Collins and Storfer, 2003; Hilman *et al*., 2009). Salamanders in the Plethodontidae family are especially useful as indicators of water quality. Many species of Plethodontid salamanders are typically found in close association with stream habitats. They are lungless and respiratory gas exchange happens entirely through their skin (Hilman *et al*., 2009).

Total Dissolved Solids

This thesis focused on the sub-lethal effects of total dissolved solids (TDS) on Plethodontid salamanders. TDS is a measurement of the concentration of dissolved materials in the water including salts, metals and minerals (Environmental Protection

Agency, 2010). Sources of total dissolved solids (TDS) in Pennsylvania include natural gas extraction in the Marcellus shale formation and abandoned mine drainage (AMD). Elevated TDS has been shown to have adverse effects on aquatic biosystems. Total dissolved solids, its sources, and its effects on aquatic biosystems are discussed below.

Total dissolved solids can be determined directly by measuring the mass of residue left after evaporation of the liquid (Southern University and A&M College of Engineering). Alternatively, TDS can be estimated by measuring conductivity, which is the amount of electrical current passing through the water (Environmental Protection Agency, 2011a). Conductivity is typically measured in μs/cm whereas TDS is measured in parts per million or milligram per liter (Environmental Protection Agency, 2010). Salinity is another term related to TDS, but refers specifically to the salt content of the water. Salinity is measured in parts per million or milligrams per liter (Environmental Protection Agency, 2010).

Sources of Total Dissolved Solids – Natural Gas Extraction

The Marcellus shale is a rock formation created during the Devonian Age that is on average located around 5,000 feet below portions of New York, Pennsylvania, Ohio, West Virginia, and Maryland (Arthur *et al*., 2008; Kargbo *et al*., 2010). Estimates suggest that recoverable gas from the shale could reach close to 500 trillion cubic feet (Arthur *et al*.,2008; Kargbo *et al*., 2010). Until recently the gas was thought to be irretrievable, but the use of horizontal drilling and hydraulic fracturing has proved otherwise (Arthur *et al*., 2008). Horizontal drilling involves drilling vertically and then drilling at an angle so that the well is parallel to the surface. Hydraulic fracturing involves pumping millions of gallons of water along with sand and other additives under high pressure into the gas well

(Arthur *et al*., 2008). Hydraulic fracturing fluid may contain sand, quartz, gels, acids, biocides, scale inhibitors, and surfactants (Arthur et al., 2008). This process cracks the rock and allows the gas to be retrieved. The use of hydraulic fracturing and horizontal drilling has led to rapid growth in the Marcellus Shale gas extraction industry in Pennsylvania. In 2008 there were over 400 Marcellus wells in Pennsylvania (Authur *et al*., 2008). Figure 1 shows permitted and completed wells in Pennsylvania as of December 2010.

Natural gas extraction using hydraulic fracturing can potentially impact the environment in several ways, including habitat destruction, air pollution, water withdrawals, potential ground and surface water contamination, and increased TDS in waterways (Kargbo *et al*., 2010). Building a well pad involves leveling off the area for the pad, constructing gravel roads leading to the pad, installing pipelines to and from the pad, and freshwater impoundments (Kargbo *et al*., 2010, Department of Conservation and Natural Resources). This can cause a disruption in the ecosystem and potentially destroy and fragment animal habitats (Kargbo *et al*., 2010). Gas extraction in the Barnett shale in Texas has been similar to that in the Marcellus Shale. A report summarizing air emissions from drilling in the Barnett Shale showed that drilling procedures produced greenhouse gases such as methane and carbon dioxide, smog-forming compounds such as nitrous oxides and volatile organic compounds, and toxic air chemicals (Armendariz, 2009).

Hydraulic fracturing requires 3-4 million gallons of water per well, with a large portion of the water coming from surface withdrawals (Kargbo *et al*., 2010). This could lead to altered flow patterns. Wells are required to have a cement casing to prevent groundwater contamination. However, at great depths, increased temperature and pressure along with brackish water conditions and acidic gases make the maintenance of the cement casings difficult (Kargbo *et al*., 2010). The water that comes back up from the well is referred to as flowback water and contains high levels of total dissolved solids (TDS).

Flowback water can have a TDS concentration of over 100,000 ppm (Blauch *et al*., 2009). The Environmental Protection Agency set the secondary maximum contaminant level (SMCL) for TDS at 500 ppm (Environmental Protection Agency, 2011c). SMCL are non-enforceable levels meant to guide treatment plants. The dominant ions found in Marcellus shale flowback water are sodium, calcium, and chloride (Kirby, 2011, Gaudlip and Paugh, 2008). Flowback water also typically contains barium and strontium (Kirby, 2011) and is generally low in sulfates (Gaudlip, and Paugh, 2008). Treatment of the flowback water proves difficult for waste water treatment plants. To reduce the TDS concentration the water is generally diluted and then dispersed into a waterway. This causes the overall TDS level of the waterway to increase.

Sources of Total Dissolved Solids - Abandoned Mine Drainage

Abandoned Mine Drainage (AMD) is another source of elevated TDS in waterways. When surface water flows over sulfur-bearing rocks, such as pyrite, the water increases in acidity, and this is referred to as abandoned mine drainage (Environmental Protection Agency, 2011b). This acidic water can now further dissolve metals such as copper and lead, causing an increase in TDS (Environmental Protection Agency, 2011b). AMD is common in areas, such as Pennsylvania, with a history of heavy coal mining (Environmental Protection Agency, 2011b). Common constituents of TDS from AMD are iron, sulfate, calcium, and aluminum (Barnes, 1968).

Figure 1

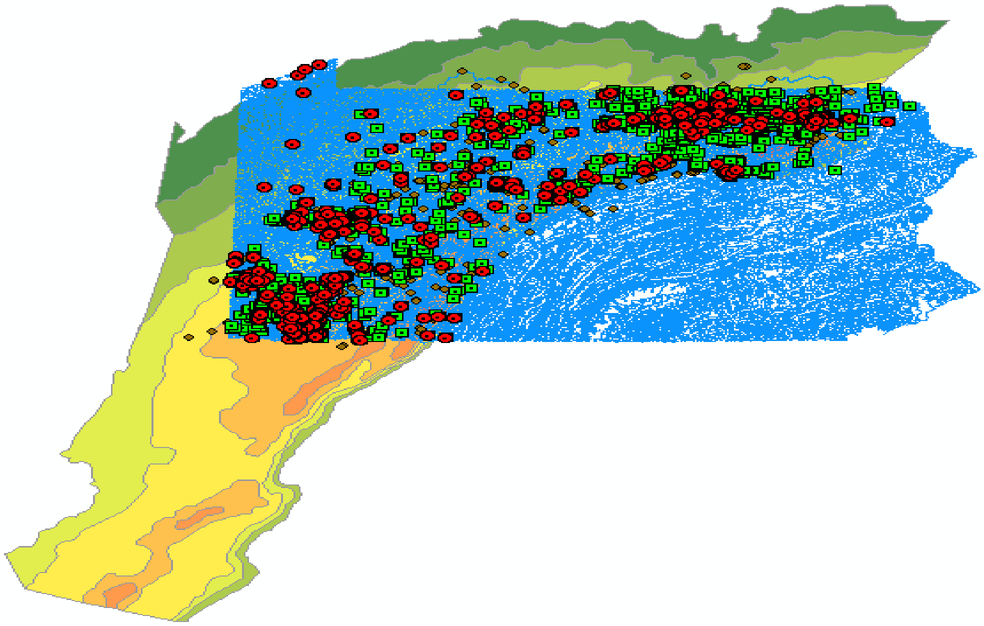

Figure 1: Marcellus Shale gas extraction-related activities in Pennsylvania as of December 2010. Red circles indicate completed Marcellus Shale wells. Green circles indicate active permits, which means that preparation to begin gas extraction is imminent. In December of 2010 there were 758 completed wells and 5176 active permits. This figure and data was compiled by Lucas Eastham using information provided by the United States Geological Survey and the PA Department of Environmental Protection.

Many studies have been done on the effects of AMD on amphibians, particularly on embryos and larval stages because these are typically considered the most sensitive forms (Freda, 1991). However, these studies generally focus on the pH and aluminum content of the water (Freda, 1991) and not the overall TDS concentration or composition.

Total dissolved solids effect on aquatic biota

Some studies have examined the effects of TDS on fish, invertebrates, and amphibians (Webber-Scannell and Duffy, 2007). Chapman *et al*. (2000) studied the effects of TDS on both chironomid larvae and rainbow trout embryos. They used synthetic effluent prepared to match the TDS constituents of two mines (Kensington and Red Dog) that had concentrations of up to 2,000 ppm. They observed no adverse affects of TDS exposure on trout embryo morphogenesis. In chironomid larvae, they observed a 45% weight reduction after exposure to Red Dog synthetic effluent and a similar weight loss as well as reduced survival after exposure to Kensington synthetic effluent over 1750 ppm (Chapman et al, 2000). The ions in their TDS solution included sodium, chloride, potassium, magnesium, sulfate, and chloride (Chapman et al, 2000). They stressed that the effect was probably dependent on the identity and concentration of the individual ions in the TDS and cautioned against assuming that different ions at the same concentration will have the same effect.

Kimmel and Argent (2006) conducted a study on fish communities' responses to conductivity on Tenmile Creek. They looked at both TDS and conductivity at various stations along the Creek. At each station they surveyed species of fish present. Their results suggested that a specific conductivity above 3500µs/cm and a TDS over 2300 ppm may be toxic to fish communities.

Bowles *et al.* (2006) conducted a series of salamander surveys at eight streams in Texas. The streams were classified as either developed or undeveloped based on the percent impermeable cover in the watershed, with >10% impermeable coverage considered developed (Bowles *et al.* 2006). In addition to conducting the surveys, they measured water temperature, pH, dissolved oxygen, and conductivity. They found that the conductivity was higher and salamander abundance was lower at developed sites (Bowles *et al.* 2006). Sayim *et al*. (2009) studied water quality parameters including conductivity, pH, dissolved oxygen and chloride at breeding and non-breeding sites of the salamander *Mertensiella caucasica*. They compared the water quality parameters at each type of site and found that breeding sites had lower conductivities (Sayim *et al*., 2009). In addition, laboratory studies on spotted salamander (*Ambystoma maculatum*) embryos have shown reduced survival at conductivities over 500µs/cm (Karraker *et al*., 2008). To conclude, the studies summarized above provide some evidence that elevated TDS has adverse effects on aquatic biota.

Water with elevated TDS may affect the ability of salamanders to osmoregulate. Amphibians use osmoregulation to maintain the osmotic pressure of their bodily fluids (Shoemaker and Nagy, 1977). This requires that influxes of water and solute must balance effluxes over time. Studies have shown that anuran amphibians have a limited ability to osmoregulate in high salinity conditions (Ruibal, 1962). Romspert and McClanahan (1981) found that the salamander species *Ambystoma tigrinum* could tolerate an osmolarity up to 300 mOsm/l NaCl .This corresponds to a TDS level of about 9000 ppm NaCl (Hilman et al, 2009).

Components of the TDS in the water may also act as toxicants to the salamanders. TDS may also act as a stressor to the salamander. A general definition of a stressor is any unusual or unpredictable change in the environment that perturbs the physiological and behavioral homeostasis of an organism (Widmaier, Raff, and Strang, 2006). In response to a stressor, an animal may succumb, or may avoid, counter, or cope with the stressor to maintain health and stay alive (Widmaier, Raff, and Strang, 2006). Decreased activity is a general response to exposure to stressors in plethodontid salamanders. For example salamanders exposed to a low pH, handling, or predator cues had significant lower activity than controls (Ricciardella and Woodley, unpublished, Woodley and Lacy, 2010). Also certain tadpole species decreased activity when exposed to certain pesticides (Reylea and Edwards, 2010). Decreased activity could lead to decreased foraging and mating which would have an impact on salamander health and populations.

Summary of thesis study

This study investigated the effects of exposure to water sources with different compositions of elevated TDS on salamanders in a controlled laboratory setting. Sublethal effects of both acute and chronic TDS exposure were investigated. For the acute exposure experiment, activity and feeding were measured. For the chronic laboratory studies, activity and body weight measured. In addition to laboratory studies, a baseline survey of salamander diversity and abundance was conducted on two streams. One stream downstream from Marcellus shale wells and one without any upstream Marcellus-shale related activities.

THESIS OBJECTIVES

Objective 1: To test the hypothesis that acute exposure to water with elevated TDS causes behavioral changes.

Rationale: Water with elevated TDS may contain toxic ions and have an elevated salinity. Acute exposure to water with elevated TDS may represent an acute stressor that causes sub-lethal changes in salamander behavior such as activity and feeding.

Objective 2: To test the hypothesis that chronic exposure to water with elevated TDS causes physiological and behavioral changes.

Rationale: Water with elevated TDS may contain toxic ions and will have an elevated salinity. Chronic exposure to high TDS may cause sublethal changes in behavior such as activity, changes in body weight, and other physiological changes.

Objective 3: To conduct a baseline survey of plethodontid salamander abundance and diversity on a pair of streams, one downstream form Marcellus shale drilling activity and the other not downstream of Marcellus shale activity.

Rationale: An increase in Marcellus shale activity may impact salamander diversity and abundance. These surveys are will provide important baseline data to help determine any future Marcellus shale activity impacts on the sites.

METHODS

All procedures described were approved by Duquesne University's Institutional Animal Care and Use Committee (IACUC). Animals were collected with the appropriate permits from the Pennsylvania Fish and Boat Commission.

Objective 1: Acute Exposure to water with elevated TDS

Subjects

The salamanders used in this laboratory experiment were of the species *Desmognathus ochrophaeus*. Ten males and ten females were used. They were collected on August 30, 2010 from Lynn Run in Forbes State Forest in Westmoreland County, Pennsylvania. *D. ochrophaeus*, more commonly called Allegheny dusky salamanders are members of the Plethodontidae family (Duellman and Trueb, 1986). They are typically found within one meter of the stream. This makes them especially useful as indicators of the environmental quality of the water. The salamanders were housed individually in plastic boxes (12.7 x 12.7 x 3.81 cm) lined with moist unbleached paper towels and kept in incubators at 16ºC. They were spatially distributed evenly throughout the incubator. The incubators had a light dark cycle of 14:10, with lights out from 4pm until 2am. Salamanders were fed waxworms (*Galleria* species) every 3-4 weeks.

Treatments with different water stimuli

Each salamander was tested while exposed to each of six different treatments for one hour. Water stimuli were derived from streams with different levels of TDS. Water stimuli were: 1) water from Whitely Creek (N39 49 16.4, W79 57 6.3), at 2000 mg/l, 2) water from Whitely Creek diluted with distilled water to 1000 mg/l, 3) water from Whitely Creek diluted with distilled water to 500 mg/l, 4) water from Bates Fork, with a TDS measured at 229 mg /l 5) water from Fonner Run with a TDS measured at 169 mg/l and 6) distilled deionized water. TDS was measured indirectly using an EC/TDS/TEMP COM-100 meter (HM Digital) which converts conductivity to TDS using a non-linear scale (COM-100 meter User's Guide). For KCl, the mode used for this study, the conversion factor is 0.50 to 0.57.

Water from Whitely Creek was chosen as a stimulus because it had a relatively elevated TDS concentration of 2000 ppm. A concentration of 2000 ppm is commonly found in natural systems and has been used in other TDS studies (Chapman et al, 2000). Elevated levels of sulfate (Table 1), indicate that water from Whitely Creek is likely to be impacted by Abandoned Mine Drainage. Bates Fork and Fonner Run were chosen because they are the sites at which the field population survey (Objective 3) was conducted.

Water from Whitely Creek, Bates Fork, and Fonner Run was filtered through a 0.2μm filter within 24 hours and stored at 4ºC in 50 ml vials with no headspace. Distilled water was also stored in vials at 4ºC. Before use in the behavioral studies the water was allowed to reach room temperature. To describe the ionic composition of the water samples, anions were measured at Duquesne University using an Ion Chromatography System ICS-1100 and cations were analyzed at the University of Pittsburgh using a Perkin Elmer NexION 300X ICP Mass Spectrometer. Table 1 shows the anion and cation analysis for each sample as well as the pH and conductivity.

Romspert and McClanahan (1982) determined that the plasma osmolarity of the salamander species *Ambystoma tigrinum* was 262.2 mOsm/l NaCl. This corresponds to a TDS concentration of about 8000 ppm (NaCl) (Hillman et al, 2009). Presumably, *D. ochrophaeus* would have similar plasma osmolarity, meaning that a TDS of 2000 ppm would be a hypotonic environment for the animal.

Behavioral Measurements

Animals were tested on the water stimuli using a repeated measures design. Each subject was placed into a testing chamber (bioassay tray: 23.5 x 23.5 x 2 cm) lined with unbleached paper towel moistened with 15 ml of a water stimulus. A Sony 3CCD Progressive Scan, 48X Digital Zoom camcorder was used to record salamander activity. Salamanders were recorded for 2 seconds every minute for a total of 75 minutes. The first fifteen minutes were not scored because it represents an acclimation period to the testing chambers. Filming took place at room temperature during the salamanders' dark phase between the hours of 4 pm and 8 pm. Humidifiers were used to increase humidity and temperature and humidity were recorded. Salamanders were filmed under dim lighting conditions. During filming, salamanders were visually isolated from one another. Testing occurred on each of six nights with at least four nights between each test. Recording occurred in two batches each night. On a given night, all treatments were represented. Sexes and treatment were evenly distributed within and across batches. After filming, fifteen frozen fruit flies were randomly scattered into the testing chambers and salamanders were placed back in the incubator. The next morning the number of flies eaten were counted and the salamanders were returned to their home boxes.

To score activity, videos were viewed and the number of times a salamander moved from one quadrant to another was counted. A salamander was considered to be in a quadrant if half of its body was across the line.

Table 1

Water Analysis for Water Stimuli used in Acute Exposure Experiment

Ion	Distilled Deionized Water	Bates Fork	Fonner Run	Whitely Creek
Chloride	5.52	16.2	7.7	293.8
Sulfate		42.5	25.6	1184
Sodium		6.2	3.8	563.4
Magnesium		6.2	5.1	45.9
Potassium		2.7	1.5	4.2
Strontium				1.7
Calcium		42.8	40.1	99.6
pH	8.21	8.06	8.13	7.85
TDS (ppm)	2.9	229	169	2010
Date Collected		10/15/10	10/15/10	11/10/11
GPS coordinates		N39° 57’ 39.8’’ W80° 15’ 29.6’’	N39° 57’ 42.8’’ W80° 15’ 24.5’’	N39° 49’ 16.4’’ W79° 57’ 6.3’’

Table 1: Anion and cation concentrations of water stimuli used in Objective 1: acute effects of elevated TDS. Values are in ppm. pH was taken using a YSI Professional Plus. TDS was measured using a COM - 100 Meter.

Objective 2: Chronic Exposure to Water with Elevated TDS

Chronic Experiment 1*: *Exposure to Whitely Creek Water, TDS 2000 ppm

Thirty-one salamanders (including the 20 from the acute experiment) were divided into two exposure groups; undiluted Whitely Creek water (2000 ppm) and distilled deionized water. Males and females and similarly sized salamanders were distributed equally among the groups. Animals were housed in petri dishes (14 cm diameter, 2 cm in height) lined with an unbleached paper towel moistened with 5 ml of either Whitely Creek water (2000 ppm) or distilled deionized water.

Each day close to 3 pm, subjects were exposed to a fresh treatment of their designated stimuli. To do so, the petri dish bottom was replaced with a new bottom lined with a fresh paper towel moistened with a new solution of the water stimulus.

This experiment was designed to last at least 21 days, and activity and body weight data were to be at collected at the half way point. However, the study was halted on the ninth day due to the unexpected deaths of three salamanders in the Whitely Creek water (2000 ppm) group. After halting the experiment, all animals were returned to their home boxes. The animals from the experiment were monitored for an additional week and all animals appeared healthy.

Chronic Experiment 2: Chronic Exposure to Whitely Creek Water, TDS 1000 ppm

Rationale

Because of the deaths that occurred in chronic experiment 1, exposure to a TDS of 2000 ppm the TDS concentration was reduced for chronic experiment 2. For chronic experiment 2, subjects were exposed chronically to Whitely Creek water diluted to a TDS of 1000 ppm. The Whitely Creek water, initially at 2000 ppm, was diluted to 1000 ppm at the time of use. Water was diluted by placing 2.5 ml of distilled water in the petri dish and then 2.5 ml of the 2000 ppm TDS, resulting in a new concentration of 1000 ppm. Animals were exposed to a fresh treatment every day as described in Chronic Experiment 1. The same treatment groups were used as in the previous chronic experiment.

The animals were checked every day for signs of toxicity. These signs included the overall appearance of the skin, posture, righting response, and perceived rate of gular flapping (Johnson *et al*., 2004). They were checked at least every other day by someone who was blind to the treatment groups. If an animal appeared moribund or had lost more than 30% body weight, they were removed from the study and returned to clean home boxes. Animals were considered moribund if they were very lethargic, showed gaping behavior, or had very dull skin.

Data Collection

Salamanders were weighed at the start of the experiment and every other day until the end of the experiment. To weigh salamanders, they were gently placed onto an absorbent wipe to remove excess moisture and then weighed. To quantify locomotory activity, animals were video recorded every 3 or 4 days. Salamanders behavior was recorded as described in the acute experiment, but instead of being recorded in bioassay trays they were left in their petri dishes for recording.

After 4 weeks, the subjects were weighed and euthanized. Blood samples were collected at the time of euthanasia for analysis of plasma corticosterone (a stress hormone). Also, blood smears were collected in order to measure relative numbers of white blood cells as a measure of immune function. Fat body, spleen, liver, and gonads were dissected and weighed. Carcasses were weighed, dried in a 60ºC oven for five days, and weighed again to determine dry carcass masses.

Chronic Experiment 3: Chronic Exposure to Synthetic Ion Solutions and Blacklick Creek Water, TDS 1000 ppm

Rationale

To better understand the effects found in chronic experiment 2, a synthetic ion solution was made to mimic the TDS composition of Whitely Creek water. This would determine if the effects found in chronic experiment 2 were due to a component of the water besides TDS. Water collected from Blacklick Creek (N40° 28' 50.1", W79° 10' 8.8") and a synthetic ion solution of Blacklick Creek were included to determine the effects of ion composition. Blacklick Creek water was collected immediately downstream from the outflow at the Pennsylvania Brine Treatment facility in Josephine, Pennsylvania. Elevated levels of chloride and strontium (Table 2) indicate that water from Blacklick Creek is potentially impacted by Marcellus shale flowback water.

Subjects

Fifty nine male *D. ochrophaeus* were used for this experiment. Salamanders used were collected at State Game Lands 51, Pennsylvania in June of 2011. They were housed on different water stimuli as described in chronic experiments 1 and 2. They were fed 5 days before the start of the experiment and on day 14 of the experiment.

Water Stimuli

Salamanders were exposed for 21 days to one of five solutions. 1) water containing the major ions of the Whitely Creek water, 2) water collected from Blacklick Creek, 3) water containing the major ions found in the Blacklick Creek water, 4) synthetic spring water, and 5) distilled deionized water. Solutions 1, 2, and 3 had similar high TDS (1120 ppm) but solution 1 had a different ionic composition than solutions 2 and 3. Solutions 4 and 5 were controls with low TDS. Both distilled deionized water and synthetic spring water were used as controls to determine if responses to control solution depended on the mineral content. Synthetic spring water was made from 100 mg $NaSiO_3$, 12 mg NaCl, 6 mg Na_2SO_4, 6.5 mg $CaCl_2$, 3.5 mg $MgCl_2$, and 4 mg $FeCl_3$ per liter of distilled deionized water (Presnell and Schreibman, 1997). Synthetic solutions were made based on the anion and cation analysis. Cations and anions found in concentrations under 0.9 mg/l were not included in the synthetic solutions. The cation and anion analysis for all five solutions are shown in Table 2. Synthetic solutions were made by dissolving salts into deionized distilled water. The amount of salts used was calculated from the anion and cation analysis. pH was adjusted to near neutral levels by adding HCl or NaOH. The solutions were then stored in the in 20 ml propylene bottles at 4ºC.

Salamanders were placed on a fresh sample of their designated substrate every day typically between 2 and 4 pm as described in the previous chronic experiments. They were monitored for signs of toxicity as described in the previous chronic experiments.

Data Collection

Salamander weight was collected every other day for the first week and every third day for the following weeks. Weight data were collected as described in chronic experiment 2. Salamander activity was digitally recorded every three to four days as described above. Treatments were coded so that the investigator was blind to exposure groups. Activity data was analyzed as described above. Unlike chronic experiment 2, animals were not euthanized at the end of the experiment.

Objective 3: Baseline Survey of plethodontid salamander diversity and abundance.

Population Survey

Population surveys of a pair of stream sites were done on seven occasions from September 2010 until October 2011. The sites surveyed were Fonner Run and Bates Fork located in Greene County, Pennsylvania. GPS coordinates are shown in Table 3. The sites were chosen because they are in close geographic proximity and share many similar features, but there was Marcellus shale gas extraction activity upstream of Bates Fork and not upstream of Fonner Run. Both Bates Fork and Fonner Run are third order streams. The salamander survey was part of a larger study of potential impacts of Marcellus Shale activity on water quality and biota that involved study of the biogeochemistry, ecology, and geomorphology of the streams. Surveys were done using a method appropriate for patchy habitat like that found at Bates Fork and Fonner Run. (Rocco and Brooks, 2000). The method involved placing a 2X2 meter quadrat spanning the land-water interface of suitable habitat, with one meter on land and one meter on water (Figure 2 and 3). After the quadrat was selected the area was searched thoroughly by hand and dip net. The age (adult, juvenile, or larvae), sex, snout to vent length, and weight of each salamander collected were recorded. Animals were released where they were found. Sex was determined by checking the salamanders for eggs and hypertrophied premaxillary teeth. Eggs indicated an adult female, teeth indicated an adult male, and the absence of both eggs and teeth indicated a juvenile. Larvae were identified by the presence of gills and were typically collected while dip netting. Density of

Table 2

Water Analysis for Stimuli used in Chronic Experiment 3

Ion	Synthetic Whitely Creek	Blacklick Creek Field Water	Synthetic Blacklck Creek	Synthetic Spring Water	DD Water
Chloride	206.2	682.8	688.7	120.5	0.18
Sulfate	849.5	6.59	7.549	6.53	
Bromide		5.53	5.54		
Sodium	384.2	283.9	302.1	66.57	1.156
Magnesium	25.74	5.679	5.375	2.595	0.5966
Potassium	3.568	3.245	0.5045		0.2229
Strontium	6.876	13.82	11.03		0.0232
Calcium	68.59	122.0	115.0	3.710	3.791
Lithium	0.002	1.116	0.8373		0.003
Iron	0.395	0.583	0.868	2.309	0.1474
Barium	1.43		0.061		0.102
pH	7.94	8.13	8.17	7.8	7.21
TDS (ppm)	1120	1120	1120	179	2.9

Table 2: shows the ion analysis, pH, and TDS of water samples used in chronic experiment 3. Anions were analyzed at Duquesne University using an Ion Chromatography System ICS-1100. Cations were analyzed at the University of Pittsburgh using a Perkin Elmer NexION 300X ICP Mass Spectrometer. pH was measured using an Ultra Basic -10 pH/mV meter and TDS was measured using a COM-100 meter.

animals was calculated by dividing the abundance of salamanders by the total number of m^2 searched. The TDS concentration, pH, and GPS coordinates were also recorded. Three quadrats were chosen at each stream site (Bates and Fonner). The same quadrats were searched during each survey except the April survey. Rain and high water allowed only two quadrats on Fonner Run and one quadrat on Bates Fork to be searched. Figures 4 and 5 provide maps of the sites.

Habitat Quantification

To characterize the habitat at each site, quantification methods from Grossman and Skyfield (2009) and Wolman (1954) were modified and used. Each of the three 4 m^2 quadrats at each stream surveyed was divided into 25 X 25cm squares. Twenty five percent of the squares were randomly selected and quantified. Percent sand (< 0.2cm) gravel (0.3-4.5cm), course gravel (4.6- 6.4cm), cobble (6.5-26cm), boulder (>26cm), silt and clay, and vegetation were determined. Measurements are based on the longest axis (length) of the rock. Each of the above categories was then given a code number: 1=sand, 2=gravel, 3= course gravel, 4=cobble, 5=boulder, 6=silt and clay, and 7=vegetation. The dominant type of the above substrates was recorded for each square searched. In addition to the habitat quantification, an analysis of the two streams was done using the United States Geological Survey's (USGS) stream stats interactive map for Pennsylvania (USGS,2010).

Table 3

Field Survey Quadrat GPS Coordinates

Site	Quadrat 1	Quadrat 2	Quadrat 3
Fonner Run	N39° 57’ 41.0’’ W80° 15’ 25.4’’	N39° 57’ 42.7’’ W80° 15’ 24.7’’	N39° 57’ 45.3’’ W80° 15’ 23.5’’
Bates Fork	N39° 57’ 39.4’’ W80° 15’ 23.5’’	N39° 57’ 41.5’’ W80° 15’ 32.2’’	N39° 57’ 42’’ W80° 15’ 32.5’’

Table 3: GPS coordinates where the quadrats for the field surveys were placed. During each survey quadrats were placed as close to these locations as possible. GPS coordinates were determined by a Garmin GPSmap 60CSx.

Figure 2

Figure 2: Quadrat at Fonner Run. Illustrates the 2X2m quadrat used in field studies which extends one meter onto the land and one meter into the stream. Photo courtesy of Dr. Sarah Woodley.

Figure 3

Figure 3: Quadrat at Bates Fork. Illustrates the 2X2m quadrat used in field studies. Photo courtesy of Dr. Sarah Woodley.

Figure 4

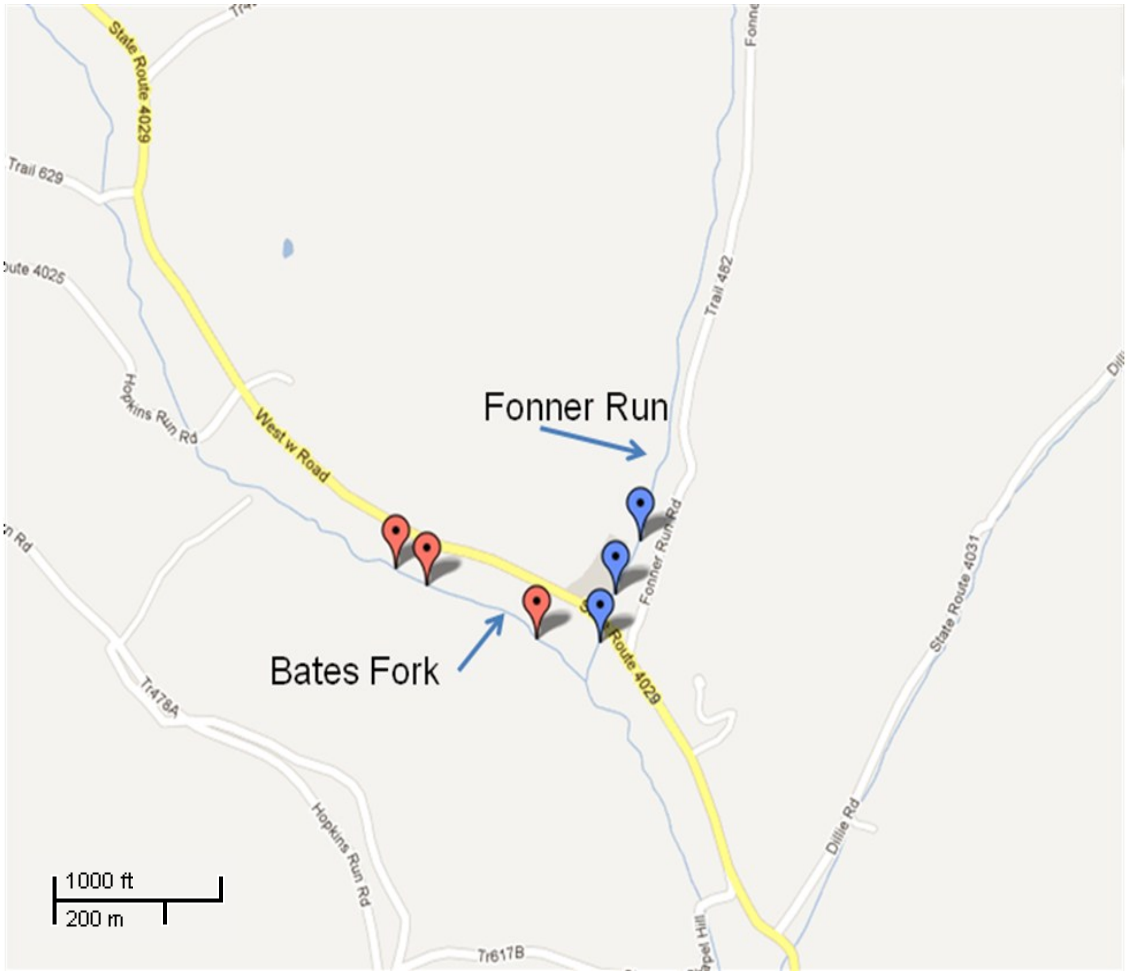

Figure 4: Image obtained from Google Maps, on 12/20/11. The pink pins show the quadrats searched at Bates Fork. The blue pins show the quadrats searched at Fonner Run. On the left is Bates Fork. On the right is Fonner Run.

Figure 5

Figure 5: Image obtained from Google Earth, obtained 4/21/2011. The yellow pins show the transects searched at Bates Fork Creek and Fonner Run. The green pins show the streams. On the left is Bates Fork Creek. On the right is Fonner Run.

STATISTICAL ANALYSIS

Statistical analyses were done using PAWS Statistics 18 (SPSS Inc.). All data analyzed satisfied the assumptions for parametric statistics (test of normality and test of homogeneity of variance).

Objective 1: Acute Exposure to Water with Elevated TDS

Acute activity and feeding were analyzed using a repeated measures ANOVA with water treatment as the within subjects factor and sex as the between-subjects factor.

Objective 2: Chronic Exposure to Water with Elevated TDS

Weight and Activity Data

Weight and activity were measured repeatedly during the course of the experiment. Therefore data were analyzed using a repeated measures ANOVA with date of sampling as the within-subjects factor. Sex and water treatment were the between-subject factors depending on the experiment. For chronic experiment 2, one male subject exposed to the Whitely Creek water was found moribund on the tenth day of the experiment and was therefore removed from the study and euthanized. His data excluded from the analysis. For chronic experiment 3, activity data were only analyzed for those days when overall activity was high and satisfied assumptions of parametric statistics. One animal in the distilled water control group for chronic experiment 3 died four days before the end of the experiment. The data for this animal was excluded from analysis. Data from animals that died were not analyzed because incomplete data sets cannot be analyzed in a repeated measures ANOVA.

Post-Euthanasia Data

Organ weights, white blood cell ratios, plasma corticosterone, and carcass water weight were analyzed using a univariate analysis of variance with both sex and treatment as between-subjects factors. Organ weight analysis was corrected for body weight at time of euthanasia by including body weight as a covariant in the analysis.

Objective 3: Baseline Field Survey

Habitat quantification data were analyzed by determining the mean, standard error of the mean, and mode for dominant substrates found at each quadrant searched at each stream. This type of habitat substrate analysis was used by The American Fisheries Society (Bain, 1999).

RESULTS

Objective 1: Acute Exposure to Water with Elevated TDS

There was no effect of treatment ($F_{5,85}$=1.8, P=0.11), sex by treatment interaction ($F_{5,85}$=0.41, P=0.83), or sex ($F_{1,17}$=2.9, P=0.11) on activity of subjects acutely exposed to water sources with different TDS. There was also no effect of treatment ($F_{5,85}$=0.34, P= 0.89), sex by treatment interaction ($F_{5,85}$=0.82, P=0.54), or sex ($F_{1,17}$=0.32, P=0.58) on feeding of animals acutely exposed to water with different TDS. Results are shown in figures 6 and 7.

Objective 2 : Chronic Exposure to Water with Elevated TDS

Chronic Experiment 1*: *Exposure to Whitely Creek Water, TDS 2000ppm

As stated above, chronic experiment 1 was halted due to unexpected deaths. Three subjects exposed to Whitely Creek water with a TDS of 2000 ppm died or were

Figure 6

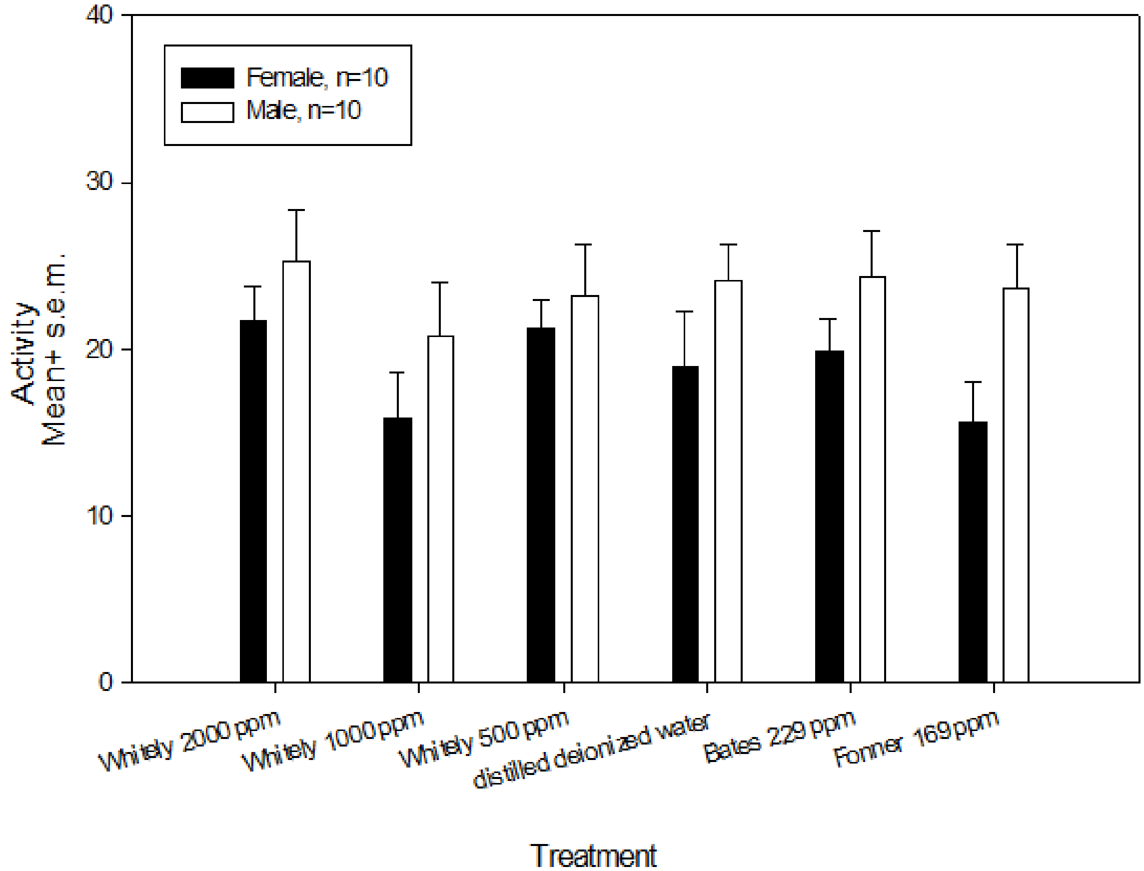

Figure 6: Levels of locomotory activity of salamanders during a 1 hour acute exposure to substrates moistened with different water stimuli. No significant difference in activity was found among exposure groups (P=0.113) or between sexes (P=0.838).

Figure 7

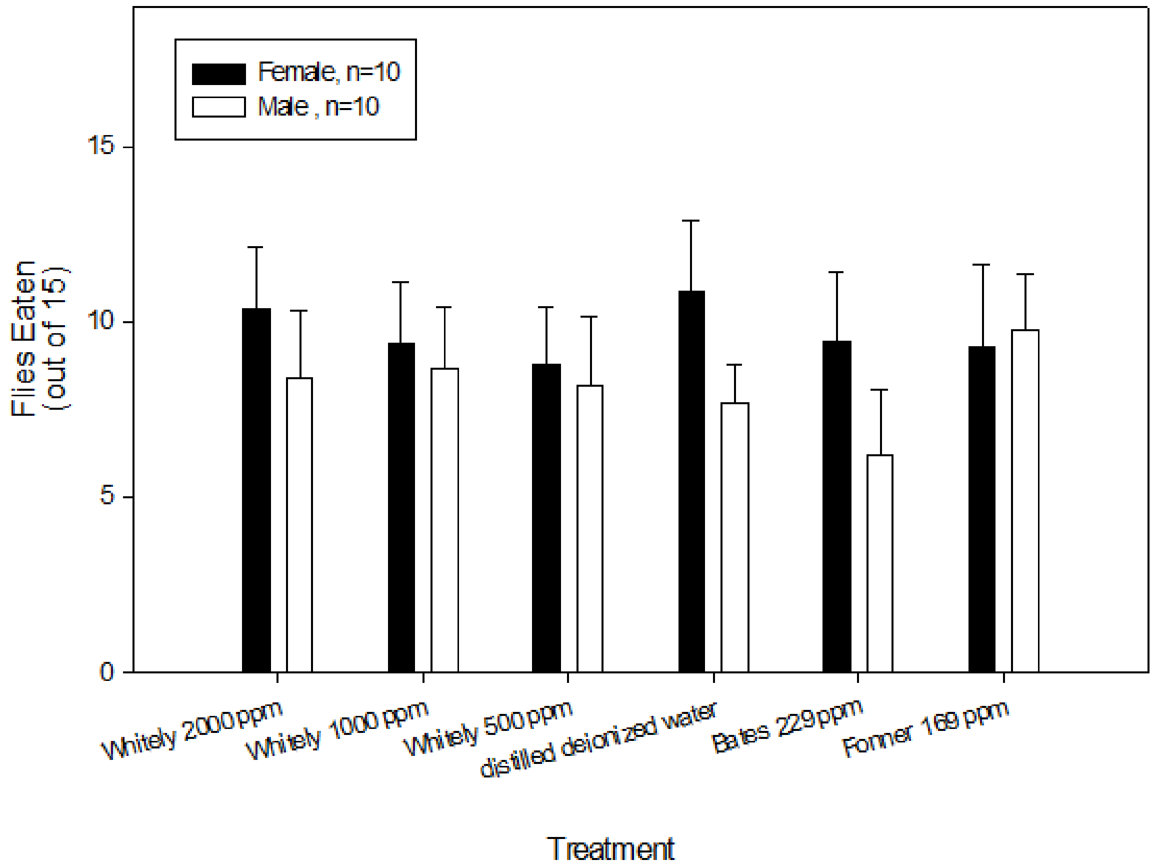

Figure 7: Flies eaten by salamanders after a 1 hour acute exposure to substrates moistened with different water stimuli. No significant difference was found among groups in flies eaten among sources (P=0.885) or between males and females (P=0.539).

moribund and therefore euthanized. A female subject in the high exposure group was lethargic on the second day and died on the third day. Another female subject in the high exposure group was moribund on the seventh day and had no righting response, gaping, and no gular flapping and was therefore euthanized. After a third death of a male in the high exposure group on the eighth day, the experiment was halted. A female control animal was found moribund on the tenth day with no righting response or movement and was euthanized. All other animals were monitored for an additional week and no further deaths occurred.

Chronic Experiment 2*: *Exposure to Whitely Creek Water, TDS 1000ppm

Body Weight

There was a significant difference in the percent change in body weight between subjects exposed chronically to Whitely Creek water (TDS of 1000 ppm) and controls ($F_{1,22}$=7.01, P=0.02). No sex by treatment interaction ($F_{1,22}$=1.1, P=0.30) or sex differences ($F_{1,22}$=1.2,P=0.28) were observed. There was an effect of date, indicating that animals lost weight over the course of the experiment ($F_{13,286}$=60.7, P=0.001) (Figure 8). Organ weight data was analyzed relative to the body weight at time of euthanasia. No significant effect of water treatment was found (Table 4), although, there was a significant sex by treatment interaction in the liver weights ($F_{1,21}$=4.4, P=0.05). There was no significant effect of water treatment ($F_{1,22}$=0.65,P=0.43) in the percent water weight of animal carcasses and no sex by treatment interaction ($F_{1,22}$=1.6, P=0.22).

Figure 8

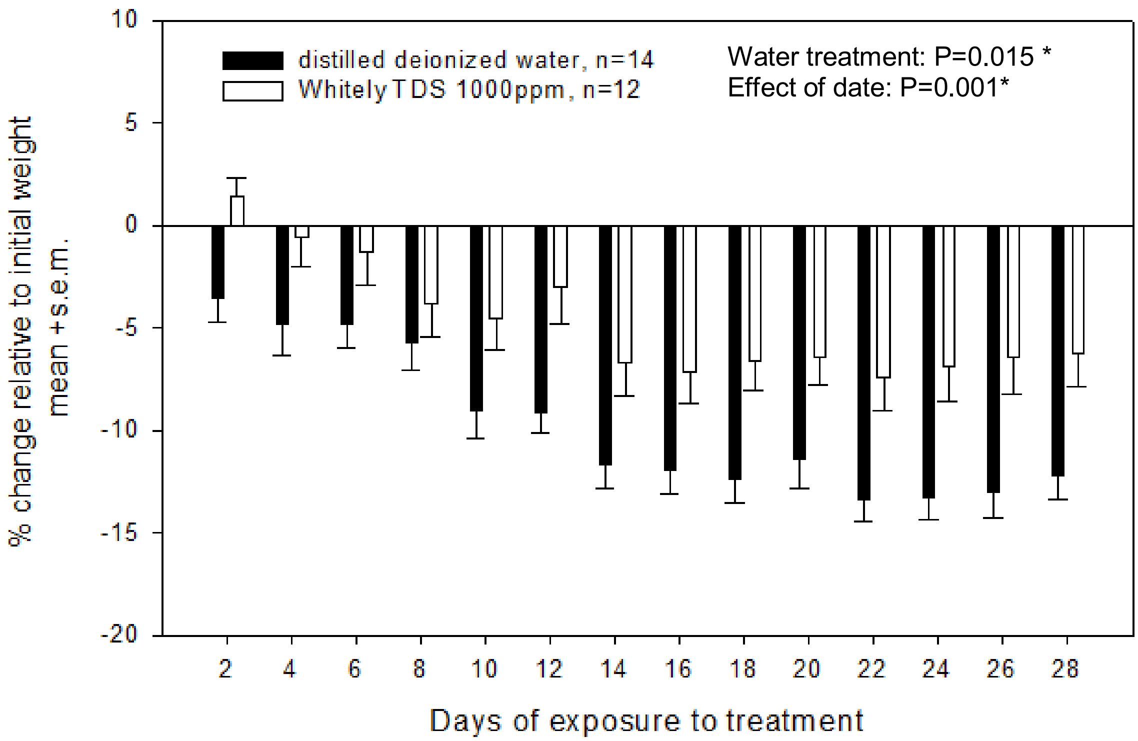

Figure 8: The average percent change in body weight relative to initial body weights of salamanders during chronic exposure to distilled deionized water and Whitely Creek water with a TDS of 1000 ppm. Animals exposed to Whitely Creek water lost significantly less body weight over the course of the experiment than did control animals (P=0.015). No treatment by gender interaction was observed. *indicates a significant effect.

Table 4

Organ Weight Data and Plasma Corticosterone Levels for Subjects Exposed Chronically to Whitely Creek Water (1000 ppm)

	Distilled Deionized Water (Control)			**Whitely Water with TDS 1000ppm (High)**			**Statistics**
	Mean	**s.e.m.**	**n**	**Mean**	**s.e.m.**	**n**	**P-value, treatment effect**
Fat Bodies*	2.13	0.24	13	1.45	0.34	12	0.260 $F_{1,20}$=1.3
Spleen*	0.07	0.01	14	0.12	0.04	11	0.122 $F_{1,20}$=2.6
Liver*	2.75	0.13	14	2.29	0.14	12	0.405 $F_{1,21}$=0.72
Corticoste-rone ug/l)	1.811	.550	14	.830	.273	12	0.13 $F_{1,23}$=2.5
Neutrophil: lymphocyte	0.085	0.023	13	0.037	0.016	7	0.44 $F_{1,20}$=0.62

Table 4: No significant difference in water treatment was observed in the organs measured, plasma corticosterone levels, or neutrophil lymphocyte ratio. *Organ weight is percent of body weight at time of euthanasia.

Locomotory Activity

There was a marginally significant effect of water treatment ($F_{1,22}$=3.9, P= 0.06) and a significant sex by treatment interaction ($F_{1,22}$=5.3, P=0.03) on locomotory activity. To better understand the sex by treatment interaction, males and females were analyzed separately. A significant effect of water treatment was observed in males ($F_{1,10}$=9.3, P=0.01) (Figure 9) but not in females ($F_{1,12}$=0.5,P=0.82) (Figure 10).

Corticosterone and White Blood Cell

Analysis of plasma corticosterone showed no significant effect of water treatment ($F_{1,23}$=2.5, P=0.13) or sex ($F_{1,23}$=0.90, P=0.35) (Table 5). There was also no effect of water treatment on the neutrophil:lymphocyte ratio ($F_{1,20}$=0.62, P=0.44).

Chronic Experiment 3: Chronic Exposure to Synthetic Ion Solutions and Blacklick Creek Water, TDS 1000 ppm

Comparison of Controls

This study included two types of controls: distilled deionized water and synthetic spring water. Two controls were used to determine whether differences in the presence or absence of minerals in the control solutions affected response variables. There was no significant difference in body weight ($F_{1,19}$=1.1,P=0.32) (Figure 11) or activity ($F_{1,20}$=1.2,P=0.29) (Figure 12) between controls. Because distilled deionized water is not a natural type of water, subsequent analyses used the synthetic spring water as the most appropriate control.

Body Weight

An initial analysis that included data from all dates revealed an interaction between water treatment and date of sampling ($F_{32,416}$= 2.1, P =0.001). Because the

Figure 9

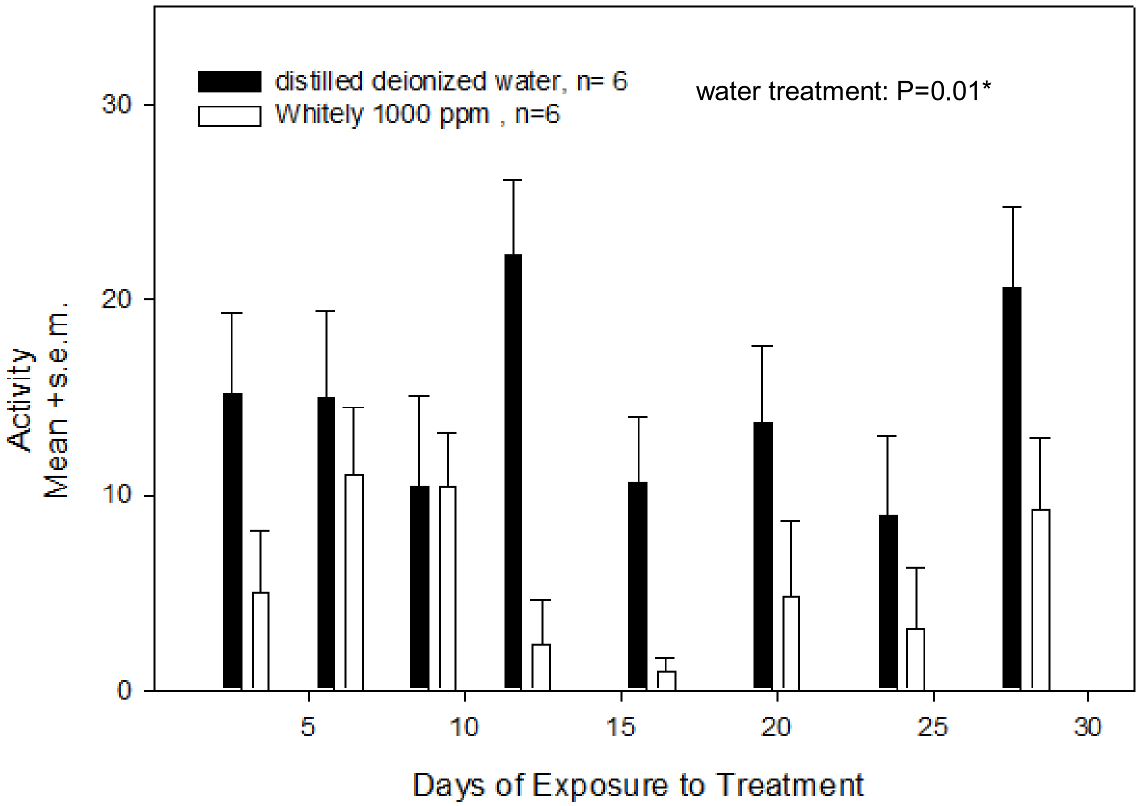

Figure 9: Levels of locomotory activity in male salamanders during chronic exposure to substrates moistened with distilled deionized water or Whitely Creek water with a TDS of 1000 ppm. Males exposed chronically to Whitely Creek water were significantly less active than were controls (P=0.01). *indicates a significant effect.

Figure 10

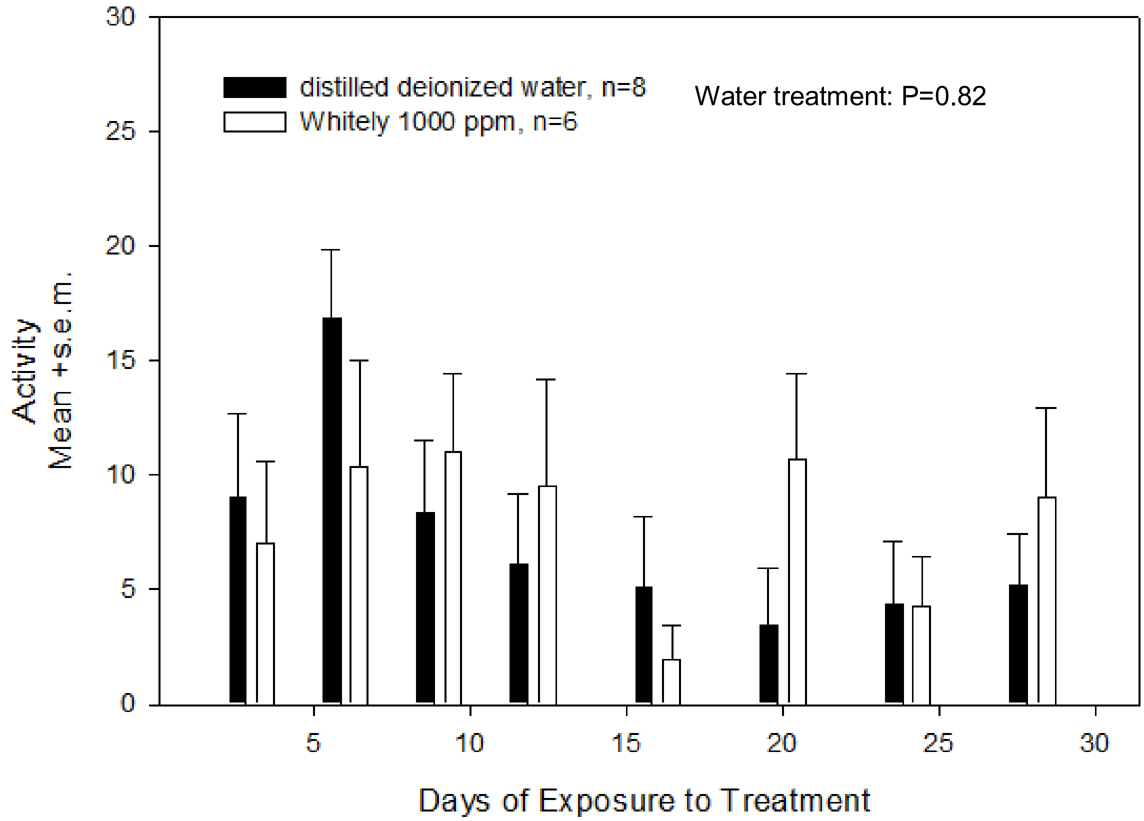

Figure 10: Levels of locomotory activity in female salamanders during chronic exposure to substrates moistened distilled water or Whitely Creek water with a TDS of 1000 ppm. No significant difference in the activity of females exposed to Whitely Creek water was observed (P=0.82).

Figure 11

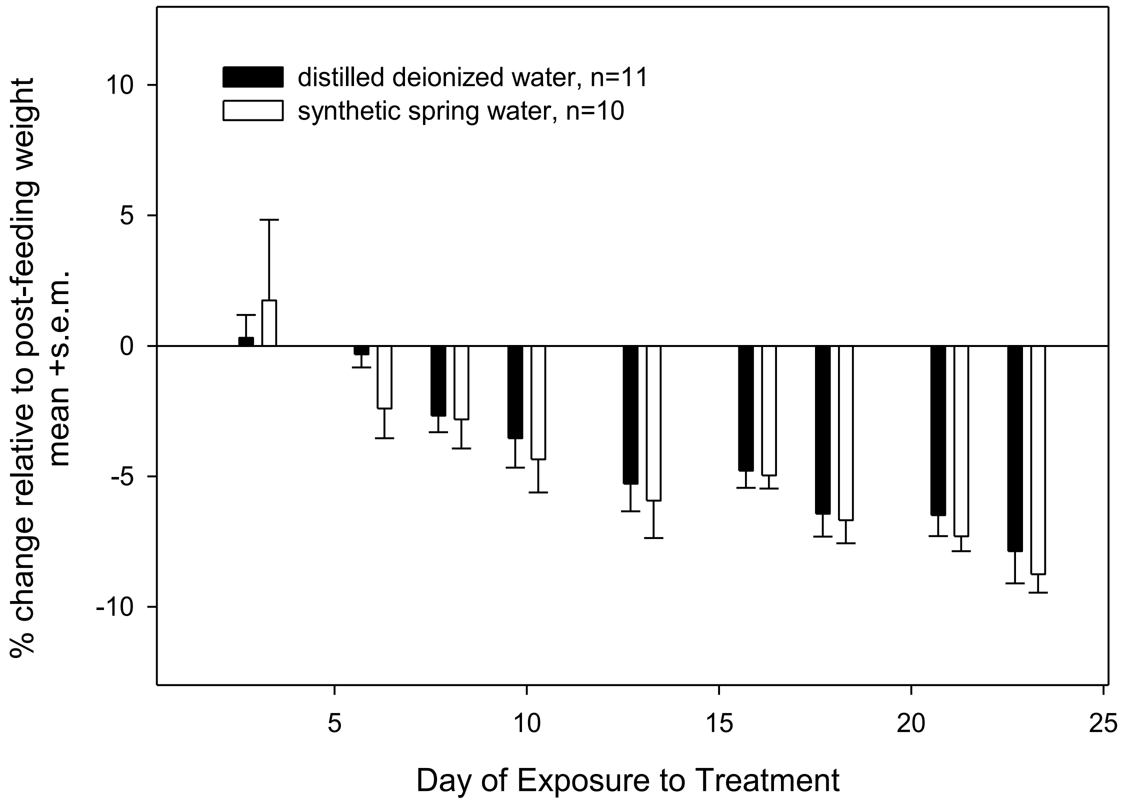

Figure 11: The average percent change in body weight relative to initial body weights of salamanders during chronic exposure to distilled deionized water and synthetic spring water. No significant difference in weight was observed between the two types of controls (P=0.32).

Figure 12

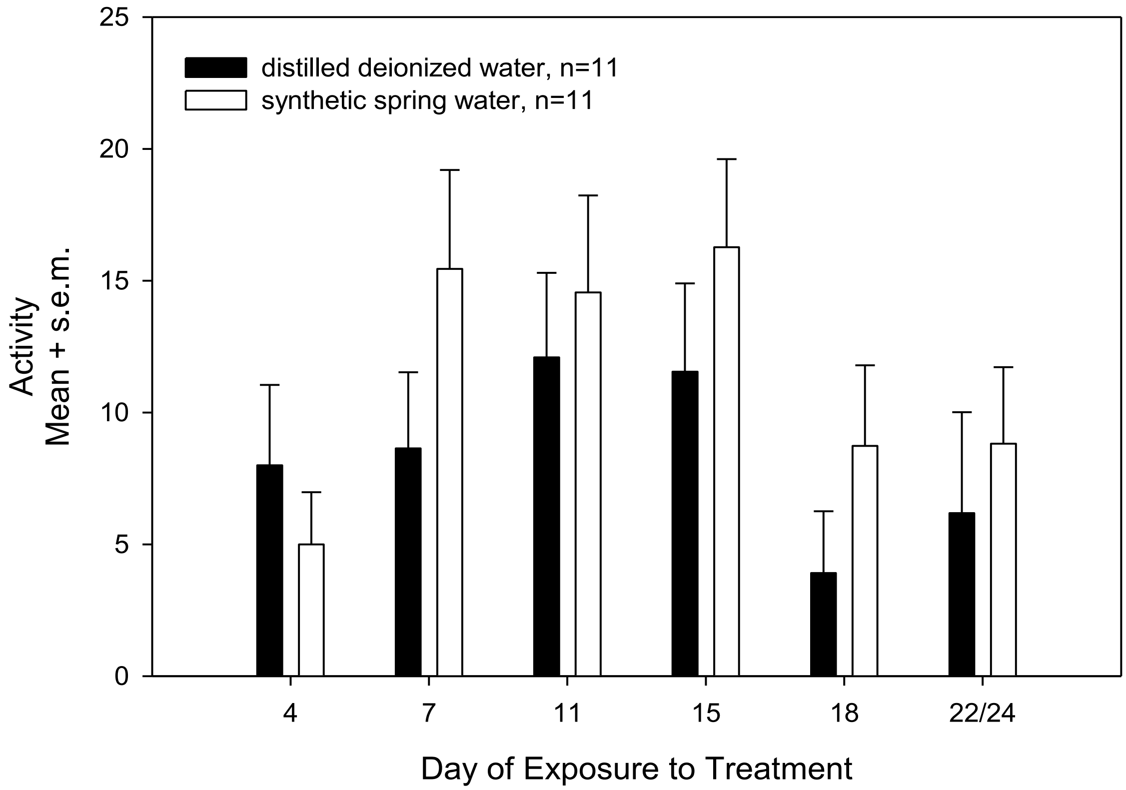

Figure 12: Levels of locomotory activity in salamanders chronically exposed to substrates moistened with distilled deionized water or synthetic spring water. No significant difference was observed in activity between subjects exposed to distilled deionized water versus synthetic spring water (P=0.29).

subjects were fed midway through the experiment, the data obtained before feeding was analyzed separately from data collected after feeding. There was a significant effect of water treatment ($F_{3,43}$ = 2.9, P = 0.045) on body weight before feeding. To better understand the water treatment effect, contrast tests were done comparing each water type to the control treatment of synthetic spring water. Subjects exposed chronically to Blacklick field water and synthetic Blacklick water lost less body weight (Blacklick field: P = 0.009; synthetic Blacklick: P = 0.025) than control subjects exposed to synthetic spring water (Figure 13). There was no difference in body weight loss between subjects exposed to synthetic Whitely and controls (P = 0.14) (Figure 13). As seen before, there was a significant effect of date on body weight ($F_{4,172}$ = 41.8, P = 0.001), indicating that animals lost body weight over the course of the experiment. However, there was no interaction between date and water treatment ($F_{12,172}$ = 0.80, P = 0.65) before feeding.

Finally, analysis of data from the final 4 sampling dates (after feeding) showed there was no effect of water treatment on body weight loss ($F_{3,42}$=1.5, P=0.22) nor a time by treatment interaction ($F_{3,42}$=0.85, P=0.47).

Locomotory Activity

Statistical analysis was restricted to data collected on days 7, 11, and 15 because data from these dates satisfied the assumption of parametric statistics test. However, as seen in Figure 13, a similar pattern of results was evident on all sampling dates. An initial analysis showed a marginally significant effect of treatment ($F_{3,43}$=2.3, P= 0.09), a marginally significant effect of date ($F_{2,86}$=2.5, P=0.09), and no treatment by date interaction ($F_{6,86}$=0.63, P=0.70).

Figure 13

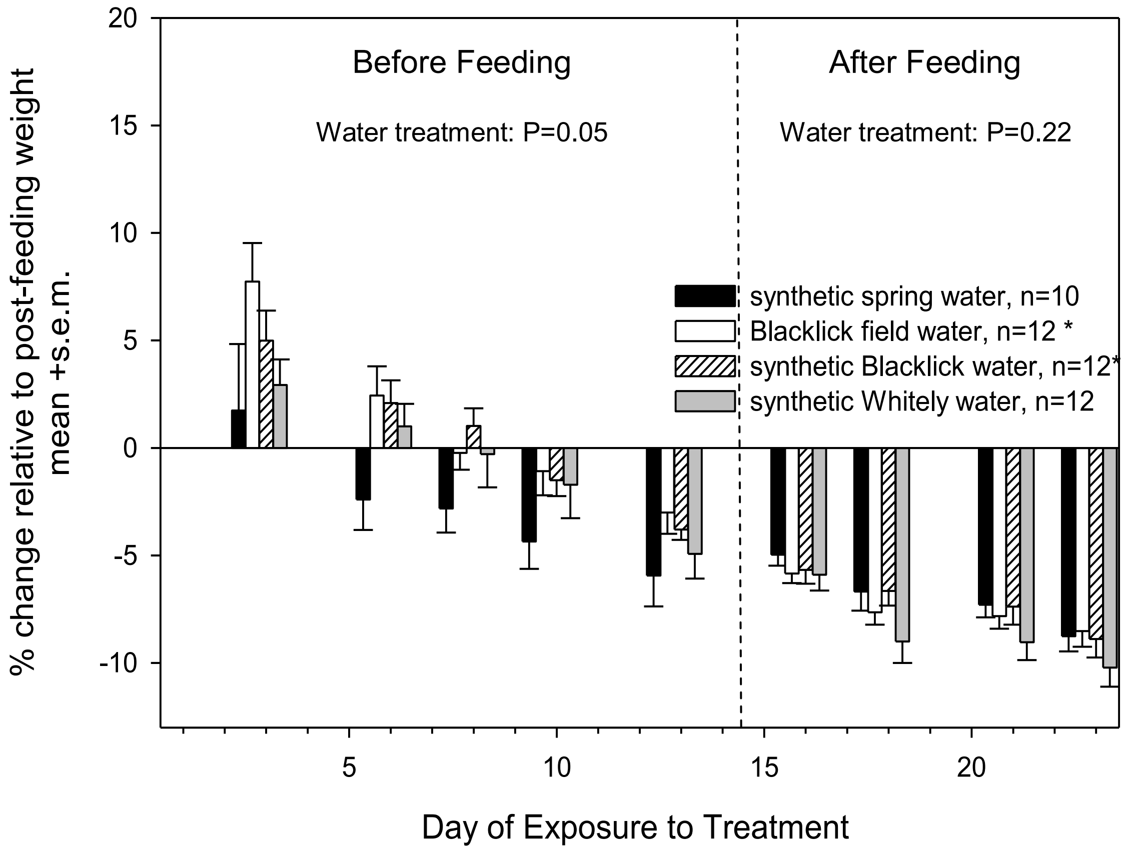

Figure 13: Percent change in body weight of salamanders during chronic exposure to substrates moistened with different water stimuli. Prior to feeding, there was a significant effect of water treatment on body weight. Compared to control subjects, animals chronically exposed to Blacklick field water (P=0.01) and to synthetic Blacklick water (P=0.03) lost significantly less body weight. After feeding there was no effect of water treatment (P=0.22). The date that salamanders were fed is represented by the dotted line. *indicates a significant difference relative to control subjects.

To better understand the marginal effect of water treatment on activity, each water type was contrasted to the control treatment of synthetic spring water. There was a significant difference of activity on synthetic Whitely Creek water ($P=0.02$) with subjects exposed to synthetic Whitely Creek water moving less than controls. (Figure 14). Levels of activity after exposure to Blacklick field water ($P=0.82$) and synthetic Blacklick water ($P=0.39$) were similar to activity of subjects exposed to controls.

Objective 3: Baseline Field Survey

Field Surveys

Salamander surveys were conducted at Bates Fork and Fonner Run on seven dates from September 2010 until October 2011. *Eurycea bislineata* (the two-lined salamander) was the most prevalent salamander found at either site, but one *Eurycea longicauda* (long tailed salamander) was found at Bates Fork and one *Plethodon glutinosus* (slimy salamander) was found at Fonner Run.

There were more *Eurycea. bislineata* at Fonner Run than at Bates Fork (average ± sem: 0.14 ±0.07 salamanders/m^2). The number of males and females at each site was comparable, but Fonner Run had over 3 times as many juveniles as Bates Fork Creek. There were also an average density of 1.4 +/- 0.57 larvae/m^2 found at Fonner Run and zero larvae found at Bates Fork Creek (Table 5). Figure 15 shows a larvae collected while surveying.

Habitat Quantification

The aquatic habitat (Figure 16) at Bates Fork Run was largely sand and gravel with little silt and clay (mean = 2.09+/-0.27, mode=2). Fonner Run's aquatic habitat was

Figure 14

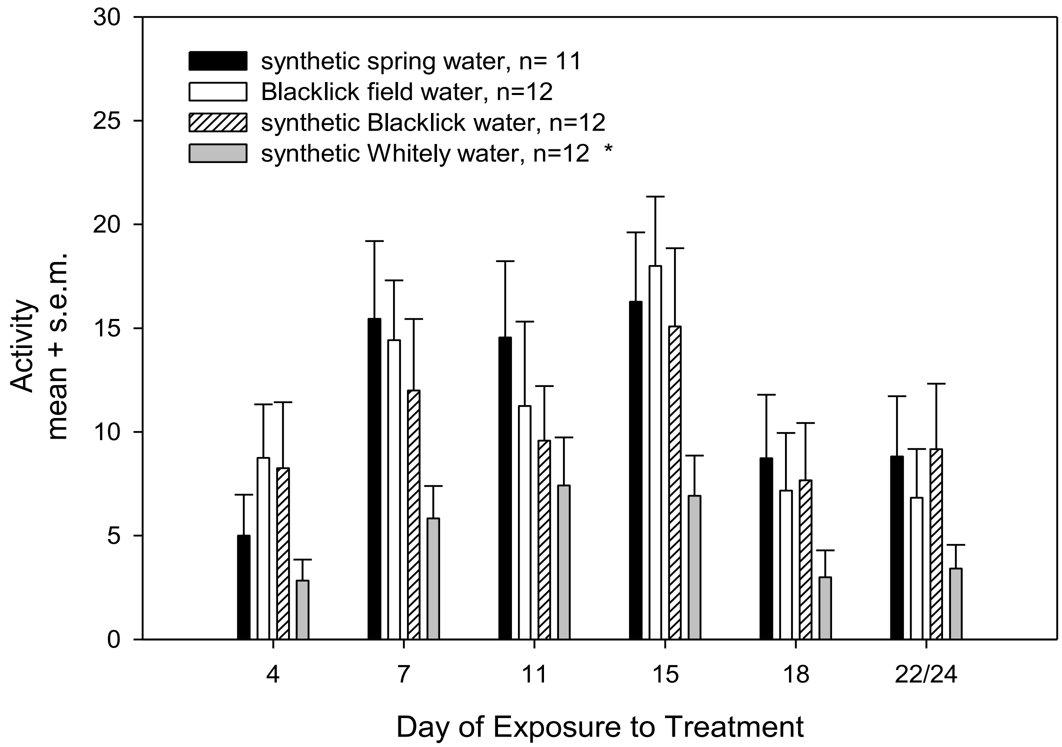

Figure 14: Levels of locomotory activity of salamanders exposed chronically to substrates moistened with different water stimuli. Compared to salamanders exposed to synthetic spring water, salamanders exposed to synthetic Whitely water were less active (P=0.02). No significant difference in activity was found between salamanders exposed to control and Blacklick field water (P=0.82) or the control and synthetic Blacklick water (P=0.39). * indicates a significant effect of treatment relative to controls.

Table 5

Euryceabislineata Abundance at Bates Fork and Fonner Run

	Bates Fork					
Date	M	F	Juv.	L	**Total**	**Density ($/m^2$)**
9/29/11	2	1	1	0	**4**	**0.33**
10/20/11	2	3	1	0	**6**	**0.5**
4/13/11	0	0	0	0	**0**	**0**
5/26/11	0	0	0	0	**0**	**0**
6/30/11	0	0	1	0	**1**	**0.08**
9/16/11	1	0	0	0	**1**	**0.08**
10/26/11	0	0	0	0	**0**	**0**
Mean +/- s.e.m.	0.71 +/- 0.36	0.57 +/-0.43	0.43 +/-0.20	0.0 +/-0.0	1.71 +/-0.89	0.14 +/-0.07
	Fonner Run					
Date	M	F	Juv.	L	**Total**	**Density ($/m^2$)**
9/29/11	0	1	3	3	**7**	**0.58**
10/20/11	5	3	3	1	**12**	**1**
4/13/11	0	0	1	0	**1**	**0.08**
5/26/11	0	0	0	1	**1**	**0.08**
6/30/11	1	0	2	0	**3**	**0.25**
9/16/11	0	1	2	1	**4**	**0.33**
10/26/11	0	0	0	4	**4**	**0.33**
Mean +/- s.e.m.	0.86+/- 0.70	0.71+/- 0.42	1.6 +/-0.48	1.4 +/-0.57	4.6 +/-1.46	0.38 +/-0.12

Table 5: M=adult male, F=adult female, Juv=juvenile, L= larvae

Figure 15

Figure 15: *Eurycea. bislineata* larvae collected during a field survey. Photo courtesy of Dr. Sarah Woodley.

a majority of sand and gravel, but also contained some cobble, boulders, and silt and clay (mean =2.58+/- 0.26, mode =2). The terrestrial habitat (Figure 17) at Bates Fork was nearly homogeneous sand and gravel with little silt and clay and grass (mean = 2.82+/- 0.46, mode = 2), whereas Fonner Run was primarily a heterogeneous mixture of silt and clay and grass with some sand and gravel, and little boulders and cobble (mean=4.96+/- 0.46, mode =7). Thus, overall, Bates Fork was determined to be largely composed of gravel and sand with little silt and clay, and grass, whereas Fonner Run was determined to be an intermediate mixture of all types of substrates. The USGS analysis of Bates Fork and Fonner Run showed that Fonner Run had a smaller drainage area (3.3 $miles^2$) than Bates Fork (6.1 $miles^2$). Also, Fonner Run's river basin was more heavily covered by forest (84%) than Bates Fork (61%)

DISCUSSION

Laboratory experiments showed that chronic exposure to water with elevated TDS (1000 ppm) had effects on both salamander locomotion activity and body weight, with the effect dependent on the ion composition. A TDS concentration of 1000 ppm represents a realistic, relatively low exposure. It should be noted that this TDS is believed to be hypotonic relative to salt levels in salamander plasma. Despite the relatively low levels of TDS, sub-lethal effects were observed. Chronic exposure to water from Whitely Creek and a synthetic solution mimicking the ions in Whitely Creek water both decreased male activity. Exposure to Blacklick Creek water at 1000 ppm and a solution mimicking ions in Blacklick Creek had no effect on salamander activity, but caused slower weight loss than controls. In contrast to the results of chronic exposure,

Figure 16

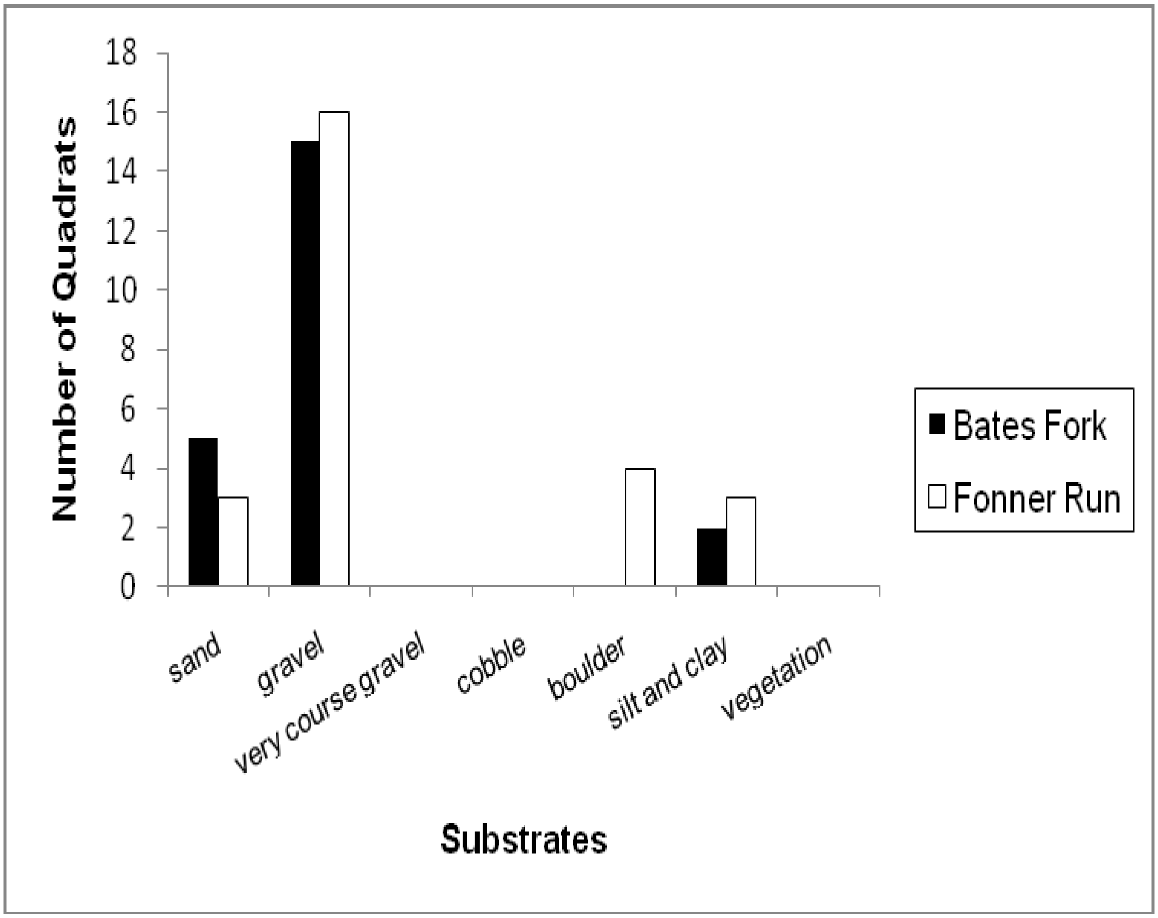

Figure 16: Aquatic habitat quantification. 1=sand (<0.2cm), 2=gravel (0.2-4.5cm), 3= very course gravel (4.6-6.4cm), 4=cobble (6.5-26cm), 5= boulder (26cm+), 6= silt and clay (silt and clay), 7=vegetation

Figure 17

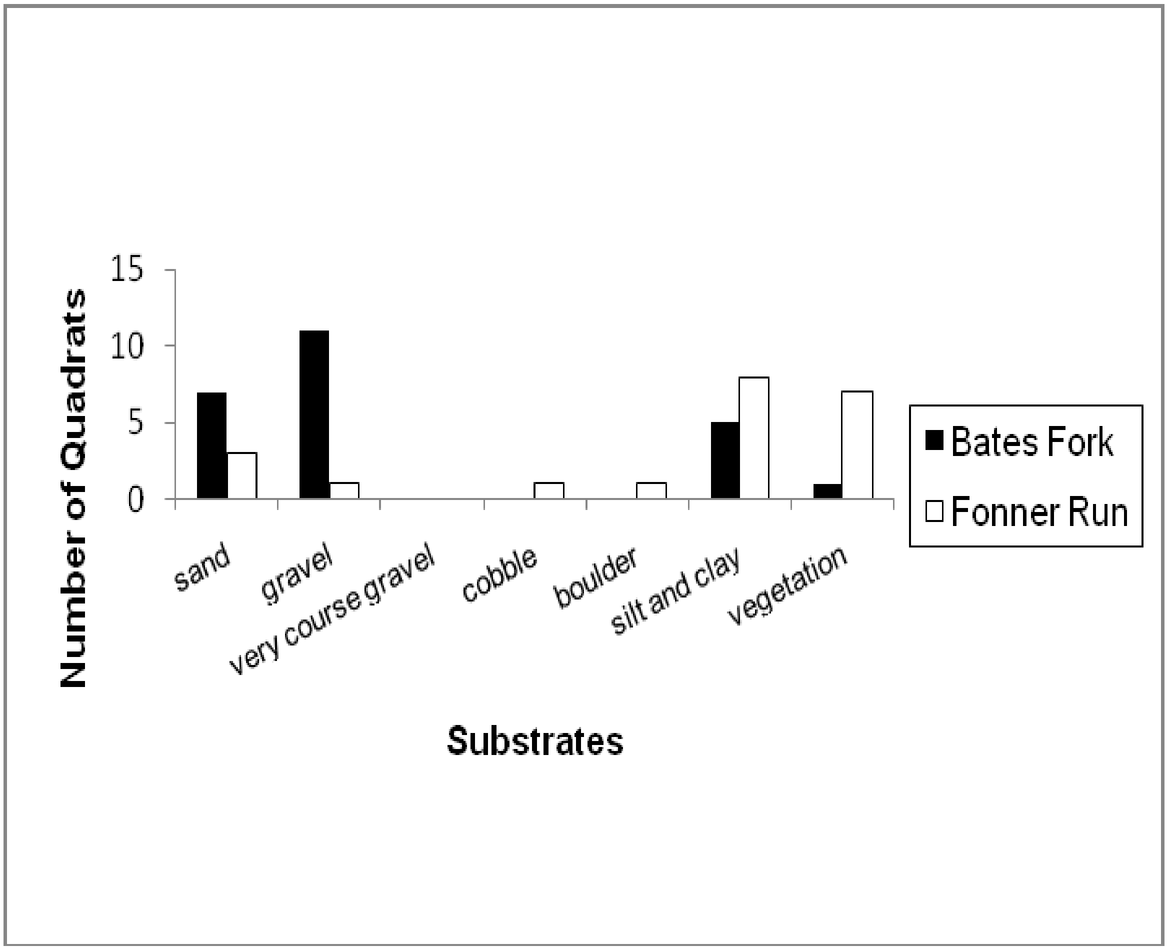

Figure 17 : Terrestrial habitat quantification. 1=sand (<0.2cm), 2=gravel (0.2-4.5cm), 3= very course gravel (4.6-6.4cm), 4=cobble (6.5-26cm), 5= boulder (26cm+), 6= silt and clay (silt and clay), 7=vegetation

acute exposure to water samples with elevated TDS had no effect on salamander locomotory activity or feeding. Finally, field surveys of salamander abundance were completed to provide baseline data with which to assess future impacts of potential TDS influxes to aquatic biosystems.

Acute Exposure:

Previous lab experiments have shown that activity and feeding are useful measurements of acute stress. For example when exposed to certain pesticides tadpoles, another amphibian, have been shown to decrease activity (Reylea and Edwards, 2010). Also the capture and handling of amphibians, including *Desmognathus* salamanders, resulted in reduced activity (Woodley and Lacy, 2010). A decrease in activity may result in a decreased ability to find mates or to forage, which might affect survivorship and recruitment to future generations. My results suggest that acute exposure to the water stimuli used in the acute experiment did not cause stress because acute exposure to elevated TDS had no effect on salamander activity. Thus, a sudden quick spike in TDS to levels approximating those used in the acute experiment may not alter salamander activity. This conclusion applies only to TDS concentrations and ionic compositions used in this study. Increasing the overall TDS concentration or changing the identity of ions present may have a different effect.

Chronic Experiment 1:

The first experiment that I conducted on the effects of chronic exposure to TDS used a concentration of 2000 ppm. The water was collected from Whitley Creek, a stream located in southwestern Pennsylvania. Overall, three animals exposed to elevated TDS and one control animal died. Due to these unexpected deaths, the experiment was halted because the experiment was meant to investigate sub-lethal effects of elevated TDS and was not designed to have death as an endpoint.

Chronic Experiment 2:

A second experiment was conducted in which animals were chronically exposed to a TDS of 1000 ppm. The same water collected from Whitely Creek for chronic experiment 1 was used, but diluted to a TDS concentration of 1000 ppm. Chronic exposure to Whitely Creek water at a TDS of 1000 ppm caused a statistically significant decrease of activity in males, but not females. The cause of the sex effect is unclear, but it should be noted that there was also a sex-specific effect on liver weights.

In this and the other chronic experiments, all animals lost body weight over the course of the experiment. For example, control subjects lost an average of about 12% body weight over the course of the one month experiment. It is possible that the static renewal design of the experiment was somehow stressful and caused the body weight loss. However, a previous experiment found that a similar static renewal design did not increase body weight loss compared to subjects not subjected to a static renewal of substrates (Fonner and Woodley, unpublished data). Thus, the decrease in body weight in all animals found in the current studies must have been due to the frequent weighing and behavioral testing. One possibility is that the recurrent exposure to room temperature when weighing and conducting behavioral tests may have increased metabolic rate and in that way reduced body weight. Future experiments should be conducted at a constant temperature to prevent an increase in the salamanders' metabolic rate.

Regardless of the overall changes in body weight, there was a significant effect of water treatment, such that salamanders exposed to Whitely Creek water lost less weight than did controls relative to body weight at the start of the experiment. The difference was apparent within two days of the start of the experiment. This rapid response suggests that some characteristic of the Whitely Creek water might have affected the salamanders' water balance and their ability to osmoregulate. However, post-euthanasia data analysis showed there were no significant differences between the treatment groups in the percent of carcass weight that was water when measured at the end of the experiment. This is perhaps due to the large amount of individual variation in body weights and carcass weight, precluding an ability to discern differences between groups. The removal of organs before determining carcass water content may also have altered the results.

The control used in this experiment was distilled deionized water. Since aquatic organisms are not normally exposed to distilled deionized water, it could be argued that differences reflected response to the lack of trace minerals in the water. This is unlikely because, as shown in chronic experiment 3, salamanders responded similarly to distilled deionized water as they did to synthetic spring water.

As with acute stress, locomotory activity is a useful indicator of chronic stress. Previous lab experiments showed that salamanders handled chronically had significantly lower activity than controls (Bliley and Woodley, submitted). Corticosterone is typically believed to play a role in regulating vertebrate responses to stress (Widmaier, and Strang, 2006). However, subjects exposed chronically to Whitely Creek water (1000 ppm TDS) had significantly decreased activity, with no change in plasma corticosterone levels. This suggests that corticosterone may not be the physiological mediator responsible for the decreased activity. Another potential marker of physiological stress is the neutrophil: lymphocyte ratio. The N:L ratio has been shown to increase with exposure to stress in amphibians (Davis et al. 2008). However, there was no effect of water treatment on the N:L ratio in this experiment.

To conclude, this experiment showed subjects exposed chronically to Whitely Creek water with a TDS of 1000 ppm lost weight slower and were less active than the controls. The Whitely Creek water used in this experiment was filtered to remove particulate matter. However, it likely contained other small contaminants such as hormones and pharmaceuticals in addition to the TDS. Thus, the results cannot be attributed to TDS on the basis of this experiment.

Chronic Experiment 3:

Chronic experiment 3 was designed to determine whether the effects of the Whitely Creek water on body weight and activity were due to the elevated TDS or to another aspect of the water. Thus, responses to a synthetic ion solution that mimicked the ion composition observed in the Whitely Creek water were examined. In addition, responses to water from a water source (Blacklick Creek) that also had elevated TDS, but of a different ionic composition than that observed in Whitely Creek was examined. Finally, to determine whether the use of distilled deionized water was an appropriate control to use, an additional control was included, synthetic spring water, which contained trace minerals.

Compared to exposure to synthetic spring water, chronic exposure to synthetic Whitely Creek water (1000 ppm TDS) caused a significant decrease in male activity, but had no effect on weight loss. This indicates that the TDS composition of Whitely Creek water had an effect on activity, but not body weight. Thus, the decreased activity in response to Whitely Creek field water seen in chronic experiment 2 was likely due, at least in part, to the elevated TDS. In contrast, the body weight changes in response to Whitely Creek field water were probably not due to the elevated TDS, but to some other factor.

Both the Blacklick Creek field water and the synthetic ion solution mimicking the ionic composition of Blacklick Creek water caused salamanders to lose weight slower than controls, but had no effect on activity. This suggests that the TDS composition of Blacklick Creek was responsible for the weight loss differences.

The effect of water treatment on body weight loss depended on the feeding status of the animals. That is, there was no difference in body weight loss in animals once they had been fed during exposure. A previous study found that feeding alleviated the detrimental effects of exposure to hypertonic salinities in tiger salamanders (Romspert and McClanahan,1981). Amphibians exposed to hypersaline condition use urea to increase body osmolarity to reduce dehydration. Fed animals could use urea from food sources whereas unfed animals used urea derived from protein catabolism which could potentially affect body weight. Thus perhaps something about the TDS affected urea production and thereby body weights. Future experiments should adjust feeding schedule to prevent feeding during the experiment.

The differential effects of Whitely Creek versus Blacklick Creek water highlight an important concept when studying biological effects of TDS. The ionic solutions mimicking Blacklick Creek and Whitely Creek had the same concentration, but different ionic compositions. The main differences in ionic compositions were in the amount of the anions sulfate and chloride in each source. Blacklick Creek water possessed high levels of chloride and sodium whereas Whitely Creek water was largely sulfate and sodium. Blacklick Creek also contained small, but detectable amounts of lithium, iron, barium, and bromide while Whitely Creek did not. Blacklick Creek is likely an example of water impacted by Marcellus shale activity whereas Whitely Creek is likely an example of water impacted by abandoned mine drainage (AMD). The difference in the effects of these two samples of water suggests that water impacted by Marcellus activity may have a different impact on salamanders than water impacted by AMD. It is important to remember the results from this study may not be the same for a solution with the same concentration, but different ions or the same ions but a different concentration.

Part of chronic experiment 3 was also to determine whether the type of control water (distilled deionized water versus synthetic spring water) affected responses. Synthetic spring water has a low concentration of ions where as distilled deionized water has almost zero. Results indicated that there was no significant difference on activity or weight loss between the two types of controls. Subsequent analyses used synthetic spring water control because it more accurately represents the type of water salamanders are exposed to in the wild. As explained above, the results validated the finding from chronic experiment 2, in which distilled deionized water was used as the control.

Field Surveys:

More *Eurycea. bislineata* salamanders were found in Fonner Run than in Bates Fork. Differences in abundance could be due to a number of factors, such as the differences in habitat that I documented, or the differences in the amount of upstream Marcellus Shale related activity.

The baseline data will be useful to assess potential future impacts of TDS and Marcellus Shale drilling. If drilling continues to occur upstream of Bates Fork, baseline data can be used to track any changes to salamander populations. Should drilling begin above Fonner Run, the baseline data can be used to observe any impacts on salamander populations. Thus, field surveys should be continued at these locations.

CONCLUSION

Results showed that chronic exposure to relatively low levels of TDS altered salamander activity and body weight, although the affects were dependent on the composition of the ions present. Future experiments should continue examining effects of different ion compositions and levels of TDS on salamanders both in the laboratory and the field. It would be useful to determine the thresholds at which particular ion compositions are detrimental. Studies should also determine whether there are other effects of these ion compositions on amphibians and whether other organisms found in stream habitats are similarly affected.

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APPENDIX A – HABITAT QUANTIFICATION RAW DATA

Bates Fork									
Terrestrial (t) /aquatic (a)	dominant substrate	% sand	%gravel	% course gravel	% cobble	% boulders	% silt and clay	% vegetation	% roots
Quadrat 1 (N39° 57'39.4'' W80° 15' 28.2'')									
a	1	70	25		5			5	
a	1	60	20		10			10	
a	1	60	25		15				
a	2	5	85		10				
a	2	25	70		5				
a	1	75	20					5	
a	2	20	70		10				
a	2	20	80						
a	2	40	60						
t	1	70	20					10	
t	1	40	30				15	15	
t	1	40	35	5	17			3	
t	1	70	10		10			10	
t	1	60	25		15				
Quadrat 2 (N39° 57' 40.7'' W80° 15' 30.5'')									
a	2	10	60	10	20				
a	2	10	80		10				
a	2	10	75	5	10				
a	2	5	80	10	5				
a	2		95		5				
a	2	5	65	30					
t	1	40	35	10	15				
t	2	35	50		13			2	
t	2	14	50	5	20		10	1	
t	1	70	20		5			5	
t	2	10	70	17				3	
t	2	5	70	10	15				
t	2	8	60	10	20			2	
t	7	10	40					50	
t	2	5	80	3	7				
t	2	10	65	10	15				
Quadrat 3 (N39° 57' 42'' W80° 15' 32.3'')									
a	2	25	65	3	7				
a	6	5	30	25			40		
a	2	10	90						
a	1	50	20	10			20		
a	2	5	90				5		
a	6		15				85		

a	2		90	5			5		
t	6						75	25	
t	2	20	80						
t	2		60	10			30		
t	2		75				25		
t	6		6				64	30	
t	6			5			90	5	
t	6						80	20	
t	6		10				80	10	
t		10	80		10				

1=sand, 2=gravel,3= course gravel, 4=cobble, 5=boulder, 6=silt and clay, 7=grass, 8= roots

Fonner Run									
Terrestrial (t) /aquatic (a)	dominant substrate	% sand	%gravel	% course gravel	% cobble	% boulders	% silt and clay	% vegetation	% roots
Quadrat 1 (N39° 57’ 41.0’’ W80° 15’ 25.4’’)									
a	5					100			
a	6		5			95			
a	2		100						
a	1	90	10						
a	1	75	25						
a	5	10	35		55				
a	5	5	30		65				
a	2	5	90				5		
a	2		70	5	25				
a	1	90		10					
t	5	5				85			10
t	6.5						50	50	
t	6		10				50	40	
t	1	60			10		30		
t	1	50	15				30		
t	1	40		20			25	5	10
Quadrat (N39° 57’ 42.8’’ W80° 15’ 24.8’’)									
a	5		5		25	70			
a	2		60		10			30	
a	2		40		30			30	
a	2		60				40		
a	2		45		20		35		
a	2		100						
a	2		80		20				
a	6				30		70		
a	2		60		40				
t		20	30		20			30	

t	6.5		20				40	40	
t	6		5				50	45	
t	6.5						50	50	
t	4				50		25	25	
t	6		10				80	10	
t	2		50				20	20	
Quadrat 3 (N 39° 57’ 45.4’’ W80° 15’ 23.5’’)									
a	2	5	70	10	15				
a	2		80					20	
a	2	10	70		20				
a	6		30		70				
a	2		90		10				
a	2		60	10			30		
a	2	15	75		10				
t	7		20				20	60	
t	7		20				20	60	
t	6	20	10				40	30	
t	7		35				25	40	
t	7		10				40	50	
t	7		10				40	50	
t	6.5		20				40	40	
t	6		20	3	7		50	20	
t	6		35				45	20	

1=sand, 2=gravel,3= course gravel, 4=cobble, 5=boulder, 6=silt and clay, 7=grass, 8= roots