Use of an environmentally realistic laboratory test organism and field bioassessments to determine the potential impacts of active coal mining in the Dumps Creek subwatershed on the Clinch River, Virginia

by

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ABSTRACT

This research was divided into four objectives for assessing the impacts of coal mining on ecosystem health. The first objective was to provide an ecotoxicological assessment in the upper Clinch River using standard bioassessment techniques. Analysis of sediments and interstitial water (porewater) indicate higher concentrations of trace metals in samples from sites located above both a power plant (CRP) and Dumps Creek mining influences. The furthest sampling site located near Pounding Mill, Virginia (CR-PM) had higher concentrations of aluminum (2,250.9 mg/kg), copper (5.9 mg/kg) and iron (12,322.6 mg/kg) compared to samples collected directly below the Dumps Creek confluence (site CR-2). Similar results were obtained from bioaccumulation *in-situ* tests with the Asian clam (*Corbicula fluminea*) in 2009. Aluminum (7.81 mg/kg), Fe (48.25 mg/kg) and Zn (7.69 mg/kg) were accumulated in higher concentrations at CR-PM site than CR-2. However, the site located below the CRP effluent discharges (CR-3L) on the left bank had substantially higher concentrations of Al (14.19 mg/kg), Cu (6.78 mg/kg), Fe (88.78 mg/kg) and Zn (7.75 mg/kg) than both CR-PM and samples collected directly opposite of this site at CR-3R

To further understand the potential impact active mining on the Clinch River, a more comprehensive ecotoxicological evaluation was conducting in the Dumps Creek subwatershed. Field bioassessments determined that biological impairment occurred directly below a deep mine discharge (CBP 001), which was characterized by a distinct hydrogen sulfide odor. Total

abundance and richness of benthic macroinvertebrates decreased to 3.5-20 and 1.25-2.3, respectively at DC-1 Dn. The discharge also caused the proliferation of a sulfur-oxidizing bacterium, *Thiothrix nivea*. During continuous discharge of the effluent, the bacteria was observed coating all surfaces at DC-1 Dn and may also contribute to an Fe-encrusted biofilm observed on *in-situ* clams at downstream site, DC-2 Dn. Toxicity tests with mining effluents indicate some potential toxicity of the 001 discharge, but this was variable between test organisms.

Selecting the most appropriate test species for sediment and water column assays has been a primary goal for ecotoxicologists. Standard test organisms and established test guidelines exist, but US EPA recommended species may not be the most sensitive organisms to anthropogenic inputs. Therefore, Chapter Three and Four addressed the use of mayflies in routine laboratory testing. Preliminary results of toxicity tests with the mayfly, *Isonychia sp*. (Ephemeroptera) suggested that *Isonychia* were moderately sensitive to NaCl after 96-hr with an average LC_{50} value of 3.10 g NaCl/L. When exposed to a coal-mine processed effluent, *Isonychia* generated LC_{50} values that ranged from 13 to 39% effluent and were more sensitive to the effluent than *Ceriodaphnia dubia*. Based on results of the feasibility study in presented in Chapter Four, field collected organisms appear to be too unpredictable in test responses and therefore, such tests would be unreliable as stand-alone indicators of effluent toxicity.

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Table of Contents

ABSTRACT ii
Acknowledgments iv
Table of Contents v
List of Tables viii
List of Figures xi
General Introduction 1
Literature Cited 9

Chapter 1. An Evaluation of the Clinch River below Carbo, Virginia to determine the Effects of Past and Present Mining Activities on Freshwater Mussels (Bivalvia: Unionidae) 14
Introduction 15

Materials and Methods 18

Study Location 18
Water and Sediment Quality 19
Habitat Assessment 20
Benthic Macroinvertebrate Surveys 20
Asian Clam *In-Situ* Studies 21
Interstitial (Pore) Water Sampling and Analysis 23
Statistical Analysis 23

Results 24

Water and Sediment Quality 24
Interstitial (Porewater) Analysis 25
Habitat Assessment 26
Benthic Macroinvertebrate Surveys 26
Asian Clam *In-Situ* Studies 29

Discussion 31

Summary and Conclusions 39

Acknowledgements 40

Literature Cited 40

Chapter 2. An Ecotoxicological Assessment of the Dumps Creek Watershed to Determine Potential Impacts from Active and Historical Coal-Mining Activities on Aquatic Biota 63
Introduction 64

Materials and Methods 67

Site Locations 67
Habitat Assessment 67
Water and Sediment Quality 67
Benthic Macroinvertebrate Surveys 69
Asian Clam *In-Situ* Studies 70
Interstitial (Pore) Water Sampling and Analysis 71
Toxicity Testing 72
Sediment Toxicity Tests with *Daphnia magna* 76
Stream Condition Index Scoring Methodology 77
Statistical Analysis 78
Results 79
Habitat Assessment 79
Water Quality 80
Sediment Quality 85
Benthic Macroinvertebrate Surveys 87
Asian Clam *In-Situ* Studies 97
Interstitial (Pore-water) Analysis 107
Toxicity Testing of Mining Effluents 108
Sediment Toxicity Tests with *Daphnia magna* 120
Evaluation of an Epibiotic Bacterium 123
Discussion 124
Summary and Conclusions 134
Acknowledgments 134
Literature Cited 134
Appendix I. Benthic Macroinvertebrate Data 180
Appendix II. Multiple Comparisons 184
Chapter 3. Preliminary Results of Laboratory Toxicity Tests with the Mayfly, *Isonychia bicolor* (Ephemeroptera: Isonychiidae) for Development as a Standard Test Organism for Evaluating Streams in the Appalachia Coalfields of Virginia and West Virginia 188
Introduction 189
Materials and Methods 191
Description of the test organisms 191

Isonychia toxicity tests 192
Ceriodaphnia dubia toxicity tests 194
Trace metal analysis of CPI effluent 195
Statistical analysis 195
Results and Discussion 195
Isonychia reference tests with sodium chloride 195
Isonychia sp. CCW CPI effluent tests 198
Summary and Conclusions 199
Literature Cited 201
Chapter 4. Seasonal Availability and Sensitivity of Two Field Collected Mayflies for the Development of a Standardized Toxicity Test: A One Year Feasibility Study 213
Introduction 214
Materials and Methods 216
Test Organisms 216
Collection of Test Organisms 217
Laboratory Acclimation 218
Toxicity Testing 218
Statistical Analysis 219
Results 220
Collection of Test Organisms 220
Laboratory Acclimation 221
Toxicity Testing 228
Summary and Conclusions 237
Acknowledgments 241
Literature Cited 242

List of Tables

Table 1.1. Clinch River sampling site locations and corresponding GPS coordinates. **46**
Table 1.2. Mean values (± standard deviation) for water chemistry parameters at Clinch River sites. Water samples were collected from November 2007 through November 2009. **47**
Table 1.3. Mean total recoverable metals (± SD) in Clinch River site sediments collected during summer 2008 and 2009. Differences in means compared using Tukey's HSD (α= 0.05). **48**
Table 1.4. Total concentrations (µg/L) of select metals in interstitial water samples from Clinch River sampling sites. **49**
Table 1.5. Habitat assessment scores at Clinch River study sites including one site in the Guest River. Scores were based on a 200-point scale. **50**
Table 1.6. Qualitative benthic macroinvertebrate samples taken at sampling sites in the Clinch River. **51**
Table 1.7. Average concentrations of select metals (mg/kg) in Asian clam (*Corbicula fluminea*) tissue samples from Clinch River sampling sites after 60 days *in-situ*. Site location for CR-1 was moved downstream from Cleveland, VA during the 2009 study. **52**
Table 1.8. Estimated Bioaccumulation Factors (BAF) for determining the relationship between select metals in clam tissues (C_t, mg/kg wet wt.) and sediments (C_s, mg/kg dry wt.) from sampling stations in the Clinch River. **53**
Table 1.9. Estimated Bioconcentration Factors (BCF) to determine the relationship between accumulated metals in Asian clam (*Corbicula fluminea*) tissues and metal concentrations in interstitial water (porewater). Tissue and water concentrations expressed as moles per unit (g, ml). **54**
Table 2.1. Routine sampling locations in Dumps Creek and Hurricane Fork, Russell County, Virginia. **141**
Table 2.2. Benthic macroinvertebrate metrics used for Stream Condition Index scoring according to Virginia and West Virginia guidance documents (Map Tech, Inc. 2003 and 2000, respectively). **142**
Table 2.3. Average water chemistry measurements for select parameters from 2007-2010 for sampling sites in the Dumps Creek watershed. **143**
Table 2.4. Average trace metal concentrations of water column samples collected during summer 2010. Highlighted values indicate measurements were above the National Recommend Water Quality Criteria (US EPA 2009). **144**
Table 2.5. Average sediment trace metal concentrations for Dumps Creek watershed sampling sites from 2008-2010. Highlighted values indicate exceedence of the US EPA Mid-Atlantic Risk Assessment Sediment Screening Benchmarks for Ecological Risk Assessments. **145**
Table 2.6. Community Loss Index (CLI) for Dumps Creek sampling sites in 2010. **146**
Table 2.7. Virginia Stream Condition Index (VA SCI) scores generated for winter benthic macroinvertebrates samples in 2009 and 2010 and comparison to summer scores. Site identifications were provided as used for the TMDL study (Map Tech 2004) and the current study. **146**
Table 2.8. (a) Bioassessment results for winter 2009 sampling in Dumps Creek and (b) corresponding biometric scores and biological condition ratings. **147**
Table 2.9. (a) Bioassessment results for winter 2010 sampling in Dumps Creek and (b) corresponding biometric scores and biological condition ratings. **148**

Table 2.10. Mean Asian clam (*Corbicula fluminea*) growth after 60 days *in-situ* at select sites in the Dumps Creek subwatershed. **149**
Table 2.11. *Corbicula fluminea* tissue concentrations of aluminum after 60 days *in-situ.* **150**
Table 2.12. *Corbicula fluminea* tissue concentrations of copper after 60 days *in-situ.* **151**
Table 2.13. *Corbicula fluminea* tissue concentrations of iron after 60 days *in-situ.* **152**
Table 2.14. *Corbicula fluminea* tissue concentrations of zinc after 60 days *in-situ.* **153**
Table 2.15. Mean concentrations of additional analytes included in the 2010 analysis of *Corbicula fluminea* tissues after 60 days *in-situ*. **154**
Table 2.16. Bioconcentration Factors (BCF) for metal accumulation in Asian clam (*Corbicula fluminea*) tissues compared to water column concentrations. **155**
Table 2.17. General water chemistry for porewater samples collected during summer 2008. **155**
Table 2.18. Trace metal concentrations of porewater samples collected at available sites in Dumps Creek and Hurricane Fork during summer 2008. **156**
Table 2.19. Survival (%) and growth (mm) of *Hyalella azteca* (amphipod) and *Villosa iris* (unionid) after 28 days of exposure to two coal mining effluents. **156**
Table 2.20. Distribution of male versus female *Hyalella azteca* in the 28-day chronic toxicity test. **157**
Table 2.21. *Villosa iris* Moss 2 (019) chronic test summary water chemistry without sediment. **157**
Table 2.22. *Villosa iris* Moss 2 (019) chronic test summary water chemistry with sediment substrate. **158**
Table 2.23. *Hyalella azteca* Moss 2 (019) chronic test summary water chemistry. **158**
Table 2.24. *Villosa iris* Moss 3 (003) chronic test summary water chemistry without sediment. **159**
Table 2.25. *Villosa iris* Moss 3 (003) chronic test summary water chemistry with sediment substrate. **159**
Table 2.26. *Hyalella azteca* Moss 3 (003) chronic test summary water chemistry. **160**
Table 2.27. Survival and growth of *Villosa iris* and *Hyalella azteca* after 28 days of exposure to the coal mining effluent, Moss 3 004. Control water was obtained from the Columbia Environmental Research Center (CERC) in Columbia, Missouri. **161**
Table 2.28. Water chemistry measurements from the *Villosa iris* Moss 3 (004) 28-day chronic toxicity test. **162**
Table 2.29. Water chemistry measurements from the *Hyalella azteca* Moss 3 (004) 28-day chronic toxicity test. **163**
Table 2.30. Chronic, 10-day sediment toxicity test with *Daphnia magna* survivorship and mean reproduction. Sample and control reproduction comparisons made using Dunnett's Method (α=0.05). **164**
Table 2.31. Summary of mean water chemistry of renewal water for the *Daphnia magna* sediment toxicity test, March 2010. **164**
Table 2.32. Average conductivity and dissolved oxygen measurements for sediment test out-water. **165**
Table 2.33. Chronic, 10-day sediment toxicity test with *Daphnia magna* survivorship and mean reproduction. Sample and control reproduction comparisons made using Dunnett's Method (α=0.05). **165**
Table 2.34. Trace metal concentrations (mg/kg) in site sediments versus test sediments after 10-day toxicity test with daily changeovers. **166**

Table 2.35. Least Squares Fit model responses for daphnid survivorship (%), mortality (# dead) and reproduction. Analysis conducted in JMP 8.0 (SAS Inst., 2010). **167**
Table A1. Summary of benthic macroinvertebrate data collected summer (July) 2008 from the Dumps Creek subwatershed. **181**
Table A2. Summary of benthic macroinvertebrate data collected summer (June) 2009 from the Dumps Creek subwatershed. **182**
Table A3. Summary of benthic macroinvertebrate data collected summer (June) 2010 from the Dumps Creek subwatershed. **183**
Table 3.1. Comparison of test organism lethal concentration (LC_{50}) values for sodium chloride (g NaCl/L) at 24, 48, 72 and 96 hr. **206**
Table 3.2. *Isonychia sp.* survivorship and *Ceriodaphnia dubia* survivorship and reproductive responses in 7-day chronic toxicity tests with CCW CPI effluent. **207**
Table 3.3. Trace metals and other elements measured in the coal processing impoundment effluent in Test 1. **207**
Table 4.1. Field collection summary of collection attempts for *Isonychia bicolor* and *Maccaffertium sp*. nymphs from Sinking Creek, Giles, County, Virginia during 2009. **245**
Table 4.2. Comparison of control survivorship for *Isonychia bicolor* over 7-14 day test duration and relative collection and test temperatures (°C). **247**
Table 4.3. Multivariate correlation and linear regression of collection and test temperatures (°C) relative to *Isonychia bicolor* control survivorship at 7, 10 and 14 days (JMP 8.0). **248**
Table 4.4. Comparison of control survivorship for *Maccaffertium sp.* over 7-14 day test duration and relative collection and test temperatures (°C). **248**
Table 4.5. Multivariate correlation and linear regression of collection and test temperatures (°C) relative to *Maccaffertium sp.* control survivorship at 7, 10 and 14 days (JMP 8.0). **249**
Table 4.6. Geometric means for NOEC values calculated by season for *Isonychia bicolor* nymphs exposed to NaCL over a 7-day period. **250**
Table 4.7. Geometric means for NOEC values calculated by season for *Maccaffertium sp.* nymphs exposed to NaCL over a 7-day period. **250**
Table 4.8. Exuvia results from *Isonychia bicolor* toxicity tests conducted in 2009. Gray areas indicate no data generated for that concentration during the test. **251**
Table 4.9. Exuvia results from *Maccaffertium sp.* toxicity tests conducted in 2009. Gray areas indicate no data generated for that concentration during the test. **252**

List of Figures

Figure 1.1. Sampling site locations in the Clinch River. **55**
Figure 1.2. Mean conductivity (µS/cm) values (± standard deviation) for sites in the Clinch and Guest Rivers, collected from 2007-2009. **56**
Figure 1.3. Mean (a) taxa richness, (b) mayfly abundance and (c) EPT richness of quantitative benthic macroinvertebrate samples, summer 2008. Means connected by different letters are statistically different based on Tukey's HSD comparison of means (α=0.05). **57**
Figure 1.4. Functional feeding group distribution of organisms collected on multi-plate artificial substrate samplers during summer, 2008. **58**
Figure 1.5. Functional feeding group distribution of organisms collected during single replicate qualitative sampling in the Clinch River, fall 2008. **58**
Figure 1.6. Mean (a) taxa richness, (b) mayfly abundance and (c) EPT richness of quantitative benthic macroinvertebrate samples, 2009. Means connected by different letters are statistically different based on Tukey's HSD comparison of means (α=0.05). **59**
Figure 1.7. One-way analysis of Asian clam (*Corbicula fluminea*) survivorship (%) in summer 2008 after 60 days *in-situ*. **60**
Figure 1.8. One-way analysis of Asian clam (*Corbicula fluminea*) growth (mm) in summer 2008 after 60 days *in-situ*. **60**
Figure 1.9. One-way analysis of Asian clam (*Corbicula fluminea*) survivorship (%) in fall 2009 after 60 days *in-situ*. **61**
Figure 1.10. One-way analysis of Asian clam (*Corbicula fluminea*) growth (mm) in fall 2009 after 60 days *in-situ*. **61**
Figure 1.11. Comparison of Bioaccumulation Factors (BAF) and Bioconcentration Factors (BCF) at sampling sites in the Clinch and Guest Rivers. **62**
Figure 2.1. Routine sampling site locations in the Dumps Creek subwatershed. **168**
Figure 2.2. Habitat assessment scores at sites in the Dumps Creek subwatershed. **169**
Figure 2.3. Mean taxa richness at sampling sites in the Dumps Creek subwatershed from 2008-2010. **169**
Figure 2.4. Mean Ephemeroptera-Plecoptera-Trichoptera (EPT) richness at sites in the Dumps Creek subwatershed from 2008-2010. **170**
Figure 2.5. Mean Percent Ephemeroptera at sites in the Dumps Creek subwatershed from 2008-2010. **170**
Figure 2.6. Mean Percent Chironomidae at sites in the Dumps Creek subwatershed from 2008-2010. **171**
Figure 2.7. Stream Condition Index (SCI) scores for sites in the Dumps Creek subwatershed using Virginia and West Virginia methodologies. **172**
Figure 2.8. Comparison of VA and WVA Stream Condition Index scores using geometric averages of scores generated from 2008-2010. **173**
Figure 2.9. Asian clams (*Corbicula fluminea*) retrieved from DC-2 Dn (bottom row) with distinct covering after 60-day *in-situ* during the 2008 study year, compared to control clams. Photograph by Brandi Echols. **173**
Figure 2.10. Chronic, 28-day test results with Camp Branch (CB Pump) 001 effluent using juvenile stage *Villosa iris*. **174**
Figure 2.11. Chronic toxicity tests results with the mayfly, *Isonychia bicolor*, exposed to CB Pump 001 effluent. **174**

Figure 2.12. Chronic toxicity tests results with a Heptageniidae mayfly, *Maccaffertium* sp., exposed to CB Pump 001 effluent. **175**
Figure 2.13. *Isonychia bicolor* chronic test survivorship after 21-day exposure to Moss 3 (003) effluent. **175**
Figure 2.14. *Maccaffertium* chronic test survivorship after 21-day exposure to Moss 3 (003) effluent. **176**
Figure 2.15. Exuvia produced in the Moss 3 (003) effluent test with *Isonychia bicolor*. **177**
Figure 2.16. Total number of exuvia produced after 14 and 21 days for the *Isonychia bicolor* Moss 3 (003) effluent chronic test. **177**
Figure 2.17. Exuvia produced in the Moss 3 (003) effluent test with *Maccaffertium sp.* **178**
Figure 2.18. Total number of exuvia produced after 14 and 21 days for the *Maccaffertium* Moss 3 (003) effluent chronic test. **178**
Figure 2.19. *Thiothrix nivea* coating the substrate as the CBP 001 effluent discharge merges with Dumps Creek. Photograph by Brandi Echols. **179**
Figure 3.1. Lethal concentration (LC_{50}) values during a 7-day test period for *Isonychia sp.* exposed to NaCl. **208**
Figure 3.2. *Isonychia sp.* survivorship in 7-day NaCl chronic toxicity tests. Dashed lines indicated significant difference from the control. **209**
Figure 3.3. *Isonychia sp.* chronic Test # 1 with CCW CPI effluent over 7 days (A) and 14 days (B). Dashed lines indicate significant difference from the control. **210**
Figure 3.4. *Isonychia sp.* chronic Test #2 with CCW CPI effluent over 7 days (A) and 14 days (B). Dashed lines indicated significant difference from the control. **211**
Figure 3.5. *Isonychia sp.* chronic Test #3 with CCW CPI effluent over a 10-day test period. Dashed lines indicated significant difference from the control. **212**
Figure 4.1. Collection of *Isonychia* vs. *Maccaffertium* nymphs from Sinking Creek, Giles County, VA 2009. **253**
Figure 4.2. *Isonychia* versus *Maccaffertium* availability from Sinking Creek, Giles Co., VA during 2009 collection attempts. When multiple collections were conducted within the same month, numbers were averaged per species. **253**
Figure 4.3. Seasonal availability of *Isonychia bicolor* from Sinking Creek, Giles County, VA. **254**
Figure 4.4. Seasonal availability of *Maccaffertium sp.* from Sinking Creek, Giles County, VA. **254**
Figure 4.5. Test control comparisons for *Isonychia* acclimated for 24 (A) and 48 (B) hrs. **255**
Figure 4.6. No Effect Concentration (NOEC) values for *Isonychia bicolor* reference toxicant tests using NaCl during each season in 2009. Test temperature (°C) also included for reference. Lower graph excludes first two test NOECs along with the geometric mean of tests 3-12. **256**
Figure 4.7. No Effect Concentration (NOEC) values for *Maccaffertium sp.* reference toxicant tests using NaCl during each season in 2009. Test temperature (°C) also included for reference. Lower graph excludes the second test NOEC, and shows the modified geometric mean for tests 1 and 3-7. **257**
Figure 4.8. Geometric means per test concentration of NaCl for each test conducted in 2009. **258**
Figure 4.9. Inhibition concentration (IC_{25}) values for *Isonychia* exuvia over 7-day test periods for reference toxicity tests conducted in 2009. Summer test denoted with an asterik (*) was terminated after six days due to significant control mortality. **258**

Figure 4.10. Seasonal comparison of average IC_{25} values for exuvia in *Isonychia* 7-day tests. **259**
Figure 4.11. Exuvia IC_{25} values compared to *Isonychia* 7-day survivorship NOECs. **259**
Figure 4.12. Exuvia IC_{25} values compared to *Isonychia* 7-day control survivorship. **260**
Figure 4.13. Comparison of molted exuvia from *Maccaffertium* and *Isonychia* nymphs exposed to varying NaCl concentrations over seven days. **261**

General Introduction

Extensive habitat deterioration in North American streams is contributing to alarming reductions in freshwater fauna diversity and abundance. Numerous species are currently listed as threatened, many are extinct and others are in jeopardy. Ricciardi and Rasmussen (1999) project that future extinction rates for freshwater organisms will be approximately five times greater than that of terrestrial fauna and will fall within range of estimated extinction rates for tropical rainforest communities (1-8% loss/decade, Reid 1997). Since the turn of the 20th century, a conservative estimate of 123 freshwater organisms have gone extinct, not including organisms that may have been extinct prior to documentation of existence (Miller et al. 1989, Taylor et al. 1996, Neves et al. 1997, Turgeon et al. 1998, Ricciardi and Rasmussen 1999). The extinction of freshwater fishes, mollusks, gastropods, crayfish and amphibians can be attributed to human induced influences such as sediment loading, organic pollution, toxic substances, channelization and the introduction of exotic species (Benke 1990, Allan and Flecker 1993, Dynesius and Nilsson 1994, Neves et al. 1997, Ricciardi et al. 1998, Ricciardi and Rasmussen 1999). Of the freshwater organisms threatened to go extinct, freshwater mussels stand out with the highest incidence of recent extinction (1.2% per decade) and projected extinction (6.4 % per decade over the next 100 years) rates (Ricciardi and Rasmussen 1999).

Freshwater mussels (Bivalvia: Unionidae) are a key component in the ecological functioning in freshwater ecosystems. Mussels filter bacteria, algae and other small particles, essentially improving the water quality of their environment (VA Game and Inland Fisheries 2010). Additionally, empty mussel shells function as both a habitat for aquatic insects and spawning ground for small fish. However, their complex life cycle, which involves a parasitic larval stage and long life span, increase their vulnerability to perturbations.

The Clinch River, one of three river systems making up the Upper Tennessee River Basin, is recognized internationally as an epicenter for freshwater mussel diversity. A.E. Ortmann (1918) once termed the assemblage the "Cumberlandian fauna" due to number of endemic species specific to this area of the Appalachian Mountains, the Cumberland–Plateau Region. Historical surveys conducted in the Upper Clinch River (UCR), Virginia, documented a combined total of 55 unionid species (Ortmann 1918, Stansbery 1973). Other, unsubstantiated claims include unionid species as high as 79 in the UCR (USGS NAWQA, 2005). More recent surveys performed by Bates and Dennis (1978), Ahlstedt (1984) and Ahlstedt and Tuberville (1997) report a decreased species number of 39. A recent draft report by Jones et al. (2009) listed 45 extant species in the Clinch River, of which 16 are listed as federally endangered. These estimates follow the global trend of diminishing unionid diversity whereby ten percent of approximately 300 global species are declared extinct, while nearly half of the remaining species are considered threatened or rare (USFWS 2002). Furthermore, approximately 40% of unionid species in the mainstem Tennessee River are not reproducing, virtually making them functionally extinct (Neves et al. 1997).

The diminishing populations of unionids in the Clinch River can be attributed to several anthropogenic perturbations which include chemical spills, acid-mine drainage, introduction of exotic species, non-point source runoff such as sedimentation from agriculture and logging practices, urbanization and active mining processes (Ahlstedt 1984, Goudreau et al. 1993, Jones et al. 2001, Diamond et al. 2002, Zimmerman 2003). In fact, the Clinch River system is considered to be one of the "more biologically threatened river systems in the country" (USGS NAWQA 2005, Master et al. 1998). Although several factors have likely contributed to unionid demise in the Clinch River, specific causation for the faunal decline has not been identified.

Two well documented industrial spills originating from a coal-fired power plant (Clinch River Plant) operated by American Electric Power (AEP) in Carbo, Virginia, occurred in the watershed in 1967 and 1970 (Crossman et al. 1974). The first spill occurred in June 1967 when a retaining system holding fly-ash failed. The alkaline waste (pH 12.0-12.7) poured into Dumps Creek and the Clinch River, equaling a discharge 40% of the river's daily flow (Crossman et al. 1974) and continued downstream at a rate of 1.5 km per hr. The alkaline waste had deleterious effects on fish (Cairns et al. 1971, 1973, Crossman et al. 1973, 1974), benthic macroinvertebrate assemblages, and snail and mussel populations (Hull 2002). It was anonymously reported that mussel populations were eliminated for approximately 18 km downstream (1967). Benthic macroinvertebrate abundance and richness recovered by 1969, but mussel populations remained impaired downstream (30 km) below the impact site (Crossman et al. 1973).

In June 1970, a second, more isolated event occurred at the Clinch River Plant (CRP), resulting in the release of an undetermined amount of sulfuric acid. Not only did this spill interrupt recovery, an additional 5300 fish were killed and aquatic fauna were impacted downstream for approximately 22 km (Crossman et al. 1974). Although some studies reported a recovery of benthic macroinvertebrates within six weeks of the second CRP incident (Cairns et al. 1971, Crossman et al. 1974), mollusks were still absent from the stream sections affected by the spills. However, the term mollusks used in these "assessments" referred to combined mussel and snail taxa, not unionids alone (Van Hassel 2007). Some speculation (Hull 2002) has been reported that unionid fauna did not actually exist in the stream reach influenced by CRP effluent due to insufficient mussel surveys conducted after the CRPs initial construction/operation, indicating unionid absence in the stream section of interest may be due to the overall presence of the power plant and not necessarily the result of the two spills. Intensive surveys conducted by

Ahlstedt (1984) and Stansbery et al. (1986) officially reported the non- existence of unionids in the section of the river influenced by the CRP and approximately 19 km (12 miles) downstream, thereby creating a baseline for subsequent studies. Furthermore, this study also reported the colonization of mussels below the CRP was limited to the opposite side of the river from the effluent discharges.

Effluent discharges from the CRP are also thought to be a contributor to faunal decline in the Clinch River. From 1977 to 1984, effluent high in copper (average of 857 μg /L) leached from pipes into the river (Van Hassel and Gaulke 1986, Cherry et al. 1996) resulting in macroinvertebrate and bivalve impairment (Farris et al. 1988, Clements et al. 1992). Clean-up efforts from 1986 to 1989 successfully reduced copper levels in the effluent to 260 μg /L. Measured copper concentrations in aqueous samples collected directly below the discharge in the river were 52.2 μg Cu/L (Clements et al. 1992); however, earlier studies (Clements et al. 1988) showed insect communities were still adversely affected (diversity and abundance) at 12 μg Cu/L. As a result of these findings, the CRP constructed a waste-water treatment plant facility (1993) to further reduce copper concentrations in the effluent to less than 12 μg /L (Cherry et al. 1996). Cherry et al. (2002) examined Cu toxicity to freshwater biota indigenous to the Clinch River watershed using laboratory acute toxicity tests. The culmination of these tests, which utilized several unionid (7) and benthic [insect] species (2), resulted in a site-specific Cu criterion for the watershed. Genus Mean Acute Values (GMAV) ranged from 37 μg/L for the Wavy-rayed Lampmussel (*Lampsilis fasciola*), to 4,040 μg/L (*Lepomis macrochirus,* Blue-gill sunfish). The average GMAV for the seven mussels tested was 76 μg/L, with four genera having values of 60 μg/L or lower (37-60 μg/L). Results of this research are necessary for predicting the lethal response of mussels during a one-time event; however, it is difficult to estimate the

responses of mussels to low-level, long-term exposure. Wang et al. (2007) tested juveniles of three unionid species (*Villosa iris*, *Lampsilis siliquoidea* and *Epioblasma capsaeformis*) in chronic laboratory bioassays to establish a chronic value [ChV] for Cu. The ChV, which is generated using the geometric mean of the no-observed effect concentration (NOEC) and the lowest-observed effect concentration (LOEC), ranged from 8.5-9.8 μg/L for survivorship and 4.6-8.5 μg/L for growth. Raj and Hameed (1991) determined that sub-lethal concentrations of copper decreased body weight after 30 days of exposure, while Hameed and Raj (1989) and Lasee (1991) found exposure to copper resulted in the "dissolution of the crystalline style in *Lamellidens marginalis* and *L. ventricosa*" (Keller et al. 2007), which disrupts the digestion of food. Considering the relatively high levels of Cu reported in Clements et al. (1992) compared to chronic thresholds presented in the literature, it is plausible that repeated exposure to Cu in the water column could have affected unionid populations downstream of the CRP.

Previous researchers have postulated that decline in species richness could be due to coal mining, pre-Clean Water Act industrial and municipal discharges and nonpoint source runoff from agricultural and urban sources (Ahlstedt 1983, Hampson et al. 2000, Van Hassel 2007). According to the Clinch-Powell Valley Watershed Ecological Risk Assessment (2002), only one major industrial discharge, the CRP, is present in the Clinch River, while numerous (119) municipal discharges are listed for the combined Clinch-Powell River watersheds. However, most sewage treatment plants (STPs) have implemented secondary treatment standards during recent upgrades. Impacts related to nonpoint sources have been considered, but not thoroughly investigated. Locke et al. (2006) examined the influences of 19 major tributaries in the Clinch River watershed using ecotoxicological assessments. Tributaries were grouped based on percent land-use and included forest, agriculture, urban development and mining. Results of this study

indicated that mining-influenced tributaries had lower Ecotoxicological Rating scores (ETRs); a multimetric index which combines chemical, biological and toxicological testing components to determine the overall health of a particular site (Cherry et al. 2001). Dumps and Coal Creeks received the two lowest ETR scores (< 50). Although both creeks were mining dominant, Coal Creek contained > 1% developed land.

The Dumps Creek subwatershed is located in Russell County, Virginia and confluences with the Clinch River at Carbo, near the AEP CRP. The watershed covers approximately 20,300 acres, dominated by 71% forest and 26% mining related uses (as of 1997, in Map Tech 2004). Dumps Creek was placed on Virginia 303(d) List of Impaired Waters due to violations of the general (benthic) standard in 1994 and a Total Maximum Daily Load (TMDL) has been developed to reduce total dissolved solids (TDS) and total suspended solids (TSS) in the impaired section of Dumps Creek. The TMDL states both point and nonpoint source contributions are linked to the impairment. According to the 2004 TMDL document, there are currently 74 permitted point source discharges located in the Dumps Creek watershed and abandoned mine land (AML) is listed as the primary nonpoint source, which includes mine spoils, benches and disturbed areas. Point source discharges include sedimentation basins to control losses from surface mining and deep mine discharges.

This research focused on evaluating the Clinch River below the confluence with Dumps Creek by conducting biological, toxicological and physical/chemical assessments at various sites. Comparisons between bioassessment parameters at sites located directly below the AEP CRP influence (left bank facing downstream) and Dumps Creek confluence (right bank) were also made to determine if potential impacts were discernable. Additionally, the Dumps Creek watershed was thoroughly evaluated using both field and laboratory methods to determine the

probability that active and historical mining activities in this subwatershed of the Clinch River could be having deleterious effects on river fauna. Laboratory toxicity tests of point source discharges from active mining were conducted to determine the potential toxicity from effluent using both standard US EPA test organisms as well as surrogate test organisms that may be more representative of natural fauna. Using multiple test organisms also allowed for a broader determination of effluent toxicity to naturally occurring stream organisms. Test organisms used in this research are the US EPA WET test organisms, *Ceriodaphnia dubia* (water flea), *Pimephales promelas* (fathead minnow), a freshwater mussel (*Villosa iris*), *Hyalella azteca* (scud) and a mayfly species (*Isonychia bicolor*).

The use of mayfly larvae in laboratory testing has occurred intermittently over the past three decades (Sherberger et al. 1977; Peters et al. 1985; Diamond et al. 1992; Dobbs et al. 1994; Beketov 2004; Kennedy et al. 2004; Hassell et al. 2006; Brinkman and Johnston 2008; O'Halloran et al. 2008). To date, one of the most common species used for toxicity testing has been the burrowing mayfly, *Hexagenia limbata,* which has been used extensively in sediment toxicity tests. Water column testing has utilized a variety of mayfly species and has been used to study a wide range of ecotoxicological issues. Sherberger et al. (1977) examined the effects of thermal shock on *Isonychia sadleri* nymphs in laboratory simulations. Peters et al. (1985) determined responses of *Isonychia bicolor* nymphs exposed to alkaline pH in laboratory toxicity tests. Kennedy et al. (2004) used *Isonychia sp*. to evaluate the potential toxicity of coal-mining discharges with high ionic composition. This study found *Isonychia* exhibited a high dose-dependent response to specific conductivity, and better represented the responses of sensitive native organisms; however, test results were not as consistent as those conducted with *C. dubia.* Still, the lowest observable effects concentration (LOEC) to conductivity from a coal mining

effluent was 1,562 µS/cm for *Isonychia* and more than twice as high (3,730 µS/cm) for *C. dubia*. In the development of site-specific acute Cu criteria for the Clinch River, Cherry et al. (2002) ranked sensitivities of the 17 organisms tested. *Ceriodaphnia dubia* and *P. promelas* ranked 6th and 14th, while the top four organisms included *Lampsilis, Medionidus, Villosa* (unionids) and *Isonychia*. Hence, *Isonychia* would be more protective to aquatic life against copper stress than the two US EPA test species, *C. dubia* and *P. promelas.*

Some researchers (Kennedy et al. 2004) suggest the development of a standardized toxicity test using mayflies may be more beneficial for assessing potential adverse effects of point source discharges on aquatic organisms. Interest in mayfly testing has increased in recent years as the desire to use more relevant test organisms in coal mining impacted streams has increased; however, standardization of test methodology has yet to be established. Additionally, the selection of the most suitable test species that would be sensitive enough to predict toxicity, but tolerant of handling stress in the laboratory setting, has also not been determined. Therefore, this research also explores the use of a locally abundant mayfly species, *Isonychia bicolor*, for use in routine laboratory tests. Research objectives included sensitivity comparisons between *I. bicolor* nymphs and other common test organisms, appropriate field to laboratory acclimation techniques and establishment of test methodology.

This dissertation is comprised of four chapters. Research conducted for Chapter One involved an ecotoxicological evaluation of the Clinch River at select sites downstream of the CRP and Dumps Creek confluence. Chapter One focuses specifically on the trace metal dissipation downstream of these two upstream influences, in an attempt to differentiate the degree of impact each is having on unionid assemblages downstream. Chapter Two research is focused specifically within the Dumps Creek subwatershed. Using laboratory toxicity tests with

standard and ecologically relevant test organisms, potential toxicity of coal-mining effluents was evaluated. Additionally, field bioassessments of benthic assemblages and Asian clam field studies were conducted to determine stream condition at sites in Dumps Creek and Hurricane Fork. Chapters Three and Four focus on the use of mayflies, specifically *Isonychia bicolor* and *Maccaffertium* sp., for use in standard laboratory toxicity tests. This research first examined (Chapter Three) whether *Isonychia bicolor* nymphs were more sensitive than routinely used USEPA test organisms, such as *C. dubia*, to both a reference toxicant (NaCl) and a coal mining effluent with high TDS. Chapter Four is an extension of this research and primarily focuses on the feasibility of using field collected *Isonychia* and *Maccaffertium* in routine toxicity tests. This final chapter includes a one-year study that evaluated ease of collection on a seasonal basis, control survivorship responses, test temperature preferences, sublethal endpoints and seasonal sensitivity to a reference toxicant.

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Chapter 1. An Evaluation of the Clinch River below Carbo, Virginia to determine the Effects of Past and Present Mining Activities on Freshwater Mussels (Bivalvia: Unionidae)

Introduction

The Clinch River system, located in southwestern Virginia, is internationally recognized for significant endemic and sometimes endangered populations of freshwater fauna. In recent decades, surveys of freshwater mussel (Bivalvia: Unionidae) populations and fish community structure have indicated alarming declines in abundance and biodiversity (USGS 2010). Such declines have been loosely linked to changing land-use practices, particularly coal mining. A study by Locke et al. (2006) evaluated land-use practices in the Clinch River watershed by assessing 19 tributaries characterized as forested, agriculture, developed (residential), mining and other (open water, wetlands, etc.). Ecotoxicological rating scores (ETRs) were used to delineate impacted vs. non-impacted streams. The multimetric ETR provides a numerical ranking of a sampling site, similar to an academic grading scale. Tributaries dominated by coal mining had substantially lower (44-63) rankings than those streams dominated by forest (64-91) or agriculture (57-86) when evaluated on a 100-point scale. In addition to declining species richness and density, mussel surveys have also indicated a shift in community age structure with little to no evidence of recruitment (USGS 2009).

Numerous studies have evaluated the Clinch River basin and identified the major stressors most likely contributing to unionid decline as: untreated and treated wastewater, chemical spills, acid-mine drainage, exotic species, impoundments, runoff and sedimentation from agriculture and deforestation, and runoff from active and abandoned mining (Ahlstedt 1984, Goudreau et al. 1993, Jones et al. 1991, Diamond et al. 2002, Zimmerman 2003, Locke et al. 2006). Two well documented industrial spills originating from a coal-fired power plant (Clinch River Plant) operated by American Electric Power (AEP) in Carbo, Virginia, occurred in the watershed in 1967 and 1970 (Crossman et al. 1974). The first spill, which occurred in June

1967, caused alkaline fly-ash waste (pH 12.0-12.7) to pour into Dumps Creek subsequently, the Clinch River, equaling a discharge 40% of the river's daily flow (Crossman et al. 1974). The alkaline spill caused major impairment to fish (Cairns et al. 1971, 1973, Crossman et al. 1973, 1974), benthic macroinvertebrate assemblages, and snail and mussel populations (Hull 2002). An anonymous source reported mussel populations were eradicated for approximately 18 km downstream (1967). The second insult occurred nearly three years later in June 1970, when a second, more isolated event occurred at the AEP Clinch River Plant (CRP), resulting in the release of an undetermined amount of sulfuric acid. This spill inhibited recovery of aquatic organisms from the previous alkaline spill and killed an additional 5,300 fish, impacting aquatic fauna for approximately 22 km downstream (Crossman et al. 1974). Benthic assemblages reportedly recovered within six weeks, (Cairns et al. 1971, Crossman et al. 1974); however, mussels and snail taxa were still absent from the stream sections affected by the spills. Hull et al. (2002) speculated that unionid fauna did not actually exist in the stream reach influenced by the CRP effluent due to insufficient mussel surveys conducted after the CRPs initial construction/operation, indicating unionid absence in the stream section of interest may be due to the overall presence of the power plant and not necessarily the result of the two spills. Mussel surveys conducted in the Clinch River in the 1970-80's below the CRP, report the "non-existence" of unionids for approximately 19 km downstream (Ahlstedt 1984, Stansbery et al. 1986). Therefore, multiple point and non-point sources of pollution are contributing to contamination in the Clinch River.

Due to the mining activities and other land use practices in the Clinch River watershed, high concentrations of metals have historically been associated with biological impairment (Cherry and Guthrie 1977). For example, the CRP effluent is known to discharge high

concentrations of metals, specifically Cu. Reportedly, Cu concentrations averaged 436 µg/L from 1985-1989, but modifications to the wastewater treatment facility reduced these averages to 12µg/L (Cherry et al. 1996). Van Hassel and Gaulke (1986) linked changes in Ephemeroptera composition downstream with CRP effluent, while Clements et al. (1988) reported increased Cu concentrations caused a shift in benthic taxa downstream. This study showed that pollution sensitive taxa (Ephemeroptera, Plecoptera, and Trichoptera) were replaced by the more tolerant dipteran, Chironomidae. A more recent evaluation of metal availability and unionids at select mussel preserves in the Clinch River indicated a negative correlation between mussel density and bioaccumulated Cu (r=0.993) and Pb (r=0.995) in Asian clam (*Corbicula fluminea*) tissues after 30-day *in-situ* (Cherry et al. 2001). Therefore, the greater the bioaccumulation of these two metals, the lower the mussel density. Additional studies which used *C. fluminea* as a surrogate test organism have shown that exposure to Cu and Zn can cause reduced endo-and exocellulase activity (Farris et al. 1994) and a loss of shell size (Belanger et al., 1986, 1990).

The objective of this research was to evaluate trace metal concentrations in sediment, interstitial water and clam tissues, and their dissipation in the Clinch River, downstream of the Dumps Creek confluence. Heavy mining activities in the Dumps Creek subwatershed, which includes Hurricane Fork and Chaney Creek, may be contributing to the lack of unionid recruitment downstream. Because the confluence with Dumps Creek is close to the Clinch River power plant, operated by AEP, it is important to differentiate between metal contributions from each source. Trace metals associated with these coal mining facilities and the AEP plant will be examined in interstitial water, sediments and *in-situ* Asian clam.

Materials and Methods

Study Location

The Clinch River originates in Tazewell County, Virginia and flows southwesterly ~322 km (200 mi) to northeastern Tennessee at Norris Lake (Goudreau et al. 1993, Cherry et al. 1996) (Fig. 1.1). The watershed primarily drains the Valley and Ridge province with some westernmost tributaries draining the Cumberland Plateau. Overall, the watershed drains ~ 7,542 km or 2,912 sq. miles. Characterized primarily by steep slopes and poor riparian forest cover, land use is composed largely of forest or agricultural land (US EPA 2002).

Eleven sampling sites were selected in the Clinch River, with the uppermost site located near Pounding Mill, Virginia (CR-PM) (Table 1.1). Additional sampling sites were located above the AEP Clinch River Plant (CRP) and Dumps Creek influences (CR-1), below Dumps Creek (CR-2), below the CRP/Dumps Creek confluences (CR-3 Right and CR-3 Left, CR-4, CR-5) and above and below the town of St. Paul, VA (CR-6 and CR-7). Two sites were located much further downstream below the towns of Dungannon (CR-8) and Clinchport, VA (CR-9). These sites were chosen based upon results of previous studies (Cherry et al. 2002, Hull 2002), and to discern possible influences of the CRP versus active mining impacts in the Dumps Creek subwatershed. The original CR-1 site was located in the town of Cleveland, Virginia, approximately 2.8 miles east of Carbo, VA and the CRP. This site location was revised during the middle of the 2008 sampling season due to poor habitat and accessibility issues. An additional site, located in the Guest River near Coeburn, Virginia was also included in the study. Previous research in the watershed (Diamond et al. 2002) reported significantly lower Ephemeroptera-Plecoptera-Trichoptera (EPT) and index of biotic integrity (IBI) scores in the Guest River, which is also known to have substantial coal mining activity.

Water and Sediment Quality

General water chemistry parameters were monitored at each sampling station in the Clinch River watershed. Samples were collected as grab samples using clean, 1-L Nalgene® bottles and transported to the laboratory at VA Tech on ice (< 4°C). Measurements of conductivity (μS/cm), pH (su), dissolved oxygen (mg/L), temperature (°C), were determined in the laboratory followed standard operating procedures (SOPs). Measurements were obtained using a Yellow Springs, Inc. model 54 dissolved oxygen meter calibrated for elevation (YSI, Inc., Yellow Springs, OH), a YSI 30 conductivity (accuracy ± 0.05%) meter, and an Accumet® AB15 (Fisher Scientific, Pittsburgh, PA) pH meter with an Accumet gel-filled combination electrode (accuracy < ±0.05 pH at 25°C). Total alkalinity and hardness (expressed as mg $CaCO_3$/L) were measured through colorimetric titrations following APHA (1998).

Site sediments were collected from each sampling site in the Clinch River in 2008 (n=2) and 2009 (n=1). An acid-washed polyurethane cup was used to scoop sediments from the top layer (< 2 cm) of substrate and then transferred into sterile plastic bags. Sediments were collected from various locations within the sampling reach. Samples were maintained on ice at ~ 4°C for transport to the laboratory. Homogenized sediments were divided into equal replicates for each site, weighed and dried overnight at 60°C. Acid digestion of sediments followed methods outlined in US EPA Method 200.7 (2001) for sample preparation. One gram (±0.01 g) of dried sediment was transferred to a 250-ml glass beaker, with 4 ml of (1+1) HNO_3 and 10 ml of (1+4) HCl for acid extraction. Beakers were then placed on a hot plate adjusted to a constant temperature of 95°C, and heated for approximately 30 min. After cooling, the sample was transferred to a clean, acid-washed 100-ml volumetric flask and diluted to volume using Milli-Q water. Extracted samples were allowed to stand overnight to allow insoluble material to

separate. Sediment samples were analyzed for total Al, Cr, Cu, Fe, Mn, Sr and Zn (mg/kg dry weight) at the Inductively Coupled Plasma (ICP) laboratory at VA Tech (Dept. Crop and Soil Environmental Sciences). These metals were chosen as they are commonly associated with coal mining effluents in this region of Virginia. In addition to metals analysis, sediment samples were analyzed for Ash-free dry weight and percent solids were calculated for sediment subsamples and used for sediment characterization.

Habitat Assessment

A habitat assessment was conducted at each benthic sampling site and included ten parameters as outlined in the US EPA Rapid Bioassessment Protocols for low gradient streams (Barbour et al. 1999). These include: 1) epifaunal substrate/ available cover, 2) pool substrate characterization, 3) pool variability, 4) sediment deposition, 5) channel flow status, 6) channel alteration, 7) channel sinuosity, 8) bank stability, 9) vegetative protection and 10) riparian vegetative zone width. A rating of 0-10 to 0-20 was developed for each parameter, and the higher the score (up to 200 points), the more pristine the station.

Benthic Macroinvertebrate Surveys

Quantitative benthic macroinvertebrate studies were conducted in the Clinch River during summer 2008 and 2009. Multiplate Hester-Dendy (Hester and Dendy 1962) artificial substrate samplers were deployed at each sampling station for six weeks to allow for colonization by aquatic invertebrates. Samplers were constructed following DeShon (1995), using 3mm thick hardboard, cut into 76.2 mm squares. Plates were separated using nylon spacers (3 mm thick), and constructed together with long stainless steel eyebolts. The 8-plate design was used for this study, which consisted of three single spaces (3 mm each), three double spaces (6 mm each) and one triple space (9 mm). This design provides a 0.09 m^2 surface area for colonization. At

retrieval, plates were carefully removed from the eyebolt, and placed in sample jars with approximately 80 % ethyl alcohol (ETOH). Organisms were carefully scraped from plates in the laboratory and identified to lowest practical taxonomic level following standard keys (Pennak 1989, Merritt et al. 2008).

In addition, a qualitative sample of all available habitat types (multihabitat) was taken using a standard D-frame dip net. A single-replicate sample was taken at each sampling location following routine sampling protocols (RBP III, Barbour et al. 1999) methods. Sample contents were placed in sample jars and covered with ~ 80% ETOH. Samples were sorted in the laboratory and organisms identified as described above.

Asian Clam *In-Situ* Studies

Growth Studies

The Asian clam (*Corbicula fluminea*) was used for *in- situ* survival and growth studies in the Clinch River. Asian clams have been a successful indicator of toxicity in previous studies (Soucek et al. 2001, Hull et al. 2002, Echols et al. 2009). Clams were collected using clam rakes from the New River, Ripplemead, Virginia, and kept in Living Streams® (Frigid Units, Toledo, OH). Clams were measured for initial width using ProMax® digital calipers and marked on the exterior shell using a file. For this study, clams measuring between 9 and 11 mm were used. Five clams were placed into 18 x 36 cm mesh bags (~0.5 cm^2 mesh size) with five replicate bags at each sampling station. After 60 days, clams were retrieved and returned to the laboratory where mean survival and final growth were measured. Mortality was determined if clams were found gaping or easily opened, or failed to close when the visceral mass was probed. Survival, change in growth, and average growth for each replicate/site were determined. Statistical analyses were conducted using JMP® (SAS Institute 2008).

Metal Bioaccumulation

Bioaccumulation studies were conducted at all study sites in the Clinch River to estimate the accumulation potential of select trace metals (Al, Cu, Fe and Zn) in bivalve tissue. This was a crucial aspect in the trace metal dissipation study as it is important to link levels of pollutants in water and sediment with what is ultimately bioavailable to sediment-dwelling biota. Previous studies have utilized clams as surrogates for unionids because of their capability of bioaccumulating dissolved trace metals from field exposure (Adams et al. 1981, Graney et al. 1983, ASTM 2007b).

Asian clams used for this study were collected from a reference area in the Clinch River near Pounding Mill, VA using clam rakes. Clams were held in Living Streams® (Toledo, OH) at the Ecosystem Simulation Laboratory, Virginia Tech, until used. Fifty clams were placed at each site using mesh produce bags tied to rebar. Produce bags are 18 cm wide by 36 cm long with a mesh size of ~0.5 cm. After 60 days, clams were collected from each site and transported on ice to the laboratory. Clams were depurated in clean water for 24 hr in living streams, then placed in the freezer for a minimum of 24 hr. Tissues samples were split into equal 5 g replicates (4) and prepared for analysis following US EPA Method 200.3 (McDaniel 1991). Metal analyses were conducted at the ICP laboratory at Virginia Tech.

Results were converted from mg/L to mg/kg wet weight and statistical comparisons between sites were determined using JMP 8.0 (SAS Institute, 2008). Differences between sites was determined using Tukey-Kramer HSD (α=0.05) means comparison test. Bioconcentration Factors (BCF) and Bioaccumulation Factors (BAF) were calculated to determine the relationship between tissue concentrations of metals and ambient concentrations interstitial water and sediments. Bioconcentration factors are a ratio of the chemical (metal) concentration in an

organism to the concentration in the water, whereby: $BCF = (C(\text{"tissue.)"})/(C(water))$. Tissue concentrations (g /kg wet weight) and water concentrations (g/L) are converted to moles based on the atomic weight of the metal. BCF values > 1,000 are considered high; the 250-1,000 range is moderate and values <250 are low. The BAF is a similar ratio of the chemical concentration in an organism to the chemical concentration in sediments, whereby BAF=C_{tissue} (mg/kg wet weight)/ $C_{sediment}$ (mg/kg dry weight). Both values are unitless estimations of the relationship between these concentrations.

Interstitial (Pore) Water Sampling and Analysis

Interstitial water, also known as pore-water, was collected from depositional zones at each sampling station in the Clinch River, including a site in the Guest River, using diffusion samplers (peepers). Peepers were constructed using 250-ml amber glass jars with 32 mm diameter holes cut out of the plastic lids. Amber glass was used to prevent photolysis of PAH compounds (Miller and Olejnik, 2001). Prior to deployment, peepers were completely filled with distilled water and 105 μm nylon mesh was inserted under the lid, allowing fine and suspended particles to pass through by osmosis. Nine peeper bottles were placed lid side down at each site, approximately 10 cm into the sediment and allowed to equilibrate for a minimum of 21days (Vroblesky et al. 2002; Webster et al. 1998). Upon collection, water was transferred into 1-L Nalgene® containers, and transported back to the laboratory on ice. Samples were acid-digested in the laboratory according to US EPA Method 200.7 (2001) for aqueous samples and analyzed in the VA Tech ICP laboratory for selected metals.

Statistical Analysis

Statistical differences of bioassessment parameters between study sites were determined using JMP IN® statistical software (SAS Institute, 2008-2009). Normally distributed means for

clam growth and survivorship were compared using analysis of variance (ANOVA). Pairwise analysis was conducted using the Tukey-Kramer honestly significant post-hoc test (HSD) at α=0.05 level.

Results

Water and Sediment Quality

Water quality characteristics were consistent between sampling events for most parameters, with only minor fluctuations (Table 1.2). Since samples were collected at various times during the year, temperature reflects the sample measurement in the laboratory after gradual warm-up. Dissolved oxygen levels were at or above saturation for all sites. Mean pH was above neutral and increased slightly from the upstream reference site (CR-PM) near Pounding Mill, Virginia (8.10 su) to the furthest downstream site in the Clinch River (CR-9, 8.34 su), with minor fluctuations occurring at sites in between. Alkalinity ranged from 94 mg $CaCO_3$/L at CR-7 to 201 mg $CaCO_3$/L at CR-2. Hardness was lowest (112 mg $CaCO_3$/L) at site CR-7 and highest at the Guest River site (260 mg $CaCO_3$/L).

Mean conductivity was lowest at the three upstream reference locations (CR-PM, CR-1 Cleveland, CR-1), ranging from 314 to 369 µS/cm (Fig. 1.2). Values increased at CR-2 (635 µS/cm), but decreased at remaining downstream sites. Comparisons between conductivity measurements at site CR-3 indicated a higher mean conductivity at the left bank site (plant side) (458 µS/cm) than at the right bank (430 µS/cm), which is influenced by Dumps Creek. Mean conductivity was much higher (646 µS/cm) for the Guest River, with values ranging from 398 to 792 µS/cm.

Total recoverable metals in river sediments were variable between sites with no apparent discernable trends (Table 1.3). Metal concentrations were elevated at all sites, including

upstream sites used as a reference (CR-PM, CR-1 Cleveland, CR-1). In fact, the highest mean (12,323 mg/kg) level of Fe occurred at CR-PM, while Al (2,251 mg/kg) and Cu (6 mg/kg) were second highest at this site, in comparison to the other sampling sites. CR-3R samples were higher for all metals except Cr, compared to CR-3L. Strontium, which is a known constituent in mining effluents originating from facilities in Dumps Creek, was twice as high in sediment samples collected at CR-3R (11 mg/kg) than at CR-3L (5 mg/kg). Manganese levels were also higher in downstream sediments than upstream (136.3). Sites CR-2 and CR-5 had the highest mean Mn values (327 and 331 mg/kg, respectively), while samples at CR-3 left and right bank sides had similar Mn concentrations (161 and 200 mg/kg). Zinc was highest (30 mg/kg) for sediments collected in the Guest River, although total metal concentrations were generally much lower for this site than those in the Clinch River.

Interstitial (Porewater) Analysis

Interstitial water samples were analyzed for total Al, Fe, Sr, Zn and Cu after a 21-day equalization period at Clinch River sampling sites (Table 1.4). For general comparison purposes and to quantify high metal concentrations, results were compared to the US EPA Water Quality Criteria (WQC) for available trace metals (USEPA 2009). The highest levels observed occurred for Al (91-5,330 µg/L) and Fe (2,021-15,919 µg/L). For total Al, each Clinch River station was high and exceeded the US EPA acute (criterion maximum concentration or CMC) criteria of 750 µg/L. Guest River samples were substantially lower (91 µg/L), but still exceeded the US EPA chronic (criterion continuous concentration or CCC) criteria of 87 µg/L. All sites also exceeded the US EPA WQC for Fe (1,000 µg/L). Total concentrations of Cu and Zn were much lower, ranging from 3 to 50 µg/L and were generally consistent between sites. The only clear trend observed in these data was the highest concentrations of all but one metal (Sr) occurred at CR-1

Cleveland. In comparing CR-3R and CR-3L, the only major difference in metal concentration was for Fe, which was higher for the right bank site, while Sr was slightly higher for the left bank site (plant side).

Habitat Assessment

Qualitative habitat assessment scores were consistent between most sites (Table 1.5). The furthest upstream site near Pounding Mill, Virginia scored the highest (182) as all parameters evaluated were within optimal range. Sites CR-1 Cleveland and CR-2 scored the lowest (118 and 109) and were characterized as having diminished benthic habitat, heavy sedimentation and poor riparian vegetation due to human activities. Habitat at the Guest River site was optimal for most habitat categories, but scored in the marginal range for riparian zone parameters.

Benthic Macroinvertebrate Surveys

Quantitative benthic assessments conducted in 2008 indicated upstream sites had overall greater diversity compared to sites located downstream of the power plant (Fig. 1.3a). CR-2 had the highest mean taxa richness (17.3) followed by CR-7 (14.4) and CR-1 (13.8). Taxa richness was significantly lower (7.0) at site CR-3, directly below the CRP and at the Guest River site (8.8). Caddisfly and Stonefly abundance was minimal at all sites and ranged from 33.5 to 0.0 and 3.6 to 0.0, respectively. Mayfly abundance was slightly greater (41.5-5.8), with the highest mean occurring at CR-2 (Fig.1.3b). Lowest mayfly abundance occurred at CR-3. Overall EPT richness at CR-2 (8.5) was significantly higher than the upstream reference site, CR-1 (3.2) (Fig.1.3c). The distribution of trophic groups was also determined and as expected with artificial substrate samplers such as Hester-Dendy's, scrapers were most predominant (Fig. 1.4). Site CR-5 had the most proportionate distribution of feeding groups, while CR-2 and CR-3 were the

only two other sites in which organisms belonging to each of the five groups (filterers, collectors, predators, shredders and scrappers) were collected.

Single replicate qualitative samples (dip-net) were taken at each site at the time of Hester-Dendy retrieval (fall 2008) (Table 1.6). Taxa richness was highest at CR-5 (16.0) and CR-3 (15.0), but lowest at CR-9 (3.0). CR-2, which had the highest richness for quantitative samples, had the second lowest richness value of 6.0. Mayfly abundance was similar between sites, ranging from 1.0-7.0 total mayflies. Overall EPT richness was consistent between sites, ranging from 1.0-10.0 organisms, with site CR-3 having the highest diversity of EPT taxa. The Guest River site had a high abundance of caddisflies (60.0) and mayflies (21.0), which contributed to an overall percentage of EPT organisms of 80.2 %. However, all caddisflies collected at this site were in the family Hydropsychidae.

Functional feeding groups were better represented with the qualitative samples (Fig. 1.5). Collector filterers were the most dominant group at all sampling sites, except for site CR-8 which was dominated by predators (40%) and gatherers (36%).

Quantitative results of the 2009 benthic study were somewhat similar, with overall means for taxa richness similar to those from the previous year (Fig 1.6a). One major change however, was observed at site CR-2, in which taxa richness was substantially lower (7.5) than 2008 results and much lower than averages at downstream sites. Common to the 2008 study, site CR-3 had the lowest overall taxa richness (5.3) average. Mayfly abundance was similar at sites CR-6 (33.3) and CR-4 (8.3) to that of 2008. Remaining sampling sites had much lower means, including CR-2 which had an average of 3.3 mayflies colonizing Hester-Dendy plates after the 6-week sampling period (Fig.1.6b). Site CR-3 had no mayflies present on samplers. EPT Richness in general was similar between 2008 and 2009, with grand means of 5.2 and 4.8,

respectively. Site CR-2, which had the highest EPT richness in 2008, only had the second to lowest overall average in 2009 (2.5) (Fig.1.6c). The number of midge larvae present on samplers was drastically higher in 2009 (2.7) compared to both the previous year and all other sampling stations in 2009, indicating a shift in community structure at this site. Differences in benthic results between the two sampling years was most likely due a later sampling period in 2009, in which Hester-Dendy's were not deployed until early September due to high flow/flooding conditions that occurred throughout the summer. High river flow was also an issue at the time of retrieval, which prevented site access and sample retrieval at sites CR-1, 3R, and CR-5. Samples were also not retrieved at the Pounding Mill site and CR-5, but may have been vandalized. Two additional attempts to locate these samples were made, but samples were not located.

Qualitative samples were taken at the time of Hester-Dendy deployment in September 2009, and results were comparable to those collected in 2008 (Table 1.6). Taxa richness was highest at CR-3L (20), while CR-3R was similar (13) to results from upstream and downstream sites which ranged from 11-17 taxa. Caddisfly abundance was variable between sites, but was generally lowest at upstream sites (0-6 total) and higher downstream of the CRP and Dumps Creek influences (1-46 total). However, Hydropsychidae caddisflies were the predominant taxa collected. Mayflies were most predominant at sites CR-4 (40%) followed by CR-6 (32.8 %), and were absent from samples taken from the upstream site, CR-1. Overall EPT taxa richness was highest at CR-6 (8) and CR-4 (6), while all other sites ranged from 2-5 taxa. The Guest River site was only sampled qualitatively in 2009, and results were similar to those in 2008. Mean taxa richness was comparable (11.3) to sites in the mainstem Clinch River, but total mayflies was much higher with an average of 37.8 organisms collected. Total EPT richness; however, was low

(4.3), as the majority of EPT taxa collected were Hydropsychidae caddisflies and *Isonychia* mayflies.

Asian Clam *In-Situ* Studies

Growth Studies

Clam survival was significantly lower at upstream sampling sites (CR-1 Cleveland, 2 and 3L) in the Clinch River after 60 days *in-situ* during summer 2008 (Fig. 1.7). Mean percent survivorship was 44 ± 51% for CR-1 Cleveland and 52 ± 51% at CR-2. Clam survivorship was slightly higher at CR-3R (72 ± 46%) than at CR-3L (60 ± 50 %). Survival averaged 80% (± 41%) for clams placed in the Guest River. Generally, trends for growth were similar, with significantly higher growth at the furthest downstream sites (Fig.1.8). Average growth was significantly reduced at CR-3L (0.18 ± 0.17 mm). Mean growth was slightly higher at CR-3R (0.29 ± 0.20 mm), which was similar to growth at CR-1 Cleveland (0.28 ± 0.30 mm). Site CR-7 had the highest average growth after 60 days (1.06 ± 0.41 mm).

Survivorship (%) was less variable for the 2009 *in-situ* study (Fig.1.9). An additional upstream reference site, CR-PM was included in the study, which had 92 ± 27% clam survivorship after 60 days. The CR-1 site was moved downstream from Cleveland, to just above the CRP, which provided a more suitable habitat for clams, resulting in significantly higher (100 %) survivorship compared to the previous year. Lowest survivorship values occurred for the Guest River, with an overall mean of 84 ± 37.4 % which was similar to 2008 results. Growth means were less variable between sites in 2009 than in 2008 (Fig. 1.10). However, averages were substantially lower, ranging from 0.07to 0.21 mm. Upstream sites (CR-PM, 1, 2, 3R/L) had the lowest growth (0.07-0.11 mm), while growth averages incrementally increased at downstream sites. Differences in growth averages between 2008 and 2009 were most likely due

to a later *in-situ* period in the fall 2009, after water temperatures began to decrease. Clam growth studies were not carried out at two sites, CR-2 and CR-7 due to accessibility issues (construction and high water).

Metal Bioaccumulation

The most concentrated metal bioaccumulated in 2008 was Fe (15.5 to 33.6 mg/kg) followed by Zn (5.40 to 13.76 mg/kg) (Table 1.7). Concentrations of accumulated Al and Cu were somewhat lower and ranged from 0.82 to 4.18 mg/kg and 2.90 to 6.40 mg/kg, respectively. Slight increases in all four metals occurred at CR-2 compared to averages at CR-1 Cleveland. All metal concentrations were higher for CR-3L than CR-3 R, especially for Fe (33.6 mg/kg) and Cu (6.40 mg/kg). Overall Zn concentrations increased at sites below the CRP, and remained well above background level (9.82 mg/kg) at the furthest downstream site (CR-9) by Clinchport, VA.

Similar trends were observed in the 2009 bioaccumulation study, as Fe concentrations were the highest (20.1 to 88.8 mg/kg) (Table 1.7). Zinc concentrations were similar to those in 2008, ranging from 5.21 to 11.0 mg/kg, and were highest for Guest River clams. CR-3L metal concentrations were significantly higher than CR-3R and were substantially higher than those observed the previous year. The major difference observed in 2009 was the concentration of Al in clam tissues which was substantially higher and varied between sites (3.60 to 14.2 mg/kg). Copper concentrations were consistent between most sites, with the highest value occurring at CR-3L, below the CRP discharges.

Bioaccumulation Factors (BAF) were low for Al and Fe, with values ranging from 0.0008 to 0.0065 and 0.0020 to 0.0081, respectively (Table 1.8). Values for Cu and Zn were much higher, indicating a greater bioaccumulation potential. BAF values for Cu ranged from

0.616 to 2.22, with the highest values (> 1) occurring at sites CR-1 (2.22), CR-3L (1.45) and the Guest River (1.37). BAF values for Zn were highest at CR-7 (0.69) and CR-6 (0.58).

Similar estimations of accumulation relationships between tissue concentrations and interstitial (porewater) were also determined using Bioconcentration Factors (BCF) (Table 1.9). BCF values for Al and Fe followed the same trend as seen with BAF values, indicating a low bioaccumulation potential for these two metals (Fig. 1.11). Copper BCF values were high (> 1,000) for CR-1 (2,143.3), CR-3L (2,196.7) and remaining sampling sites downstream of the CRP to the town of St. Paul (CR-7). All other sites, including the upstream sites in Cleveland, VA had values in the moderate range (251- 898). The fourth metal included in the comparison, Zn, also showed a moderate relationship between concentrations in tissue samples and porewater. Nine out of the eleven Clinch River sites had values in the moderate range (253-780), while the Guest River had the highest BCF for Zn (4,117).

Discussion

The eradication of many freshwater mussel species in North American rivers may be due in part to low-level exposure to toxic metals (Naimo 1995). Although this relationship between trace metals and sublethal toxicity is not yet understood, high concentrations of metals used in laboratory toxicity tests have caused mortality, growth impairment and changes in enzyme activity and can also impair filtration rates and behavior in bivalves (Rodgers et al. 1980, Belanger et al. 1986, Doherty and Cherry 1988, Farris et al. 1988, Lasee 1991, Jacobson et al. 1993, Naimo 1995). The purpose of this study was to attempt to differentiate the potentially detrimental influences of active coal mining in the Dumps Creek subwatershed from those of the Clinch River Power plant (CRP), using various ecotoxicological approaches and specifically trace metal dissipation downstream.

Using standard accepted techniques for assessing ecosystem health, variability was observed between sampling locations with no discernable, continuous trends. Metals highest in site sediments were Fe (7,507-12,322 mg/kg) and Mn (136-331), which were both, well below predicted toxicity thresholds provided by the freshwater sediment screening benchmarks (USEPA 2010a) of 20,000 and 460 mg/kg, respectively. Although results for Al appear high (1,224 -2,666 mg/kg), it is difficult to discern a value that is critically high to aquatic organisms as no sediment toxicity benchmarks for Al have been identified. Additionally, Al commonly occurs as a component of mineral materials that comprise river sediments. Comparisons between right (Dumps Creek) and left bank (CRP) sediments indicated a greater influence was occurring downstream of Dumps Creek at CR-3R, than the CRP side of the river (CR-3L); however, these differences are minor compared to background or upstream sediment concentrations which are also high. Analysis of sediment interstitial or porewater showed consistent results, as values were generally greater for right bank samples than left. One discrepancy was determined in that sediment Sr concentrations were double for right bank samples than left; however, the opposite results were seen with porewater samples when concentrations were nearly double (295 µg/L) for CR-3L than CR-3R (161 µg/L). Overall, porewater Al and Fe concentrations were quite high. Currently, criteria do not exist for maintaining contaminant levels in porewater; however, when using the ambient WQC (USEPA 2009) as a general benchmark for the protection of aquatic organisms, both Al and Fe exceed protective levels of 87 and 1,000 µg/L.

Although tracking the dissipation of trace metals downstream of both the mining and CRP influences was an important objective of this research, inherent problems with sediment and porewater analysis prevents the determination of direct links between elevated trace metal

concentrations and bioavailability to unionids. Particle size, sediment composition and water quality greatly affect metal partitioning and thus the availability of uptake by organisms (Luoma and Bryan 1979, Naimo 1995). Secondly, areas of the river that facilitate collection of sediments that are ~ 2mm in size are often quiescent depositional areas where pockets of metals may settle out. Although every attempt was made to collect sediments from various areas and habitats within a sampling reach, in swift flowing waters such as those of the Clinch River, sediment collection can be biased toward areas where collection is feasible. Furthermore, these areas of sediment accumulation are rarely occupied by adult bivalves that require greater flow, and prefer gravel and cobble substrata (Gardner et al. 1976, Yeager et al. 1999); although vulnerable juvenile mussels inhabit depositional areas characterized by low-flow (Neves and Widlak 1987). Similar issues apply to porewater samples collected using peepers, which must be inserted several cm into the sediments. Although these samples provide researchers with a ball-park estimate of trace metals at a site, they are often a worst-case scenario approach to determining what actually is bioavailable to aquatic organisms. For these reasons, *in-situ* testing with a surrogate bivalve (*Corbicula fluminea*) may have provided more realistic data regarding the bioavailability of trace metals to unionids.

Results of bioaccumulation tests were opposite of trace metals results discussed above. After 60 days of exposure, clams accumulated higher concentrations of metals at CR-3L below the CRP discharges, than at CR-3R, which ideally is influenced primarily by Dumps Creek. Although results for all metals were high upstream at CR-PM and CR-1 Cleveland/Carbo, increases in accumulated concentrations were still evident at CR-3L, not CR-3R. Site CR-2 was only evaluated in 2008 due to accessibility issues; however, all four metals (Al, Cu, Fe, Zn) increased below the Dumps Creek confluence, but not to the degree observed at CR-3L. The

differences between sediment/porewater metal concentrations and accumulated metals in clam tissues between sites, may also be due to the modes by which the contaminant is taken in, such as through a food source or by water column filtration (Naimo 1995). Conversely, Yeager et al. (1994) demonstrated that juvenile freshwater mussels fed on bacteria, detritus and colloidal particles in porewater, and although they typically burrowed less than 8 cm (Neves and Widlak 1987), they have a greater association with elevated metal concentrations found in sediments or porewater than dissolved metals in overlaying water. Therefore, porewater concentrations of trace metals may be a contributing factor to the depressed unionid recruitment (USGS 2009, Jones et al. 2009) in the Clinch River.

To better understand the bioavailability potential of Al, Cu, Fe and Zn to bivalves, accumulation results were used to calculate both Bioconcentration and Bioaccumulation Factors (BCF and BAF) using porewater and sediments, respectively. Strongest relationships for sediments and clam tissues were observed for Cu, followed by Zn. BAF values showed a significant (<1.0) relationship for Cu both upstream of the CRP and Dumps Creek confluences (1.11 and 2.22) and at CR-3L (1.45), while sites influenced predominantly by Dumps Creek (CR-2 and CR-3R) indicated only a minor relationship between sediment and tissue concentrations of Cu. Trends were variable downstream, indicating no discernable pattern of dissipation or association with upstream influences. In contrast, Hull et al. (2006) determined Cu body burdens were ~160% higher in *in-situ* clam tissues compared to nominal levels and four times higher in *V. iris* in studies conducted below the CRP. Although Zn BAF values were higher than those of Al and Fe, only minor relationships between the two variables were determined, with results generally higher at downstream sites (0.331-0.689) than sites nearest to the Dumps Creek confluence (0.205-0.294), while CR-3L was higher with a BAF of 0.512.

Caution should be taken with BAF values as these estimates do not account for the variability observed in bioaccumulation due to the lipid and organic carbon content of tissues and sediments, which affect uptake. The more appropriately used Biota-Sediment Accumulation Factors (BSAF) is based on the relationship of lipid normalized tissues and total organic carbon normalized sediments. However, BSAF values were not used in this study as they are considered to be unreliable when evaluating the relationships of elemental metals. According to the US Army Corps of Engineers (USACE) BSAF database (USACE 2010); reliable lipid to TOC relationships have not been established for elemental metals and attempts to establish BSAF has yielded variable results.

Similar trends were observed using BCF values, which compared tissue and porewater concentrations. BCF values were low for Al and Fe, despite seemingly high accumulated levels in tissues. Copper BCF values followed the same pattern as seem with the BAF values. High BCF values (>1,000) occurred at six of the 12 Clinch River sampling stations, with the remaining sites falling with the moderate range (250-1,000) for bioavailability potential. According to these results, all sites below Cleveland, VA had moderate to high bioavailability of Cu, including the sampling station above the CRP and Dumps Creek confluence, although these results varied between sites, indicating no signs of dissipation. Zinc BCF values indicated a more defined pattern, with an increase evident at CR-3L which remained elevated at or above the moderate range at all sites downstream.

These results do not imply that trace metal concentrations observed in sediments, porewater or in *C. fluminea* tissues are at levels that are toxic. In fact, no correlation (r= -0.061to 0.301) was observed between Asian clam growth after 60 days and BAF values for the four metals analyzed, or between growth and individual metals concentrations (r=-0.0.048to -0.1249).

According to Naimo 1995, freshwater organisms, including unionids, possess mechanisms which inhibit toxic metal accumulation in tissues. Several researchers (Hamilton and Mehrle 1986, Steinert and Pickwell 1988, Garvey 1990) have studied how proteins called methallothioneins (MT) bind certain metals, such as Cd, Cu, Hg and Zn. Coullard et al. (1993) determined that MT is also involved in Cd detoxification in the freshwater mussel, *Anodonta grandis* (Giant Floater). However, direct elimination of metals from mussel tissues may be a lengthy process. Millington and Walker (1983) found that mussels exposed to Zn in laboratory experiments failed to eliminate significant amounts of the metal after a 42 day depuration period. Despite the extensive research on accumulation of metals in bivalve tissues, little is understood on the chronic effects of long-term exposure. Farris et al. (1988) determined cellulolytic activity was significantly reduced in *Corbicula* exposed to 16 µg/L Cu and 87 µg/L Zn. Salanki and Varanka (1976) found that *Anodonta cygnea* had decreased (10%) periodic activity of abductor mussels at 0.1 µg/L Cu and a 50% reduction at 1.0 µg/L Cu. Unfortunately, the effects of metals on reproduction is not well understood. Myint and Tyler (1982) found that Cu suppressed both the growth of oocytes and vitellogenesis in larger oocytes in the marine bivalve, *Mytilus edulis*. Furthermore, Zn also caused the inhibition of oocyte development in test organisms, which resulted in severe lysis of gametes. Minimal research has evaluated the reproductive responses of freshwater bivalves to chronic metal exposure.

In-situ tests with *Corbicula* are beneficial to understanding the response of indigenous biota of concern to the factors which may be influencing community structure and health (Doherty and Cherry 1988, Doherty 1990, Soucek et al. 2001, Hull et al. 2002). The addition of benthic macroinvertebrate surveys can provide valuable information regarding ecological health in ecosystems impacted by anthropogenic stressors (Rosenberg et al., 1986, Lenat and Barbour

1993, Resh et al. 1995, Wallace et al. 1996, Smith and Beauchamp 2000). However, benthic macroinvertebrate surveys conducted for this study were not adequate measures of ecosystem condition. Standard sampling methods for wadeable streams were not possible at all sampling sites due to water depth, channel size and flow velocity. For these reasons, quantitative samplers (Hester-Dendy's) were used to collect benthic invertebrates over a six-week period. Based on these results, the Hester-Dendy samplers did not adequately represent community structure at each site, which were primarily colonized by scrapers and filter feeders only. Taxa richness, EPT richness and mayfly abundance were significantly higher at site CR-2 compared to other sampling sites; however, it is important to note that the majority of mayflies collected belonged in the family Heptageniidae (*Maccaffertium* sp.). *Maccaffertium* are characterized by their affinity for larger substrate such as coarse cobble or boulders, slower current (Kondratieff and Voshell 1980) and morphological adaptations for grazing or scraping bacteria and detrital matter from surfaces (Cummins and Klug 1979). Quantitative results also indicate some impairment occurred below the CRP, as taxa richness decreased significantly at CR-3, and remained lower than richness total observed upstream at CR-1.

Qualitative, dip-net results showed an opposite trend with taxa richness totals higher than other sampling sites. During both 2008 and 2009, samples at CR-3 had the highest number of taxa collected with a total of 15 at CR-3 (combined banks) in 2008 and 20 taxa at CR-3L in 2009. In fact, CR-3L had substantially more taxa collected compared to CR-3R (13), which was comparable to the total taxa collected at all other Clinch River sites. However, the types of taxa collected is important to note as many of the taxa observed at CR-3L belonged in the Order Diptera (Ephydridae, Tiplulidae, Athericidae) and Megaloptera (Corydalidae). Although these organisms are considered in-tolerant to organic pollution based on tolerance values (USEPA

2010b, Tetra Tech 2003), the presence of these organisms at this site indicate a change in benthic community composition which could possibly be attributed to the addition of inorganic pollution or subtle changes in water quality and habitat. Additionally, mayfly abundance may have been higher (9) at CR-3L than CR-3R (3), but only one taxon, *Isonychia*, was identified, and the difference in totals was minimal. Overall, the use of benthic macroinvertebrate data to determine impacts in the river was difficult. The Clinch River, which is a fourth order stream near the Dumps Creek confluence, becomes much wider and deeper as sites progress downstream. Habitat also varies at downstream sites, making site to site comparisons difficult. Furthermore, sampling in 2009 was conducted much later in the season compared to 2008 due to dangerously high flow conditions at every sampling site. Seasonal differences in community structure are attributed to changes in water temperature, photoperiod, flow regime, nutrient availability, dissolve oxygen levels and individual life history patterns (Resh and Rosenberg 1984).

Overall results of this research showed that the Clinch River is not only being influenced by the CRP effluent and active mining in Dumps Creek, but also by unknown sources upstream. Upstream sites had total metal concentrations higher than some downstream sites, which complicates any conclusions that can be made in determining a major source of contamination. Furthermore, it is inconclusive based on these results that mining in Dumps Creek is a major contributor to unionid decline. Because chronic, sub-lethal effects of trace metal exposure are so poorly understood, it is difficult to say any of these influences are impacting unionid assemblages. Because mussel surveys were not performed in the initial years following the construction of the CRP (1958), it is unclear whether unionids were even present in this section of the Clinch River prior to the 1967 and 1970 chemical spills (Hull 2002). Previous investigations (Hull 2002, Hull et al. 2006) in the Clinch River have been inconclusive in pin-

pointing a more definitive source of contamination. Perhaps it is not a single source of contamination, but rather a history of devastating insults such as the spills of 1967 and 1970 and continuous inputs from agriculture, logging, coal mining and urbanization. As suggested in Nalepa et al. (1991) and Brogan (1993), a single source or impact may not be the cause of unionid declines, but rather the result of long term cumulative effects from multiple impacts.

Summary and Conclusions

Concentrations of trace metals in sediments and porewater are indicative of continual contamination in the Clinch River. Active coal mining in the Dumps Creek subwatershed undoubtedly contribute to this metal loading; however, accumulated concentrations of Al, Fe, Cu and Zn were significantly higher in Asian clam tissues below the CRP effluent discharges than those below the Dumps Creek. Further, trace metal concentrations in sediments and porewater and tissues indicate higher concentrations upstream near Pounding Mill, Virginia, than the site directly below the confluence with Dumps Creek, indicating upstream sources of contamination. Certain bioassessment tools used in this study were more predictive of in-stream contamination, than others. Sediment and porewater metal concentrations were not consistent with bioaccumulation results; however, *in-situ* Asian clam studies provide more thorough results chronic low-level exposure. Analysis of site sediments can be limited as this type of grab-and-go sampling can provide only a "snap-shot" of contamination at a given time. Porewater samplers used in this study collected composite samples over a 21-day period; however, these too were limited as the location by which the samplers were placed were often quiescent depositional zones in which trace metals could accumulate over time. In general, Cu and Zn remain bioavailable to bivalves, based on the 60 day *in-situ* clam test results and BAF /BCF calculations, and were much higher below the CRP discharge than Dumps Creek. Water quality

measurements that are often useful in tracking pollutants in stream (i.e., conductivity and pH) were consistent between sites, with only one site (CR-2) showing any substantial increase in conductivity. However, conductivity dissipated quickly in-stream. Both quantitative and qualitative benthic macroinvertebrate surveys were not useful in evaluating ecosystem health, as samples were compromised by both habitat limitations at downstream sites and seasonal differences in community structure.

Multiple sources of impact, including unidentified sources upstream, active coal mining in the Dumps Creek subwatershed and discharges from the CRP power plant are contributing to continual metal contamination in the Clinch River. This chronic exposure may be contributing to depressed unionid recruitment at downstream reaches.

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Table 1.1. Clinch River sampling site locations and corresponding GPS coordinates.			
Sampling Site	**Location**	**Coordinates**	**Notes**
Sites located off of Route 664			
CR-1 PM	Located off of VA-19 near Pounding Mill, VA.		Added in 2009
CR-1 Cleveland	Located 4.8 km upstream of Dumps Creek at community ball park.	36° 56.436 N 082° 09.393 W	
CR-1	Located 1.0 km upstream of Dumps Creek.	36° 55.86 N 082° 11.75 W	
CR-2	Sampled below Dumps Creek confluence.	36°56.087 N 082° 11.802 W	Site occasionally inaccessible due to construction.
Sites located off of Route 665			
CR-3 R	Right bank (Dumps Creek) sampling site, located ~ 3 km downstream of CRP and Dumps Creek influences.	36° 55.719 N 082° 12.082 W	
CR-3 L	Left bank (Plant) sampling site, located ~ 3 km downstream of CRP and Dumps Creek influences.	36° 55.719 N 082° 12.082 W	
CR-4	Located ~ 4 km downstream from CR-3.	36° 55.248 N 082° 12.785 W	
CR-5	Site located 100 m above Carterton boat ramp, off of Rt. 855.	36° 54.899 N 082° 13.286 W	
CR-6	Site above St. Paul, VA off River Road.	36° 53.810 N 082° 17.514 W	
CR-7	Located below St. Paul, VA- cross Rt. 58 and left toward industrial park. Park above boat ramp and walk down dirt ramp toward river. Sampling reach located below major riffle area in calmer region.	36° 54.292 N 082° 19.217 W	Site inaccessible at times due to high flow.
CR-8	Below Guest River influence near Dungannon, VA. Site located off of Wilder Ave. at boat launch.	36° 49.869 N 082° 27.732 W	
CR-9	Furthest downstream site. Located off of 65 N (Clinch River Hwy.). Site located off of Bridge St.	36° 40.411 N 082° 44.816 W	
Guest River	Near (~0.80 km) Coeburn, VA at Grace Covenant Church at RM 6.3.	36° 55.769 N 082° 27.385 W	

Table 1.2. Mean values (± standard deviation) for water chemistry parameters at Clinch River sites. Water samples were collected from November 2007 through November 2009.

Site Name	Temp. (°C)	pH (su)	Alkalinity (mg $CaCO_3$/L)	Hardness (mg $CaCO_3$/L)
CR-PM	25.0 ± 2.0	8.10 ± 0.21	110.0 ± 0.0	158.0 ± 0.0
CR-1 Cleveland	25.0 ± 2.0	8.19 ± 0.24	136.0 ± 20.4	158.7 ± 25.0
CR-1	25.0 ± 2.0	8.28 ± 0.14	148.0 ± 0.0	162.0 ± 0.0
CR-2	25.0 ± 2.0	8.51 ± 0.08	201.0 ± 49.0	153.0 ± 3.0
CR-3 R	25.0 ± 2.0	8.38 ± 0.13	172.5 ± 41.6	171.0 ± 16.6
CR-3 L	25.0 ± 2.0	8.41 ± 0.16	160.7 ± 41.5	174.0 ± 11.8
CR-4	25.0 ± 2.0	8.33 ± 0.20	174.0 ± 36.0	182.5 ± 6.5
CR-5	25.0 ± 2.0	8.35 ± 0.14	175.3 ± 26.0	194.7 ± 13.6
CR-6	25.0 ± 2.0	8.36 ± 0.16	174.0 ±36.0	189.5 ± 19.5
CR-7	25.0 ± 2.0	8.43 ± 0.24	94.0 ± 0.0	112.0 ± 0.0
CR-8	25.0 ± 2.0	8.54 ± 0.24	106.0 ± 0.0	130.0 ± 0.0
CR-9	25.0 ± 2.0	8.34 ± 0.17	96.0 ± 10.0	113.0 ± 13.0
Guest River	25.0 ± 2.0	8.35 ± 0.29	144.7 ± 50.1	260.0 ± 85.2

Table 1.3. Mean total recoverable metals in Clinch River site sediments collected during summer 2008 and 2009. Means compared using Tukey's HSD (α=0.05).

Site	Aluminum	Chromium	Copper	Iron	Manganese	Strontium	Zinc
CR-PM	2250.9 b	.	5.9 a b	12322.6 a	.	.	21.9 c d e
CR-1 (Cleveland)	1622.8 e f	6.7 c d e	3.4 d e	7566.4 d e	136.3 e	10.2 a b	21.9 c d e
CR-1	1758.8 d e	.	2.9 e	7976.7 c d e	.	.	16.4 g
CR-2	2246.6 b	4.9 e f	5.8 a b	9112.8 c	327.0 a	12.8 a	27.6 a b
CR-3 R	2666.3 a	6.7 c d e	5.2 a b c d	8689.1 c d	200.1 c d e	10.8 a b	25.8 a b c
CR-3 L	1224.0 g	8.1 b c d	4.6 b c d e	7507.3 e	160.8 d e	5.4 c d	16.7 f g
CR-4	1780.1 d e	10.0 b	5.4 a b c	8954.1 c	245.2 b c	11.2 a b	20.9 d e f
CR-5	2110.2 b c	14.4 a	5.1 a b c d	10787.5 b	331.0 a	10.4 a b	21.5 c d e
CR-6	1747.7	9.2	3.6 c d e	7977.0 c d e	230.0 b c d	11.0 a b	16.2 g
CR-7	1559.1 e f	6.7 c d e	4.2 b c d e	7527.0 d e	202.7 b c d e	8.3 b c	18.7 e f g
CR-8	1963.6 c d	6.0 d e	6.9 a	7620.7 d e	225.9 b c d	6.3 c d	21.3 d e
CR-9	2098.8 b c	13.9 a	4.0 b c d e	10567.8 b	283.0 a b	5.3 c d	24.7 b c d
Guest River	1445.0 f g	1.9 f	3.7 c d e	5511.4 f	244.8 b c	4.7 d	29.9 a

[a] Means connected by different letters are statistically different (α=0.05).

Table 1.4. Total concentrations (µg/L) of select metals in interstitial water samples from Clinch River sampling sites.

Site	Al	Fe	Sr	Zn	Cu
CR-1 Cleveland	5,330	15,919	127	50	15
CR-1	1,247	5,428	119	20	3
CR-2	961	4,815	311	21	5
CR-3R	1,215	5,185	161	21	4
CR-3L	1,083	3,428	295	17	3
CR-4	1,360	4,711	142	19	3
CR-5	2,221	6,889	84	25	5
CR-6	991	2,021	154	12	3
CR-7	1,149	3,093	82	21	3
Guest River	91	3,875	385	3	3
CR-8	1,386	5,652	266	25	5
CR-9	2,269	8,940	219	49	10

Table 1.5. Habitat assessment scores at Clinch River study sites including one site in the Guest River. Scores were based on a 200-point scale.

Site	Habitat Assessment Score
CR-Pounding Mill	182
CR-1 Cleveland	118
CR-1	166
CR-2	109
CR-3R	159
CR-3L	145
CR-4	172
CR-5	163
CR-6	166
CR-7	149
CR-8	133
CR-9	165
Guest River	154

Table 1.6. Qualitative benthic macroinvertebrate samples taken at sampling sites in the Clinch River.

Parameter	Qualitative Results 2008									
	CR-1	CR-2	CR-3*	CR-4	CR-5	CR-6	CR-7	CR-8	CR-9	Guest River
Taxa Richness	8.0	6.0	15.0	7.0	16.0	10.0	11.0	14.0	3.0	13.0
Caddisfly Abundance	2.0	0.0	22.0	5.0	25.0	1.0	20.0	0.0	0.0	60.0
Stonefly Abundance	0.0	0.0	7.0	1.0	4.0	0.0	0.0	0.0	0.0	0.0
Mayfly Abundance	1.0	2.0	7.0	3.0	3.0	4.0	2.0	4.0	2.0	21.0
Percent EPT	27.3	12.5	61.0	64.3	46.4	22.7	68.8	16.0	10.0	80.2
EPT Richness	3.0	1.0	10.0	4.0	7.0	4.0	4.0	2.0	2.0	6.0
Midge/EPT Ratio	0.000	3.500	0.000	0.111	0.094	0.000	0.136	1.000	0.000	0.123
Total Hydropsychidae	1	0	16	3	21	1	20	0	0	60

Parameter	Qualitative Results 2009									
	CR-1	CR-2	CR-3L	CR-3R	CR-4	CR-5	CR-6	CR-7	CR-8	Guest River
Taxa Richness	12.0	12.0	20.0	13.0	12.0	11.0	16.0	13.0	17.0	11.3
Caddisfly Abundance	0.0	0.0	4.0	6.0	11.0	3.0	13.0	46.0	1.0	87.5
Stonefly Abundance	7.0	0.0	4.0	9.0	0.0	1.0	3.0	0.0	0.0	0.25
Mayfly Abundance	0.0	3.0	9.0	3.0	14.0	4.0	19.0	18.0	2.0	37.8
Percent Mayfly	0.0	15.0	18.0	9.7	40.0	22.2	32.8	20.0	2.2	26.8
Percent EPT	23.3	15.0	34.0	58.1	71.4	44.4	60.3	71.1	3.3	86.7
EPT Richness	2.0	2.0	5.0	3.0	6.0	5.0	8.0	3.0	3.0	4.25
Midge/EPT Ratio	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.333	0.007
Total Hydropsychidae	0.0	0.0	3.0	6.0	9.0	3.0	13.0	46.0	1.0	87.5

* Site CR-3 was sampled mid-river due to accessibility at the CR-3L location.

Table 1.7. Average concentrations of select metals (mg/kg wet weight) in Asian clam (*Corbicula fluminea*) tissue samples from Clinch River sampling sites after 60 days *in-situ*. Values listed as n/d were not determined because of site inaccessibility due to high river flow.

	2008 Results (mg/kg wet weight)			
	Aluminum	**Copper**	**Iron**	**Zinc**
CR-1	2.06 [b]	3.76 [b c]	19.27 [c d]	6.64 [e f]
CR-2	4.18 [a]	4.49 [b]	26.35 [a b c]	8.11 [d e f]
CR-3R	1.63 [b]	2.90 [c]	15.47 [d]	5.40 [f]
CR-3L	1.78 [b]	6.40 [a]	33.56 [a]	9.34 [c d e]
CR-4	1.74 [b]	4.38 [b]	30.64 [a b]	11.93 [a b c]
CR-5	1.43 [b]	6.38 [a]	32.39 [a b]	12.34 [a b c]
CR-6	1.34 [b]	4.83 [b]	28.21 [a b c]	10.97 [a b c d]
CR-7	0.75 [b]	3.50 [b c]	20.26 [c d]	7.96 [d e f]
Guest River	1.55 [b]	4.38 [b]	31.28 [a b]	13.66 [a]
CR-8	1.20 [b]	3.69 [b c]	25.83 [a b c]	12.89 [a b]
CR-9	0.82 [b]	4.49 [b]	23.30 [b c d]	9.82 [b c d e]
	2009 Results (mg/kg wet weight)			
	Aluminum	**Copper**	**Iron**	**Zinc**
Control*	1.01 [f]	4.14 [c d]	20.3 [c]	5.86 [c d]
Pounding Mill	7.81 [b c]	4.96 [b c]	48.25 [b c]	7.69 [b c d]
CR-1**	3.85 [d e f]	6.43 [a]	32.81 [b c]	6.30 [c d]
CR-2	**n/d**	**n/d**	**n/d**	**n/d**
CR-3R	3.60 [e f]	3.49 [d]	20.10 [c]	5.21 [c d]
CR-3L	14.19 [a]	6.78 [a]	88.78 [a]	7.75 [b c d]
CR-4	5.04 [c d e]	3.81 [c d]	27.34 [b c]	7.23 [b c d]
CR-5	6.45 [b c d e]	4.32 [c d]	38.29 [b c]	8.04 [b c]
CR-6	9.29 [b]	4.13 [c d]	55.49 [b]	7.75 [b c d]
CR-7	**n/d**	**n/d**	**n/d**	**n/d**
Guest River	7.66 [b c d]	5.69 [a b]	43.98 [b c]	11.04 [a]
CR-8	7.47 [b c d e]	4.84 [b c]	44.07 [b c]	9.18 [a b]
CR-9	4.56 [c d e f]	3.81 [c d]	27.98 [b c]	6.56 [b c d]

[a] Means not connected by the same letter are significantly different (α=0.05), Tukey-Kramer HSD.

*A subset of clams were retained for analysis of pre-exposure condition and are included as a control in the 2009 study.

** Site location for CR-1 was moved downstream from Cleveland, VA during the 2009 study.

Table 1.8. Estimated Bioaccumulation Factors (BAF) for determining the relationship between select metals in clam tissues (C_t, mg/kg wet wt.) and sediments (C_s, mg/kg dry wt.) from sampling stations in the Clinch River.

Site	Al			Cu			Fe			Zn		
	C_t	C_s	*BAF*	C_t	C_s	*BAF*	C_t	C_s	*BAF*	C_t	C_s	*BAF*
Pounding Mill	7.81	2250.9	*0.0035*	4.96	5.90	*0.84*	48.25	12322.6	*0.0039*	7.69	21.85	*0.352*
CR-1 Cleveland	2.06	1622.8	*0.0013*	3.76	3.38	*1.11*	19.27	7566.4	*0.0025*	6.64	21.88	*0.304*
CR-1	3.85	1758.8	*0.0022*	6.43	2.90	*2.22*	32.81	7976.7	*0.0041*	6.30	16.43	*0.384*
CR-2	4.18	2246.6	*0.0019*	4.49	5.83	*0.77*	26.35	9112.8	*0.0029*	8.11	27.58	*0.294*
CR-3R	2.615	2666.3	*0.0010*	3.20	5.18	*0.62*	17.785	8689.1	*0.0020*	5.31	25.83	*0.205*
CR-3L	7.985	1224.0	*0.0065*	6.59	4.55	*1.45*	61.17	7507.3	*0.0081*	8.55	16.70	*0.512*
CR-4	3.39	1780.1	*0.0019*	4.10	5.43	*0.76*	28.99	8954.1	*0.0032*	9.58	20.90	*0.458*
CR-5	3.94	2110.2	*0.0019*	5.35	5.10	*1.05*	35.34	10787.5	*0.0033*	10.19	21.50	*0.474*
CR-6	5.315	1747.7	*0.0030*	4.48	3.55	*1.26*	41.85	7977.0	*0.0052*	9.36	16.23	*0.577*
CR-7	0.75	1559.1	*0.0005*	3.50	4.18	*0.84*	20.26	7527.0	*0.0027*	7.96	18.70	*0.426*
CR-8	4.335	1963.6	*0.0022*	4.27	6.93	*0.62*	34.95	7620.7	*0.0046*	11.04	21.28	*0.519*
CR-9	2.71	2098.8	*0.0013*	4.15	4.03	*1.03*	25.64	10567.8	*0.0024*	8.19	24.73	*0.331*
Guest River	4.625	1445.0	*0.0032*	5.04	3.68	*1.37*	37.63	5511.4	*0.0068*	12.35	29.93	*0.413*

Table 1.9. Estimated Bioconcentration Factors (BCF) to determine the relationship between accumulated metals in Asian clam (*Corbicula fluminea*) tissues and metal concentrations in interstitial water (porewater). Tissue and water concentrations expressed as moles per unit (g, ml).

	Aluminum			**Copper**			**Iron**			**Zinc**		
BCF Values	**Tissue**	**Water**	***BCF***	**Tissue**	**Water**	***BCF***	**Tissue**	**Water**	***BCF***	**Tissue**	**Water**	***BCF***
CR-1 Cleveland	7.63E-05	0.000198	***0.39***	5.92E-05	2.36E-07	***250.7***	0.000345	0.000285	***1.2***	0.000102	7.65E-07	***132.8***
CR-1	0.000143	4.62E-05	***3.09***	0.000101	4.72E-08	***2,143.3***	0.000588	9.72E-05	***6.0***	9.64E-05	3.06E-07	***315.0***
CR-2	0.000155	3.56E-05	***4.35***	7.07E-05	7.87E-08	***898.0***	0.000472	8.62E-05	***5.5***	0.000124	3.21E-07	***386.2***
CR-3R	9.69E-05	4.5E-05	***2.15***	5.03E-05	6.29E-08	***798.8***	0.000318	9.28E-05	***3.4***	8.11E-05	3.21E-07	***252.6***
CR-3L	0.000296	4.01E-05	***7.37***	0.000104	4.72E-08	***2,196.7***	0.001095	6.14E-05	***17.8***	0.000131	2.6E-07	***502.6***
CR-4	0.000126	5.04E-05	***2.49***	6.44E-05	4.72E-08	***1,365.0***	0.000519	8.44E-05	***6.2***	0.000147	2.91E-07	***504.2***
CR-5	0.000146	8.23E-05	***1.77***	8.42E-05	7.87E-08	***1,070.0***	0.000633	0.000123	***5.1***	0.000156	3.82E-07	***407.6***
CR-6	0.000197	3.67E-05	***5.36***	7.05E-05	4.72E-08	***1,493.3***	0.000749	3.62E-05	***20.7***	0.000143	1.84E-07	***780.0***
CR-7	2.78E-05	4.26E-05	***0.65***	5.51E-05	4.72E-08	***1,166.7***	0.000363	5.54E-05	***6.6***	0.000122	3.21E-07	***379.0***
CR-8	0.000161	5.14E-05	***3.13***	6.71E-05	7.87E-08	***853.0***	0.000626	0.000101	***6.2***	0.000169	3.82E-07	***441.4***
CR-9	0.0001	8.41E-05	***1.19***	6.53E-05	1.57E-07	***415.0***	0.000459	0.00016	***2.9***	0.000125	7.49E-07	***167.1***
Guest River	0.000171	3.37E-06	***50.8***	7.92E-05	4.72E-08	***1,678.3***	0.000674	6.94E-05	***9.7***	0.000189	4.59E-08	***4,116.7***

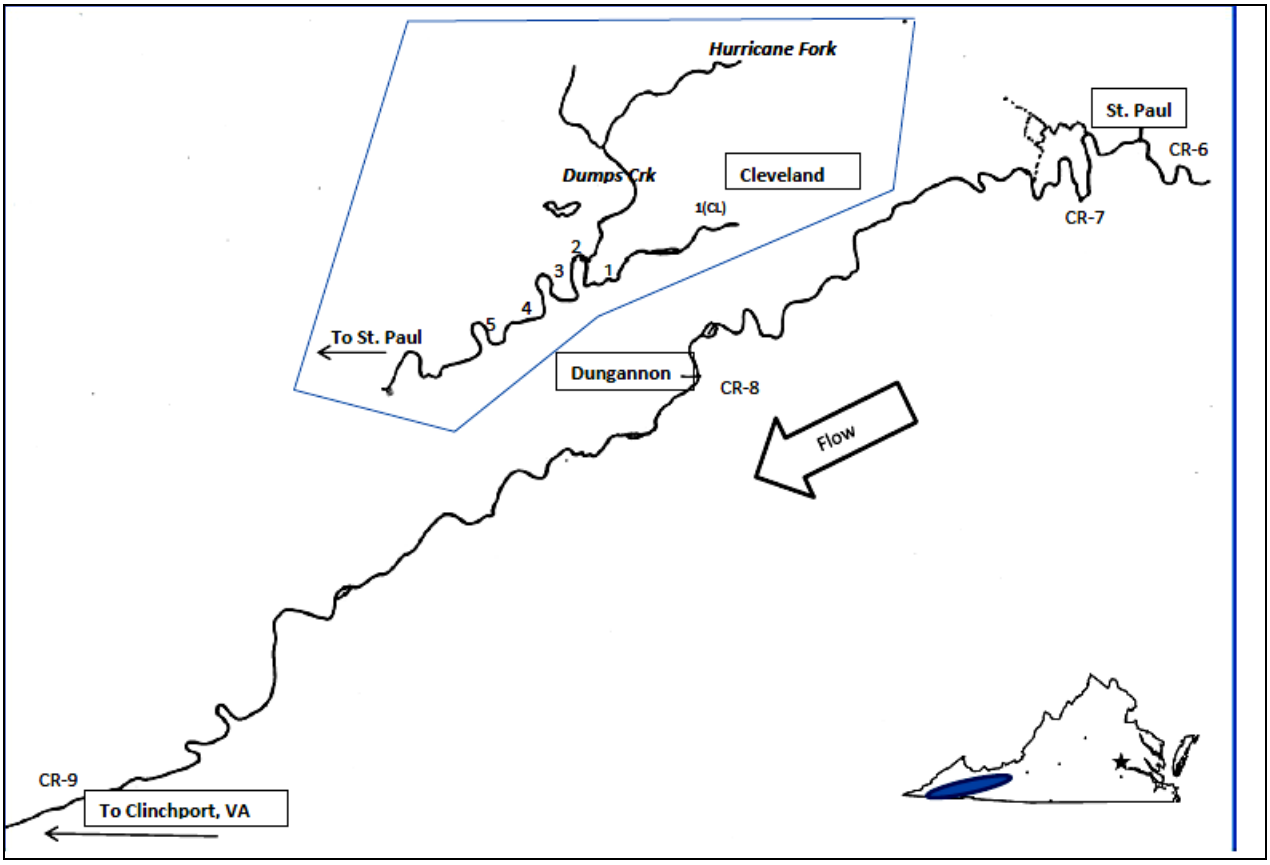


Figure 1.1. Sampling site locations in the Clinch River. Map not drawn to scale.

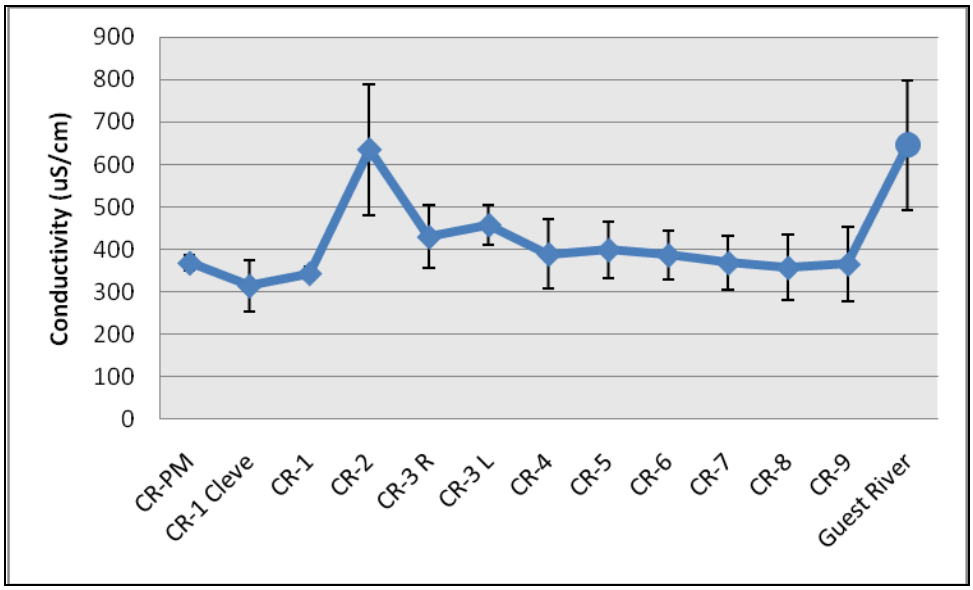
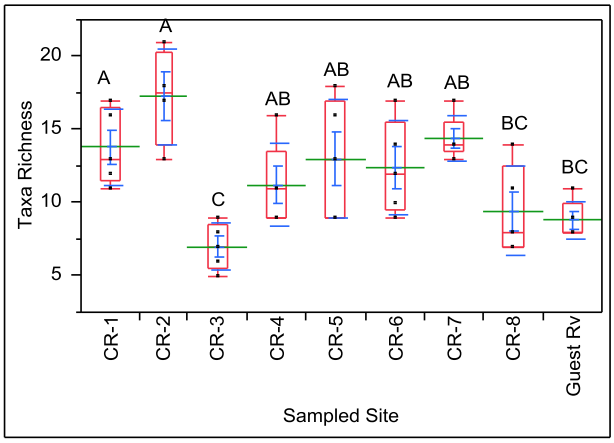
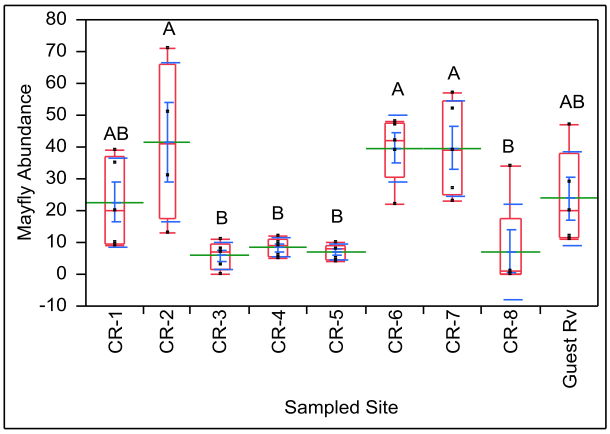


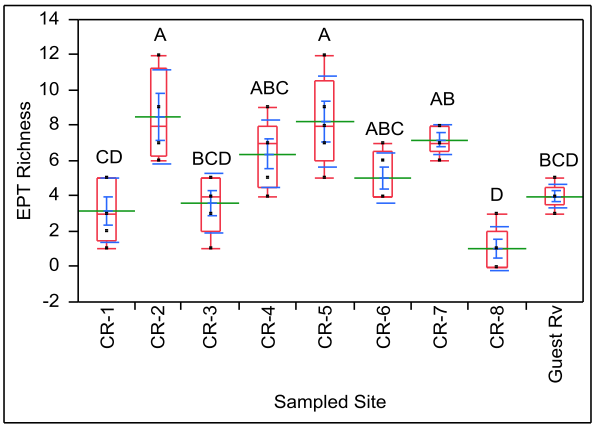
Figure 1.2. Mean conductivity (µS/cm) values (± standard deviation) for sites in the Clinch and Guest Rivers, collected from 2007-2009.



(a)



(b)



(c)

Figure 1.3. Mean (a) taxa richness, (b) mayfly abundance and (c) EPT richness of quantitative benthic macroinvertebrate samples, summer 2008. Means connected by different letters are statistically different based on Tukey's HSD comparison of means (α=0.05). Site CR-3 was located mid-river due to inaccessibility at CR-3L.

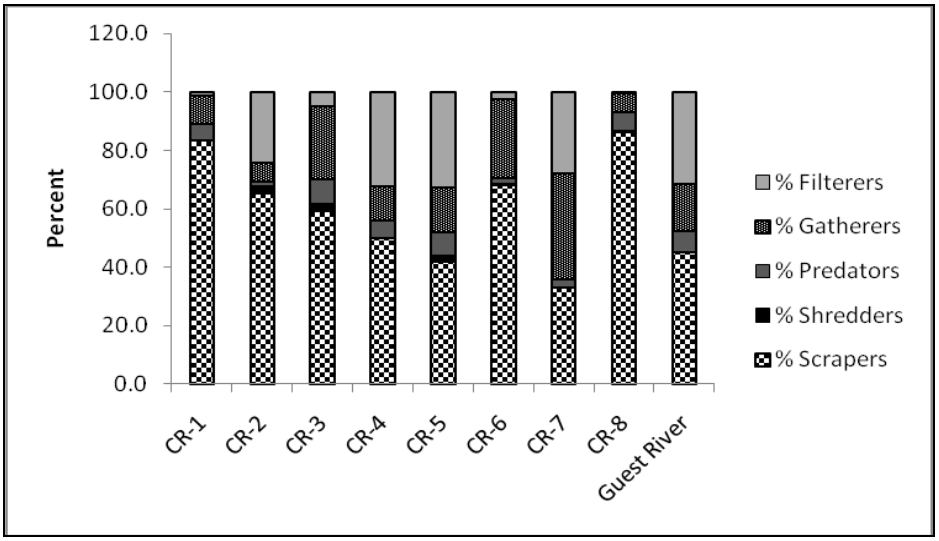


Figure 1.4. Functional feeding group distribution of organisms collected on multi-plate artificial substrate samplers during summer, 2008.

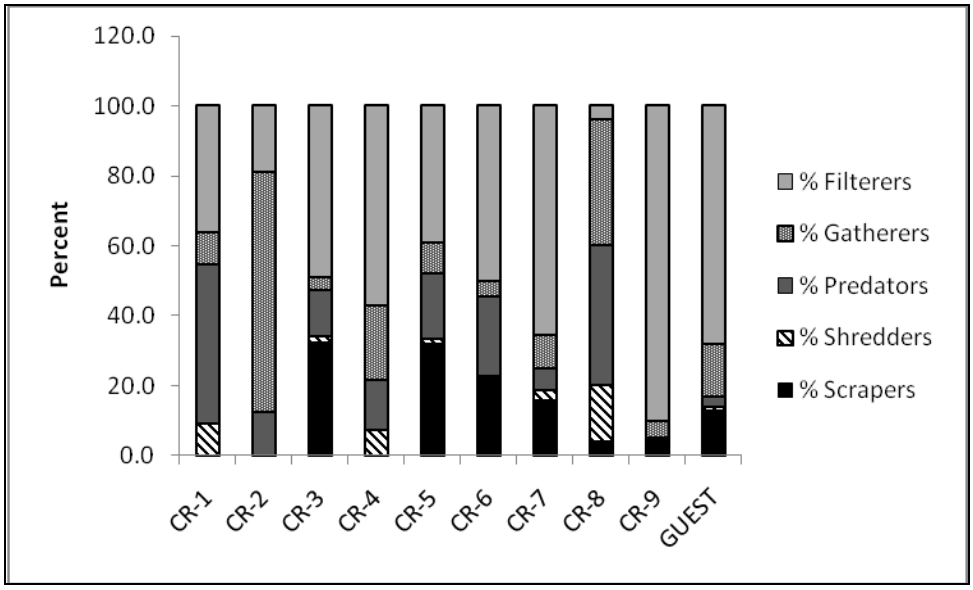
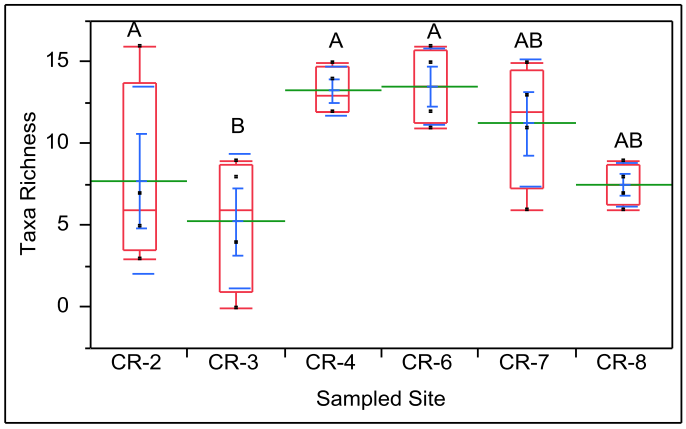
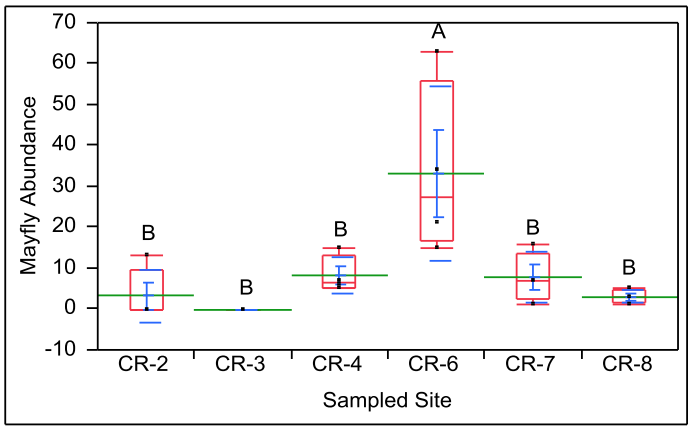


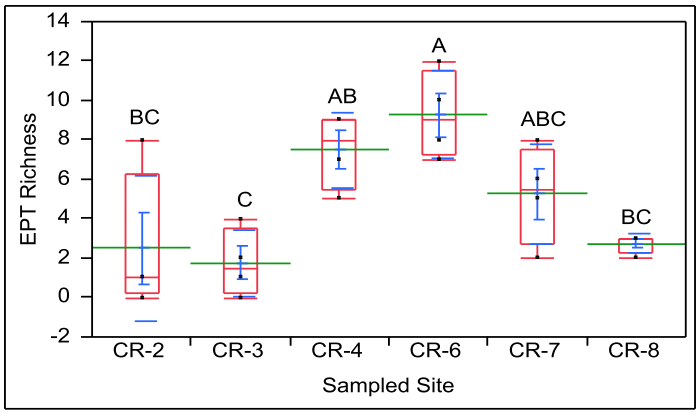
Figure 1.5. Functional feeding group distribution of organisms collected during single replicate qualitative sampling in the Clinch River, fall 2008.



(a)



(b)



(c)

Figure 1.6. Mean (a) taxa richness, (b) mayfly abundance and (c) EPT richness of quantitative benthic macroinvertebrate samples, 2009. Means connected by different letters are statistically different based on Tukey's HSD comparison of means (α=0.05).

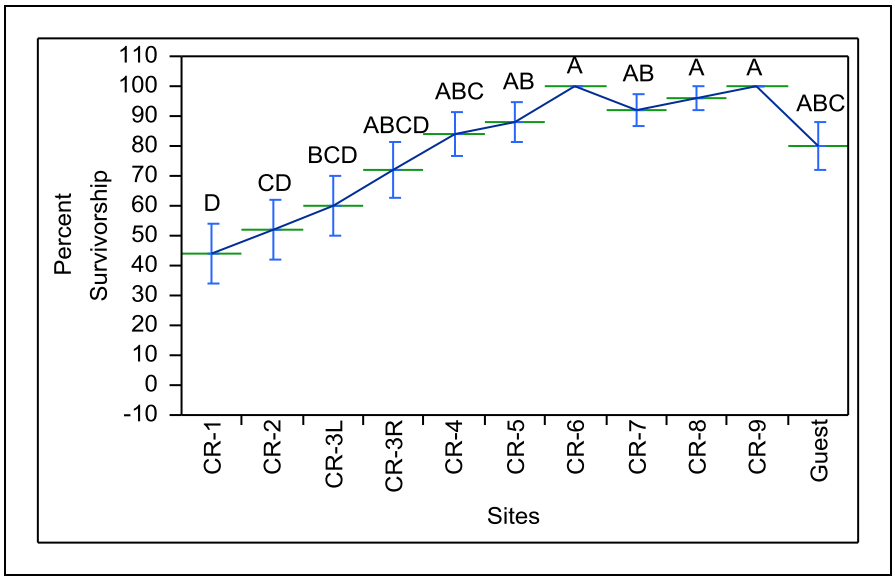


Figure 1.7. One-way analysis of Asian clam (*Corbicula fluminea*) survivorship (%) in summer 2008 after 60 days *in-situ*. Sites connected by the same letter are not significantly different based on Tukey-Kramer HSD means comparison (α=0.05).

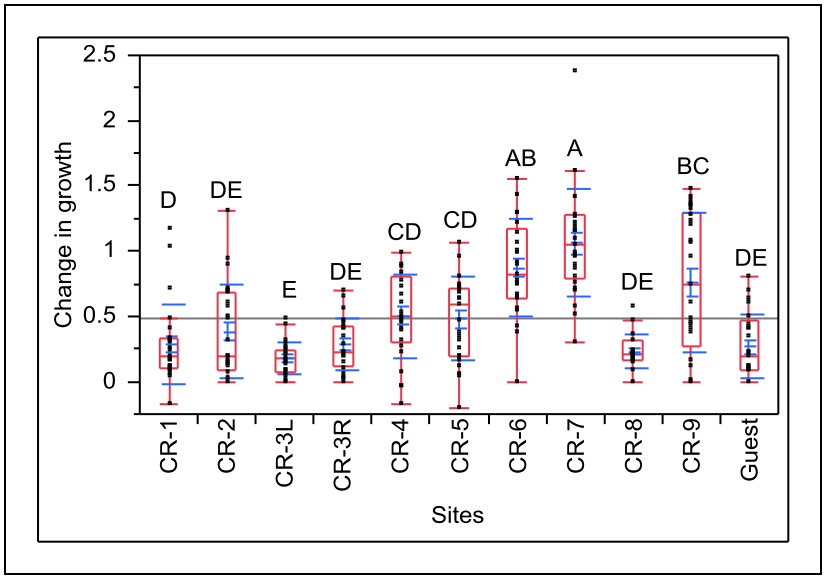


Figure 1.8. One-way analysis of Asian clam (*Corbicula fluminea*) growth (mm) in summer 2008 after 60 days *in-situ*. Sites connected by the same letter are not significantly different based on Tukey-Kramer HSD means comparison (α=0.05).

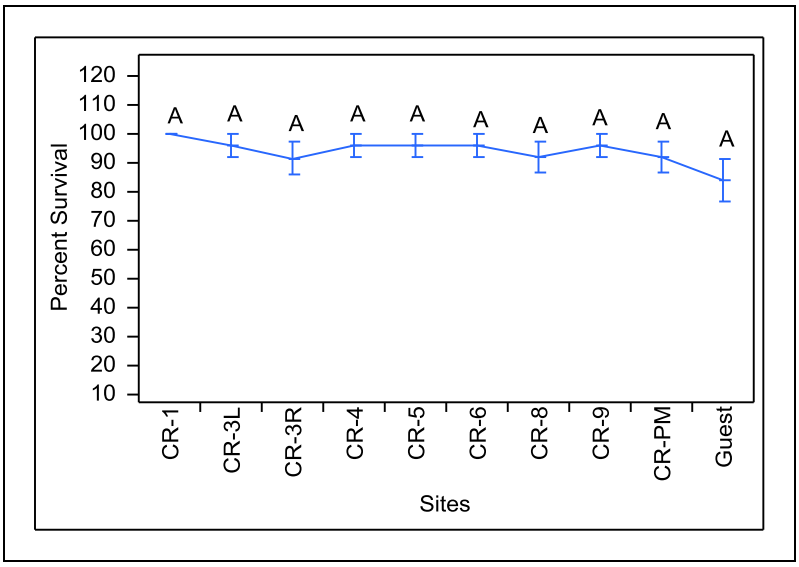


Figure 1.9. One-way analysis of Asian clam (*Corbicula fluminea*) survivorship (%) in fall 2009 after 60 days *in-situ*. Sites connected by the same letter are not significantly different based on Tukey-Kramer HSD means comparison (α=0.05).

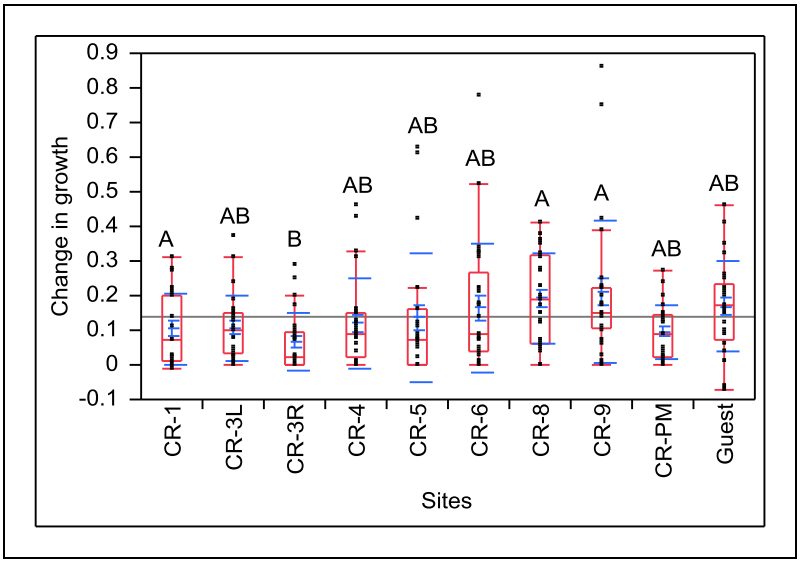


Figure 1.10. One-way analysis of Asian clam (*Corbicula fluminea*) growth (mm) in fall 2009 after 60 days *in-situ*. Sites connected by the same letter are not significantly different based on Tukey-Kramer HSD means comparison (α=0.05).

Figure 1.11. Comparison of Bioaccumulation Factors (BAF) and Bioconcentration Factors (BCF) at sampling sites in the Clinch and Guest Rivers.

Chapter 2. An Ecotoxicological Assessment of the Dumps Creek Watershed to Determine Potential Impacts from Active and Historical Coal-Mining Activities on Aquatic Biota

Introduction

The demise of diverse unionid (Bivalvia: Unionidae) assemblages in the Tennessee and Cumberland River systems has prompted many research projects aimed at determining the cause of mussel mortality and lack of recruitment. Both point and nonpoint sources of contaminants have been suggested as potential contributors to reduced mussel diversity in these systems. Major tributaries, such as the Clinch and Powell Rivers which drain into the upper Tennessee River, contain some of the most diverse mussel assemblages in the world. Of the 81 known unionid species in Virginia, 37 are listed as threatened or endangered, with 32 of these species occurring in the Clinch, Powell and Holston rivers (Eckert et al. 2007). Historical sampling preformed throughout the latter part of the 20th century, found a total of 39 mussel species in the Clinch River. Of these, 14 species were endemic to the Cumberland Plateau region and six were included on the endangered species list (Ahlstedt and Tuberville 1997). Protection of these imperiled organisms has become a priority as both mussel abundance and diversity continue to decline (Ahlstedt and Tuberville 1997, Hull 2002).

Coal mining is considered to be a major source of contamination potentially affecting benthic communities and in particular, sensitive unionids (Diamond et al. 2002). When not properly controlled, discharges originating from active mining can release a variety of constituents into streams and rivers, some of which are toxic and can impact aquatic species and communities. A study by Cohen and Gorman (1991) documented more than 66,000 active and abandoned coal mining related discharges in the Appalachia region of Virginia which encompasses the Clinch River watershed.

Determining specific causes of unionid decline has been difficult. In addition, pin-pointing the direct effects of mining discharges on unionids can be more daunting as knowledge

of sub-lethal effects of these discharges is limited. Point source contamination can easily be linked with localized mortality, while widespread decreases in unionid diversity and density may be due to chronic exposure to low-level contamination (Naimo 1995). In an effort to understand the impact of active coal mining on freshwater mussels, the US Fish and Wildlife Service (USFWS) partnered with the US Geological Survey (USGS) and developed a research project in 2007-2008, to assess the sensitivity of freshwater mussels to various coal mining effluents. The goal of this research was to evaluate organism responses to different types of coal mining discharges, such as coal processing effluents and deep mine discharges. Effluents would be collected from various mining operations in the Clinch and Powell River watersheds, including facilities located in the Dumps Creek subwatershed (Dickenson-Russell Coal Company), and operated by Alpha Natural Resources (ANR). These mining operations were chosen as they were representative of the majority of effluent types needed for a thorough evaluation. In addition, ANR and collaborators at Virginia Tech conducted simultaneous toxicity tests on mining effluents.

The Dumps Creek sub-watershed is located in Russell County, VA, just north of the town of Cleveland and confluences with the Clinch River at Carbo, VA. In 1994, Dumps Creek was placed on Virginia's 303(d) List of Impaired Waters because of benthic impairment. The impaired stream segment extends a length of 3.4 stream miles (sm) from the Hurricane Fork confluence to the mouth of the Dumps Creek confluence with the Clinch River at Carbo. Primary land use within the Dumps Creek watershed included forested land and mining and is in close proximity (~50 m above, opposite bank) to the CRP (power plant). Sources of impairment in this sub-watershed are believed to be point and non-point contributions related to the mining activities (MapTech, Inc., Inc. 2004). A study by Locke et al. (2006) examined how

ecotoxicological conditions in tributaries of the Clinch River in Virginia varied with primary land use practices (mining, urbanization, agriculture). The authors used a multimetric index, the Ecotoxicological Rating System (ETR) used in several other watersheds influenced by mining activities (Soucek et al. 2000, Cherry et al. 2001, Schmidt et al. 2002, Simon et al. 2006), to rate the major tributaries. Overall, the mining influenced tributaries had the lowest ETR scores (< 60), which indicated poor ecotoxicological conditions. Dumps Creek received the lowest ETR, 44, of all tributaries studied (Locke 2005).

This research was conducted to evaluate ecotoxicological influences of coal mining facilities in the Dumps Creek watershed. This study was originally developed to provide an evaluation of the mining effluents from coal mining facilities in Dumps Creek and Hurricane Fork. Initial testing of effluents focused on the toxicity potential of effluents to the juvenile stage of a freshwater mussel (*Villosa iris*) and a benthic organism (*Hyalella azteca*). Objectives were broadened to include an ecotoxicological assessment of the subwatershed to determine if these tributaries could potentially be a major influence in unionid decline in the mainstem Clinch River. Although Dumps Creek has been determined to be ecologically impaired based on benthic impairment (TMDL) and ETR scores (Locke et al. 2006), the degree of impact on the Clinch River has not been determined or quantified. Additional testing, using standard US EPA test organisms (*Ceriodaphnia dubia* and *Pimephales promelas*) and ecological relevant test organisms (mayflies) were also included in the assessment of major effluent discharges into the system.

Materials and Methods

Site Locations

Dumps Creek watershed is located in Russell County, Virginia and drains into the Clinch River near the town of Carbo (Fig. 2.1). The watershed encompasses approximately 20, 300 acres of land, primarily consisting of forest and mined land (Map Tech, Inc. 2004). Fifteen routine study sites were selected to assess the impacts of active mining in the Dumps Creek watershed, which included the tributaries of Dumps Creek (DC) and Hurricane Fork (HF) (Table 2.1). Both DC and HF receive point-source mining effluents from both deep mine and coal-processing plant discharges. In addition, Dumps Creek and its tributaries receive non-point source discharges from abandoned mines and facilities that remain as a legacy of unregulated mining. Of the fifteen sites, some sites were excluded between sampling years due to changes in study plan and/or inaccessibility. Two additional site locations, Chaney Creek Reference and DC at Hurricane Fork, were also included in the TMDL comparison study in 2009 and 2010.

Habitat Assessment

A qualitative assessment of habitat suitability for benthic assemblages was conducted from 2008-2010 following US EPA Rapid Bioassessment Protocols for high gradient streams (Barbour et al. 1999). A rating of 0-10 to 0-20 was developed for each parameter, and the higher the score (up to 200 points), the more pristine the station.

Water and Sediment Quality

General water chemistry parameters were monitored seasonally from 2008- 2010, at each sampling station in the Dumps Creek watershed. Grab samples were taken mid-stream, using clean, 1-L Nalgene® bottles and transported to the laboratory at VA Tech on ice (< 4°C). Measurements of conductivity (μS/cm), pH (su), dissolved oxygen (mg/L), temperature (°C),

were determined in the laboratory following standard operating procedures. Measurements were obtained using a Yellow Springs, Inc. model 54 dissolved oxygen meter calibrated for elevation (YSI, Inc., Yellow Springs, OH), a YSI 30 conductivity (accuracy ± 0.05%) meter, and an Accumet® AB15 (Fisher Scientific, Pittsburgh, PA) pH meter with an Accumet gel-filled combination electrode (accuracy < ±0.05 pH at 25°C). Total alkalinity and hardness (expressed as mg $CaCO_3$/L) were measured through colorimetric titrations following APHA (1998). Additionally, water samples were preserved (pH < 2.0) using trace metal grade nitric acid and analyzed for select trace metals using Inductively Coupled Plasma (ICP) spectrophotometry at the soil testing laboratory at VA Tech (Dept. Crop and Soil Environmental Sciences).

Site sediments were collected from sampling sites in Dumps Creek and Hurricane Fork in 2008-2010. Sediment sampling normally occurred in conjunction with *in-situ* Asian clam studies to allow for reliable statistical comparisons between clam growth/survivorship and bioaccumulation of trace metals. An acid-washed polyurethane cup was used to scoop sediments from the top layer (< 2 cm) of substrate and then transferred into sterile plastic bags. Samples were maintained on ice at ~ 4°C for transport to the laboratory. Homogenized sediments were divided into equal replicates for each site, weighed and dried overnight at 60°C. Acid digestion of sediments followed methods outlined in US EPA Method 200.7 v. 5 (2001) for each sample. One gram (±0.01 g) of dried sediment was transferred to a 250-ml glass beaker, with 4 ml of (1+1) HNO_3 and 10 ml of (1+4) HCl for acid extraction. Beakers were then placed on a hot plate adjusted to a constant temperature of 95°C, and heated for approximately 30 min. After cooling, the sample was transferred to a clean, acid-washed 100-ml volumetric flask and diluted to volume using Milli-Q water. Extracted samples were allowed to stand overnight to allow

insoluble material to separate. Sediment samples were analyzed at the soil testing laboratory at VA Tech (Dept. Crop and Soil Environmental Sciences) using ICP spectrophotometry.

Benthic Macroinvertebrate Surveys

Qualitative assessment of biological health was conducted at select sites in Dumps Creek and Hurricane Fork from 2008-2010. Samples were taken during late spring (May) or early summer (June) each year. Benthic macroinvertebrate sampling methods followed US EPA Rapid Bioassessment Protocols (Barbour et al. 1999). Riffle, run, pool and shoreline habitats were sampled and four replicates were collected at each site using 800 μ*m* mesh dipnets. Specimens collected were transferred into 1-liter plastic jars and preserved in ~80% ethyl alcohol (ETOH). Each sample was sorted in the laboratory until either no remaining organisms were observed in five minutes or only redundant organisms with a count >100 were observed within a 20-minute period. Organisms were identified to lowest practical taxonomic level and community indices determined using the RBP tier III approach and standard taxonomic keys (Pennak 1989, Merritt et al. 2008). Indices included but were not limited to: total taxonomic abundance, taxonomic richness, and Ephemeroptera-Plecoptera-Trichoptera (EPT) abundance and richness. An additional metric, Community Loss index, was included in site comparisons for 2010 sample results only. The Community Loss index estimates the difference in taxa composition between a targeted site and a reference. The Community Loss index calculated for this study was based upon Taxa Richness using the following equation:

$$Community\ Loss = \frac{\#\ of\ taxa\ in\ a\ reference\ sample - \#\ of\ taxa\ common\ in\ both\ samples}{\#\ of\ taxa\ in\ target\ sample}$$

The higher the index score, the greater the dissimilarity between the targeted sample and the reference sample.

Stream Condition Index (SCI) scores were also generated following protocols developed for both Virginia and West Virginia (see *Stream Condition Index Scoring Methodology* section below). Statistical differences between sites were also determined using JMP® (SAS 2008) statistical software (see *Statistical Analysis* section).

Asian Clam *In-Situ* Studies

Asian clam (*Corbicula fluminea*) *in-situ* growth and survivorship tests were conducted at sites in Dumps Creek as well as Hurricane Fork from 2008-2010, to determine potential impacts of mining activities on bivalve organisms. Asian clams have been a successful indicator of toxicity in previous studies (Soucek et al. 2001, Hull et al. 2002, Echols et al. 2009a) and may be useful in determining potential stressors to other bivalves, such as freshwater mussels (Unionidae). Clams were collected using clam rakes from the New River, Ripplemead, Virginia, and kept in Living Streams® (Frigid Units, Toledo, OH). Clams were measured for initial width using ProMax® digital calipers and marked on the exterior shell using a file. For this study, clams measuring between 9 and 11 mm were used. Five clams were placed into 18 x 36 cm mesh bags (~0.5 cm^2 mesh size) with five replicate bags at each sampling station. After 60 days, clams were retrieved and returned to the laboratory where mean survival and final growth were measured. Mortality was determined if clams were found gaping or easily opened, or failed to close when the visceral mass was probed. Survival, individual change in size, and average growth for each replicate/site were determined. Statistical analyses were conducted using JMP® (SAS Institute 2008).

Additional studies with *C. fluminea* were conducted to estimate the bioaccumulation of select trace metals in bivalve tissue. Previous studies have utilized clams as surrogates for unionids because of their capability of bioaccumulating dissolved trace metals from field

exposure (Adams et al. 1981, Graney et al. 1983, ASTM 2007a). Asian clams used for this aspect of the study were collected from the Clinch River near Pounding Mill, VA using clam rakes. Fifty clams were placed at each site using mesh produce bags (18 cm wide by 36 cm long with a mesh size of ~0.5 cm) tied to rebar. After 60 days, clam bags were retrieved from each site and transported to the laboratory in cool, dechlorinated tap water as used in Living Streams® at the lab. After a 24-hr depuration period, surviving clams were frozen for a minimum of 24 hours prior to tissue dissection. Prior to acid-digestion for metals analysis, the total visceral mass was removed from each clam and subdivided into two replicates (5 grams each). Acid digestion followed US EPA methodology for biological tissue preparation for trace metal analysis (Method 200.3, McDaniel 1991). Tissues were analyzed for select trace metals at the Soil, Crop and Environmental Science Inductively-Coupled Plasma spectrophotometry (ICP) laboratory at Virginia Tech.

Interstitial (Pore) Water Sampling and Analysis

Interstitial water, also known as pore-water, was collected from depositional zones at select sites in Dumps Creek and Hurricane Fork using diffusion samplers (peepers). Peepers were constructed using 250-ml amber glass jars with 32-mm diameter holes cut out of the plastic lids. Amber glass is used to prevent photolysis of PAH compounds (Miller and Olejnik, 2001). Prior to deployment, peepers were completely filled with distilled water and 105 μm nylon mesh was inserted under the lid allowing fine and suspended particles to pass through by osmosis. Peepers were placed lid side down, approximately 10 cm into the sediment and allowed to equilibrate for a minimum of 21days (Vroblesky *et al*. 2002; Webster *et al*. 1998). Due to the substantial bedrock at each stream sampling site, replicates and depth of insertion varied. Upon collection, water was transferred into 1-L Nalgene® containers, and transported back to the

laboratory on ice. Samples were digested in the laboratory according to US EPA Method 200.7 (2001) for aqueous samples and analyzed in the VA Tech ICP laboratory for selected metals.

Toxicity Testing

Acute Toxicity Tests

Acute toxicity of various coal mining effluents from the Dumps Creek watershed (Russell County, VA) was determined using US EPA endorsed test organisms, *Ceriodaphnia dubia and Pimephales promelas*. Specific test procedures are outlined below for each test organism. Water chemistry was analyzed for temperature (°C), conductivity (μS/cm), pH (standard units) and dissolved oxygen (mg/L) at the beginning and end of each test. Alkalinity (mg $CaCO_3$/L) and hardness (mg $CaCO_3$/L) was also determined by titration on the highest and lowest test concentrations at the start of the toxicity tests (APHA 1998). Lethal concentration values (LC_{50}) were calculated by the Trimmed Spearman-Karber Method 1.5 (US EPA 1993).

Ceriodaphnia dubia

Ceriodaphnia dubia have been cultured for more than 20 years in this laboratory and are considered the most sensitive standard toxicity test organism (US EPA 2002 a and b). Acute, 48-hr static toxicity tests were conducted following US EPA protocol (US EPA 2002a). Tests were conducted in 50-ml glass beakers, with four replicates per test concentration and five, < 24-hr old neonates per replicate. Test temperature and survivorship was assessed after 24 hr and survivorship was reassessed at the test termination after 48 hr.

Pimephales promelas

Fathead minnows (*P. promelas*) < 14 d were obtained from Chesapeake Cultures, Inc., located in Hayes, VA and shipped overnight via Federal Express after hatching. Upon arrival,

fish were placed into 2.5 L aquaria with EPA [100] moderately hard synthetic freshwater and allowed a minimum of two hours for lab acclimation at 22±2°C. Acute, 48-hr toxicity tests were conducted in 300-ml bioassay jars containing 250 ml of test solution. Two replicates with 10 fish per replicate were used. Fish mortality along with temperature, pH and dissolved oxygen were checked at 24-hour increments and recorded.

Chronic Toxicity Tests

Hyalella azteca

Chronic survivorship and growth toxicity tests with the amphipod, *Hyalella azteca*, were conducted using organisms obtained from reserve cultures maintained in this laboratory. Approximately seven-day old amphipods were isolated from adults by sieving the mass culture using a US Standard Sieve No. 25 (710- μm mesh screen) as described by Greer (1993), Tomasovic et al. (1995) and Ingersoll (1998) and placed in a beaker with culture water until testing. Toxicity tests were conducted in 300-ml glass beakers following ASTM (2005) protocols for testing with freshwater invertebrates. Each test included five effluent concentrations plus a control; four replicates per concentration. Diluent used for these tests was filtered Sinking Creek Water (SCW), a reference water used in this laboratory for >20 years. Each test replicate contained a fine layer (<2ml) of sterile aquarium sand as substrate for the amphipods. At the start of each test, amphipods were randomly selected and pipetted into test replicates using plastic transfer pipettes. Test duration was 28 d with mortality being assessed weekly. Tests were fed 1 ml of Yeast-Cereal Leaves-Trout Chow (YCT) daily. In addition, each test replicate contained one dried sugar maple leaf (*Acer saccharum*) which provided both an additional nutritional source as well as substrate. Due to the presence of sand, dissolved oxygen (DO_2, mg/L) was monitored daily to assure DO_2 levels did not drop below 2.5 mg/L (ASTM

2007b), or the test was required to be aerated at a rate of 1 bubble/sec. Temperature was also monitored daily to ensure amphipod tests maintained at 23±1°C. Water quality parameters (pH, conductivity, alkalinity and hardness) were measured for each batch of renewal and out-water, and ammonia was measured for each concentration of out-water. A subset of 20 amphipods were retained and measured at the start of the test so change in growth can be estimated. At the end of the test, living amphipods were removed and placed in an 8% sugar formalin solution until measurements for length were completed. Length was measured using a microscope and digitizing software.

Villosa iris

The sensitivity of *V. iris* juveniles to the various coal mining discharges was tested over a 28-day period. Mussels (2-3 month old) were obtained from the Freshwater Mollusk Conservation Center (FMCC) at VA Tech and from cultures maintained at the White Sulphur Springs Fish Hatchery, White Sulphur Springs, WVA and tested following procedures outlined in ASTM (2006). Preliminary toxicity tests with 100 % effluent were conducted in 300-ml glass beakers with no aeration and no substrate. Full chronic tests were conducted in a similar manner with four replicates per effluent. Additional replicates were also run with ~ 2 ml of fine sediments (< 200 μm) as substrate to provide a more realistic environment for the mussels. Control water used for toxicity testing was SCW, as used with the other toxicity tests being conducted in this study. Tests were conducted in a Fisher Isotemp® Low-Temp incubator at 21 ± 2° C.

Mussels used for testing were placed in a Petri dish and observed under magnification (x 10) for viability. Initial shell length measurements were taken using an ocular micrometer and converted to millimeters prior to loading five mussels into each test chamber. Organisms were

fed a daily mixture (3ml/L) of *Nannochloropsis* and Shellfish Diet (Instant Algae®). Water renewal occurred on Monday, Wednesday and Friday by siphoning and replacing 90 % of the test water. Water chemistry was analyzed for the renewed water (in-water) and out-water after each changeover. Parameters measured included, conductivity (µS/cm), DO_2 (mg/L), pH (su) and temperature (°C). After 28 days, organisms were removed and assessed for survivorship and growth. Survivorship was determined by assessing pedal movement or ciliated activity.

Ceriodaphnia dubia

Chronic, seven day toxicity tests were conducted using the water flea, *C. dubia*, to evaluate potential toxicity of selected coal mining effluents. Test procedures followed US EPA protocol (US EPA 2002b). Fifty- milliliter, glass beakers were used as test chambers with ten replicates per effluent concentration. Moderately-hard, synthetic lab water (EPA^{100}) was used as the diluent and control water. *Ceriodaphnia* neonates (< 24 hr) were individually transferred into each test replicate at the start of the test using a plastic, transfer pipette. To ensure a constant temperature of 25±1°C was maintained, tests were conducted in a Fisher Isotemp® Low Temperature incubator (25 ± 1° C, Fisher Scientific, Pittsburg, PA, USA). Water renewal was conducted on a daily basis and survivorship/reproduction recorded. Organisms were fed a daily mixture of *Pseudokirchneriella subcapitata* and YCT (0.40ml/50 ml). Conductivity (µS/cm), DO_2 (mg/L), pH (su) and temperature (° C) were measured on the in and out-water after each changeover and alkalinity and hardness were measured by titration (APHA 1998) on the highest concentration and control for each batch of renewal water.

Pimephales promelas

Fathead minnows were obtained from Chesapeake Cultures., located in Hayes, Virginia. Only juvenile fathead minnows <24 hr old were used for testing according to US EPA protocol

(US EPA 2002b). Fish were shipped to the laboratory overnight by Federal Express upon hatching. Fathead minnows were acclimated to a constant 25°C in moderately hard synthetic freshwater (EPA^{100}) following standard operating procedures (Cherry Lab SOP's, Virginia Tech) upon arrival.

At the start of each test, fish were gently pipetted using a cut, plastic transfer pipette and placed in 300-ml bioassay jars containing 250 ml of test solution. Chronic tests consisted of four replicates with 10 fish per replicate. Effluent concentrations ranged from 6.25 to 100% plus a control. Diluent and control water for these tests was moderately-hard synthetic freshwater (EPA^{100}). Fish mortality along with temperature, pH and dissolved oxygen was checked at 24-hour increments and recorded. All water chemistry parameters were measured at the start and end of the test as described above.

Sediment Toxicity Tests with *Daphnia magna*

Sediment samples were collected from depositional areas at study locations in Dumps Creek and transported to Virginia Tech for testing. Sediments were collected from the top 2 cm using a polyethylene cup and placed in 4-L freezer bags, placed in coolers and chilled to 4° C. Toxicity tests were conducted according to ASTM (1995) and Nebeker et al. (1984) protocols. Test chambers consisted of 250-ml beakers with approximately 100 ml of site sediment (v/v) and 150 ml of filtered reference water. Reference water was collected from Sinking Creek (SC), Newport, VA, vacuum filtered through 0.45µm Whatman® glass microfiber filters, then aerated overnight to ensure adequate dissolved oxygen (DO2). There were five replicates with three, 5-day old *Daphnia magna* in each. *D. magna* have been cultured in the Aquatic Ecotoxicology Laboratory at Virginia Tech for 20+ years. Organisms used for testing were raised in filtered

Sinking Creek water and fed 5ml/L of *P. subcapitata* and 5ml/L YCT daily until they were five days old.

Sediments were added to beakers, followed by overlaying reference water, and then placed in an incubator to allow the sediments to settle out overnight. Prior to loading the daphnids, dissolved oxygen was checked to ensure adequate saturation (>4.0 mg/L). *D. magna* were randomly selected and placed one at a time into the beakers. Mortality and reproduction were checked and organisms were fed 2 ml of *P. subcapitata* YCT mixture after each 24-hr interval. Tests were changed over daily by siphoning 90% of overlaying water and renewing with filtered SCW. Water chemistry was analyzed on renewal (in-water) water and on out-water for each changeover. Parameters measured for renewal included temperature, conductivity, DO_2, pH, alkalinity and hardness. Out-water was measured for conductivity, DO_2 and pH. Sediment tests ran for 10 days and were maintained at 25± 2° C. Survivorship and fecundity were totaled for test replicates and averages were calculated for each site. Pairwise comparisons (Tukey's HSD, α=0.05) was used to determine statistical differences between sampling sites. Statistical differences between site sediments and control organisms were calculated using Dunnett's test. All analyses were conducted using JMP IN® statistical software (SAS Institute, 2008).

Stream Condition Index Scoring Methodology

Stream condition index scores were used as an assessment tool for determining biological impairment at sampling locations in the Dumps Creek subwatershed in 2008-2010. This type of multimetric index can be useful for discerning the biological health of a site compared to reference conditions. Two index methodologies were used to determine ecological health, the Virginia SCI (Tetra Tech 2003) and the West Virginia SCI (WV SCI, Tetra Tech 2000). According to the VA SCI guidance document, VA SCI scores in the Central Appalachians

(Ecoregion 69) score lower than other regions used in the index development for the state, with a majority of the reference sites scoring below the 25th percentile of the other four ecoregions. This is supported by a reference study conducted in Tazewell County, Virginia which indicated unimpaired sites still scored lower compared to sites in other regions of the state (Passmore et. al, personal communication in Tetra Tech 2003). Therefore, conditions in the Central Appalachians could be different based upon past disturbance at reference locations that may not be reflected in water quality measurements (pH, conductivity, etc.). It is recommended in Tetra Tech (2003) that the West Virginia index be used as it may be more appropriate for sites located in this region of Virginia.

Benthic macroinvertebrate data collected from stream sites was used to calculate defined metrics (Table 2.2). Each metric is designed to represent a specific community characteristic, such as taxonomic diversity, taxonomic composition (i.e. % Ephemeroptera), functional feeding group, habit and tolerance to pollutant exposure.

Some metrics included a decrease with stress, while some increased. The VA SCI includes eight metrics, while the WV SCI includes only six. Calculated metrics are then converted to unit-less scores and averaged to produce a single numerical index. Since four replicates of benthic macroinvertebrate samples were collected at each site, each replicate index score was averaged to produce a final index score for each site.

Statistical Analysis

Statistical differences of bioassessment parameters between study sites were determined using JMP IN® statistical software (SAS Institute, 2008). Normally distributed means for clam growth and survivorship were compared using analysis of variance (ANOVA). Pairwise analysis was conducted using the Tukey-Kramer honestly significant post-hoc test (HSD) at α=0.05 level.

Pairwise mean comparisons was used to determine significant differences between study sites for clam growth, metal bioaccumulation, sediment metals levels and benthic macroinvertebrate parameters.

Results

Habitat Assessment

Except for a few sites, habitat assessment scores (HAS) were consistent at sites between sampling years (Fig.2.2). Site DC-2 Dn scored lower during 2008 (104) compared to 2009 (123) and 2010 (119) due to impaired flow. Site HF-1 scored higher during the 2008 sampling season (160) versus the latter two years (134 and 149), in which habitat had been altered for the construction/deconstruction of a low-water bridge. Site CH-1 also scored lower during 2009 (89) compared to 2008 (129) and 2010 (121), due to impaired channel flow when water levels failed to extend to the base of each bank and only 25-75% of the available channel was filled. However, this site is located in a stream that is dominated by mining effluent; therefore, flow reduction is dependent on discharge from the mining operation, although comparisons to discharge rates for 2009 were not made. Overall, DC-1 Up had the highest HAS ($\bar{x}$ =181.5±0.71), while DC-6 was substantially lower ($\bar{x}$ -=92±8.5) due to the homogenous nature of the site, which lacked riffles, epifaunal substrate and available cover, and was heavily impacted by sedimentation. Scores for the Gob Pile site (149), HF-4 Up (151) and Dn (149), were also determined once, and were consistent with those from the other Hurricane Fork sites (not shown in figure). The original DC-4 site used in 2008 (not shown) had a HAS of 116. Due to heavy sedimentation and lack of available habitat for benthic colonization, this site was moved to a more representative location upstream.

Water Quality

General water chemistry parameters were monitored at sampling sites in the Dumps Creek subwatershed on a routine basis from fall 2007 through fall 2010 (Table 2.3). Background conductivity levels measured at the upstream reference site (DC-1 Up) in Dumps Creek were lowest, with an average of 290 µS/cm. The influence of a deep mine discharge (Camp Br. 001) with elevated conductivity (1,173 µS/cm) located just below DC-1 Up, caused an increase of in-stream conductivity. The average conductivity at this site (DC-1 Dn) was 1,035 µS/cm. Conductivity levels remained elevated without any noticeable variation at the next three downstream sites, ranging from 1,176 at DC-2 Dn to 1,001 at DC-3 which is located above the Hurricane Fork confluence. Hurricane Fork conductivity was consistent between sites and ranged from 553 µS/cm at HF-1 (reference) to 441 µS/cm at HF-5.

Two potential impact areas in Hurricane Fork, the Gob Pile (coal refuge area) and the Moss 2 (019) deep mine discharge located at HF-4 Dn did not appear to contribute to an increase in conductivity (Table 2.3). In fact, conductivity decreased at sites located below these two influences. The remaining Dumps Creek sites (DC-4 and 6), located below the Hurricane Fork confluence, had a moderate decrease in conductivity with means of 596 and 891 µS/cm, respectively. However, the original DC-4 location, used in 2007-2008, had a similar conductivity average (1,162 µS/cm) compared to upstream locations. This discrepancy was most likely due to influences from the American Electric Power Bottom Ash holding pond and/or road runoff due to the specific location of this site. Therefore, the new location, located closer to the Hurricane Fork confluence, allowed a more realistic gauge of conditions in Dumps Creek without outside influences not included within the scope of this research. One additional site,

referred to as CH-1 had an average conductivity of 974 µS/cm. This site, also referred to as the Moss 3 effluent stream, predominately carried the 003 effluent from the coal prep plant to its discharge point in Chaney Creek.

Average pH in Dumps Creek ranged from 7.56 to 8.65, with the lowest measurements occurring in the headwater region of the stream (Table 2.3). The pH of CB Pump effluent (7.39) was consistent with that of the receiving system (7.59 at DC-1 Up), and therefore, had no discernable impact on in-stream pH. Average pH values; however, did increase at DC-2 Up (8.45) and remained above pH 8.0 at remaining Dumps Creek sites. The average pH at Hurricane Fork sites ranged from 7.97 at the upstream reference (HF-1) to 8.15 at HF-5. In general, averages remained consistent to that of HF-1; however, the in-stream pH at the base of the Gob Pile was substantially higher (8.29) as well as the sites (HF-4 Dn and HF-5) located below the Moss 2 effluent discharge. In addition, a slightly acidic (6.83) seep, located directly across the stream from the Gob Pile may have had some influence on stream pH, but the low, intermittent flow of the seep was not a major concern and did not appear to have any significant impact on stream pH.

Average water hardness and alkalinity (expressed as mg $CaCO_3$/L) were lowest at DC-1 Up at 94 and 58, respectively (Table 2.3). Both parameters increased downstream of the CB Pump discharge, which had hardness and alkalinity means of 110 and 294 mg $CaCO_3$/L. Alkalinity increased to 417 mg $CaCO_3$/L at DC-1 DN and was even higher at DC-2 Dn and DC-3 (520 and 555). Average hardness increased as well at DC-1 Dn (104 mg $CaCO_3$/L) but remained at similar or lower levels at DC-2 Up/ Dn and DC-3 (90, 99 and 84). Alkalinity was similar for Hurricane Fork upstream sites (HF-1 and 2) at 69 mg $CaCO_3$/L, compared to upstream Dumps Creek sites. However, hardness was substantially higher at 240 and 263 mg

$CaCO_3$/L at HF-1 and 2. Mean alkalinity increased downstream of the Moss 2 (019) effluent discharge to 278 mg $CaCO_3$/L at HF-4 Dn, but decreased slightly at HF-5 (153 mg $CaCO_3$/L). Hardness decreased slightly at these two sites, compared to averages for upstream sampling sites (178 and 201 mg $CaCO_3$/L). Remaining sites in Dumps Creek (DC-4 through DC-6) had variable averages for these two parameters. Again, there was a substantial difference between the DC-4 site used in 2008 (496 and 107 mg $CaCO_3$/L) for alkalinity and hardness, versus the site used for the 2009-2010 sampling seasons (144 and 146 mg $CaCO_3$/L). The lowest site in Dumps Creek, DC-6, indicated a moderate increase in alkalinity, compared to DC-4 (321 mg $CaCO_3$/L) but average hardness was similar at 141 mg $CaCO_3$/L.

Water samples were analyzed for select trace metals in summer 2010 (Table 2. 4). Metals selected during analysis were based on those that are generally associated with coal mining in this region of Virginia and have been previously detected in sediments and clam tissue samples in Dumps Creek and the Clinch River. Average aluminum concentrations were highly variable and showed no discernable trend between sites. Measurements ranged from < 6.0 (detection limit) to 401 µg/L. Lowest values (<6.0 µg/L) detected were found in the CBP effluent (001) and downstream below the settling pond at DC-2 DN. The upstream reference area (DC-1UP) had one of the highest concentrations (96 µg Al/L), but showed a steady decrease at sites downstream until DC-3, in which the average concentration jumped to 245 µg Al/L. Hurricane Fork sites were generally low, ranging from 27 µg Al/L at the upstream reference station, to 54 µg Al/L at HF-3. The Gob Pile, located between HF-2 and HF-3 did not appear to have any influence on stream Al concentrations; however, the small seep located near the Gob Pile (opposite bank) may have contributed to the slightly elevated Al concentration (54 µg Al/L) at HF-3. The remaining two Dumps Creek sites (DC-4 and 6) indicated mean aluminum

concentration was still elevated compared to upstream locations (179 µg Al/L) but had dissipated substantially from DC-3. DC-6 levels were consistent with background levels. Five sites (DC-1 UP, DC-3, Seep by Gob Pile, DC-4 and CH-1) were above the National Recommended Water Quality Criteria (WQC) chronic threshold (87 µg/L) (US EPA 2009), including the upstream reference site.

Water column copper concentrations do not appear to be an area of concern as levels were below detection limits (<5.0 µg/L) for all sampling stations, including the effluent discharge (Table 2.4). Average iron concentrations appeared to follow a more predictable trend with lowest measurements occurring for upstream reference sites, DC-1 UP (121 µg Fe/L) and HF-1 (62 µg Fe/L). Dumps Creek sites showed an increase in ambient Fe following effluent inputs. The CBP outfall averaged 742 µg Fe/L during this sampling event, which increased water column concentrations to 431 µg Fe/L at DC-1 DN, located directly below the discharge. This level increased slightly at the next site (494 µg Fe/L at DC-2 UP); however, the settling pond appears to have allowed for some reduction as levels below the pond were nearly half (262 µg Fe/L) those observed upstream. Iron levels increased at DC-3, a trend also observed with Al. Hurricane Fork levels quadrupled between HF-1 and HF-2, although no known point-sources enter the creek in this area. The highest concentration was observed in the yellow-boy seep (Seep by Gob Pile) that was 19 times the recommended WQC for Fe (1,000 µg/L). This may have contributed to the slight increase at HF-3 (339 µg Fe/L); however, the concentration of Fe returned to near background level (91 µg Fe/L) at the last site before confluence with Dumps Creek (HF-5). Iron measurements continued to decrease in Dumps Creek, with DC-6 having the lowest concentration of Fe in the stream at 110 µg Fe/L.

Manganese levels fluctuated between sites in both Dumps Creek and Hurricane Fork, with highest levels observed in the CBP 001 effluent (116 µg/L), Seep by Gob Pile (328 µg/L) and the Moss 3 effluent stream (CH-1, 315 µg/L) (Table 2.4). The lowest observed level occurred at HF-1 (6 µg/L) followed by HF-5 (7 µg/L). Although the range appears quite large (6-328 µg/L), these levels do not appear to be close to the documented toxic thresholds reported in the literature (LC_{50}=9,100 µg/L; Boucher and Watzin 1999) at a similar water hardness (75-100).

Strontium levels were lowest at DC-1 Up (197 µg/L) and fluctuated at downstream sites in Dumps Creek, ranging from 290-763 µg/L (Table 2.4). Strontium was included in analysis, as it is commonly used as a tracer in environmental studies involving coal mining and it stands out as one of the primary constituents in the CBP 001 discharge, which during this analysis was 813 µg/L. The discharge increased the in-stream concentration to 468 µg/L at DC-1 Dn; however, a substantial spike occurred at the next sample station downstream (DC-2Up) at 773 µg/L. Hurricane Fork reference (HF-1) had the highest measured Sr concentration (963 µg/L), with levels decreasing slightly at downstream locations. The exception was HF-3 which had a nearly identical measurement to that observed at HF-2, but may have been due to the seep influence.

The last metal included in analysis was zinc, which also did not appear to be at levels considered to be problematic for aquatic biota (Table 2.4). Levels observed in Dumps Creek were near or below detection limits, except for DC-6 (9 µg/L). Concentrations were higher for Hurricane Fork sites, ranging from < 5 to 22 µg/L, with the highest measurement occurring at HF-1. Average zinc concentrations were substantially lower than the recommended WQC level of 120 µg/L (US EPA 2009).

Sediment Quality

Sediments were collected at sampling stations in the Dumps Creek watershed from spring 2008 until fall 2010. Due to site access limitations periodically during the study period, some sites were not sampled each year. Sediments were analyzed for select trace metal content based on metals generally associated with coal mining discharges in this region. Metal concentrations were averaged for each site in order to determine any possible trends between sampling sites (Table 2.5). When applicable, averages or specific measurements were compared to the US EPA Mid-Atlantic Risk Assessment, Freshwater Sediment Screening Benchmarks (US EPA 2010).

The highest average Al concentration occurred for CH-1 (4,725.2 mg/kg), while the highest levels in the mainstem Dumps Creek were detected at DC-2 Dn (4,594 mg/kg) and at the upstream reference site, DC-1 Up (3,367 mg/kg) (Table 2.5). Average Al in sediments was slightly lower at DC-1 Dn (3,066 mg/kg, although during the spring 2010 analysis sediment Al was slightly higher at DC-1 Dn (3,593 mg/kg) compared to DC-1 Up (2,542 mg/kg). Downstream sites in Dumps Creek showed a decrease in sediment Al compared to upstream reference levels, ranging from 2,641 mg/kg at DC-3 to 2,936 mg/kg at DC-6. Hurricane Fork sediment Al concentrations were fairly consistent between sites with the highest average (3,480 mg/kg) occurring for HF-3, located below the Gob Pile. A spike in sediment Al occurred during the fall 2009 at this site (5,101 mg/kg), although all sites showed elevated Al compared to other years analyzed. Overall Al averages for Hurricane Fork sediments ranged from 2,024 at HF-2 to 2,928 mg/kg at HF-5.

Sediment iron levels were elevated at all sites in the Dumps Creek watershed , with only a minor increase in average sediment Fe at DC-1 Dn (13,776 mg/kg) compared to DC-1 Up (12,739 mg/kg) (Table 2.5), even though the CBP 001 discharge is known to be high in Fe (see

Table 2.4). The second highest average occurred for the DC-2 Dn site (17,349 mg/kg), located below a settling pond designed to mitigate some of the effects from upstream loading. Remaining averages indicate Fe levels decrease to below upstream levels for remaining Dumps Creek sites, ranging from 11,101 mg/kg at DC-3 to 8,976 mg/kg at DC-4. A slight increase was seen for the last sampling site in Dumps Creek (DC-6) at 11,918 mg/kg; however, this average was still lower than the reference site average. Average sediment Fe appeared to be consistent for Hurricane Fork sites compared to those for Dumps Creek. Iron ranged from 11,210 mg/kg at HF-1 (reference) to 9,713 mg/kg at HF-5. Although not consistent between all sampling stations, Fe levels appeared to be highest during spring 2008 and fall 2009, with highest spikes occurring specifically at DC-2 Dn (22,090 mg/kg in Sp. 2008) and CH-1 (25,689 mg/kg in Sp. 2008). Both measurements exceed the US EPA sediment screening benchmark (US EPA 2010) of 20,000 mg/Kg. Average sediment Fe also exceeds this level at CH-1 (21,684 mg/kg), the Moss 3 effluent stream that discharges into Chaney Creek.

Sediment copper levels appeared to fluctuate between sites with the majority of averages being similar to those at the reference sites in Dumps Creek (DC-1 Up, 7.3 mg/kg) and Hurricane Fork (HF-1, 5.0 mg/kg) (Table 2.5). Unlike Al and Fe, a drastic increase in average sediment Cu (207.0 mg/kg) was observed at DC-1 Dn compared to DC-1 Up. The substantial increase in Cu was only observed during fall 2009 when average sediment Cu was 520.9 mg/kg, compared to 52.5 and 47.7 mg/kg for spring 2008 and fall 2010, respectively. Average sediment Cu returned to the background level (7.9 mg/kg) at DC-2 Up. A slight increase was observed at DC-2 Dn, which followed the same trend as Al and Fe at this site. Copper concentrations returned to similar levels as observed at the upstream reference for all remaining sites, and were generally lower for Hurricane Fork sites than in Dumps Creek. Only the DC-1 Dn site had a sediment Cu

average that exceeded the US EPA sediment screening benchmark of 31.6 mg/kg (US EPA 2010).

Average sediment manganese levels ranged from 166.7 mg/kg at DC-1 Dn to 362.2 mg/kg at DC-6 for Dumps Creek sampling stations and 196.5 mg/kg at HF-3 to 240.9 mg/kg at HF-5 (Table 2.5). One exception occurred at DC-2 Dn, which had an average of 2,099.8 mg/kg with a range of 1,416.3 to 2,783.3 mg/kg. In addition, CH-1 sediments were also high in Mn with an average of 6,118.6 mg/kg. Although several sites came close to the US EPA screening benchmark of 460 mg/kg (US EPA 2010), including DC-1 Up (347.1 mg/kg), only DC-2 Dn and CH-1 exceeded this toxicological reference point.

Sediment Zn was the only metal tested that did not exhibit levels at any sampling site that were in exceedence of the benchmark level of 121 mg/kg (Table 2.5). The highest averages occurred at the two sampling stations most impacted by effluent discharges, DC-1 Dn (60.1 mg/kg) and CH-1 (95.7 mg/kg). Averages in Dumps Creek ranged from 19.7 mg/kg at DC-4 to 42.7 mg/kg at DC-1 Up. Hurricane Fork averages were similar, ranging from 19.8 mg/kg at HF-4 Dn to 32.8 mg/kg at HF-5.

Benthic Macroinvertebrate Surveys

Benthic macroinvertebrates were sampled in Dumps Creek and Hurricane Fork on an annual basis from 2008-2010. Select metrics and multimetric indices (MMI) were used to determine site health based on benthic structure.

Taxa richness was used as a key parameter in determining site health in comparison to reference conditions (Fig. 2.3). Average taxa richness was highest at HF-5 (23.0) in 2008, DC-1 Up (20.5) in 2009 and HF-1 (22.8) in 2010. Mean taxa richness during 2008 and 2010 were similar; however, several sites indicated a major drop in benthic invertebrate diversity during

2009, including the Hurricane Fork reference site (HF-1) in which taxa richness was cut by almost half compared to results in 2008 and 2010 (Fig. 2.3). Mean taxa richness was similar at DC-1 Up during 2009 and 2010 with 20.5 and 20.8 organisms, respectively. DC-1 Dn had the lowest observed taxa richness in the subwatershed, but showed a slight improvement in 2010 (2.3 organisms) compared to 2009 (1.3). The point of impact most likely associated with benthic decline occurs just above DC-1 Dn and therefore; a gradual increase in taxa richness can be observed as sites progress downstream. Taxa richness improved slightly at DC-2 Up, with an average of 4.3 in 2009 and 8.8 organisms in 2010. Means almost doubled at DC-2 Dn [compared to DC-2 Up] with an average taxa richness of 8.3 in 2009 and 15.0 in 2010. Site DC-3 taxa richness remained similar betwe all three sampling years with the highest average occurring in 2008 (19.3) and slightly lower means in 2009 and 2010 (18.8 and 18.3).

Hurricane Fork was both similar among sites and years, except the reference site HF-1 which had drastically lower taxa richness in 2009 (11.0) compared to 2008 (20.8) and 2010 (22.8) (Fig. 2.3). This reduction in benthic diversity may have been due to habitat alteration which was observed at the location in spring 2009, in which a low-water bridge was constructed and later removed during late summer 2009. With this exception, average taxa richness in Hurricane Fork ranged from 17.5 at HF-3 (2008) to 23.0 (HF-5 in 2008 and HF-3 in 2010). Although sampling at sites located above and below the Moss 2 effluent discharge (HF-4 Up and Dn) only occurred during 2008, no significant differences in taxa richness were observed (Tukey's HSD, α=0.05) between these two sites. Remaining sites in Dumps Creek (DC-4 and DC-6) had variable results. Taxa richness at the original (08) DC-4 site was much lower at 13.8 compared to upstream sites above the Hurricane Fork confluence, but this was most likely due to poor benthic habitat, including heavy sedimentation. Mean taxa richness at the DC-4 site used

during 2010, was more comparable to that of DC-3 and reflected a continued improvement in benthic structure. However, average taxa richness was second lowest at the DC-6 site, although this site was only sampled for benthic macroinvertebrates in 2010 due to restricted site access (water level too high in 2008 and 2009). The CH-1 site located in the 003 effluent dominated stream, had nearly identical averages in 2008 and 2010 with 11.5 and 11.3 taxa, showing the consistency in the effluent (Moss 3, 003) which dominants this streams flow.

During the 2008 sampling year, average EPT taxa richness was significantly higher at HF-5 (11.0) compared to all sites in Dumps Creek, except DC-3 (Tukey's HSD, α=0.05) (Fig. 2.4). Means were lowest at DC- 4 (3.0), DC-2 Dn (3.5) and CH-1 (4.0) (Fig. 2.4). Average EPT richness was again highest at HF-5 during 2009 (11.0) followed by HF-3 (10.8). The DC-1 Up reference site was slightly lower with 9.5 EPT taxa. Sites DC-1 Dn, DC-2 Up and DC-2 Dn were significantly different from the reference site according to Tukey's HSD levels differences. Means at these three sites averaged 0.0 at DC-1 Dn to 0.8 at DC-2 Dn. The same pattern was observed during the 2010 benthic survey, whereby EPT taxa were absent from DC-1 Dn, but increased with distance downstream (0.5-7.5 organisms) through DC-4. Compared to 2009 data, mean EPT richness improved in 2010 at DC-2 Up and Dn with a minimal improvement at DC-2 Up (0.5) but substantial increase at DC-2 Dn (0.8 in 2009 vs. 3.3 EPT taxa in 2010). Average EPT richness was statistically similar between sites in Hurricane Fork, except for HF-1, which also had a significantly lower average for EPT taxa compared to other sites in Hurricane Fork during 2009 (4.8).

The percent of mayflies (Ephemeroptera) relative to the total number of organisms collected at each site was substantially lower for sites located in Dumps Creek compared to Hurricane Fork (Fig. 2.5). During 2008, mean percent mayfly abundance was highest at HF-3

(48.9) and HF-5 (29.4 %). Average percentages ranged from 2.1 to 11.0% for Dumps Creek sites. The lowest percent mayfly (1.5%) for 2008 occurred at the designated reference site, HF-1. During the 2009 sampling season, mayflies were highest at the Dumps Creek reference site, DC-1 Up with 18.6 %, which decreased to zero mayflies present at the next site downstream (DC-1 Dn), directly below the CBP 001 outfall. A slight recovery of mayfly taxa occurred at DC-2 Up (2.1 %), which consisted entirely organisms in the predominantly facultative family, Baetidae. Mayflies were again absent at DC-2 Dn during 2009, located below the settling pond although *Maccaffertium* sp. (Heptageniidae) were collected here in 2008. Compared to 2008, the percent mayfly abundance at DC-3 decreased slightly to 6.0 %, which was comprised only by Baetidae mayflies during both sampling years. During 2009, percent mayflies increased at HF-1 (5.4 %) but decreased at HF-3 and HF-5 (8.1 and 7.2%). Although the number of mayflies collected at these sites decreased in 2009, similar taxa were represented during both years and included genera in the families of Ephemeridae (*Ephemera*), Heptageniidae (*Maccaffertium*), Isonychiidae (*Isonychia*), Ephemerellidae (*Ephemerella*), Baetidae (*Baetis*) and Ameletidae (*Ameletus*).

During 2010, percent mayflies was highest at HF-2 (25.7 %), which was significantly higher than the HF-1 reference site (10.1 %), according to Tukey's HSD comparison (α=0.05) (Appendix I, Table A3). Although HF-1 showed some improvement compared to the two previous years of sampling (Fig. 2.5), genera represented at the site remained relatively unchanged with Baetidae, *Ephemera* (Ephemeridae), *Maccaffertium* still present with the addition of *Drunella* and *Attenella* species (Ephemerellidae) in 2010. Percent mayfly abundance decreased at DC-1 Up (9.4 %), DC-2 Up (0.2 %) and DC-3 (2.8 %) compared to 2009, but increased at Hurricane Fork sites. Although not significant, a substantial decrease in mayflies

occurred at a site located at the base of the Gob Pile (13.7 %) compared to sites located directly above (HF-2, 25.7 %) and below (HF-3, 22.3 %) the coal refuse pile.

The percent of organisms belonging to the tolerant group, Chironomidae (Diptera) was used to further distinguish stressed sites from those that represent a healthy benthic structure. As expected, DC-1 Dn had the highest percentages (83.3 and 56.6%) of Chironomidae relative to total abundance in 2009 and 2010 (Fig. 2.6). These averages were similar (83.3%) or slightly higher (73.7%) at DC-2 Up during these two sampling years. Site DC-2 Dn averages for all three years remained significantly higher compared to reference conditions, but were substantially lower than DC-2 Up (Appendix I, Tables A2 and A3; Fig. 2.6). Mean percent Chironomidae was similar at DC-3 to that of the reference sites in Hurricane Fork and Dumps Creek, but was much lower in 2009 (4.2 %) compared to 2008 and 2010 (10.5%). All Hurricane Fork sites showed an increase in Chironomidae in 2010 compared to previous sampling years, especially HF-5, which increased from 0.6% in 2009 to 23.8 % in 2010. Remaining sites in Dumps Creek showed a higher percentage of Chironomidae were present in benthic macroinvertebrate samples compared to the upstream DC-3 site and were significantly higher than DC-1 Up (Tukey's HSD, α=0.05), including the last Dumps Creek site prior to the confluence with the Clinch River (DC-6), which had an average of 73.3 % Chironomidae during 2010.

Additionally, Community Loss index (CLI) scores were used to estimate loss of taxa between Dumps Creek samples and reference composition (Table 2.6). The higher the index value, the more dissimilar the site is when compared to reference conditions. Samples collected in 2010 were used to determine community loss at sites located in the mainstem Dumps Creek only. Sites DC-1 DN and DC-6 were the most dissimilar with scores of 12 and 7.2, respectively.

Loss indices gradually improved as sites progressed downstream, ranging from 1.76 at DC-2 Up to 0.63 at DC-4.

Family-level based Stream Condition Index (SCI) scores were generated using both Virginia and West Virginia methodologies (Fig. 2.7) for sampling sites from 2008-2010. Scores were used to compare both annual changes in benthic macroinvertebrate structure at sites and differences between scores generated using the two slightly different MMI's. In 2008, only three out of ten sampled sites were at or above the impairment threshold of 61.3 for the Virginia SCI. These three sites included the reference site used for 2008, HF-1 (62.6), HF-3 (66.2) and HF-5 (65.5). All Dumps Creek sites were substantially lower than the impairment threshold, with DC-3 having the highest score at 38.6. DC-2 Dn and DC-4 had scores of 26.4 and 25.8, respectively. In comparison, seven out of ten sites were at or above the "impairment threshold" for West Virginia. The WV SCI impairment threshold is based on a rating system that places a site scores in five categories: highly comparable to reference conditions (> 78-100); comparable to below average reference sites (>68-78); increasingly difference from reference conditions (>45-68; >22-45; 0-22). Of the seven sites, five sites scored in the "highly comparable" category. These sites included, HF-1 (77.5), HF-2 (76.9), HF-3 (80.7), HF-4 Up (79.1) and HF-5 (86.4) (Appendix I, Table A1). Sites DC-3 (68.4) and HF-4 Dn (73.4) were in the second category or comparable to below average reference sites. The lowest scoring site was DC-2 Dn, which with a score of 39.8 was in the second to lowest category, well below the impairment threshold.

Scores were much lower in 2009 and only one site (DC-1 Up) scored above the Virginia impairment threshold with a score of 74.9 (Fig. 2.7). Site DC-1 Dn was much lower with a VA SCI score of 14.8. The lowest SCI occurred at DC-2 Up (11.8), while scores at DC- 2 Dn and DC-3 showed incremental improvement at 21.3 and 54.0, respectively. The three Hurricane

Fork sites included in 2009 were all below 61.3, with scores ranging from 47.2 at HF-1 to 55.3 at HF-3. West Virginia SCI scores were more favorable with five sites scoring greater than 68. The Dumps Creek reference site, DC-1 Up had the highest score of 92.5, followed by DC-3 (80.9). Although scores were lower compared to 2008, HF-1 (70.4), HF-3 (79.8) and HF-5 (77.4) were still categorized as either highly comparable to reference conditions (HF-3) or comparable to below average reference sites.

Overall, Virginia SCI scores improved in 2010, resulting in four sites at or above the 61.3 threshold for impairment (Fig. 2.7). DC-1 Up was slightly lower at 71.1 than the previous year, as was DC-1 Dn (7.6). Sites DC-2 Up and DC-2-Dn showed substantial improvement over 2009 with scores of 16.8 and 30.5, although still well below the impairment line. Site DC-3 VA SCI was also lower for 2010 at 43.4, which may have been driven by the reduction in mayflies at this site (see Percent Mayfly results). Hurricane Fork sites showed the most improvement compared to 2009 results, with four of the five sampled sites scoring < 61.3, including HF-1 (68.6) and the Gob Pile site (65.1).

Although scores appeared to be higher based on WV SCI protocol compared to 2009, all sites that scored above 68 in 2010 were also above this threshold in 2009 (Fig. 2.7). Site DC-1 Up had the highest WV SCI score of 94.3, followed by HF-3 (92.7) and HF-1 (91.9). Lowest scores occurred at DC-1 Dn (35.7) and DC-6 (42.0). Gradually, scores increased at sites downstream of DC-1 Dn, with scores ranging from 46.3 at DC-2 Up to 81.2 at DC-4.

Stream Condition Index scores were averaged for all three sampling years to determine if both scoring systems indicate similar trends (Fig. 2.8). Comparisons show identical trends between sites, although WVA scores show fewer sites below the regulatory threshold. Using the WVA SCI, reference sites DC-1 Up and HF-1 scored well above the impairment threshold (>68)

with geometric averages of 93.4 and 79.4, while VA SCI averages for these two sites are 73.0 and 58.7; meaning HF-1 averages conditions that are below the state impairment level of 61.3. The differences between the two MMI's are the parameters used to generate the final score; the VA SCI includes Percent Ephemeroptera and Percent Scrapers while, the WVA SCI excludes the functional feeding metric and includes Ephemeroptera in calculations with remaining EPT taxa. Therefore, there is less emphasis on mayflies, which may not be as abundant in Central Appalachian streams due to historical disturbances not reflected in current water quality conditions (VA SCI, Burton and Gerritsen 2003).

Pairwise Correlations (JMP 8.0) were conducted to determine the potential relationship between selected benthic parameters (% Ephemeroptera, Taxa Richness, EPT Richness and WVA SCI), trace metal levels (sediment and tissues [BCF]), habitat assessment scores (HAS) and conductivity from each site (Appendix II). Only 2010 results were used in analysis, as this dataset was the most complete for all sampling sites. WVA SCI scores were moderately, negatively correlated (r=-0.5528) with the Bioconcentration Factor (BCF) of Cu for Asian clam tissues. BCF values for Fe and Zn had no significant relationship (r=0.0116, r=0.0018) to the WVA SCI. Individual benthic metrics (% Ephemeroptera, Taxa Richness, EPT Richness) were also moderately correlated (r=-0.5688, -0.6037, -0.4964) to the BCF for Cu. Relationships of sediment trace metals to benthic parameters were also analyzed, and all correlations were found to be non-significant. Habitat assessment scores were determined to be significantly correlated to benthic parameters, with correlations ranging from r=0.6232 (% Ephemeroptera) to r=0.8370 (taxa richness) and 0.8376 for WVA SCI scores. Mean conductivity (µS/cm) was moderately but significantly correlated with WVA SCI scores (r= -0.7849; p=0.0123), taxa richness (r=-

0.7040, p=0.0343) and % Ephemeroptera (r=-0.7362, p=0.0237), while a strong negative correlation was determined between EPT richness and conductivity (r=-0.8842, p=0.0015).

TMDL Comparison Benthic Survey

Benthic macroinvertebrates were also collected during winter (February/March) 2009 and 2010 as a comparison to benthic data used to develop a General Standard TMDL for the Dumps Creek watershed (Map Tech 2004), which was obtained during late winter months (January/February 2002). Results were used to calculate Virginia SCI scores that could be compared to scores generated with summer benthic data, as well as a biometric used in the TMDL study.

Stream Condition Index scores were similar for winter and summer samples at the four included sites, except for DC-2 Dn which had a substantially lower SCI score during winter 2009 (13.73), compared to the following winter (Table 2.7). However, summer 2009 was also lower compared to summer 2010 results. Site DC@ HF was not sampled during summer 2009 or 2010 because this site (along with the Chaney Ck. Reference) were not included in the original scope of the project and therefore, comparisons to summer results could not directly be made. However, the VA SCI score for site DC-4 (49.7) was included as it is located approximately ¼ mile downstream of the Hurricane Fork confluence and no known point-source discharges are known to come in between these two points, thereby affecting the stream condition.

Bioassessment scores and biological condition ratings were also compared to those listed at corresponding sites in the TMDL study. Biological condition categories were based on the percent comparable (% Comp.) to a reference site [Chaney Creek upstream] and the categories were as follows: not impaired [NI], moderately impaired [MI] and severely impaired [SI].

Eight biometrics were used to assess the biological community at three targeted sites in Dumps Creek which were then compared to results from the Chaney Creek upstream reference area, as used in the TMDL study (Tables 2.8a and 2.9a). During the winter 2009 study, taxa richness ranged from 10.5 at DC@ HF to 14.5 at DC-1 Up (Table 2.8a). Modified Family Biotic Index (MFBI) scores, which increase with benthic community stress, were highest DC-2 Dn (5.98), but lowest at DC-1 Up (2.61) and DC@ HF (2.95), while the reference station surprisingly had a much higher MFBI score of 3.73. The ratio of scraper to collector filterer organisms was also highest at DC-1Up (1.66). Ephemeroptera-Plecoptera-Trichoptera (EPT) organisms were better represented though at the Chaney Creek reference site, as EPT index (abundance) was highest with 163.3 organisms, compared to 43.0 at DC-1 Up. The ratio of these invertebrates compared to Chironomidae (midge) larvae was also highest for the reference site (52.9), followed by DC-1 Up (17.7). Community Loss index scores were highest at DC-2 DN (3.36), indicating the most difference in benthic community structure compared to Chaney Creek, while DC-1 Up appeared to be similar to reference conditions with an index of 0.96.

Biometric scores were then generated based on the quantile distributions of the bioassessment results (Appendix I). For parameters that increase with benthic health, scoring distributions were as follows: 90^{th}-100^{th} quantile=6; 25^{th}-75^{th} quantile=3, 0-10^{th} quantile=0. For, parameters that decrease with good benthic health, scoring distributions were opposite with 90^{th}-100^{th} quantile=0; 25^{th}-75^{th} quantile=3, and 0-10^{th} quantile=6. Total scores were then compared to the Chaney Creek reference site to determine the percent comparable, which was then used to rate the biological condition of the site (Table 2.8b). DC-1 Up was 84.6% comparable to Chaney Ck. and considered not impaired [NI], while DC-2 Dn was 0% comparable and severely impaired. In comparison to the TMDL rankings, both the overall score and biological condition

were highly comparable for DC@ HF and Chaney Creek, while DC-1 Dn and DC-2 Dn did not correspond at all with results given in the TMDL study, which found both sites to be moderately impaired.

Similar trends were observed during winter 2010, although taxa richness was higher at all sites (Table 2.9a). Taxa richness was highest at the reference site (20.0), followed by DC-1 Up (18.3), and lowest at DC-2 Dn (11.5). Modified-Family Biotic Index scores were higher at all sites except for DC-2 Dn (5.94) which was almost identical to the MFBI for 2009 (5.98). Although scores were higher at all other sites, ranging from 3.14 (DC-1 Up) to 4.15 (Chaney Creek), they followed the same trend as observed in 2009. Community Loss Index scores were much lower in 2010 compared to 2009, ranging from 0.26 at DC-1 Up to 1.06 at DC-2 Dn, indicating some overall improvement in biological conditions. Biometric scores were similar between 2009 and 2010, as were biological condition ratings which still rated the DC-2Dn site as severely impaired and DC@ HF as moderately impaired compared to the Chaney Creek reference (Table 2.9b).

Asian Clam *In-Situ* Studies

Growth and Survivorship

Asian clam (*Corbicula fluminea*) 60-day growth studies were carried out at selected sites in the Dumps Creek subwatershed from 2008 through 2010. Clams were measured for initial size prior to deployment. Small to medium sized clams were used for the growth study. The overall mean length (mm) for clams used during the 2008 study was 10.91 mm, with site averages ranging from 10.19 to 11.45 mm. The overall average initial size for clams used in 2009 was similar ($\bar{x}$ =10.92 mm) with site means ranging from 9.91 to 11.79 mm. Clams used for

the 2010 growth study were larger ($\bar{x}$ =11.82 mm) than those used in the two previous years, with site averages ranging from 11.36 to 12.23 mm.

The survivorship endpoint did not appear to be a sensitive indicator of ecological condition (Table 2.10). The percent of surviving clams varied slightly at sites between sampling years, with median ranges of 68-100 %. The lowest percentages occurred for DC-6, with average survivorship ranging from 64 to 98%.

Growth averages were also variable at sampling sites between years (Table 2.10). During the 2008 study, growth was significantly (Tukey's HSD, α=0.05) higher (1.07 mm) at site DC-6, despite having the lowest clam survivorship. Similar growth averages occurred at DC-4 (0.95 mm) and HF-4 Dn (0.94 mm). Growth and survivorship measurements were compromised at DC-2 Dn, as clams were covered with thick, epiphytic/iron deposits after the 60-day *in-situ* period. This hardened covering inhibited individual growth calculations as markings on clam shells, used to identify individuals, were no longer discernable. An estimate of growth determined to be 0.59 mm, similar to the majority of sites included in the study.

The 2009 study included to additional upstream sites, DC-1 Up and DC-2 Up. A third site located below the CBP 001 discharge (DC-1 Dn) was also used, but clams placed at this site died within the first two weeks of the *in-situ* study, and therefore were removed from statistical analysis. Growth averages at DC-1 Up were similar (0.16 mm) to those downstream at DC-3 (0.31 mm), DC-4 (0.40 mm) and DC-6 (0.38 mm), which were all lower compared to averages from the 2008 growth study (Table 2.10). Clam growth was lowest at DC-2 Up (0.08 mm), but the highest mean growth occurred at the next site downstream, DC-2 Dn (1.95 mm).

Similar growth trends were observed during the 2010 sampling season (Table 2.10). Growth was again, significantly higher at DC-2 Dn (1.97 mm), and lowest at DC-2 Up (0.11

mm). Average growth was substantially higher for Hurricane Fork sites in 2010, compared to those in 2008 and 2009. For example, site HF-5 growth in 2010 (0.52 mm) was much higher compared to averages the previous two years at 0.23 and 0.11 mm, respectively.

Overall, growth averages were highest during the 2008 and 2010 sampling seasons, but substantially lower during 2009, when growth outliers (DC-2 Dn) were excluded from consideration (Table 2.10). Highest growth averages overall occurred at DC-2 Dn (1.95 and 1.97mm) during 2009 and 2010. These averages were followed by DC-6 (1.07 mm, 2008). Survivorship trends were similar between all years, with the lowest average survivorship occurring at site DC-6 in 2008 (64%), which also had the second lowest average of 68%, which occurred during 2009. All other sites ranged from 76% survivorship (CH-1, 2009-10) to 100%.

Pairwise correlations between clam growth and BCF values for bioaccumulated metals in clam tissues were not significant for Cu (r=-0.0930), Zn (0.0018) or Fe (0.0116) (Appendix II). Clam growth was also not significantly correlated to trace metals found in site sediments. The only moderate correlation (r=-0.5771) determined was between clam growth and habitat assessment scores (HAS), which was not significant (p=0.1017) according to a Spearman's *p* nonparametric test (α=0.05).

Bioaccumulation

Average bioaccumulation of aluminum (Al) was significantly higher at HF-3 (19.5 mg/kg) compared to all other sampled sites during 2008 (Table 2.11). This was followed by a statistically lower mean of 12.8 mg/kg at DC-2 Dn. Bioaccumulation of Al decreased substantially at downstream sites in Dumps Creek with concentrations in 2008 ranging from 5.57 mg/kg at DC-3 to 2.99 mg/kg at DC-4; however, Al increased to 5.80 mg/kg at the lowest Dumps Creek sampling site (DC-6). Mean Al in clam tissues varied at sites in Hurricane Fork,

but in general, decreased significantly from sites below HF-3, with HF-5 having the lowest mean of 3.38 mg/kg.

Bioaccumulated levels of Al were lower at most sites during 2009 compared to the previous year (Table 2.11). Sites DC-2 Dn (8.09 mg/kg) and HF-3 (7.93 mg/kg) again had the highest tissue Al averages. Mean Al concentrations were higher at the upstream reference site, DC-1 Up (4.37 mg/kg) compared to downstream concentrations, with Al measurements ranging from 3.88 to 2.35 mg/kg. Due to construction activities at DC-6 during summer 2009, *in-situ* clam data were not available. A similar pattern of results was observed in Hurricane Fork with Al tissue concentrations higher at HF-1 (5.25 mg/kg) than the lowest site, HF-5 (4.47 mg/kg). Tissue samples were also compared to control clams using Dunnett's Method (α=0.05). All sites were statistically higher than tissue concentrations for unexposed clams ($p=<0.0001$-0.0127).

Mean bioaccumulated Al was substantially higher in 2010 with concentrations ranging from 4.17-33.6 mg/kg for Dumps Creek sites and 20.9-30.0 mg/kg in Hurricane Fork (Table 2.11). The highest average (37.4 mg/kg) occurred for clams at site CH-1, the Moss 3 (003) effluent stream. Sites DC-4 and HF-2 were statistically similar with means of 33.6 and 30.0 mg/kg, respectively. Upper Dumps Creek sites (DC-1 Up, DC-2 Up, and DC-2 Dn) had similar results, with a mean Al concentration higher at DC-1 Up (13.3 mg/kg) compared to DC-2 Up (9.61 mg/kg), located below the 001/016 effluent discharges which enter the stream just below the upstream reference site. In Hurricane Fork, average tissue Al concentrations were lowest at the upstream site, HF-1 (20.9 mg/kg), but highest at the following site downstream (HF-2, $\bar{x}$ =29.6 mg/kg). Mean Al in tissue decreased thereafter, including at the Gob Pile location (27.3 mg/kg) and below the Moss 2 (019) deep-mine discharges (HF-5, $\bar{x}$ =24.6 mg/kg). Clam tissues

from DC-6 had the lowest mean Al at 4.17 mg/kg). All site tissues were statistically higher than control clams (p=<0.0001-0.0255), except for DC-6 (p=0.9757).

Average bioaccumulated copper (Cu) was determined at select sites from 2008-2010 (Table 2.12). In 2008, the highest average for Cu in tissue samples occurred at HF-1 (5.69 mg/kg), followed by HF-2 (5.64 mg/kg). No discernable trends were apparent for Dumps Creek sites as means were statistically similar and ranged from 3.95 mg/kg (DC-3) to 4.22 mg/kg (DC-2 Dn). Although DC-6 average tissue Cu was second lowest for Dumps Creek sites (4.11 mg/kg), the mean was determined to be statistically similar to higher levels according to a Tukey's HSD means comparison (α=0.05). This was most likely due to the high variability between sample replicates at DC-6 (SD=1.12), with individual measurements ranging from 3.00-5.24 mg/kg. Large variability in sample replicates was also noted from HF-2 (SD=1.76), with measurements ranging from 3.28-7.18 mg/kg Cu.

Mean Cu concentrations were higher for clams in 2009 (Table 2.12). As observed with 2008 samples, no discernable trend was apparent in the Dumps Creek results. The DC-1 Up reference site had an average tissue concentration of 5.58 mg/kg, which increased to 6.72 mg/kg at DC-2 Up, the highest average measured during 2009. Average Cu decreased to 4.88 mg/kg at the next downstream site (DC-2 Dn), and further decreased at DC-3 (4.36 mg/kg). Copper concentrations were highest in Hurricane Fork at the upstream site, HF-1 (6.36 mg/kg), but varied downstream with HF-3 averaging slightly less (4.10 mg/kg) than the control clams (4.14 mg/kg). Copper increased to 5.29 mg/kg for the last sampling site in Hurricane Fork (HF-5). The remaining site in Dumps Creek (DC-4) had an average of 5.72 mg/kg for clam tissues. When compared to control clam tissues, four sites were statistically higher; DC-2 Up (p=<0.0001), HF-1 (p=0.0006), DC-4 (p=0.0242) and DC-1 Up (p=0.0483).

Tissue samples analyzed in 2010 generally had higher Cu concentrations compared to results from the previous studies conducted in 2008 and 2009 (Table 2.12). The upstream reference location, DC-1 Up had the highest bioaccumulated Cu (11.55 mg/kg). Concentrations decreased at the following two downstream sites, DC-2 Up (7.51 mg/kg) and DC-2 Dn (6.94 mg/kg). Mean Cu increased at DC-3 (8.20 mg/kg) and DC-4 (9.17 mg/kg), but decreased to 7.49 mg/kg at DC-6. Measurements in Hurricane Fork were similar to results from 2009, with the highest average occurring for clams placed at HF-1 (6.63 mg/kg) and decreasing downstream. Results from sites located at the base of the Gob Pile (4.82 mg/kg) and downstream (HF-3, $\bar{x}$ =4.85 mg/kg) show no indication that the large pile of coal tailings are contributing to Cu availability to aquatic biota; in fact, these two sites had the lowest averages of all sites included in the 2010 study. Compared to control clam tissue, all sites were statistically higher ($p=<0.0001$-0.0142) when $\alpha=0.05$. Control clams had an average tissue concentration of 2.13 mg/kg, much lower compared to control clams used during 2009.

Tissue samples were also analyzed for bioaccumulated iron (Fe) during the three year study (Table 2.13). Average Fe was highest at the site located just below the Gob Pile, HF-3 (78.9 mg/kg), followed by the upstream site, HF-1 (68.5 mg/kg). All sites in Dumps Creek were significantly lower, ranging from 44.4 mg/kg at DC-2 Dn to 21.4 mg/kg (DC-4). Clams placed in the Moss 3 (003) effluent stream (CH-1) had an average of 36.2 mg/kg Fe. The last site in Dumps Creek, DC-6 averaged 29.2 mg/kg Fe for clam tissues. Concentrations of Fe in clam tissues increased at HF-3 compared to HF-2 (50.1 mg/kg), which is located above the Gob Pile. Sites further downstream of the Gob Pile continued to show a decrease in bioaccumulated Fe, including sites located below the Moss 2 (0.19) deep mine discharges (HF-5, $\bar{x}$ =23.1 mg/kg).

Bioaccumulation results for Fe were slightly lower in 2009 (Table 2.13). Site DC-2 Dn had the highest average Fe (46.7 mg/kg) for clam tissues. Means at DC-1 Up, HF-3 and HF-1 were similar at 41.8, 41.2 and 40.6 mg/kg Fe. Site DC-3 had the lowest tissue concentration of Fe (29.2 mg/kg). All sites were statistically higher when compared to the control clam tissues ($p=<0.0001$-0.0168) which averaged 20.3 mg/kg Fe.

The highest measured concentrations of Fe occurred during the 2010 sampling season, in which some averages doubled compared to the previous year, ranging from 151.8 to 40.3 mg/kg (Table 2.13). Site CH-1 had the highest mean accumulation of Fe, followed by DC-4 (139.4 mg/kg). Surprisingly, tissue concentrations of Fe were higher at DC-1 Up (82.5 mg/kg) compared to the next site located below the 001/016 discharges which are known to be high in Fe (70.9 mg/kg). The site located below the settling pond had a higher average of 92.1 mg/kg, therefore, following a similar trend to that which was seen in 2009 where DC-2 Dn was higher in Fe compared to the site above the pond. Bioaccumulation rates were also higher for Hurricane Fork sites, ranging from 85.6 mg/kg at HF-1, up to 122.5 at HF-2 and 90.47 mg/kg at HF-5. During 2010 analysis, the Gob Pile did not appear to have any effect on Fe accumulation as concentrations at this site and at HF-3 were less than that of HF-2. The lowest average Fe occurred at DC-6 (40.3 mg/kg) and was the only site not statistically different from the control ($p=0.3630$). All other sites were statistically higher compared to control tissues ($p=0.0001$).

The fourth element measured during all three sampling seasons was zinc (Zn) (Table 2.14). Averages during 2008 ranged from 7.33 mg/kg (DC-3) to 10.8 mg/kg (DC-6). Both the highest and lowest averages occurred at sampling sites located in Hurricane Fork. The furthest upstream site, HF-1 had a mean tissue concentration of 12.1 mg/kg, which was significantly higher than downstream sites. The lowest measurement occurred at HF-4 Up (6.43 mg/kg),

while sites above and below ranged from 7.66 mg/kg at HF-4 Dn to 11.9mg/kg Zn at HF-2. The Moss 3 effluent stream (CH-1) was statistically similar to all other sampling sites, with an average accumulated Zn of 6.94 mg/kg.

Averages of Zn were similar during 2009, with the lowest observed concentration at the reference site in Dumps Creek, DC-1 Up (5.90 mg/kg) (Table 2.14). Mean Zn in clam tissues increased downstream of the 001/016 outfalls to 7.20 mg/kg at DC-2 Up, but continued to increase slightly at DC-2 Dn (7.31 mg/kg) and DC-3 (7.86 mg/kg). The lowest downstream site used during this study year, DC-4, had a slightly lower mean of 7.12 mg/kg. The highest average occurred at CH-1 at 9.17mg/kg Zn. Overall measurements in Hurricane Fork were lower compared to the previous year, with the highest average occurring again at HF-1 (8.29 mg/kg) but decreasing at HF-3 (7.74 mg/kg) and HF-5 (6.89 mg/kg). Compared to control tissue concentrations (5.86 mg/kg), four sites were significantly higher, CH-1, HF-1, DC-3 and HF-3 ($p=<0.001$-0.0391).

Tissue Zn concentrations were much higher at all sampling sites during 2010 (Table 2.14). Site DC-1 Up had the highest mean (16.6 mg/kg), followed by DC-2 Dn (16.5 mg/kg). Means were variable between sites and did not appear to follow a clear trend. Site DC-2 Up was substantially lower than the immediate downstream sites with a mean of 12.7 mg/kg. Hurricane Fork site HF-1 again had the highest average (11.6 mg/kg) compared to remaining downstream sites, although the Gob Pile site had the lowest mean for this stream (9.75 mg/kg), which increased to 10.2 mg/kg at HF-3. Site DC-6 had the lowest average in 2010 at 9.17 mg/kg Zn. Control tissues also were much higher for Zn during 2010, compared to the previous year, and results were not the lowest recorded during analysis. Therefore, only two sites were found to be significantly higher compared to the control, DC-1 Up ($p=0.0057$) and DC-2 Dn ($p=0.0077$).

Analysis in 2010 was expanded to include arsenic (As), cadmium (Cd), chromium (Cr), manganese (Mn) and strontium (Sr) (Table 2.15). Arsenic tissue concentrations were relatively low at all sampling sites, ranging from 0.44 to 0.87 mg/kg. The highest average was measured for clams placed at DC-1 Up, and means decreased to similar levels at downstream sites in Dumps Creek. Mean As was highest in Hurricane Fork upstream sites, HF-1 and HF-2 (0.55 and 0.62 mg/kg), which also decreased to similar levels at downstream sites. Tissues from seven sites (DC-1 Up, DC-4, DC-3, HF-2, DC-2 Dn, DC-6 and DC-2 Up) were determined to be significantly different from the control tissue (0.37 mg/kg) ($p=<0.0001$-0.0236) using Dunnett's Method ($\alpha=0.05$).

Average Cd concentrations were below detection limits (<0.08 mg/kg Cd) for all sites sampled during 2010 (Table 2.15). Chromium averages for tissue samples were all significantly ($p=<0.0001$) lower than that of the control (2.32 mg/kg) and ranged from 0.82 mg/kg at DC-1 Up to 0.34 mg/kg at the Gob Pile.

Manganese concentrations were variable between sites, but an increase in levels occurred downstream of the settling pond located below DC-2 Up in Dumps Creek (8.00 mg/kg) (Table 2.15). Tissue concentrations were even higher at sites DC-4 (12.7 mg/kg) at CH-1 (15.10 mg/kg); however, the lowest mean occurred at DC-6 (1.28 mg/kg). Hurricane Fork sites were similar, with HF-1 (7.74 mg/kg) having the lowest average compared to downstream locations, including the following downstream site, HF-2 (12.4 mg/kg), which increased significantly, before declining at the Gob Pile site (10.4 mg/kg). All sites were significantly higher when compared to control tissues ($p=<0.0001$-0.0066), except for DC-6 ($p=0.9832$).

Strontium was significantly higher at sites DC-2 Dn and DC-3 (8.93 and 7.15 mg/kg) compared to all other sites in both Dumps Creek and Hurricane Fork (Table 2.15). Site DC-1

Up had an average tissue concentration for Sr of 2.12 mg/kg, which was surprisingly similar to mean Sr at DC-2 Up as the 001/016 discharges are known to have elevated levels of Sr; therefore, an increase in bioavailable Sr would be expected at DC-2 Up. Measurements were statistically similar at all Hurricane Fork sites, ranging from 1.59 to 2.01 mg/kg. Bioaccumulated Sr was also slightly elevated at CH-1 (5.08 mg/kg). Concentrations of Sr accumulation in clam tissues was similar to background levels observed at DC-1 Up for site DC-6 (2.79 mg/kg). When compared to control concentrations (0.85 mg/kg), all sites within Dumps Creek were significantly higher ($p=<0.0001$-0.0161) except DC-1 Up ($p=0.2379$), while all Hurricane Fork sites were similar ($p=0.33363$-0.8264).

Bioconcentration Factors (BCF) were calculated to compare water column concentrations of Al, Cu, Fe, Mn, Sr and Zn to those levels detected in clam tissues (Table 2.16). Values below the unitless 250 level are considered to be low, while those above 1,000 are high. All Sr levels were considered to be low, ranging from 2.0 to 22.5. BCF values for Al were also low for all sites, except for DC-2 Up (646.7) and HF-2 (611.6), which were in the moderate accumulation range. Moderate BCF values for Fe occurred for five sites, but were all well below 1,000. Copper and Zn had the highest calculated BCF values, which were in the moderate to high range for all sites. The BCF for Cu was 1,116.0 at the DC-1 Up reference site and highest (1,344.0) at the next site downstream (DC-2 Up). The lowest (818.0) BCF for Cu was at HF-3 followed by DC-3 (872.0). The BCF for Zn was moderate (981.7) at DC-1 Up but high at the following downstream sites (1,440.0-1,572.0). Although BCF's were moderate at HF-1 and HF-2 (376.8 & 616.7), they increased substantially below the Gob Pile at HF-3 (1,105.7) and the highest BCF calculated occurred at HF-5 (4,259.3). Bioaccumulation Factors (BAF) were also calculated which compared the tissue concentrations to detectable concentrations in site sediments. Trends

were similar to those of BCF values, with Cu and Zn having the highest bioaccumulation potential, compared to Al and Fe.

Interstitial (Pore-water) Analysis

Sediment porewater was collected at sites in Dumps Creek and Hurricane Fork during 2008. Due to bedrock substratum present at most sites, it was difficult to deploy the samplers, which require several inches of sediment as in depositional zones, to adequately submerge the collection container. Therefore, several sites were not able to be included in the survey because a suitable location for placement could not be found. Three sites from Dumps Creek were sampled and included DC-3, DC-4 and DC-6. Two routine sampling sites in Hurricane Fork (HF-1 and HF-2) were sampled for interstitial water as well as a small orange boy seep located above HF-3 and near the Gob Pile. Additionally, porewater samplers (Peepers) were placed at CH-1, the Moss 3 (003) effluent stream.

Peepers were collected during July 2008 after 21 days. General water chemistry was measured on combined replicates (Table 2.17). Conductivity was lowest at HF-2 (198 µS/cm) and HF-1 (465 (µS/cm) at 25°C. Highest conductivity occurred at CH-1 (1,028 µS/cm) and DC-3 (998 µS/cm). The pH of samples was generally circumneutral, ranging from 6.67 to 6.97 su for Hurricane Fork samples and 6.95 to 7.81 su for Dumps Creek. Alkalinity and hardness (as mg $CaCO_3$/L) were also determined for porewater samples and were consistent with water column values.

Porewater samples were also analyzed for select trace metal concentration (Table 2.18). Aluminum was highest at CH-1 (1,726 µg/L) followed by HF-3 Seep (891 µg/L). Mean Al was also similarly high at DC-6 (805 µg/L), while all remaining sites were substantially lower, ranging from 212 to 549 µg/L Al.

Mean Fe was highest for the HF-3 Seep (49,991 µg/L), followed by DC-4 (8,594 µg/L)(Table 2.18). Mean Fe was lowest for porewater sampled at DC-3 (1,830 µg/L). Hurricane Fork samples at sites HF-1 and HF-2 had means of 2,497 and 3,528 µg/L, respectively.

Chromium levels were below detection limits (4.0 µg/L) at all sites in Dumps Creek and Hurricane Fork (Table 2.18). Average Cu was also below detection limits (3.0 µg/L) at sites DC-3, HF-1 and HF-2, and barely detectable at remaining sites with means ranging from 5 µg/L (DC-4) to 9.0 µg/L (HF-3 Seep).

Average Mn was elevated at all sampling sites, except for DC-3, which had the lowest mean of 499 µg/L (Table 2.18). Site CH-1 had the highest Mn average (6,076 µg/L), while remaining site means ranged from 1,107 to 2,197 µg/L Mn.

Strontium levels were fairly consistent between sites, with the exception of CH-1 (1,123 µg/L) (Table 2.18). Average Sr was higher in samples collected from Dumps Creek, with means of 562 µg/L (DC-3), 521 µg/L (DC-4) and 538 µg/L at DC-6. HF-1 had the lowest average of 198 µg/L Sr.

Zinc levels were also relatively low at all sites with CH-1 (50.0 µg/L) and the HF-3 Seep (34.0 µg/L) having the highest means. DC-3 had the lowest average Zn at 4.0 µg/L (Table 2.18).

Toxicity Testing of Mining Effluents

Acute and chronic bioassays were conducted on treated mine water and coal prep effluent which routinely discharge into the Dumps Creek subwatershed in Russell County, VA. Bioassays were conducted with officially recognized test organisms (*C. dubia, P. promelas* and *H. azteca*), juvenile (2-mo old) freshwater mussels (*V. iris*) and mayfly species, (*I. bicolor, Maccaffertium sp.*) when both effluents and organisms were available.

Camp Branch 001 Effluent Evaluation

The Camp Branch 001 discharge is located near the headwater region of Dumps Creek and discharges intermittently. Discharge flow measurements averaged 1,516.1 g/min over ten sampling dates during spring 2009, with two of these dates (1/21/09 and 2/04/09) having no flow from the pipe. Similar measurements ($\bar{x}$ =1,840 g/min) were recorded during spring-fall 2008 (data obtained from Environmental Monitoring, Inc., Norton, Virginia). Routine National Pollutant Discharge Elimination System (NPDES) monitoring parameters for this discharge are pH, total suspended solids (TSS) and total iron, while total dissolved solids (TDS) are also monitored for groundwater parameters. Median pH on these samples was 7.3 su (n=9), with a range of 7.2 to 7.7 su. Iron ranged from 0.89 to 2.06 mg/L ($\bar{x}$ =1.58 mg/L, n=9). Mode of TSS was 2 mg/L, with only one sampling occurrence of 7 mg/L. Average TDS during this sampling period was 906.0 mg/L, ranging from 788-978 mg/L. A complete analysis of total and dissolved metals was conducted on four samples (5/27/08, 6/2/08, 6/9/08, and 6/16/08) of the 001 effluent, in conjunction with *Villosa iris* chronic (28-day) toxicity tests. Only Fe (total and dissolved) exceeded WQC (1 mg/L), with measurements ranging from 0.668-2.20 mg/L total Fe and 0.317-1.79 mg/L dissolved Fe.

The 001 discharge was evaluated using the daphnid, *C. dubia*, a juvenile stage (~ 2 months old) freshwater mussel (*V. iris*) and two mayflies (*I. bicolor*, *Maccaffertium* sp.). *Ceriodaphnia* were used to determine acute toxicity, using 100% effluent. Two acute tests resulted in 100% survivorship of test organisms after 48 hrs. Conductivity measured on test samples averaged 1,506 µS/cm (25°C) for the first test, and 1,998 µS/cm (23.5°C) for the second test. Chronic *C. dubia* tests were conducted twice. The first test, conducted during October 2008, resulted in no significant survivorship impairment after seven days exposure (70%),

compared to control organisms (100%); therefore, the No Observed Effects Concentration (NOEC) for survivorship was >100%. Reproduction; however, was significantly lower ($\bar{x}$ =29.4) compared to the controls ($\bar{x}$ =49.9), with an NOEC of 50% 001 effluent. A follow-up test conducted in November 2008 only evaluated the 100% 001 discharge, but used both filtered and unfiltered samples. Survivorship was identical for these two samples, with both only losing one daphnid each. Reproduction was greater for daphnids exposed to the unfiltered 001 effluent (27.4) compared to filtered (17.9); however, both samples were significantly lower than the control (34.7). Because concentrations were not used in these tests, NOEC's could not be determined. Water chemistry parameters were fairly consistent between renewal and out-water samples. The highest conductivity and alkalinity measurements occurred for the 100% concentration during the first chronic test (851 µS/cm and 384 mg $CaCO_3$, respectively), while conductivity was slightly lower in the unfiltered samples (1,374-1,408 µS/cm) compared to the filtered ones (1,426-1,472 µS/cm) during the second test. Dissolved oxygen (mg/L) was within normal range (>7.40 mg/L, controls), but was generally lower (6.92-7.27 mg/L) for the 100% 001 effluent renewal water, as this discharge is normally low in dissolved oxygen.

Survival and growth of the juvenile mussel, *V. iris*, was determined during the spring/summer 2008. Survival was 90% after 28-days and not significantly different than the control (97%). Growth for the 001 effluent; however, was significantly lower (0.0775 mm) compared to that of the control (0.26 mm) (Fig. 2.10). The low growth observed may not have been a true reflection of the effluent toxicity as organism quality may have been compromised and therefore, a contributing factor to the minimal growth observed. The mussels used for this test were the smallest ones received from the overall batch obtained from the hatchery that were used to test the original set of effluents during this testing quarter. Conductivity varied from

905-1,398 during the first week of the test to 1,330-1,443µS/cm and was highest in the last week at 1,395-1,510 µS/cm.

Four mayfly chronic toxicity tests were conducted to evaluate the influence the 001 discharge on mayfly survival and growth. The growth endpoint used to evaluate sub-lethal effects was exuvia production. *Isonychia bicolor* (5-7 mm) nymphs were exposed to 001 effluent over 14 days during November 2009 at an ambient test temperature of 11°C. Minimal mortality (5%) occurred within the initial 24 hr of testing, but additional mortality did not occur for another four days of exposure (10%) (Fig. 2.11). After seven days, 15% mortality (85% survivorship) had occurred in the test, which was comparable to control survivorship (90-95% [two controls]). After 14 days, five mayflies had died, resulting in 75% survivorship for the 001 effluent. Control survivorship was 80% and 95%. No exuvia were produced in the 001 effluent during the duration of the test, although control organisms had variable growth responses. One control test had a total of 20 exuvia during the 14 days of testing, while the second set of control replicates had only seven. Conductivity ranged from 898-921 µS/cm at 11°C.

A second chronic test during April 2010 used *Isonychia* that were slightly larger (7-10 mm in length) and more developed. The total duration of this test was 21 days and was conducted at 17°C after three days of laboratory acclimation (Fig. 2.11). Mortality was first observed after three days of test exposure for the 001 effluent (10%) and control organisms (5%). Additional mortality occurred on day nine (15%). After 14 days, only 50% of mayflies in the 001 effluent were still alive, while control survivorship remained at 95%. For the remaining seven days of testing, survivorship continued to decrease, with only 25% living after 21 days. Control survivorship at test termination was 90%. Exuvia production was steady during the first ten days of testing. After 14 days, 11 exuvia were produced for the 001 effluent, compared to 18

in the controls. After 21 days, mayflies in the 001 effluent produced a total of 12 exuvia, and controls had more than twice that, with a total of 30. Conductivity in this test averaged 1,102 µS/cm @ 17°C.

A second mayfly, *Maccaffertium* sp. (Heptageniidae), was tested simultaneously with *Isonychia*. Organisms used were between 5-7 mm in length and were considered to be during the middle range of development. During the first test (November 2009), survivorship was greater than that observed in the *Isonychia* test, with 85% survivorship after 14 days (Fig. 2.12). Two mayflies were dead on the second day of the test, but additional mortality (one) did not occur until Day 14. Control survivorship was 95% after 14 days, and therefore, survivorship responses were similar. As with the *Isonychia* test conducted at this time, no exuvia were produced for the 001 effluent replicates. Control organisms produced a total of nine exuvia during the 14-day test period. Conductivity ranges were the same as measured in the *Isonychia* test (898-921 µS/cm).

In April 2010, a second chronic test with *Maccaffertium* was conducted, using organisms that were much more advanced in development and close to emergence. Late stage organisms tend to be more sensitive not only pollutants, but also handling stress. Substantial mortality (30%) was observed after three days of exposure to the 001 effluent, but was less than that observed in the controls (40%) (Fig. 2.12). After seven days of testing, control survivorship was only 50%, while survivorship in the 001 effluent was also 50%. At Day 14, 20% of organisms were living. By Day 21, only 10% of mayflies were living in the 001 effluent and 20% remained in the control. Control survivorship does not reflect organisms that were lost due to emergence. On Day 17, one control organism emerged (subimago) and was therefore counted as a loss. Exuvia production was also greatest during the initial ten days of testing, with nine total exuvia being produced by Day 14. After Day 14, no additional exuvia were produced in the 001

effluent. At test termination, control organisms had produced a total of 12 exuvia. Average test conductivity was 1,105 µS/cm (17°C).

Moss 2 Deep Mine 019 Effluent Evaluation

The Moss 2 (019) effluent, which discharges into Hurricane Fork, was evaluated for chronic toxicity during summer 2007 (Table 2.19). A preliminary acute test with *C. dubia* exposed to 100% effluent indicated no acute impairment as all organisms survived after 48 hr. Chronic toxicity tests with a juvenile mussel (*V. iris*) were conducted over 28 days; one test that utilized a sediment substrate, and one which did not. Mussel survivorship was not statistically different from control growth for either test. Survivorship was 75% for *V. iris* in test beakers without sediment (control =90% survivorship), while 97.5% of mussels were surviving after 28 days in the test containing sediment, which was substantially higher than the control (82.5%). Growth was also greater (0.34 mm) in the test containing sediment, which was identical to average growth in the control. Mussel growth was similar (0.20 mm) to control (0.26 mm) growth in the test that did not contain sediment.

Survivorship and growth were also determined for the amphipod, *Hyalella azteca* (Table 2.19). After 28 days, 95% of amphipods were living in the 019 effluent, compared to 100% in the controls. Final length measurements were determined and amphipods exposed to the 019 effluent had a significantly lower average (2.34 mm) compared to the controls (3.32 mm) and additional effluents tested. To determine growth, a subset of 40 *Hyalella* were removed from available test organisms prior to test loading and measured for initial length. This average initial length was used to estimate change in average sizes between effluents. Estimated growth was significantly lower (1.02 mm) for the organisms exposed to the 019 effluent than the control (1.89 mm). One possible reason for amphipods being significantly lowest in final and estimated

growth in Moss 2 effluent could be due to the disproportionately high number of females found at the end of the test (87.5%) (Table 2.20).

Female amphipods normally do not grow as robustly as males and in the other three tests, the male/female ratio was more equally distributed. When amphipods are seven days old at the initiation of a test, it is quite difficult to determine sex differences and if tried, this approach would only stress the test organisms in the 28-day tests.

Reference toxicant tests using NaCl were conducted for both test species, 96 hr for *V. iris* and 48 hr for *H. azteca*. The acute LC_{50} values were 1.74 g/L for *V. iris* and 2.85 g/L for *H. azteca* indicating that the amphipod was more tolerant to the reference toxicant than the mussel.

Water chemistry was measured on out-water during test renewals (Tables 2.21-2.23). Conductivity was similar (699-778 µS/cm) in all three tests, with the highest measurement (804 µS/cm) occurring in the *H. azteca* chronic test (Table 2.23). The lowest (4.28 mg/L) DO_2 measurement occurred in the amphipod test, while DO_2 in the mussel chronic tests ranged from 5.40 (sediment) to 8.10 mg/L (no sediment). Generally, DO_2 was highest (7.60 mg/L) in the *V. iris* test that contained no sediment substrate (Table 2.21), compared to 6.71 mg/L (*V. iris*-sediment) and 6.08 mg/L (*H. azteca*). Average ammonia measurements were highest (2.65 mg/L) in the amphipod test, and lowest in the mussel test without sediment (0.37 mg/L).

Moss 3 Coal Prep Plant 003 Effluent Evaluation

The Moss 3, 003 effluent (also referred to as Kiser Pond 003) was evaluated using multiple test organisms to determine potential toxicity to native freshwater fauna. Survivorship and growth were determined in 28-day chronic toxicity tests using the freshwater mussel, *V. iris*, and *Hyalella azteca*, a freshwater scud during summer 2007 (Table 2.19). Two separate mussel

toxicity tests were conducted; one using a natural sediment substrate and the other with no substrate. Amphipod test beakers contained sterilized sand as a substrate.

Mussel and amphipod survivorship was not affected by the 003 effluent. Percent surviving unionids was higher for organisms that were provided the sediment substrate (Table 2.19). Mussel growth was also greater for these test organisms, with a mean of 0.21 mm, compared to 0.12 mm for mussels exposed to the 003 effluent without the sediment substrate. Although growth was greater, both mussel tests resulted in growth being statistically lower compared to control clams (0.34 and .026 mm), for the sediment versus no-sediment tests. Estimated growth (mm) for *Hyalella* was not significantly different from control growth Estimated growth for these organisms was obtained by subtracting initial size ($\bar{x}$ =1.43, n=20) measurements from averaged final measurements (length).

Water chemistry parameters were monitored during water renewals on combined replicates samples (Tables 2.24-2.26). Conductivity was consistent in the two *V. iris* chronic tests, ranging from 903-970 µS/cm (without sediment) and 884-939 µS/cm (with sediment) (Table 2.24 & 2.25). Measurements were higher in the *H. azteca* chronic test, with averages ranging from 922-1,036 µS/cm; an increase most likely caused by the food source used for this test (YCT) (Table 2.26). Dissolved oxygen measurements (DO_2) were lowest in the *V. iris* chronic test with the sediment substrate (5.01-6.91 mg/L) and the amphipod test (3.99-7.44 mg/L). The highest ammonia measurement (4.31 mg/L) was also measured in the *H. azteca* chronic test.

The Moss 003 effluent was tested over the course of ten quarters from 2008-2010 using acute and chronic methods for *C. dubia* and *P. promelas* (fathead minnow). No acute toxicity to either *C. dubia* or the fathead minnow (FHM) occurred as test organism survival was 100% for

the all test concentrations and the controls for both test organisms. Therefore, no LC_{50} values could be determined. The 003 effluent was also not chronically toxic at any effluent test concentration from 6.25 to 100%. Daphnid survival was 100% in all concentrations and reproduction was not inhibited at any effluent concentration. Fathead minnow survival was also 92.5-100% in the effluent concentrations (6.25-100 %), with only a few random mortalities occurring during the two-year testing period. Fish growth (mg) was never determined to be statistically lower than average control growth (Dunnett's Test or Steel's Many One Rank Test).

Water quality parameters were measured on renewal (in) and out-water samples from the tests and were generally stable. Test temperatures ranged from 24.0-25.0°C and dissolved oxygen measurements were always > 4.0 mg/L, with the lowest recorded value of 5.69 mg/L occurring in a FHM chronic test. Test conductivity was consistent (999-1,198 µS/cm) between quarterly tests, and was as low as 931 µS/cm in 2008.

Chronic toxicity tests with *Isonychia bicolor* and *Maccaffertium* sp. were conducted once during spring 2010. Due to limited organism availability and coinciding effluent availability, these tests could not be replicated. Tests were conducted slightly above ambient conditions (17°C) for 21 days.

Isonychia survivorship in the 003 effluent was 100% for the first 48 hr of the test, but declined to 95% on Day 3, which was comparable to control (95%) responses (Fig. 2.13). Survivorship remained at 95% for both the control and effluent until Day 8 when additional mortality (10%) occurred in the 003 effluent. Survivorship continued to gradually decline thereafter, with 65% survivorship after 14 days and 45% at test termination (21 days). Control survivorship remained at 95% through Day 20, but declined to 90% on Day 21.

Maccaffertium survivorship was greater in the 003 effluent than in the controls (Fig. 2.14). Survivorship was 100% for the first eight days of the test, but declined to 95% on Day 9. Control survivorship was 100% for the first 48 hr of testing, but declined sharply on Day 3 (60%). Normally, such low control survivorship would warrant test termination in standardized toxicity tests (following US EPAWET test guidelines); however, such protocols have not been established for mayfly testing. *Maccaffertium* survivorship continued to decline, with 70% survivorship after 14 days in the 003 effluent, while only 40% of control organisms were still living. After 21 days, 40% of mayflies in the 003 were still alive and 20% were living in the control. In comparing responses of *Isonychia* and *Maccaffertium*, both organisms had similar survivorship responses in the 003 effluent after 14 days. *Maccaffertium*, however, were much further along in development and a few organisms (3) emerged beginning on Day 9.

Growth estimates were based on the number of exuvia produced by organism molting during the test (Fig. 2.15). Exuvia were counted and removed after each day. No exuvia were observed in the *Isonychia* chronic test during the first 48 hr of testing. On Day 3, four exuvia were counted in the 003 effluent and three in the controls. Overall, daily results were quite variable. Organisms in the 003 effluent appeared to molt mostly during the middle of the test, while control mayflies continued molting through the full 21-day duration. Another way to look at these data is to consider the total exuvia production after 14 days and then again at 21 days (Fig. 2.16). Control organisms and those in the 003 effluent both produced a total of 18 exuvia after 14 days of testing, indicating no differences in "growth" had occurred between the effluent and control. However, after 21 days, the total number of exuvia in the 003 effluent had barely increased (19), while the control had a total of 30 exuvia.

Unlike the *Isonychia* chronic test, Maccaffertium exposed to the 003 effluent produced more exuvia than control organisms (Fig. 2.17). No exuvia were observed during the initial 48 hr of testing, but on Day 3, both the control (4) and 003 effluent (2) had exuvia. Total exuvia on days 9 and 10 reflected carapaces shed during emergence as one mayfly emerged on both days. Daily totals were also variable in this test, but surprisingly, the control organisms were no longer molting by Day 18.

The total number of exuvia produced after 14 days was greater for the 003 effluent (14) compared to controls (10) (Fig. 2.18). *Maccaffertium* exuvia totals increased (17) by Day 21, while control totals remained similar (11).

Overall, the Moss 3 003 effluent did not result in any chronic impairment for *C. dubia, P. promelas, H. azteca* and no survivorship impairment for *V. iris*. Survivorship impairment occurred in the *Isonychia* chronic toxicity test (65% survivorship) compared to controls (95%) after 14 days, and responses were substantiated after 21 days when mayfly survivorship in the 003 effluent declined to 45% by test termination, while control survivorship remained acceptable (90%). *Maccaffertium* survivorship results were less reliable as survivorship responses were greater for the 003 effluent, than the control. Sublethal impairment did occur for the *V. iris* chronic tests, whereby organism growth after 28-days was significantly lower (0.12 and 0.21 mm) compared to the controls (0.26 and 0.34 mm). The *Isonychia* chronic test indicated growth impairment occurred after 21 days of testing, based on the total number of exuvia produced, compared to controls. However, at 14 days, the number of exuvia was the same for the control and 003 effluent. As with survivorship, growth responses in the *Maccaffertium* test were unreliable as controls had a significant amount of mortality early in the test and produced less exuvia compared to the 003 effluent.

Moss 3 Coal Prep Plant 004 Effluent Evaluation

The Moss 3, 004 effluent was evaluated for potential toxicity during summer 2008. An acute test with *C. dubia* resulted in 100% survivorship after 48 hr. Chronic toxicity tests were also conducted with the freshwater mussel, *V. iris*, and an amphipod (*H. azteca*) (Table 2.27). Mussel tests were conducted without the addition of a sediment substrate, as used in previous mussel toxicity tests for this study. After 28 days, mussel survivorship was 97.5 % and was similar to that of the control (100%). Growth was also similar for the 004 effluent sample (0.41 mm) and control (0.48 mm).

Amphipod results also indicated no toxicological issues for this effluent as survivorship was also 97.5 %, with control survivorship of 100% (Table 2.27). Final size measurements for amphipods in the 004 effluent were very similar (3.72 mm) to the control (3.73 mm), as was the estimated growth average (2.52 mm vs. 2.53 mm).

General water chemistry parameters were measured during test changeovers and out-water chemistry is provided in Tables 2.28 and 2.29. Conductivity was higher in the mussel test ($\bar{x}$ =991 µS/cm) than the *H. azteca* chronic ($\bar{x}$ =970.2 µS/cm). Average DO_2 was also higher in the *V. iris* test (7.25 mg/L) compared to the amphipod test (6.84 mg/L). The lowest measurement, however, occurred in the *V. iris* test (2.82 mg/L). The initial measurement of ammonia in the *V. iris* chronic was substantially higher (3.8 mg/L) than subsequent measurements which ranged from 0.43-0.53 mg/L (Table 2.28). All ammonia measurements were consistent in the amphipod test (0.37-0.64 mg/L).

Reference toxicant tests using NaCl were conducted for both test species, 96 hr for *V. iris* and 48 hr for *H. azteca*. The LC_{50} values were 4.0 g/L for *V. iris* and 6.28 g/L for *H. azteca*.

Sediment Toxicity Tests with *Daphnia magna*

Toxicity tests with *D. magna* were conducted on site sediments at selected sites in the Dumps Creek subwatershed during spring 2010. Initial tests conducted in March 2010, evaluated sediments collected from sites DC-1 Up (reference), CB Pump (001) Mixing Zone (MZ), DC-1 Dn, DC-2 Dn, DC above Hurricane Fork and Chaney Creek (reference). Additional replicates of the CB Pump MZ sediments were tested using the 001 effluent as the overlaying water. Two controls were used, one with filtered Sinking Creek water (SCW) and a second with 001 effluent.

Survivorship was 100% for samples collected in Chaney Creek (100%) and the CB Pump MZ (100%) (Table 2.30). Control survivorship was slightly lower (91.7%) as one daphnid died during the test. The CB Pump MZ replicates, which used the 001 effluent as the overlaying water also, had no significant survivorship impairment (91.7 %). Daphnid survivorship was significantly ($p=0.0087$) lower at the upstream reference site (41.7 %). The lowest (8.3 %) survivorship; however, occurred for site DC-1 Dn, by which all but one daphnid were dead by Day 8.

Reproduction means were calculated based on the original number (3) of daphnids exposed in each test replicate, whereby the total number of neonates produced in each replicate was divided by three. Correlation analysis indicated a moderate correlation ($r=0.7299$) between reproduction and survivorship. Chaney Creek had the greatest number of mean neonates (75.10) followed by the SCW control (59.3) (Table 2.30). The CBP MZ with 001 overlaying water had lower (39.53) mean reproduction compared to mixing zone sediments with SCW as the overlaying water (50.50). DC-1 Up reproduction was significantly ($p=0.0031$) lower (9.67)

compared to the control. However, as seen with survivorship, the lowest average occurred for the DC-1 Dn (6.83) sediment sample.

Water chemistry parameters were monitored for each test changeover on renewal water and out-water. The two overlaying waters used were quite different (Table 2.31). Filtered SCW had an average conductivity of 117.9 µS/cm, and only one measurement (114 µS/cm) was substantially lower. The 001 effluent was also consistent between changeovers, and averaged 1,186.6 µS/cm. Dissolved oxygen was stable for the SCW (7.82-8.38 mg/L), as carboys were bubbled to maintain saturation. However, the 001 effluent is characteristically low in DO_2, often discharged at < 1 mg/L. Therefore, DO_2 was lowest during the first changeover (3.86 mg/L), but increased gradually to 9.05 mg/L due to refrigeration of effluent. Additionally, effluent used for changeovers was gradually warmed to 25°C and gently aerated prior to being used. The pH of both the SCW and 001 effluent was stable, averaging 8.27 and 7.70 respectively.

Out-water chemistry was measured on combined replicates for each sample tested (Table 2.32). Conductivity and dissolved oxygen were the most variable, while pH was stable between samples and remained consistent to that of the renewal water. The two samples with the highest conductivity were the CBP 001 effluent (water only, 1,284.3 µS/cm) and the mixing zone sediment sample with 001 effluent as overlaying water (1,240.6 µS/cm). Remaining averages were consistent to the control (227.3 µS/cm), except the CBP mixing zone sediments, which was slightly (306.2 µS/cm) higher. Due to microbial activity in the sediments, dissolved oxygen was a concern and was checked prior to loading organisms into test beakers to ensure oxygen measurements were above the minimal requirement of 4 mg/L. Because all initial measurements were well above (> 6 mg/L) this limit, test beakers were not aerated. Dissolved oxygen measurements remained well above the minimal requirement for six days of testing, after which

two samples (mixing zone sediments and DC-2 Dn) fell below 4 mg/L (3.94 and 3.68 mg/L). Since the 10-day test was near completion, test replicates for these two samples were not aerated. Even gentle aeration can be more detrimental to daphnid survivorship than the low DO_2, as oxygen becomes trapped under the organism's carapace; preventing them from swimming and causing the daphnid to become trapped in the surface film of the water.

Average DO_2 was lowest at DC-2 Dn (3.88 mg/L), as measurements remained < 4 mg/L for the remainder of the test (Table 2.32). Other samples came close to this required limit, and ranged from 5.73 (001 effluent only) to a low of 4.10 mg/L (DC-1 Dn).

A second sediment toxicity test was conducted in May 2010 (Table 2.33). Mean survivorship was highest in the control and DC-4 samples (100%). Compared to the first sediment test (41.7 %), DC-1 Up survivorship doubled (83.3 %) and DC-1 Dn survivorship was more than six times higher (50%). Three new samples were also evaluated; DC-3 and DC-4, which both had acceptable survivorship and CH-1 which was significantly lower (16.7 %) than the control.

Average reproduction was also improved (53.5) for the DC-1 site compared to that of the first test (9.67) and was similar to that of the control (57.8) (Table 2.33). DC-1 Dn had significantly lower (12.6) reproduction compared to the control, but mean neonates were approximately doubled compared to the first test. Neonate production was also significantly lower (13.6) for the CH-1 sample.

Water chemistry was consistent with that of the first test. Average conductivity of the overlaying water (filtered SCW) was 230.3 µS/cm. Since this was the only overlaying/control water used during this test, conductivity measurements on the out-water samples were also consistent, ranging between 241-483 µS/cm. The highest measurement of 483 µS/cm occurred

one time for the DC-1 Dn sample, while no other measurements exceeded 334 µS/cm. Overall DO_2 was also higher in this test, except for DC-1 Dn which had low (< 3 mg/L) dissolved oxygen measurements for the duration of the test; however, beakers were not aerated. The pH was stable, with daily averages for all samples between 8.03 and 8.14.

Some researchers believe sediment toxicity tests are not reliable as water changeovers tend to dilute the toxicity components in the sediments. For this study, trace metal concentrations were analyzed on site sediments and on test samples at the end of the 10 days (Table 2.34). Replicate samples were each analyzed and a sample mean was established for each site. Not all samples or metal concentrations became diluted with changeovers. Some samples, such as DC-2 Dn, had an increase in concentration of Mn (245 mg/kg), Sr (190 mg/kg), Cu (124 mg/kg) and Zn (23 mg/kg). Chaney Creek samples also increased for Fe (5,113 mg/kg), Mn (152 mg/kg) and Cu (9 mg/kg).

Linear regression models (least square fit) were used to estimate the relationship between daphnid mortality and reproduction (Table 2.35). Prior to analysis, sediment trace metal data were logarithmically transformed. The relationship between both percent survivorship and mortality was statistically significant ($p =< 0.0001\text{-}0.0017$, $\alpha=0.05$) with all trace metals included in analysis. For daphnid reproduction, Al, Fe, Mn and Cu were statistically significant according to the model.

Evaluation of an Epibiotic Bacterium

During reconnaissance efforts in Dumps Creek during winter 2008/2009, a bacterium was observed coating all substrates immediately below the Camp Branch 001 discharge in the headwaters region of the creek (Fig. 2.19). The filamentous masses have been observed up to 200 feet downstream along the edge of the creek and attached to fallen branches within the creek

as well. Samples of the bacterium were taken and identified as *Thiothrix nivea* based on microscopic observations and comparative 16S rRNA gene sequence analyses (Duke Institute for Genome Sciences and Policy, Genome Sequencing and Analysis Core Facility).

Discussion

The effects of coal mining on aquatic ecosystems have been well researched and are generally understood as having a major impact on the environment (Rochow 1979). Nearly the entire length of Dumps Creek is influenced by active mining, degrading both habitat and water quality. The extensive mining and reported biological impairment in Dumps Creek (Map Tech 2004) and the Clinch River (Cairns et al. 1971, Clements et al. 1988, Farris et al. 1988, Reed-Judkins et al. 1998, Hull et al. 2002, Hull et al. 2006) necessitated the need for both field bioassessments and laboratory evaluations of mining effluents in this watershed.

Coal mining activities in the headwaters of Dumps Creek influence the biological integrity of the entire system. The upstream reference site (DC-1 Up), located ~ 10m above the first active effluent discharges (CBP 001/016), provided optimal habitat for benthic colonization. Habitat quality, however, diminished downstream as attributes of the 001 discharge caused an iron-encrustation on all rocks and woody debris. Based on qualitative observation, this deep mine dewatering discharge frequently contributes to ~ 90% of channel flow during normally low-flow conditions. The discharge, which can be characterized as having a moderately elevated conductivity ($\bar{x}$ =1,172 µS/cm) and neutral pH ($\bar{x}$ =7.39 su), increases stream conductivity substantially from 290 µS/cm (DC-1 Up), to an average of 1,035 µS/cm ~ 35 m downstream at DC-1Dn. Elevated conductivity remained below DC-1 Dn, with gradual dissipation occurring after the confluence with Hurricane Fork (~ 1.6 km downstream). Additionally, alkalinity increased considerably below the discharge (316-555 mg $CaCO_3$/L). Substantial changes in

stream alkalinity can cause changes in taxa. Several studies (Almer et al. 1978, Kilham 1982, Edmondson 1990) reported changes in planktonic populations in rivers and lakes experiencing a considerable change in alkalinity. Further, lab toxicity test results suggested that higher levels of alkalinity caused a chronic effect in *Ceriodaphnia dubia* reproduction (Cowgill and Milazzo 1991). Conversely, alkalinity is generally considered a buffer which ameliorates the toxicity of heavy metals, such as Cd, Cu, Hg and Zn (Wang 1987).

A draft general standard Total Maximum Daily Load (TMDL) had identified total dissolved solids (TDS) as the major source of benthic impairment in Dumps Creek (Map Tech 2004). TDS measurements from the TMDL study ranged from 186 to 540 mg/L, which is comparable with estimates of TDS from this study (178-833 mg/L). These measurements were from measured conductivity values based on established formulas for TDS estimation (Kennedy et al. 2003, 2005). Due to benthic macroinvertebrate surveys and elevated TDS, the TMDL identified the section of stream impairment as the point of confluence with Hurricane Fork downstream to the confluence with the Clinch River (~ 5.5 stream km). However, the benthic data used in the development of the TDML were suspected as being biased due to the time of year samples were collected (winter). Selected sites that corresponded with the TMDL study were sampled for benthic invertebrates during the winter (February/March) and summer (June/July) and compared using the Virginia Stream Condition Index (VA SCI). Results indicated that while most sites had consistent scores between the two seasons, one site (DC-2 Dn) had a much lower SCI score during the winter 2009 (13.0) than the following summer (21.3). However, the following winter, the SCI score was even higher (32.8) than both the winter and summer of 2009 and was consistent in summer 2010. Therefore, our results did not support the hypothesis that winter sampling affected survey results.

Bioassessment scores, which rank site health based on reference site conditions (Chaney Creek), were also used to compare TMDL study results to those of this study. Results showed that for the four sites used in the comparison, only two sites were comparable between studies. Sites DC-1 Up and DC-2 Dn scores and impairment status were very different from those in the TMDL, which ranked both sites as being moderately impaired. Results of this study found that the upstream site, DC-1 Up, was very comparable to reference conditions in Chaney Creek (84.6-92.3% similar) and therefore not impaired, while DC-2 Dn was found to be severely impaired based on current study results.

Despite minor inconsistencies between this study and the TMDL, benthic impairment is evident in Dumps Creek. Using both the Virginia and West Virginia Stream Condition Indices, drastic reductions in scores occurred below the CBP 001 effluent and remained well below reference conditions downstream. In fact, using the more favorable WVSCI (Tetra Tech 2003) impairment threshold of 68, only two downstream sites (DC-3 & DC-4) were above this level.

The degree of impairment, observed at upstream sampling sites does not coincide with published reviews of TDS toxicity in the literature. For example, Kennedy et al. (2003) reported sensitive aquatic fauna was impaired at an average conductivity of 3,700 µS/cm, which is significantly higher than levels estimated in this study. A related study (Kennedy et al. 2005) found that sodium and sulfate dominated TDS was acutely toxicity at ~ 7,000 µS/cm (5,143 mg/L TDS). Some studies emphasize the need to consider the individual ions contributing to TDS toxicity (Kennedy et al. 2005, Echols et al. 2009b). Goodfellow et al. (2000) stated that the effects of TDS on aquatic insects is specific to the combinations of ions, which is supported by other studies that state the individual constituents must be considered when determining toxicological effects of an effluent to aquatic organisms (Dwyer *et al.* 1992; Goetsch and Palmer

1997; Mount *et al.* 1997; Goodfellow et al. 2000, Chapman *et al.* 2000; Clements 2002; Kennedy *et al.* 2005). The sampling region near the 001 and 016 discharges can be described as having a strong hydrogen sulfide odor and therefore, sulfate concentrations in the 001 discharge were evaluated. Routine monitoring data provided by the mining company showed an average of 89.4 mg/L sulfate for the 001 discharge with downstream concentrations up to 487 mg/L (unpublished data).

Soucek and Kennedy (2005) found that acute toxicity of sulfate to *Hyalella azteca* occurred at 512 mg SO_4^{2-}/L, while other invertebrates were more tolerant, with LC_{50} values ranging from 2,050 to 14,134 mg SO_4^{2-}/L. A review of sulfide toxicity (Wang and Chapman 1999) to freshwater invertebrates listed a range of acute and chronic lethal concentrations of 0.002 to 1.07 mg/L total sulfide (Oseid and Smith 1974 a, b, 1975). Specifically, Oseid and Smith (1975) found three mayfly genera*, Ephemera, Hexagenia* and *Baetis*, had LC_{50} values of 0.32, 0.11 and 0.02 mg/L. Hydrogen sulfide may also be causing additional ecotoxicological impacts in Dumps Creek. During a site reconnaissance in winter 2008, a thick filamentous substance was discovered in Dumps Creek, just below the CBP 001 discharge. The white biofilm was observed on all surfaces in the creek bed, including submerged vegetation, epifaunal and rock substratum. Subsequent analysis determined the bacteria to be the mixotroph, *Thiothrix nivea* (Winogradsky 1888).

Thiothrix is a colorless, filamentous sulfide-oxidizing bacterial species also considered an epibiont, that obtains its energy by oxidizing reduced sulfur or sulfide compounds such as hydrogen sulfide (H_2S) and iron disulfide (FeS_2) and deposits sulfur internally (Larkin and Shinabarger 1983). Single bacterium cells attach to solid surfaces by secreting a gelatinous layer, which eventually surrounds the entire basal cell. The filament develops by transverse

binary fission (Merkel 1975). *Thiothrix* has been described in habitats ranging from natural sulfide-containing waters, irrigation systems (Brigmon et al. 1994, Brigmon et al. 2003,Larkin and Strohl 1983, Gillan and Dubilier 2004), to activated sludge wastewater treatment plants (Kanagawa et al. 2000, Gillan and Dubilier 2004). Optimal growth occurs at temperatures between 25 to 30°C, with slow growth under 20°C. Growth does not occur in anaerobic conditions (Larkin and Shinabarger 1983); however, Larkin and Strohl (1983) suggest that only minimal (~10% of saturation) oxygen concentrations are necessary. The presence of the bacteria in the environment is dependent on the proper concentrations of both oxygen and sulfide and thus populations are only sustained when both oxygen and reduced sulfur compounds are replenished regularly. Sulfate concentrations averaged 89.4 mg/L in the 001 discharge from March 2006 through July 2008 (n=12), while Fe concentrations during this sampling period ranged from 1.55 to 5.13 mg/L (unpublished data). Merkel (1975) reported a "stimulatory effect" on *Thiothrix* growth with H_2S concentrations up to 30 mg/L. Farquhar and Boyle (1972) noticed an increase in *Thiothrix* growth when total sulfide concentrations in settled wastewater ranged from 0.06 to 1.9 mg/L.

The environmental effects of *Thiothrix* on aquatic biota are not fully understood based on results of this study. The formation of gliding gonidia called rosettes give the bacteria the ability to attach to objects using holdfasts, including aquatic biota. Larkin et al. (1990) reported a *Thiothrix* sp. occurring on a mayfly larvae (*Drunella grandis*) in Diamond Fork Creek, Utah. Although the bacteria covered the exoskeleton of organisms collected, it did not appear to be harmful. Ford and Scott (1996) reported *Thiothrix* growth on tadpoles collected from an aquatic breeding site in Western Mexico. *Thiothrix* was primarily attached to the tadpole mouths, adhering to the keratinized denticles, beak and wrinkles in the soft tissue. Based on observations,

the authors report no apparent adverse effects were caused by the bacteria. Additional studies have reported the bacteria on hydrothermal vent organisms (Jannasch and Wirsen 1981), intertidal sediment dwellers (Gillan and De Ridder 1997, Oeschger and Schmaljohann 1988) and a sea urchin (Temara et al. 1993, Brigmon and De Ridder 1998). After 60-day *in-situ*, Asian clams placed at DC-2 Dn were encrusted with a hard, rust-colored covering. The covering was so thick that identification based on shell markings could not be made and clam survival was difficult to be determined as both valves were sealed closed. It has been shown in other studies that microbial epibionts may contribute to iron deposition due to their ability to degrade ferric ion organic complexes (Gillan and Cadee 2000, Gillan and De Ridder 2001). Gillan and Cadee (2000) further suggest that shell epibionts on gastropods may have both positive and negative environmental consequences. It has been shown that some microorganisms in biofilms may remove toxic Fe (II) ions and sulfide that occur in sediments (Vismann 1991, Gillan and De Ridder 1997). However, bacterial microborers have been shown to decompose the periostracum and organic shell matrix in molluscs such as *Mytilus edulis* (Knauth-Köhler et al. 1996), although there is no evidence that *Thiothrix* has this capability. The iron-encrustation may also provide a mechanism of defense against predators or parasites (Wahl 1989), but could also inhibit siphoning and pedal movement in bivalves as seen in this study. The effects on invertebrates, such as mayflies or stoneflies, could include a decrease in oxygen exchange over gill surfaces, or impair swimming movement with decreased buoyancy. During 2010 benthic sampling, stoneflies collected at DC-3 were coated in a similar biofilm as observed on clam shells. The epibiont covered both mouthparts and gill surfaces of the organisms.

Laboratory toxicity test results with the CBP 001 effluent were variable which may have been more attributable to the health of test organisms than toxicity of the effluent. A *Villosa iris*

28-day chronic test resulted in growth impairment compared to control organisms, but this may have been due to inferior test organisms, not effluent toxicity. One observation made during this test was the amount of algae and other debris that appeared to be accumulating on the bivalve shells, and also seemed to affect pedal movement. Mayfly toxicity tests resulted in survivorship impairment after 3-4 days of testing for *Isonychia* and *Maccaffertium*. Survivorship stabilized for both organisms in the November test, which was conducted at 11°C, but continued to steadily decline in tests conducted in April (17°C). These results suggest that effluent was most toxic to mayflies during the first several days of testing, but constituents in the effluent contributing to toxicity diminished in refrigerated samples. Ideally, effluent samples used for test renewal should have been recollected for each changeover as recommended by the USEPA (2002a, b) to support these findings, but this was not feasible as the discharge is several hours away from the laboratory and also flows intermittently. This may be one of the few occasions when maintaining samples at <4°C inhibits toxicity issues associated with the effluent due to a lack of toxicity persistence. Standard toxicity tests with *C. dubia* may have been more reliable than toxicity tests conducted with unionids or mayflies. *Ceriodaphnia* tests resulted in reproductive impairment in both filtered and unfiltered samples, compared to controls.

Additional effluent toxicity tests from the Dumps Creek watershed did not indicate the potential for biological impairment. The coal-processing effluent from Moss 3 (003) was evaluated quarterly for two years following standard US EPA guidelines. Chronic tests with *C. dubia* and the fathead minnow (*P. promelas*) consistently had NOECs > 100% effluent for both survivorship and reproduction/growth. A 28-day chronic test with the amphipod, *H. azteca,* also resulted in survivorship and growth similar to control responses. However, *V. iris* chronic tests resulted in significant growth impairment after 28 days compared to control organisms.

Organism reliability and overall health must be considered. Although research has shown that intra- and interlaboratory variability in test results is consistent with those reported for standard test organisms, some variability is likely when organism source (i.e., culturing facility), and dilution/culture water sources change (Wang et al. 2007). Unfortunately, additional tests with the juvenile mussel were not performed as test organisms were not readily available. *In-situ* Asian clams deployed in the Moss 3 003 effluent stream (CH-1) resulted in growth impairment after 60 days in 2008, while results in 2009 and 2010 were consistent or higher than those at the upstream reference site.

Representative toxicity tests with mayflies were also used to evaluate the Moss 3 003 effluent. These tests, conducted with *I. bicolor* and *Maccaffertium,* were inconclusive based on minimal data. In general, seven-day survivorship data indicated both mayfly species were not affected by the 003 effluent in static-renewal tests. *Maccaffertium* survivorship and growth (exuvia) were greater for the 003 effluent than controls, while *Isonychia* survivorship was comparable to that of the controls through Day 8, declining thereafter. Therefore, *Isonychia* survivorship impairment after 21 days was more likely due to handling stress and not effluent toxicity (Echols et al., In Prep).

Hurricane Fork seems to have been far less impacted by mining than the upper reaches of Dumps Creek. Habitat assessment scores were consistent between sampling years and were generally lower for the uppermost site (HF-1) than downstream sites. Benthic macroinvertebrate results were generally higher for sites in Hurricane Fork compared to Dumps Creek. Alteration of the stream bed due to the construction of a low-water bridge at HF-1, coupled with fluctuating flow regimes in 2009 caused a drastic decrease in taxa richness, but sites downstream remained relatively consistent between years. A slight shift in composition was evident at HF-1 in 2009 as

EPT taxa decreased and Chironomidae increased. Additionally, Bioconcentration Factors (BCF) of metals (Al, Cu, Fe, Mn) concentrations were higher at this upstream site and gradually decreased downstream which indicated some land use activities were occurring in the headwaters of Hurricane Fork and may be contributing to some degradation in the stream. Sediment interstitial water was also higher in Sr, Zn and Mn at HF-1, implying that the suggested activities upstream are influencing metal concentrations more so than the Gob Pile (coal refuse) or Moss 2 deep mine processing facility and 019 effluent discharge. Toxicity test results were variable between tests organisms. *Hyalella* experienced growth impairment after 28 days compared to control responses, while *V. iris* growth was similar to the control. Growth response of *Hyalella*, however, were most likely influenced by the predominance of females in the 019 effluent (87%) compared to the equal distribution in the controls (50 %) and 003 effluent (53%) replicates.

Through the course of this research project, a large database was established for the Dumps Creek watershed, that included both field and laboratory assessments. Field bioassessments in Dumps Creek indicate that benthic invertebrate communities are severely impaired below the CBP 001 discharge. Although the upstream reference site DC-1 Up supports a benthic assemblage representative of most headwater tributaries, the addition of the CBP 001 discharge quickly transforms habitat and eliminates most benthic organisms. Additionally, *in-situ* studies with *Corbicula* resulted in total mortality of organisms in less than two weeks, indicating that the constituents or nature of the discharge has drastic effects on the integrity of the system. During seasonal low-flow conditions, the effluent contributes to 90% of stream flow; therefore, dilution of the effluent is not possible. Further, the discharge potency is not well understood as it discharges intermittently and is dependent on water levels in the underground mine. Effluent constituents may be more concentrated during the first several days of effluent

discharge, increasing toxicity potential. However, laboratory toxicity tests were inconclusive in validating these results.

The influence of the CBP 001discharge caused an increase in stream conductivity, alkalinity and the concentration of select metals. A draft general standard TMDL for TDS was developed based on 2002 data (Map Tech 2004), but field and laboratory data collected in 2008, 2009 and 2010 do not support TDS as a sole source of impairment. Sulfate, a major contributor to TDS, was elevated above reported toxicity endpoints in the literature downstream of the CBP 001 discharge, based on routine monitoring data provided by the mining company. The bacterium, which can be linked to Fe-encrustation and biofilm formation on aquatic organisms, may also inhibit benthic colonization at downstream sites. However, the specific influences on tributary health from these bacteria are not yet understood.

Based on VA and WVA SCI scores, results indicated that some improvement in benthic composition occurred below the confluence with Hurricane Fork, but additional land-use practices further downstream contributed to impairment at the lowest site (DC-6). This site was heavily impacted by sedimentation, which is most likely enhanced due to road runoff. Site DC-6 also receives effluent from the Moss 3 coal prep plant facility and has higher conductivity than sites upstream that have higher benthic scores, such as site DC-4. Assessments in the Hurricane Fork tributary do not indicate active mining at the Moss 2 deep mine are having any noticeable effect on aquatic biota downstream. In fact, trace metal concentrations and benthic composition improve at downstream sites relative to conditions at the upstream reference site, which appears to be impacted from an unknown source. Further, the coal refuse pile, also referred to as the Gob Pile, does not have any apparent influence on stream integrity. Although the pile contributes coal particles to the stream bed over time, no discernable increases in metal accumulation were

apparent in clam tissues placed at or below the Gob Pile site. Both VA and WVA SCI scores recorded at and immediately below (HF-3) the Gob Pile were among the highest recorded in the entire watershed.

Summary and Conclusions

Overall results of this study indicate biological impairment is occurring in Dumps Creek; the primary source of impairment is mostly likely due to the influence of the CBP 001 discharge in the headwater region. This discharge, which has a distinctive hydrogen-sulfide odor, alters water quality and decimates benthic populations immediately. Additionally, the nature of the discharge facilitates the proliferation of the epibiotic bacteria, *Thiothrix*, which may further impair the system and hinder recolonization at downstream sites. Although TDS is considered the primary driver for ecological impairment in Dumps Creek, additional research should consider the individual ion contributions to TDS.

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Table 2.1. Routines sampling locations in Dumps Creek and Hurricane Fork, Russell County, Virginia.

Sampling Site	Location	Coordinates	Notes
Dumps Creek located off of Route 600			
DC-1 Up (Ref)	Headwater region above deep mine discharge, ~ 4.2 mi above HF confluence.		Added in 2009
DC-1 Dn	Below deep mine discharge.		Added in 2009
DC-2 Up	Located above settling pond, ~ 1 mi below DC-1 Dn.		Added in 2009
DC-2 Dn	Below settling pond, ~ 1.3 mi below DC-1 Dn.		
DC-3	Above HF confluence (~1/10th mi).	36° 58.522N 082° 11.919W	
Hurricane Fork located off of Route 621			
HF-1 (Ref)	Located 1/10th mi past bridge. Road descending to creek on left.	36° 59.834N 082° 08.874W	
HF-2	Above Gob Pile. Access road just past Moss 2 prep plant; park at gate and walk ¼ mi up road to concrete bridge to sample.		
Gob Pile	Access creek at Gob Pile, midway between HF-2 and gate.	36° 59.323N 082° 10.3044W	
Seep	Small orange-boy seep, located on the opposite bank from Gob Pile, near the end of the Gob Pile site.		Not routinely sampled.
HF-3	Located ~ 50 m below Gob Pile. Sample in creek diagonally across from gate.		
HF-4 Up	Located on Moss 2 prep plant property. Above 002 discharge into creek. Park and walk around settling ponds and down rocky embankment to creek.		Dropped after 2008
HF-4 Dn	Sample below 002 discharge. Same access as above.		Dropped after 2008
HF-5	Lowest sampling site in HF ~1/10th mi above HF/DC confluence. Sampling station located by pull off.	36° 58.264N 082° 11.499W	
DC-4 (2008)	Sampling site located where Rt. 600 bridge crosses creek. Pull over just before bridge.		Dropped after 2008 due to poor habitat
DC-4 (2009-2010)	Located just above the Dickenson-Russell mining office on Rt. 600. Pull off and walk short dirt path to creek.	36° 57.971N 082° 11.057W	
CH-1	Site located below bridge on Rt. 615 below Moss 3 discharge into Chaney Creek.	36° 57.067N 082° 11.757W	Located in Chaney Creek
DC-6	Park by AEP Carbo plant entrance and walk down path to river/creek confluence.	36° 56.087N 082° 11.802W	

Table 2.2. Benthic macroinvertebrate metrics used for Stream Condition Index scoring according to Virginia and West Virginia guidance documents (MapTech, Inc. 2003 and 2000, respectively).

VA SCI		WVA SCI		Trend	Community Characteristic
Parameter	**Standardization (score=)**	**Parameter**	**Standardization (score=)**		
Total Taxa	=100 x (X/22)	**Total Taxa**	=100x(X/21)	Decreases with stress	Taxonomic richness.
EPT Taxa	=100 x (X/11)	**EPT Taxa**	=100x(X/13)		Taxa richness in pollution-sensitive orders.
% Ephemeroptera	= 100 x (X/58.9)	**% EPT**	=100x(X/91.9)		Composition (mayfly nymphs only for VA)
% Plecoptera + Trichoptera Less Hydropsychidae	= 100 x (X/34.8)	**---**	--		Composition (stonefly and Caddisfly nymphs excluding pollution tolerant Hydropsychidae)
% Scrapers	= 100 x (X/49.1)	**---**	--		Trophic or functional feeding group
% Chironomidae	= 100 x [(100-X)/(100-0)]	**% Chironomidae**	=100x[(100-X)/(100-0.98)]	Increases with stress	Composition
% Top 2 Dominant	= 100 x [(100-X)/(100-29.5)]	**% Top 2 Dominant**	=100x[(100-X)/(100-36.0)]		Diversity (top 2 most abundant taxa)
HBI (Family Level)	=100 x [(10-X)/(10-3.2)]	**HBI (Family Level)**	s=100x[(10-X)/(10-2.9)]		Diversity

Table 2.3. Average water chemistry measurements for select parameters from 2007-2010 for sampling sites in the Dumps Creek watershed.

Sampling Site	Means (±SD)			
	Conductivity (µS/cm)	pH (su)	Alkalinity (mg CaCO3/L)	Hardness (mg CaCO3/L
DC-1 Up	290.3 ± 106.6	7.59 ± 0.14	58 ± 14.0	94 ±31.5
CBP 001	1,172.5 ± 94.7	7.39 ± 0.12	294 ± 188.5	110 ± 24.0
DC-1 Dn	1,035.4 ± 275.5	7.56 ± 0.10	417 ± 207.2	104 ± 26.6
DC-2 Up	1,081.2 ± 171.3	8.45 ± 0.09	316 ±0.0	90 ±0.0
DC-2 Dn	1,175.6 ± 266.2	8.39 ± 0.09	520 ± 165.9	99 ± 9.9
DC-3	1,000.7 ± 267.8	8.61 ± 0.13	555 ± 87.4	84 ± 4.0
HF-1	553.3 ± 193.2	7.97 ± 0.24	69 ± 7.3	240 ± 87.9
HF-2	446.4 ± 141.8	7.80 ± 0.20	69 ± 5.7	263 ± 3.0
Gob Pile	419.0 ± 0.0	8.29 ± 0.00	74 ±0.0	168 ± 0.0
Seep	505.0 ± 0.0	6.83 ± 0.37	210 ± 0.0	234 ± 0.0
HF-3	438.5 ± 106.7	7.97 ± 0.17	80 ±6.5	244 ± 2.0
HF-4 UP	565.3 ± 145.1	7.96 ± 0.10	n/d	160 ± 0.0
HF-4 DN	468.0 ± 82.0	8.10 ± 0.22	278 ± 4.9	178 ± 0.0
HF-5	440.9 ± 103.8	8.15 ± 0.15	153 ± 2.5	201 ± 11.0
DC-4	596.2 ± 161.0	8.52 ± 0.25	144 ± 0.0	146 ± 12.0
DC-4 (08)	1,161.8 ± 103.4	8.65 ± 0.11	496 ± 65.3	107 ± 3.0
DC-5	974.1 ± 147.4	8.35 ± 0.15	295 ± 13.9	184 ± 12.0
DC-6	890.8 ± 248.1	8.51 ± 0.17	321 ± 158.9	141 ± 2.5

Table 2.4. Average trace metal concentrations of water column samples collected during summer 2010. Highlighted values indicate measurements were above the National Recommend Water Quality Criteria (US EPA 2009).

	Average Concentration (µg/L)					
Analyte	**Al**	**Cu**	**Fe**	**Mn**	**Sr**	**Zn**
Detection Limit	**6.0**	**5.0**	**9.0**	**3.0**	**2.0**	**5.0**
DC-1 UP	96.0	< 5.0	121.0	17.0	197.0	6.0
CBP 001	<6.0	< 5.0	742.0	116.0	813.0	<5.0
DC-1 DN	77.0	< 5.0	431.0	64.0	468.0	8.0
DC-2 UP	<6.0	< 5.0	494.0	46.0	763.0	<5.0
DC-2 DN	61.0	< 5.0	262.0	57.0	397.0	<5.0
DC-3	245.0	< 5.0	364.0	32.0	360.0	<5.0
HF-1	27.0	< 5.0	62.0	6.0	963.0	22.0
HF-2	49.0	< 5.0	249.0	26.0	521.0	18.0
Gob Pile	44.0	< 5.0	204.0	15.0	488.0	< 5.0
Seep by Gob Pile	252.0	< 5.0	19,042.0	328.0	731.0	<5.0
HF-3	54.0	< 5.0	339.0	15.0	489.0	7.0
HF-5	41.0	< 5.0	91.0	7.0	477.0	15.0
DC-4	179.0	< 5.0	248.0	22.0	290.0	<5.0
CH-1	401.0	< 5.0	674.0	315.0	772.0	13.0
DC-6	76.0	< 5.0	110.0	32.0	512.0	9.0

Table 2.5. Average sediment trace metal concentrations for Dumps Creek watershed sampling sites from 2008-2010. Highlighted values indicate exceedence of the US EPA Mid-Atlantic Risk Assessment Sediment Screening Benchmarks for Ecological Risk Assessments.

Site	Average Trace Metals				
	Al (mg/Kg)	**Fe (mg/Kg)**	**Cu (mg/Kg)**	**Mn (mg/Kg)**	**Zn (mg/Kg)**
DC-1 Up	3,366.7	12,738.7	7.3	347.1	42.7
DC-1 Dn	3,066.0	13,775.7	207.0	166.7	60.1
DC-2 Up	2,987.5	11,941.8	7.9	n/d	n/d
DC-2 Dn	4,593.5	17,348.8	13.1	2,099.8	37.4
DC-3	2,640.6	11,100.8	7.5	358.3	30.7
HF-1	2,470.3	11,209.6	5.0	211.6	26.8
HF-2	2,023.5	8,872.7	3.2	214.5	24.0
HF-3	3,480.0	10,498.3	7.8	196.5	25.6
HF-4 Up	2,518.7	12,021.5	n/d	n/d	n/d
HF-4 Dn	2,692.9	10,477.5	5.5	142.5	19.8
HF-5	2,927.7	9,712.9	6.7	240.9	32.8
DC-4 (08)	2,196.7	10,658.4	5.6	156.5	29.5
DC-4	2,225.9	8,976.1	6.1	157.6	19.7
CH-1	4,725.2	21,684.0	16.7	6,118.6	95.7
DC-6	2,936.1	11,918.1	9.1	362.2	32.6

Table 2.6. Community Loss Index (CLI) for Dumps Creek sampling sites in 2010.

Community Loss Index	
DC-1 Up	Reference
DC-1 Dn	12
DC-2 Up	1.76
DC-2 Dn	1.13
DC-3	0.76
DC-4	0.63
DC-6	7.2

Table 2.7. Virginia Stream Condition Index (VA SCI) scores generated for winter benthic macroinvertebrates samples in 2009 and 2010 and comparison to summer scores. Site identifications were provided as used for the TMDL study (Map Tech 2004) and the current study.

TMDL Site ID (Map Tech 2004)	Current Study Site ID	2009		2010	
		Winter	Summer	Winter	Summer
CH-1	DC-1 Up	70.25	74.9	75.69	71.1
DC-6	DC-2 Dn	13.73	21.3	32.88	30.5
DC-4	DC@HF	55.07	ND	55.44	49.7*
DC-10	Chaney Ck. Reference	70.51	ND	72.51	ND

* VA SCI score from site DC-4 (current summer study), located ¼ mi downstream from Hurricane Fork confluence.

Table 2.8. (a) Bioassessment results for winter 2009 sampling in Dumps Creek and (b) corresponding biometric scores and biological condition ratings.

(a)

Metrics	DC-1 Up	DC-2 Dn	DC @ HF	Chaney Ref.
Taxa Richness	14.5	6.3	10.5	19.3
MFBI	2.61	5.98	2.95	3.73
Scraper to Filter Ratio	1.66	0.00	0.21	1.30
EPT/Midge Ratio	17.7	0.01	11.8	52.9
% Contr. Dom Family	22.99	77.84	57.67	27.46
EPT Index	43.0	0.50	116.8	163.3
Shredder to Total Ratio	0.27	0.03	0.16	0.39
Community Loss Index	0.96	3.36	1.48	0.00

(b)

Metrics	DC-1 Up	DC-2 Dn	DC @ HF	Chaney Ref.
Taxa Richness	3	0	3	6
MFBI	6	0	3	3
Scraper to Filter Ratio	6	0	0	3
EPT/Midge Ratio	3	0	3	6
% Contr. Dom Family	6	0	3	3
EPT Index	3	0	3	6
Shredder to Total Ratio	3	0	3	6
Community Loss Index	3	0	3	6
Total Score	33	0	21	39
% Comp to Chaney	84.6	0	53.8	100
Biologial Condition	NI	SI	MI	NI
Corresponds to TMDL	No	No	Yes	Yes

Table 2.9. (a) Bioassessment results for winter 2010 sampling in Dumps Creek and (b) corresponding biometric scores and biological condition ratings.

(a)

Metrics	DC-1 Up	DC-2 Dn	DC @ HF	Chaney Ref.
Taxa Richness	18.3	11.5	14.5	20.0
MFBI	3.14	5.94	3.94	4.15
Scraper to Filter Ratio	1.65	1.51	0.52	2.17
EPT/Midge Ratio	11.92	0.53	4.62	133.48
% Contr. Dom Family	18.36	46.84	31.44	34.14
EPT Index	47.8	12.8	95.3	239.8
Shredder to Total Ratio	0.24	0.03	0.04	0.07
Community Loss Index	0.26	1.06	0.35	0.00

(b)

Metrics	DC-1 Up	DC-2 Dn	DC @ HF	Chaney Ref.
Taxa Richness	3	0	3	6
MFBI	6	0	3	3
Scraper to Filter Ratio	3	3	0	6
EPT/Midge Ratio	3	0	3	6
% Contr. Dom Family	6	0	3	3
EPT Index	3	0	3	6
Shredder to Total Ratio	6	0	3	3
Community Loss Index	6	0	3	6
Total Score	36	3	21	39
% Comp to Chaney	92.3	7.69	53.8	100
Biological Condition	NI	SI	MI	NI
Corresponds to TMDL	No	No	Yes	Yes

Table 2.10. Mean Asian clam (*Corbicula fluminea*) growth after 60 days *in-situ* at select sites in the Dumps Creek subwatershed.

Site	Growth (mm)		
	2008	2009	2010
DC-1 Up		0.16 b c d	0.12 d
DC-2 Up		0.08 d	0.11 d
DC-2 Dn	0.59 b c d	1.95[a]	1.97[a]
DC-3	0.72[a] b c	0.31 b c d	0.39 c d
HF-1	0.33 d e	0.26 b c d	0.50 c
HF-2	0.45 c d e		0.61 c
HF-3	0.35 d e	0.35 b c	0.52 c
HF-4 Up	0.34 d e		
HF-4 Dn	0.94[a] b		
HF-5	0.23 e	0.11 c d	0.52 c
DC-4 (2008)	0.95[a]		
DC-4		0.40 b	0.49 c
CH-1	0.16 e	0.18 b c d	0.55 c
DC-6	1.07[a]	0.38 b c	0.98 b

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α=0.05)

Table 2.11. *Corbicula fluminea* tissue concentrations of aluminum after 60 days *in-situ.*

Site	Aluminum (mg/kg)		
	2008	2009	2010
Control		1.00 e	1.98 g
DC-1 Up		4.37 b c	13.30 e f
DC-2 Up		3.88 c	9.61 f g
DC-2 Dn	12.79 b	8.09 a	15.58 e f
DC-3	5.57 d e	3.46 c d	21.60 c d e
HF-1	10.98 b c	5.25 b	20.85 d e
HF-2	9.27 b c d		29.97 a b c
Gob Pile			27.26 b c d
HF-3	19.50 a	7.93 a	26.75 b c d
HF-4 Up	8.10 c d		
HF-4 Dn	8.97 b c d		
HF-5	3.38 e	4.47 b c	24.55 c d
DC-4 (2008)	2.99 e		
DC-4		3.21 c d	33.55 a b
CH-1	5.52 d e	2.35 d	37.38 a
DC-6	5.80 d e		4.17 g

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α=0.05).

Table 2.12. *Corbicula fluminea* tissue concentrations of copper after 60 days *in-situ.*

Site	Copper (mg/kg)		
	2008	2009	2010
Control		4.14 c	2.13 f
DC-1 Up		5.58 a b c	11.55 a
DC-2 Up		6.72 a	7.51 b c d e
DC-2 Dn	4.22 a b c	4.88 b c	6.94 b c d e
DC-3	3.95 c	4.36 c	8.20 b c d
HF-1	5.69 a	6.36 a b	6.63 c d e
HF-2	5.64 a b		6.48 c d e
Gob Pile			4.82 e f
HF-3	3.84 c	4.10 c	4.85 e f
HF-4 Up	3.99 c		
HF-4 Dn	4.50 a b c		
HF-5	4.20 b c	5.29 a b c	5.85 d e
DC-4 (2008)	4.17 b c		
DC-4		5.72 a b c	9.17 a b c
CH-1	3.76 c	5.28 a b c	9.50 a b
DC-6	4.11 a b c		7.49 b c d e

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α=0.05).

Table 2.13. *Corbicula fluminea* tissue concentrations of iron after 60 days *in-situ.*

Site	Iron (mg/kg)		
	2008	2009	2010
Control		20.27 d	21.36 g
DC-1 Up		41.79 a b	82.48 e f
DC-2 Up		33.06 b c c	70.94 f g
DC-2 Dn	44.35 c	46.71 a	92.10 c d e
DC-3	30.25 c d	29.18 c d	101.11 c d e
HF-1	68.54 a b	40.57 a b	85.55 d e
HF-2	50.06 b c		122.50 a b c
Gob Pile			112.08 b c d
HF-3	78.94 a	41.18 a b	109.23 b c d
HF-4 Up	44.19 c		
HF-4 Dn	30.91 c d		
HF-5	23.05 d	33.91 b c	90.47 c d e
DC-4 (2008)	21.41 d		
DC-4		33.12 b c	139.37 a b
CH-1	36.21 c d	29.46 c	151.81 a
DC-6	29.17 c d		40.30 f g

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α=0.05).

Table 2.14. *Corbicula fluminea* tissue concentrations of zinc after 60 days *in-situ.*

Site	Zinc (mg/kg)		
	2008	2009	2010
Control		5.86 c	12.11 b c
DC-1 Up		5.90 c	16.61 a
DC-2 Up		7.20 a b c	12.65 a b c
DC-2 Dn	9.27 b c	7.31 a b c	16.48 a
DC-3	7.33 c d	7.86 a b c	14.64 a b
HF-1	12.10 a	8.29 a b	11.64 b c
HF-2	11.91 a		11.10 b c
Gob Pile			9.75 c
HF-3	8.02 c d	7.74 a b c	10.19 c
HF-4 Up	6.43 d		
HF-4 Dn	7.66 c d		
HF-5	8.50 b c d	6.89 b c	9.91 c
DC-4 (2008)	7.88 c d		
DC-4		7.12 a b c	12.39 a b c
CH-1	9.64 a b c	9.17 a	10.28 c
DC-6	10.79 a b		9.17 c

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α=0.05).

Table 2.15. Mean concentrations of additional analytes included in the 2010 analysis of *Corbicula fluminea* tissues after 60 days *in-situ*.

Site	Results (mg/kg)				
	Arsenic	Cadmium	Chromium	Manganese	Strontium
Control	0.37 [d]	BDL	2.32 [a]	0.50 [g]	0.85 [e]
DC-1 Up	0.87 [a]	BDL	0.82 [b]	4.98 [d e]	2.12 [d e]
DC-2 Up	0.57 [b c d]	BDL	0.56 [c d e f]	3.80 [e f]	2.94 [c d]
DC-2 Dn	0.60 [b c]	BDL	0.56 [c d e f]	8.00 [c d]	8.93 [a]
DC-3	0.66 [a b c]	BDL	0.70 [b c]	8.38 [c]	7.15 [a]
HF-1	0.55 [b c d]	BDL	0.42 [e f g]	7.74 [c d]	1.89 [d e]
HF-2	0.62 [b c]	BDL	0.44 [d e f g]	12.43 [a b]	1.91 [d e]
Gob Pile	0.48 [c d]	BDL	0.34 [g]	10.40 [b c]	1.59 [d e]
HF-3	0.44 [c d]	BDL	0.43 [e f g]	10.72 [b c]	1.76 [d e]
HF-5	0.45 [c d]	BDL	0.40 [f g]	8.12 [c d]	2.01 [d e]
DC-4	0.71 [a b]	BDL	0.60 [c d]	12.70 [a b]	4.48 [b c]
CH-1	0.49 [c d]	BDL	0.58 [c d e]	15.10 [a]	5.08 [b]
DC-6	0.57 [b c d]	BDL	0.44 [d e f g]	1.28 [f g]	2.79 [c d e]

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α=0.05).

Table 2.16. Bioconcentration Factors (BCF) for metal accumulation in Asian clam (*Corbicula fluminea*) tissues compared to water column concentrations.

Site	Al	Cu	Fe	Mn	Sr	Zn
DC-1 Up	45.4	1,116.0	345.4	292.9	10.8	981.7
DC-2 Up	646.7	1,344.0	66.9	82.6	3.8	1,440.0
DC-2 Dn	132.5	976.0	178.3	140.4	22.5	1,462.0
DC-3	14.1	872.0	80.2	261.9	19.9	1,572.0
HF-1	194.4	1,272.0	654.4	1,290.0	2.0	376.8
HF-2	611.6	1,120.0	492.0	478.1	3.7	616.7
HF-3	146.9	818.0	121.5	714.7	3.6	1,105.7
HF-5	108.8	1,058.0	372.6	1,160.0	4.2	4,259.3
DC-4	17.9	1,144.0	133.5	576.8	15.4	1,424.0
CH-1	5.9	1,054.0	43.7	47.9	6.6	705.4
DC-6	54.9	1,498.0	366.4	40.0	5.4	1,018.9
< 250= Low; > 1,000= High; <250<1,000=moderate						

Table 2.17. General water chemistry for porewater samples collected during summer 2008.

Site	Temperature (°C)	Conductivity (µS/cm)	pH (su)	Alkalinity (mg $CaCO_3$/L)	Hardness (mg $CaCO_3$/L)
HF-1	25.0	465	6.84	110	134
HF-2	25.0	198	6.67	80	82
HF-3	25.0	687	6.97	200	240
Seep					
DC-3	25.0	998	7.81	416	84
DC-4	25.0	689	6.95	230	180
CH-1	25.0	1,028	7.50	336	220
DC-6	25.0	631	7.32	262	134

Table 2.18. Trace metal concentrations of porewater samples collected at available sites in Dumps Creek and Hurricane Fork during summer 2008.

Site	Results (µg/L)						
	Al	**Cr**	**Cu**	**Fe**	**Mn**	**Sr**	**Zn**
Detection Limit	**35**	**4.0**	**3.0**	**5.0**	**1.0**	**1.0**	**3.0**
HF-1	297	< 4.0	3.0	2,497	2,129	467	13.0
HF-2	212	< 4.0	3.0	3,528	1,107	198	6.0
HF-3 Seep	891	< 4.0	9.0	49,991	1,911	1,062	34.0
DC-3	260	< 4.0	3.0	1,830	499	562	4.0
DC-4	549	< 4.0	5.0	8,594	1,518	521	15.0
CH-1	1,726	< 4.0	9.0	6,377	6,076	1,123	50.0
DC-6	805	< 4.0	6.0	5,401	2,197	538	18.0

Table 2.19. Survival (%) and growth (mm) of *Hyalella azteca* (amphipod) and *Villosa iris* (unionid) after 28 days of exposure to two coal mining effluents.

Villosa iris-No Sediment in Test Chambers		
Sample	Survival (%)	Growth (mm)
Control	90[a]	0.26[a]
Moss 2 (019)	75.0[a]	0.20[a b]
Moss 3 (003)	67.5[a]	0.12 [b]
Villosa iris-Sediment Substrate Used in Chambers		
Control	82.5[a]	0.34[a]
Moss 2 (019)	97.5[a]	0.34[a]
Moss 3 (003)	82.5[a]	0.21 [b]
Hyalella azteca		
Control	100[a]	1.89[a b]
Moss 2 (019)	95[a]	1.02 [c]
Moss 3 (003)	95[a]	1.66 [b]

[a] Means with the same letter are not significantly different (α=0.05) from each other.

Table 2.20. Distribution of male versus female *Hyalella azteca* in the 28-day chronic toxicity test.

Sample/Replicate	Number Recovered	Number Male	Number Female	Percent Male	Percent Female	Conc. % Male	Conc. % Female
Control A	10	7	3	70	30		
Control B	10	4	6	40	60	50	50
Control C	10	4	6	40	60		
Control D	10	5	5	50	50		
Moss 2 A	10	3	7	30	70		
Moss 2 B	10	2	8	20	80	12.5	87.5
Moss 2 C	9	0	9	0	100		
Moss 2 D	9	0	9	0	100		
Moss 3 A	9	5	4	56	44		
Moss 3 B	10	6	4	60	40	47	53
Moss 3 C	10	4	6	40	60		
Moss 3 D	9	3	6	33	67		

Table 2.21.*Villosa iris* Moss 2 (019) chronic test summary water chemistry without sediment.

Date	Ammonia	Temp.	Cond.	DO_2	pH	Alk.	Hard.
	mg/L	°C	µS/cm	mg/L	su	mg $CaCO_3$/L	
7/30/2007	0.51	20.8	712	7.92	8.36	N/A	N/A
8/1/2007	N/A	19.9	N/A	8.03	N/A	262	188
8/3/2007	N/A	20.1	N/A	7.19	N/A	N/A	N/A
8/6/2007	0.97	21.1	717	7.23	8.45	270	190
8/8/2007	N/A	19.4	N/A	7.15	N/A	N/A	N/A
8/10/2007	N/A	20.4	N/A	7.29	N/A	N/A	N/A
8/13/2007	0.01	21.9	725	7.32	8.43	264	210
8/15/2007	N/A	21.9	N/A	8.10	N/A	N/A	N/A
8/17/2007	N/A	20.3	N/A	8.09	N/A	N/A	N/A
8/20/2007	0.01	20.0	758	7.79	8.56	370	200
8/22/2007	N/A	22.2	N/A	7.75	N/A	N/A	N/A
8/24/2007	N/A	23.6	735	7.31	8.57	N/A	N/A
Average	**0.37**	**21.0**	**729.4**	**7.60**	**8.47**	**291.5**	**197**

Table 2.22. *Villosa iris* Moss 2 (019) chronic test summary water chemistry with sediment substrate.

Date	Ammonia	Temp.	Cond.	DO_2	pH	Alk.	Hard.
	mg/L	°C	µS/cm	mg/L	su	mg $CaCO_3$/L	
7/30/2007	2.35	20.6	699	6.88	8.04	N/A	N/A
8/1/2007	N/A	20.1	N/A	6.91	N/A	248	196
8/3/2007	N/A	19.8	N/A	5.89	N/A	N/A	N/A
8/6/2007	0.17	20.3	732	6.76	8.24	270	172
8/8/2007	N/A	19.6	N/A	5.40	N/A	N/A	N/A
8/10/2007	N/A	20.4	N/A	6.06	N/A	N/A	N/A
8/13/2007	0.11	20.2	730	7.02	8.33	258	200
8/15/2007	N/A	21.5	N/A	7.14	N/A	N/A	N/A
8/17/2007	N/A	20.5	N/A	7.32	N/A	N/A	N/A
8/20/2007	0.01	20.0	734	7.03	8.37	270	204
8/22/2007	N/A	22.7	N/A	7.04	N/A	N/A	N/A
8/24/2007	N/A	21.6	716	7.11	8.34	N/A	N/A
Average	**0.66**	**20.6**	**722.2**	**6.71**	**8.26**	**261.5**	**193**

Table 2.23. *Hyalella azteca* Moss 2 (019) chronic test summary water chemistry.

Date	Ammonia	Temp.	Cond.	DO_2	pH	Alk.	Hard.
	mg/L	°C	µS/cm	mg/L	su	mg $CaCO_3$/L	
7/30/2007	2.45	20.6	710	6.45	8.00	N/A	N/A
8/1/2007	N/A	20.7	N/A	6.90	N/A	250	194
8/3/2007	N/A	20.3	N/A	4.83	N/A	N/A	N/A
8/6/2007	4.40	20.1	755	4.60	8.02	252	174
8/8/2007	N/A	20.9	N/A	4.28	N/A	N/A	N/A
8/10/2007	N/A	20.2	N/A	4.38	N/A	N/A	N/A
8/13/2007	2.71	21.8	767	5.25	8.08	272	200
8/15/2007	N/A	20.0	N/A	8.14	N/A	N/A	N/A
8/17/2007	N/A	18.9	N/A	7.97	N/A	N/A	N/A
8/20/2007	1.05	19.4	804	7.97	8.41	288	210
8/22/2007	N/A	21.1	N/A	5.53	N/A	N/A	N/A
8/24/2007	N/A	20.5	778	6.65	8.24	N/A	N/A
Average	**2.65**	**20.4**	**762.8**	**6.08**	**8.15**	**265.5**	**194.5**

Table 2.24. *Villosa iris* Moss 3 (003) chronic test summary water chemistry without sediment.

Date	Ammonia	Temp.	Cond.	DO_2	pH	Alk.	Hard.
	mg/L	°C	µS/cm	mg/L	su	mg $CaCO_3$/L	
7/30/2007	0.35	20.7	903	8.00	8.31	N/A	N/A
8/1/2007	N/A	20.0	N/A	7.87	N/A	274	192
8/3/2007	N/A	20.3	N/A	6.94	N/A	N/A	N/A
8/6/2007	0.19	21.3	907	9.80	8.39	272	196
8/8/2007	N/A	19.5	N/A	7.48	N/A	N/A	N/A
8/10/2007	N/A	20.4	N/A	7.19	N/A	N/A	N/A
8/13/2007	0.15	22.3	927	7.23	8.41	268	174
8/15/2007	N/A	21.9	N/A	8.17	N/A	N/A	N/A
8/17/2007	N/A	20.3	N/A	8.10	N/A	N/A	N/A
8/20/2007	0.02	20.0	970	7.75	8.53	288	170
8/22/2007	N/A	22.2	N/A	7.61	N/A	N/A	N/A
8/24/2007	N/A	23.6	952	7.27	8.34	N/A	N/A
Average	**0.18**	**21.0**	**931.8**	**7.78**	**8.40**	**275.5**	**183**

Table 2.25. *Villosa iris* Moss 3 (003) chronic test summary water chemistry with sediment substrate.

Date	Ammonia	Temp.	Cond.	DO_2	pH	Alk.	Hard.
	mg/L	°C	µS/cm	mg/L	su	mg $CaCO_3$/L	
7/30/2007	2.25	20.6	884	6.88	8.04	N/A	N/A
8/1/2007	N/A	20.4	N/A	6.21	N/A	264	192
8/3/2007	N/A	20.1	N/A	5.01	N/A	N/A	N/A
8/6/2007	0.23	20.1	904	5.85	8.14	260	170
8/8/2007	N/A	19.6	N/A	5.88	N/A	N/A	N/A
8/10/2007	N/A	20.4	N/A	6.42	N/A	N/A	N/A
8/13/2007	0.01	20.5	912	6.67	8.29	260	180
8/15/2007	N/A	21.6	N/A	6.86	N/A	N/A	N/A
8/17/2007	N/A	20.5	N/A	6.88	N/A	N/A	N/A
8/20/2007	0.01	20.0	925	6.91	8.35	270	172
8/22/2007	N/A	22.9	N/A	6.90	N/A	N/A	N/A
8/24/2007	N/A	21.6	939	6.83	8.30	N/A	N/A
Average	**0.62**	**20.7**	**912.8**	**6.44**	**8.22**	**263.5**	**178.5**

Table 2.26. *Hyalella azteca* Moss 3 (003) chronic test summary water chemistry.

Date	Ammonia	Temp.	Cond.	DO_2	pH	Alk.	Hard.
	mg/L	°C	µS/cm	mg/L	su	mg $CaCO_3$/L	
7/30/2007	2.10	20.6	922	6.65	8.11	N/A	N/A
8/1/2007	N/A	20.9	N/A	7.03	N/A	268	194
8/3/2007	N/A	20.4	N/A	4.53	N/A	N/A	N/A
8/6/2007	4.31	20.0	944	4.04	7.98	272	168
8/8/2007	N/A	20.8	N/A	4.09	N/A	N/A	N/A
8/10/2007	N/A	20.2	N/A	3.99	N/A	N/A	N/A
8/13/2007	2.44	22.0	981	4.62	8.06	270	184
8/15/2007	N/A	20.0	N/A	8.48	N/A	N/A	N/A
8/17/2007	N/A	18.9	N/A	8.59	N/A	N/A	N/A
8/20/2007	1.03	19.6	1031	7.44	8.36	280	180
8/22/2007	N/A	21.1	N/A	5.47	N/A	N/A	N/A
8/24/2007	N/A	20.8	1036	7.30	8.33	N/A	N/A
Average	**2.47**	**20.4**	**982.8**	**6.02**	**8.17**	**272.5**	**181.5**

Table 2.27. Survival and growth of *Villosa iris* and *Hyalella azteca* after 28 days of exposure to the coal mining effluent, Moss 3 004. Control water was obtained from the Columbia Environmental Research Center (CERC) in Columbia, Missouri.

Villosa iris

Sample	Survival (%)	Sample	Growth (mm)
Control (CERC)	100 a	004	0.48 a
004	97.5 a	Control (CERC)	0.41 a

Hyalella azteca

Sample	Survival (%)	Sample	Final size (mm)	Sample	Estimated Growth (mm)
Control (CERC)	100 a	Control (CERC)	3.73 a	Control (CERC)	2.53 a
004	97.5 a	004	3.72 a	004	2.52 a

* Numbers with the same letter are not significantly different ($\alpha = 0.05$) from each other.

Table 2.28. Water chemistry measurements from the *Villosa iris* Moss 3 (004) 28-day chronic toxicity test.

	Date	Ammonia	Temp	Cond.	DO_2	pH	Alk.	Hardness
		(mg/L)	(°C)	(µS/cm)	(mg/L)	(su)	mg $CaCO_3$/L	
Week 1	5/21/2008	N/A	20.5	924	8.44	8.26	220	266
	5/23/2008		20.0	917	4.26	8.02	N/A	N/A
	5/26/2008		20.6	N/A	3.15	N/A	N/A	N/A
Week 2	5/28/2008	3.8	20.4	953	2.82	7.88	N/A	N/A
	5/30/2008		20.4	1,015	7.27	8.43	200	270
	6/2/2008		19.5	986	8.30	8.52	N/A	N/A
Week 3	6/4/2008	0.43	19.4	N/A	8.82	N/A	N/A	N/A
	6/6/2008		20.3	1,026	9.64	8.47	N/A	N/A
	6/9/2008		20.5	978	8.57	8.44	230	256
Week 4	6/11/2008	0.53	19.9	N/A	7.56	N/A	N/A	N/A
	6/13/2008		20.0	1,038	7.87	8.46	N/A	N/A
	6/16/2008		19.4	N/A	9.36	N/A	N/A	N/A
	6/18/2008	0.47	19.8	1,082	8.23	8.39	220	280
Average		**1.59**	**20.1**	**991**	**7.25**	**8.32**	**215**	**263**
Standard Deviation		1.66	0.43	54.65	2.30	0.223	12.58	9.93
Standard Error		0.74	0.19	24.44	1.03	0.100	5.63	4.44

Table 2.29. Water chemistry measurements from the *Hyalella azteca* Moss 3 (004) 28-day chronic toxicity test.

	Date	Ammonia	Temp	Cond.	DO_2	pH	Alk.	Hardness
		(mg/L)	(°C)	(µS/cm)	(mg/L)	(su)	mg $CaCO_3$/L	
Week 1	5/21/2008	N/A	20.5	924	8.44	8.26	220	266
	5/23/2008		20.1	932	7.47	8.44	210	290
	5/26/2008		20.7	N/A	7.12	N/A	N/A	N/A
Week 2	5/28/2008	0.59	20.4	942	7.25	7.92	N/A	N/A
	5/30/2008		20.6	904	6.90	8.20	200	270
	6/2/2008		20.1	935	6.29	8.20	N/A	N/A
Week 3	6/4/2008	0.37	19.7	N/A	5.74	N/A	N/A	N/A
	6/6/2008		20.5	1,018	6.53	8.24	N/A	N/A
	6/9/2008		19.9	982	6.51	8.30	240	242
Week 4	6/11/2008	0.49	20.6	N/A	6.47	N/A	N/A	N/A
	6/13/2008		20.0	1,026	7.07	8.40	N/A	N/A
	6/16/2008		19.5	N/A	6.37	N/A	N/A	N/A
	6/18/2008	0.64	20.2	1,069	6.75	8.28	216	278
Average		**1.59**	**20.2**	**970.2**	**6.84**	**8.25**	**216.5**	**270**
Standard Deviation		0.119	0.374	56.25	0.667	0.148	14.81	17.75
Standard Error		0.053	0.167	25.15	0.298	0.066	6.62	7.94

Table 2.30. Chronic, 10-day sediment toxicity test with *Daphnia magna* survivorship and mean reproduction. Sample and control reproduction comparisons made using Dunnett's Method (α=0.05).

Sample	Survivorship (%)	Reproduction	
		Means	Diff. Control (p-value)
SCW (Control)	91.7	59.3 (±13.27)	1.000
CB Pump 001 (water only)	75.0	47.33 (±32.33)	0.9003
DC-1 Up (reference)	41.7*	9.67 (±12.20)	0.0031*
CB Pump MZ	100	50.50 (±25.40)	0.9791
CB Pump MZ (with 001 eff.)	91.7	39.53 (±6.48)	0.4953
DC-1 Dn	8.3*	6.83 (±10.20)	0.0017*
DC-2 Dn	75.0	52.60 (±7.65)	0.9961
DC above Hurricane Fork	83.4	42.35 (±21.48)	0.6529
Chaney Creek (reference)	100	75.10 (±10.16)	0.7121
* Significantly different from control.			

Table 2.31. Summary of mean water chemistry of renewal water for the *Daphnia magna* sediment toxicity test, March 2010.

Sample	Conductivity (µS/cm)	DO_2 (mg/L)	pH (su)
Filtered Sinking Creek Water	177.9 (114-193)	8.13 (7.82-8.38)	8.27 (8.10-8.38)
CBP 001 Effluent	1,186.6 (1,154-1,195)	6.91 (3.86-9.05)	7.70 (7.40-8.33)

Table 2.32. Average conductivity and dissolved oxygen measurements for sediment test out-water.		
Sample	**Conductivity (µS/cm)**	**Dissolved Oxygen (mg/L)**
Filtered SCW	227.3	5.42
DC-1 Up	237.4	4.89
CBP 001 Effluent	1,284.3	5.73
CBP Mixing Zone	306.2	4.23
CBP Mixing Zone w/ effl.	1,240.6	4.10
DC-1 Dn	277.2	4.34
DC-2 Dn	294.3	3.88*
DC above Hurricane Fork	282.1	4.39
Chaney Creek	262.4	4.61

Table 2.33. Chronic, 10-day sediment toxicity test with *Daphnia magna* survivorship and mean reproduction. Sample and control reproduction comparisons made using Dunnett's Method (α=0.05).			
Sample	**Survivorship (%)**	**Reproduction**	
		Means	**Diff. Control (p-value)**
SCW (Control)	100	57.8 (±15.65)	1.000
DC-1 Up (reference)	83.3	53.5 (±28.09)	0.9918
DC-1 Dn	50*	12.6 (±6.35)	0.0015*
DC-3	91.7	56.0 (±5.71)	0.9999
DC-4	100	61.0 (±3.24)	0.9975
CH-1	16.7*	13.6 (±11.73)	0.0018*
* Significantly different from control.			

Table 2.34. Trace metal concentrations (mg/kg) in site sediments versus test sediments after 10-day toxicity test with daily changeovers.

	Al			Fe			Mn		
Sample	**Site**	**Test**	**Difference**	**Site**	**Test**	**Difference**	**Site**	**Test**	**Difference**
DC-1 UP	2,974	2,571	404	10,264	8,327	1,937	609	487	122
DC-1 DN	2,979	2,365	614	13,660	11,798	1,862	273	242	31
DC-2 DN	3,929	2,637	1,292	13,288	11,456	1,832	1,416	1,661	-245
DC@HF	3,427	1,952	1,475	13,675	8,198	5,477	324	198	126
Chaney Ck	4,642	4,198	444	17,140	22,253	-5,113	625	777	-152
CBP MZ	3,310	2,121	1,189	15,222	9,294	5,928	293	145	148
CBP MZ (w/ Eff.)	3,310	2,235	1,074	15,222	11,368	3,854	293	188	105
	Sr			**Copper**			**Zinc**		
Sample	**Site**	**Test**	**Difference**	**Site**	**Test**	**Difference**	**Site**	**Test**	**Difference**
DC-1 UP	10	5	5	6	4	2	47	39	8
DC-1 DN	19	12	7	47	100	-53	60	43	17
DC-2 DN	818	1,008	-190	15	138	-124	34	57	-23
DC@HF	111	68	43	9	48	-40	37	43	-6
Chaney Ck	12	12	0	10	19	-9	51	26	25
CBP MZ	22	5	17	152	5	147	72	23	49
CBP MZ (w/ Eff.)	22	13	10	152	14	138	72	58	14

Table 2.35. Least Squares Fit model responses for daphnid survivorship (%), mortality (# dead) and reproduction. Analysis conducted in JMP 8.0 (SAS Inst., 2010).

Percent Survivorship	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Intercept	712.3069	374.4919	1.90	0.0703
Log(Al (mg/kg))	1430.7755	199.5481	7.17	<.0001*
Log(Fe (mg/kg))	-1188.622	187.0406	-6.35	<.0001*
Log(Mn (mg/kg))	-199.5825	30.80487	-6.48	<.0001*
Log(Sr (mg/kg))	11.217907	3.1436	3.57	0.0017*
Log(Cu (mg/kg))	66.389329	9.880709	6.72	<.0001*
Log(Zn (mg/kg))	0	0	.	.
Mortality	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-18.35431	11.23773	-1.63	0.1166
Log(Al (mg/kg))	-42.91479	5.98803	-7.17	<.0001*
Log(Fe (mg/kg))	35.65045	5.612704	6.35	<.0001*
Log(Mn (mg/kg))	5.9862857	0.924391	6.48	<.0001*
Log(Sr (mg/kg))	-0.336325	0.094333	-3.57	0.0017*
Log(Cu (mg/kg))	-1.991318	0.2965	-6.72	<.0001*
Log(Zn (mg/kg))	0	0	.	.
Reproduction	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-638.5309	318.847	-2.00	0.0577
Log(Al (mg/kg))	519.07323	169.8977	3.06	0.0058*
Log(Fe (mg/kg))	-342.1159	159.2486	-2.15	0.0430*
Log(Mn (mg/kg))	-60.86124	26.22764	-2.32	0.0300*
Log(Sr (mg/kg))	4.3552954	2.676499	1.63	0.1179
Log(Cu (mg/kg))	20.620507	8.412556	2.45	0.0227*
Log(Zn (mg/kg))	0	0	.	.

* indicates statistical significance.

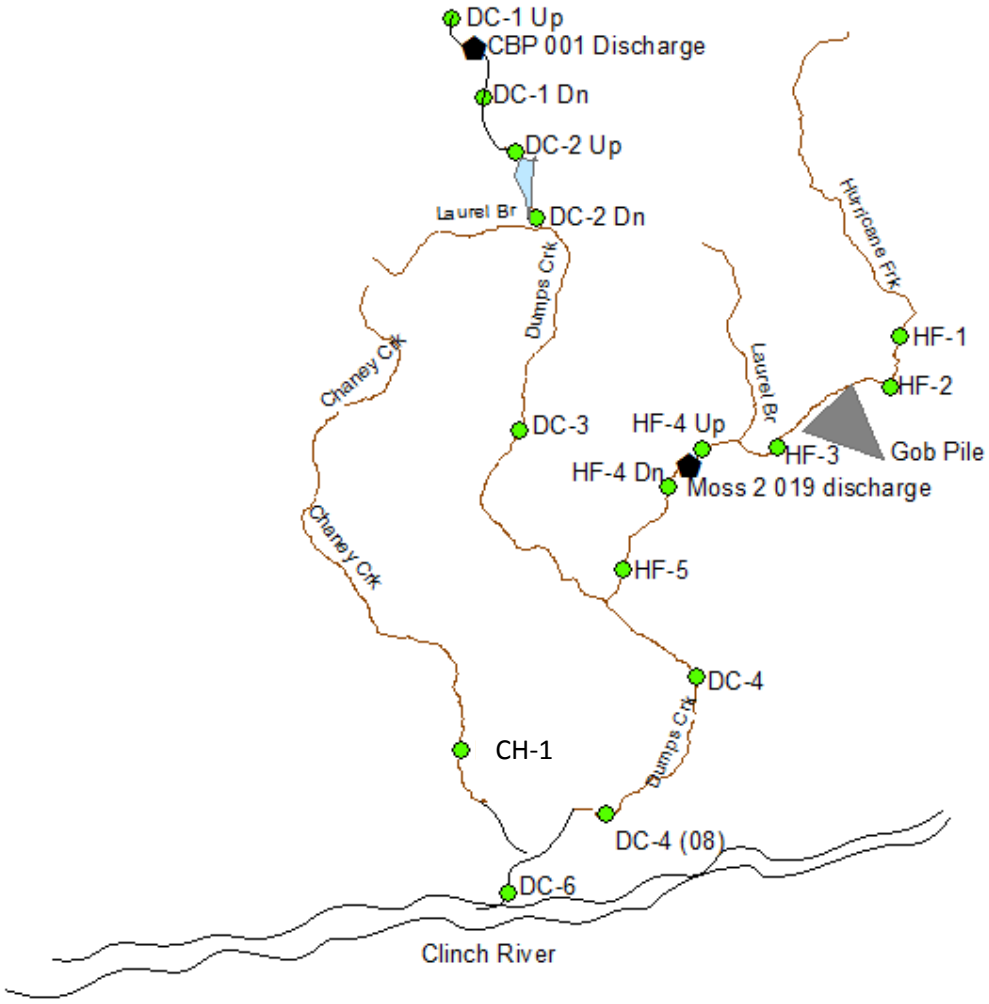


Figure 2.1. Routine sampling site locations in the Dumps Creek subwatershed.

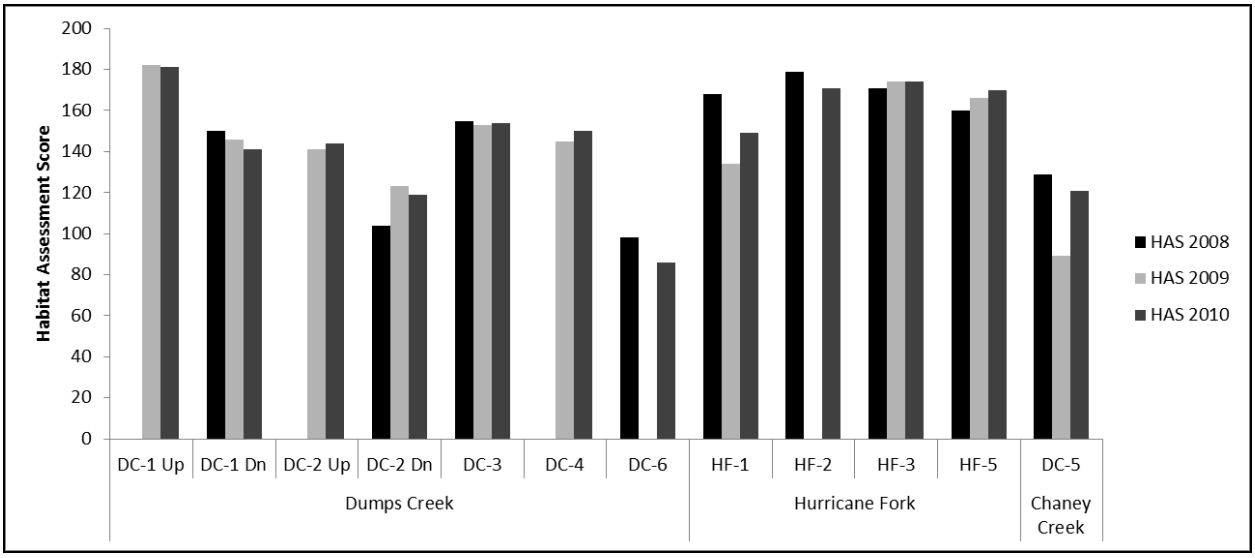
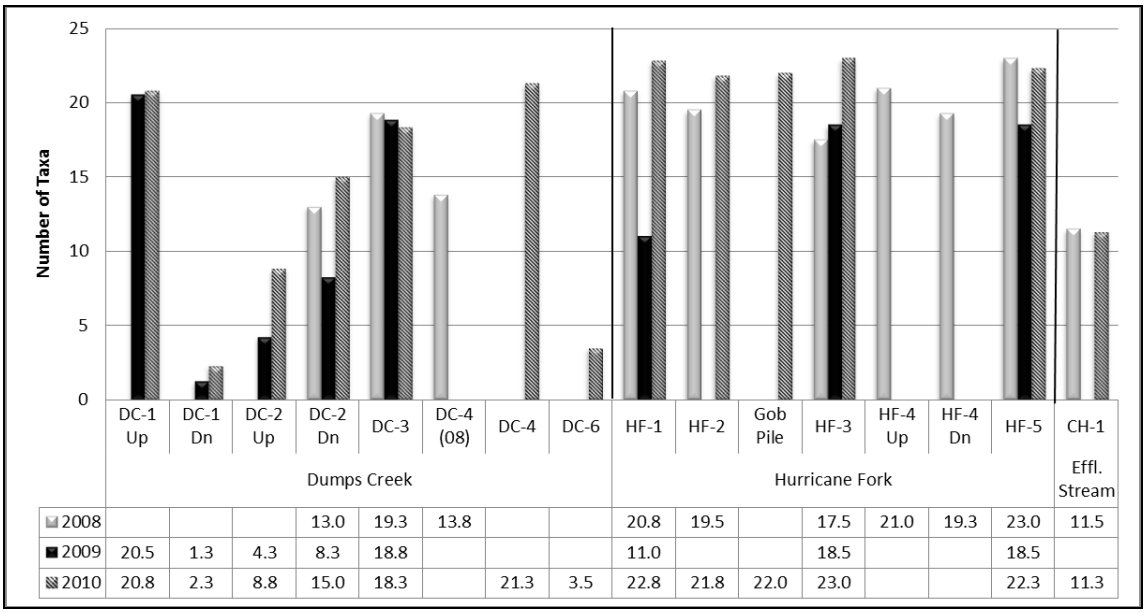


Figure 2.2. Habitat assessment scores at sites in the Dumps Creek subwatershed.



	DC-1 Up	DC-1 Dn	DC-2 Up	DC-2 Dn	DC-3	DC-4 (08)	DC-4	DC-6	HF-1	HF-2	Gob Pile	HF-3	HF-4 Up	HF-4 Dn	HF-5	CH-1 Effl. Stream
2008				13.0	19.3	13.8			20.8	19.5		17.5	21.0	19.3	23.0	11.5
2009	20.5	1.3	4.3	8.3	18.8				11.0			18.5			18.5	
2010	20.8	2.3	8.8	15.0	18.3		21.3	3.5	22.8	21.8	22.0	23.0			22.3	11.3

Figure 2.3. Mean taxa richness at sampling sites in the Dumps Creek subwatershed from 2008-2010.

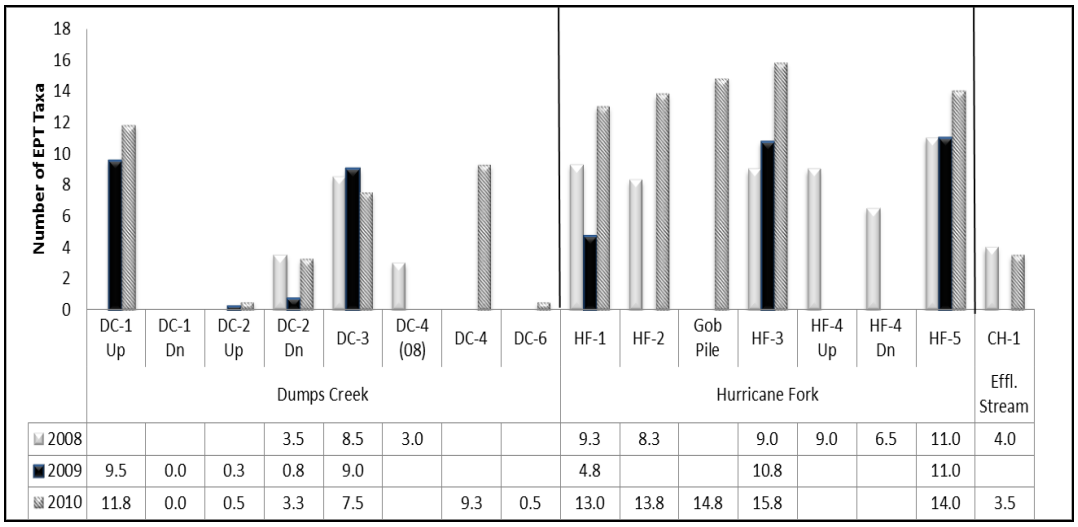


Figure 2.4. Mean Ephemeroptera-Plecoptera-Trichoptera (EPT) richness at sites in the Dumps Creek subwatershed from 2008-2010.

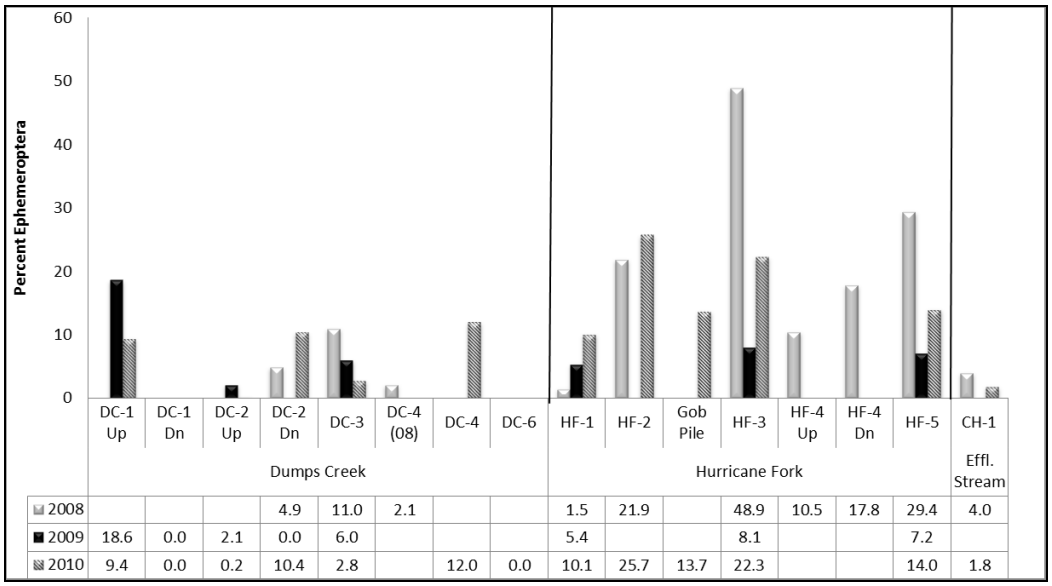


Figure 2.5. Mean Percent Ephemeroptera at sites in the Dumps Creek subwatershed from 2008-2010.

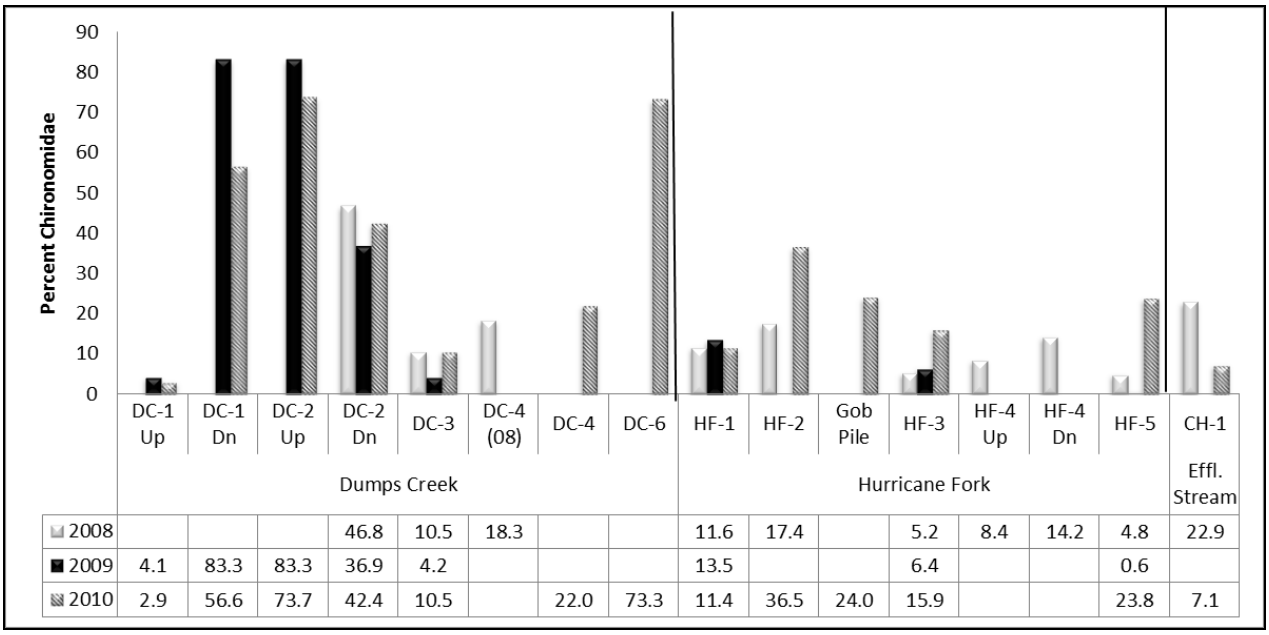


Figure 2.6. Mean Percent Chironomidae at sites in the Dumps Creek subwatershed from 2008-2010.

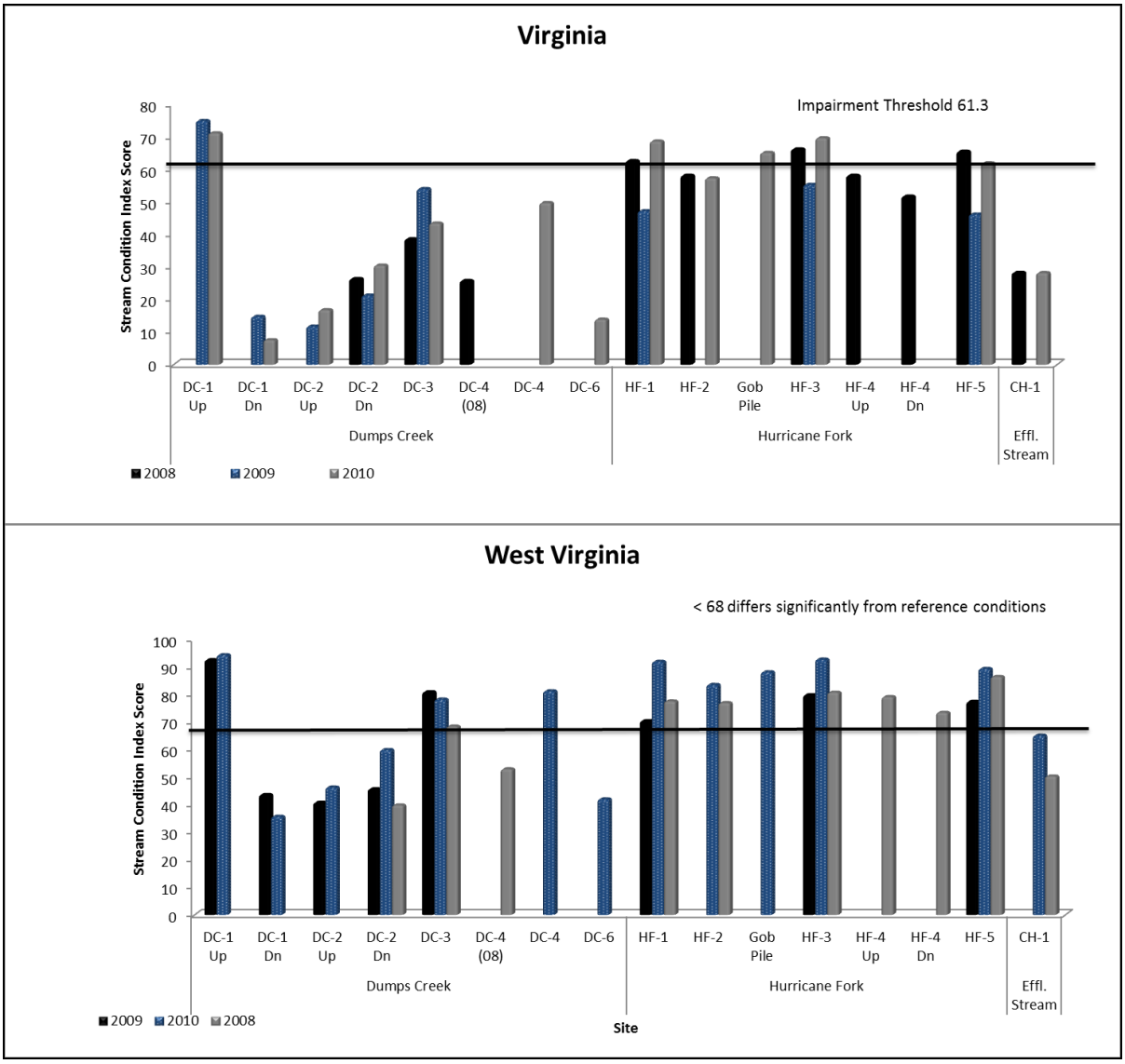


Figure 2.7. Stream Condition Index (SCI) scores for sites in the Dumps Creek subwatershed using Virginia and West Virginia methodologies.

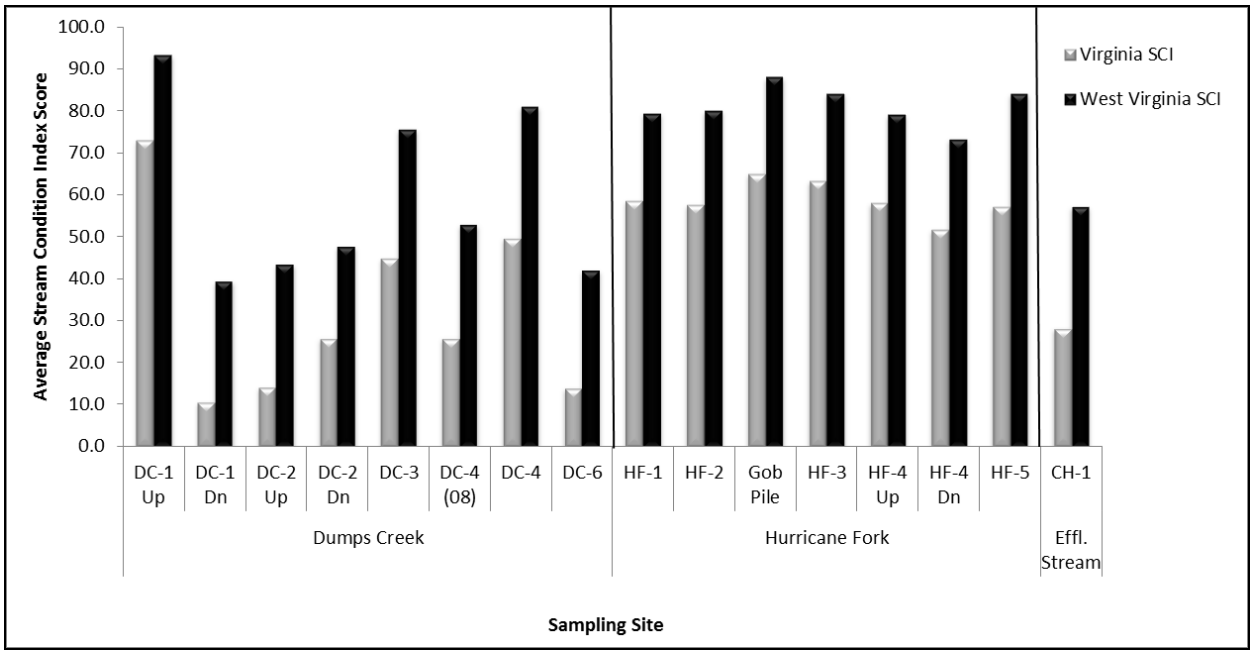


Figure 2.8. Comparison of VA and WVA Stream Condition Index scores using geometric averages of scores generated from 2008-2010.

Figure 2.9. Asian clams (*Corbicula fluminea*) retrieved from DC-2 Dn (bottom row) with distinct covering after 60-day *in-situ* during the 2008 study year, compared to control clams. Photograph by Brandi Echols.

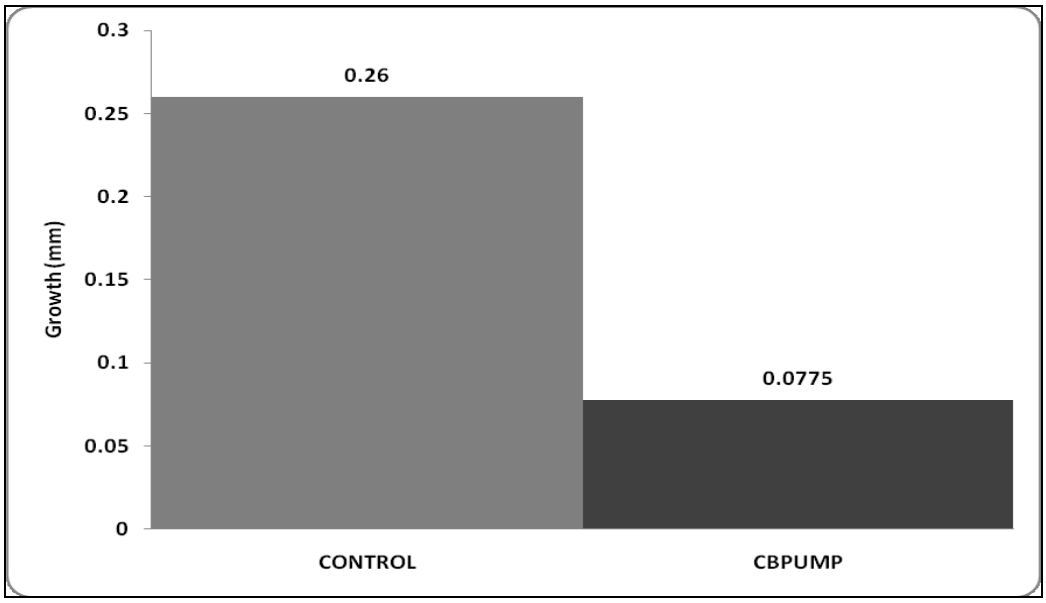


Figure 2.10. Chronic, 28-day test results with Camp Branch (CB Pump) 001 effluent using juvenile stage *Villosa iris*.

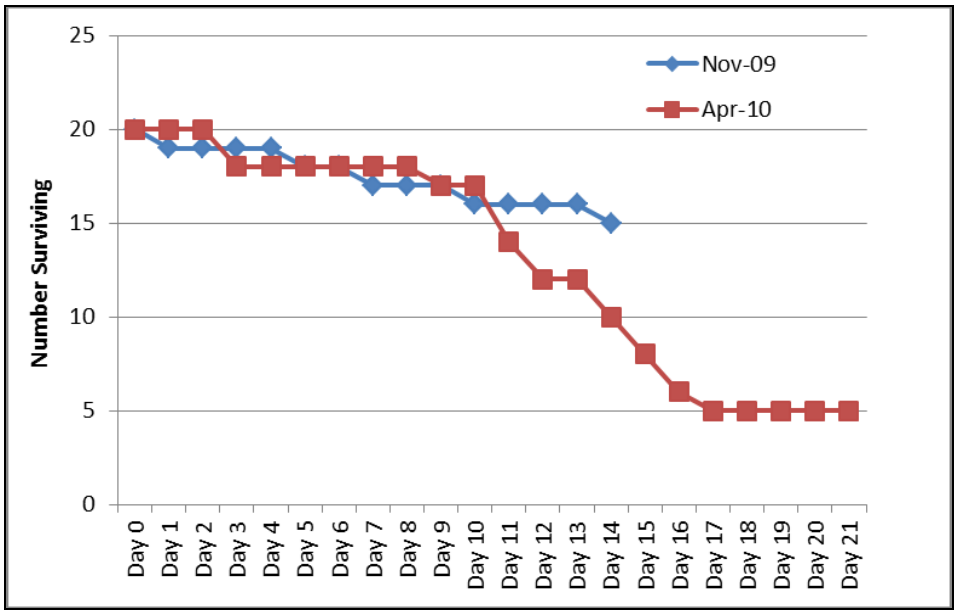


Figure 2.11.Chronic toxicity tests results with the mayfly, *Isonychia bicolor*, exposed to CB Pump 001 effluent.

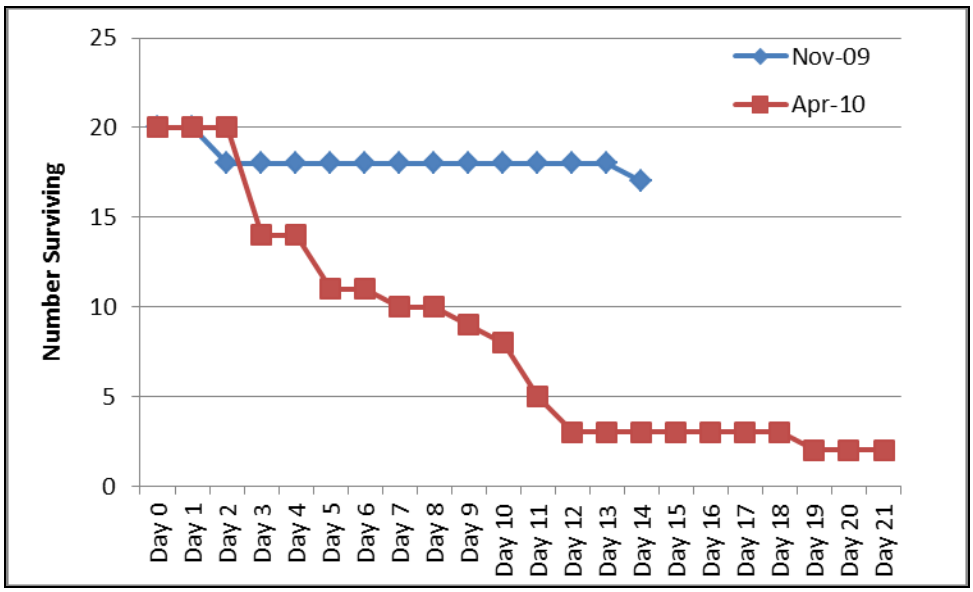


Figure 2.12. Chronic toxicity tests results with a Heptageniidae mayfly, *Maccaffertium* sp., exposed to CB Pump 001 effluent.

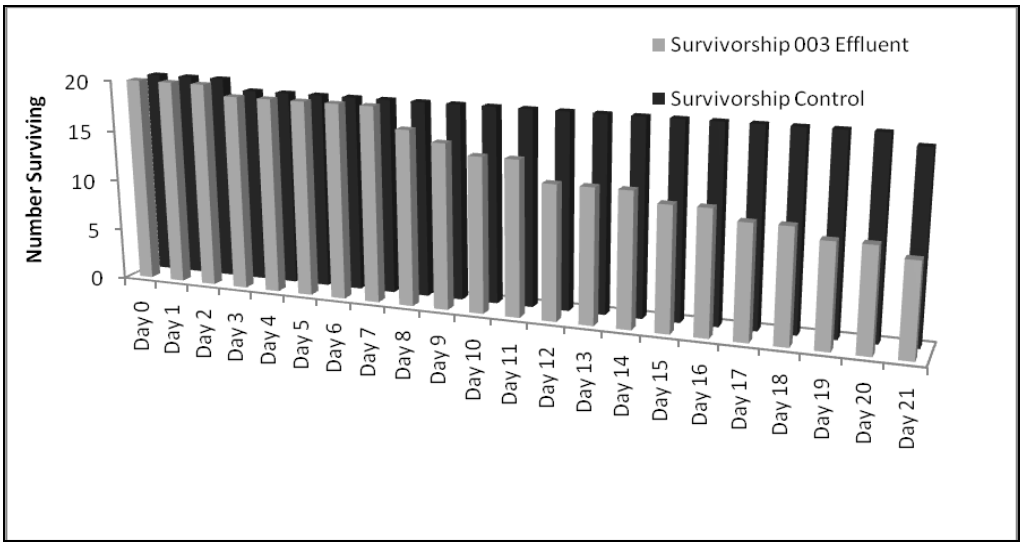


Figure 2.13. *Isonychia bicolor* chronic test survivorship after 21-day exposure to Moss 3 (003) effluent.

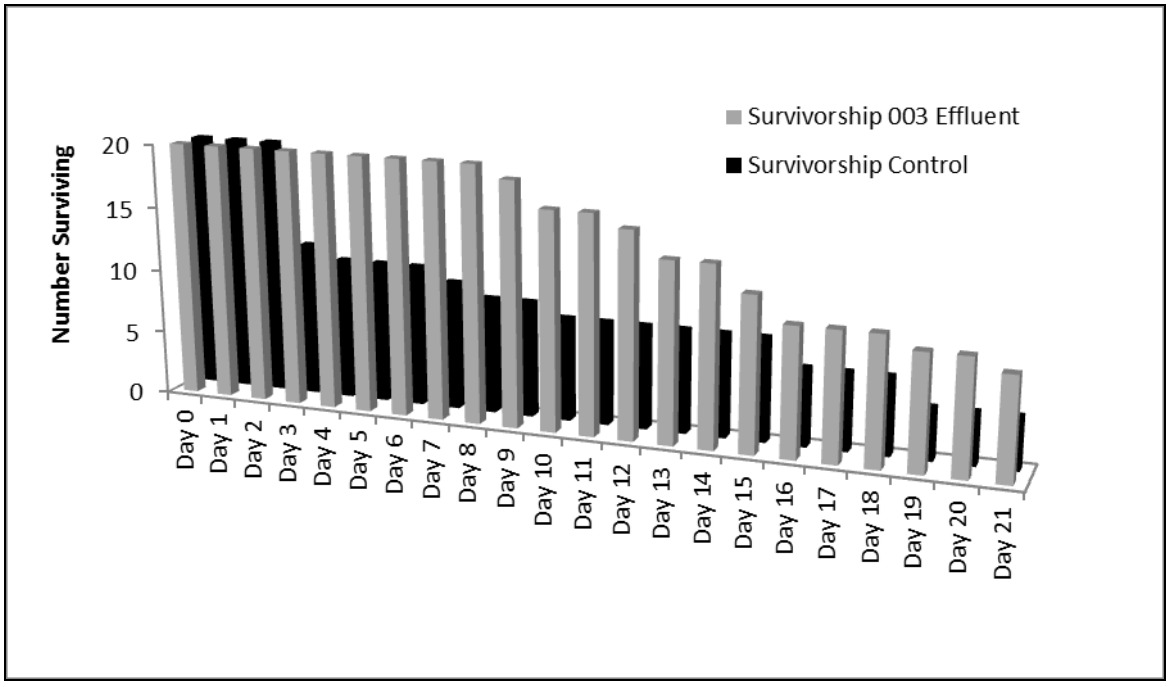


Figure 2.14. *Maccaffertium* chronic test survivorship after 21-day exposure to Moss 3 (003) effluent.

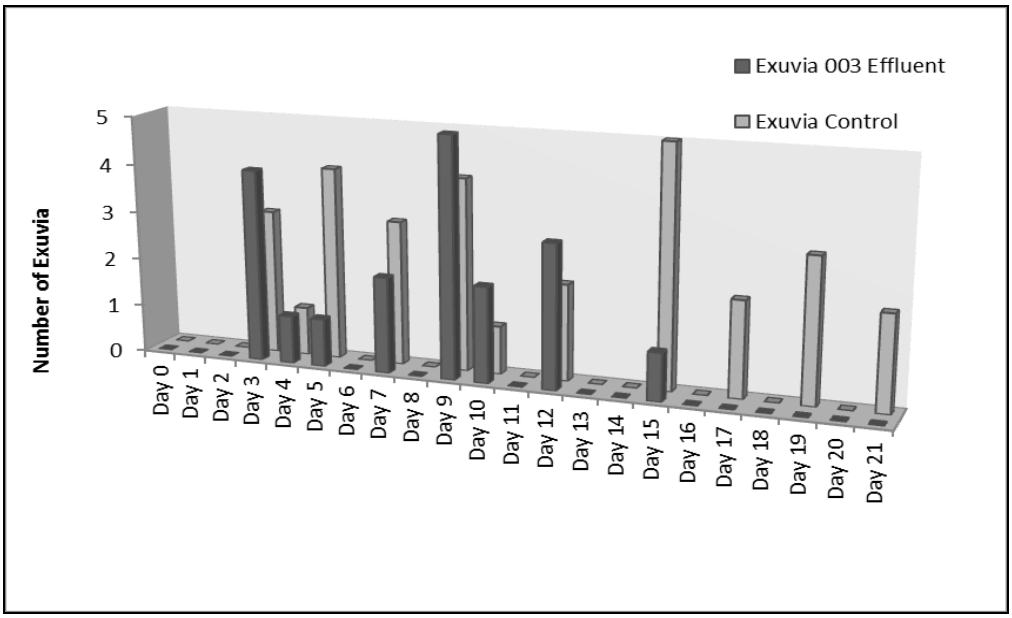


Figure 2.15. Exuvia produced in the Moss 3 (003) effluent test with *Isonychia bicolor*.

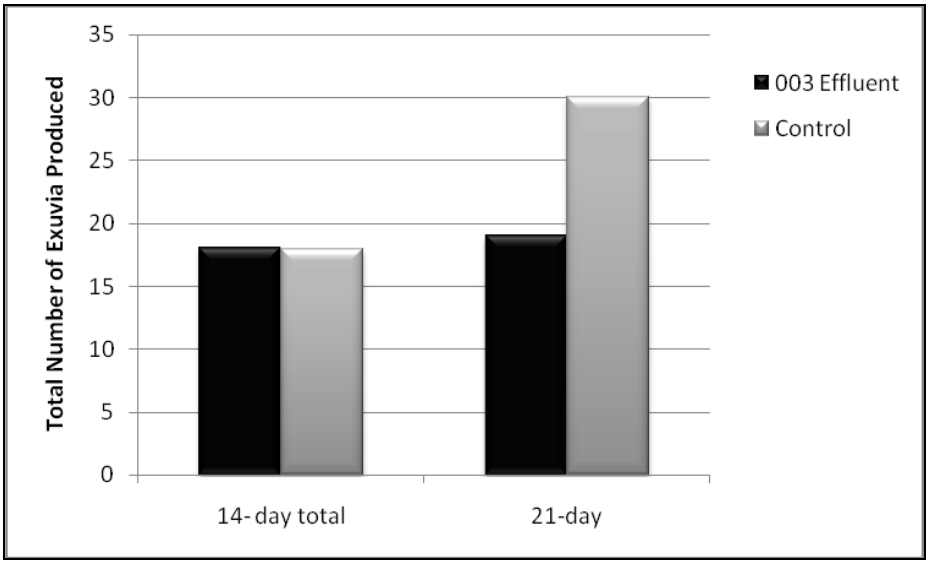


Figure 2.16. Total number of exuvia produced after 14 and 21 days for the *Isonychia bicolor* Moss 3 (003) effluent chronic test.

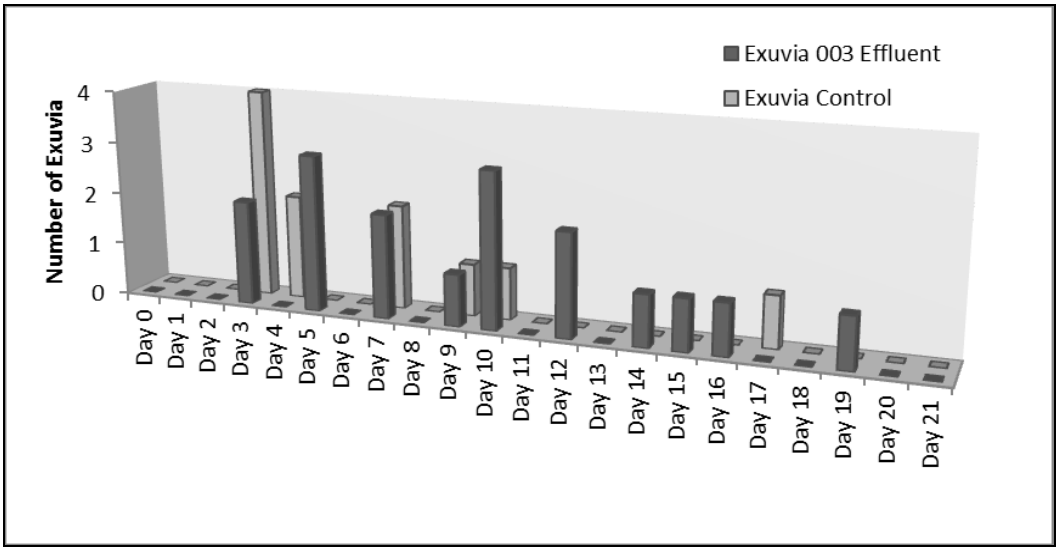


Figure 2.17. Exuvia produced in the Moss 3 (003) effluent test with *Maccaffertium sp.*

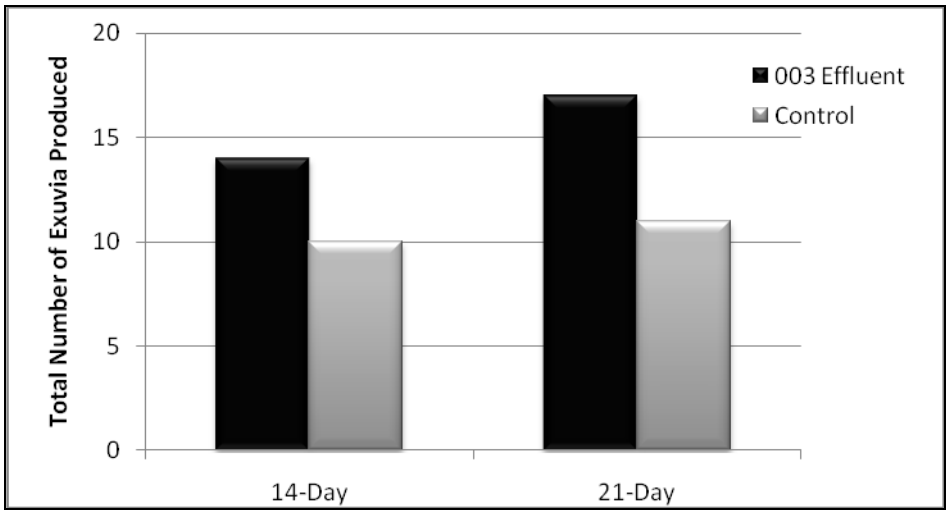


Figure 2.18. Total number of exuvia produced after 14 and 21 days for the *Maccaffertium* Moss 3 (003) effluent chronic test.

Figure 2.19. *Thiothrix nivea* coating the substrate as the CBP 001 effluent discharge merges with Dumps Creek. Photograph by Brandi Echols.

Appendix I. Benthic Macroinvertebrate Data

Table A1. Summary of benthic macroinvertebrate data collected summer (July) 2008 from the Dumps Creek subwatershed.

Site	Parameter								
	Total Abund.	Taxa Richness	% Mayfly	EPT Abund.	EPT Richness	EPT Abund Less Hydro.	Midge/ EPT Ratio	VA SCI	WV SCI
DC-2 Dn	166.0[a] [b]	13.0 [c] [d] [e]	4.9 [c] [d] [e]	10.5 [b]	3.5 [c] [d] [e]	8.5 [b] [c]	9.95[a]	**26.4**	**39.8**
DC-3	84.5[a] [b]	9.3[a] [b] [c] [d]	11.0 [c] [d] [e]	45.3 [b]	8.5[a] [b] [c]	17.0 [b] [c]	0.18 [b]	**38.6**	**68.4**
HF-1 (Ref)	145.3[a]	20.8[a] [b]	1.50 [e]	96.3[a] [b]	9.3[a] [b]	89.5[a] [b]	0.18 [b]	**62.6**	**77.5**
HF-2	101.0[a] [b]	19.5[a] [b] [c]	21.9 [b] [c]	53.0[a] [b]	8.3[a] [b] [c] [d]	47.3[a] [b] [c]	0.23 [b]	**58.1**	**76.9**
HF-3	121.5[a] [b]	17.5[a] [b] [c] [d]	48.9[a]	87.5[a] [b]	9.0[a] [b]	82.0[a] [b]	0.05 [b]	**66.2**	**80.7**
HF-4 Up	83.0[a] [b]	21.0[a] [b]	10.5 [c] [d] [e]	34.8 [b]	9.0[a] [b]	30.8[a] [b] [c]	0.25 [b]	**58.1**	**79.1**
HF-4 Dn	99.3[a] [b]	19.3[a] [b] [c] [d]	17.8 [b] [c] [d]	45.3 [b]	6.5 [b] [c] [d] [e]	29.0[a] [b] [c]	0.35 [b]	**51.7**	**73.4**
HF-5	189.3[a]	23.0[a]	29.4 [b]	141.8[a]	11.0[a]	104.0[a]	0.08 [b]	**65.5**	**86.4**
DC-4	81.3[a] [b]	13.8 [d] [e]	2.1 [e]	39.3 [b]	3.0 [e]	2.0 [c]	1.67 [b]	**25.8**	**52.9**
CH-1	55.8 [b]	11.5 [e]	4.0 [d] [e]	14.3 [b]	4.0 [d] [e]	4.75 [c]	0.95 [b]	**28.3**	**50.3**

[a] **Means denoted by different letters indicate statistical difference (Tukey's HSD, α= 0.05).**

Table A2. Summary of benthic macroinvertebrate data collected summer (June) 2009 from the Dumps Creek subwatershed.

Site	Parameter								
	Total Abundance	**Taxa Richness**	**% Mayfly**	**EPT Abundance**	**EPT Richness**	**EPT Abund. less Hydropsych.**	**Midge/ EPT Ratio**	**VA SCI**	**WV SCI**
DC-1 Up (Ref)	106.5[a] [b]	20.5[a]	18.6[a]	87.8[a]	9.5[a]	77.0[a]	0.05 [b]	**74.9**	**92.5**
DC-1 DN	3.5 [c]	1.25 [d]	0.0 [b]	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [b]	**14.8**	**43.6**
DC-2 Up	35.3 [b] [c]	4.25 [c] [d]	2.1 [b]	1.0 [b] [c]	0.25 [c]	1.0 [c]	9.75[a]	**11.8**	**40.8**
DC-2 Dn	42.0 [b] [c]	8.25 [c] [d]	0.0 [b]	0.75 [c]	0.75 [c]	0.5 [c]	7.5[a]	**21.3**	**45.7**
DC-3	129.5[a]	18.8[a]	6.0 [b]	90.5[a]	9.0[a]	66.0[a] [b]	0.065 [b]	**54.0**	**80.9**
HF-1 (Ref)	39.8 [b] [c]	11.0 [b] [c]	5.4 [b]	19.8 [b] [c]	4.75 [b]	16.3 [c]	0.32 [b]	**47.2**	**70.4**
HF-3	81.8[a] [b] [c]	18.5[a] [b]	8.1 [b]	58.3[a] [b]	10.75[a]	32.3 [b] [c]	0.09 [b]	**55.3**	**79.8**
HF-5	106.8[a] [b]	18.5[a] [b]	7.6 [b]	87.8[a] [b]	11.0[a]	35.8 [b] [c]	0.008 [b]	**46.2**	**77.4**

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α= 0.05).

Table A3. Summary of benthic macroinvertebrate data collected summer (June) 2010 from the Dumps Creek subwatershed.

Site	Parameter								
	Total Abundance	**Taxa Richness**	**% Mayfly**	**EPT Abund.**	**EPT Richness**	**EPT Abund. Less Hydropsych.**	**Midge/EPT Ratio**	**VA SCI**	**WV SCI**
DC-1 Up (Ref)	151.5[a b c d]	20.8[a b]	9.40[b c d]	126.0[a]	11.8[a]	109.5[a b c]	0.04[b]	**71.1**	**94.3**
DC-1 DN	20.0[d]	2.3[e]	0.00[d]	0.00[f]	0.00[f]	0.00[f]	0.00[b]	**7.6**	**35.7**
DC-2 Up	67.3[b c d]	8.8[c d e]	0.23[d]	0.50[f]	0.50[f]	0.50[f]	33.8[a]	**16.8**	**46.3**
DC-2 Dn	141.0[a b c d]	15.0[a b c d]	10.4[b c d]	55.0[c d e f]	3.25[d e f]	18.5[d e f]	1.4[b]	**30.5**	**59.9**
DC-3	170.3[a b c]	18.3[a b c]	2.8[c d]	112.5[a b c d e]	7.5[c d e]	44.8[c d e f]	0.16[b]	**43.4**	**78.2**
HF-1 (Ref)	195.8[a b]	22.8[a]	10.1[b c d]	152.5[a b c]	13.0[a b c]	138.8[a b]	0.15[b]	**68.6**	**91.9**
HF-2	162.0[a b c d]	21.8[a]	25.7[a]	90.5[b c d e f]	13.8[a b c]	76.8[b c d e]	0.69[b]	**57.3**	**83.5**
Gob Pile	141.8[a b c d]	22.0[a]	13.7[a b c]	97.5[b c d e f]	14.8[a b]	87.3[b c d]	0.35[b]	**65.1**	**88.1**
HF-3	256.0[a]	23.0[a]	22.3[a b]	201.3[a]	15.8[a]	171.0[a]	0.21[b]	**69.6**	**92.7**
HF-5	275.0[a]	22.3[a]	14.0[a b c]	185.8[a b]	14.0[a b]	138.0[a b]	0.36[b]	**61.9**	**89.3**
DC-4	237.3[a]	21.3[a]	12.0[b c d]	141.8[a b c d]	9.3[b c d]	78.8[b c d e]	0.37[b]	**49.7**	**81.2**
CH-1	64.5[b c d]	11.3[b c d e]	1.8[c d]	41.8[d e f]	3.5[d e f]	7.5[f]	0.15[b]	**28.2**	**65.1**
DC-6	8.5[d]	3.5[d e]	0.0[d]	0.50[f]	0.50[f]	0.50[f]	4.0[a b]	**13.9**	**42.0**

[a] Means denoted by different letters indicate statistical difference (Tukey's HSD, α= 0.05).

Appendix II. Multiple Comparisons

Pairwise Correlations based on 2010 Results

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
BCF Zn	BCF Cu	-0.1896	10	-0.7319	0.4997	0.5999
BCF Fe	BCF Cu	0.4294	10	-0.2744	0.8336	0.2156
BCF Fe	BCF Zn	-0.0029	10	-0.6314	0.6279	0.9936
2010 Clam Growth (mm)	BCF Cu	-0.0930	10	-0.6827	0.5700	0.7983
2010 Clam Growth (mm)	BCF Zn	0.0018	10	-0.6285	0.6307	0.9961
2010 Clam Growth (mm)	BCF Fe	0.0116	10	-0.6226	0.6366	0.9747
WVA SCI	BCF Cu	-0.5528	10	-0.8771	0.1178	0.0974
WVA SCI	BCF Zn	0.1533	10	-0.5272	0.7140	0.6723
WVA SCI	BCF Fe	0.3300	10	-0.3782	0.7946	0.3517
WVA SCI	2010 Clam Growth (mm)	-0.3544	10	-0.8045	0.3543	0.3150
Taxa Richness	BCF Cu	-0.6037	10	-0.8936	0.0419	0.0646
Taxa Richness	BCF Zn	0.2297	10	-0.4675	0.7508	0.5232
Taxa Richness	BCF Fe	0.2583	10	-0.4435	0.7637	0.4712
Taxa Richness	2010 Clam Growth (mm)	-0.2313	10	-0.7515	0.4662	0.5203
Taxa Richness	WVA SCI	0.9567	10	0.8225	0.9900	<.0001*
% Mayfly	BCF Cu	-0.5688	10	-0.8824	0.0947	0.0862
% Mayfly	BCF Zn	0.2423	10	-0.4570	0.7565	0.4999
% Mayfly	BCF Fe	0.1555	10	-0.5256	0.7151	0.6680
% Mayfly	2010 Clam Growth (mm)	0.0719	10	-0.5842	0.6711	0.8435
% Mayfly	WVA SCI	0.7353	10	0.1967	0.9330	0.0154*
% Mayfly	Taxa Richness	0.7978	10	0.3378	0.9501	0.0057*
EPT Richness	BCF Cu	-0.4964	10	-0.8579	0.1938	0.1445
EPT Richness	BCF Zn	0.2479	10	-0.4523	0.7590	0.4899
EPT Richness	BCF Fe	0.3816	10	-0.3265	0.8153	0.2766
EPT Richness	2010 Clam Growth (mm)	-0.3244	10	-0.7922	0.3836	0.3605
EPT Richness	WVA SCI	0.9598	10	0.8343	0.9907	<.0001*
EPT Richness	Taxa Richness	0.9275	10	0.7159	0.9830	0.0001*
EPT Richness	% Mayfly	0.8356	10	0.4346	0.9601	0.0026*
HAS	BCF Cu	-0.5295	10	-0.8693	0.1501	0.1155
HAS	BCF Zn	0.2988	10	-0.4075	0.7814	0.4016
HAS	BCF Fe	0.0469	10	-0.6005	0.6571	0.8977
HAS	2010 Clam Growth (mm)	-0.5771	10	-0.8851	0.0825	0.0807
HAS	WVA SCI	0.8376	10	0.4401	0.9606	0.0025*
HAS	Taxa Richness	0.8370	10	0.4384	0.9605	0.0025*

HAS	% Mayfly	0.6232	10	-0.0106	0.8998	0.0542
HAS	EPT Richness	0.8135	10	0.3770	0.9543	0.0042*
Sediment Al	BCF Cu	-0.4565	10	-0.8437	0.2429	0.1847
Sediment Al	BCF Zn	-0.2372	10	-0.7542	0.4613	0.5094
Sediment Al	BCF Fe	-0.3325	10	-0.7956	0.3758	0.3478
Sediment Al	2010 Clam Growth (mm)	0.3147	10	-0.3928	0.7882	0.3758
Sediment Al	WVA SCI	-0.0189	10	-0.6409	0.6181	0.9586
Sediment Al	Taxa Richness	-0.1124	10	-0.6930	0.5566	0.7572
Sediment Al	% Mayfly	0.0654	10	-0.5885	0.6675	0.8575
Sediment Al	EPT Richness	-0.0804	10	-0.6758	0.5785	0.8252
Sediment Al	HAS	-0.1615	10	-0.7181	0.5211	0.6558
Sediment Cu	BCF Cu	-0.3705	10	-0.8109	0.3380	0.2919
Sediment Cu	BCF Zn	-0.3130	10	-0.7875	0.3943	0.3785
Sediment Cu	BCF Fe	-0.5120	10	-0.8633	0.1735	0.1303
Sediment Cu	2010 Clam Growth (mm)	0.3693	10	-0.3391	0.8105	0.2935
Sediment Cu	WVA SCI	-0.2168	10	-0.7448	0.4781	0.5474
Sediment Cu	Taxa Richness	-0.2898	10	-0.7776	0.4156	0.4166
Sediment Cu	% Mayfly	-0.0903	10	-0.6812	0.5718	0.8040
Sediment Cu	EPT Richness	-0.3063	10	-0.7846	0.4006	0.3894
Sediment Cu	HAS	-0.3003	10	-0.7821	0.4061	0.3991
Sediment Cu	Sediment Al	0.9448	10	0.7779	0.9872	<.0001*
Sediment Fe	BCF Cu	-0.2677	10	-0.7679	0.4353	0.4546
Sediment Fe	BCF Zn	-0.4261	10	-0.8324	0.2781	0.2195
Sediment Fe	BCF Fe	-0.2750	10	-0.7711	0.4289	0.4420
Sediment Fe	2010 Clam Growth (mm)	0.2151	10	-0.4795	0.7440	0.5507
Sediment Fe	WVA SCI	-0.1425	10	-0.7086	0.5351	0.6945
Sediment Fe	Taxa Richness	-0.2527	10	-0.7612	0.4483	0.4813
Sediment Fe	% Mayfly	-0.3452	10	-0.8008	0.3635	0.3287
Sediment Fe	EPT Richness	-0.3127	10	-0.7873	0.3946	0.3789
Sediment Fe	HAS	-0.2897	10	-0.7775	0.4158	0.4169
Sediment Fe	Sediment Al	0.8520	10	0.4798	0.9643	0.0017*
Sediment Fe	Sediment Cu	0.8526	10	0.4815	0.9645	0.0017*
Sediment Zn	BCF Cu	-0.1511	10	-0.7129	0.5288	0.6769
Sediment Zn	BCF Zn	-0.0834	10	-0.6774	0.5765	0.8189
Sediment Zn	BCF Fe	-0.1756	10	-0.7251	0.5105	0.6275
Sediment Zn	2010 Clam Growth (mm)	-0.1328	10	-0.7036	0.5422	0.7146
Sediment Zn	WVA SCI	0.0270	10	-0.6130	0.6457	0.9409

Sediment Zn	Taxa Richness	-0.1521	10	-0.7134	0.5281	0.6749
Sediment Zn	% Mayfly	-0.2359	10	-0.7536	0.4624	0.5118
Sediment Zn	EPT Richness	-0.0946	10	-0.6835	0.5689	0.7949
Sediment Zn	HAS	-0.0543	10	-0.6613	0.5957	0.8817
Sediment Zn	Sediment Al	0.7743	10	0.2824	0.9438	0.0086*
Sediment Zn	Sediment Cu	0.7179	10	0.1611	0.9280	0.0194*
Sediment Zn	Sediment Fe	0.8042	10	0.3537	0.9519	0.0050*
conductivity	BCF Cu	0.1380	9	-0.5792	0.7348	0.7233
conductivity	BCF Zn	-0.2952	9	-0.8021	0.4589	0.4407
conductivity	BCF Fe	-0.4581	9	-0.8604	0.2961	0.2149
conductivity	2010 Clam Growth (mm)	0.3802	9	-0.3798	0.8338	0.3128
conductivity	WVA SCI	-0.7846	9	-0.9524	-0.2516	0.0123*
conductivity	Taxa Richness	-0.7040	9	-0.9323	-0.0750	0.0343*
conductivity	% Mayfly	-0.7362	9	-0.9405	-0.1410	0.0237*
conductivity	EPT Richness	-0.8842	9	-0.9755	-0.5332	0.0015*
conductivity	HAS	-0.6183	9	-0.9091	0.0778	0.0760
conductivity	Sediment Al	0.2376	9	-0.5064	0.7788	0.5382
conductivity	Sediment Cu	0.4410	9	-0.3155	0.8548	0.2348
conductivity	Sediment Fe	0.5333	9	-0.2025	0.8843	0.1392
conductivity	Sediment Zn	0.1762	9	-0.5526	0.7523	0.6502
pH	BCF Cu	0.1378	10	-0.5385	0.7062	0.7042
pH	BCF Zn	0.0891	10	-0.5727	0.6805	0.8067
pH	BCF Fe	-0.4986	10	-0.8586	0.1910	0.1425
pH	2010 Clam Growth (mm)	0.2939	10	-0.4120	0.7793	0.4099
pH	WVA SCI	-0.6739	10	-0.9152	-0.0768	0.0326*
pH	Taxa Richness	-0.5400	10	-0.8729	0.1357	0.1071
pH	% Mayfly	-0.5216	10	-0.8666	0.1609	0.1221
pH	EPT Richness	-0.6667	10	-0.9130	-0.0639	0.0353*
pH	HAS	-0.6196	10	-0.8986	0.0165	0.0561
pH	Sediment Al	-0.1671	10	-0.7209	0.5169	0.6445
pH	Sediment Cu	0.0053	10	-0.6264	0.6328	0.9883
pH	Sediment Fe	-0.0201	10	-0.6416	0.6174	0.9561
pH	Sediment Zn	-0.2376	10	-0.7544	0.4610	0.5085
pH	conductivity	0.6862	9	0.0405	0.9276	0.0413*

Chapter 3. Preliminary Results of Laboratory Toxicity Tests with the Mayfly, *Isonychia bicolor* (Ephemeroptera: Isonychiidae) for Development as a Standard Test Organism for Evaluating Streams in the Appalachia Coalfields of Virginia and West Virginia

Introduction

The overall goal of aquatic ecotoxicology is the assessment and ultimate protection of aquatic ecosystems. This goal is accomplished through the process of risk assessment and the derivation of ambient water quality guidelines that provide a regulatory format for protecting ecosystem integrity and biodiversity. In order to develop appropriate guidelines the development of toxicity tests that use test organisms that will provide the maximum amount of protection for naturally occurring species is necessary. Determining the appropriate species, organism age and most sensitive test endpoints has been a challenging task for researchers and has lead to the acceptance of a battery of specific organisms outlined in current US EPA regulatory guidelines (US EPA 2000; US EPA 2002 a, b). The organisms selected by the US EPA have proven to be useful in regulating the toxicity of effluents to receiving systems as well as for monitoring and maintaining healthy ecosystems, but they may not serve as appropriate surrogates for protection of the most sensitive species within receiving systems.

There are many benefits to utilizing these established standard test organisms, which include *Ceriodaphnia dubia*, *Daphnia magna, Pimephales promelas, Chironomus dilutus* (formerly *C. tentans*)*, Hyallela azteca* and *Lumbriculus variegatus*. These organisms are relatively easy to culture, require a minimal amount of labor to use in tests, provide limited variability between individuals and have been used extensively, following standard guidelines, so a large database of toxicological responses is available. However, many of these standard test organisms are not widely distributed in aquatic systems although results of tests with these species drive the derivation of water quality criteria (WQC) and are used as required species in national pollutant discharge elimination system (NPDES) permits. For example, *C. dubia* is

native to Australia and occurs in lentic habitats although it is used for testing effluents that enter lotic systems. Standard test organisms may not be representative of the most sensitive species found in specific habitats making extrapolation of laboratory results to in-stream benthic assemblages difficult (Rosenberg and Resh 1996). Cherry et al. (2002) examined the acute toxicity of copper to 17 organisms for the development of site-specific criteria in the Clinch River, Virginia. Results of this study were used to determine a ranking of sensitivities in which *C. dubia* and *P. promelas* ranked 6th and 14th, while the top four organisms included *Lampsilis, Medionidus, Villosa* and *Isonychia*. Hence, *Isonychia* would be more protective to aquatic life against copper stress than the 2 US EPA test species, *C. dubia* and *P. promelas.*

There are numerous aquatic organisms that can be selected as test species, but finding the most appropriate organism that meets the necessary criteria is challenging. Suitable test species should be ecologically important, sensitive enough to a range of pollutants to provide relevant protection to aquatic habitats, easily cultured in the laboratory or collected in the field and should allow for the development of standard testing protocols with adequate control survivorship. A number of benthic macroinvertebrates have been suggested as standard toxicity tests species, but few of them have the attributes that qualify them as a possible indicator organism in laboratory tests. Due to the well documented sensitivity of insects in the order Ephemeroptera (Pontasch and Cairns 1988; Short et al. 1991; Williams and Williams 1998; Hickey and Clements 1998; Clements et al. 2002) to both natural and anthropogenic stressors, some researchers (Kennedy et al. 2004) feel the development of a standardized toxicity test using mayflies may be more beneficial for assessing potential adverse effects of point source discharges on aquatic organisms.

To date, one of the most common species used for toxicity testing has been the burrowing mayfly, *Hexagenia limbata,* which has been used extensively in sediment toxicity tests. The extension of such tests to include other genera of mayflies in water column toxicity tests has occurred intermittently over the past 3 decades (Sherberger et al. 1977; Peters et al. 1985; Diamond et al. 1992; Dobbs et al. 1994; Beketov 2004; Kennedy et al. 2004; Hassell et al. 2006; Brinkman and Johnson 2008; O'Halloran et al. 2008), with a variety of mayfly species, and variable test designs.

Kennedy et al. (2004) used *Isonychia sp*. to evaluate the potential toxicity of coal-mining discharges with high ionic composition. This study found *Isonychia* exhibited a high dose-dependent response to specific conductivity, greater ecological relevance compared to standard test organisms and were overall more protective of sensitive biota; however, test results were not as consistent as those conducted with *C. dubia.* Still, the lowest observable effects concentration (LOEC) to conductivity from a coal mining effluent was 1562 µS/cm for *Isonychia* and more than twice as high (3730 µS/cm) for *C. dubia*. The objective of this research project was to examine the potential usefulness of *Isonychia sp*. as a standard aquatic test organism by evaluating the response of this mayfly to a standard reference toxicant and to an environmentally relevant stressor. Results of this research were published in Environmental Monitoring and Assessment and co-authored by B.S. Echols, R.J. Currie and D. S. Cherry (2010).

Materials and Methods

Description of the test organisms

The order Ephemeroptera along with Plecoptera and Trichoptera represent some of the most sensitive aquatic macroinvertebrates. These benthic macroinvertebrates are widely used in

field studies to evaluate the environmental effects of point and nonpoint source pollution (Barbour et al. 1999). Ephemeroptera are well documented as sensitive indicators of water quality (Pontasch and Cairns 1988; Short et al. 1991; Williams and Williams 1998) particularly to contaminants such as metals and ammonia (Peckarsky and Cook 1981; Leland et al. 1989; Clements 1994; Clements and Kiffney 1995; Hickey and Clements 1998; Hickey et al. 1999; Beketov 2004) and have been regarded as the most sensitive order of aquatic invertebrates. *Isonychia* (Ephemeroptera: Isonychiidae) nymphs were selected for this research because of their ecological relevance, wide distribution and use in previous studies (Sherberger et al. 1977; Peters et al. 1985; Sibley and Kaushik 1991; Diamond et al. 1990; Dobbs et al. 1994; Kobuszewski and Perry 1994; Cherry et al. 2002; Kennedy et al. 2004; Cherry and Soucek 2006). *Isonychia* mayflies are a bivoltine insect having two generations per year with an over-wintering cohort that emerges in the spring and a smaller summer/fall cohort that develops and emerges quickly in late summer and early fall (Kondratieff and Voshell 1984). These organisms are regarded as strong swimmers, but are often collected in leaf packs or clinging to cobble where they seek cover (Kondratieff and Voshell 1984). Uniquely, these mayflies feed by filtering fine suspended particles in the 0.1 to 0.7 μm size range (Wallace and O'Hop 1979) from the water column using long setae on their forearms. Filter feeding organisms are an integral part of an aquatic ecosystem and contribute to the overall structure and function by filtering fine suspended particles from the water column.

Isonychia **toxicity tests**

Isonychia nymphs were collected from a riffle area in Sinking Creek, Newport, Virginia (Giles County), a reference area used in this laboratory for more than 25 years and used as a

collection source in previous studies (Peters et al. 1985; Cherry et al. 2002; Kennedy et al. 2004; Cherry and Soucek 2006). *Isonychia* were collected using D-frame dip-nets (Wildco, 425-A40, 800 X 900 µm mesh), sub-sorted in plastic trays, and gently transferred into coolers filled with aerated Sinking Creek water (SCW) using BioQuip® soft-touch forceps. Organisms were acclimated to laboratory conditions for ~ 5 to 14 days, depending on water temperature at time of collection. If water temperature was less than 8°C, then mayflies were allowed to acclimate slowly to testing temperature for a minimum of two weeks (~14 days). When collection temperature was greater than 8°C, time of acclimation was dependent upon the degree difference between collection temperature and desired testing temperature. Temperature was increased by ≤ 2° C daily (Kennedy et al. 2004). During this acclimation period, *Isonychia* remained in the coolers (5 L) with SCW, to minimize the stress of additional handling/transfer, and aerated using standard aquarium aerators. Water was renewed daily by siphoning approximately 75% of the water and replenishing with unfiltered SCW. Mesh screen was placed into the coolers to provide a substrate for the mayflies to cling to. Mayflies were initially fed a mixture of ground Tetra-min® flakes and Yeast-Cereal Leaves-Trout Chow (YCT), sieved to < 200 µm). Later tests utilized a food mixture of ground dog chow, cereal leaves and green algae (*Pseudokirchneriella subcapitata*) sieved to < 150 µm, based upon gut-analysis and food preferences outlined in Wallace and O'Hop (1979).

Isonychia bioassays were conducted in 600-ml glass beakers containing a minimum of 500 ml of test solution or control water. Each beaker contained a 10.2 x 5 cm mesh screen, which served as a substrate for the mayflies. To provide flow and maintain DO_2 saturation, beakers were aerated (~ 2 bubbles per second). Air-stones were used to gently agitate the surface water, creating water column flow. Chronic toxicity tests were conducted with sodium chloride

(NaCl, A.C.S. certified) a common salt used as a reference toxicant. Use of NaCl allowed results to be compared to a database of test results with other aquatic organisms. In addition, a coal mine processing impoundment (CPI) effluent obtained from the Callahan Creek Watershed (CCW) in Wise Co., Virginia with moderately high conductivity and TDS was used to further test the response of *Isonychia* in a more realistic scenario. Results of these tests were compared to responses of *C. dubia,* also exposed to the coal processing effluent.

***Ceriodaphnia dubia* toxicity tests**

Ceriodaphnia dubia were used in chronic toxicity tests to determine the comparative sensitivity of this daphnid relative to *Isonychia* mayflies. *Ceriodaphnia* seven day chronic toxicity tests were conducted using the same CPI effluent obtained from the CCW, following US EPA protocols (US EPA 2002b). Daphnids (< 24 hr old) were isolated from cultures that were less than 14 d old, and that had < 20% adult mortality. Bioassays were conducted in 50-ml glass beakers with a minimum of 40 ml test solution. Test concentrations ranged from 6.25 to 100 % effluent, consisting of ten replicates per concentration, with one daphnid per replicate. Control and diluent water used for testing was moderately hard synthetic-freshwater. Test concentrations were renewed daily. Daphnid survivorship and neonate production were also observed and recorded daily and organisms were fed a mixture (0.40 ml/50ml) of green algae (*Pseudokirchneriella subcapitata*) and yeast-cereal leaves-trout chow (YCT). Water chemistry was measured daily on renewal (in) and outgoing (out) water for temperature (°C), pH (standard units), conductivity (µS/cm), and dissolved oxygen (DO_2, mg/L). Alkalinity and hardness (mg $CaCO_3$/L) were also measured on renewal water for the highest and lowest (control) concentrations.

Trace metal analysis of CPI effluent

Trace metals known for toxic consequences were analyzed by Induced Coupled Plasma-Mass Spectrophotometry (ICP-MS) from the coal processing impoundment effluent. Dissolved metals in the effluent included arsenic, cadmium, cobalt, chromium, copper, iron, lead, mercury, nickel, selenium and zinc along with benign trace elements such as calcium, magnesium, potassium, sodium and strontium.

Statistical analysis

Toxicity test endpoints (survivorship and reproduction for daphnids only), were analyzed with TOXSTAT® (3.3/ 1996, University of Wyoming Department of Zoology and Physiology, Laramie, WY) using appropriate parametric (Dunnett's Test) and nonparametric (Steels-Many One Rank Test) procedures ($\alpha = 0.05$). Lethal concentration values (LC_{50}) were determined using trimmed Spearman-Karber Method (Hamilton et al. 1977). Correlation analyses were performed using JMP IN (7.0.2/2008, SAS, Cary, NC).

Results and Discussion

Isonychia reference tests with sodium chloride

Mayfly survivorship was observed every 24 hr and LC_{50} values were determined at 48, 72 and 96 hr and at the end of seven days of exposure to NaCl (Figure 3.1). All four tests were similar at 48 hr with LC_{50} values > 8.00 g/L NaCl. After 72 hr, LC_{50} values ranged from 3.50 to > 8.00 g/L NaCl with Tests 1 and 2 being the most similar. Tests 3 and 4 had the highest (8.0 g/L) and lowest (3.5 g/L) LC_{50} values. This wide range of toxicity values may be attributed to the varying age and developmental stage of the mayflies. *Isonychia* used in tests 1, 2, and 3 were collected in January 2007 (Test 1) and February 2008 (Tests 2 and 3) during which they were

several months away from their first emergence (May). Mayflies from Test 4, which resulted in an LC_{50} value of 3.50 g/L after 72 hr, were in the latter stages of development having been collected in April 2008.

After four days of exposure (96 hr), *Isonychia* LC_{50} values began to decrease substantially, ranging from 2.25 to 3.78 g/L NaCl with a mean LC_{50} of 3.10 g/L. Test 3 had the greatest change in mortality from the 72-hr interval to the 96-hr interval (Figure 3.1). After seven days, values ranged from 1.29 to 2.28 g/L NaCl with a mean LC_{50} value of 1.73 g/L NaCl.

Minimal control mortality was observed with ≥ 80% of the mayflies surviving through seven days of exposure for each of the four reference (NaCl) tests (Figure 3.2). Values for conductivity in the lowest concentration of NaCl tested ranged from 1147 to 1878 us/cm (mean= 1373 μS/cm) and had ≥ 60% survival at test termination. The highest conductivity tested ranged from 14,240 to 14,270 μS/cm (mean = 14,247 μS/cm) in Test 1, 2, 3 and resulted in 100% mortality of test organisms by test day 6. Test 4 had similar results with a high test conductivity of 12,000 μs/cm, which resulted in 100% mortality by day 6. The second highest conductivity values were 7370, 7200, 7411 and 6460 μS/cm in Test 1, 2, 3 and 4, respectively. This is the highest conductivity tested that did not result in 100% mortality in any of the four tests by test termination. Mayfly survival was negatively correlated with conductivity in all four reference tests (Figure 3.2).

Isonychia sensitivity to NaCl was also compared to 15 additional aquatic test organisms (Table 3.1). This comparison included three routinely tested daphnids, the fathead minnow (*P. promelas*), an amphipod (*Hyalella azteca*), a freshwater snail (*Physa heterostropha*) and eight benthic macroinvertebrates, including two additional mayflies in the families Heptageniidae and Ameletidae. Data were obtained from the PAN Pesticides Database (http://pesticideinfo.org/

2008) and consisted of previously published LC_{50} values for each species. LC_{50} values were averaged for 24, 48, 72 and 96 hr, where applicable. Species sensitivity to NaCl ranged from 3.38 g/L for the daphnid (*C. dubia*) to 32 g/L for the damselfly (*Argia sp.)* after 24 hr of exposure. *Isonychia* had a similar LC_{50} value to another mayfly, *Ameletus*, with an LC_{50} value of > 8g/L after 24 hr.

Many of the organisms included in Table 3.1 were only tested for 48 hr which represents the exposure duration for many acute tests. Mayfly sensitivity to NaCl increased after 48 hr, so data generated after 48 hr data were compared to 72 and 96 hr LC_{50} values for *Physa*, *Ameletus*, fathead minnows, *Hyalella*, *Hydropsyche* and *Argia*. At 72 hr, LC_{50} values generated for *Isonychia* were lower than those for fathead minnows and *Argia* and after 96 hr *Isonychia* had the lowest LC_{50} value at 3.10 g NaCl/L, half the reported value for *Hyalella azteca* and a third of the LC_{50} value reported for *Hydropsyche sp*. Organisms in the Family Hydropsychidae, particularly *Hydropsyche sp*. have been reported to have a high tolerance to salinity (Williams and Williams 1998; Blinn and Ruiter 2006; Kennedy et al. 2003).

A comparison of alkaline pH exposure between *Isonychia* (96 hr LC_{50}) and *Ceriodaphnia* (48 hr LC_{50}) indicated that both test organisms had the same tolerance with an LC_{50} value of 10.3 g NaCl/L in static laboratory tests (Peters et al. 1985; Belanger and Cherry 1990). However, when *Isonychia* were tested in a continuously-flowing artificial stream system receiving New River, Virginia water, the 96 hr LC_{50} value declined to 9.5 g NaCl/L under more environmentally realistic conditions for the mayfly. Therefore, mayflies appear to be more sensitive to alkaline pH than *Ceriodaphnia dubia*.

***Isonychia* sp. CCW CPI effluent tests**

Data from CCW CPI effluent tests with *Isonychia* were analyzed at seven and 14 days to determine differences in mayfly response during the duration of testing and for comparison with other test organisms (Figures 3.3-3.5). Conductivity in Test 1 ranged from 493 to 4101 µS/cm and seven day results showed a poor correlation between survival and conductivity (r=-0.4077) that improved by the end of the 14 day exposure period (r=-0.7717) (Figure 3.3). In Test 2, a stronger relationship was observed between survival and conductivity after seven days (r=-0.8177), that increased after an additional seven days of exposure (r=-0.8948) (Figure 3.4). Test 3 was conducted for ten days with a strong negative correlation between mayfly survival and conductivity (r=-0.8049) (Figure 3.5). In each of the three tests, 100% mortality occurred in the highest test concentrations (>4000 µS/cm) by test termination. Calculated LC_{50} values for the CCW CPI effluent ranged from 13 to 39% effluent.

In Tests 1 and 2, (Figures 3.3 and 3.4), impairment was not observed until after 48 hr of exposure to CCW CPI effluent, while mortality occurred in Test 3 after the first 24 hr (Figure 3.5). Test 3 mayflies were collected from the summer cohort in July 2006, which may explain the more sensitive response seen in these organisms. Developing standard guidelines including standard collection methods, holding conditions and acclimation, seasonality of collection, size of mayflies and overall general condition of the organisms will help to reduce variability in sensitivity that may occur.

Five chronic tests were conducted with *Ceriodaphnia* exposed to the CPI effluent. Percent survival in 100% effluent ranged from 0 to 100% (Table 3.2). Reproduction impairment was observed in two of the five tests with LOEC values for reproduction reported at 75 and 50% effluent for Tests 3 and 4, respectively. Mortality in 100% effluent and reproductive

impairment were not correlated with conductivity values. Test 4 had varying mortality in 100% effluent and reduced reproduction, but the effective values for conductivity were similar to these reported in the other 4 tests.

Significant reductions for mayfly survival and *Ceriodaphnia* reproduction in 100% CPI effluent were considered to be primarily from the ionic salts in the TDS and not due to trace metal influence (Table 3.3). In Test 1, the CPI 100% effluent had conductivity >4000 µS/cm and TDS of 2900 mg/L but ten of the 11 trace metals analyzed had results that were equal to or lower than non-detectable limits (Table 3.3). Other than iron (100 µg/L), most detection limits were ≤ 40 µg/L (Ni) to 0.20 µg/L (Hg) with most of them being between 7-20 µg/L. The TDS comprising the effluent basically came from strontium, magnesium, potassium, calcium, and sodium with concentrations that ranged from 7020-957,000 µg/L.

Summary and Conclusions

Our toxicity testing results indicated that *Isonychia* were substantially more sensitive to the CCW CPI mining effluent than the 7-day *Ceriodaphnia* test although the mayfly tests lasted 10-14 days. The LOEC for mayfly 10-14 day survivorship ranged from 1429 to 2251 µS/cm relative to the *Ceriodaphnia* results (~4000 µS/cm). These test result trends were similar (1562 µS/cm for *Isonychia* and 3730 µS/cm for *C. dubia*) to those reported by Kennedy et al. (2004) when testing a coal mining effluent from Leading Creek, Ohio. The CCW CPI effluent had minimal trace metal presence, which were below water quality criteria or non-detection limits, so the major factor influencing mayfly toxicity was the ionic salts in the TDS. The conductivity (~200 µS/cm) and TDS (~150 mg/L) in the copper toxicity test results reported by Cherry et al. (2002) for mayflies (LC_{50} = 52 µg/L) were not a factor when copper sulfate was a measurable

component in the TDS that caused the acute toxicity while no one would suspect 150 mg/L TDS to be problematic. Therefore, when TDS limits are being developed in coal mining influenced watersheds, the potential presence of trace metals that override TDS influence should be considered. In the Callahan Creek, Virginia watershed where the CPI effluent is discharged, some higher level of allowable TDS needs to be considered that incorporates the characteristics of this effluent. The question is how high can TDS levels be, without trace metal influence and still be safe for aquatic life. For the CPI effluent, which is comprised of non-toxic trace elements and very low trace metal concentrations, impairment levels of TDS may occur at ~ 1400 mg/L. Additional testing with *Isonychia* is needed to determine the most appropriate upper TDS level. An update to the Water Quality Standards (WQS) in Alaska was recently proposed with a recommendation for revision of the TDS limit from 500 to 1500 mg/L based upon a site specific criterion (SSC) developed in Red Dog Creek (http://www.dec.state.ak.us/water/wqsar/wqs/pdfs/reddogfactsheet092605.pdf 2005). The higher SSC proposed for the TDS limit was based upon a study by Chapman et al. (2000) whereby they found no toxicity to embryos or fry of rainbow trout at greater than 2000 mg/L TDS. They also emphasized that TDS toxicity is influenced by ionic content of the test solution especially that of mining effluents. The authors found that chironomid larvae showed no toxic effects at 1134 mg/L when exposed to an artificially prepared effluent and that reduced growth (45% reduction in dry weight) occurred at 2089 mg/L, while a second artificial effluent generated reduced survival at 1750 and 2240 mg/L, but no effects were observed at 1220 mg/L TDS. Revising the existing TDS standard of 500 mg/L to 1500 mg/L is a substantial increase.

Isonychia are thought to be a more tolerant mayfly than some other types found in Central Appalachia streams (e.g. ephemerellids, heptageniids) according to Pond et al. (2008).

Studies have demonstrated that mayflies may exhibit significant differences in sensitivity to toxicants not only between families and genera, but also between species within the same genus. Beketov (2004) researching the sensitivities of mayflies to ammonia, nitrite and nitrate reported that *Baetis vernus* was more sensitive than *B. fuscatus*. Choosing the most sensitive mayfly would provide the most conservative data, but the most sensitive species are not always well suited for laboratory testing. The goal in establishing a mayfly bioassay is to provide predictive data that can be used to protect the integrity of aquatic systems, but the chosen species must be reliable enough for repetitive testing in multiple laboratories, handling stress must be tolerated and control survival must meet a specific criteria. *Isonychia* has a history of research efforts over the past 30 years starting with Sherberger et al. (1977) and then Peters et al. (1985) and others thereafter to allow us to use a sensitive mayfly with minimal to no laboratory induced stress when conducting a test for 14 days.

Few mayflies in the families Ephemereliidae and Heptageniidae were collected in the Callahan Creek, Virginia watershed where mining activities are ongoing although *Isonychia* are present. Therefore, the use of *Isonychia* has merit as a new laboratory testing organism for evaluating coal mining effluents and determining safe TDS limits because of its extensive laboratory database, presence in the watershed of coal mining activity and being substantially more sensitive than the current, most widely used test species, *C. dubia*.

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Table 3.1. Comparison of test organism lethal concentration (LC_{50}) values for sodium chloride (g NaCl/L) at 24, 48, 72 and 96 hr.

Test Organism		LC_{50} Values (g NaCl/L)				Citation
Taxonomic Group	**Genera**	**24-hr Mean (range)**	**48-hr Mean (range)**	**72-hr Mean (range)**	**96-hr Mean (range)**	
Cladocera: Daphniidae	*Ceriodaphnia dubia*	3.38 (3.08-3.54)	1.96 (1.77-2.33)	----	----	Mount et al. 1997
Cladocera: Daphniidae	*Daphnia pulex*	----	2.26 (1.47-3.05)	----	----	Birge et al. 1985
Ephemeroptera: Heptageniidae	*Stenonema rubrum*	----	2.50 (2.50-2.50)	----	----	Roback 1965
Cladocera: Daphniidae	*Daphnia magna*	6.38 (6.38-6.38)	4.88 (3.31-6.03)	----	----	Dowden and Bennett 1965; Hoke et al. 1992; Harris 1994; Arambasic et al. 1995; Mount et al. 1997
Gastropoda: Physidae	*Physa heterostropha*	5.53 (4.20-7.5)	5.13 (3.70-6.95)	4.89 (3.5-6.2)	4.72 (3.5-6.20)	Wurtz and Bridges 1961
Diptera: Chironomidae	*Cricotopus trifasciatus*	----	6.22 (6.22-6.22)	----	----	Hamilton et al. 1975
Tricoptera: Hydroptilidae	*Hydroptila angusta*	----	6.62 (6.62-6.62)	----	----	Hamilton et al. 1975
Ephemeroptera: Ameletidae	*Ameletus sp.*	> 8.00	6.96 (5.91-8.0)	5.14 (4.07-6.20)	4.13 (3.25-5.01)	Echols, unpublished data
Cypriniformes: Cyprinidae	*Pimephales promelas*	7.92 (7.1-9.0)	7.69 (7.05-8.7)	7.65 (7.65-7.65)	7.62 (7.62-7.62)	Adelman and Smith 1976
Diptera: Chironomidae	*Chironomus attenuatus*	9.82 (9.82-9.82)	7.99 (7.99-7.99)	----	----	Thornton and Sauer 1972
Ephemeroptera: Isonychiidae	*Isonychia sp.*	> 8.00	> 8.00	5.95 (3.50-8.00)	3.10 (2.25-3.78)	------
Amphipoda: Hyalellidae	*Hyalella azteca*	----	----	----	6.51 (6.51-6.51)	Lasier et al. 1997
Tricoptera: Hydropsyhiidae	*Hydropsyche sp.*	----	----	----	9.00 (9.00-9.00)	Roback 1965
Diptera: Culicidae	*Culex sp.*	10.50 (10.50-10.50)	10.20 (10.20-10.20)	----	----	Dowden and Bennett 1965
Odonata: Coenagrionidae	*Argia sp.*	32 (32-32)	29 (26-32)	25 (24-26)	23.5 (23-24)	Wurtz and Bridges 1961

Table 3.2. *Isonychia sp.* survivorship and *Ceriodaphnia dubia* survivorship and reproductive responses in 7-day chronic toxicity tests with CCW CPI effluent.

TEST Number	Percent Survivorship 100% Effluent	Survival/Reprod. LOEC	Mean Conductivity for LOEC (μS/cm)
Mayflies			
Test 1	65	100	4,101
Test 2	65	100	3,451
Test 3	10	25	1,508
Daphnids			
Test 1	80	100	4,250
Test 2*	100	100	4,014
Test 3	100	75	3,132
Test 4	0	50	2,132
Test 5*	90	100	3,403

*Test concentrations were based on conductivity range and not serial dilution.

Table 3.3. Trace metals and other elements measured in the coal processing impoundment effluent in Test 1.

Parameter	Result	Detection Limit (µg/L)
Arsenic	ND	10.0
Cadmium	ND	2.0
Cobalt	ND	7.0
Chromium	ND	5.0
Copper	ND	25.0
Iron	ND	100.0
Lead	ND	3.0
Mercury	ND	0.20
Nickel	ND	40.0
Selenium	8.5	5.0
Zinc	ND	20.0
	Other Trace Elements	
Calcium	68,800	5,000
Magnesium	50,700	5,000
Potassium	31,800	5,000
Sodium	957,000	25,000
Strontium	7020	50.0

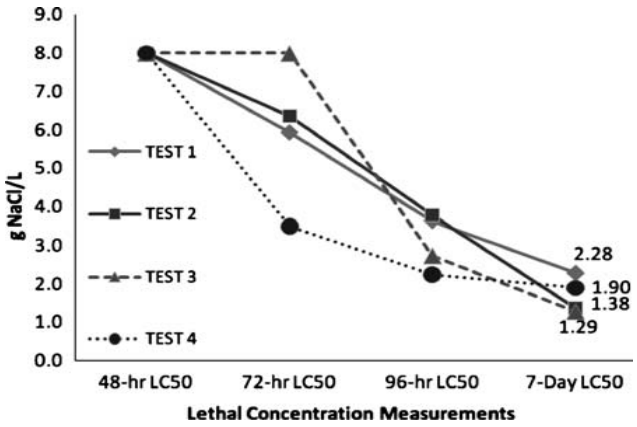

Figure 3.1. Lethal concentration (LC_{50}) values during a 7-day test period for *Isonychia sp.* exposed to NaCl.

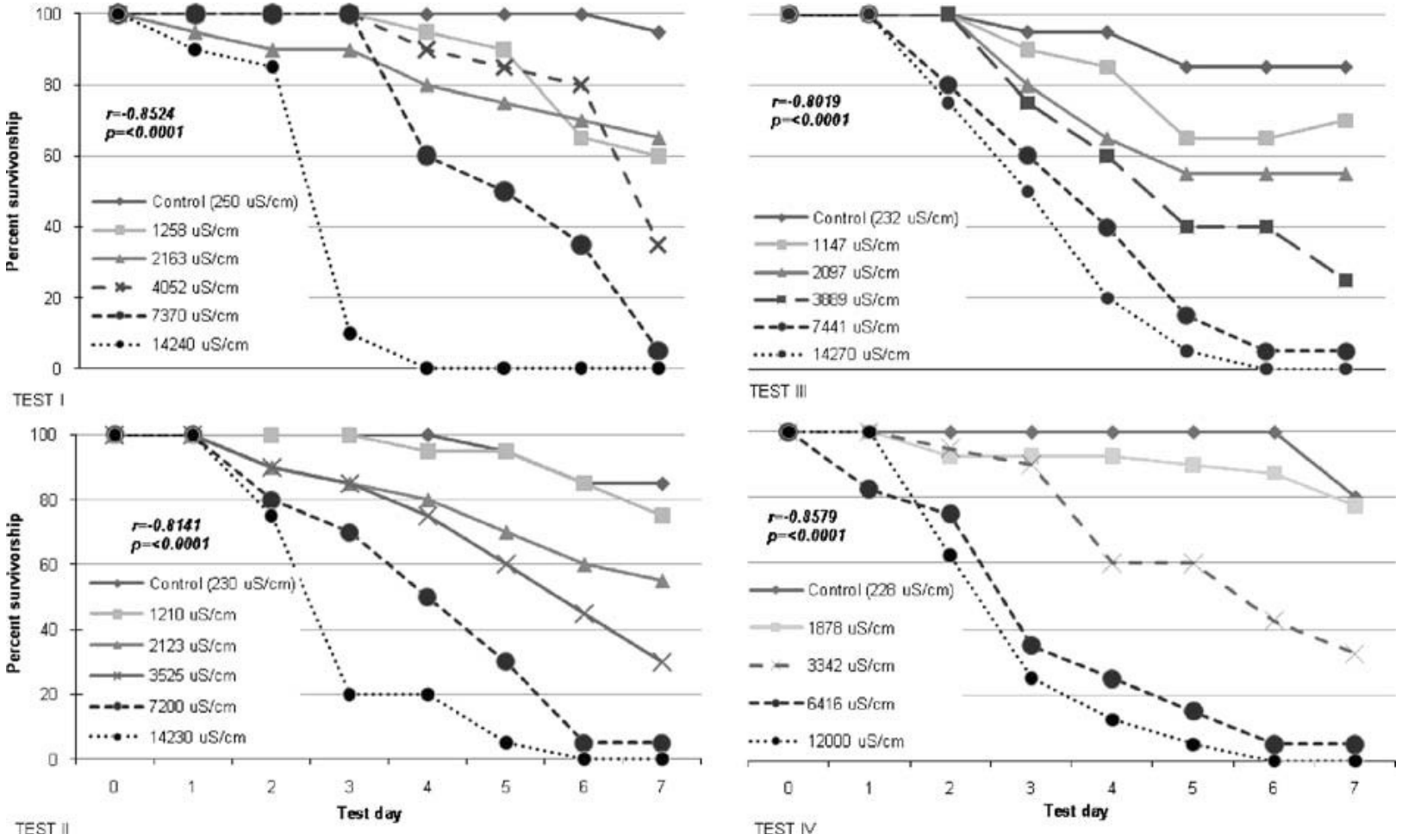

Figure 3.2. *Isonychia sp.* survivorship in 7-day NaCl chronic toxicity tests. Dashed lines indicated significant difference from the control.

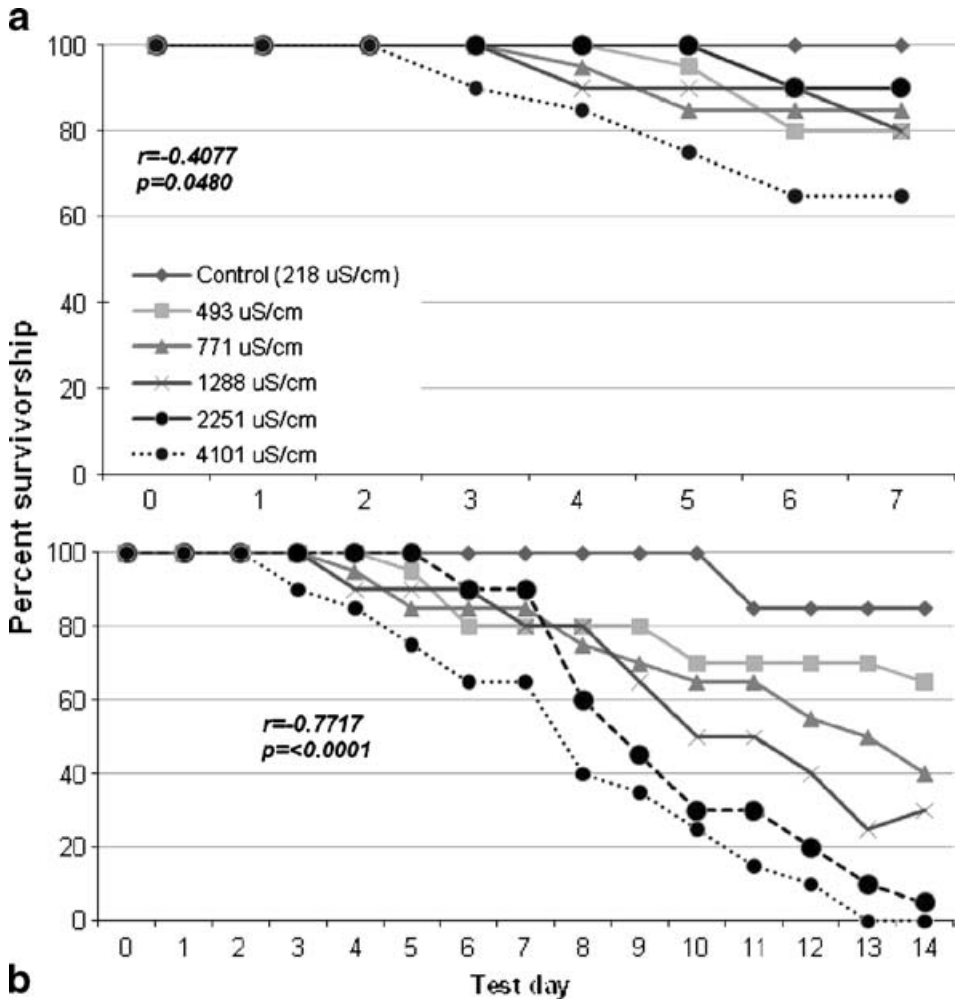

Figure 3.3. *Isonychia sp.* chronic Test # 1 with CCW CPI effluent over 7 days (A) and 14 days (B). Dashed lines indicate significant difference from the control.

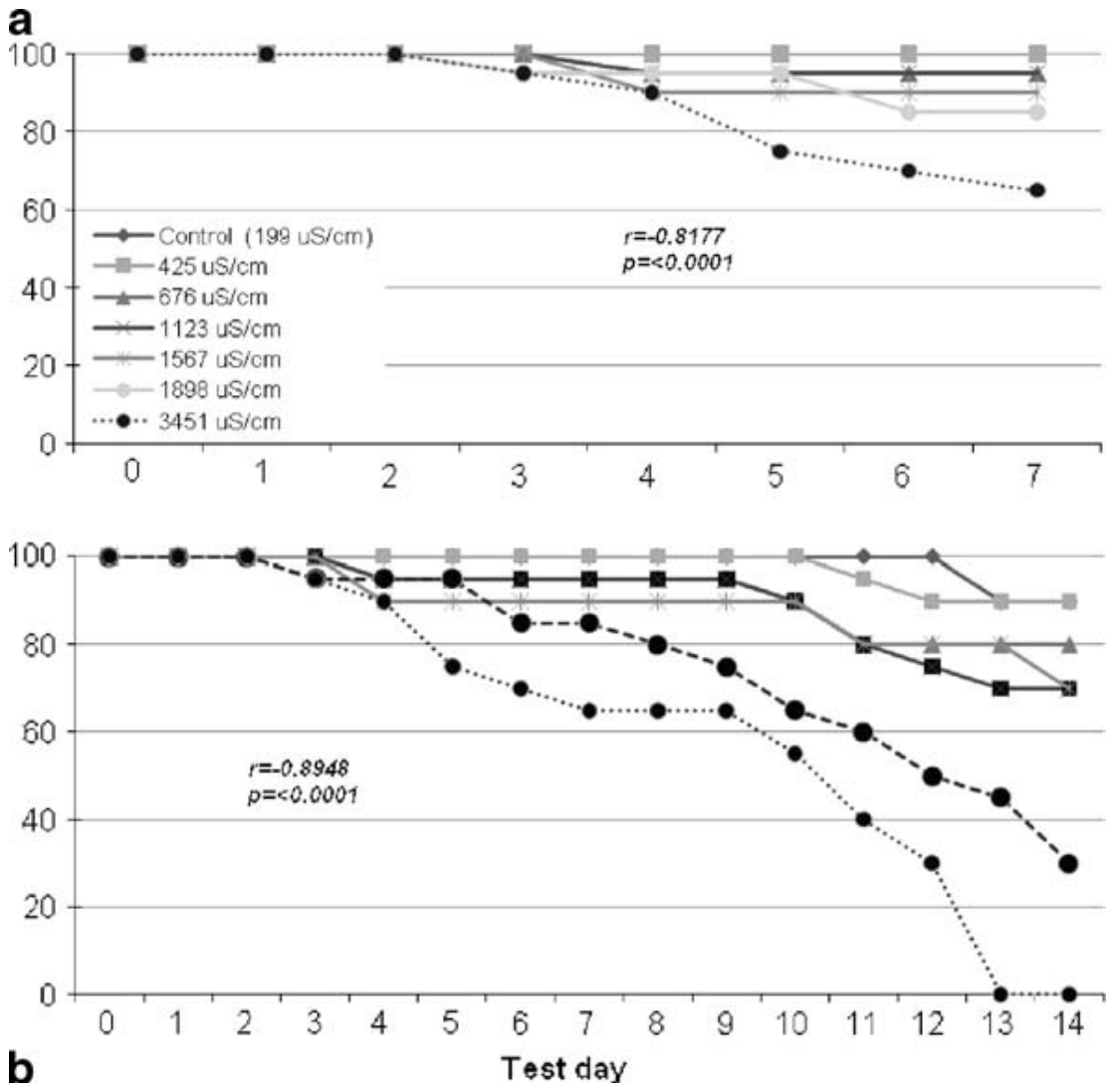

Figure 3.4. *Isonychia sp.* chronic Test #2 with CCW CPI effluent over 7 days (A) and 14 days (B). Dashed lines indicated significant difference from the control.

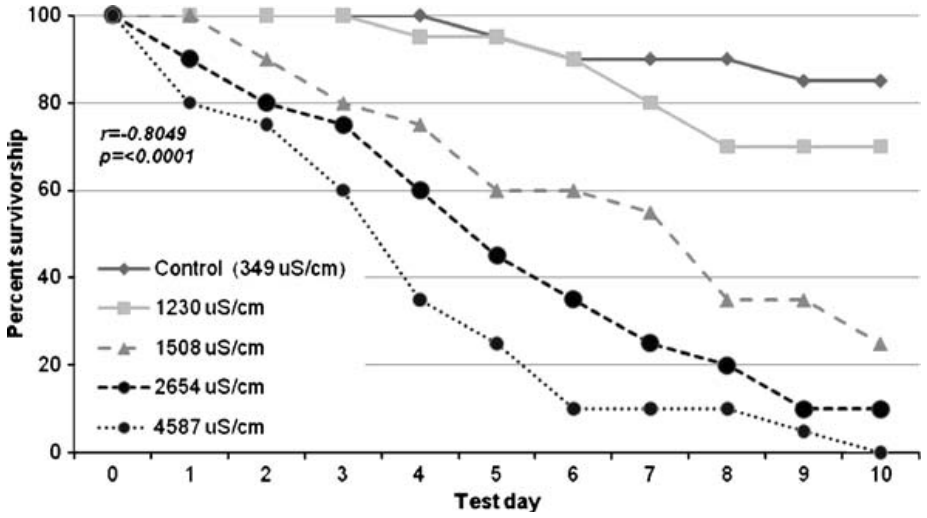

Figure 3.5. *Isonychia sp.* chronic Test #3 with CCW CPI effluent over a 10-day test period. Dashed lines indicated significant difference from the control.

Chapter 4. Seasonal Availability and Sensitivity of Two Field Collected Mayflies for the Development of a Standardized Toxicity Test: A One Year Feasibility Study

Introduction

The protection of freshwater receiving systems is accomplished most often by the combined use of field bioassessment and laboratory bioassays. Field assessments of aquatic biota, specifically benthic macroinvertebrate, have long been used to determine an ecosystem's health prior to or after environmental degradation has occurred. Degradation to aquatic systems can include sedimentation and habitat alteration, which transform the structure and subsequently the function of the ecosystem, as well as chemical alterations which can lead to the eradication of specific organisms within the system. The impact of chemical alteration on a freshwater system can often be more difficult to quantify due to the lack of predisturbance data, which provide regulatory agencies and scientists with an idea of naturally occurring populations within a given creek or river. In order to determine the potential impacts originating from point-source discharges, laboratory toxicity tests are used, which most often utilize standard test organisms, such as the Cladocera, *Ceriodaphnia dubia*, or the fathead minnow (*Pimephales promelas).* These organisms, along with several others, have been used for more than two decades to protect freshwater receiving systems. However, more recent research questions the validity of such bioassays that utilize organisms not indigenous to most freshwater systems in North America, particularly those impacted by active coal mining (Echols et al. 2010). The complexity of ion mixtures associated with coal mining discharges may be more toxic to freshwater invertebrates naturally occurring in Appalachia streams than test organisms such as *C. dubia*.

The use of aquatic insects in field bioassessments has been an important tool for determining ecosystem health following an environmental impact. The presence or absence of certain organisms can indicate a system is environmentally stressed. Organisms in the order Ephemeroptera (mayflies) are considered to be the most sensitive freshwater invertebrate. Often,

their absence from a sampling site is an indicator that the physical and chemical properties of the system are not meeting the specific needs required from this diverse group of organisms. Because of this sensitivity, mayflies have been used in many laboratory bioassays (Sherberger et al. 1977, Peters et al. 1985, Diamond et al. 1990, Diamond et al. 1992, Dobbs et al. 1994, Beketov 2004, Kennedy et al. 2004, Hassell et al. 2006, Brinkman and Johnston 2008, O'Halloran et al. 2008, Echols et al. 2010) for more than two decades. Although mayflies have been used as laboratory test organisms, testing methodology has not yet been developed.

The objective of this one-year study was to determine the feasibility of routine testing with two genera of mayflies, *Isonychia bicolor* (Isonychiidae) and *Maccaffertium sp.* (Heptageniidae), over four seasons of collection, acclimation and testing and the evaluation of basic testing methodology which is based on US EPA whole effluent toxicity test (WETT) methods (US EPA 2002 a, b) . This evaluation considered important test parameters including test duration, sublethal endpoints and test chamber design. A key component of chronic toxicity testing is appropriate test duration. Short term acute tests (48 to 96 hr) assess survivorship, but do not evaluate sublethal effects. Long term (chronic) tests ranging from 7, 10 to 14 days are needed to test mayfly sensitivity allowing for the inclusion of sublethal effects such as growth (head capsule length, biomass) and molting. Test duration must also consider validity criteria such as acceptable control survivorship ($\geq$ 75%). The test results are also contingent upon the difference in stream collection temperature, duration and rate of acclimation in the laboratory, and time testing interval thereafter. The test chamber needs to have some degree of environmental realism whereby aeration provides water current and acceptable dissolved oxygen levels. Also, the type of substrate provided as a hold-fast or clinging apparatus in the test chamber is important as is the food type selected. These criteria were addressed and tested over

four seasons (winter, spring, summer then fall) in 2009 using the standard reference toxicant (sodium chloride) bioassays.

Materials and Methods

Test Organisms

Two mayfly genera were used for this project, the brush-legged mayfly, *Isonychia bicolor* (Isonychiidae) and the flat-headed mayfly, *Maccaffertium sp*. (Heptageniidae). These mayflies have been used in previous laboratory toxicity tests and exhibit important characteristics, including abundance in local streams, year-long availability, larger physical size to minimize handling stress and relative sensitivity to many anthropogenic stressors. These characteristics make them suitable candidates for standardized laboratory testing.

Isonychia mayflies are a bivoltine insect having two generations per year with an over-wintering cohort that emerges in the spring and a smaller summer/fall cohort that develops and emerges quickly in late summer and early fall. These organisms are regarded as strong swimmers, but are often collected in leaf packs or clinging to cobble where they seek cover (Kondratieff and Voshell 1984). Uniquely, these mayflies feed by filtering fine suspended particles in the 0.1 to 0.7 μm size range (Wallace and O'Hop 1979) from the water column using long setae on their forearms. Filter feeding organisms are an integral part of aquatic ecosystems and contribute to the overall ecosystem structure and function by filtering fine suspended particles from the water column.

Maccaffertium (formerly *Stenonema*) mayflies are commonly found throughout eastern North American streams and rivers (Kondratieff and Voshell 1980), inhabiting all sizes of flowing water bodies, both fast and slow currents. Heptagenid mayflies are considered primarily

lotic-erosional, preferring the swift water current found in riffle habitats. They are normally found clinging to the underside of rocky substrate, including boulders, bedrock and cobble, but are also found on large woody debris and leaf packs. Habitat preference is given to areas which are sheltered from the rapidly moving water. These organisms primarily feed by scraping algae and fine organic material (FPOM < 1mm) from the top of the substrate surface. *Maccaffertium* in general, spend the majority of their life as nymphs, progressing through a minimum of 25 instars, which are present in streams at various stages of development throughout the year (Lewis 1974). Certain species of *Maccaffertium* are multivoltine, while the majority of organisms within this genus are bivoltine, producing only two generations per year. Kondratieff and Voshell (1980) state that *Stenonema modestum* (now known as *Maccaffertium modestum*) vary in their life cycle due to some over-wintering larvae that are less developed than others which creates a cohort of organisms at various stages of development. Ide (1935) reported similar findings for *Stenonema* species. Therefore, this organism can be readily available, at differing life stages, throughout the year.

Heptagenid mayflies and specifically, *Maccaffertium* species can be variable in pollution tolerance, ranging from intolerant to facultative (Lewis 1974). Facultative can be defined simply as the capability of an organism to function under varying environmental conditions. *Maccaffertium modestum* is listed as pollution-sensitive by the US EPA (Hubbard and Peters 1978; Barbour et al. 1999).

Collection of Test Organisms

Both *Isonychia bicolor* and *Maccaffertium sp*. were collected from a riffle area in Sinking Creek, Newport, VA (Giles County); a reference area used in this laboratory for more than 25

years and used as a collection source in previous studies (Peters et al. 1985, Cherry et al. 2002, Kennedy et al. 2004, Cherry and Soucek 2006, Echols et al. 2009). Mayfly nymphs were collected using D-frame dip-nets (Wildco, 425-A40, 800 X 900 μm mesh), sub-sorted in plastic trays, and gently transferred into separate coolers filled with aerated Sinking Creek water (SCW) using BioQuip® soft-touch forceps. Mayflies were transported back to the laboratory at field collected temperatures. In order to evaluate collection feasibility across seasons, the amount of time spent for field collection was 2.5-3 hrs.

Laboratory Acclimation

Mayflies were acclimated to laboratory conditions using several different approaches. Due to the variable water temperatures at the time of collection, laboratory acclimation methods had to be modified depending on both the collection temperature and desired test temperature (9 versus 20°C). Specific acclimation techniques can be found in the Results section of this report.

Toxicity Testing

Toxicity testing methods were based on US EPA WETT protocols for chronic toxicity testing (USEPA 2002b) with some modifications. Static, mayfly bioassays were conducted in 600-ml glass beakers containing a minimum of 500 ml of test solution or control water. Each beaker contained a single maple leaf, which served as a substrate for the mayflies. Maple leaves provide a more realistic substrate for clinging, versus polyethylene mesh substrate, which has been used previously in this lab. In addition, natural bacterial growth and decomposition/breakdown of the leaves provide an additional food source for both *Maccaffertium* and *Isonychia.* To provide flow and maintain DO_2 saturation, beakers were aerated using air diffusers, which provide both oxygen and gentle agitation to the water surface,

mimicking flow. Two types of air diffusers were used during the course of this one-year study. Initially, low-cost air diffusers (air flow range 0.01-0.06 cfm) were used; however, these did not seem to provide consistent air/flow to all test beakers. Sweetwater® glass-bonded silica diffusers were then used, which provided more consistent air-flow and fine bubbles (1-3 mm; 0.35 cfm). This simplistic design was preferred to that of a larger, flow-through or flowing set-up as routine tests should be easy to replicate and repeat.

Chronic toxicity tests were conducted with sodium chloride (NaCl, A.C.S. certified) a common salt used as a reference toxicant. Tests were checked for mortality and exuvia or molts every 24 hours and test water was renewed every other day. During changeovers, ~ 80% of water was removed by siphoning, which aided in removing debris from the bottom of the test beaker, but prevented any unnecessary handling of the mayflies. Test duration was dependent on control survivorship.

Statistical Analysis

Toxicity test endpoints were analyzed with TOXSTAT® (3.3/ 1996, University of Wyoming Department of Zoology and Physiology, Laramie, WY) using appropriate parametric (Dunnett's Test) and nonparametric (Steels-Many One Rank Test) procedures ($\alpha = 0.05$). Lethal concentration values (LC_{50}) were determined using trimmed Spearman-Karber Method (Hamilton et al. 1977, US EPA 1993). Multivariate correlation and linear regression analyses were performed using JMP IN (8.0.2/2009, SAS, Cary, NC).

Results

Collection of Test Organisms

Field collection of mayflies was attempted 20 times during 2009 for *I. bicolor* and *Maccaffertium sp.* with four attempts being completely unsuccessfully due to habitat inaccessibility (Table 4.1). Collection attempts were not made during the months of March or December. In addition to collection of these two test organisms, a third mayfly species, *Ameletus sp*. (Ameletidae) was collected on February 6, 2009. *Ameletus* mayflies (only North American genus) normally occur in lotic-erosional habitats, but occasionally are found in seasonal or intermittent streams or seeps (Voshell 2002). These mayflies have a long egg diapause during dry conditions and develop quickly during the winter months, emerging middle to late February. Unfortunately, these organisms did not survive the laboratory acclimation process. More than 75% mortality occurred within the first 48 hr of laboratory acclimation, in which the gradual (2°C daily) temperature increase was lethal.

The process of test organism collection was equally as important in this study as the actual collection of organisms to be used in laboratory bioassays. It was important to establish the feasibility of routine collection for these organisms during the course of a year. Therefore, the time spent attempting to collect test organisms was limited to 2.5 to 3 hr. This allowed us to make quantitative comparisons of organism availability and ease of collection on a monthly basis.

Although both *Isonychia bicolor* and *Maccaffertium* nymphs are abundant in Sinking Creek, *Isonychia* were more readily abundant in each dip net sample and were generally more available throughout the year (Fig. 4.1). Except for the two months during 2009 when collection

was not attempted, *Isonychia* were noted as being present at this site each month of the year. This coincides with frequency distributions presented by Kondratieff and Voshell (1984) of *I. bicolor* from Sinking Creek. *Maccaffertium* were virtually absent in samples collected from the end of May until September, although a few organisms were collected during July (Fig. 4.1).

A breakdown of the number of organisms collected by season shows both *Isonychia* and *Maccaffertium* nymphs were most abundant during the winter and fall of 2009 (Fig. 4.1 & 4.4). The average number of *Isonychia* collected per sampling trip during winter months (January-February) was 251.7, while the average number of *Maccaffertium* collected per sampling trip was 175, resulting in 31 and 49% of the total organisms (per species) collected in winter 2009. Organism collection during the fall of 2009 (Sept.-November) was similar, comprising 30 and 33% of the total *I. bicolor* and *Maccaffertium* collected, respectively. The average number of *I. bicolor* nymphs collected during these months was 165.0, while *Maccaffertium* averaged 93.8 organisms per collection attempt. Summer proved to be the most difficult time to collect both species, with *I. bicolor* averaging only ~100 organisms per sample (n=5), and < 100 total *Maccaffertium* (6%).

Laboratory Acclimation

Laboratory acclimation encompassed two objectives during this one year study. First, the role of collection temperature at the time of organism collection, versus ideal test temperature had to be addressed. Secondly, during warmer months when temperature acclimation was no longer an issue, the time period of appropriate lab acclimation (or holding time) to reduce or prevent handling stress also had to be evaluated. Therefore, acclimation to lab conditions was

handled in two separate ways, depending on the time of year that collection and testing took place.

Temperature Acclimation

Laboratory acclimation during the first and fourth quarter was influenced by the stream temperature at the time of organism collection at Sinking Creek. These were the only times during the one-year study in which the temperature difference between the field and test temperatures were factors that may have affected the performance of the mayflies in the bioassays.

During the first quarter (January and February, 2009) of mayfly testing, organisms were collected at 4-5°C. Testing at such low temperatures (~freezing) is not recommended due to increased tolerance of test organisms to toxicants. Therefore, toxicity tests were conducted at a low temperature of 9°C, which was slightly above ambient conditions but still much lower than a routine test temperature (20-25°C) which is more defendable for establishing routine testing guidelines.

Initial toxicity tests with *Isonychia* and *Maccaffertium* (conducted during January 2009) were tested at 9°C in a walk-in temperature control room. This low temperature mimicked average day-time air temperatures at the time of collection and was only double that of in-stream temperature conditions. Because of this low testing temperature, mayflies only required a brief acclimation period. Mayflies were maintained for the initial 24-hr period at 5°C, after which the temperature was increased in the temperature control room to 7°C for an additional 24-hr period. After this total 48 hr of lab acclimation, organisms were loaded into test replicates and the temperature was increased the additional two degrees to the 9°C test temperature. Therefore,

mayflies were initially introduced into test conditions at 7°C and temperatures gradually increased overnight to 9°C. The rushed acclimation period was due to the limited availability of the temperature control room and the attempt to run the test for 14 days. During the laboratory acclimation, mayflies were maintained in coolers, aerated and provided leaves as a realistic substrate. We found that when test chambers were provided both leaves and artificial mesh substrate, the mayflies preferred to cling to the leaves. Mayflies were fed 3 ml of ground Tetramin® and Yeast-Cereal Leaves-Trout Chow (YCT) mixture once daily.

Additional mayflies were collected for testing the following week (January 12, 2009) during similar, seasonal conditions (water temperature = 5°C). These organisms were immediately placed in the walk-in temperature control room (9°C) and allowed to acclimate up to this temperature for 48 hr prior to test initiation. Acclimation procedures, including holding apparatus, substrate and feeding regime followed those used for the previous test organisms.

Isonychia and *Maccaffertium* were once again collected (January 23, 2009) from Sinking Creek (4°C) the following week for additional toxicity tests. Tests with these organisms were conducted at 20°C or approximately room temperature and therefore, thermal acclimation was double that of previous tests. Upon initial arrival at the laboratory, organisms were allowed to acclimate up to 9°C in the walk-in temperature room for 48 hr. After this initial acclimation period, the organisms were moved to the main laboratory in 2006 Derring Hall and allowed to acclimate to 20 ± 2°C for an additional 48 hr. This shortened acclimation period was used to compare the testability of these organisms at a higher temperature, but with similar acclimation procedures as used for the previous tests conducted at 9°C.

For the final round of testing during the first quarter, mayflies were again collected at 5°C and transported back to the laboratory for acclimation and testing on February2, 2009. To test the

hypothesis that longer acclimation would improve test results, mayflies were placed in a refrigeration unit, maintained at 5°C for 48 hr. After 48+ hours, mayflies were moved to the loading dock of our Ecosystem Simulation Laboratory (ESL), where nightly temperatures ranged from 11-14°C. Mayflies were moved during cooler, night-time temperatures so that the air temperature change would be less than that of the daytime. Over the next 72 hr, water temperature in the mayfly holding tanks (coolers) gradually increased to 15°C. Mayflies were monitored during this period for possible increases in exuvia production and/or mortality, but no such activity was observed. After 72 hr, mayflies were finally moved to 2006 Derring Hall and maintained at room temperature for 48 hr, allowing a gradual warming from 15°C to 20°C, prior to test initiation. Total acclimation time was seven days.

During the second and third quarters, stream temperature was > 12°C and not considered an issue for laboratory acclimation; however, the water temperature was substantially lower during the fourth quarter testing. By mid-October, stream temperature conditions at Sinking Creek were almost half of those in September, although they remained quite stable during the duration of the fourth quarter, and ranged from 10-11°C. Mayflies collected in October (October 21, 2009 at 10°C) were acclimated to laboratory test conditions over a 5-day period. Upon collection and return to the laboratory, mayflies were kept in a Fisher Isotemp® low-temperature incubator, which was adjusted 2°C daily until the desired temperature (20°C) was reached. Organisms were monitored daily for mortality and unusual exuvia production. During the acclimation period, ≤ 1% mortality occurred, which was more than acceptable. *Isonychia* were fed ground Tetramin® and water was renewed once daily, during the acclimation process.

Acclimation Duration

When temperature acclimation was not an issue, proper laboratory acclimation focused on the appropriate duration that would not negatively influence the outcome of toxicity tests.

During the second quarter of testing (April-June), field conditions were more conducive to lab acclimation and higher testing temperatures. Stream temperatures at time of collection ranged from 12°C in April to 16°C in May 2009. These higher temperature conditions decreased the amount of time needed for proper temperature acclimation in the laboratory.

Due to the minimal degree difference required for this acclimation/testing, we conducted a modified range finding test with organisms after only 24 hr of laboratory acclimation time. By exposing organisms to control water and a NaCl test concentration of 8 g/L we were able to test the response of *Isonychia* to test conditions with only a minimal lab acclimation period. After 48 hours, a full chronic toxicity test was initiated and conducted side-by-side to determine if the additional 24- hour period decreased organism stress in test conditions.

Survivorship was 100% for the first three days of the 24-hr test using controls only. After five days, 50% of *Isonychia* were dead, while all were dead on day 7 of testing. These results were much different compared to control results after mayflies acclimated to lab conditions for a 48-hr period. Survivorship remained at 100% for five days for this test and 75% were living after 7 days of test exposure. Mayfly mortality was more rapid in the 8 g/L concentration as 30% mortality occurred within the first 24 hr of testing followed by 45 % mortality on day 2. All mayflies were dead after three days of exposure. After one day of exposure to the 8 g/L salt concentration in the full chronic test initiated after 48 hr acclimation time, mortality was only 5%. Mortality after 48 hr of testing was only slightly greater at 35% than mortality occurring after only one day of testing for the less acclimated test organisms. In fact, complete mortality in

the 8 g/L test concentration did not occur until day 6 of the chronic test. These results show a dramatic difference in organism responses when acclimation time is rushed, despite the minimal temperature change.

During the third quarter, we again compared the results of *Isonychia* which were allowed 48 hr to acclimate versus only 24 hr and observed improved control survivorship in the mayflies with the longer acclimation period. After 24 hr of testing, 15% mortality had occurred for the 24-hr acclimation controls, compared to only 5% for the 48-hr acclimated ones. However, after 48 hr of testing, survivorship dropped substantially to 70% for the longer acclimated controls while survivorship remained at 85% in the 24 hr acclimation test. Survivorship was the same (70%) in both tests after 72 hr and comparable after 96 hr (55 & 60%) for the 24 and 48-hr acclimated organisms, respectively. Additionally, organisms were compared using survivorship responses in the highest NaCl concentration (8 g/L). Initial mortality was the same after 24 hr (35%), then more substantial (85%) after 48 hr in the controls initiated after 48 hr of acclimation. Complete mortality occurred in the 24 hr acclimation test after 72 hr and after 96 hr in the 48- hr acclimation test.

During the fourth quarter of testing in the fall of 2009, stream temperatures at collection were much lower (10-11°C). An acclimation test was conducted in November 2009 at ambient (11°C) conditions and therefore, temperature was not an additional factor influencing mayfly survivorship. In contrast to previous results, *I. bicolor* exposed after only 24 hr acclimation had greater overall survivorship with little fluctuation during the 14-day test period. Survivorship was 100% for the first six days, dropping to 95% on day 7, which remained for the duration of the test (14 days). *Isonychia* which acclimated for an additional 24 hr (48 hr total) appeared to be more sensitive to test conditions and subsequent handling stress. After five days, 10%

mortality had occurred, with additional mortality occurring on day 12 (15%) and day 13 (20% total).

Although *Maccaffertium* were unavailable for acclimation tests during previous quarters of testing, their availability in November made it feasible to understand if they too were affected by the length of acclimation time. These results were much more dramatic as those organisms exposed to test conditions after 24 hr had much better survivorship compared to those which were acclimated for 48 hr. Control survivorship was 100% for the first nine days of the test, only declining to 95% for the remainder of the 14-day test. Minimal mortality (5%) occurred after two days in the 48-hr acclimation test and continually increased for the duration of the 14-day test with only 45% of organisms living at the end of the test.

The appropriate duration for laboratory acclimation appears to vary when comparing the results of three tests, conducted in 2009 when temperature acclimation was not necessary (Fig. 4.5). Although *Isonychia* collected during July and November were from two different cohorts (summer and overwintering), they both performed better under test conditions after only 24 hr of laboratory acclimation, compared to subsequent control organisms exposed to the test conditions after the additional time (48 hr) of acclimation. Organisms used for the May tests were far more sensitive in survivorship with the reduced acclimation time. After seven days, all test organisms were dead that had only 24 hr for acclimation, while organisms that were allowed the additional 24 hr had significantly greater survivorship after 14 days of testing. The developmental stage and time in which these organisms were collected may have played a large role in their susceptibility for handling stress. As reported by Kondratieff and Voshell (1984), *I. bicolor* for this cohort normally begin emergence in Sinking Creek around early May, with stragglers continuing to emerge through June. Mayflies used for testing varied in developmental stage so it

could be determined which stage of development appeared to be the most sensitive to handling stress. As expected, mayflies that were in the latter stages of nymphal development (dark swollen wing pads) were the first to die in both the 24 hr and 48-hr acclimation tests. As these organisms approach adulthood and transformation to the winged stage (subimago and imago), it is possible that the major developmental changes occurring, make them more sensitive to stress. In addition, the minor shift in temperature from 16°C, at collection, to 20 ± 2°C in the lab may have been too drastic of a temperature change during this particular stage of development. The best control survivorship results for these comparison studies occurred during November 2009, with survivorship of 95 and 80 % after 14 days for the 24 vs. 48 hr acclimated organisms. The 24-hr acclimated organisms responded better in test conditions, but these differences were minor. This could possibly be due to absolutely no change in temperature from field to laboratory conditions (11°C).

Toxicity Testing

Control Survivorship

Mayfly survivorship in Sinking Creek control beakers served two purposes, the first was to validate toxicity test results and the second was to determine sensitivity differences between mayflies collected during each season in 2009. Under routine testing conditions in toxicity tests with well-developed methodologies, test validation is based on acceptable (>80 or 90%) survival of control organisms; however, for the purpose of determining appropriate test duration, mayfly tests remained running past the point of acceptable control survivorship.

Isonychia survivorship was variable, even within each quarter of testing and was directly linked to test temperature (Table 4.2). Control survivorship was the highest (100%) for the entire 14-day test period during the first toxicity test conducted (January 2009). A second test

conducted that month yielded similar survival data with 95% of the organisms surviving over the 11-day test. These tests were conducted at 9°C, only five degrees warmer than ambient conditions at the time of collection. Tests that were conducted at 20°C in January had much lower survivorship (75 and 85%), and were terminated after the seventh day. Test organisms collected and tested the following month in February had significantly lower survivorship (35%) after seven days. This low survivorship was surprising considering tests conducted only a few weeks prior in January at 20°C were twice as high for control survivorship. Therefore, even during similar collection and testing conditions, survivorship was variable.

During the second quarter of testing, control survivorship was much higher than that which was observed in February (Table 4.2). During the April test, survivorship was 90% after seven days, and 80% at days 10 and 14. Organisms were tested at 8°C above ambient conditions at the time of collection; however, mayflies tested in May with a slight 4°C difference in temperature (collection vs. test), had only 75% survivorship after seven days, and dropped to 70 and 65% at 10 and 14 days. Testing during the third quarter was more difficult due to the limited availability of organisms, particularly in June. Additionally, organisms used for testing in July and August were from the summer cohort. Control survivorship was the lowest observed during the year-long study at 25% in the July test after six days and 20% in the August test after seven days. Organisms collected on July 31, 2009 had significant mortality during laboaratory acclimation and therefore, no testing was initiated. *Isonychia* from the summer cohort may inherently be more sensitive due to the rapid development they undergo during the warm summer months. These organisms generally hatch in late June-early July and develop quickly during July and August, with typical emergence in late August and early September.

Testing conducted during the fourth quarter, once again utilized organisms from the over-wintering cohort (Table 4.2). Seven-day control survivorship was improved (50%) for the September test, but still quite low compared to established acceptable ranges (80-90%). Survivorship was much better in the following test, conducted in October with 7-day survivorship at 75%. Temperature acclimation was again a factor for this round of testing as the difference between stream and test temperature was ~10°C.

Control survivorship for tests conducted in November was substantially higher, ranging from 90-95% after seven days, and 80-95% after 14 days. Two of these tests were conducted at ambient conditions (11°C). A third modified test was conducted at 20°C. Survivorship was 90% after the initial seven days, but declined quickly (55%) by day 10 and day 12, after which the test was terminated.

Temperature was thought to play a major role in control survivorship and was therefore, examined more closely. The relationship between collection and test temperature and control survivorship after 7, 10 and 14-day intervals was examined using mutivariate analysis (Table 4.3). Correlation analysis (correlation coefficients or r-value) measures the strength and direction of the relationship between two variables, whereby $-1 \leq r \leq +1$. Correlation coefficients for collection temperature and survivorship showed a moderate negative relationship, while test temperature had a much stronger negative correlation with control survivorship. This relationship was strongest for 10-day survivorship ($r= -0.8797$), meaning that as test temperature increased, control survivorship decreased. Coefficients of determination (R^2) values explain the variation in this relationship and help make predictions based on the data. These values were again low for collection temperature comparisons ($r=0.1879$ and 0.3897) at seven and 10 days, but much higher at 0.7729 for 14-day survivorship. In contrast, test

temperature and 10-day control survivorship had an r^2 value of 0.7845, meaning ~78% of the variance in 10-day survivorship could be explained by the test temperature. These results support our assumption that the test temperature plays a vital role in test acceptability, regardless of the temperature at the time of collection. However, these results raise some interesting questions regarding mayfly testing and temperature. Of the 14 tests conducted, 10 tests had greater than 75% control survivorship after seven days, while two tests had less than 30% survivorship. The two tests resulting in the lowest control survivorship were conducted when collection and test temperatures only differed by ~ 4°C. Although temperature adjustments seem to be a problematic factor in these types of tests in which field collection is necessary for obtaining test organisms, tests conducted under seemingly ideal temperature conditions, appear to be more unrealiable.

Maccaffertium control test survival was more consistent compared to *Isonychia* (Table 4.4). Overall, 7-day percent survivorshp ranged from 75-100%, while 10-day survivorship was 80-95% except for one occassion, in which survivorship after 10 days was 55%. Test survivorship was more variable past ten days, ranging from 95 to 45%. Although fewer tests could be conducted with *Maccaffterium*, due to limited availability, control survivorship was much more reliable and consistent between quarters, regardless of test temperature.

Correlation coefficients for *Maccaffertium* tests indicated a weak negative (r=-0.1645) relationship between control survivorship and collection temperature after seven days of testing and moderate negative relationships (r=-0.5413) between survivorship and collection temperature at days 10 and 14 (Table 4.5). *Maccaffertium* control survivorship was more strongly correlated with test temperature, especially after 10 days of testing (r=-0.9096), which was the trend also observed with *Isonychia*. The percent of variation (R^2) explained in both the

collection and test temperature comparisons to control survivorship were low, ranging from 2-25% for collection temperature and 4-25% for test temperature, showing that very little linear association between the variables.

Sensitivity Comparisons to a Reference Toxicant (NaCl)

Reference toxicant (NaCl) tests were used to gauge mayfly sensitivity under test conditions. No Observed Effect Concentrations or NOEC values for survivorship were used to compare organism responses during each season of testing. Seven-day NOECs for *Isonychia* nymphs were highest (>8 g NaCl/L) during winter testing when test temperature was 9°C; however, when tests were conducted at 20°C (tests 3-5), NOECs were dramatically lower (1-2 g NaCl) (Fig.4.6). Results were similar for tests conducted in spring 2009 and for the first test (#8) during summer 2009 (1-2 g NaCl/L), but results of the second summer test (test # 9) had an NOEC at the control.

Both of these tests utilized organisms of the summer cohort and although they were conducted less than a month apart, nymphs were at different developmental stages which may have contributed to the difference in test response. Organisms used for the earlier tests in July (# 8) were classified as early instar (< 5-6 mm in length); while organisms used in the August test (#9) were closer to mid-development (7-9-mm). Remaining tests during the fall 2009 again used the overwintering cohort of organisms, but test NOECs remained low. The NOEC for a test conducted in October 2009 resulted in an NOEC at the control. Test results were improved for the final test conducted in November 2009 at ambient temperature (11°C); however, they were still substantially lower than NOECs from the initial tests conducted in January 2009.

Geometric means were calculated and used to determine the central tendency for 7-day NOEC values (Table 4.6). Average NOECs were highest during the winter at 3.03 mg/L (± 3.49

mg/L) due to the unually high NOEC for the first two toxicity tests. Values were lowest during the fall testing season (1.0 ± 1.06 mg/L). Means calculated for the summer (< 2) and fall did not reflect NOECs at the control level. The overall geometric mean of NOECs calculated for the 7-day exposure to NaCL for *Isonychia* was 2.0 mg/L (± 2.77 mg/L). When the first two tests with the high NOECs (> 8g/L) were excluded from calculations, the geometric mean decreased to 1.41 mg/L (± 0.62 mg/L), a more reliable average.

Fourteen-day NOECs were much less reliable than those for 7-day survivorship results. One test conducted in winter 2009 at 9°C had a NOEC of 4 gNaCl/L, while a subsequent test conducted the following week had a 11-day NOEC of 4 gNaCl/L, but was terminated early due to loss of the testing chamber. Fourteen-day testing only occurred five times in 2009 and three of those tests resulted in NOECs at the control level.

Maccaffertium sp. were also evaluated to determine sensitivity to NaCl using NOEC (Fig. 4.7). Survivorship NOECs were highest for the first two tests conducted (1 and 2), although endpoint values were very different for the two tests as the NOEC for the first test was 4 g NaCl/L, while the following test conducted less than two weeks at the same temperature (9°C) later had a 7-day NOEC of 8 g NaCl. With the exception of test 7, which was conducted at 11°C, all remaining tests were conducted at 20-21°C. Therefore, test temperature did not appear to be a factor in NaCl sensitivity. Survivorship NOECs for tests 3-7 ranged from 0.5-2 g NaCl/L with the majority of endpoints being at 2 g NaCl/L.

Geometric means were also calculated to determine a centralized average for survivorship NOECs. The overall average, which included all test endpoints, was 2 g NaCl/L (± 2.55 SD) (Table 4.7). Since the second test NOEC (8 g NaCl/L) conducted during the winter was substantially higher compared to the other tests, the geometric mean was recalcuated,

excluding this test, which lowered the average to 1.59 (± 1.2 SD). When averaged by season, NOECs were highest (3.36) for winter tests, and consistent (1.0) for tests conducted during the spring and fall, although fewer tests were able to be conducted during the spring and no tests were conducted in the summer due to the lack of mayfly availability. Addionally, the surviorship NOEC for the spring (1 g NaCl/L) also reflected the emergence of several test organisms during the test.

Fourteen-day NOECs were unreliable as only four tests were carried out for the additional seven days. Three out of the four tests had survivorship NOECs at the control concentration.

Exuvia as a Sublethal (Growth) Endpoint

Quantitative measurements of subchronic exposure responses are necessary to establish a sub-lethal toxicity test endpoint; however, establishing the appropriate and accurate method of measurment can be difficult. Standard toxicity tests with readily used test organisms such as fathead minnows *(P. promelas*) or the amphipod (*Hyalella azteca*) utilize a growth measurement, either as final weight or change in length, as a sublethal endpoint. Some researchers propose the use of molting, or exuvia production as a method of determining subletal responses in laboratory mayfly tests (Henry et al. 1986, Pontasch et al. 1989, Giesy et al. 1990, Diamond et al. 1990, Diamond et al. 1992), while others observed molting response to be a poor indicator of dose-dependent responses (Kennedy et al. 2004). It is also suggested that molting could be a response to handling stress, temperature change (Reece Voshell, personal communication)or a mechanism for shedding certain pollutants which may be absorbed onto the exoskeleton or gill surfaces (Kormonday 1965, Smock 1983, Row et al. 1989, Diamond et al. 1992). The number of exuvia

produced by *Isonychia* over a 7-day test period were calculated, along with geometric means and incipient response values at the concentration by which 25% of organisms showed an inhibited growth (IC_{25}) (Table 4.8). The number of exuvia observed per concentration for each test conducted was not useful in establishing dose-dependent response trends or seasonal sensitivity. In fact, the number of exuvia appear to be random between test concentrations and toxicity tests in general.

In order to determine potential trends, geometric means of exuvia for all *Isonychia* tests in 2009 were calculated and compared amongst test concentrations (Table 4.9 and Fig. 4.8). Overall geometric means indicated a dose-dependent reponse occurred, with averages being highest (6.93) in the controls, followed by 5.94 (0.5 g NaCl/L) and 4.66 in the 1 g NaCl/L concentration. Averages decreased in the remaining test concentrations from 3.89 (2 g NaCl/L) to 1.52 (8 g NaCl/L). Test concentrations greater than 8 g NaCl/L were used on four occasions, and were 10, 12 and 16 g NaCl/L. Only the 10 g NaCl/L concentration had a calculated mean (1.0) as no exuvia were observed in the 12 or 16 g NaCl/L concentrations due to complete mortality which occurred within the initial 24 hr of testing.

Growth inhibition, as IC_{25} values, were calculated for each test and then averaged for each season (Table 4.8, Fig. 4.9). These values were quite variable between tests and ranged from 0.185 to 5.50 g NaCl/L.

Exuvia IC_{25}'s were also compared between seasons in order to determine any seasonal changes in *Isonychia* sensitivity (Fig. 4.10). Using geometric averages of IC_{25} data, exuvia were grouped by season. Spring and summer tests had the highest (2.71 and 1.41) averages, while winter and fall were substantially lower (0.979 & 0.61, respectively). There appears to be no

direct relationship between molting and either survivorship NOECs or control survivorship between seasons.

Because no observable trend was apparent between IC_{25} values during the 2009 study, these values were statistically compared to survivorship NOECs and control survivorship (%) (Figs. 4.11 & 4.12). No substantial correlation between exuvia IC_{25} values and survivorship NOECs (r=0.2758) was observed. There was a moderate correlation (r=0.4620) between control survivorship and the exuvia $IC_{25's.}$ In several tests, as control survivorship increased, so did the number of exuvia produced; however, this was not consistent throughout all the tests. Linear regression models also indicated no significant relationship between IC_{25}'s and survivorship NOECs (r^2= 0.1233, p=0.3198) or IC_{25}'s and control survivorship (r^2=0.041, p=0.5233).

The number of exuvia observed in *Maccaffertium* tests were substantially less than that which was observed in the *Isonychia* bioassays (Fig. 4.13). Geometric means were used to determine if any dose-dependent molting response to increased NaCl concentration was occuring for *Maccaffertium* nymphs, as the number of exuvia produced per concentration/test did not indicate any observable trends (Table 4.9). Average exuvia produced was highest in the 0.5 g NaCl/L concentration (4.93) followed by 3.80 for control organisms. Geometric means dropped to 2.24 at the 1 g NaCl/L concentration, but increased to 3.09 average exuvia for the 2 g NaCl/L concentration. The highest concentrations tested (4-10 g NaCl/L) had an average range of 1.15-0.0 exuvia/ concentration. Unlike *Isonychia*, *Maccaffertium* test organisms did not exihibit a dose-dependent response to NaCl. Inhibition concentrations (IC_{25}) values averaged 1.52 g NaCl/L overall for the 2009 tests, but fluctuated between 0.64 and 3.5 g NaCL/L for the seven tests conducted. Data were not compared seasonally due to the lack of results from the spring and summer seasons of testing.

Statistical comparisions of *Maccaffertium* exuvia results (IC_{25} values)were compared to several test parameters to determine possible correlations. Exuvia IC_{25} values were weakly correlated with control survivorship (r=0.1725) and moderately correlated with NOEC survivorship (r=0.4875). The most significant relationship was between exuvia IC_{25}'s and test temperature, which had a strong negative correlation (r= -0.9119). As test temperature increased, the number of exuvia decreased.

Summary and Conclusions

The overall goal of this study was to determine the feasibilty of using field collected mayflies in laboratory bioassays. *Isonychia bicolor* and *Maccaffertium* sp. nymphs were collected locally from Sinking Creek (Giles County, Virginia) and tested using a reference toxicant (NaCL) and protocols based on WET test guidelines (USEPA 2002 a, b). The one-year study examined seasonal availability, acclimation techniques, test duration, sensitivity and exuvia production as sublethal endpoints.

In comparison, *Isonychia* nymphs were more readily available than *Maccaffertium i*n both abundance and seasonal availability. Both organisms were most abundant during the winter of 2009, resulting in 31 and 49% of total organisms collected. Summer was the most difficult time to collect both species with ~ 100 *Isonychia* (n=5) and < 100 *Maccaffertium* collected per sampling trip. *Maccaffertium* were unavailable beginnning in May due to April emergence and were not observed during sampling attempts until late July when early instar (< 5mm) nymphs were obtained in low abundance (50 per sampling trip). *Maccaffertium* were not readily available for testing again until August. Therefore, during summer months, collection of both species was difficult which prevented routine testing.

Laboratory acclimation was comprised of temperature acclimation during winter months and duration during spring and summer months when ambient and testing conditions were similar. During winter months, when collected at cold tempertures, mayflies required a longer acclimation period by which temperature was gradually increased no more than three degrees in a 24-hr period. Acclimation duration during the warmer summer months was not dependent on this incremental temperature change, and therefore, acclimation duration (24 vs. 48 hr) was compared using control survivorship. Acclimation to increasing temperatures was necessary during the winter months; however, organisms appeared more tolerant to handling stress at colder temperatures as fewer organisms died during this holding period. Summer cohort nymphs were more sensitive to handling stress resulting in increased mortality during laboratory holding times. Variability in acclimation responses may be due to differences in development rates.

Seven-day NOEC values were used for determining differences in NaCl sensitivities. The NOEC values for *I. bicolor* were highest during winter months when tested at 9°C. At higher test temperatures (i.e., 20°C), NOECs dropped substantially and remained low. The overall geometric mean was 2 g NaCl/L (±2.77). When the two highest NOEC values were omitted from statiscial analysis, averages dropped to 1.41 g NaCl/L.

Maccaffertium sensitivity responses were similar to *I. bicolor* with the two highest NOECs generated at 9°C. Although test temperature would appear to have contributed to decreased NOEC values in subsequent testing, test temperature was not significantly correlated with control survivorship. Additionally, a chronic test conducted in November 2009 at ambient conditions (11°C) had a similar 7-day NOEC for survivorship (2 g NaCl/L) to those conducted previously at higher temperatures (20-21°C). Therefore, life history factors, such as stage of development could have a greater impact on organism sensitivity than test factors such as

temperature. The overall geometric mean for *Maccaffertium* survivorship NOECs was 2 g NaCl/L (±2.55), but was much lower at 1.59 (±1.2) when the average was recalculated to exclude test 2 which had a 7-day NOEC of 8 g NaCl/L.

Fourteen-day toxicity tests were conducted five times in 2009 for *Isonychia* and four times for *Maccaffertium*. Three of the 14-day NOECs for *Isonychia* and *Maccaffertium* survivorship were at the control level. These levels are considered unreliable due to the limited number of tests conducted at this extended duration.

The number of exuvia produced per concentration was also examined to determine the appropriateness of using this quantitative measurement as a sublethal endpoint. Although some authors advocate its inclusion as an endpoint, others have argued that exuvia could be more of an indication of handling stress and not growth. The number of exuvia per concentration per test as well as IC_{25} values indicated no dose-dependent response was occuring as exuvia production was variable; however, geometric means [which calculates a centralized average], for all *Isonychia* tests conducted in 2009 indicate a dose-dependent respose did occur. Averages were highst for control organisms (6.93) followed by the 0.5 g NaCL/L concentration (5.94) and so on to the highest concentration tested reguarly (8 g NaCl/L, 1.52 exuvia). No exuvia were observed in higher concentrations of 12 and 16 g NaCl/L as 100% mortality occurred within the first 24 hr of testing.

The number of exuvia produced by *Maccaffertium* was substantially less than those observed in *Isonychia* tests and no dose-dependent response of exuvia per concentration was observed. Overall geometric means for all 2009 *Maccaffertium* tests also indicated no dose-dependent response was occuring as averages varied from the highest (4.93) in the 0.5 g NaCl/L concentration to 3.80 for control organisms followed by 2.24 at 1 g NaCl/L. The average again

increased to 3.09 in the 2 g NaCl/L concentration, and then declined in the highest concentrations (4-10 g NaCl/L). Inhibition concentrations (IC_{25}) also indicate no discernable trends; however, these values did have a strong negative correlation with test temperature (r=-0.9119).

Based on the results of this feasibility study, mayflies in general would be good test organisms for determinig the potential toxicological effects of an effluent on coal-mining impacted streams. However, field collected organisms appear to be too unpredictable in test responses and, therefore, such tests would be unreliable as stand-alone indicators of effluent toxicicity. Although both organisms chosen for this study, *Isonychia bicolor* and *Maccaffertium* sp., are bivoltine, having two generations per year, availability throughout the year was still difficult. There was too much variability in seasonal availability to allow for routine collection and testing. Additionally, differences in developmental rates between cohorts (summer vs. winter) may be a contributing factor in handling stress and also sensitivity responses.

Isonychia bicolor may not be the most suitable mayfly to be used in laboratory testing due to susceptibility of handling stress and flow requirements. Due to the stream-lined physical characteristic and lateral gill placement, damage can easily occur during handling. Different tools were used during this study to minimize this stress, including modified (cut) pipettes, soft-touch forceps, fish nets and turkey basters to transfer organisms from holding apparatus to test beakers; however, the only items that worked sufficiently were the combination of the fish net and soft-touch forceps to catch the mayflies and gently transfer them to the beakers. *Maccaffertium* were much hardier when it came to handling stress and were also easier to work with as they are not as proficient at swimming as *Isonychia;* therefore, they were less likely to be injured during handling. *Isonychia* nymphs also have a greater flow/current demand which is

difficult to maintain in static-aerated only beakers. Stir-plate tests that were not included in this study (Echols unpublished data) show improved control survivorship (95-100%) after 14-days, despite warmer test temperatures (21-23°C). Although *Maccaffertium* were used less frequently for laboratory testing due to lack of availability, control survivorship for these organisms on average was better (87.9%) than *Isonychia* (67.9%) even when outliers were removed from calculations (77%). Geometric averages for survivorship NOECs were also comparable, but somewhat lower for *Maccaffertium* (1.20 g NaCl/L) than *Isonychia* (1.41 g NaCl/L). Additional Heptageniidae mayflies, such as *Epeorus* sp. or *Stenacron* sp. could be examined as potential test organisms as they are more prevalent in headwater streams where concern for coal-mining impact is greatest.

Mayflies serve a great purpose in field bioassessments and utilization in laboratory toxicity assessments should continue to be pursued as an additional tool for the protection of freshwater ecosystems, especially those impacted in coal-mining impacted watersheds. However, based on these results, mayfly test results should be used in conjunction with field bioassessments and additional, standardized toxicity tests with the routine test organisms, such as *C. dubia*. Future research in this area should focus on the development of culturing methodology, which would allow researchers to conduct more frequent tests and would also help to eliminate the variability between seasonal/quarterly testing.

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Table 4.1. Field collection summary of collection attempts for *Isonychia bicolor* and *Maccaffertium sp*. nymphs from Sinking Creek, Giles, County, Virginia during 2009.

Collection Date	Water Temperature (°C)	Species	Number Collected	Size Class (Length in mm)	Development	Comments
1/3/2009	4.0	*Isonychia*	200	7-10	Mid-development	
1/3/2009	4.0	*Maccaffertium*	150	5-10	Mid-development	
1/12/2009	5.0	*Isonychia*	300	7-10	Mid-development	Easily collected due to abundant availability.
1/12/2009	5.0	*Maccaffertium*	150	5-10	Mid-development	
1/23/2009	4.0	*Isonychia*	260	7-10	Mid-development	Easily collected.
1/23/2009	4.0	*Maccaffertium*	250	5-10	Mid-development	More plentiful in each dip net sample compared to previous collection attempts.
2/7/2009	4.0	*Isonychia*	250	7-10	Mid-development	
2/7/2009	4.0	*Maccaffertium*	150	5-10	Mid-development	
4/16/2009	12.0	*Isonychia*	300	5-12	Various-head capsule width 0.5-1.0mm	Easily obtainable.
4/16/2009	12.0	*Maccaffertium*	150	12-20	Mostly late instar with dark swollen wing pads	Sparse in samples. Much further along in development compared to *Isonychia.*
5/20/2009	16.0	*Isonychia*	350	10-17	Various- some with light wing pads and some with dark swollen wing pads	Water level at SC elevated and turbid. No *Maccaffertium* collected because only a few organisms were collected in dip net samples.
6/19/2009	18.0	n/c	--	--	--	Water at Sinking Creek too high for collection due to high precipitation amounts in June.
6/24/2009	18.0	*Isonychia*	13	14-17	Late instar- dark swollen wing pads	No *Maccaffertium* observed in any dip net sample and only 13 total Isonychia collected after 2+ hrs of collection attempts.
7/17/09	19.0	*Isonychia*	30	< 5	Early instar	Riffle area disturbed at Sinking Creek sampling location (man-made dam); Some late instar (~10) *Isonychia* also collected with dark, swollen wing pads, most likely stragglers from over-wintering cohort.
7/17/09	19.0	*Maccaffertium*	30	< 5	Early instar	
7/24/09	18.5	*Isonychia*	200	< 5-13.5	Mostly early instar with few larger mature nymphs	Deconstructed dam before collection, which facilitated collection.
7/24/09	18.5	*Maccaffertium*	50	<5	Early instar nymphs	Not readily available.
7/31/09	18.5	*Isonychia*	200	6.5	Early-mid instar summer nymphs	Most collected w/o wing pads, suggesting the available organisms are all from the summer cohort.
8/12/09	19.0	*Isonychia*	165	7-9	Mid-development	
9/2/09	17.0	*Isonychia*	155	11-13	Wing pads and some late instar nymphs	Sample size per dip net sample < 20 (low). *Maccaffertium sp*. also low in number.

Table 4.1. Continued

Collection Date	Water Temperature (°C)	Species	Number Collected	Size Class (Length in mm)	Development	Comments
9/16/09	17.5	*Isonychia*	120	<5-14	Mostly late instar nymphs with dark/swollen wing pads; very few early instar	Presence of small (< 5mm) nymphs indicate over-wintering cohort has begun to hatch.
9/23/09	19.5	*Isonychia*	140	3-6	Early instar	Some late-instar nymphs present in collection, but very few. Majority of *Isonychia* were small, early instar nymphs.
9/23/09	19.5	*Maccaffertium*	100	<2-6	Early instar	Not as plentiful as *Isonychia* but definitely more prevalent than in previous sampling events over the past 3+ months.
10/21/09	10.0	*Isonychia*	300	≤3-9	Some very early instar to mid-development	Organism availability very high. Only organisms 5-9 mm collected.
10/21/09	10.0	*Maccaffertium*	50	4-9	Some very early instar but mostly mid-development	Not readily available. Organism density per sample very low.
11/14/09	11.0	n/c	--	--	--	Sinking Creek at bank-full; water too high for collection.
11/17/09	10.5	n/c	--	--	--	Water level receding but still too high (3/4 bank-full) for collection.
11/21/09	10.8	n/c	--	--	--	Water levels still receding, still turbid with high flow.
11/24/09	11.0	*Isonychia*	275	5-11	Wide range of size classes, a few with light colored wing-pads	
11/24/09	11.0	*Maccaffertium*	175	5-10	Mid-development	Organism availability very high; density in dip-net samples consistent with collections earlier in 2009.

* n/c= not collected/not accessible/available.

Table 4.2. Comparison of control survivorship for *Isonychia bicolor* over 7-14 day test duration and relative collection and test temperatures (°C).

Month	Collection Temp (°C)	Test Temp (°C)	*Temp. Difference*	Control Survivorship		
				7 day	10 day	14 day
January	4	9	*5*	100	100	100
January	4	9	*5*	95	95	95*
January	4	20	*16*	75	n/a	n/a
January	5	20	*15*	85	n/a	n/a
February	4	20	*16*	35	n/a	n/a
April	12	20	*8*	90	80	80
May	16	20	*4*	75	70	65
July	18.5	23	*4.5*	25**	n/a	n/a
August	19	23	*4*	20	n/a	n/a
Sept.	19.5	21	*1.5*	50	n/a	n/a
October	10	20	*10*	75	n/a	n/a
November	11	11	*0*	90	90	80
November	11	11	*0*	95	95	95
November	11	20	*9*	90	55	25***

*Test terminated at 11 days due to loss of test chamber.

** Test terminated at 6 days due to mortality.

*** Test terminated at 12 days due to mortality.

Table 4.3. Multivariate correlation and linear regression of collection and test temperatures (°C) relative to *Isonychia bicolor* control survivorship at 7, 10 and 14 days (JMP 8.0).

Test Day	Collection Temperature (°C)					Test Temperature (°C)				
	n	**Mean Temp (range)**	**Correlation (r-value)**	**R^2**	**Prob. > |t|**	***n***	**Mean Temp (range)**	**Correlation (r-value)**	**R^2**	**Prob. > |t|**
7	14	10.6 (4.0-19.5)	-0.4477	0.1879	0.1389	14	17.6 (9.0-23.0)	-0.6565	0.4271	0.0154*
10	7		-0.5995	0.3897	0.1340	7		-0.8797	0.7845	0.0080*
14	6		-0.5219	0.7729	0.0495*	6		-0.7541	0.6462	0.1012

*indicates statistical difference (α=0.05)

Table 4.4. Comparison of control survivorship for *Maccaffertium sp.* over 7-14 day test duration and relative collection and test temperatures (°C).

Month	Collection Temp (°C)	Test Temp (°C)	Control Survivorship		
			7 day	**10 day**	**14 day**
January	4	9	95	95	95
January	4	9	90	90	90*
January	4	20	95	n/a	n/a
February	4	20	75	n/a	n/a
April	12	20	95	80	60**
Sept.	19.5	21	80	n/a	n/a
November	11	11	100	95	95
November	11	11	85	55	45

*Test terminated at 11 days due to loss of test chamber.
** Survivorship impairment reflects emergence of three individuals.

Table 4.5. Multivariate correlation and linear regression of collection and test temperatures (°C) relative to *Maccaffertium sp.* control survivorship at 7, 10 and 14 days (JMP 8.0).

Test Day	Collection Temperature (°C)					Test Temperature (°C)				
	n	**Mean Temp (range)**	**Correlation (r-value)**	**R^2**	**Prob. > |t|**	***n***	**Mean Temp (range)**	**Correlation (r-value)**	**R^2**	**Prob. > |t|**
7	14	10.6 (4.0-19.5)	-0.1645	0.0271	0.6970	14	17.6 (9.0-23.0)	-0.4013	0.1610	0.3245
10	7		-0.5413	0.2510	0.3899	7		-0.9096	0.0445	0.7334
14	6		-0.5413	0.2500	0.6667	6		-0.9096	0.2500	0.6667

Table 4.6. Geometric means for NOEC values calculated by season for *Isonychia bicolor* nymphs exposed to NaCL over a 7-day period.

Season	NOEC (g NaCl/L)	GeoMean/Season	STDEV
Winter	> 8		
Winter	> 8		
Winter	2	3.03	3.49
Winter	1		
Winter	2		
Spring	1	1.41	0.71
Spring	2		
Summer	2	<2	0.87
Summer	Control		
Fall	0.5		
Fall	Control	1.0	1.06
Fall	2		
Overall Geometric Mean		**2.0**	**2.77**
Geometric Mean (exclude. first two tests)		**1.41**	**0.62**

Table 4.7. Geometric means for NOEC values calculated by season for *Maccaffertium sp.* nymphs exposed to NaCL over a 7-day period.

Season	NOEC (g/L)	GeoMean/Season	STDEV
Winter	4		
Winter	8	3.36	2.83
Winter	2		
Winter	2		
Spring	1	1.0	n/a
Fall	0.5		1.06
Fall	2	1.0	
Overall Geometric Mean		**2.0**	**2.55**
Geometric Mean (exclude. Test 2)		**1.59**	**1.20**

Table 4.8. Exuvia results from *Isonychia bicolor* toxicity tests conducted in 2009. Gray areas indicate no data generated for that concentration during the test.

	# of Exuvia per Concentration (g NaCl/L) after 7 Days									IC_{25}	IC_{25} Geometric Means
Season	**Control**	**0.5**	**1**	**2**	**4**	**8**	**10**	**12**	**16**		
Winter	0	2	1	1	0	0				2.50	
Winter	1	1	4	3	0	1				2.64	
Winter	10	16	10	6	3	0				1.06	**0.979**
Winter	10	12	4	3	1	2				0.696	
Winter	9	2	3	3	1	1				0.185	
Spring	9	14	10	6	4	2				1.34	
Spring	4	7	5	8	6	2			0	5.50	**2.71**
Summer*	8			2	4	0		0		0.80	
Summer	7	6	5	8	2		0			2.50	**1.41**
Fall	7	11	4	4						0.704	
Fall	20	11	10	6	1	0				0.278	
Fall	7			4	3	0	1			1.167	**0.61**
GEOMEAN	**6.93**	**5.94**	**4.66**	**3.89**	**2.29**	**1.52**	**1.00**	**n/d**	**n/d**	**1.10**	
STDEV	**4.7**	**5.4**	**3.2**	**2.3**	**1.7**	**0.5**	**n/d**	**n/d**	**n/d**	**1.49**	

Table 4.9. Exuvia results from *Maccaffertium sp.* toxicity tests conducted in 2009. Gray areas indicate no data generated for that concentration during the test.

	# of Exuvia per Concentration (g NaCl/L) after 7 Days							IC_{25}
Season	**Control**	**0.5**	**1**	**2**	**4**	**8**	**10**	
Winter	2	2	1	4	1	0		2.90
Winter	1	3	0	4	0	0		2.50
Winter	6	6	2	2	1	1		0.69
Winter	5	5	4	3	1	0		1.25
Spring	7	8	7	3	1	0		1.34
Fall	9	10	1	0				0.64
Fall	3			3	2	0	0	3.50
Overall GEOMEAN	**3.80**	**4.93**	**2.24**	**3.09**	**1.15**	**1.00**	**0.00**	**1.52**
STDEV	**2.9**	**3.0**	**2.5**	**0.8**	**0.4**	**n/a**	**n/d**	**1.13**

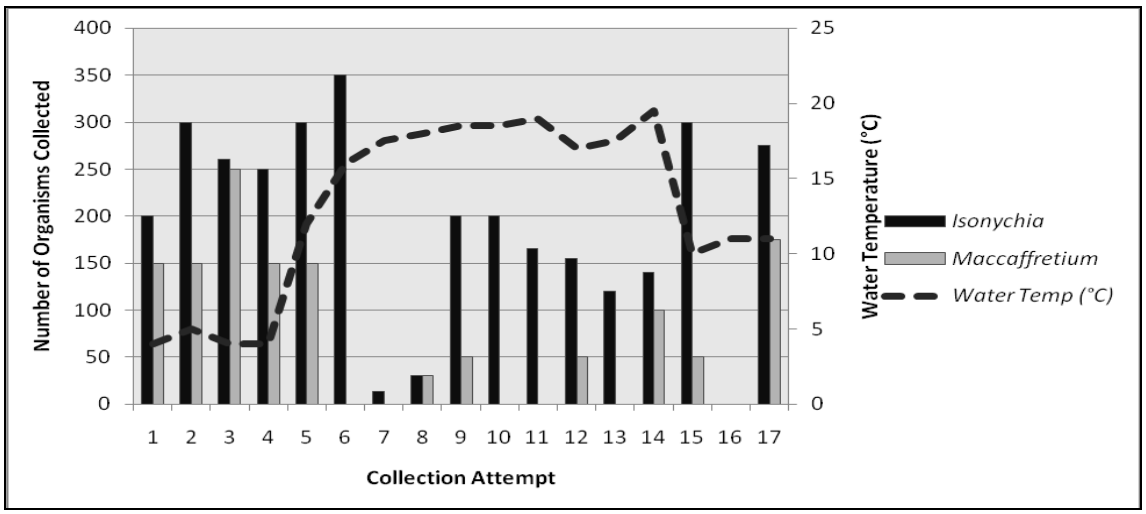

Figure 4.1. Collection of *Isonychia* vs. *Maccaffertium* nymphs from Sinking Creek, Giles County, VA 2009.

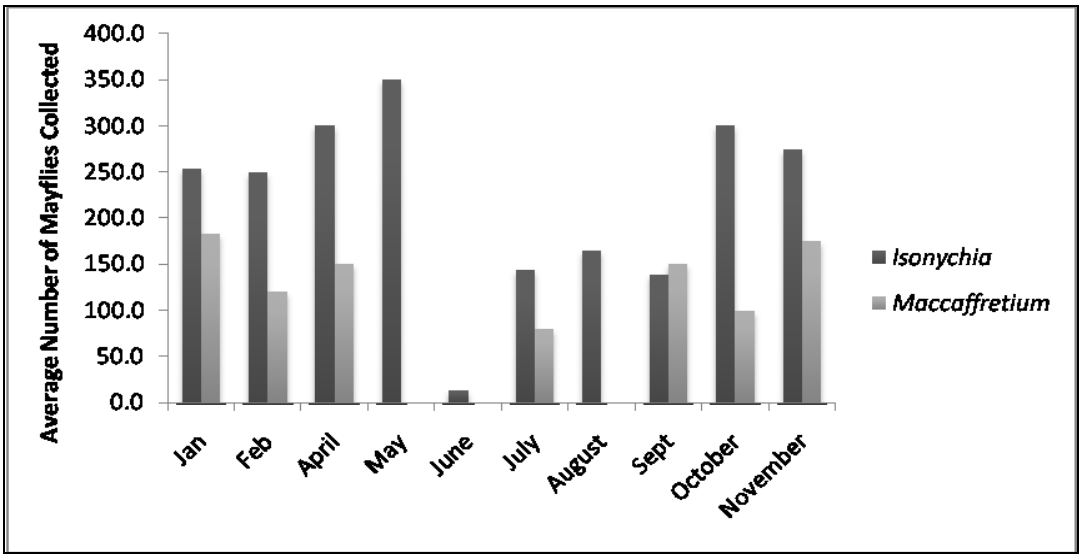

Figure 4.2. *Isonychia* versus *Maccaffertium* availability from Sinking Creek, Giles Co., VA during 2009 collection attempts. When multiple collections were conducted within the same month, numbers were averaged per species.

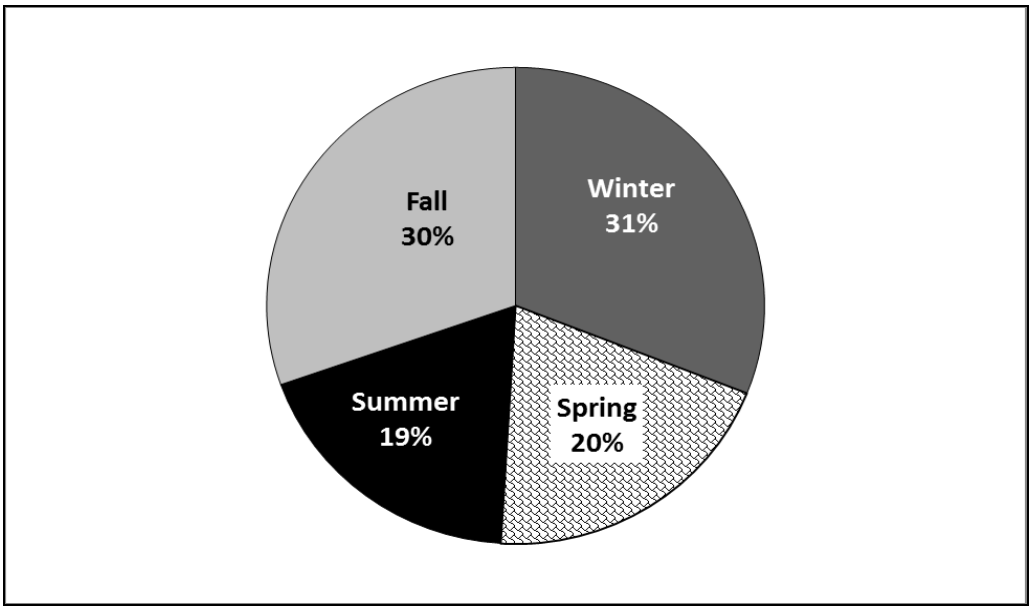

Figure 4.3. Seasonal availability of *Isonychia bicolor* from Sinking Creek, Giles County, VA.

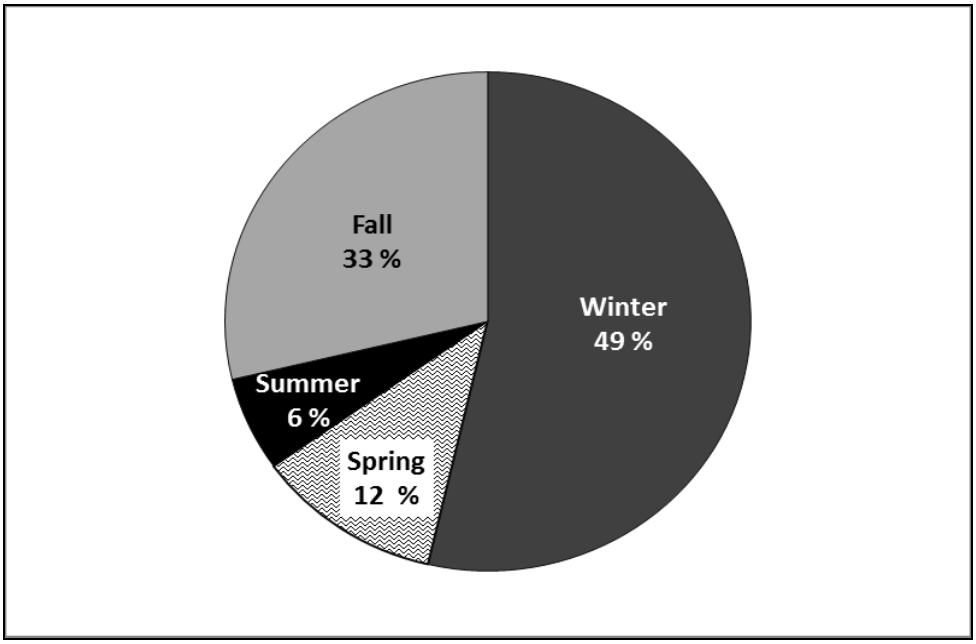

Figure 4.4. Seasonal availability of *Maccaffertium sp.* from Sinking Creek, Giles County, VA.

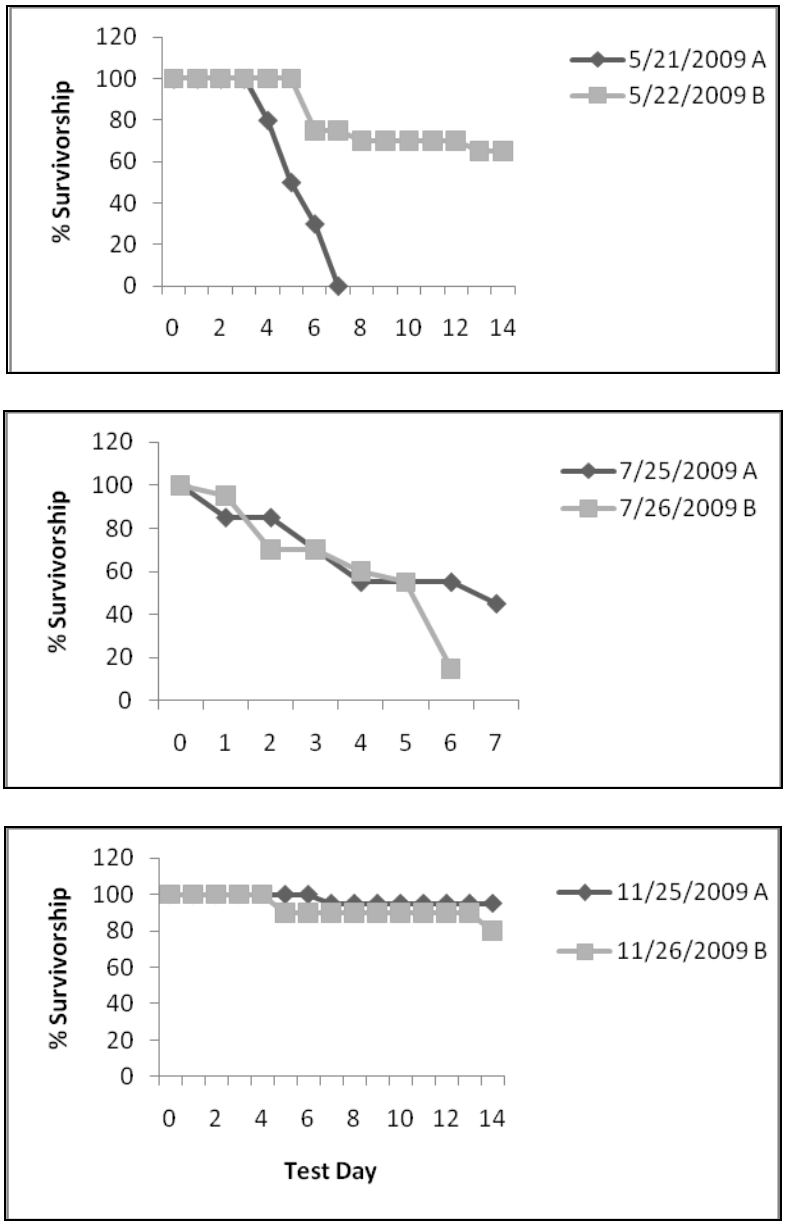

Figure 4.5. Test control comparisons for *Isonychia* acclimated for 24 (A) and 48 (B) hrs.

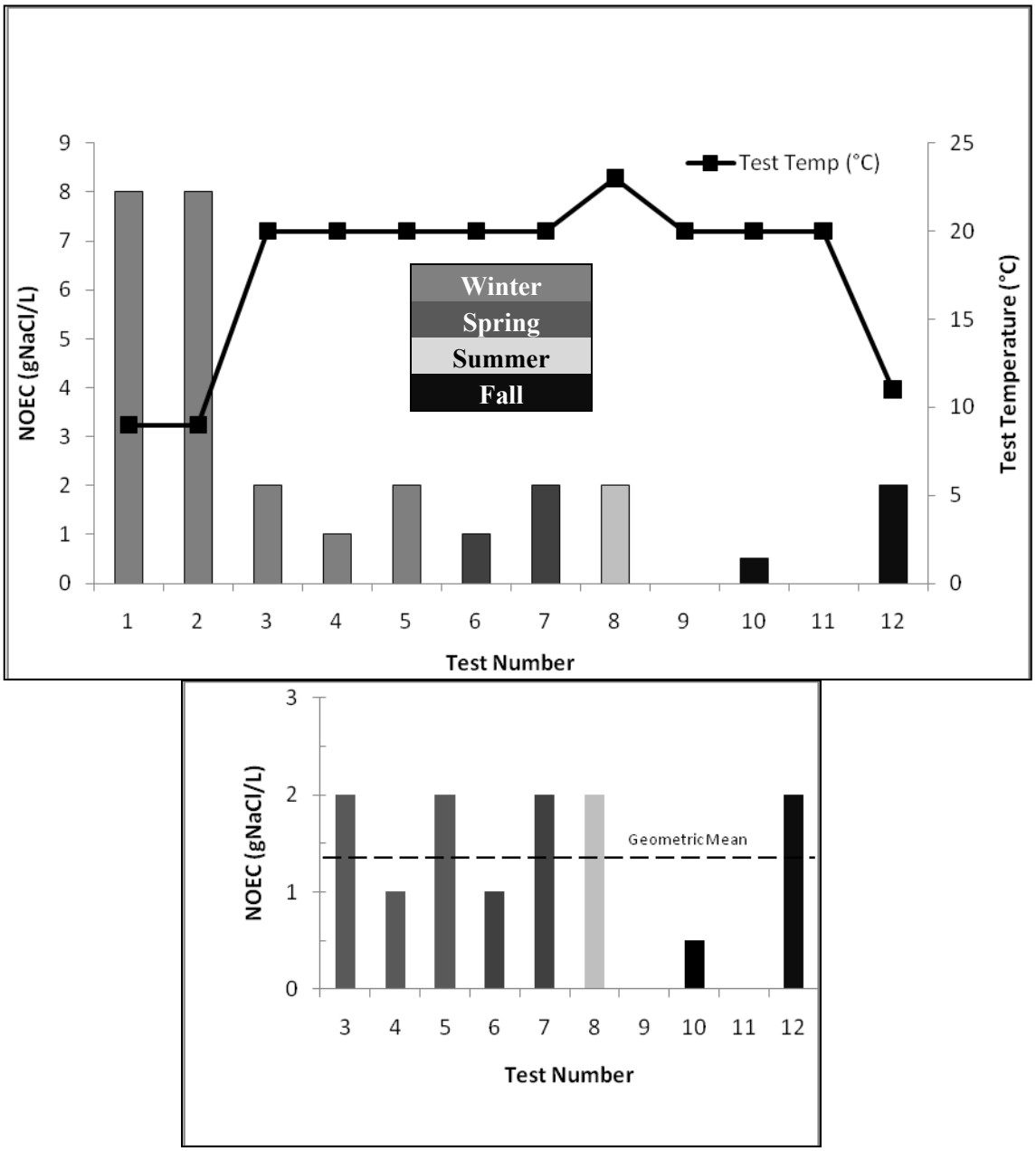

Figure 4.6. No Effect Concentration (NOEC) values for *Isonychia bicolor* reference toxicant tests using NaCl during each season in 2009. Test temperature (°C) also included for reference. Lower graph excludes first two test NOECs along with the geometric mean of tests 3-12.

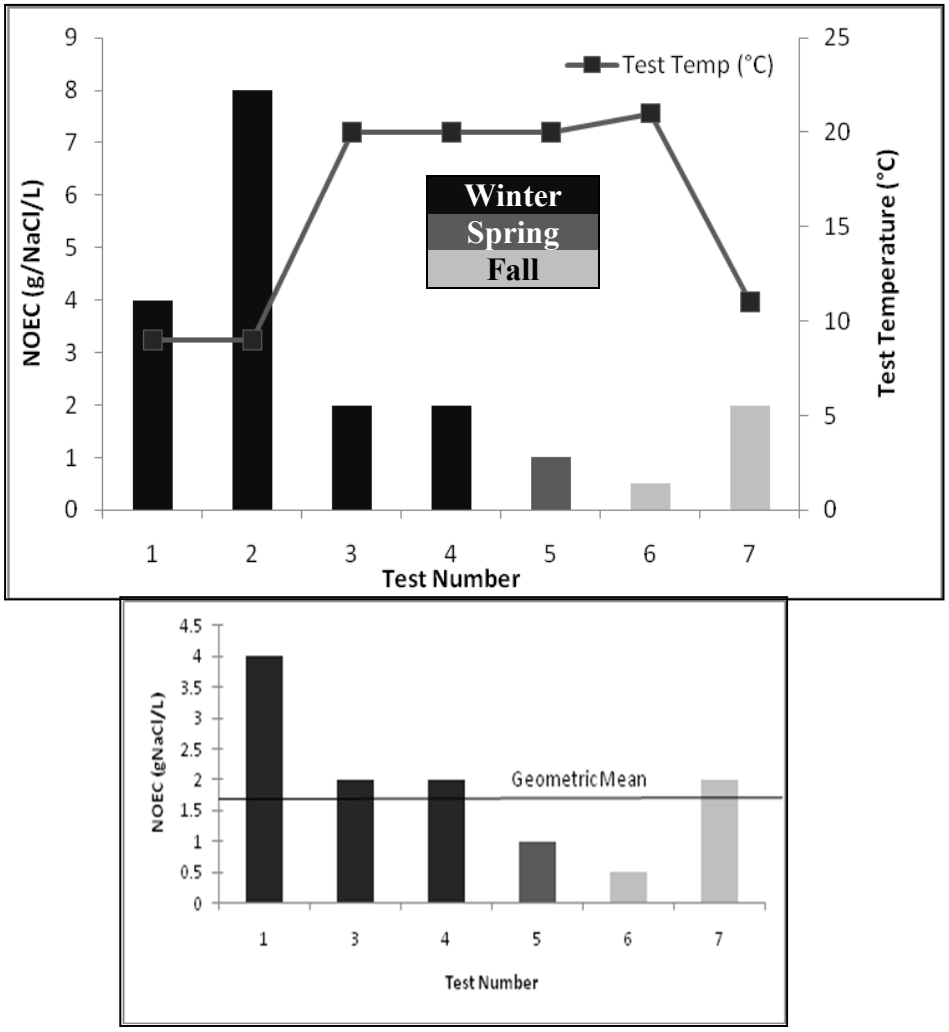

Figure 4.7. No Effect Concentration (NOEC) values for *Maccaffertium sp.* reference toxicant tests using NaCl during each season in 2009. Test temperature (°C) also included for reference. Lower graph excludes the second test NOEC, and shows the modified geometric mean for tests 1 and 3-7.

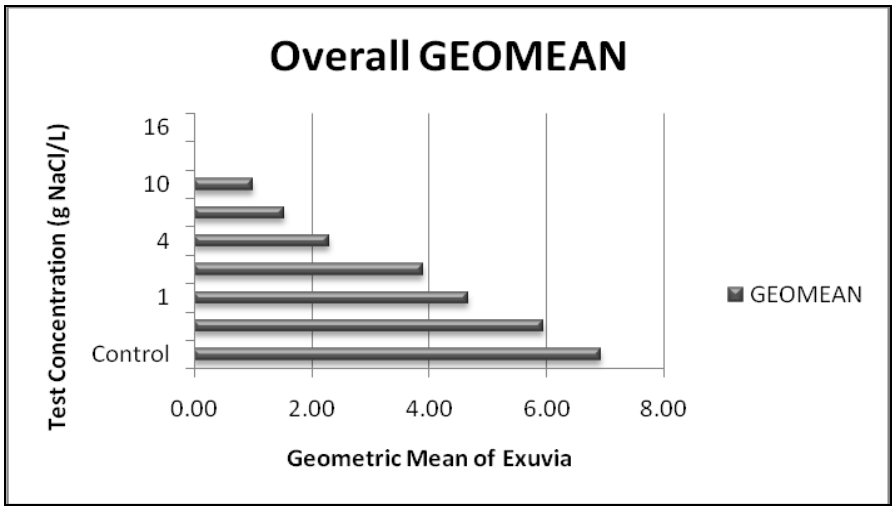

Figure 4.8. Geometric means per test concentration of NaCl for each test conducted in 2009.

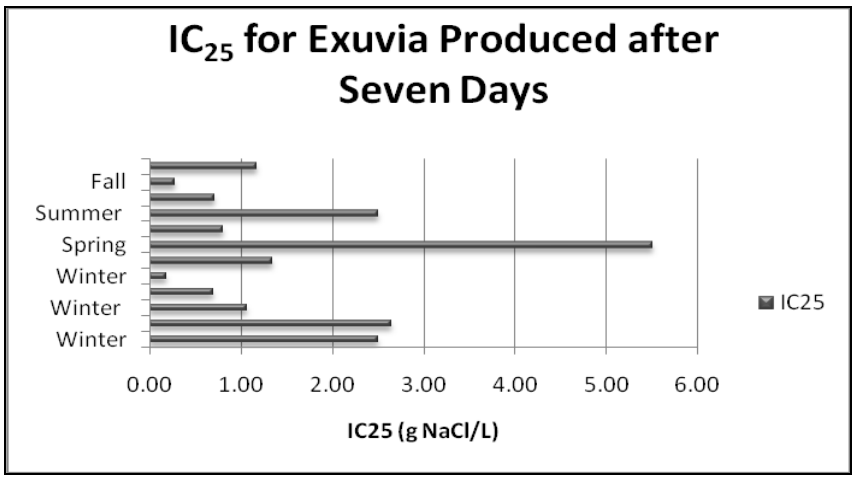

Figure 4.9. Inhibition concentration (IC_{25}) values for *Isonychia* exuvia over 7-day test periods for reference toxicity tests conducted in 2009. Summer test denoted with an asterik (*) was terminated after six days due to significant control mortality.

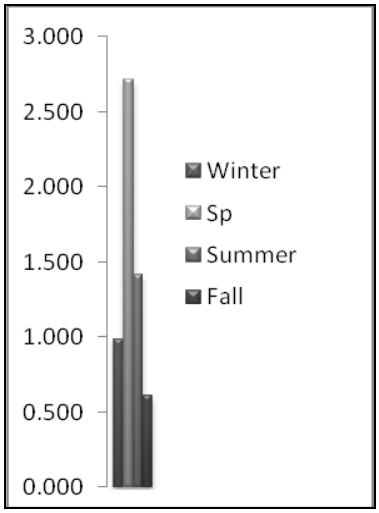

Figure 4.10. Seasonal comparison of average IC_{25} values for exuvia in *Isonychia* 7-day tests.

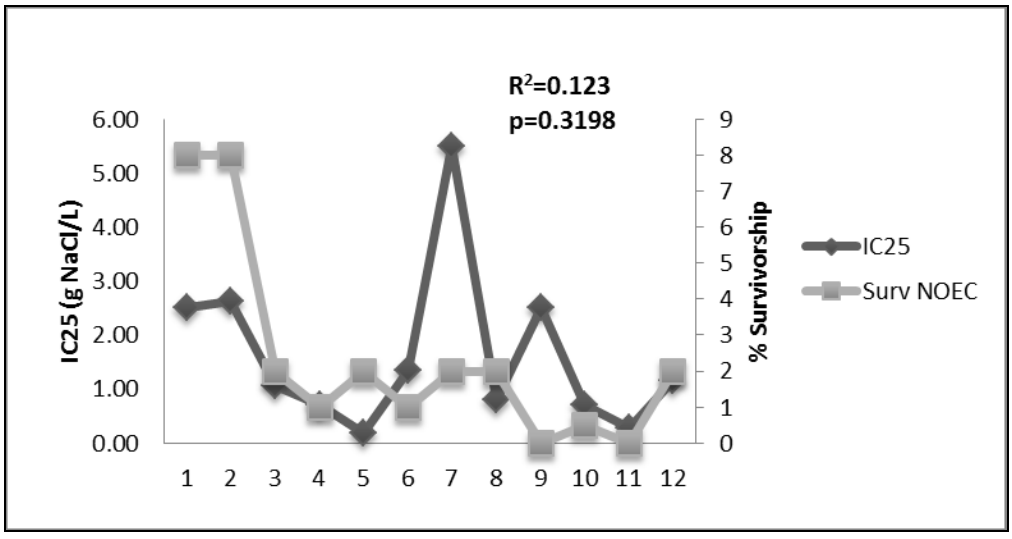

Figure 4.11. Exuvia IC_{25} values compared to *Isonychia* 7-day survivorship NOECs.

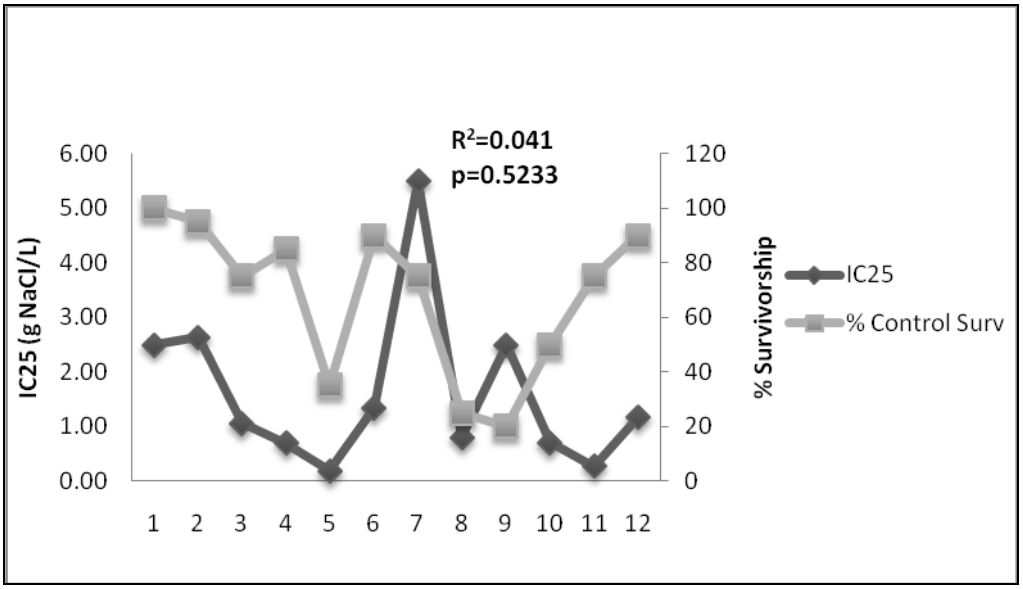

Figure 4.12. Exuvia IC_{25} values compared to *Isonychia* 7-day control survivorship.

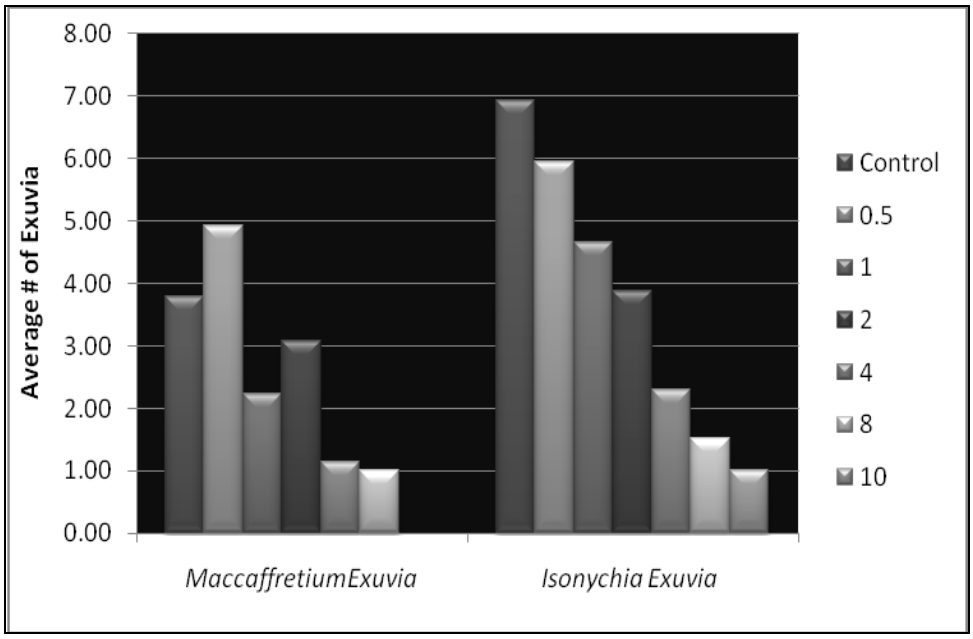

Figure 4.13. Comparison of molted exuvia from *Maccaffertium* and *Isonychia* nymphs exposed to varying NaCl concentrations over seven days.