

**Water Resources Research Institute of the  
University of North Carolina  
Annual Technical Report  
FY 2015**

# Introduction

During 2015-2016 (Fiscal Year 2015), the Water Resources Research Institute (WRRI) of The University of North Carolina System was responsible for fostering and developing a research, training, and information dissemination program responsive to the water problems of the state and region. To develop its programs, the Institute maintains an aggressive effort to interact and communicate with federal, state, and local water managers and other relevant stakeholders. The close contact with these individuals is the tool used to ensure our research priorities stay at the forefront of an ever-changing landscape. NC WRRI continued its strategic planning efforts during this period by engaging stakeholders in helping to identify priority areas for Institute growth and hosting a facilitator-led strategic planning retreat among staff. WRRI's inaugural strategic plan was presented to the WRRI Advisory Committee in January 2016 in draft form and has been finalized during this reporting period.

Research priorities continue to be identified and refined by the WRRI Advisory Committee, composed of 16 representatives of several federal and state agencies, local governments, industries, and non-governmental organizations (NGOs). Other water resource experts in the state with whom WRRI has close relationships are also consulted informally. A technical review committee is also convened on an annual basis to advise WRRI staff on the scientific merit of research proposals submitted for funding. Full-time faculty members from all North Carolina institutions of higher education are eligible to receive grants from WRRI.

In response to the RFP issued in FY15, WRRI received a total of 40 faculty pre-proposals from 8 institutions with a total request of \$3,933,625. Of these, 21 were invited to submit full proposals and WRRI ultimately selected 5 proposals to award, totaling \$281,800. In response to the student RFP, WRRI received 17 proposals from five institutions with a total funding request of \$99,992. Five of these were selected for funding, totaling \$49,994. Funds for these projects comes from USGS, state funds, and funds from two research consortia (the Urban Water Consortium and the Stormwater Group) administered by WRRI. Projects resulting from the FY15 annual call will be funded from March 1, 2016 to February 28, 2017 and details from those supported by USGS funds will be reported in the next USGS Annual Report.

From the FY14 RFP, 7 new research projects totaling \$489,223 began during this reporting period. Of these, 4 were USGS-funded projects totaling \$239,305, and are reported in the following sections. The remaining projects were supported by the Urban Water Consortium and the Stormwater Group (for more information about WRRI's activities with these two groups, please see the progress report for the Information Transfer Program).

WRRI funding was used to support a total of four post-docs, four PhD students, six master's students and six undergraduate students. Ten faculty were supported through Institute-funded projects during this cycle, including one visiting professor from China. An additional 47 students participated in the WRRI annual conference.

The information transfer program continued to focus on disseminating results of sponsored research and providing information on emerging water issues, solutions, and regulations. Results of research are disseminated by publication of technical completion reports, peer reviewed manuscripts, summary posts on the newly revamped (August 2015) WRRI website, and presentations by investigators at the WRRI Annual Conference and individual group meetings. Two peer-reviewed publications and six internal research reports from previous WRRI projects were published during this period.

WRRI team members are actively engaged in board and committee activities around the state where they bring expertise and perspective to efforts to address NC's water issues. WRRI is represented on the following:  
- NC Water Resources Association Board of Directors - NC Sedimentation Control Commission - NC

Nutrient Criteria Implementation Committee - NC Defense Coastal/Estuarine Research Program Regional Coordinating Committee - National NIWR-USGS Partnership Committee - Greater Triangle Stewardship Development Association Board of Directors

Through the WRRI Center for Watershed Excellence (CEWM), the NC Watershed Stewardship Network (WSN) continued its engagement of watershed professionals and volunteers across the state. The WSN finalized a strategic plan and logic model to help guide the network's efforts into the future, launched a website and developed an online mapping and database tool to help connect watershed stakeholders from around the state with each other as well as provide access to local watershed data. In FY 2015, the NCWSN engaged 58 participants in training and networking forums. Two community watershed restoration efforts, supported by EPA 319 grants and cost-sharing by partnering organizations, continue to be managed under the CEWM. Through these efforts, 73 people including 18 K-12 students, were involved in community projects to protect and restore watersheds. Black Creek watershed volunteers donated 58 hours of time in 2015, for a value of \$1,245. Local contractors were employed to install stormwater control measures in Black Creek. An additional grant was received in February 2016 for \$143,870, provided by US EPA through the NC Department of Environmental Quality, to partner with a school community, the Town of Cary and homeowners to continue improvements in Black Creek for an additional 2.5 years. Additional collaborative efforts are under way in the Walnut Creek community.

WRRI continues to be a sponsor of continuing education credits by the NC Board of Examiners of Engineers and Surveyors and the NC Board of Landscape Architects. This allows WRRI to offer Professional Development Hours (PDHs) and contact hours for attendance at the WRRI Annual Conference and other workshops and seminars that WRRI sponsors. During this reporting year, WRRI provided 46.5 PDHs and 32.5 CEUs to 996 people at 11 workshops, seminars, and other events described in the following pages. WRRI is also expanding its reach by offering webinar options for many of its events.

WRRI continues to adapt to changes in the landscape of its home institution, NC State University, and the UNC System as a whole, by consolidating its operations and maximizing staff efficiencies and outputs. The program continues to leverage funds from a variety of sources such as the Urban Water Consortium, the Stormwater Group, and grants received by the Center of Excellence for Watershed Management. These additional inputs help WRRI to expand the reach and impact of research and outreach activities, and grow involvement in and support of water-related research and outreach across the state.

## Research Program Introduction

During 2015-2016 (Fiscal Year 2015), WRRRI continued its focus of fostering research, training, and information transfer that is responsive to water issues of the state and region. Results from Institute-supported research efforts are expected to assist local, municipal, state, regional and federal agencies improve their decision-making in the management and stewardship of their water resources. WRRRI expanded its engagement of students through a new graduate student request for proposals (RFP) and more targeted tracking of student activities.

To help it chart and sponsor a research program responsive to the water resource issues and opportunities in North Carolina, WRRRI interacts closely with state agencies such as the NC Department of Environmental Quality, water and power utilities, and an array of research and outreach programs within the UNC system and at private higher educational institutions across North Carolina. The Institute's advisory committee provides input, guidance, and review of the research priorities that are used in developing our Requests for Proposals (RFPs) and directing other research activities. This committee is composed 16 representatives of several federal and state agencies, local governments, industries, and non-governmental organizations (NGOs). In early 2016, the committee convened in person in Raleigh for a thorough discussion of the state's most pressing water issues and how WRRRI's research priorities and programs could address these issues.

Based on in-depth discussions with stakeholders and advisory committee members regarding the most significant water research needs and priorities in NC, as well as considerations for recruiting the best proposals, WRRRI made substantial revisions and improvements to its annual request for proposals (RFP), consolidating its list of research priorities into four main RFP focus areas and refining the overall document. Research priorities are incorporated into our Section 104b Objectives on an annual basis. The RFP is sent to relevant offices of sponsored research at colleges and universities as well as an email distribution list of approximately 180 university faculty across North Carolina. Full-time faculty members from all North Carolina institutions of higher education are eligible to receive grants from WRRRI. During this reporting cycle, WRRRI conducted its regular solicitation for faculty research proposals, and also initiated a new student research proposal solicitation.

The proposals received are sent to external peer reviewers to determine the relevance, need for the proposed research and relative strengths and weaknesses. Then a Technical Committee convenes to review all comments made by reviewers, advise WRRRI staff on the scientific merit of proposals, and make recommendations regarding proposal funding. This year, select members from the WRRRI Advisory Committee with expertise matching research proposals served on the technical committee alongside other experts. Student proposals were reviewed by an internal panel of experts.

In response to the RFP issued in FY15, WRRRI received a total of 40 faculty pre-proposals from 8 institutions with a total request of \$3,933,625. Of these, 21 were invited to submit full proposals and WRRRI ultimately selected 5 proposals to award, totaling \$281,800. In response to the student RFP, WRRRI received 17 proposals from five institutions with a total funding request of \$99,992. Five of these were selected for funding, totaling \$49,994. Funds for these projects comes from USGS, state funds, and funds from two research consortia (the Urban Water Consortium and the Stormwater Group) administered by WRRRI. Projects resulting from the FY15 annual call will be funded from March 1, 2016 to February 28, 2017 and details from those supported by USGS funds will be reported in the next USGS Annual Report.

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## Research Program Introduction

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WRI published six internal research reports during this reporting period. Two previously funded USGS projects had peer reviewed publications during this reporting year. These are reported in the information transfer program progress report.

The FY 2015-2016 RFP research focus areas were:

### FOCUS AREA 1: STORMWATER MANAGEMENT

**LOW IMPACT DEVELOPMENT:** How do the lifecycle costs and benefits of low impact development (LID) compare to conventional development in new, retrofit, and redevelopment applications, particularly regarding LID for stormwater treatment in urban settings? What are the short-term and long-term implementation and maintenance cost and benefits of LID for developers, municipalities, communities, and individuals compared to that of conventional stormwater control measures (SCMs)? What is the short-term and long-term effectiveness of low impact development, specifically as related to stormwater treatment, costs and benefits, and water quality improvement? How can low impact development be encouraged and incentivized in North Carolina? For the questions above, how do costs and benefits for LID and conventional development compare across the different regions of the State?

**IMPERVIOUS COVER IMPACTS & MITIGATION:** How can we quantifiably mitigate the effects of impervious cover on water quality and aquatic life in different urban stream settings and stormwater systems? What realistic management measures (including stream restoration practices, riparian buffers, and floodplain-stream reconnection) exist or can be further evaluated to address effects of impervious cover? How can watershed restoration activities be implemented to achieve macroinvertebrate recovery and recolonization?

**POLLUTANT REMOVAL PROCESSES AND CREDITS:** How should pollutant removal credits be determined and evaluated for urban stormwater control measures (SCMs) and stream restoration practices, in particular those aimed at managing nutrients, pathogens, and sediment? How can we better understand the processes by which SCMs remove contaminants from stormwater and reduce impacts to receiving streams? Specifically for the state of North Carolina, and its physiographic regions (mountains, piedmont, and coastal plain), what location-based methods and criteria can be developed for evaluating SCM and stream restoration performance, credit accounting, and removal rates for pollutants (particularly nutrients, pathogens, and sediment)?

### FOCUS AREA 2: DRINKING WATER, WASTEWATER & WATER INFRASTRUCTURE

**RISK AND UNCERTAINTY:** In the face of changing population, land use, climate, and regulations, how can we quantify and manage risks and uncertainties in public water supplies? How should rate setting and financing capital improvements for water and sewer utilities be determined in the face of these risks and the changing physical and regulatory landscapes? How can utilities increase their resilience to these changes?

**CUSTOMER BEHAVIOR AND UTILITY RELATIONS:** Using social science and economic valuation methodologies, how can water/wastewater utilities better understand customers' level-of-service expectations, motivations for behaviors, willingness to pay for services, and customer perceptions, attitudes, opinions and beliefs related to drinking water, wastewater, and reclaimed water? How can this information be applied to utility management?

**ALTERNATIVE WATER SOURCES:** What alternative sources (graywater, harvested rainwater, reclaimed water) exist for differing consumptive uses (e.g. home irrigation)? What are the health risks of these

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alternatives sources? What are the impacts of alternative water use on overall water supply and demand?

**INNOVATIVE PROCESSES:** What/how can innovative processes and technologies be applied to NC utilities for water and wastewater treatment, plant operation, energy production, distribution systems, waste discharge management, potable and reclaimed water supply, and the repair, management and planning of infrastructure?

### FOCUS AREA 3: SURFACE WATER & GROUNDWATER HYDROLOGY

What are the human impacts to groundwater and surface water availability and quality in North Carolina? What fundamental hydrogeological interactions of surface water and groundwater resources do we need to further understand in order to support the sustainable use of water resources?

### FOCUS AREA 4: WATERSHED MANAGEMENT

In NC watersheds where Total Maximum Daily Loads (TMDLs) and nutrient management plans have been implemented, what changes in water quality have been observed? What are the sources, transport and fate of nutrients and sediments in surface waters in these watersheds? What physical, hydrological, biological and/or community dynamics need to be understood to enhance watershed management approaches?

# Quantification of Fecal Bacteria Removal by Micro-zooplankton Grazing in Stormwater BMPs

## Basic Information

<b>Title:</b>	Quantification of Fecal Bacteria Removal by Micro-zooplankton Grazing in Stormwater BMPs
<b>Project Number:</b>	2014NC186B
<b>Start Date:</b>	5/1/2014
<b>End Date:</b>	2/28/2016
<b>Funding Source:</b>	104B
<b>Congressional District:</b>	NC7
<b>Research Category:</b>	Water Quality
<b>Focus Category:</b>	None, None, None
<b>Descriptors:</b>	None
<b>Principal Investigators:</b>	Michael A. Mallin, Lawrence B. Cahoon

## Publications

There are no publications.

# **Quantification of Fecal Bacteria Removal by Microzooplankton Grazing in Stormwater BMPs**

**Draft Final Report  
UNC Water Resources Research Institute Project 14-02-W**

**May, 2016**

**By**

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**Abstract:** A high priority for environmental managers in general is the control of stormwater runoff pollution, especially stormwater that contains high concentrations of fecal microbes. It is of clear value to understand and design stormwater best management practices (BMPs) to achieve optimal removal of fecal microbial pollution from stormwater. Grazing by micro-zooplankton (rotifers, protozoans and heterotrophic and myxotrophic phytoplankton) is believed to be a major factor in fecal bacterial removal in BMPs (especially stormwater wetlands) but this process has been rigorously tested. This research was designed to determine; 1) if micro-zooplankton grazing on fecal bacteria is significant in aquatic BMPs; 2) if grazing frequency differs between a wet detention pond and a constructed wetland; and 3) if various environmental factors enhance grazing. Both three-day grazing tests and 24-hr dilution assays were used to determine micro-grazing differences between the two types of BMP. Our seasonal experiments support the contention that micro-zooplankton grazing is a stronger fecal bacteria removal mechanism in stormwater wetlands rich in aquatic vegetation compared to a standard wet detention pond, although micro-zooplankton grazing is clearly important in wet detention ponds as well. Furthermore, our experiments indicated that the vast majority of grazers that fed on fecal bacteria were very small, in the 10-20  $\mu\text{m}$  size range. Correlation analyses indicated that grazing rates were positively related to fecal coliform abundance, and increased water temperatures. Fecal coliform bacterial abundances were likewise positively correlated with water temperature and were also correlated with rainfall amount. Thus, grazing on fecal bacteria in BMPs is enhanced by aquatic vegetation, and aquatic BMPs in warmer climates are likely to experience greater fecal bacteria loss through grazing than in cooler climates.

## **Acknowledgements**

We thank the UNC Water Resources Research Institute for funding; Project 14-02-W. This research formed a portion of Jade M. Burtchett's M.S. in Marine Science thesis. We thank Mr. Travis Nelson and Mr. David Huffman, UNCW graduate students that were partially funded on this project during summer to provide field and laboratory support to the P.I.s and Ms. Burtchett in carrying out grazing experiments. We also thank Matthew R. McIver of UNCW for field and laboratory assistance.

## 1. Introduction:

Stormwater runoff is a major source of pollution to coastal waters of the United States. The type of pollution within stormwater runoff that most directly impacts human health and the economy is excessive fecal microbial abundance, especially fecal bacteria (NRC 2009). Some of this fecal pollution is sourced by human infrastructure defects (Whitlock et al. 2002; Cahoon et al. 2006) while some portion is sourced from wildlife or pets (Whitlock et al. 2002; Ram et al. 2007; Nugent et al. 2008). Regardless, human urbanization and the hydrological changes it brings is a major driver of such pollution. North Carolina researchers have determined that the amount of fecal coliform bacterial pollution in coastal creeks is strongly correlated with human development in the watershed (Mallin et al. 2001), especially impervious surface coverage ( $r = 0.975$ ,  $p = 0.005$ ); this relationship has similar statistical strength in creek watersheds in South Carolina (Holland et al. 2004) and the Gulf Coast (Sanger et al. 2013).

Fecal microbial runoff pollution is especially problematic in coastal waters for two major human health-related reasons. First, when shellfishing areas are polluted by fecal bacteria, they are closed to harvest by state regulators to avoid serious illness or even death through consumption of contaminated shellfish. In addition to shellfish consumption issues and economic loss, microbiologically-polluted stormwater runoff is a direct health threat to humans involved in water contact activities (Alexander et al. 1992). Such activities include swimming, waterskiing, surfing, diving and even wading. Thus, reduction of fecal microbial pollution to coastal waters is a critical management need for North Carolina and the southeast in general.

State regulators, municipalities and academic researchers have made strong efforts to combat such fecal pollution using Best Management Practices (NCDWQ 2007; Pennington et al. 2003). Wet detention ponds are the most commonly used form of stormwater treatment in the coastal zone (SCDHEC 2007). However, such ponds differ greatly from natural wetlands in water chemistry, organic material type and quantity, and invertebrate diversity and productivity (Woodcock et al. 2010). Constructed wetlands are an important and increasing part of the arsenal used by managers to reduce such microbial pollution. However, their efficacy is mixed (Pennington et al. 2003), and depends upon size, vegetation and design. Some small wetlands perform poorly in fecal microbial treatment (Hathaway and Hunt 2012) while large, properly designed systems can show excellent fecal bacterial reductions exceeding 95% (Mallin et al. 2012). Reduction of fecal bacteria in BMPs is a function of settling, filtration, attack by bacteriophages, deactivation by UV radiation, plant exudation of substances with antimicrobial properties, and presumably grazing by micro-zooplankton, especially protozoans and rotifers (Gerba et al. 1999; Stenstrom and Carlander 2001; Vymazal 2005). Wetland vegetation has been demonstrated to provide more efficient fecal microbe removal than bare sediments in ponds (Davies and Bavor 2000), likely by enhancing settling of fine particles and associated bacteria (Gerba et al. 1999) and also possibly by providing increased surface area and physical contact between the pathogens and wetland plant material and other substrata harboring protozoan and rotifer grazers. However, such grazing has been largely assumed to occur rather than experimentally tested and reported in the BMP literature.

Studies have demonstrated that grazing by micro-zooplankton can reduce fecal bacteria in open water estuarine situations (Enzinger and Cooper 1976; Menon et al. 2003). Grazing of fecal

microbes by protozoans and rotifers has long been an integral part of wastewater treatment in activated sludge plants, trickling filters and waste stabilization ponds (Clark et al. 1977). A variety of micro-zooplankton taxa groups that are present in treatment facilities as well as open natural waters are known to ingest bacteria. These include heterotrophic microflagellates (Azam et al. 1983), ciliated protozoans and amoeboid protozoans (Clark et al. 1977), rotifers (Starkweather 1980, Turner and Tester 1992), copepod nauplii (Turner and Tester 1992), gastrotrichs (Strayer and Hummon 1991) and nematodes (Poinar 1991). Some of the larger taxa likely do not target bacteria but consume bacteria incidentally while grazing larger food items such as phytoplankton.

Our previous studies demonstrated that micro-zooplankton grazing on fecal bacteria does occur in a constructed wetland (Chudoba et al. 2013). These grazing rate experiments were accomplished in the laboratory in flasks using the dilution method developed by Landry and Hassett (1982). Such assays involved making a series of dilutions of the raw water to reduce microorganism density in the samples, which in turn reduces the encounter rate of microzooplankton grazers and their bacterial prey. Our team modified this method to successfully account for grazing in a study that demonstrated the positive impact of P loading on fecal bacteria survival and growth (Chudoba et al. 2013).

Thus, in order to design BMPs that optimize fecal bacterial removal, removal processes need to be understood and quantified. *This project thus focused on providing experimental evidence of fecal bacterial removal by grazing, and testing whether it is optimized by type of BMP (wet pond versus constructed wetland), absence or presence of aquatic vegetation, and seasonal or other environmental effects.*

In order to achieve maximal pollutant removal, especially where space for BMPs may be limited, wet detention ponds and constructed wetlands need to be designed for optimal performance. Thus, statistically-sound research results are needed for such design optimization. Presently, nutrients and fecal microbes are considered priority agents for removal from stormwater (Field et al. 2006; England and Stein 2007; NRC 2009). Removal of fecal microbes is especially desired in the coastal environment where humans can be exposed to infection both from body contact in coastal waters and consuming contaminated shellfish. Overall, the removal of fecal microbes from stormwater is considered to be under-researched (England and Stein 2007).

This research was designed to provide experimentally-derived information on a number of related factors in BMP design, use and ecology. First, was to determine if micro-zooplankton grazing is indeed a significant factor in fecal bacterial removal from stormwater, as either suggested (Gerba et al. 1999; Stenstrom and Carlander 2001; Vymazal 2005) or experimentally determined by previous research (Chudoba et al. 2013). Secondly, this research was designed to verify that the presence of aquatic vegetation increases micro-zooplankton grazing on fecal bacteria by testing grazing differences between a constructed wetland with abundant aquatic vegetation and a relatively bare wet detention pond. Third, ancillary hydrological, chemical and biological information were collected concurrently with the experiments and statistically analyzed to determine what environmental factors are associated with enhanced grazing, or if some factors deter grazing. Ultimately it was hoped that our results could provide practical guidance for design and construction of future wetlands (or modified wet detention ponds) that

will increase efficacy of fecal microbial removal from stormwater. This guidance may include choosing between use of wet ponds or constructed wetlands, how much aquatic vegetation to plant, and eventually what species will optimize fecal bacterial removal.

### **1.1. Objectives of Research**

Our overarching goal was to quantify the impact micro-zooplankton grazers have on removal of fecal microbes carried by stormwater into two types of BMPs. This was accomplished by performing two different types of grazing experiments seasonally on waters from a constructed wetland and a wet detention pond.

### **1.2. Hypotheses:**

*Our main hypotheses are:* 1) Micro-zooplankton grazing upon fecal bacteria is enhanced by substrata for grazers, especially submersed and emergent aquatic vegetation, thus constructed wetlands will provide an environment more suited to promoting grazing as a loss factor for fecal bacteria in stormwater; 2) Micro-zooplankton grazing is enhanced seasonally by warm temperatures due to the presence of elevated micro-grazers in summer, and 3) Such grazing is enhanced by chemical and biological variables that influence bacteria and/or zooplankton abundance, and meteorological factors that influence stormwater inputs.

## **2. Methods:**

### **2.1. Study Site Description:**

The test stormwater treatment wetland is the JEL Wade Wetland in Wilmington, N.C. This large facility contains diverse aquatic plant species (Fig. 1A). Inflow versus outflow testing has demonstrated that this wetland achieves excellent pollutant removal, including fecal coliform bacteria (Mallin et al. 2012). This facility was previously used in experiments demonstrating that individual macrophyte species significantly differ in the amounts of denitrification that occurs among their rhizomes (Song et al. 2014). A comparison test facility is a large stormwater wet detention pond located behind a shopping center near the corner of College and Carolina Beach Roads in Wilmington, of similar depth as the wetland but lacking the emergent and submersed aquatic macrophyte vegetation (Fig. 1B). Kings Highway Pond is located behind a retail parking lot, and it accepts drainage from significant run-off of impervious surfaces. There is little vegetation, and not much variance in the species. There is a small resident population of geese that inhabit the area as well, which is suggested to be because there isn't a natural vegetative littoral shelf; it's maintained with grassy vegetation which is more appealing to certain waterfowl.



Figure 1A, Left – Diverse aquatic vegetation near the inflow of the JEL Wade constructed wetland. Figure 1B, Right – Kings Highway wet detention pond with lack of aquatic vegetation.

## **2.2. Field Collections:**

Water for the experiments was collected at the BMP inflow areas in 10L carboys. Concurrently with collection, a YSI 6820 Multiparameter Water Quality Probe linked to a YSI 650 MDS display unit was used to measure surface temperature, conductivity, salinity, pH, dissolved oxygen and turbidity at both locations. Water was collected among vegetation when available. Distinction between rain and dry sampling was noted. After use, carboys were filled with 10% bleach solution left overnight and rinsed the next day.

## **2.3. Chemical Analyses**

Water samples collected in conjunction with the grazing experiments were analyzed for chlorophyll *a*, since phytoplankton are an important food source for micro-zooplankton grazers (Landry and Hassett 1982). Across a series of Florida Lakes chlorophyll *a* has been positively correlated with the abundance of ciliated protozoans in general as well as specific taxa groups (Beaver and Crisman 1990). Thus, increased chlorophyll concentrations (as a food source) may lead to higher protozoan counts in BMPs, leading to higher grazing rates on fecal bacteria. Chlorophyll *a* measurements were performed using EPA Method 445.0, based on the Welschmeyer (1994) fluorometry method. Dissolved organic carbon (DOC) is a major food resource for bacteria in general (Azam et al. 1983) as well as for fecal bacteria specifically (Surbeck et al. 2010). Thus, it might be expected that higher DOC concentrations impact fecal bacteria growth rates, and potentially grazing rates through increased encounters. Additionally, in pelagic situations dissolved organic matter released by live and dead phytoplankton is an important food resource to bacteria (Azam et al. 1983) thus elevated chlorophyll concentrations may be indirectly indicative of support for fecal bacteria. Dissolved organic carbon (DOC) concentrations were analyzed using a Shimadzu TOC-L analyzer.

## **2.4. Fecal Coliform (FC) 24-hr Dilution Assay:**

One series of grazing rate experiments was accomplished using the dilution method developed by Landry and Hassett (1982) and refined by Chudoba et al. (2013). Four different treatments were made with ratios of 1:0, 3:1, 1:1, and 1:3 filtered to unfiltered water, and each had two replicates. To produce the filtered water, water from the carboy was filtered through a Whatman 0.45  $\mu\text{m}$  filter and collected in a clean flask. The samples were 300mL each, held in 500mL

bottles and kept on shaker tables overnight for continual agitation. Sub-samples were taken initially and 24hrs after set-up. There were two different amounts taken initially from each sample to determine fecal coliform concentrations. Sub-sample amounts varied from 0.1mL-100mL, depending on initial count. Sub-samples were filtered through a sterile filtration funnel, than placed in sterile petri dishes with a pad containing around 1.5ml of MFC media. Plates were then put in two Ziploc bags, left in a bath at 44.5C for 24hr, and then read. Dark blue colonies formed after incubation represented valid colony forming units (CFU). Pink and light blue colonies were not used in calculations, but were recorded. All glassware used in the process was rinsed with DI water, soaked in a contrad bath for at least 12hr and autoclaved 15min at 121°C. After the data were collected, the one-day growth rates for each dilution bottle were calculated using the following formula:

Specific growth rate ( $\mu$ ,  $\text{day}^{-1}$ ) =  $\ln(\text{Day 2 concentration}/\text{Day 1 concentration})$ .

The specific growth rates were then plotted against Day 1 concentrations for each bottle.

### **2.5. 3-Day Grazing Experiment (Mean Fecal Coliforms):**

The second series of grazing experiments were 3-day experiments designed to test for differences in grazing between unfiltered water (containing microzooplankton) and water filtered through a net to remove most of the zooplankton community; thus each site had two treatments, filtered and unfiltered water. To make the filtered water, water from the field collection carboy was filtered through a 20  $\mu\text{m}$  mesh net and collected in a clean flask. The samples (in triplicate) were 700ml each, held in 1L bottles and kept on shaker tables for continual agitation, under a fume hood in the dark for the duration of the experiment. There were 2 different amounts taken initially from each sample to determine fecal coliform concentrations. Sub-sample amounts varied from 0.1mL-100mL, depending on initial count. The FC analysis procedure followed Method 9222D (APHA, 2005) for total fecal coliforms. Sub-samples were filtered through a sterile filtration funnel, and then placed in sterile petri dishes with a pad containing around 1.5ml of MFC media. Plates were then put in two Ziploc bags, left in a bath at 44.5C for 24hr, and then read. The process was repeated for a total of 4 days, (3 not including the initial). All glassware used in the filtration process was washed in DI water, soaked in an acid bath for at least 12h and autoclaved for 15 min at 121 °C. After the first several months the experimental procedure was altered so that the “filtered” treatment was passed through a 10  $\mu\text{m}$  mesh net as opposed to the 20  $\mu\text{m}$  mesh.

### **2.5. Statistical Analysis:**

Fecal coliform growth rates for the dilution experiments were plotted against initial cell densities for data interpretations. Regressions were calculated from the growth rates. If the slope of the line was significantly negative, then microzooplankton grazing was assumed to have an impact on the reduction of fecal coliform concentrations.

Regarding the 3-day grazing experiments, the generated data were tested for normality using the Shapiro-Wilk test and log-transformed if appropriate, and t-tests were used to test for significant differences in fecal coliform abundances between the filtered and unfiltered treatments. If average fecal coliform counts were significantly higher in the filtered samples it was presumed that this was due to the fecal bacteria being freed from micro-zooplankton grazing (all other

environmental factors equal). Statistical tests were performed using SAS (Schlotzhaeur and Littell 1997).

Chemical, biological and meteorological factors impacting micro-zooplankton grazing rates provide additional information on interpretation of results. Thus, correlation analyses were performed to examine different environmental factors' impact on the efficacy of micro-zooplankton grazing. The 24-hr grazing rates were correlated against water temperature, turbidity, pH, conductivity, chlorophyll *a*, specific growth rate of fecal coliforms, and dissolved organic carbon (DOC), that were collected when the experiments were ran. The amount of rainfall used for statistical purposes was rain that fell on the day of sampling plus rainfall for the two days prior, collected at the Wilmington airport and accessed from the Weather Underground ([www.wunderground.com](http://www.wunderground.com)).

### 3. Results:

#### 3.1. 24-hr Dilution Assay Microzooplankton Grazing Experiments

The 24-hr dilution assays demonstrated that micro-zooplankton grazing was frequently a significant factor in reduction of fecal bacteria in the constructed wetland (Fig. 2), with significant grazing occurring in 9 of 12 dilution assays (Table 1). Significant grazing occurred in the wet detention pond as well, but in only 5 of 11 dilution assays (Fig.3); note however that the negative slopes from some of the other experiments were nearly significant (Table 1). Thus, the dilution assays tend to support Hypothesis 1 above, that vegetated ponds or wetlands are more likely to enhance micro-zooplankton grazing a means to reduce fecal bacteria abundance, although grazing can also be an important factor in wet detention ponds.

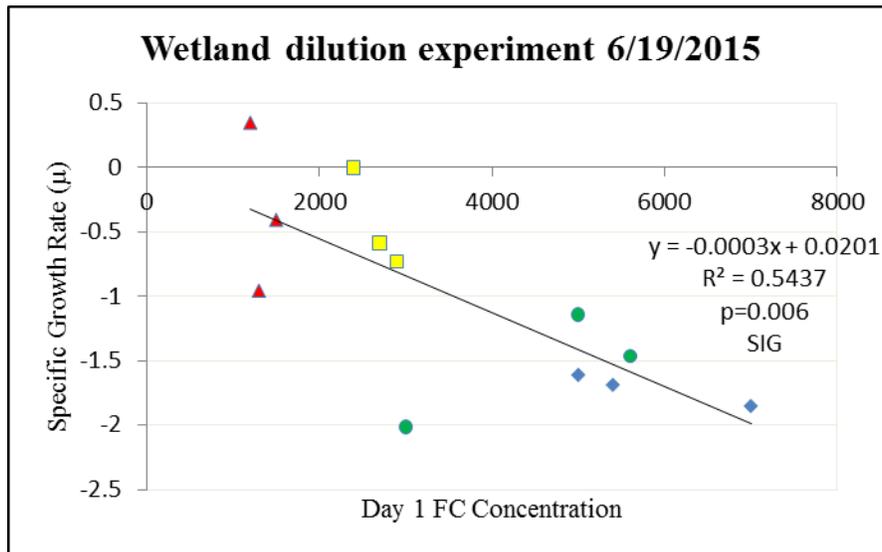


Figure 2. Results of microzooplankton grazing experiment showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in JEL Wade constructed wetland, summer 2015. Blue diamonds: 100% whole water, Green circles: 75% whole water, 25%

filtered, Yellow squares: 50% whole water, 50% filtered, Red triangles: 25% whole water, 75% filtered

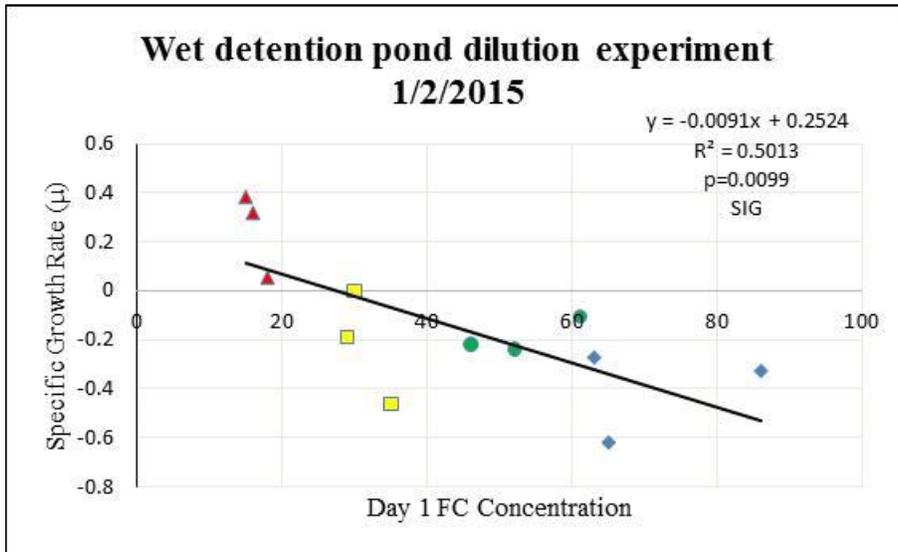


Figure 3. Results of microzooplankton grazing experiment showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in KHP constructed wet detention pond, winter 2015. Blue diamonds: 100% whole water, Green circles: 75% whole water, 25% filtered, Yellow squares: 50% whole water, 50% filtered, Red triangles: 25% whole water, 75% filtered

Table 1. Statistical results from 24 hour dilution experiments in JEL Wade constructed wetland and King’s Highway wet detention pond. The intercept shows the bacterial growth rate coefficient; a positive growth rate coefficient indicates projected growth rate under hypothetical “grazing free” conditions. The slope represents the grazing rate coefficient. The p value specifies a significant negative slope ( $p < 0.05$ ), indicating that grazing is a significant factor in removing fecal bacteria.

Site	Date	Intercept	Slope	P	Sig. (-) slope	Grazing sig.?
Wetland	8/12/2014	0.9115	-0.0011	0.003	Yes	Yes
Wetland	8/26/2014	0.3543	-0.0004	0.022	Yes	Yes
Wetland	9/2/2014	0.2203	-0.001	0.168	No	No
Wetland	12/31/2014	0.6958	-0.0008	0.304	No	No
Wetland	1/25/2015	0.2161	-0.0005	0.526	No	No
Wetland	6/8/2015	0.2007	-0.0025	0.0002	Yes	Yes
Wetland	6/19/2015	0.0201	-0.0003	0.006	Yes	Yes
Wetland	12/8/2015	0.8268	-0.0189	9.03E-05	Yes	Yes
Wetland	2/10/2016	0.902	-0.0147	1.16E-06	Yes	Yes
Wetland	2/15/2016	0.2558	-0.0016	0.018	Yes	Yes
Wetland* forebay	2/25/2016	0.6006	-0.0206	1.81E-05	Yes	Yes
Wetland* Outfall	2/25/2016	0.5739	-0.0185	0.015	Yes	Yes

Site	Date	Intercept	Slope	P	Sig. (-) slope	Grazing sig.?
Pond	8/6/2014	0.6221	-0.0047	0.249	No	No
Pond	8/11/2014	0.7889	-0.0028	0.0006	Yes	Yes
Pond	8/19/2014	-1.580	0.0101	0.357	No	No
Pond	9/18/2014	0.0166	-0.0044	0.069	No	No
Pond	12/16/2014	0.3614	0.0038	0.779	No	No
Pond	1/2/2015	0.2524	-0.0091	0.009	Yes	Yes
Pond	1/19/2015	0.1976	-0.0184	0.072	No	No
Pond	12/9/2015	0.8268	-0.0189	9.03E-05	Yes	Yes
KHP	3/28/16	0.8206	-6E-05	0.079	No	No
KHP	4/6/16	0.4345	-0.0309	0.003	Yes	Yes
KHP	4/8/16	0.3118	-0.0018	0.005	Yes	Yes

### 3.2. 3-Day Grazing Experiments:

The 3-day experiments at JEL Wade wetland comparing non-filtered water against water filtered through a 20  $\mu$ m mesh net were all negative (Fig. 4), i.e. they showed no significant reduction in

fecal coliform counts through grazing. Presumably the mesh size was large enough to permit sufficient grazers to enter the “filtered” treatment to graze down the fecal bacteria.

Table 2. Results of t-tests ( $\alpha = 0.05$ ) comparing fecal coliform counts from filtered vs unfiltered 3-day experiments using JEL Wade constructed wetland (JEL) and King’s Highway wet detention pond (KHP) waters using 20 $\mu$ m mesh for filtration. Means shown for overall whole water, then filtered.

Site	Date	Whole mean	Filtered mean	p-value	no
wetland	7/15/2014	172	150	0.90	No
wetland	7/29/2014	611	456	0.47	No
wetland	9/01/2014	125	155	0.53	No
wetland	1/06/2015	41	53	0.88	No
wetland	2/11/2015	58	70	0.71	No
Pond	7/23/2014	2622	2622	0.92	No
Pond	7/29/2014	123	105	0.75	No
Pond	9/01/2014	20	17	0.89	No
Pond	1/06/2015	9	9	0.99	No
Pond	2/11/2015	18	16	0.90	No

Beginning August 2015, 10  $\mu$ m mesh was used to further ensure all microzooplankton were filtered from samples. The results showed a very different picture than the experiments conducted using the 20  $\mu$ m mesh filtration (Figs. 6 and 8 versus Figs. 7 and 9). Of the five experiments run at the constructed wetland, three experiments showed significant grazing impacts (Table 3), one specifically on August 23, 2015 (Fig. 10). Using water from Kings Highway wet detention pond, the 3-day experiments yielded four significant micro-zooplankton grazing results in six experiments ran (Table 3), one specifically on September 25, 2015 (Fig. 11).

Table 3. Results of t-tests ( $\alpha = 0.05$ ) comparing average fecal coliform counts from filtered vs unfiltered treatments in 3-day experiments using JEL Wade constructed wetland (JEL) and King’s Highway wet detention pond (KHP) waters, using 10 $\mu$ m mesh for filtration. Means shown for whole water, then filtered water.

Site	Date	Whole mean	Filtered mean	p-value	Sig. grazing?
<b>JEL</b>	<b>8/12/2015</b>	<b>5250</b>	<b>21275</b>	<b>0.04</b>	<b>Yes</b>
<b>JEL</b>	<b>8/23/2015</b>	<b>1187</b>	<b>2522</b>	<b>0.02</b>	<b>Yes</b>
JEL	8/28/2015	878	1246	0.35	No
JEL	9/25/2015	5355	9383	0.29	No
JEL	10/6/2015	3375	3050	0.96	No
<b>KHP</b>	<b>8/12/2015</b>	<b>225</b>	<b>755</b>	<b>0.01</b>	<b>Yes</b>
KHP	8/19/2015	44	54	0.48	No
<b>KHP</b>	<b>8/23/2015</b>	<b>888</b>	<b>3517</b>	<b>0.02</b>	<b>Yes</b>
<b>KHP</b>	<b>8/28/2015</b>	<b>81</b>	<b>335</b>	<b>0.03</b>	<b>Yes</b>
<b>KHP</b>	<b>9/25/2015</b>	<b>2583</b>	<b>5975</b>	<b>0.01</b>	<b>Yes</b>
KHP	10/6/2015	1971	2008	0.97	No

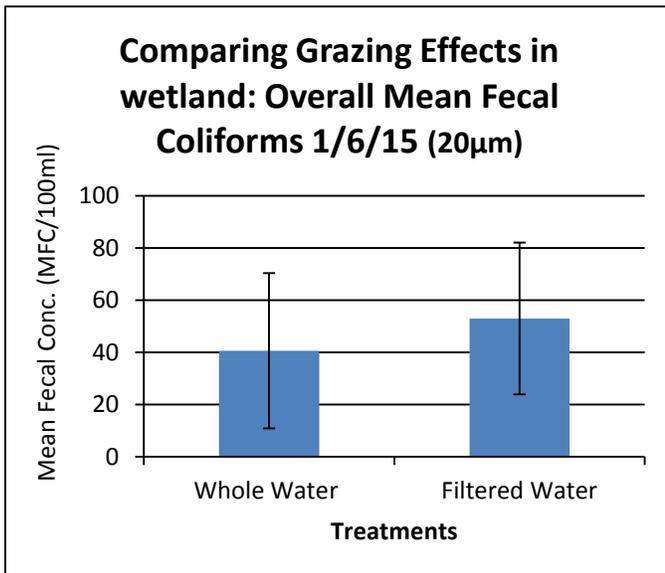


Figure 6. Mean total fecal coliform concentrations for 3-day assay at the wetland, whole water vs filtered with 20  $\mu$ m mesh.

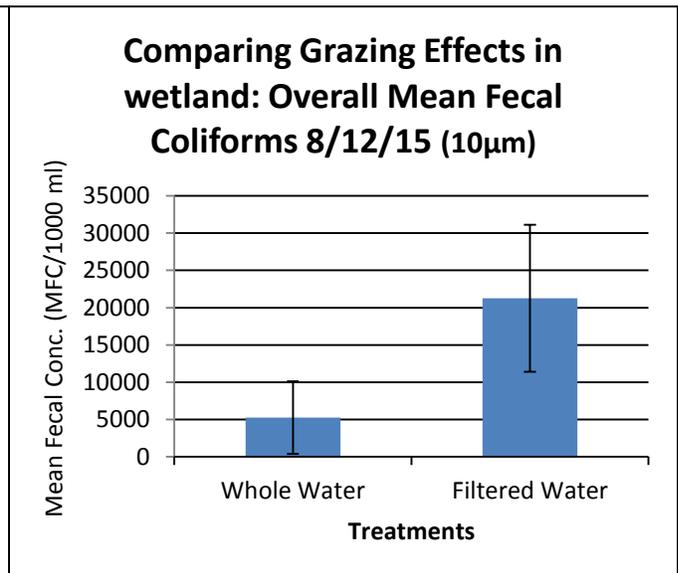


Figure 7. Mean total fecal coliform concentrations for 3-day assay at the wetland, whole water vs filtered with 10  $\mu$ m mesh, significant difference  $p = 0.04$ .

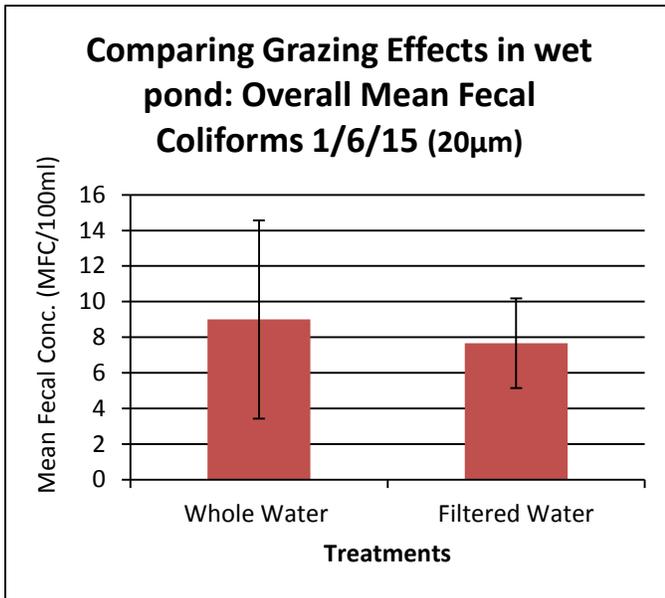


Figure 8. Mean total fecal coliform concentrations for 3-day assay at the wet detention pond, whole water vs filtered with 20 µm mesh.

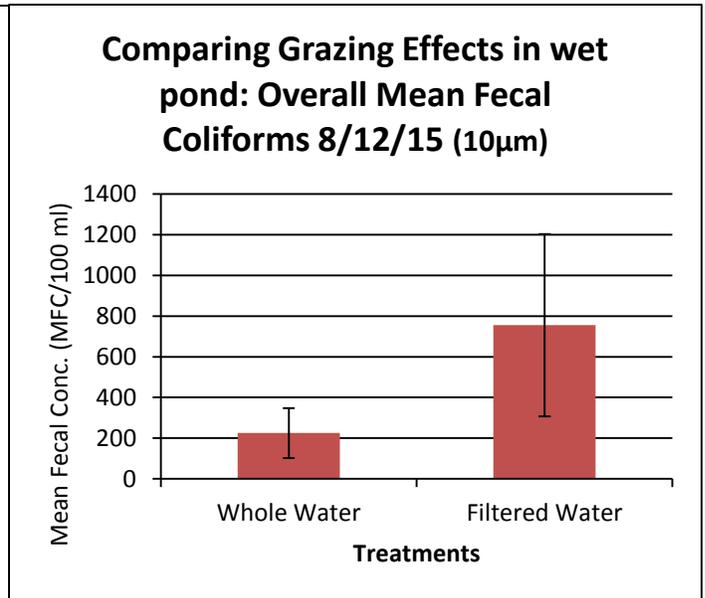


Figure 9. Mean total fecal coliform concentrations for 3-day assay at the wet detention pond, whole water vs filtered with 10 µm mesh, significant difference p = 0.01.

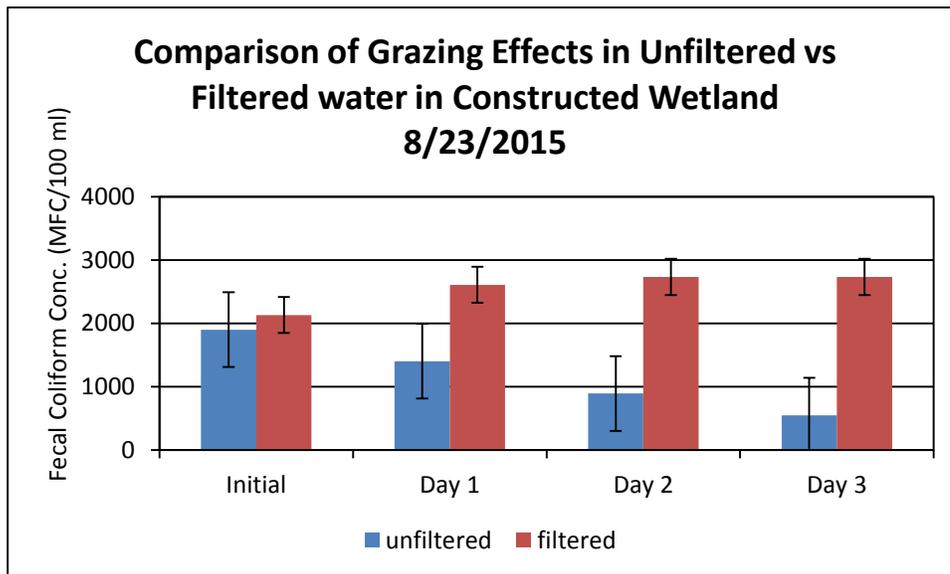


Figure 10. Results of 3-day microzooplankton grazing experiment using 10µm mesh showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in JEL Wade constructed wetland, summer 2015, significant difference p = 0.02.

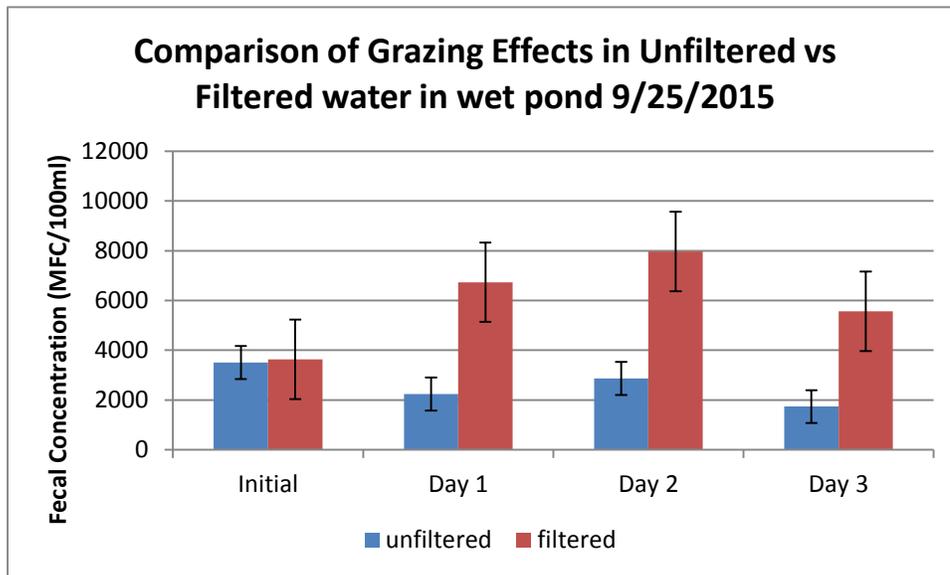


Figure 11. Results of 3-day microzooplankton grazing experiment using 10 $\mu$ m mesh showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in the wet detention pond, fall 2015, significant difference  $p = 0.01$ .

Thus, the revised 3-day grazing experiments again demonstrate that significant micro-zooplankton grazing occurs in both the constructed wetland and the wet detention pond. Further, these experiments demonstrate that the vast majority of grazing occurs by micro-zooplankton in the 10-20  $\mu$ m size range.

### 3.3 Grazing in relation to environmental factors

As noted, data were also collected in conjunction with the experiments for a number of potentially-related environmental factors (Table 4). These data indicate that the BMPs were prone to occasional algal blooms, while turbidity was generally low. Dissolved organic carbon was generally low compared to that of blackwater Coastal Plain streams (Mallin et al. 2015), but the DOC in the wetland was double that of the standard wet pond. Most of the rain events captured were in the 0.5 inch range, but a few large events also occurred (Table 1). Note the high variability among fecal coliform bacteria counts (Table 4).

Table 4. Summary data for environmental variables collected in conjunction with the grazing experiments for the wet detention pond, as mean  $\pm$  standard deviation / range.

Parameter	Constructed wetland	Wet detention pond
Water temperature (°C)	20.3 $\pm$ 7.0 (9.8 – 27.9)	21.6 $\pm$ 8.0 (9.7 – 30.6)
pH	6.5 $\pm$ 0.3 (5.9 – 7.2)	7.2 $\pm$ 0.5 (6.5 – 8.2)
Conductivity ( $\mu$ S)	164.7 $\pm$ 29.0 (100 – 218)	145.9 $\pm$ 36.1 (60 – 199)
Dissolved oxygen (mg/L)	5.9 $\pm$ 2.0 (2.5 – 9.1)	9.0 $\pm$ 1.7 (7.0 – 12.2)
Turbidity (NTU)	3.6 $\pm$ 2.0 (0.1 – 8.1)	2.4 $\pm$ 1.9 (0.1 – 8.0)
Chlorophyll <i>a</i> ( $\mu$ g/L)	36.5 $\pm$ 50.3 (0.8 – 167.1)	19.1 $\pm$ 8.7 (7.0 – 33.1)
Fecal coliforms (CFU/100 mL)	1,968 $\pm$ 3,133 (57 – 10,600)	888 $\pm$ 1,536 (39 – 5,760)
Dissolved organic carbon (mg/L)	11.3 $\pm$ 2.4 (8.5 – 17.0)	5.4 $\pm$ 0.9 (3.5 – 6.9)
Rainfall (inches)	0.6 $\pm$ 0.7 (0 – 2.9)	0.6 $\pm$ 0.6 (0 – 2.3)

Correlation analyses were performed to examine different environmental factors' impact on the efficacy of micro-zooplankton grazing. In the constructed wetland, initial fecal coliform concentrations were positively correlated with water temperature ( $r = 0.639$ ,  $p = 0.0002$ ) and with the 48 hr rainfall ( $r = 0.468$ ,  $p = 0.028$ ). Grazing rate was strongly correlated with initial fecal coliform concentrations ( $r = 0.829$ ,  $p = 0.0009$ ), suggesting the higher the concentration of bacteria, the more effectively the micro-zooplankton graze. Grazing rate was also positively correlated with water temperature ( $r = 0.577$ ,  $p = 0.049$ ). Bacterial growth rate was negatively correlated with micro-zooplankton grazing rate ( $r = -0.639$ ,  $p = 0.025$ ) and positively correlated with turbidity ( $r = 0.671$ ,  $p = 0.017$ ). In the wet detention pond, like the wetland, initial fecal coliform concentrations were positively correlated with water temperature ( $r = 0.404$ ,  $p = 0.062$ ) and rainfall ( $r = 0.696$ ,  $p = 0.0003$ ) and also with DOC concentration ( $r = 0.786$ ,  $p = 0.021$ ).

Correlation analyses were also run for all experiments combined from both systems (Table 5). For all experiments combined, initial fecal coliform counts were positively correlated with water temperature and rainfall. Micro-zooplankton grazing rate was positively correlated with water temperature, while bacterial growth rates were negatively correlated with grazing rate.

Table 5. Correlation analyses among micro-zooplankton grazing and environmental factors. Results presented as person correlation coefficient (r) / probability (p). Non-significant (p > 0.05) not shown.

Parameter	Grazing rate	FC count	growth rate	temperature	turbidity	rainfall
Grazing rate		0.496 0.016	-0.583 0.004	0.492 0.017		
FC count	0.496 0.016			0.443 0.003		0.533 0.0002
Turbidity				-0.348 0.027		

#### 4. Discussion:

The 24-hr dilution grazing experiments demonstrated that grazing by micro-zooplankton is important in removing fecal bacteria in the constructed wetland, with 75% of the experiments showing significant grazing. Grazing appeared to be less a factor in the standard wet detention pond, being a significant factor in 45% of the dilution experiments. Thus, by this metric the wetland appeared to create an environment more conducive to micro-zooplankton grazing than did the standard wet detention pond. As to the 3-day filtered vs non-filtered experiments, when a 20 µm mesh net was used to remove micro-zooplankton there was no significant grazing detected. We note that with use of the 10 µm mesh filtration 3/5 experiments in the wetland and 4/6 experiments in the wet detention pond indicated micro-zooplankton grazing as a significant fecal bacteria removal mechanism. Thus, the most intense grazing comes from small micro-zooplankton, i.e. between 10 and 20 µm in size. Rotifers range considerably in size according to species, but the vast majority are in the 60–250 µm size range (Wallace and Snell 1991) and well outside the above range. Metazoans such as copepods and their nauplii are likewise far larger. Nematodes, which are roundworms, can and do consume bacteria but freshwater species are larger than 20 µm in size (Poinar 1991) so they would not be significant grazers in the waters of these BMPs. Gastrotrichs are a related taxa group that readily, even preferentially consume bacteria but are generally 50–800 µm long, again not in the 10–20 µm size range (Strayer and Hummon 1991). Thus, fecal bacteria are not appreciably grazed by copepod nauplii, rotifers, nematodes or gastrotrichs in these BMPs. Bacteria-feeding taxa groups that contain species that can pass a 20 µm mesh net include flagellated, amoeboid, and some ciliated protozoans, as well as phagotrophic and myxotrophic algae (Jahn and Jahn 1949). It has been noted elsewhere in experiments run on ambient estuarine waters that the greatest micro-zooplankton grazing impact occurred with the smallest protozoan grazers such as flagellates and ciliates (Enzinger and Cooper 1976; Menon et al. 2003).

Correlation analyses indicated that grazing rate in the wetland was strongly related to initial fecal coliform concentrations, and for all experiments combined there was a strong correlation between initial fecal coliform counts and grazing rate (Table 5). This relationship is possibly a result of 1) increased encounter rates due to increased prey densities, and/or 2) potentially increased micro-zooplankton abundance as a response to more food availability (untested in this research).

Water temperature was positively correlated with initial fecal bacterial counts in both BMPs combined (Table 5). Increased warm-season fecal bacteria counts in stormwater have been noted in a number of studies (Whitlock et al. 2002; Coulette and Noble 2008; Parker et al. 2010; Hathaway and Hunt 2012). This may have been a result of greater animal activity in the warmer season, greater seasonal human use of the watershed area, or greater rainfall occurring (note that there was a near-significant correlation between water temperature and rainfall,  $r = 0.257$ ,  $p = 0.092$ ). Regardless of cause, there was more feces subject to runoff in the warm season that entered the BMPs. Grazing rate was also positively correlated with water temperature; this may be a response to greater bacterial abundance as noted above, or increased encounter rates that were possibly due to elevated micro-zooplankton counts in the warm season (untested). Protozoan abundance has been positively correlated with water temperature in Florida lakes (Beaver and Crisman 1990) and zooplankton abundance in general has been positively correlated with water temperature in coastal North Carolina (Mallin and Paerl 1994). Rainfall was positively correlated with initial fecal coliform abundance at these two BMPs, as is often the case (Whitlock et al. 2002; Coulette and Noble 2008; Mallin et al. 2009). Initial fecal coliform abundance was positively correlated with DOC in the pond but not the wetland. Note that the detention pond drained a lot of impervious rooftop and parking lot and had little vegetation; its DOC concentration was well below that of the wetland. Surbeck et al. (2010) found DOC limitation of fecal bacteria in streamwater so possibly the low DOC concentrations in the pond were periodically limiting but the higher concentrations in the wetland were not.

## **5. Summary:**

The potential of micro-zooplankton grazing on fecal bacteria was tested seasonally in water from a standard wet detention pond and a constructed wetland. Two types of test were used: a set of 24-hr dilution grazing experiments, and a set of 3-day growth tests comparing unfiltered samples with samples filtered through two sizes of mesh to remove micro-zooplankton grazers. In the dilution assays statistically-significant grazing occurred in 75% of the wetland test compared to 45% of the detention pond tests. No significant grazing was measured in the 3-day growth tests when a 20  $\mu\text{m}$  mesh was used for filtration, indicating that the primary grazers passed through the mesh. However, when a 10  $\mu\text{m}$  mesh was used, statistically-significant grazing occurred in 60% of the wetland tests and 67% of the detention pond tests, indicating that the principal grazers were retained on the 10  $\mu\text{m}$  mesh. Thus, the grazing that occurred in these BMPs was accomplished mainly by very small micro-zooplankton, < 20  $\mu\text{m}$  across. Such organisms include pigmented and colorless flagellates, small ciliates and small amoeboid protozoans.

The principal environmental factors correlated with initial fecal bacteria counts in the experiments were rainfall and water temperature, and in the pond experiments the concentration of dissolved organic carbon. Chlorophyll *a* abundance did not appear to influence micro-

zooplankton grazing rates. Micro-zooplankton grazing rates were positively correlated with initial fecal bacteria abundance and water temperature.

## 6. Conclusions and Recommendations

Micro-zooplankton grazing occurred overall more frequently in a well-vegetated constructed wetland compared with a standard wet detention pond. However, such grazing clearly occurred in the detention pond as well. Thus, to achieve increased grazing as a means of fecal bacteria removal the use of constructed wetlands should be emphasized, and wet detention ponds should be enhanced when possible with submersed and emergent vegetation. Besides enhancing grazing, aquatic vegetation will improve suspended sediment settling and enhance fecal bacterial removal by sedimentation (Stenstrom and Carlander 2001; Vymazal 2005; Mallin et al. 2012) and increase denitrification (Song et al. 2014). Micro-zooplankton grazing rates increased along with water temperature. While this is a meteorological variable and not subject to short-term human control, it likely indicates that micro-zooplankton grazing rates are greater in wetlands and ponds located in warmer climates as opposed to colder, more northerly climates.

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- Woodcock, T.S., M.C. Monaghan and K.E. Alexander. 2010. Ecosystem characteristics and summer secondary production in stormwater ponds and reference wetlands. *Wetlands* 30:461-474.

## **Appendix 1: List of abbreviations and symbols**

BMP – Best Management Practice, an installed device or active process designed to mitigate or reduce stormwater runoff pollution.

Micro-zooplankton – a group of various aquatic microscopic organisms including copepod nauplii, various ciliated and flagellated protozoans, pigmented and colorless algae, rotifers, gastrotrichs and nematodes known to consume bacteria, some of which may be important in removing fecal bacteria within stormwater BMPs.

## **Appendix 2: List of professional presentations related to this project**

Burtchett, J.M., M.A. Mallin, M.R. McIver and L.B. Cahoon. 2015. “Seasonal variation in the quantification of fecal bacteria removal by micro-zooplankton grazing in stormwater BMPs”. Meeting of the Southeastern Estuarine Research Society, Jacksonville, Fla.

Burtchett, J.M., M.A. Mallin, M.R. McIver and L.B. Cahoon. 2014. “Quantification of fecal bacteria removal by micro-zooplankton grazing in stormwater BMPs”. Meeting of the Southeastern Estuarine Research Society, Carolina Beach, N.C.

Burtchett, J.M., M.A. Mallin, L.B. Cahoon and R. Whitehead. 2016. “Quantification of fecal bacteria removal by micro-zooplankton grazing in stormwater BMPs”. Water Resources Research Institute Annual Conference, Raleigh, NC.

# Microbial Quality and Risk Assessment of Type 2 NC Reclaimed Water for Non-potable and Potable Reuse

## Basic Information

<b>Title:</b>	Microbial Quality and Risk Assessment of Type 2 NC Reclaimed Water for Non-potable and Potable Reuse
<b>Project Number:</b>	2014NC187B
<b>Start Date:</b>	3/1/2014
<b>End Date:</b>	8/31/2015
<b>Funding Source:</b>	104B
<b>Congressional District:</b>	NC4
<b>Research Category:</b>	Water Quality
<b>Focus Category:</b>	None, None, None
<b>Descriptors:</b>	None
<b>Principal Investigators:</b>	Mark D Sobsey

## Publications

There are no publications.

## WRRRI Progress Report

### **TITLE:**

Microbial Quality and Risk Assessment of Type 2 NC Reclaimed Water for Non-Potable and Potable Reuse

### **Names and Affiliations of All Investigators:**

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**Project Number:** 14-04-W

**Project Start Date and End Date (a NCE has been granted).** March 1, 2014 through Feb. 28, 2016

**Date of Report:** August 2015

## **Project Activities and Findings to date:**

### **1.1 Summary of project work and progress to date and project background**

To date, nearly 30 samples of NC Type 2 reclaimed water, and 20 raw sewage samples have been processed for inclusion in this WRI project and its overall microbial dataset. Microbes quantified include *E. coli*, coliphage viruses, *Clostridium perfringens*, and *Salmonella* spp. bacteria. With the expansion of this project by the approved WRI supplement award supported by the UWC to include protozoan parasites (*Cryptosporidium parvum* and *Giardia lamblia*), there have been changes to the detection methods for the enteric viruses, which has resulted in delays in the field sampling for these pathogens. Methodological changes are described in the addendum and the delays are described in this report. However, full sample processing of all pathogens (protozoan parasites, enteric viruses, and pathogenic bacteria), as well as the indicator organisms previously detailed and discussed in this report will be underway before the end August 2015. Samples collected previously in the late spring and early summer that were partially processed and archived will now be subjected to protozoan parasite and human enteric virus analysis, along with new samples now being collected and processed.

The results presented here indicate that several of the candidate wastewater treatment facilities, detailed below are meeting the North Carolina microbiological standards for “Type 2” reclaimed water. These standards include final effluent microbial water containing 1) a monthly geometric mean of *E. coli* or fecal coliform level of less than or equal to 3/100mL and a daily maximum of less than or equal to 25/100mL 2) a monthly geometric mean coliphage level of less than or equal to 5/100mL and a daily maximum level of less than or equal to 25/100mL and 3) a monthly geometric *C. perfringens* level of less than or equal to 5/100mL and a daily maximum of less than or equal to 25/100mL. Four of the five treatment plants evaluated in this study are meeting these standards continuously, although some may not be quite meeting the 5 log<sub>10</sub> reduction performance target for viruses (coliphages) due to the detection limits of the methods of analysis. Furthermore, one plant, plant ‘C’, is not producing Type 2 reclaimed water as defined by these guidelines, apparently because it is not using dual disinfection treatment.

### **1.2 Experimental Overview**

#### **1.2.1 Wastewater sample collection and handling.**

##### Wastewater treatment plants

Raw wastewater (sewage) and treated wastewater effluent (reclaimed water) samples were collected at five wastewater treatment (reclaimed water) plants located in central North Carolina. These facilities were: (A) the Orange Water and Sewer Authority WWTP in Chapel Hill, (B) the Raleigh Neuse River WWTP, (C) the North Durham Water Reclamation Facility, (D) the Holly Springs WWTP and (E) the North Cary Water Reclamation Facility.

##### Sample handling

Raw sewage and treated wastewater (reclaimed water) was collected from the appropriate WWTP sampling points in sterile bottles, and kept chilled in coolers with ice during transport to

Chapel Hill. The samples were stored at 4°C upon arrival at the laboratory. Coliphage and *Clostridium perfringens* assays were performed on the day of or the day following sample collection.

## 1.2.2 Microbial Analysis

To date, there has been regular periodic sampling of *E. coli*, coliphage viruses, *Clostridium perfringens* (spores and vegetative cells), and *Salmonella* spp. bacteria. As will be explained in detail later in this report, the enteric virus data collection has been delayed due to the expansion of this project to include analysis for protozoan parasites (*Cryptosporidium parvum* and *Giardia lamblia*) as a result of required changes in detection methods for these pathogenic microorganisms.

### 1.2.2.1 *E. coli*: Method, Results and Data Analysis

#### 1.2.2.1.1 *E. coli* Methods

Both total coliform bacteria and *Escherichia coli* were quantified using IDEXX Colilert with the Quanti-Tray 2000 system. As described in the IDEXX step-by-step guide for using Colilert, a 100mL sample was measured and appropriately diluted for analysis. For this analysis, the US EPA standard phosphate buffered saline (PBS) was used. Next, the Colilert reagent was added to each sample and allowed to dissolve completely. Then that sample was poured into a Quanti-Tray 2000, sealed using the Quanti-Tray sealer and incubated at  $35 \pm 2^\circ\text{C}$  for 18 to 24 hours. Positive results were scored as yellow wells = total coliforms, yellow/fluorescent wells = *E. coli*. The positive wells were counted, recorded and used to calculate the MPN based on the IDEXX MPN table for the Quanti-Tray 2000.

#### 1.2.2.1.2 Total Coliform and *E. coli* Results

The tables below present the concentrations for both total coliforms and *Escherichia coli* as most probable number (MPN) per 100mL with the upper and lower 95% confident levels. These values were computed using the MPN table provided by IDEXX, which is available online through their website.

Table 1: Total Coliform Concentrations Detected by IDEXX Colilert (MPN per 100mL) in 21 Raw Sewage Samples

Sample	Total Coliform (MPN per 100mL)	Lower 95% Confidence Interval	Upper 95% Confidence Interval
B2	$3.08 \times 10^7$	$1.95 \times 10^7$	$4.71 \times 10^7$
D2	$4.76 \times 10^7$	$3.07 \times 10^7$	$7.07 \times 10^7$
E2	$5.64 \times 10^7$	$3.69 \times 10^7$	$8.49 \times 10^7$
C1	$1.96 \times 10^7$	$1.28 \times 10^7$	$2.93 \times 10^7$
A2	$1.99 \times 10^7$	$1.42 \times 10^7$	$2.73 \times 10^7$
B3	$7.67 \times 10^7$	$5.11 \times 10^7$	$1.08 \times 10^8$
D3	$3.47 \times 10^7$	$2.21 \times 10^7$	$5.19 \times 10^7$

C2	1.60 x 10 <sup>7</sup>	1.11 x 10 <sup>7</sup>	2.28 x 10 <sup>7</sup>
E3	4.33 x 10 <sup>7</sup>	2.87 x 10 <sup>7</sup>	6.16 x 10 <sup>7</sup>
A3	2.15 x 10 <sup>7</sup>	1.92 x 10 <sup>7</sup>	3.25 x 10 <sup>7</sup>
B4	2.46 x 10 <sup>7</sup>	1.68 x 10 <sup>7</sup>	3.49 x 10 <sup>7</sup>
D4	2.99 x 10 <sup>7</sup>	1.93 x 10 <sup>7</sup>	4.47 x 10 <sup>7</sup>
C3	3.87 x 10 <sup>7</sup>	2.55 x 10 <sup>7</sup>	5.64 x 10 <sup>7</sup>
E4	5.18 x 10 <sup>7</sup>	3.34 x 10 <sup>7</sup>	7.63 x 10 <sup>7</sup>
A4	3.10 x 10 <sup>7</sup>	2.16 x 10 <sup>7</sup>	4.32 x 10 <sup>7</sup>
B5	1.94 x 10 <sup>7</sup>	1.35 x 10 <sup>7</sup>	2.75 x 10 <sup>7</sup>
E5	3.56 x 10 <sup>7</sup>	2.26 x 10 <sup>7</sup>	5.33 x 10 <sup>7</sup>
D5	4.62 x 10 <sup>7</sup>	2.93 x 10 <sup>7</sup>	6.86 x 10 <sup>7</sup>
C4	2.31 x 10 <sup>7</sup>	1.56 x 10 <sup>7</sup>	3.43 x 10 <sup>7</sup>
A5	3.03 x 10 <sup>7</sup>	2.04 x 10 <sup>7</sup>	4.42 x 10 <sup>7</sup>
D6	2.26 x 10 <sup>7</sup>	1.50 x 10 <sup>7</sup>	3.39 x 10 <sup>7</sup>

Table 2: *E. coli* Concentrations Detected by IDEXX Colilert (MPN per 100mL) in 21 Raw Sewage Samples

Sample	<i>E. coli</i> (MPN per 100mL)	Lower 95% Confidence Interval	Upper 95% Confidence Interval
B2	3.55 x 10 <sup>6</sup>	2.39 x 10 <sup>6</sup>	5.10 x 10 <sup>6</sup>
D2	1.45 x 10 <sup>6</sup>	8.10 x 10 <sup>5</sup>	2.40 x 10 <sup>6</sup>
E2	2.29x 10 <sup>6</sup>	1.41 x 10 <sup>6</sup>	3.51 x 10 <sup>6</sup>
C1	1.31 x 10 <sup>6</sup>	6.08 x 10 <sup>5</sup>	2.18 x 10 <sup>6</sup>
A2	2.91 x 10 <sup>6</sup>	1.90 x 10 <sup>6</sup>	4.20 x 10 <sup>6</sup>
B3	2.52 x 10 <sup>6</sup>	1.58 x 10 <sup>6</sup>	3.79 x 10 <sup>6</sup>
D3	1.65 x 10 <sup>6</sup>	9.30 x 10 <sup>5</sup>	2.67 x 10 <sup>6</sup>
C2	9.90 x 10 <sup>5</sup>	5.25 x 10 <sup>5</sup>	1.83 x 10 <sup>6</sup>
E3	1.82 x 10 <sup>6</sup>	1.09 x 10 <sup>6</sup>	2.92 x 10 <sup>6</sup>
A3	2.18 x 10 <sup>6</sup>	1.34 x 10 <sup>6</sup>	3.39 x 10 <sup>6</sup>
B4	2.00 x 10 <sup>6</sup>	1.20 x 10 <sup>6</sup>	2.89 x 10 <sup>6</sup>
D4	2.85 x 10 <sup>6</sup>	1.81 x 10 <sup>6</sup>	4.25 x 10 <sup>6</sup>
C3	4.89 x 10 <sup>6</sup>	3.59 x 10 <sup>6</sup>	6.48 x 10 <sup>6</sup>
E4	3.43 x 10 <sup>6</sup>	2.28 x 10 <sup>6</sup>	4.98 x 10 <sup>6</sup>
A4	3.17 x 10 <sup>6</sup>	2.06 x 10 <sup>6</sup>	4.62 x 10 <sup>6</sup>
B5	2.17 x 10 <sup>6</sup>	1.32 x 10 <sup>6</sup>	3.40 x 10 <sup>6</sup>
E5	2.04 x 10 <sup>6</sup>	1.24 x 10 <sup>6</sup>	3.14 x 10 <sup>6</sup>
D5	2.96 x 10 <sup>6</sup>	1.88 x 10 <sup>6</sup>	4.39 x 10 <sup>6</sup>
C4	1.63 x 10 <sup>6</sup>	9.25 x 10 <sup>5</sup>	2.66 x 10 <sup>6</sup>
A5	2.15 x 10 <sup>6</sup>	1.32 x 10 <sup>6</sup>	3.34 x 10 <sup>6</sup>
D6	3.06 x 10 <sup>6</sup>	1.98 x 10 <sup>6</sup>	4.52 x 10 <sup>6</sup>

Table 3: Total Coliform Concentrations Detected by IDEXX Colilert (MPN per 100mL) in 25 Reclaimed Water Samples

Sample	Total Coliforms (MPN per 100mL)	Lower 95% Confidence Interval	Upper 95% Confidence Interval
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B1	<1.0	0.0	3.7
D1	<1.0	0.0	3.7
E1	<1.0	0.0	3.7
A1	<1.0	0.0	3.7
B2	<1.0	0.0	3.7
D2	<1.0	0.0	3.7
E2	<1.0	0.0	3.7
C1	129.1	89.5	181.9
A2	<1.0	0.0	3.7
B3	<1.0	0.0	3.7
D3	<1.0	0.0	3.7
C2	111.5	80.5	151.8
E3	<1.0	0.0	3.7
A3	<1.0	0.0	3.7
B4	<1.0	0.0	3.7
D4	<1.0	0.0	3.7
C3	101.7	72.5	138.2
E4	<1.0	0.0	3.7
A4	<1.0	0.0	3.7
B5	<1.0	0.0	3.7
E5	<1.0	0.0	3.7
D5	1.0	0.1	5.5
C4	187.2	126.1	281.0
A5	<1.0	0.0	3.7
D6	13.5	7.8	23.4

Table 4: *E. coli* Concentrations Detected by IDEXX Colilert (MPN per 100mL) in 25 Reclaimed Water Samples

Sample	<i>E. coli</i> (MPN per 100mL)	Lower 95% Confidence Interval	Upper 95% Confidence Interval
B1	<1.0	0.0	3.7
D1	<1.0	0.0	3.7
E1	<1.0	0.0	3.7
A1	<1.0	0.0	3.7
B2	<1.0	0.0	3.7
D2	<1.0	0.0	3.7
E2	<1.0	0.0	3.7
C1	7.4	3.2	14.4
A2	<1.0	0.0	3.7
B3	<1.0	0.0	3.7
D3	<1.0	0.0	3.7
C2	6.85	3.05	14.05
E3	<1.0	0.0	3.7

A3	<1.0	0.0	3.7
B4	<1.0	0.0	3.7
D4	<1.0	0.0	3.7
C3	7.4	3.2	14.4
E4	<1.0	0.0	3.7
A4	<1.0	0.0	3.7
B5	<1.0	0.0	3.7
E5	<1.0	0.0	3.7
D5	<1.0	0.0	3.7
C4	6.3	2.5	12.7
A5	<1.0	0.0	3.7
D6	4.1	1.7	9.5

The figures below summarize the concentrations for total coliforms and *E. coli* in both raw sewage and reclaimed water for each wastewater treatment plant. For each wastewater treatment plant, the log<sub>10</sub> reduction for each sample was computed by subtracting the log<sub>10</sub> concentration of total coliform bacteria or *E. coli* in reclaimed water from the log<sub>10</sub> concentration of total coliform bacteria or *E. coli* in raw sewage. For samples that returned an MPN of <1.0, the value was assumed to be 1.0 for analytical purposes. This analysis is presented in section 1.2.2.1.3.

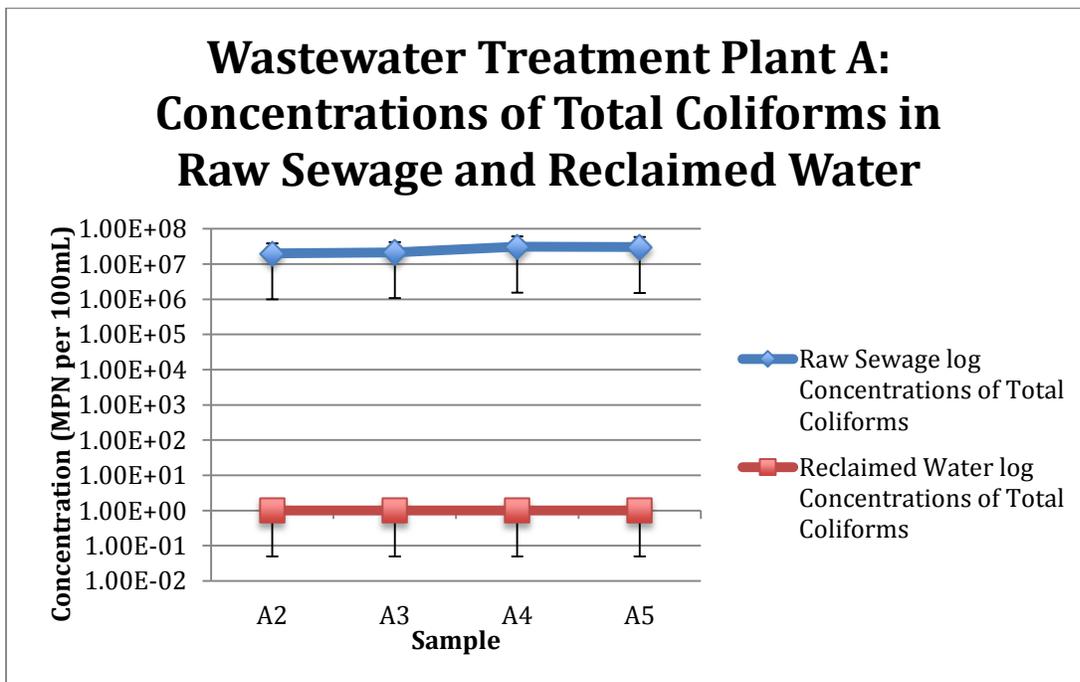


Figure 1: Wastewater Treatment Plant A: Concentrations for Total Coliforms in Raw Sewage and Reclaimed Water with 95% Confidence Intervals.

For wastewater treatment plant A, the concentration of total coliforms/100 mL in raw sewage is about  $10^7$ , whereas the concentration in reclaimed water is <1.0, or assumed 1.0 MPN/100mL for

this analysis. From to these results, the approximate  $\log_{10}$  reduction of total coliforms is about 7, as achieved by tertiary treatment and dual disinfection with both UV radiation and chlorine.

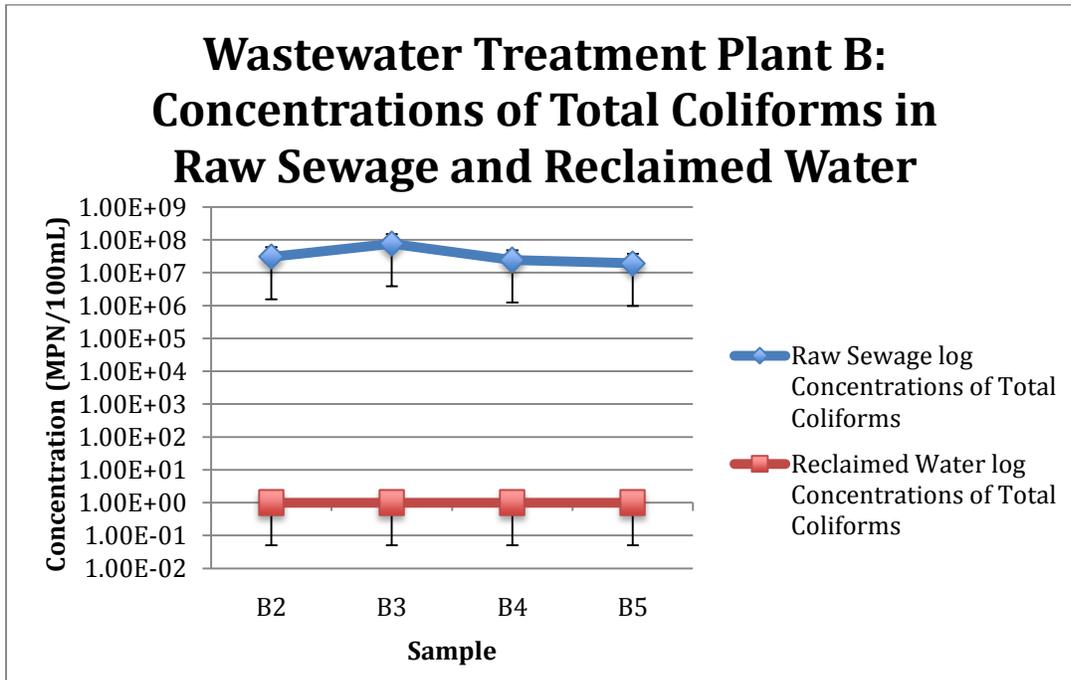


Figure 2: Wastewater Treatment Plant B: Concentrations for Total Coliforms in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant B, the concentration of total coliforms/100 mL in raw sewage is about  $10^7$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 MPN/100mL for this analysis. From to these results, the approximate  $\log_{10}$  reduction of total coliforms is 7-7.5, as achieved by tertiary treatment and dual disinfection with both UV radiation and chlorine.

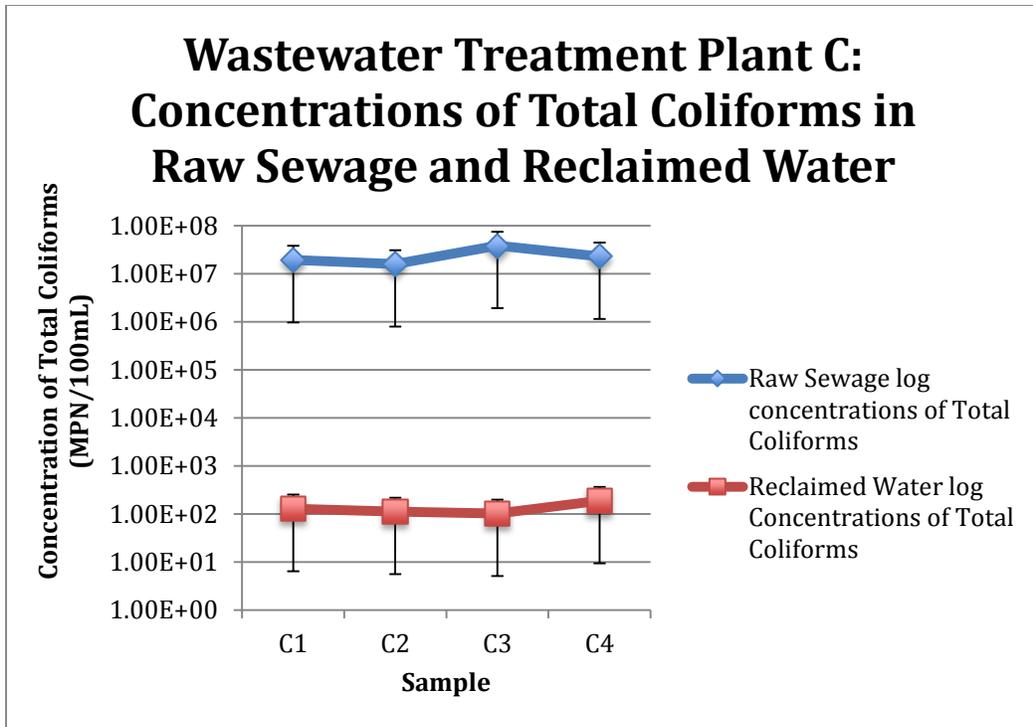


Figure 3: Wastewater Treatment Plant C: Concentrations for Total Coliforms in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant C, the concentration of total coliforms/100 mL in raw sewage is approximately  $10^7$ , whereas the concentration in reclaimed water is  $10^2$  MPN/100mL. According to these results, the approximate  $\log_{10}$  reduction is 5 for total coliforms based on tertiary treatment and disinfection.

## Wastewater Treatment Plant D: Concentrations of Total Coliforms in Raw Sewage and Reclaimed Water

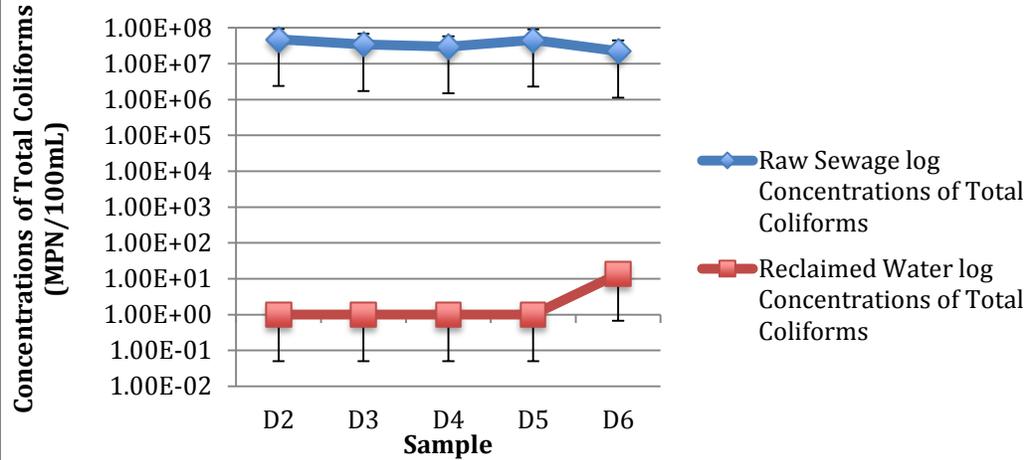


Figure 4: Wastewater Treatment Plant D: Concentrations for Total Coliforms in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant D, the MPN concentration of total coliforms/100 mL in raw sewage is about  $10^7$  and in reclaimed water it is  $<1.0$ , or assumed  $1.0/100\text{mL}$  for this analysis. According to these results, the approximate  $\log_{10}$  reduction is about 7-7.5 for total coliforms based on tertiary treatment and then dual disinfection with both UV radiation and chlorine.

## Wastewater Treatment Plant E: Total Coliform Concentrations in Raw Sewage and Reclaimed Water

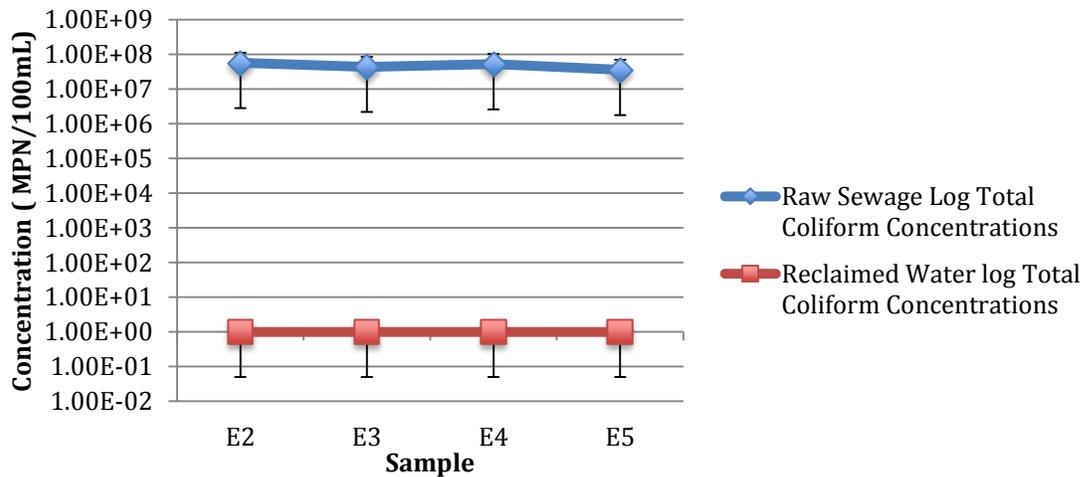


Figure 5: Wastewater Treatment Plant E: Concentrations for Total Coliforms in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant E, the MPN concentration of total coliforms/100 mL in raw sewage is approximately  $10^7$  and in reclaimed water is  $<1.0$ , or assumed  $1.0/100\text{mL}$  for this analysis. From these results, the estimated  $\log_{10}$  reduction is about 7.5 for total coliforms based on tertiary treatment followed by dual disinfection with UV radiation and chlorine.

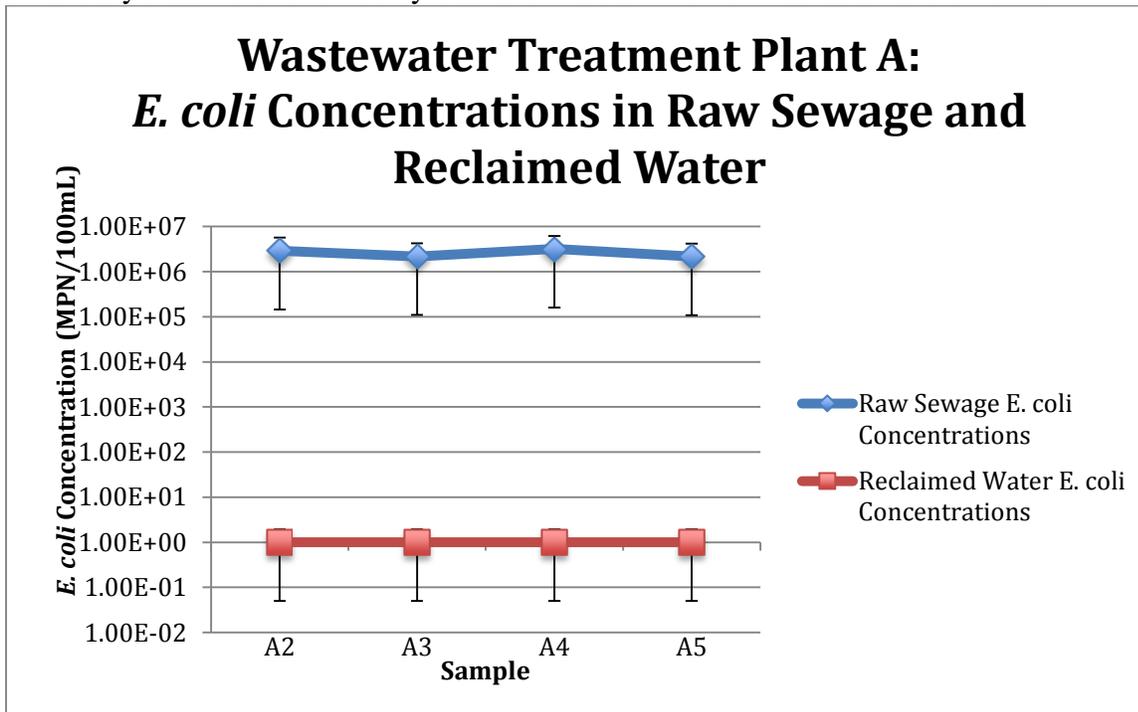


Figure 6: Wastewater Treatment Plant A: *E. coli* Concentration in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant A, the concentration of *E. coli*/100 mL in raw sewage is approximately  $10^6$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed  $1.0 \text{ MPN}/100\text{mL}$  for this analysis. According to these results, the approximate  $\log_{10}$  reduction is 6-6.5 for *E. coli* based on tertiary treatment followed by dual disinfection with UV radiation and chlorine.

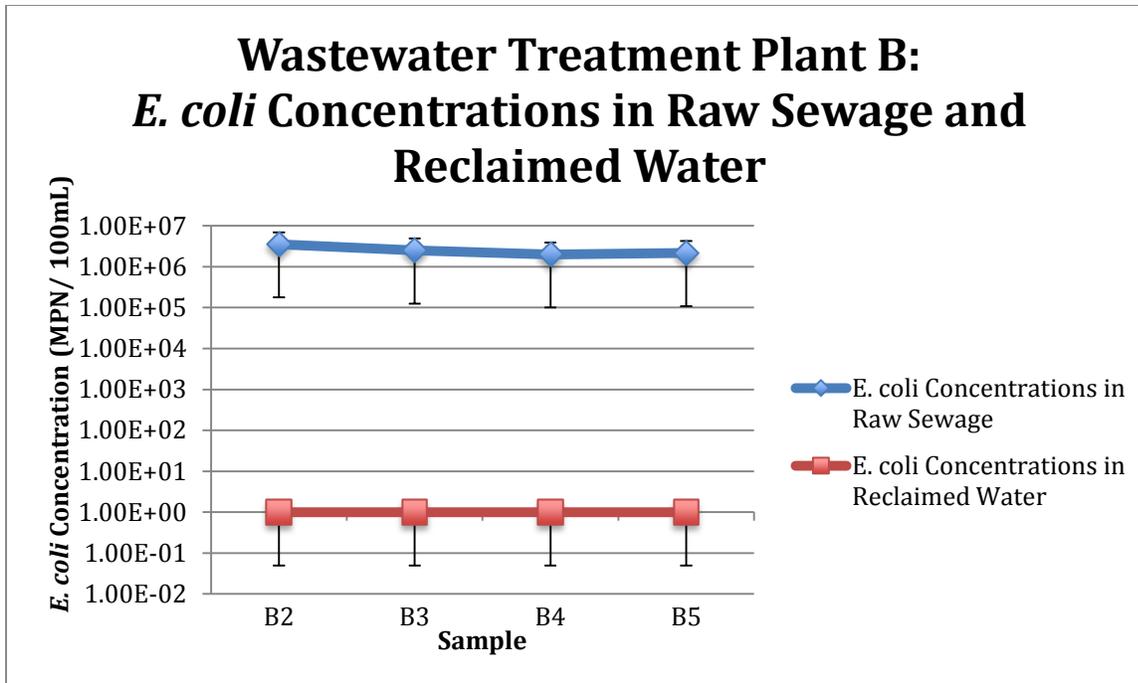


Figure 7: Wastewater Treatment Plant B: *E. coli* Concentration in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant B, the concentration of *E. coli*/100 mL in raw sewage is approximately  $10^6$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 MPN/100mL for this analysis. According to these results, the approximate  $\log_{10}$  *E. coli* reduction is 6-6.5 based on tertiary treatment followed by dual disinfection with UV radiation and chlorine.

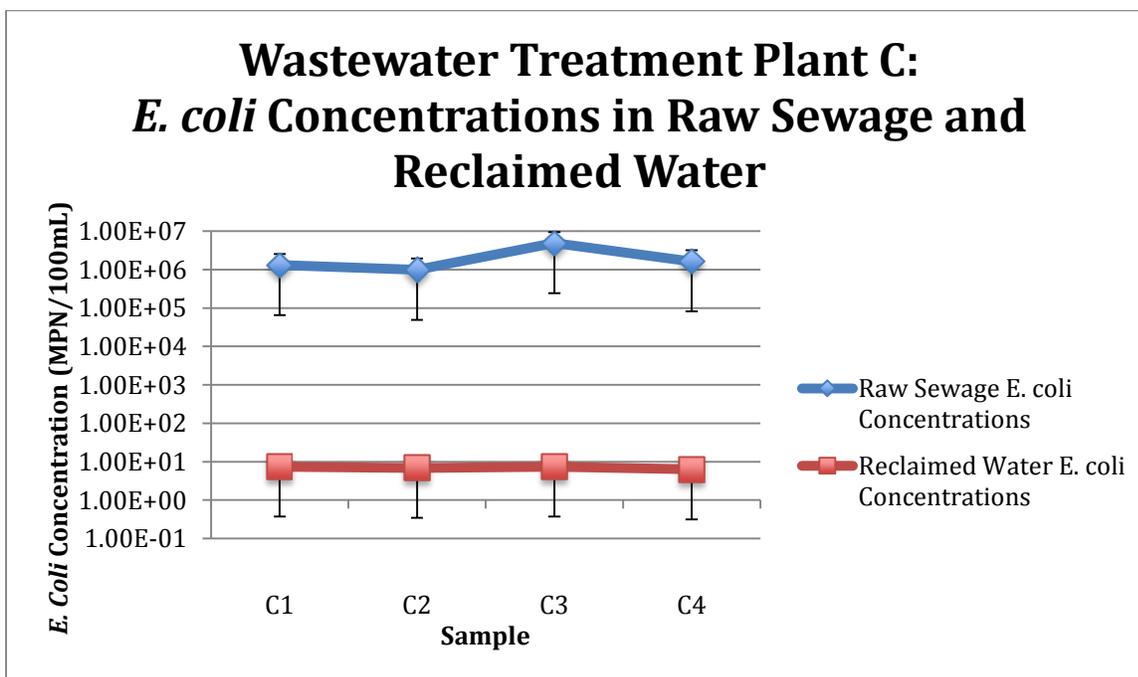


Figure 8: Wastewater Treatment Plant C: *E. coli* Concentration in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant C, the concentration of *E. coli*/100 mL in raw sewage is approximately  $10^6$ , whereas the concentration in reclaimed water is approximately 5-10 MPN/100mL. According to these results, the approximate  $\log_{10}$  reduction is about 5 for *E. coli* based on tertiary treatment and disinfection.

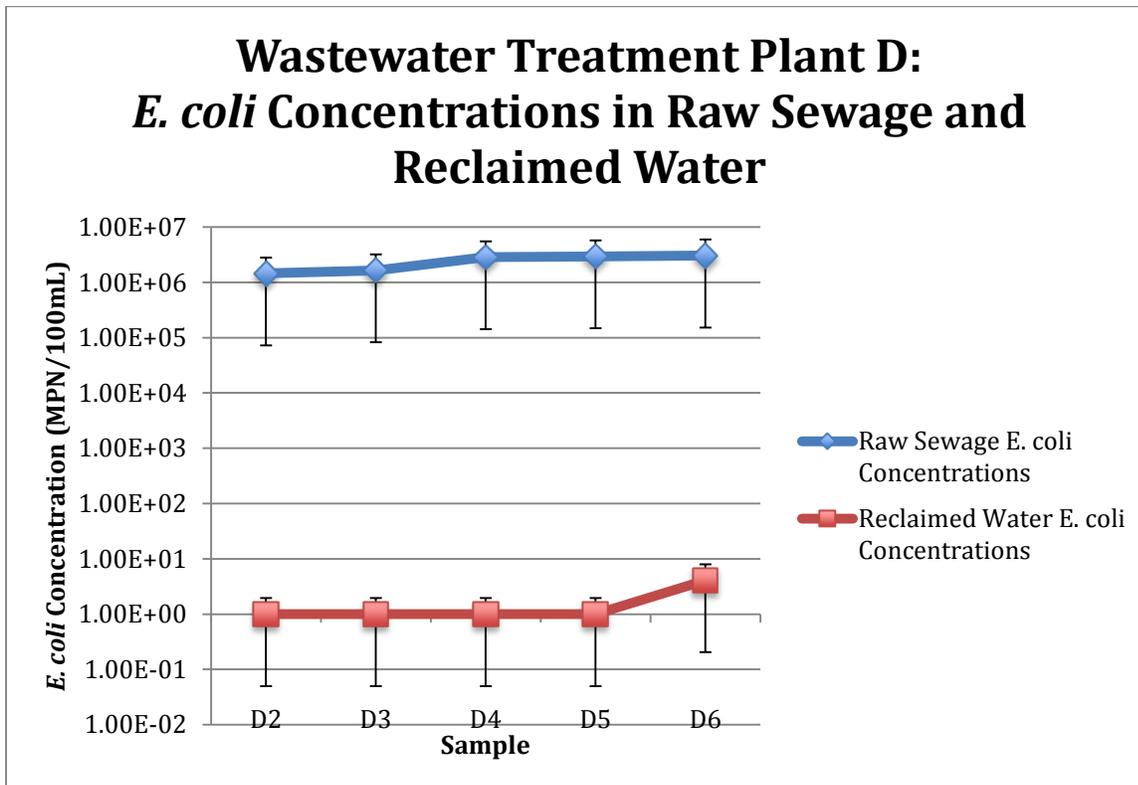


Figure 9: Wastewater Treatment Plant D: *E. coli* Concentration in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant D, the concentration of *E. coli*/100 mL in raw sewage is approximately  $10^6$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 MPN/100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction is about 6-6.5 for *E. coli* based on tertiary treatment followed by dual disinfection with UV radiation and chlorine.

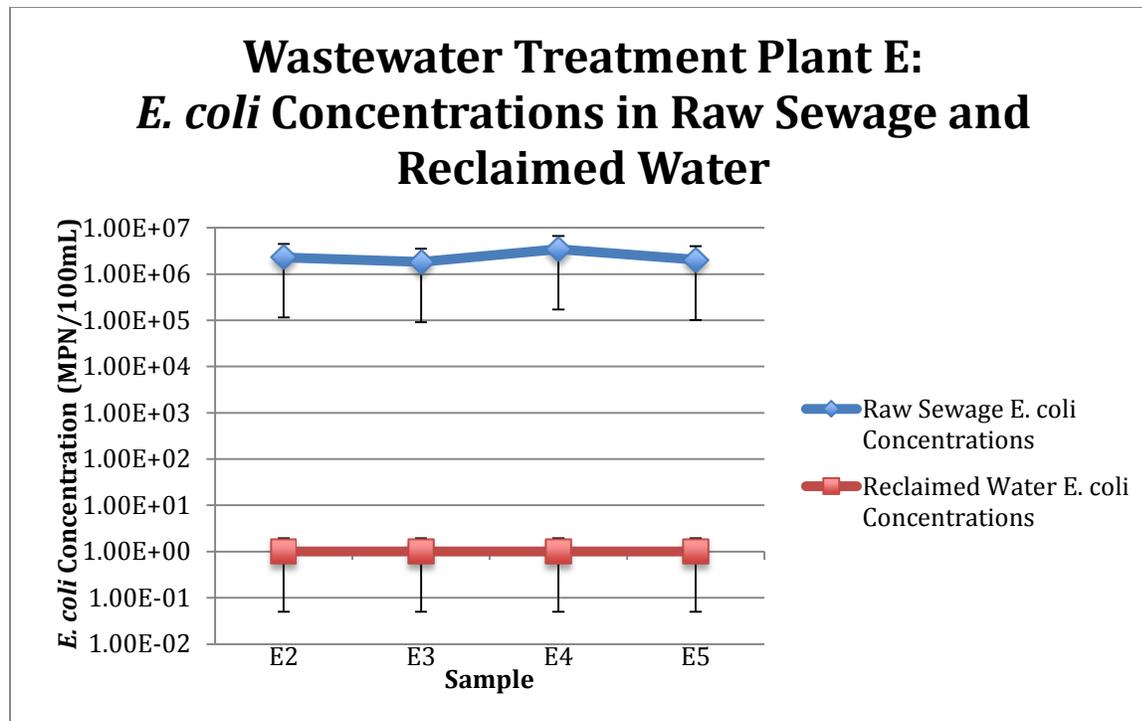


Figure 10: Wastewater Treatment Plant E: *E. coli* Concentration in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant E, the concentration of *E. coli*/100 mL in raw sewage is approximately  $10^6$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 MPN/100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction is 6-6.5 for *E. coli* based on tertiary treatment followed by dual disinfection with UV radiation and chlorine.

#### 1.2.2.1.3 Total Coliform and *E. coli* Data Analysis

In order to evaluate the microbial quality of the reclaimed water produced by each wastewater treatment facility, the average  $\log_{10}$  reductions were computed for both total coliforms and *E. coli*. The  $\log_{10}$  reduction analysis was conducted by calculating the average  $\log_{10}$  concentration in raw sewage for each treatment plant and then subtracting the average  $\log_{10}$  concentration in reclaimed water. The average  $\log_{10}$  reductions by wastewater treatment (water reclamation) plants are presented in Figures 11 and 12 below.

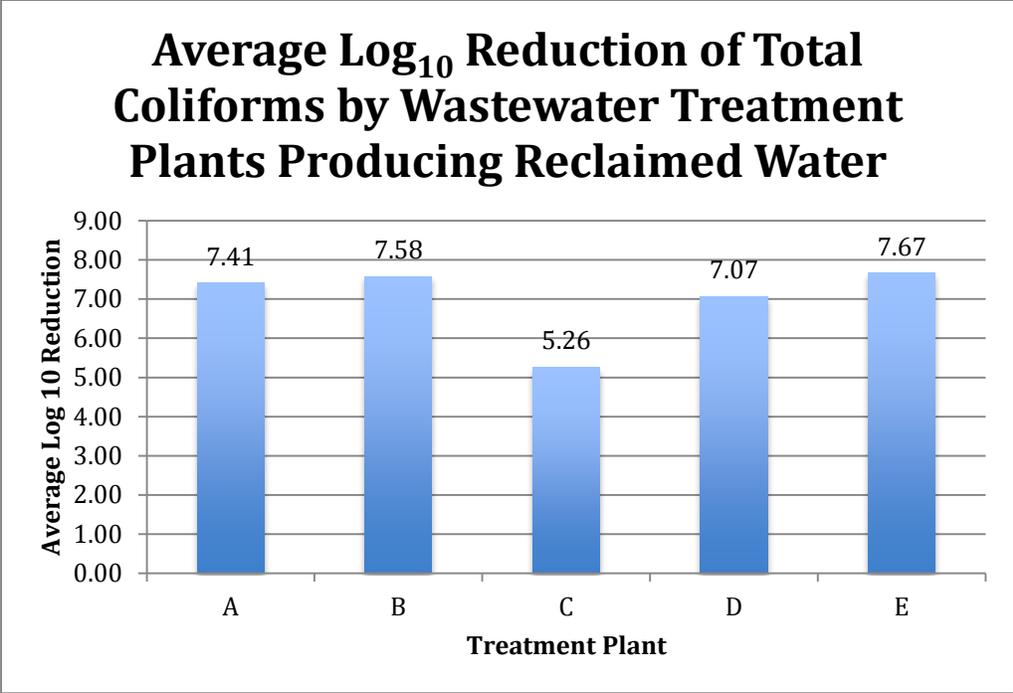


Figure 11: Average Log<sub>10</sub> Reduction of Total Coliforms by Wastewater Treatment Plant

In wastewater treatment, specifically for NC type 2 quality reclaimed water, the recommended reduction of bacteria is 6 log<sub>10</sub> from the raw, untreated influent sewage through complete treatment to produce final effluent. For total coliforms, every wastewater treatment plant, except plant C has met this requirement. Plant C is not producing Type 2 reclaimed water as it relates to this standard. As total coliforms are not regulated by NC law, the *E. coli* reductions, presented below, are of greater relevance and interest for health-related and regulatory outcomes.

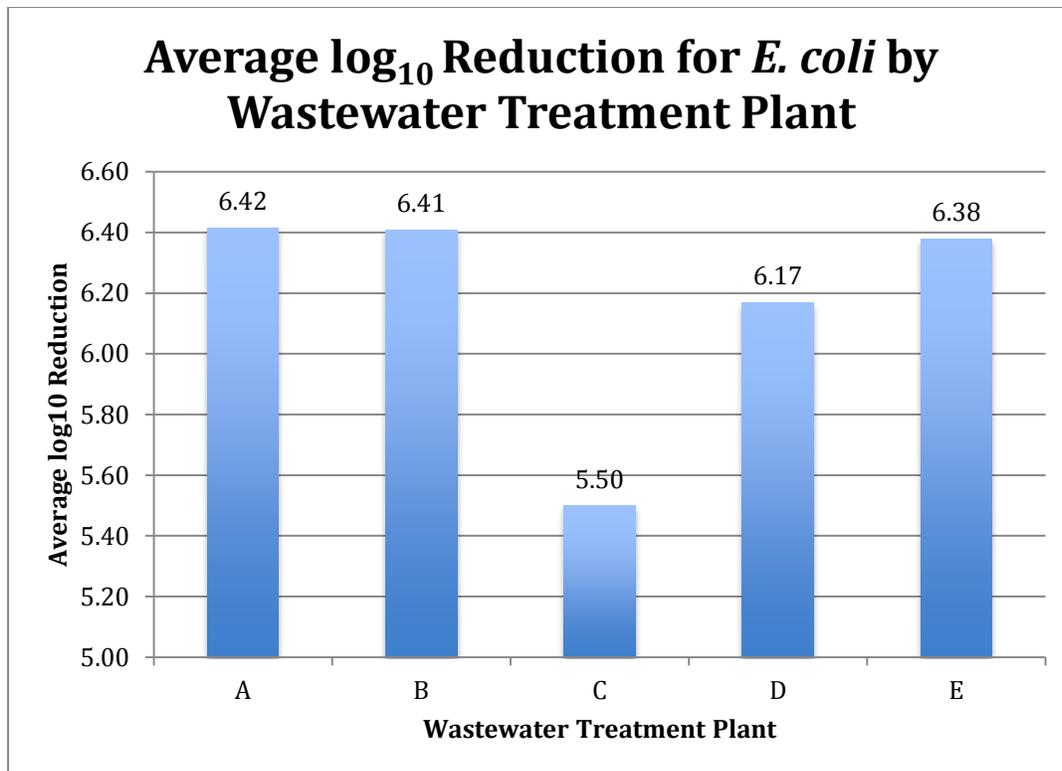


Figure 12: Average Log<sub>10</sub> Reduction of *E. coli* by Wastewater Treatment Plant

As with total coliforms, it is clear that a 6 log<sub>10</sub> reduction is being achieved in all of the wastewater treatment plants sampled by this project, except wastewater treatment plant C. The results for plant C present concerns because examination the data for both *E. coli* and total coliforms concentrations indicates that plant C had similar raw sewage concentrations of these organisms prior to tertiary treatment and dual disinfection when compared to the other wastewater treatment plants, but had higher concentrations in final effluent samples that consistently exceeded the 3 *E. coli*/100 mL mean concentration somewhat and therefore did not achieve the 6 log<sub>10</sub> reduction target. Plant is apparently not using dual disinfection treatment or having difficulties in applying these disinfection processes.

Because there is a discrepancy in performance between plant C and the other wastewater treatment (water reclamation) plants, a combined value of average log<sub>10</sub> reduction is not presented in this analysis. However, it is clear that the other four plants have achieved similar 6 log<sub>10</sub> or more reduction performance for *E. coli* over the 10-12 month period of study.

## 1.2.2.2 Coliphage Viruses: Method, Results and Data Analysis

### 1.2.2.2.1 Coliphage Viruses Methods

Coliphage viruses were detected by the single agar layer method, US EPA Method 1602. As described in US EPA Method 1602, the single agar layer method involves the combination of a 100 mL water sample with 100 mL of molten agar medium, *E. coli* coliphage host bacteria, appropriate antibiotics, and a divalent cation (as MgCl<sub>2</sub>) at a final concentration of 0.05M. This

method relies on the ability of coliphages to infect the provided *E. coli* host cells and create discrete, circular lysis zones called plaques in the solidified agar medium host cell lawn. Double strength tryptic soy agar (TSA) is prepared and tempered in a waterbath first at 55°C and then at 45°C, while a 100 mL volume of the water sample is warmed in a waterbath first at 37°C and then at 45°C for a short time period. The time for the sample to be tempered to 45°C is determined from a thermometer placed in 100 mL of Phosphate Buffered Saline (PBS) or deionized (DI) water to monitor the time required to reach 45°C. Careful temperature control is needed to prevent heat inactivation of coliphages from excessive exposure to 45°C and to prevent premature agar hardening if the water samples to be combined with the molten agar are not sufficiently high in temperature. *E. coli* coliphage host, appropriate antibiotic, and magnesium chloride (MgCl<sub>2</sub>) are added to the 100mL water sample and this mixture is added to 100 mL of molten agar medium, mixed gently (not shaken) and poured into 5 150mm diameter sterile petri dishes. These plates are allowed to harden and dry for 10-15 minutes, then inverted and incubated overnight at 37°C. The next day the plates are examined and counted (read) for plaques (discrete, circular lysis zones each produced by individual coliphages), and coliphage concentrations are reported as plaque forming (PFU) units per 100 mL.

Somatic and male-specific/F+ coliphages were detected using the US EPA recommended *E. coli* CN13 and *E. coli* Famp host bacteria, respectively, and “total” coliphages (somatic plus male-specific/F+) were detected simultaneously using the coliphage bacterial host previously validated by the research of WRRRI project 13-06-W, *E. coli* CB390.

#### 1.2.2.2.2 Coliphage Viruses Results

The tables below present the concentrations for coliphages as detected by the male-specific/ F+ coliphage host *E. coli* Famp, the somatic coliphage host, *E. coli* CN13, and the total coliphage host, *E. coli* CB390. Concentrations are presented as plaque forming units (PFUs) per 100mL.

Table 5: Coliphage Concentrations Detected by SAL (PFU per 100mL) in 20 Raw Sewage Samples on Different *E. coli* Hosts

Sample	<i>E. coli</i> CN13 (Somatic) (PFU per 100mL)	<i>E. coli</i> Famp (Male- specific/F+) (PFU per 100mL)	<i>E. coli</i> CB390 (Somatic + Male-specific/F+) (PFU per 100mL)
C1	2.6 x 10 <sup>5</sup>	6 x 10 <sup>4</sup>	3.5 x 10 <sup>5</sup>
A2	9 x 10 <sup>4</sup>	8 x 10 <sup>4</sup>	4.3 x 10 <sup>5</sup>
B2	1.2 x 10 <sup>4</sup>	2.6 x 10 <sup>4</sup>	2.2 x 10 <sup>4</sup>
D3	1.1 x 10 <sup>5</sup>	1.5 x 10 <sup>5</sup>	1.5 x 10 <sup>5</sup>
C2	9 x 10 <sup>4</sup>	5 x 10 <sup>4</sup>	3.4 x 10 <sup>5</sup>
E3	2 x 10 <sup>4</sup>	3 x 10 <sup>4</sup>	2.3 x 10 <sup>5</sup>
A3	1 x 10 <sup>5</sup>	2 x 10 <sup>4</sup>	1.5 x 10 <sup>5</sup>
B3	1.1 x 10 <sup>5</sup>	2 x 10 <sup>4</sup>	2.5 x 10 <sup>5</sup>
D4	5 x 10 <sup>4</sup>	6.6 x 10 <sup>5</sup>	7.5 x 10 <sup>5</sup>
C3	1 x 10 <sup>4</sup>	8.4 x 10 <sup>5</sup>	1.8 x 10 <sup>5</sup>
E4	1 x 10 <sup>4</sup>	2.8 x 10 <sup>5</sup>	1.5 x 10 <sup>5</sup>
A4	1.0 x 10 <sup>4</sup>	1.2 x 10 <sup>4</sup>	1.0 x 10 <sup>5</sup>
B4	2.6 x 10 <sup>4</sup>	8 x 10 <sup>3</sup>	4.6 x 10 <sup>4</sup>

E5	$3.3 \times 10^4$	$1.6 \times 10^4$	$6.7 \times 10^4$
D5	$1.3 \times 10^4$	$7 \times 10^3$	$1.9 \times 10^3$
C4	$2.8 \times 10^4$	$1 \times 10^3$	$3.4 \times 10^4$
A5	$1.9 \times 10^4$	$8 \times 10^3$	$3.5 \times 10^4$
D6	$5.7 \times 10^4$	$5.3 \times 10^4$	$5.0 \times 10^5$
A6	$6.1 \times 10^4$	$7 \times 10^3$	$7.6 \times 10^4$

Table 6: Coliphage Concentrations Detected by SAL (PFU per 100mL) in 26 Reclaimed Water Samples on Different *E. coli* Hosts

Sample	<i>E. coli</i> CN13 (Somatic) (PFU per 100mL)	<i>E. coli</i> Famp (Male-specific/F+) (PFU per 100mL)	<i>E. coli</i> CB390 (Somatic + Male-specific/F+) (PFU per 100mL)
D1	2	0	0
E1	2	2	14
A1	2	7	0
B1	0	3	2
D2	0	0	0
E2	6	2	3
C1	0	0	7
A2	6	0	0
B2	0	0	1
D3	2	0	4
C2	2	0	0
E3	0	0	0
A3	0	0	0
B3	0	0	0
D4	0	0	0
C3	0	0	10
E4	0	0	0
A4	0	0	0
B4	0	0	0
E5	0	0	0
D5	0	0	0
C4	0	0	0
A5	5	0	4
D6	14	0	12
A6	3	0	25

The figures below present the concentrations of somatic, male-specific/F+ and total coliphages for both raw sewage and reclaimed water for each wastewater treatment (water reclamation) plant as detected by the 3 *E. coli* hosts used in this study. For each wastewater treatment (water reclamation) plant, the log<sub>10</sub> reduction of coliphages for each sample was computed by subtracting the log<sub>10</sub> concentration in reclaimed water from the log<sub>10</sub> concentration in raw sewage. For samples that returned a PFU value of 0/100 mL, the log<sub>10</sub> value was assumed to be 1.0/100 mL for analytical purposes. This analysis is presented in section 1.2.2.2.3.

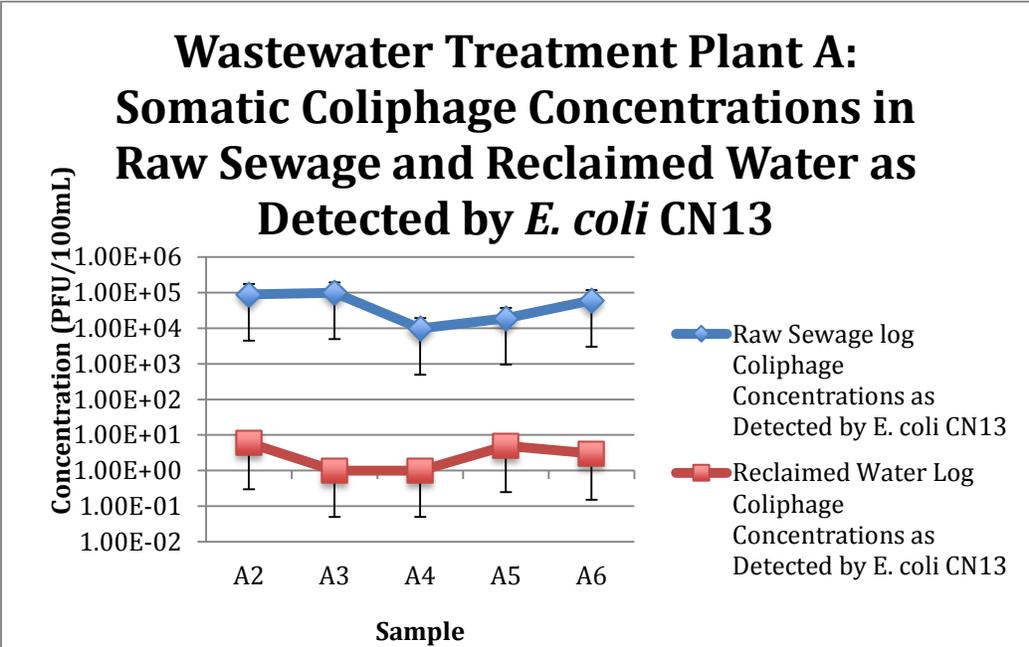


Figure 13: Wastewater Treatment Plant A: Somatic Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CN13 with 95% Confidence Intervals

For wastewater treatment plant A, the concentration of somatic coliphages per 100 mL detected in by *E. coli* CN13 in raw sewage is approximately  $10^5$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is about 4-5 for somatic coliphages, from tertiary treatment and then dual disinfection with UV radiation and chlorine.

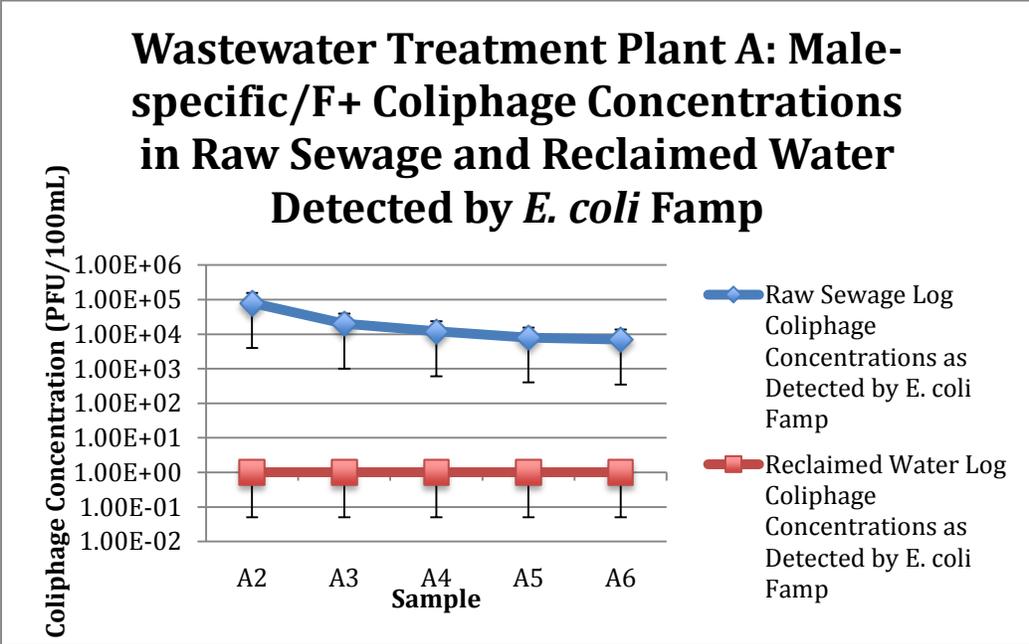


Figure 14: Wastewater Treatment Plant A: Male-specific/F+ Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* Famp with 95% Confidence Intervals

For wastewater treatment plant A, the concentration of male-specific/F+ coliphages detected by *E. coli* Famp in raw sewage is approximately  $10^4$  whereas the concentration in reclaimed water is  $<1.0$ , or assumed  $1.0$  PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is 4 for male-specific/F+ coliphages, from tertiary treatment followed by dual disinfection with UV radiation and chlorine.

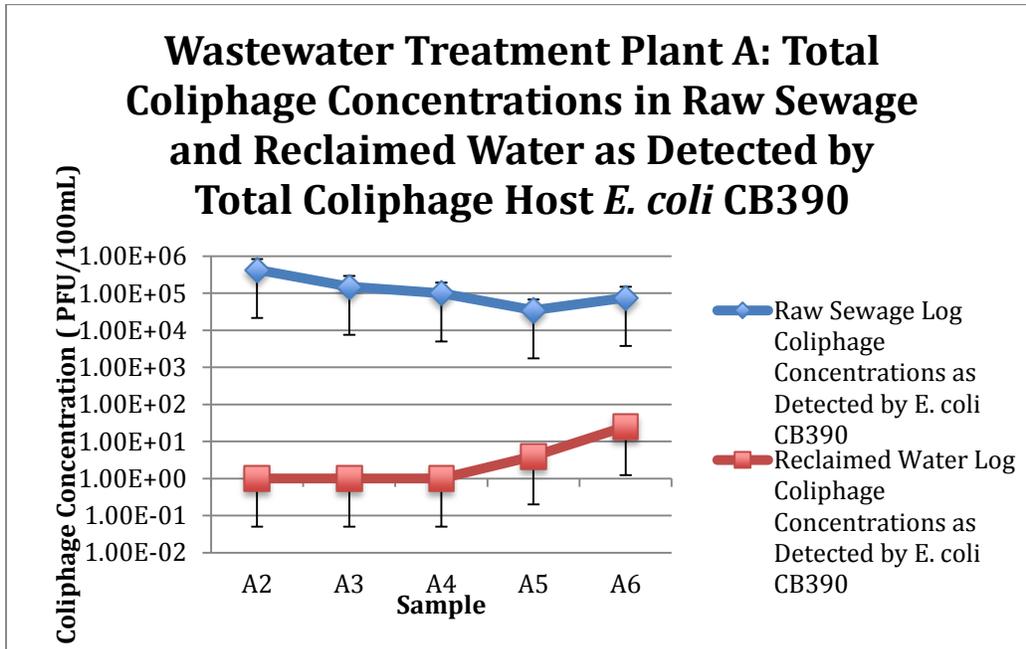


Figure 15: Wastewater Treatment Plant A: Total Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CB390 with 95% Confidence Intervals

For wastewater treatment plant A, the concentration of total coliphages/100 mL detected by *E. coli* CB390 in raw sewage is approximately  $10^5$ , whereas the  $\log_{10}$  concentration in reclaimed water is  $<1.0$  to  $1$ , or assumed  $0.3$  PFU/100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is approximately 4.5 for total coliphages, from tertiary treatment followed by dual disinfection with UV radiation and chlorine.

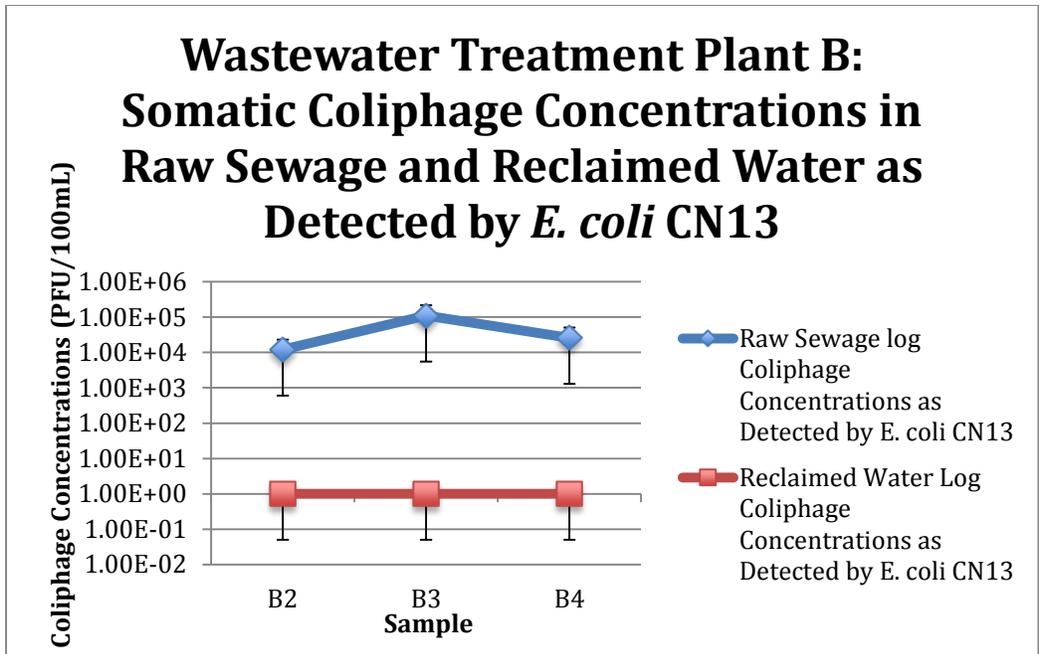


Figure 16: Wastewater Treatment Plant B: Somatic Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CN13 with 95% Confidence Intervals

For wastewater treatment plant B, the concentration of somatic coliphages detected by *E. coli* CN13 in raw sewage is approximately  $10^4$  to  $10^5$  whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is about 4.5 for somatic coliphages, from tertiary treatment followed by dual disinfection with UV radiation and chlorine.

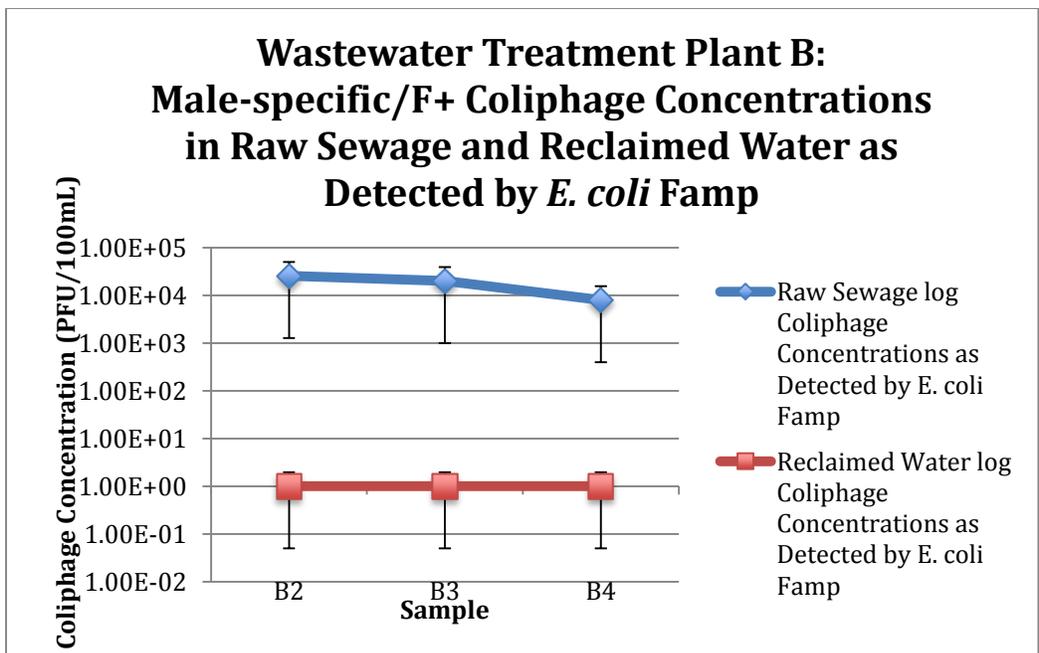


Figure 17: Wastewater Treatment Plant B: Male-specific/F+ Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* Famp with 95% Confidence Intervals

For wastewater treatment plant B, the concentration of male-specific/F+ coliphages/100mL detected by *E. coli* Famp in raw sewage is approximately  $10^4$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is about 4 for male specific/F+ coliphages, from tertiary treatment followed by dual disinfection with UV radiation and chlorine.

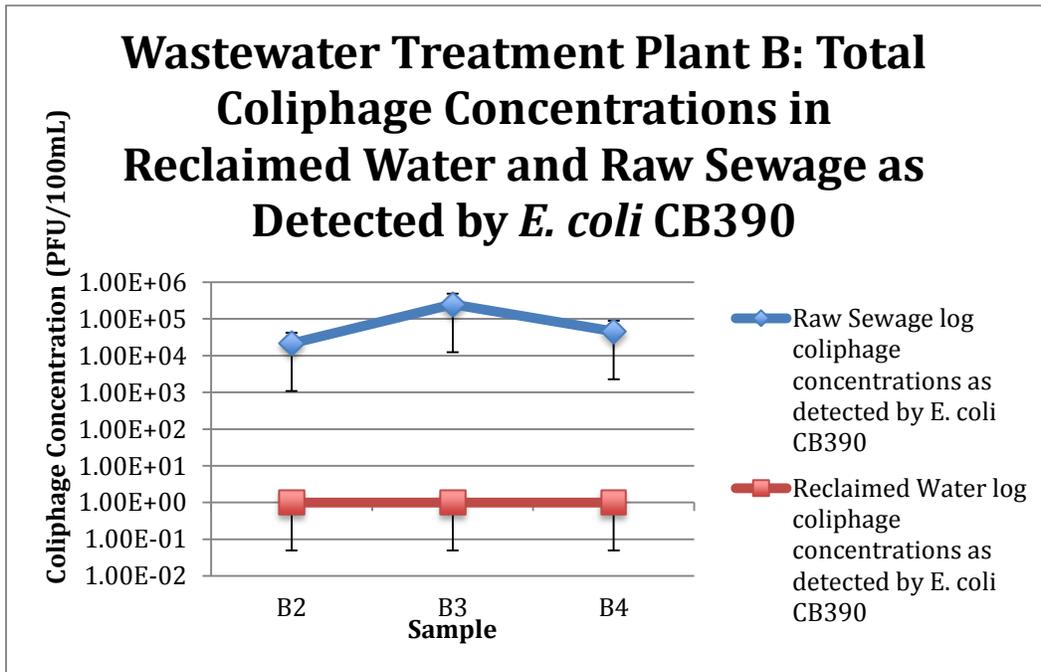


Figure 18: Wastewater Treatment Plant B: Total Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CB390 with 95% Confidence Intervals

For wastewater treatment plant B, concentration of total coliphages per 100 mL detected by *E. coli* CB390 in raw sewage is approximately  $10^5$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is approximately 5 for total coliphages, from tertiary treatment followed by dual disinfection with UV radiation and chlorine.

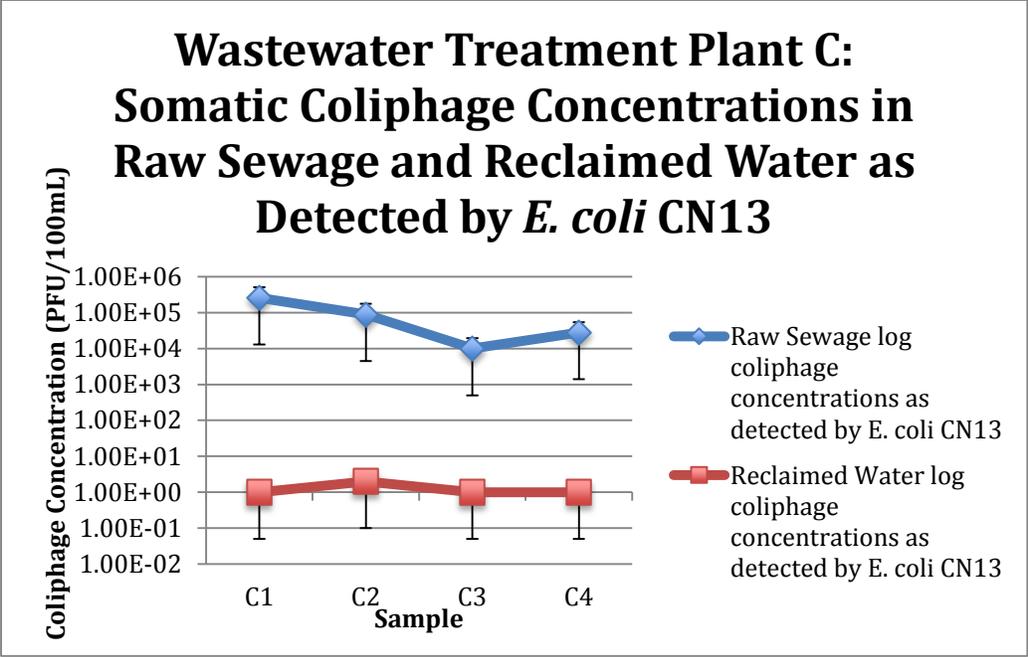


Figure 19: Wastewater Treatment Plant C: Somatic Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CN13 with 95% Confidence Intervals

For wastewater treatment plant C, the concentration of somatic coliphages per 100 mL detected by *E. coli* CN13 in raw sewage is approximately 10<sup>4</sup> to 10<sup>5</sup>, whereas the concentration in reclaimed water is <1.0, or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate log<sub>10</sub> reduction at this plant is approximately 4.5 for somatic coliphages, from tertiary treatment followed by disinfection.

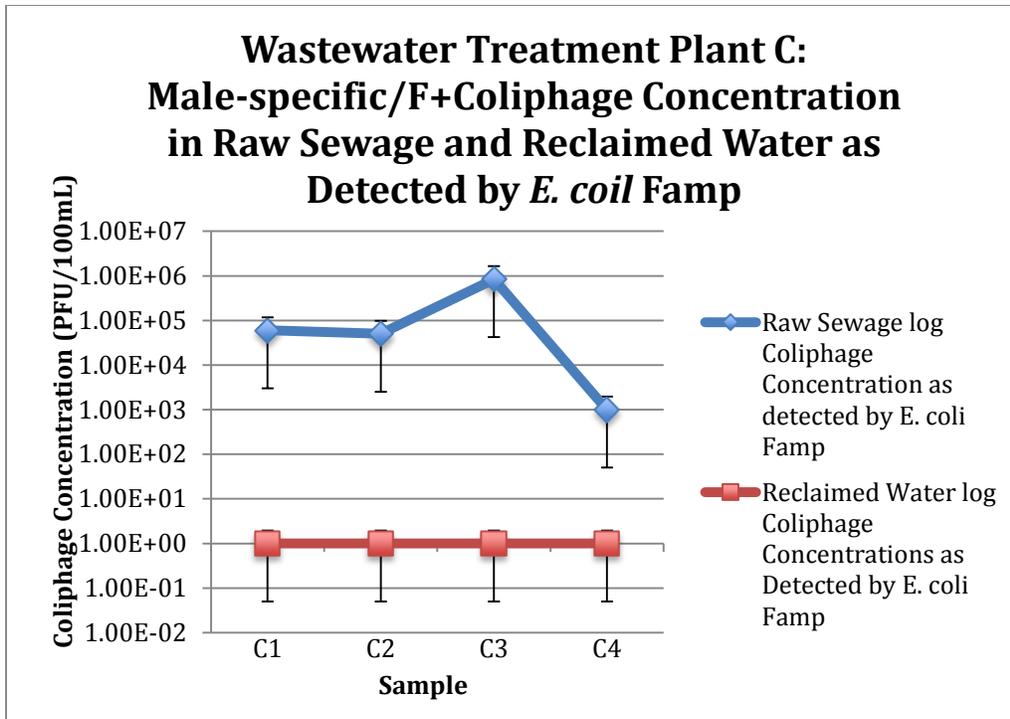


Figure 20: Wastewater Treatment Plant C: Male-specific/F+ Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* Famp with 95% Confidence Intervals

For wastewater treatment plant C, the concentration per 100 mL of male-specific/F+ coliphages detected by *E. coli* Famp in raw sewage is nearly  $10^5$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is nearly 5 for male-specific/F+ coliphages, from tertiary treatment followed by disinfection.

## Wastewater Treatment Plant C: Total Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CB390

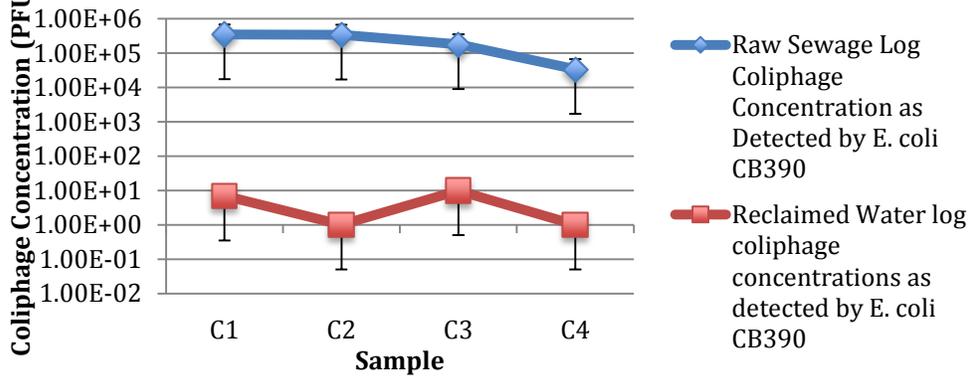


Figure 21: Wastewater Treatment Plant C: Total Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CB390 with 95% Confidence Intervals

For wastewater treatment plant C, the concentration of coliphages per 100 mL detected by *E. coli* CB390 in raw sewage is approximately  $10^{4-5}$ , whereas the concentration in reclaimed water is between  $<1.0$  and  $10$  PFU/ 100mL. According to these results, the approximate  $\log_{10}$  reduction at this plant is 4 to 5 for total coliphages, from tertiary treatment followed by disinfection.

## Wastewater Treatment Plant D: Somatic Coliphage Concentrations as Detected by *E. coli* CN13 in Raw Sewage and Reclaimed Water

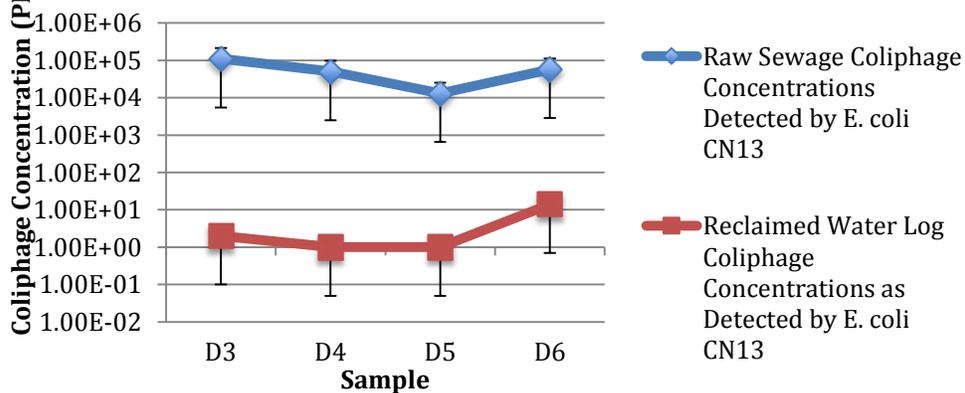


Figure 22: Wastewater Treatment Plant D: Somatic Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CN13 with 95% Confidence Intervals

For wastewater treatment plant D, the concentration of somatic coliphages detected by *E. coli* CN13 in raw sewage is approximately  $10^4$  whereas the concentration in reclaimed water is between  $<1.0$  and  $15$  CFU/100mL. According to these results, the approximate  $\log_{10}$  reduction at this plant is 4 for somatic coliphages, from tertiary treatment followed by dual disinfection with UV radiation and chlorine.

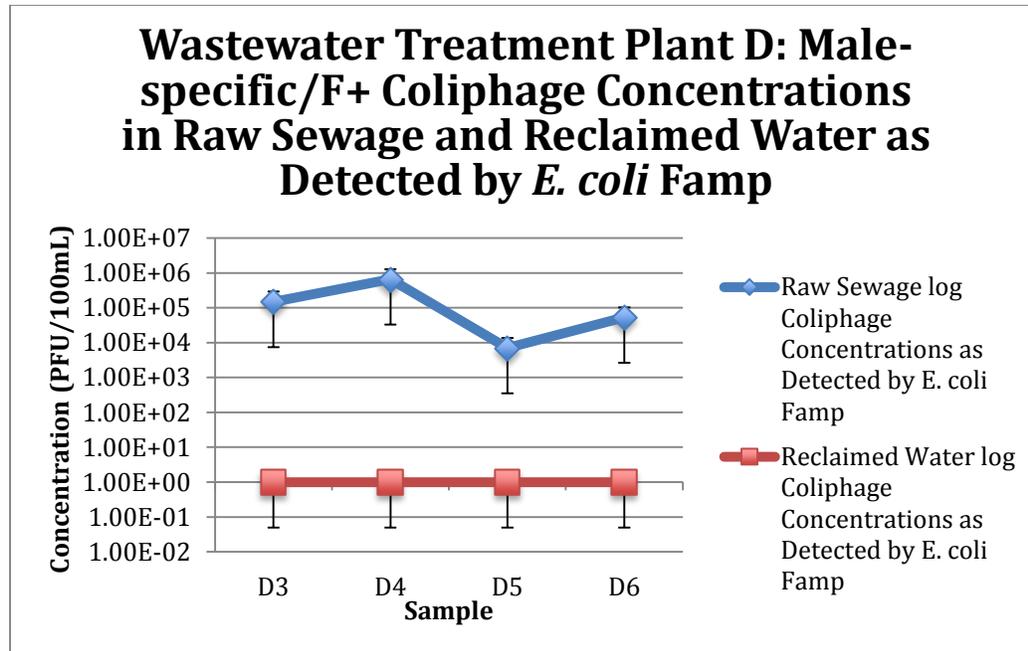


Figure 23: Wastewater Treatment Plant D: Male-specific/F+ Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* Famp with 95% Confidence Intervals

For wastewater treatment plant D, the concentration of male-specific/coliphages/100 mL detected by *E. coli* Famp in raw sewage is about  $10^5$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed  $1.0$  PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is about 5 for male-specific/F+ coliphages, from tertiary treatment followed by dual disinfection with UV radiation and chlorine.

## Wastewater Treatment Plant D: Total Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* CB390

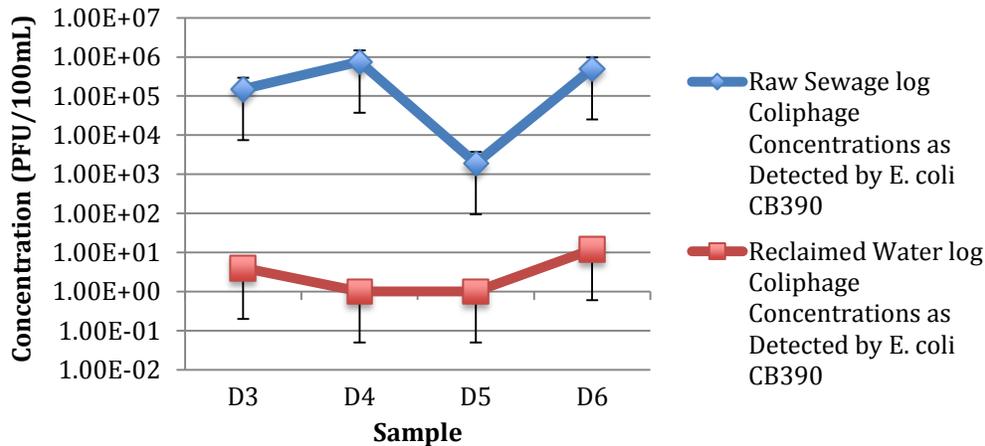


Figure 24: Wastewater Treatment Plant D: Total Coliphage Concentrations per 100 mL in Raw Sewage and Reclaimed Water as Detected by *E. coli* CB390 with 95% Confidence Intervals

For wastewater treatment plant D, concentrations of total coliphages/100 mL detected by *E. coli* CB390 in raw sewage is about  $10^5$ , and the concentration in reclaimed water is  $<1.0$  to  $10$ , or assumed  $1.0$  PFU/100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is 4 to 5 for total coliphages, by tertiary treatment followed by dual disinfection with UV radiation and chlorine.

## Wastewater Treatment Plant E: Somatic Coliphage Concentration in Raw Sewage and Reclaimed Water as detected by *E. coli* CN13

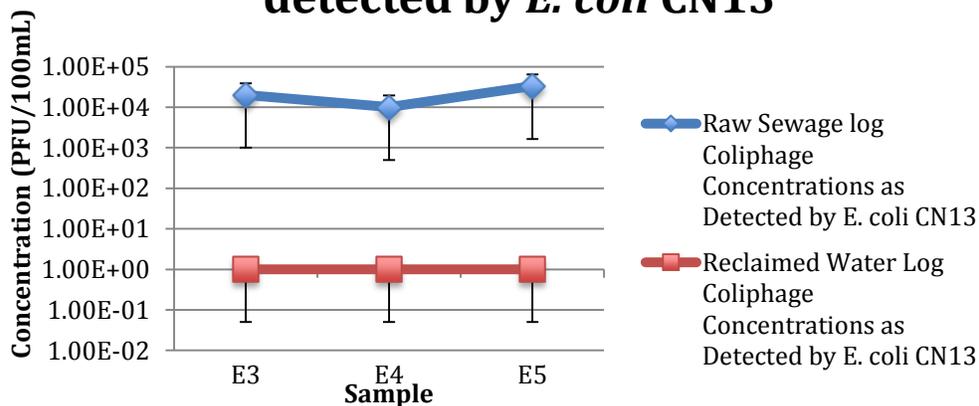


Figure 25: Wastewater Treatment Plant E: Somatic Coliphage Concentrations per 100 mL in Raw sewage and Reclaimed Water as Detected by *E. coli* CN13 with 95% Confidence Intervals

For wastewater treatment plant E, the concentration of somatic coliphages/100 mL detected by *E. coli* CN13 in raw sewage is about  $10^4$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is about 4 for somatic coliphages, from tertiary treatment and dual disinfection with UV radiation and chlorine.

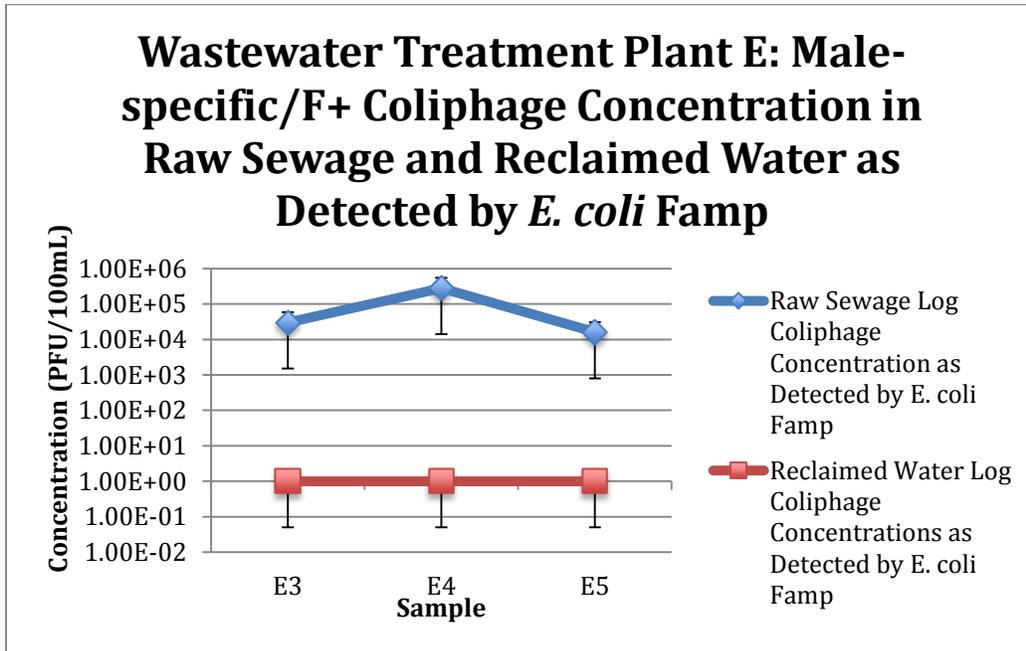


Figure 26: Wastewater Treatment Plant E: Male-specific/F+ Coliphage Concentrations in Raw Sewage and Reclaimed Water as Detected by *E. coli* Famp with 95% Confidence Intervals

For wastewater treatment plant E, the concentration of coliphages/100 mL detected by *E. coli* Famp in raw sewage is about  $10^5$ , whereas the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is approximately 4-5 for male-specific/F+ coliphages, by tertiary treatment followed by dual disinfection with UV radiation and chlorine.

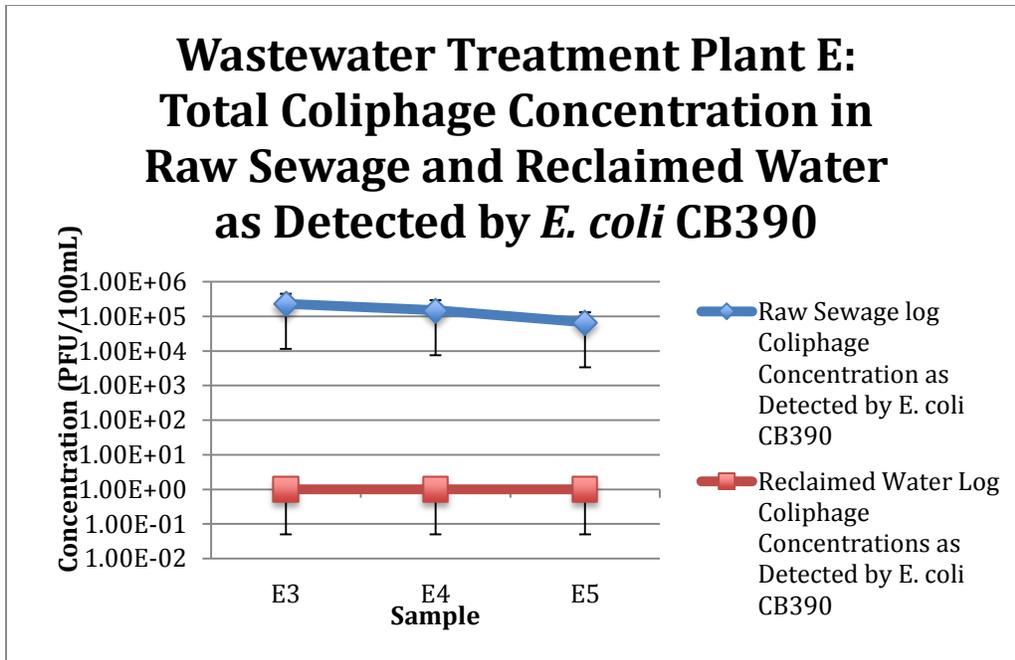


Figure 27: Wastewater Treatment Plant E: Total Coliphage Concentrations/100 mL in Raw Sewage and Reclaimed Water as Detected by *E. coli* CB390 with 95% Confidence Intervals

For wastewater treatment plant E, the concentration of total coliphages/100 mL detected by *E. coli* CB390 in raw sewage is about  $10^5$ , and the concentration in reclaimed water is  $<1.0$ , or assumed 1.0 PFU/100mL for this analysis. From these results, the approximate  $\log_{10}$  reduction at this plant is 5 for total coliphages, by tertiary treatment followed by dual disinfection with UV radiation and chlorine.

#### 1.2.2.2.3 Coliphage Viruses: Data Analysis

In order to evaluate the virological quality of the reclaimed water produced by each wastewater treatment facility,  $\log_{10}$  reductions were estimated for each coliphage host. For this analysis, the coliphage hosts were, the male specific/F+ host *E. coli* Famp, the somatic coliphage host *E. coli* CN13, and the “total” coliphage host CB390. A previous WRRRI project (13-06-W) conducted by this laboratory evaluated the use of CB390 as a total coliphage host and found that the sum of coliphages detected by somatic host *E. coli* CN13 and male-specific/F+ host *E. coli* Famp is not statistically different than the number of coliphages detected by *E. coli* CB390. The samples collected in this project will also be subjected to a similar analysis, which will be presented in the final report of the project. The  $\log_{10}$  coliphage reduction analysis was conducted by calculating the mean  $\log_{10}$  concentration in raw sewage for each treatment plant and then subtracting the mean  $\log_{10}$  concentration in reclaimed water. The mean  $\log_{10}$  coliphage reductions by the wastewater treatment plants are presented in Table 7 and Figures 28, 29 and 30 below.

Table 7: Mean  $\log_{10}$  Coliphage Reductions by Each Water Reclamation Treatment Plant

Water Reclamation Plant	Mean Log <sub>10</sub> Reduction <i>E. coli</i> CN13 (Somatic)	Mean Log <sub>10</sub> Reduction <i>E. coli</i> Famp (Male-specific/F+)	Mean Log <sub>10</sub> Reduction <i>E. coli</i> CB390 (Somatic + Male-specific/F+)
A	4.3	4.1	4.5
B	4.7	4.1	4.9
C	4.9	5.4	4.7
D	4.2	5.3	5.0
E	4.0	4.9	4.6
Average	4.4	4.8	4.7

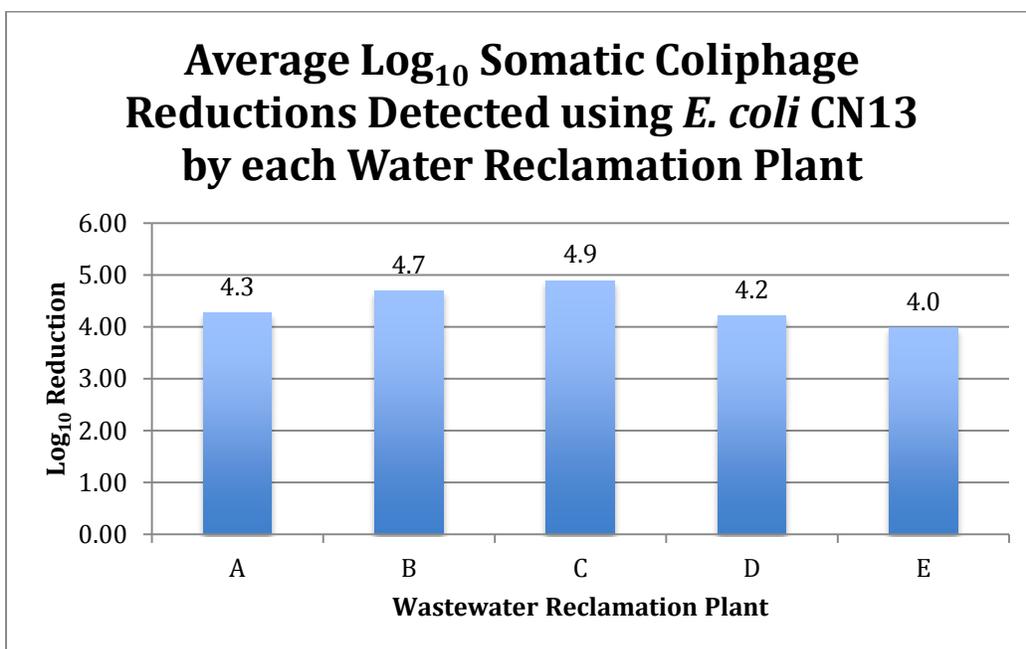


Figure 28: Average log<sub>10</sub> Somatic Coliphage Reductions Detected by *E. coli* CN13 for each Wastewater Reclamation Plant

Figure 28 presents the average log<sub>10</sub> somatic coliphage reductions using *E. coli* CN13 for each water reclamation plant. From these results it appears that each water reclamation plant is achieving at minimum a 4 log<sub>10</sub> reduction, with some exceeding this value and approaching 5 log<sub>10</sub> reduction. The lower log<sub>10</sub> bacteria reduction seen at plant C, as evident in the Total coliform and *E. coli* data, is not seen in these data for coliphages. It seems that plant C may be achieving somewhat higher log<sub>10</sub> reduction of somatic coliphages than the other wastewater reclamation plants.

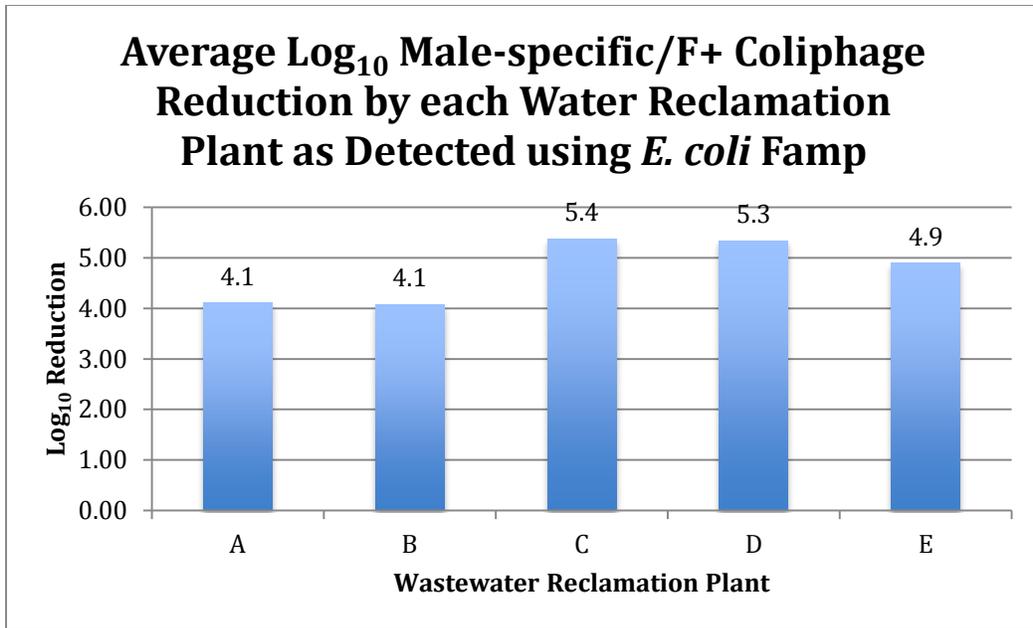


Figure 29: Average log<sub>10</sub> Male-specific/F+ Coliphage Reductions Detected by *E. coli* Famp for each Wastewater Reclamation Plant

Figure 29 presents the average log<sub>10</sub> male-specific/F+ coliphage reductions using *E. coli* Famp for each water reclamation plant. As with the somatic coliphages, for the F+/male specific coliphages, it appears that each water reclamation plant is achieving more than a 4 log<sub>10</sub> reduction, with all exceeding this value and some exceeding a 5 log<sub>10</sub> reduction. It also appears that plant C again may be achieving higher log<sub>10</sub> coliphage reductions when compared with the other wastewater reclamation plants.

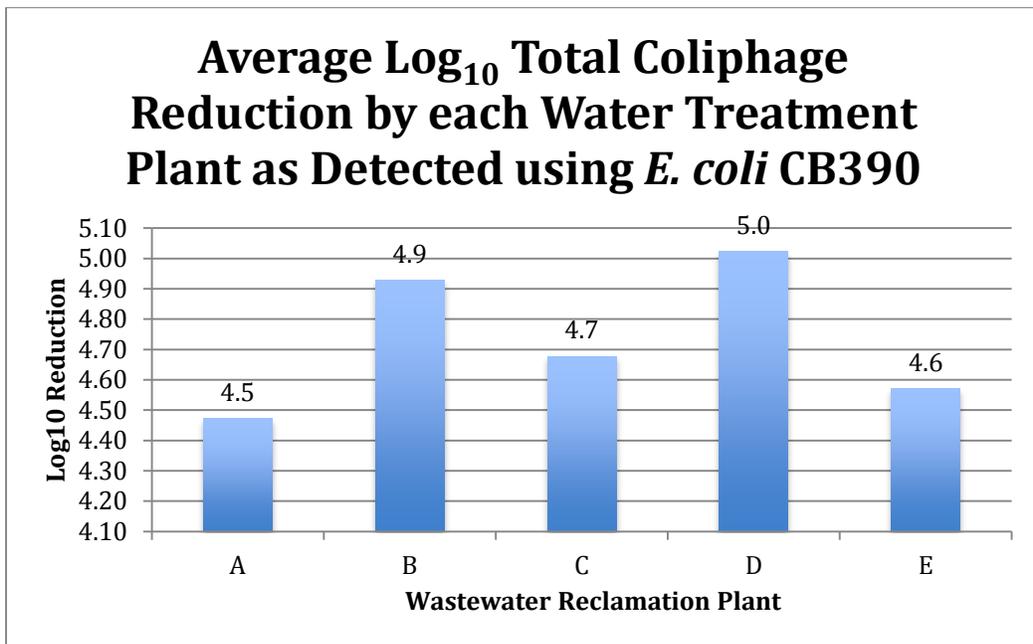


Figure 30: Average log<sub>10</sub> Total Coliphage Reductions Detected by *E. coli* CB390 for each Wastewater Reclamation Plant

Figure 30 presents the average  $\log_{10}$  total coliphage reductions using *E. coli* CB390 for each water reclamation plant. From these results, it appears that each water treatment is again achieving at minimum a 4.5  $\log_{10}$  reduction and at least one plant (D) achieves a 5  $\log_{10}$  reduction. Furthermore, the use of a total coliphage host, as expected, generally elevates the calculated  $\log_{10}$  reduction values. This is probably due to the combined detection of both somatic and male-specific/F+ coliphages, resulting in higher concentrations in raw sewage than either coliphage group alone. As a result, these coliphage  $\log_{10}$  reduction values are higher and more comparable across the various wastewater treatment plants, with a range of 4.5-5.0, a  $\log_{10}$  reduction range which is less variable than that found with the other coliphage hosts.

For coliphage viruses, it is clear that a  $>4 \log_{10}$  reduction is occurring in all of the wastewater reclamation plants sampled by this project. As treatment goals for NC type 2 reclaimed water aim for a 5  $\log_{10}$  reduction, it may be advantageous to sample for total coliphages in raw sewage and reclaimed water samples. The reason for not meeting the 5  $\log_{10}$  reduction target may be related to the initial concentration of coliphages in raw sewage as well as the lower limit of coliphage detection in the reclaimed water, rather than the inability of a wastewater reclamation plant to treat viruses to the 5  $\log_{10}$  reduction level. Therefore, increases in reclaimed water sample volumes analyzed may facilitate the ability to detect a 5  $\log_{10}$  coliphage reduction.

### 1.2.2.3 *Salmonella* spp.: Method, Results, and Data Analysis

#### 1.2.2.3.1 *Salmonella* spp. Methods

*Salmonella* spp. were detected using sequential methods of broth enrichment culture in multiple sample volumes then colony isolation of presumptive target bacteria based on appearance from each enriched samples volume to obtain most probable number (MPN) concentrations, with subsequent plating steps for confirmed identification. Initially, three sample volumes were incubated in triplicate overnight (18-24 hours) in buffered peptone water at 37°C. The 9-volume MPN test volumes for the reclaimed water sample were 300, 30, and 3 mLs; the test volumes of raw sewage samples were triplicate 1mL aliquots of serial 10-fold dilutions of the initial sewage sample. Next, a portion of the buffered peptone water was inoculated into a *Salmonella* selective broth, Rappaport Vassiliadis Broth, and incubated overnight at 37°C. Enriched samples were streak plated on Salmonella Shigella agar (BD) and incubated overnight at 37°C. Positive presumptive *Salmonella* colonies were scored as colorless colonies with black centers, as indicated by the manufacturer.

Additional streak plating and purification steps were also conducted in order to save presumptive positive *Salmonella* spp. colony isolates for further biochemical confirmation. This characterization is currently underway and the results will be reported in the final written report.

#### 1.2.2.3.2 *Salmonella* spp. Results

In the tables below are the concentrations of presumptive *Salmonella* spp. in raw sewage and reclaimed water samples. These bacteria were detected and quantified by the broth culture

enrichment most probable number (MPN) method using *Salmonella* selective peptone broth and then Rappaport Vassiliadis broth for enrichments, followed by isolation of presumptive *Salmonella* spp. colonies on differential-selective agar medium. Concentrations are reported as MPN/100mL with upper and lower 95% confidence intervals. MPNs were calculated using the MPN tables available in Standard Methods for the Examination of Water and Wastewater (SMEWW).

Table 8: *Salmonella* spp. Concentrations (MPN per 100mL) in 18 Raw Sewage Samples

Sample	<i>Salmonella</i> spp. (MPN per 100mL)	Lower 95% Confidence Interval	Upper 95% Confidence Interval
B3	4.60 x 10 <sup>5</sup>	1.00 x 10 <sup>5</sup>	2.10 x 10 <sup>6</sup>
D3	1.50 x 10 <sup>5</sup>	4.20 x 10 <sup>4</sup>	5.40 x 10 <sup>5</sup>
C2	2.40 x 10 <sup>5</sup>	6.70 x 10 <sup>4</sup>	8.60 x 10 <sup>5</sup>
E3	1.50 x 10 <sup>6</sup>	4.20 x 10 <sup>5</sup>	5.40 x 10 <sup>6</sup>
A3	1.50 x 10 <sup>5</sup>	4.10 x 10 <sup>4</sup>	5.20 x 10 <sup>5</sup>
B4	2.80 x 10 <sup>5</sup>	7.70 x 10 <sup>4</sup>	9.90 x 10 <sup>5</sup>
D4	7.50 x 10 <sup>5</sup>	1.80 x 10 <sup>5</sup>	3.20 x 10 <sup>6</sup>
C3	1.50 x 10 <sup>5</sup>	4.10 x 10 <sup>4</sup>	5.20 x 10 <sup>5</sup>
E4	9.30 x 10 <sup>5</sup>	2.30 x 10 <sup>5</sup>	3.80 x 10 <sup>6</sup>
A4	4.30 x 10 <sup>5</sup>	1.00 x 10 <sup>5</sup>	1.80 x 10 <sup>6</sup>
B5	1.50 x 10 <sup>5</sup>	4.10 x 10 <sup>4</sup>	5.20 x 10 <sup>5</sup>
E5	1.50 x 10 <sup>6</sup>	4.20 x 10 <sup>5</sup>	5.40 x 10 <sup>6</sup>
D5	2.30 x 10 <sup>5</sup>	6.60 x 10 <sup>4</sup>	8.10 x 10 <sup>5</sup>
C4	9.20 x 10 <sup>4</sup>	2.30 x 10 <sup>4</sup>	3.70 x 10 <sup>5</sup>
A5	9.30 x 10 <sup>5</sup>	2.30 x 10 <sup>5</sup>	3.80 x 10 <sup>6</sup>
D6	2.00 x 10 <sup>4</sup>	5.90 x 10 <sup>3</sup>	7.10 x 10 <sup>4</sup>
A6	2.10 x 10 <sup>5</sup>	6.10 x 10 <sup>4</sup>	7.30 x 10 <sup>5</sup>
E6	2.70 x 10 <sup>5</sup>	7.50 x 10 <sup>4</sup>	9.60 x 10 <sup>5</sup>

Table 9: *Salmonella* spp. Concentrations (MPN per 100mL) in 18 Reclaimed Water Samples

Sample	<i>Salmonella</i> spp. (MPN per 100mL)	Lower 95% Confidence Interval	Upper 95% Confidence Interval
B3	<0.1	0.012	0.82
D3	<0.1	0.012	0.82
C2	1.4	0.35	5.8
E3	<0.1	0.012	0.82
A3	<0.1	0.012	0.82
B4	<0.1	0.012	0.82
D4	<0.1	0.012	0.82
C3	3.1	0.77	13
E4	<0.1	0.012	0.82
A4	<0.1	0.012	0.82
B5	<0.1	0.012	0.82
E5	<0.1	0.012	0.82

D5	<0.1	0.012	0.82
C4	0.49	0.14	1.7
A5	<0.1	0.012	0.82
D6	<0.1	0.012	0.82
A6	<0.1	0.012	0.82
E6	<0.1	0.012	0.82

*Salmonella* spp. were found in raw sewage at concentrations ranging from about  $10^4$  to  $10^5$  per 100 mL. In reclaimed water *Salmonella* spp. were not detected in effluent samples from water reclamation plants A, B, D and E, with concentrations  $<0.1$  MPN/100 mL. However, *Salmonella* spp. were detected in the treated effluent samples of plant C, at concentrations of about 0.5 to 3.0/100 mL.

The figures below present the presumptive *Salmonella* spp. concentrations per 100 mL for both raw sewage and reclaimed water for each wastewater reclamation plant. The  $\log_{10}$  reduction for each sample was computed by subtracting the  $\log_{10}$  concentration in reclaimed water from the  $\log_{10}$  concentration in raw sewage. For samples that had an MPN of  $<0.1/100$  mL, the value was assumed to be 0.1 for analytical purposes. This analysis is presented in section 1.2.2.3.3.

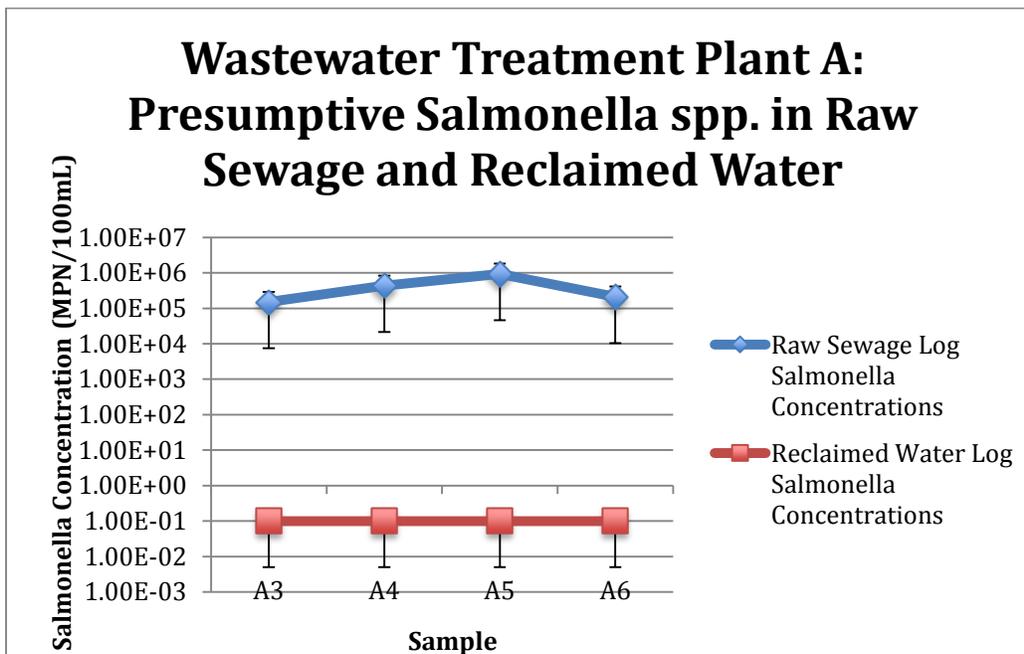


Figure 31: Wastewater Treatment Plant A: Salmonella Concentrations per 100 mL in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant A, concentrations of presumptive *Salmonella* spp./100 mL in raw sewage are approximately  $10^{5.5}$ , whereas concentrations in reclaimed water are  $<0.1$ , or assumed 0.1 MPN/ 100mL for this analysis. From these results, the approximate  $\log_{10}$  reduction is 5-6 by tertiary treatment followed by dual disinfection with UV radiation and chlorine

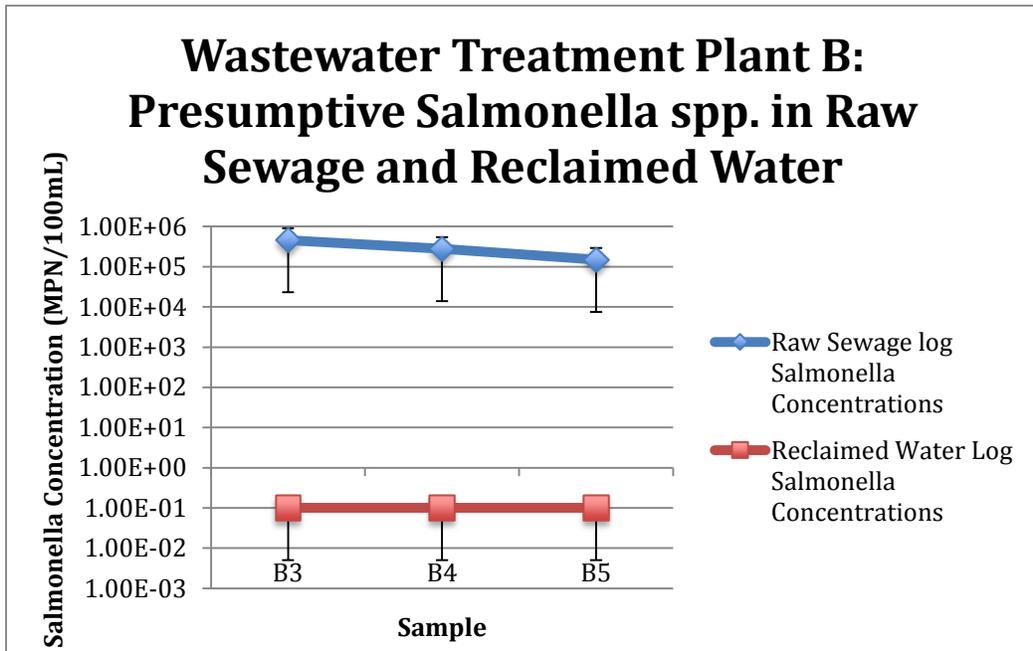


Figure 32: Wastewater Treatment Plant B: Presumptive *Salmonella* spp. Concentrations Per 100 mL in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant B, the concentration of *Salmonella* spp. detected in raw sewage is approximately  $10^{5.5}$ , whereas the  $\log_{10}$  concentration in reclaimed water is  $<0.1$ , or assumed 0.1 MPN/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction at this plant is about 6 from tertiary treatment followed by dual chlorine and UV disinfection.

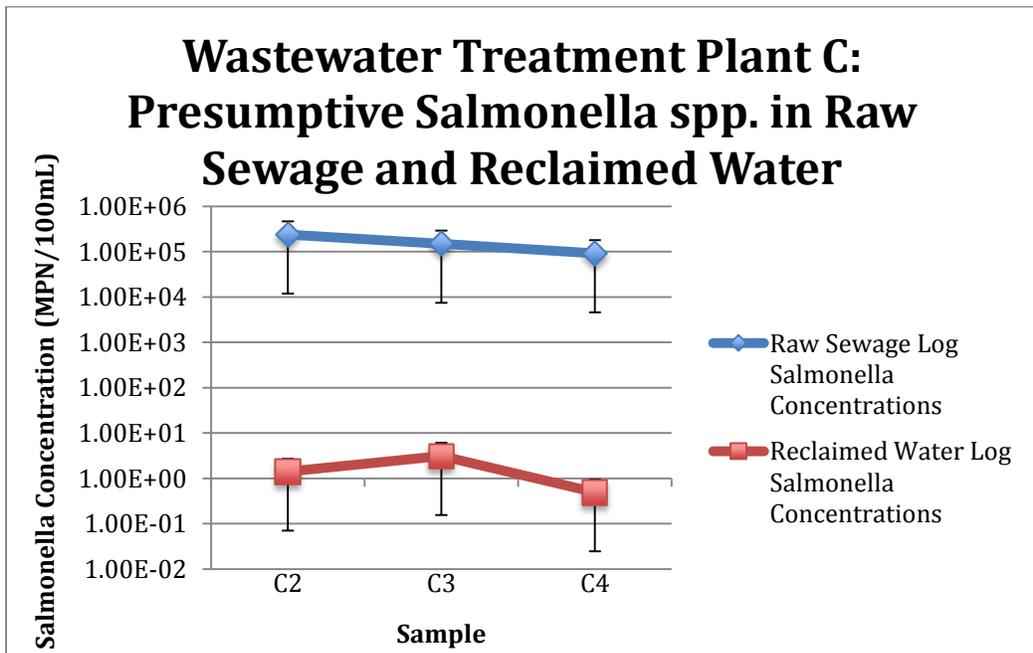


Figure 33: Wastewater Treatment Plant C: Presumptive Salmonella Concentrations per 100 mL in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant C, the concentration of presumptive *Salmonella* spp./100 mL detected in raw sewage is approximately  $10^5$ , whereas the concentration in reclaimed water is approximately 0.5-3 MPN/100mL. According to these results, the approximate  $\log_{10}$  reduction at this plant is about 4.5-5, by tertiary treatment followed by disinfection.

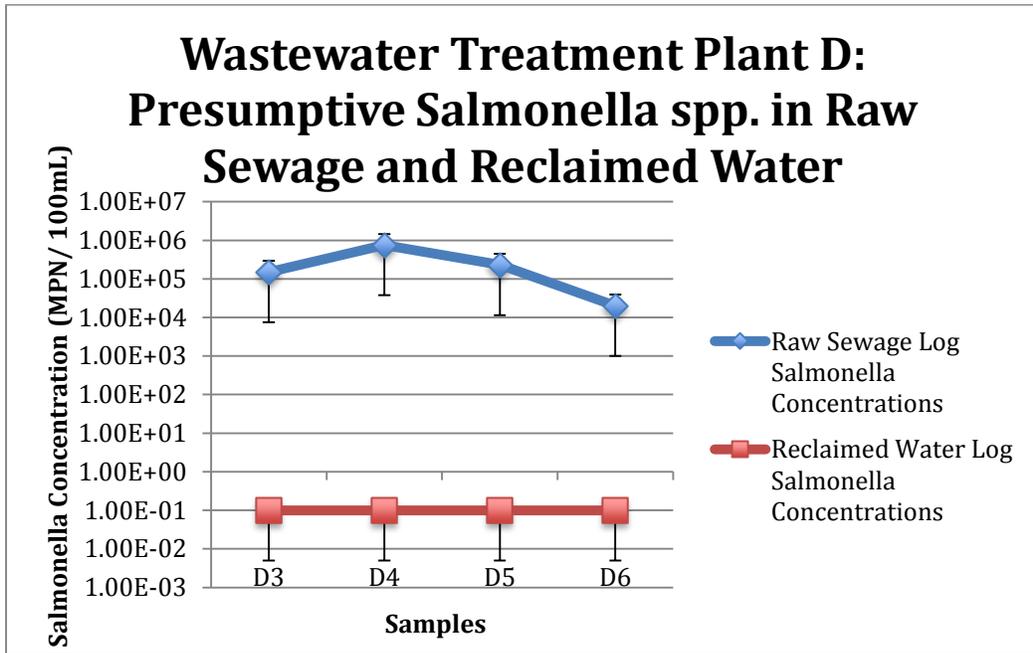


Figure 34: Wastewater Treatment Plant D: Presumptive Salmonella Concentrations Per 100 mL in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant D, the concentration of presumptive *Salmonella* spp./100 mL detected in raw sewage is about  $10^{5.5}$ , whereas the concentration in reclaimed water is  $<0.1$ , or assumed 0.1 MPN/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction is 6-6.5 by tertiary treatment and dual disinfection with UV radiation and chlorine.

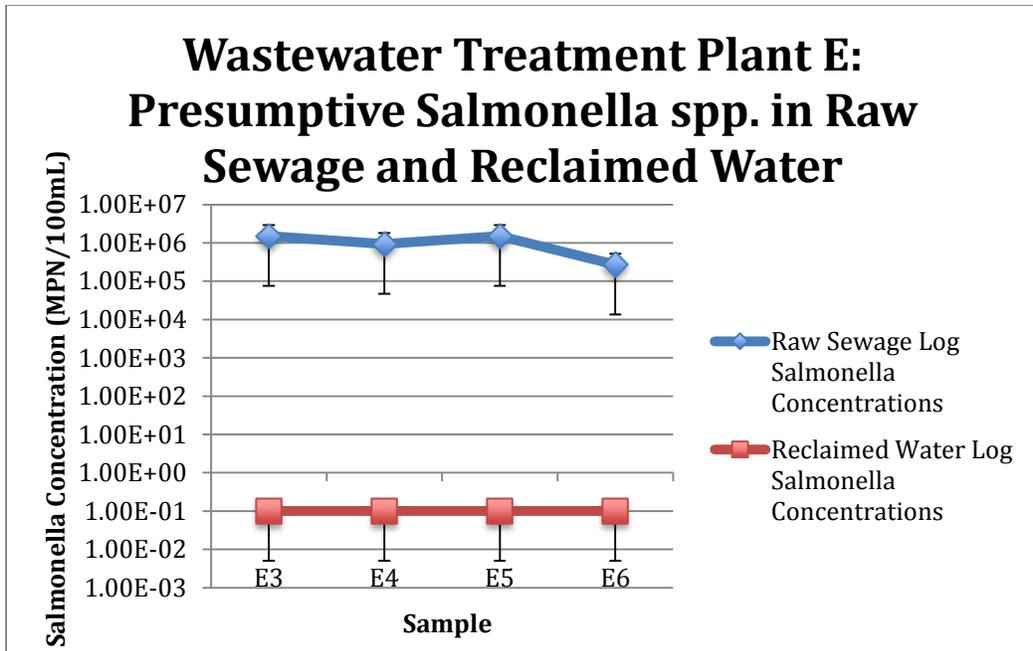


Figure 35: Wastewater Treatment Plant E: Presumptive *Salmonella* Concentrations/100 mL in Raw Sewage and Reclaimed Water with 95% Confidence Intervals

For wastewater treatment plant E, the concentration of presumptive *Salmonella* spp./100 mL detected in raw sewage is about  $10^6$ , whereas the concentration in reclaimed water is  $<0.1$ , or assumed 0.1 MPN/ 100mL for this analysis. According to these results, the approximate  $\log_{10}$  reduction is 6 by tertiary treatment and then dual disinfection with UV radiation and chlorine

#### 1.2.2.3.3 *Salmonella* spp. Data Analysis

In order to evaluate the microbial quality of the reclaimed water produced by each wastewater treatment facility, the  $\log_{10}$  reductions were obtained for presumptive *Salmonella* spp. at each wastewater treatment plant. The  $\log_{10}$  reduction analysis was done by calculating the average  $\log_{10}$  concentration in raw sewage for each treatment plant and then subtracting the average  $\log_{10}$  concentration in reclaimed water. The average  $\log_{10}$  reductions by the wastewater treatment plants are presented in Figure 36 below.

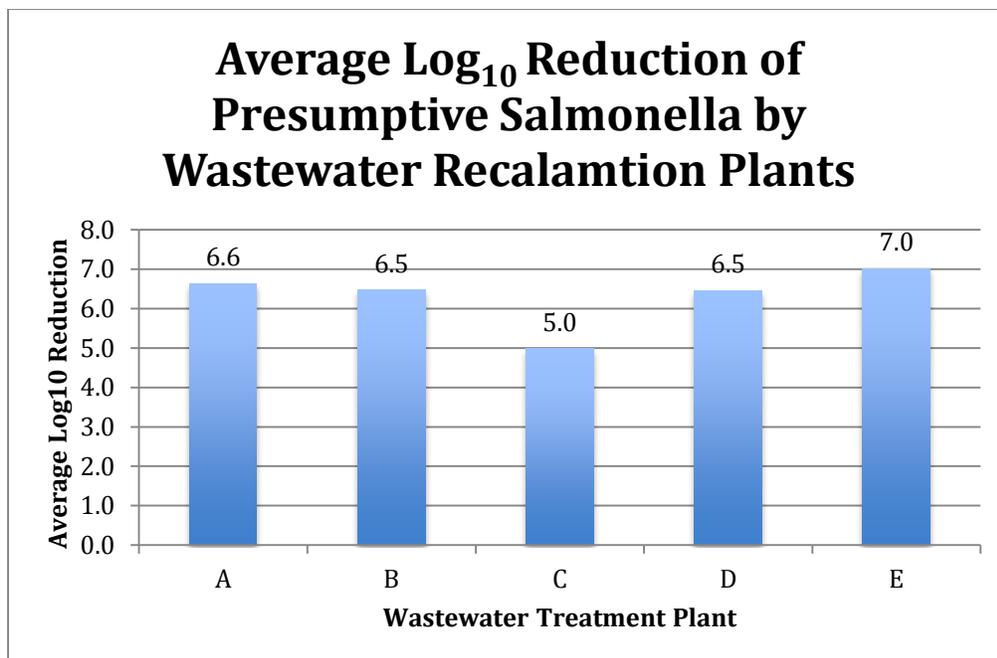


Figure 36: Average Log<sub>10</sub> Reductions of Presumptive *Salmonella* spp. by Wastewater Reclamation Plant

Figure 36 presents the average log<sub>10</sub> reductions of presumptive *Salmonella* spp. at each wastewater reclamation plant. It is clear from these results that plants A, B, D, and E, are achieving at least a 6 log<sub>10</sub> reduction in presumptive *Salmonella* spp. to meet the requirement for bacteria reduction to achieve type 2 reclaimed water quality. However, much like the trend seen in the total coliform and *E. coli* data, plant C is not achieving this level of reduction for *Salmonella* spp., with a reduction of about 5 log<sub>10</sub>. It is important to note that this lower reduction is not a result of a lack of *Salmonella* spp. in the influent raw sewage sample at plant C or at the other wastewater treatment facilities. Rather, it is indicative of continued detectable levels of *Salmonella* spp. in the reclaimed water effluent of plant C, suggesting inadequate disinfection.

Because of the performance discrepancy between plant C and the other wastewater reclamation facilities, an average log<sub>10</sub> reduction value across all of the facilities is not provided.

#### 1.2.2.4 *Clostridium perfringens*: Method, Results, and Data Analysis

##### 1.2.2.4.1 *C. perfringens* Methods

Procedures for *C. perfringens* detection and enumeration were standard membrane filter (MF) methods modified from those originally developed for US EPA by Cabelli and Bisson (1979). Prior to performing the membrane filtration (MF) method, the various media and reagents were prepared. The agar medium was CP ChromoSelect Agar (CS). CP ChromoSelect agar base (from Fluka Analytical) was prepared by adding 6.28 grams/100 mL deionized water, bringing to a boil on a hot plate and then removing to cool and keeping molten. After tempering, 0.04 grams of D-Cycloserine were added per 100 mL of molten agar medium base. This supplemented medium

was dispensed in 5-mL volumes in 50 mm diameter sterile, polystyrene Petri dishes and allowed to harden. Plates were stored at 4°C until use.

### Membrane Filtration Method

*C. perfringens* spores and total *C. perfringens* (spores plus vegetative cells) were detected in sewage and reclaimed waters by a modification of standard membrane filter (MF) methods originally developed for US EPA by Cabelli and Bisson (1979). CS media was used in MF analysis of samples of reclaimed water, untreated wastewater, and surface water. The focus was on achieving samples with *C. perfringens* concentrations to meet the NC type 2 reclaimed water treated effluent limits of 5 (as geometric mean) and 25 (as single sample maximum) per 100 mL. In the MF method a volume of sample is vacuum-filtered through a standard 47 mm diameter, approximately 0.45 µm pore size cellulose ester membrane filter. The membrane filter is placed on the surface of an agar medium for *C. perfringens* in a Petri dish, the dish is then incubated under anaerobic conditions at 44 °C overnight and then exposed to the atmosphere for 1 hour to allow for development of the diagnostic colony color. *C. perfringens* and related sulphite reducing clostridia produce characteristic green colonies that are then counted. Counted colonies of the distinctive color on the agar medium are considered total presumptive *C. perfringens* per the volume of water sample analyzed. The method was also used to detect only *C. perfringens* spores by first heating the sample to temperatures between 63 and 80 °C for 15 minutes prior to filtration in order to kill vegetative bacteria and provide colony counts of only spores. The *C. perfringens* concentrations obtained were used to calculate log reductions from tertiary treatment of wastewater.

#### 1.2.2.4.2 *C. perfringens* Results

Tabulated below are the results of *C. perfringens* detection in the raw sewage and reclaimed water samples presented as colony forming units per 100 milliliters (CFU/100mL).

Table 10: *C. perfringens* vegetative cells and spores concentrations detected by membrane filtration on CP ChromoSelect agar (CFU/100mL) in 18 raw sewage samples

Sample	Total <i>C. perfringens</i> Concentration (CFU/100 mL)	<i>C. perfringens</i> Spores Concentration (CFU/100 mL)
D2	133333	66667
E1	166667	16667
C2	173333	100000
B3	80000	43333
D3	60000	1667
C3	112833	80333
E2	47333	28833
A3	42333	27333
B4	70000	34333
D4	76833	60667

C4	88167	72667
E3	62500	51167
A4	65556	65556
B5	41111	5556
E4	51111	40000
D5	63333	65000
C5	84583	100417
A5	50167	38667

Table 11: *C. perfringens* vegetative cells and spores concentrations detected by membrane filtration on CP ChromoSelect agar (CFU/100mL) in 25 tertiary treated and dual disinfected reclaimed water samples with a lower detection limit of <1

Sample	Total <i>C. perfringens</i> Concentration (CFU/100 mL)	<i>C. perfringens</i> Spores Concentration (CFU/100 mL)
A1	<1	3
B1	<1	<1
D1	<1	<1
C1	20	17
A2	<1	<1
B2	<1	<1
D2	<1	<1
E1	<1	<1
C2	40	10
B3	<1	<1
D3	<1	<1
C3	20	32
E2	<1	<1
A3	<1	<1
B4	<1	0.166667
D4	<1	<1
C4	24.5	13.8
E3	<1	<1
A4	<1	<1
B5	<1	<1
E4	<1	<1
D5	<1	<1
C5	46	27
A5	<1	<1

The following figures depict the concentrations of total *C. perfringens* detected in the raw

sewage and reclaimed water samples from the 5 water reclamation plants, respectively. For each wastewater treatment (water reclamation) plant,  $\log_{10}$  reductions of *C. perfringens* were calculated for each sample by subtracting the  $\log_{10}$  concentration in the reclaimed sample from the  $\log_{10}$  concentration in the raw sewage sample. For reclaimed water samples in which no colonies were detected, a value of 1 CFU/100 mL was used for the purpose of performing analysis which is presented in section 1.2.2.3.3.

Treatment plant A had concentrations of *C. perfringens* about  $5 \times 10^4$ /100 mL in the raw sewage and concentrations of less than 1 CFU per 100 mL in reclaimed water. Treatment plant B had concentrations of *C. perfringens* greater than  $5 \times 10^4$  in the raw sewage and concentrations less than 1 CFU per 100 mL in the reclaimed water. Treatment plant C had *C. perfringens* concentrations of about  $10^5$  per 100 mL in the raw sewage and concentrations between  $2 \times 10^1$  and  $5 \times 10^1$  CFU per 100 mL in the reclaimed water. Treatment plant D had *C. perfringens* concentrations of about  $8 \times 10^4$ /100 mL in the raw sewage and less than 1 CFU per 100 mL in reclaimed water. Treatment plant E had concentrations of *C. perfringens* greater than  $8 \times 10^4$ /100 mL in the raw sewage and less than 1 CFU per 100 mL in reclaimed water. From this, the approximate  $\log_{10}$  reductions of total *C. perfringens* in plants A, B, C, D, and E are greater than 4.5, greater than 4.5, about 3.5, about 4.8, and about 4.8, respectively, from tertiary treatment and disinfection. Water reclamation plants A, B, D and E, which have dual disinfection with UV radiation and chlorine, had greater  $\log_{10}$  *C. perfringens* reductions than plant C which has less extensive or less efficient disinfection.

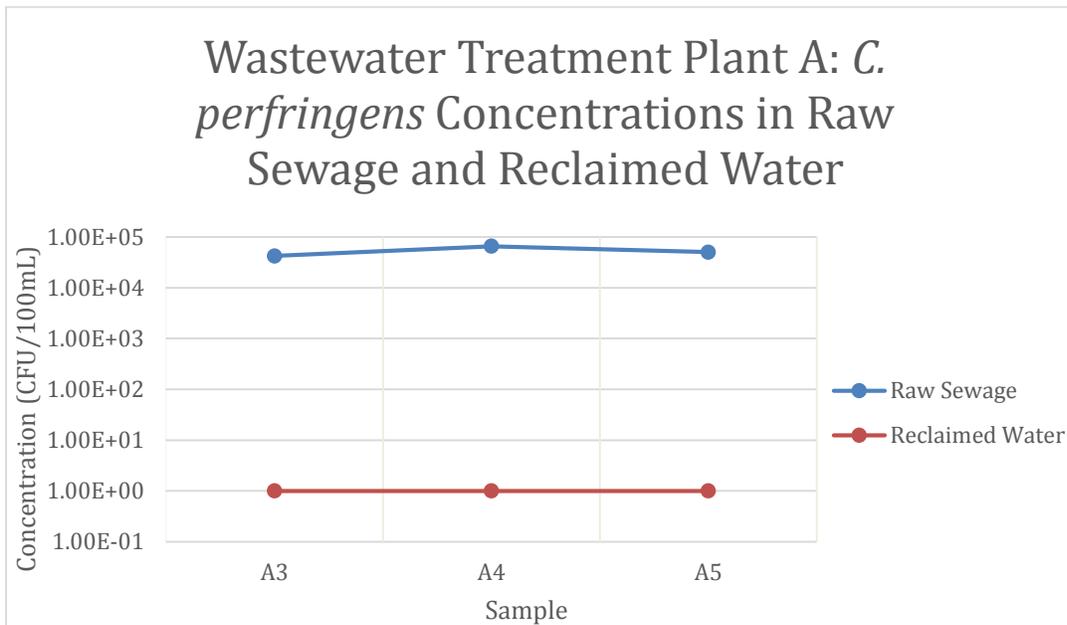


Figure 36: Wastewater treatment plant A: total *C. perfringens* concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

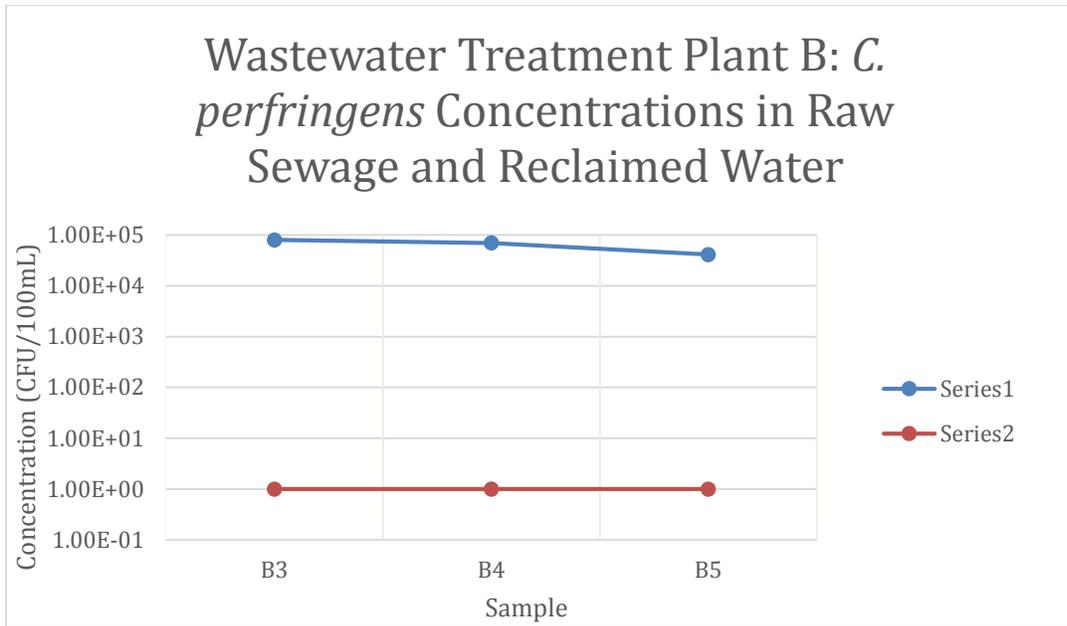


Figure 37: Wastewater treatment plant B: total *C. perfringens* concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

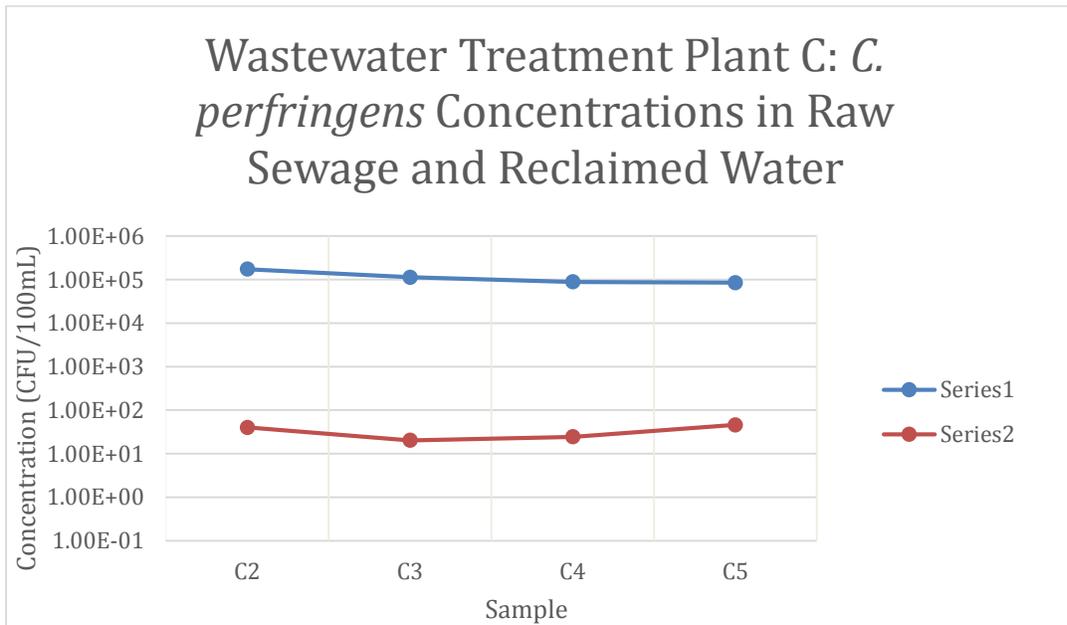


Figure 38: Wastewater treatment plant C: total *C. perfringens* concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

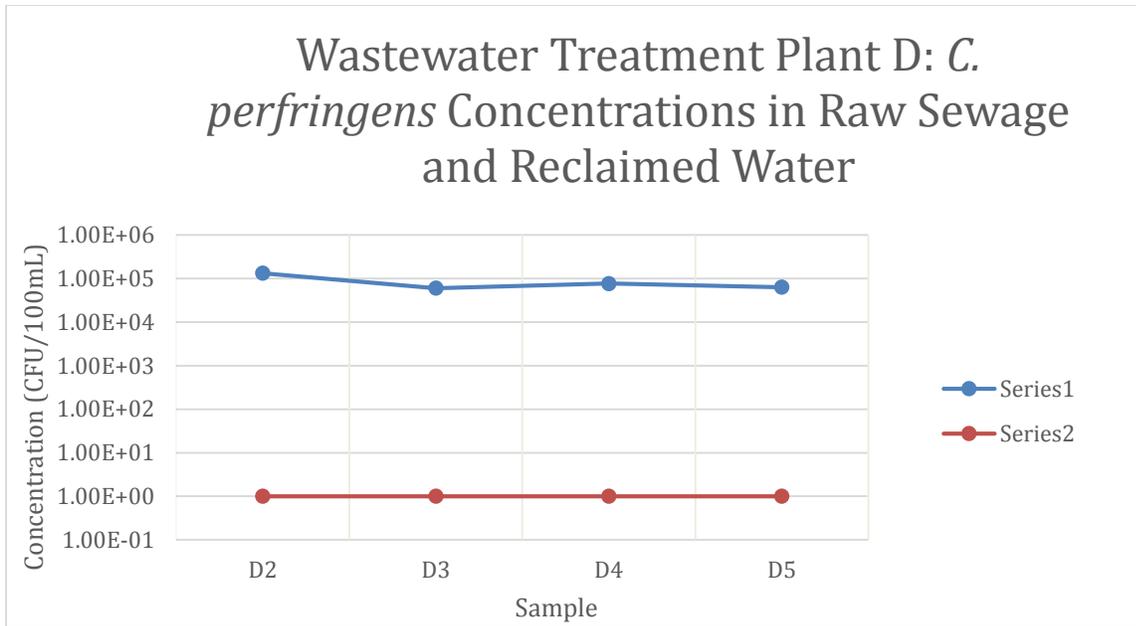


Figure 39: Wastewater treatment plant D: total *C. perfringens* concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

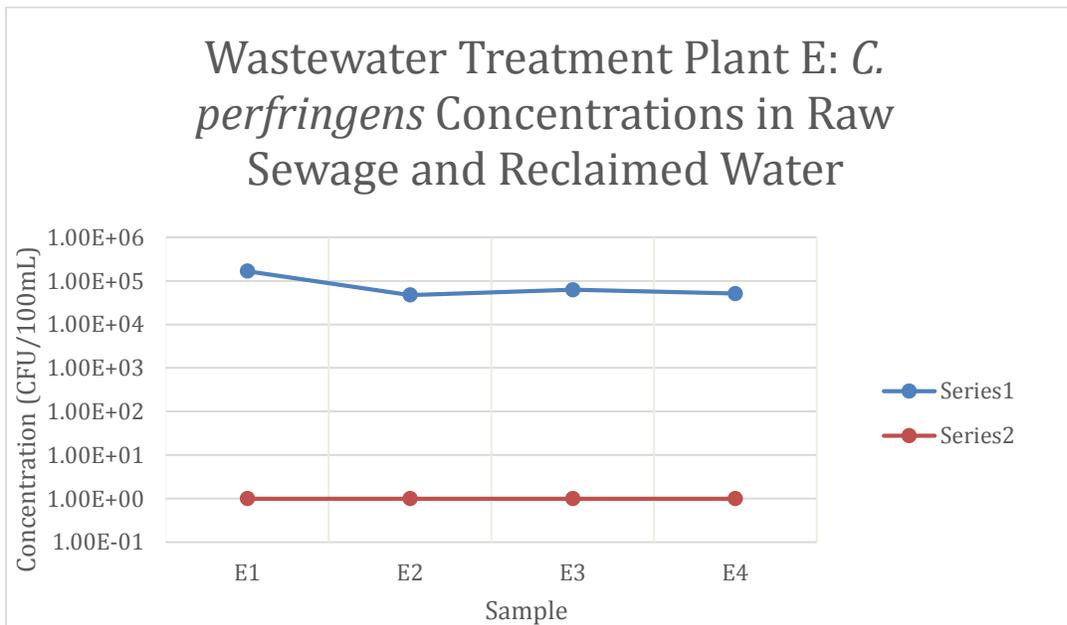


Figure 40: Wastewater treatment plant E: total *C. perfringens* concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

The following five figures depict the concentrations of *C. perfringens* spores detected after pasteurization in the raw sewage and reclaimed water samples from the 5 water reclamation plants, respectively. For each wastewater treatment (water reclamation) plant, log<sub>10</sub> reductions of *C. perfringens* spores can be calculated for each sample by subtracting the log<sub>10</sub> concentration in the reclaimed water sample from the log<sub>10</sub> concentration in the raw sewage sample. For

reclaimed water samples in which no colonies were detected, a value of 1 CFU/100 mL was used for the purpose of performing analysis which is presented in section 1.2.2.3.3.

Treatment plant A had concentrations of *C. perfringens* spores around or above  $5 \times 10^4$  per 100 mL in the raw sewage and concentrations of less than 1 CFU per 100 mL in reclaimed water. Treatment plant B had concentrations of *C. perfringens* spores greater than  $8 \times 10^3$ /100 mL in the raw sewage and concentrations less than 1 CFU per 100 mL in the reclaimed water. Treatment plant C had concentrations of *C. perfringens* spores approximately  $10^5$ /100 mL in the raw sewage and concentrations between  $10^1$  and  $5 \times 10^1$  CFU per 100 mL in the reclaimed water. Treatment plant D had concentrations of *C. perfringens* spores between  $10^3$  and  $10^5$  in the raw sewage and less than 1 CFU per 100 mL in reclaimed water. Treatment plant E had concentrations of *C. perfringens* spores greater than  $10^4$  in the raw sewage and less than 1 CFU per 100 mL in reclaimed water. For these samples, the approximate  $\log_{10}$  reductions of *C. perfringens* spores in plants A, B, C, D, and E are greater than 4.5, greater than 4.5, about 3.5, greater than about 4.5, and about 4.5, respectively, from tertiary treatment and disinfection. Water reclamation plants A, B, D and E, which have dual disinfection with UV radiation and chlorine, had greater  $\log_{10}$  *C. perfringens* reductions than plant C, which has less extensive or less efficient disinfection.

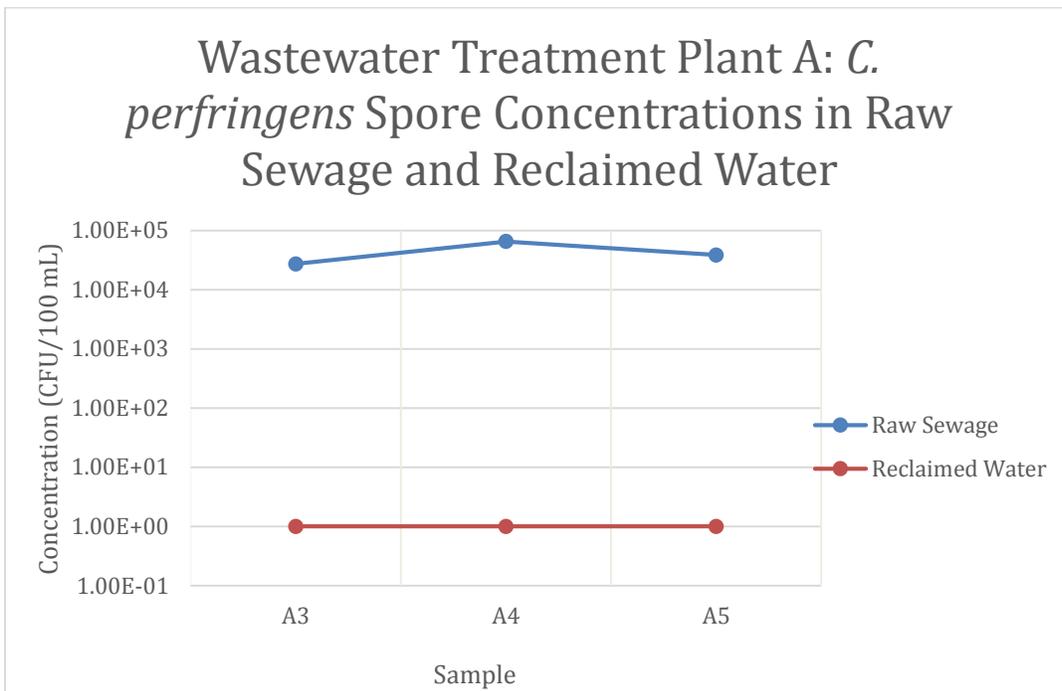


Figure 41: Wastewater treatment plant A: total *C. perfringens* spores concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

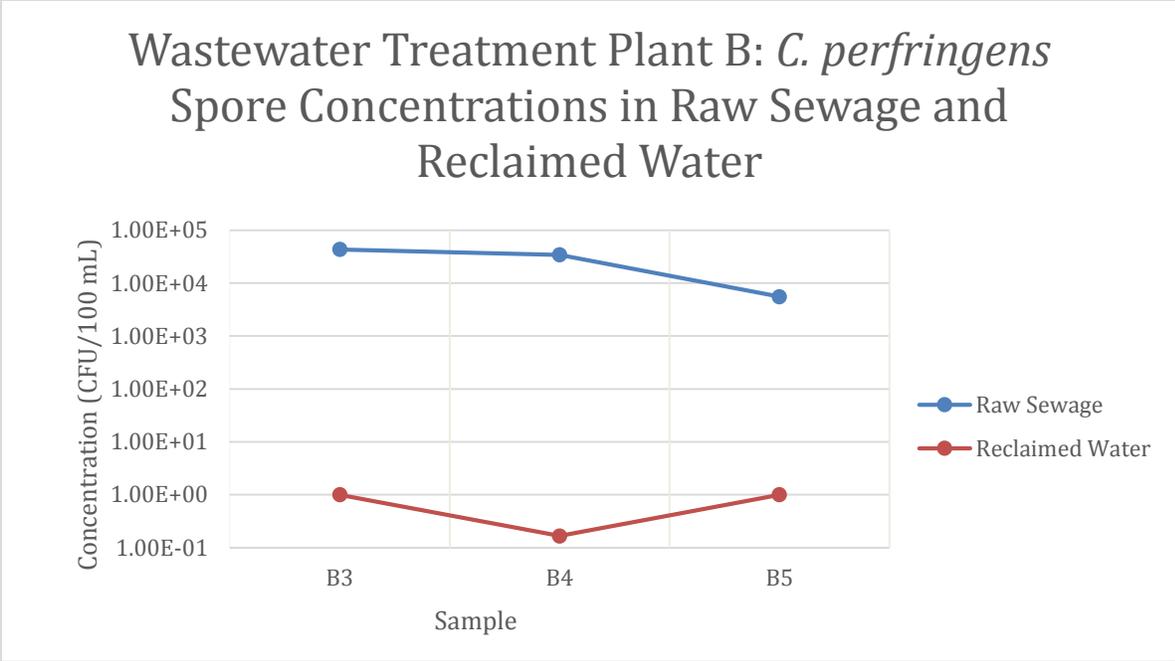


Figure 42: Wastewater treatment plant B: total *C. perfringens* spores concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

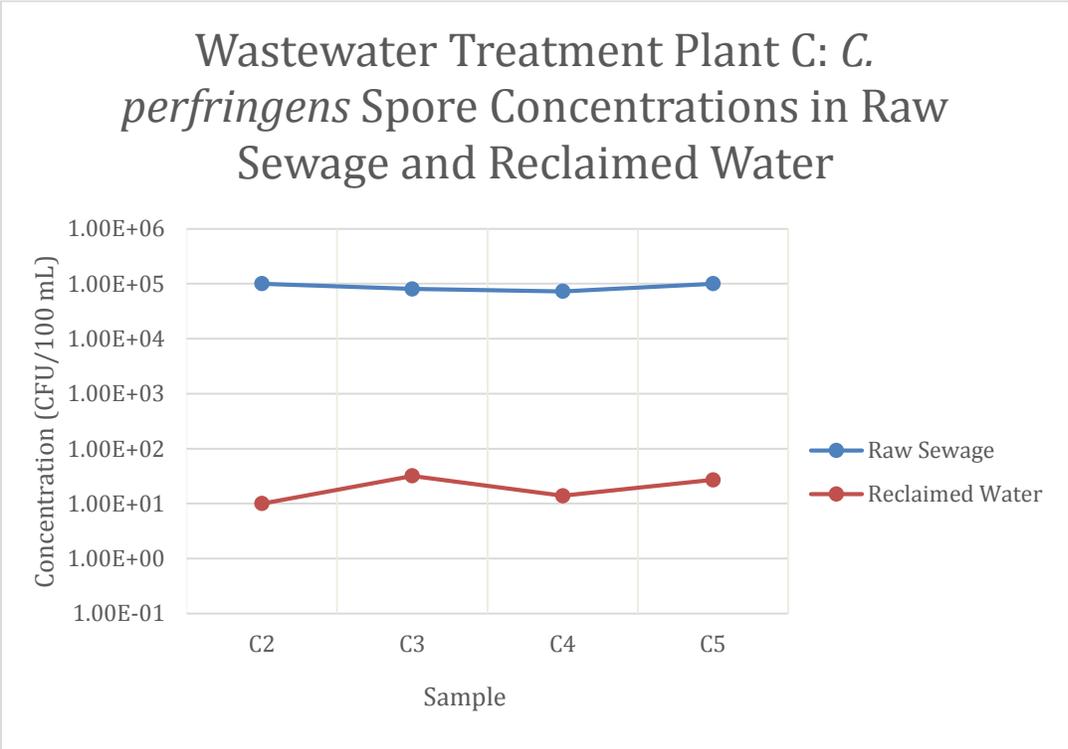


Figure 43: Wastewater treatment plant C: total *C. perfringens* spores concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

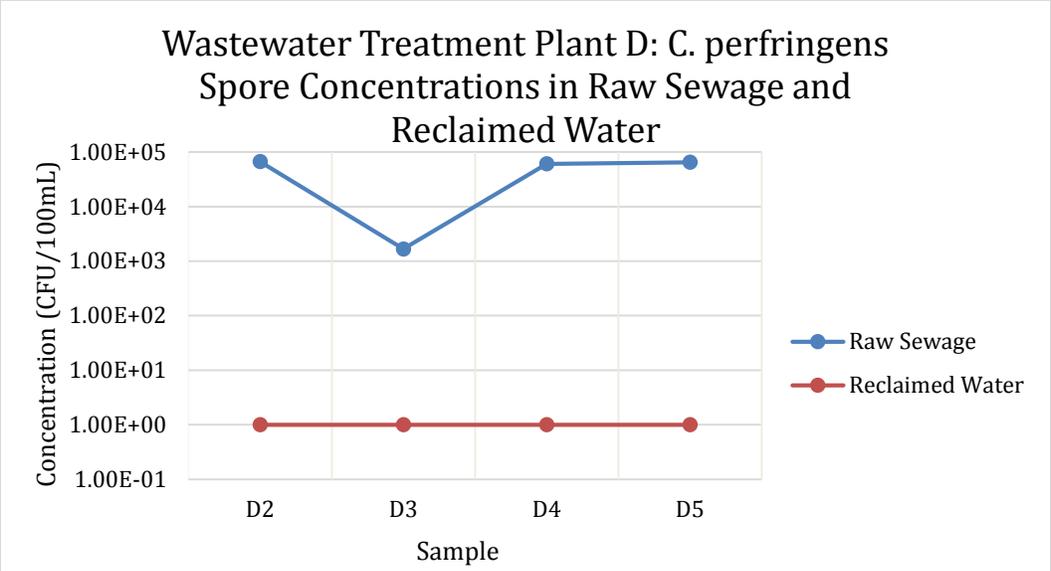


Figure 44: Wastewater treatment plant D: total *C. perfringens* spores concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

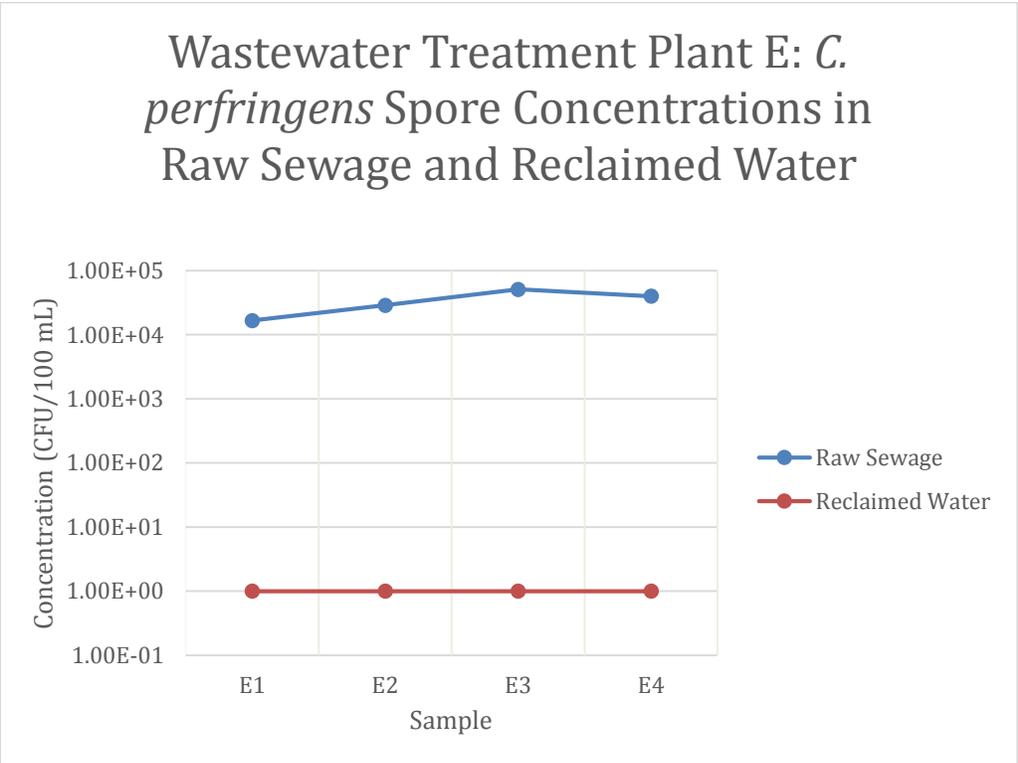


Figure 45: Wastewater treatment plant E: total *C. perfringens* spores concentrations/100 mL in raw sewage and reclaimed water as detected by membrane filtration

1.2.2.4.3 *C. perfringens* Data Analysis

To evaluate the microbial quality of the reclaimed water produced by each wastewater treatment facility with respect to *C. perfringens* vegetative cells and spores,  $\log_{10}$  reductions were estimated for both total colony counts and spore colony counts. This was done by averaging the concentrations of *C. perfringens* total colonies and spore colonies in the sewage and reclaimed water samples from each treatment plant and then subtracting the reclaimed water concentrations from the raw sewage concentrations. The average  $\log_{10}$  *C. perfringens* reductions for total bacteria and spores only by each water reclamation plant are presented in Table 12 and Figures 46 and 47 below.

From the figures below it is clear that wastewater reclamation plants A, B, D, and E are all averaging between 4-5  $\log_{10}$  reductions of total *C. perfringens* and spores only in their water reclamation processes. Wastewater treatment plant C is lower in performance, achieving only an average of 3.6  $\log_{10}$  reduction of total *C. perfringens* bacteria and spores only by treatment. Although the reductions from the other four water reclamation plants are lower for *C. perfringens* than they are for some of the other bacteria discussed in this document, this appears to be driven by the lower initial concentration of *C. perfringens* in the raw sewage. Upon examining the raw data for the *C. perfringens* concentrations in reclaimed water from treatment plants A, B, D and E, the absence of any *C. perfringens* bacteria in all but one reclaimed water sample demonstrates that the daily maximum of 25/100mL is unlikely to occur. Similarly, these values suggest that the monthly geometric mean of 5/100 mL is unlikely to be surpassed. However, treatment plant C does not appear to be meeting the requirements for  $\log_{10}$  reductions or for average or daily concentrations of *C. perfringens* per 100 mL. Therefore, water reclamation plant C does not appear to be meeting the *C. perfringens* criteria for type 2 reclaimed water.

Table 12. Average  $\log_{10}$  reductions by each water reclamation treatment plant

Treatment Plant	Average Total <i>C. perfringens</i> $\log_{10}$ Reductions	Average <i>C. perfringens</i> Spores $\log_{10}$ Reductions
A	4.7	4.6
B	4.8	4.3
C	3.6	3.7
D	4.9	4.4
E	4.9	4.5

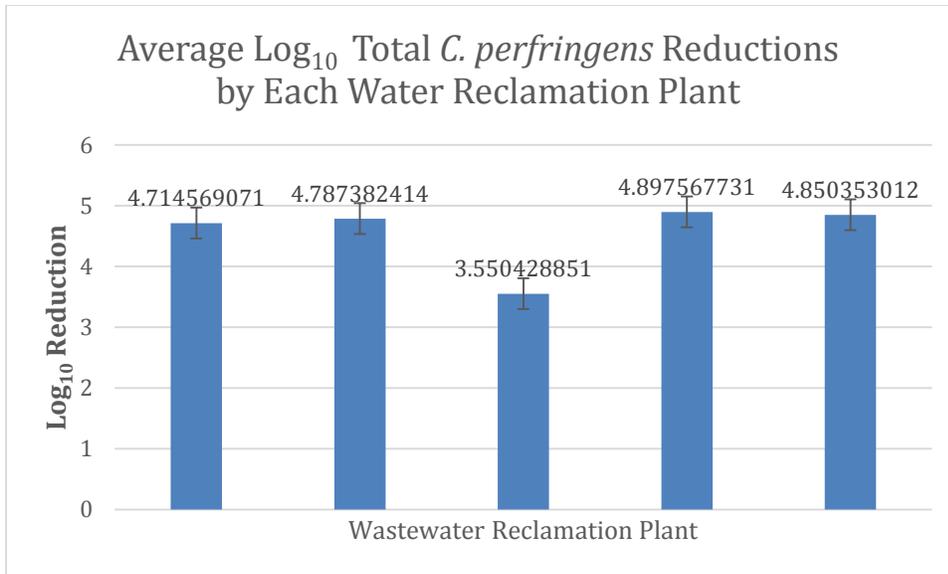


Figure 46: Average log<sub>10</sub> total *C. perfringens* reductions for each wastewater reclamation plant

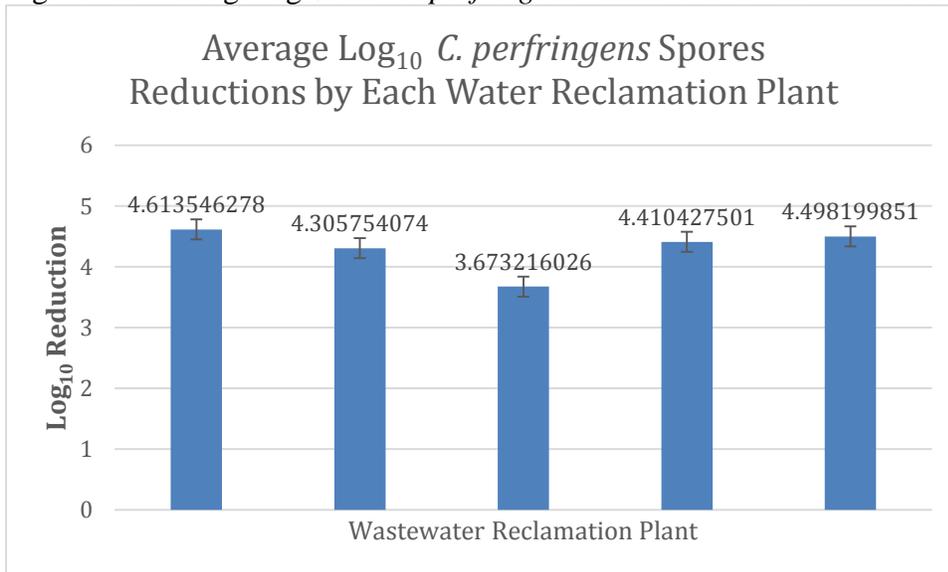


Figure 47: Average log<sub>10</sub> *C. perfringens* spores reductions for each wastewater reclamation plant

### 1.2.2.5 Enteric Virus - Progress

As this project has expanded to include the quantification of protozoan parasites in reclaimed water, the methods for the detection of enteric viruses in raw sewage and reclaimed water have also been reevaluated and revised. As a result, there has been a delay in the detection of enteric viruses in raw sewage and reclaimed water. However, raw sewage and reclaimed water samples have been collected, partially processed and archived for analysis of enteric viruses and protozoan parasites once the detection and quantification methods are validated and analyst performance has been verified by proficiency testing. We have recently completed initial method performance validation and analyst proficiency testing for these analytical methods for protozoan parasites and enteric virus analysis. Therefore, we are ready to begin processing samples for both enteric viruses and protozoan parasites very soon, probably by the end of August, 2015.

### 1.2.3 Quantitative Microbial Risk Assessment (QMRA)

As data collection is still in progress, risk models for target pathogens of concern in reclaimed water have not yet been applied to actual field data of the project. However, student researchers have been planning and drafting literature reviews with a focus on including material related to the NC Type 2 reclaimed water health risk models for pathogens of concern. We hope to include a concise, relevant review of the literature in our final report along with the results of QMRA.

#### **Student Involvement:**

To date, the following students have been involved in this ongoing project:

1 doctoral student (previously a master's student)

3 master's students

5 undergraduate students

#### **Preliminary explanation of significance of findings to date:**

Based on the results of this study to date, it appears that the 4 of the 5 wastewater reclamation plants included in this study are capable of producing reclaimed water to meet the microbial performance requirements of the NC type 2 reclaimed water regulation. The final reclaimed water effluents of these treatment plants meet or better the target mean concentrations of *E. coli*, coliphages and *C. perfringens* of 3, 5 and 5 per 100 mL and they do not exceed the single sample maximum value of 25 per 100 mL. Four of these wastewater reclamation plants achieve bacteria and protozoan parasite surrogate (*C. perfringens*) reductions of 6 and 4 log<sub>10</sub>, respectively, as specified in the regulation. However, most of these plants do not quite meet the 5 log<sub>10</sub> virus reduction requirement of the NC type 2 reclaimed water regulation, giving log<sub>10</sub> reductions ranging from 4.1 to 5.4 log<sub>10</sub>. The inability to document the 5 log<sub>10</sub> virus reduction level is due to the concentrations of coliphages in the raw sewage of these treatment plants being too low to follow this magnitude of log<sub>10</sub> reduction. The calculated log<sub>10</sub> reductions of these plants are based on the lower detection limit of the coliphage assay of the reclaimed water and therefore are "greater than" values. It is likely that the actual coliphage reductions are 5 log<sub>10</sub> or more, but this level of performance cannot be quantified based on the reclaimed water sample volumes analyzed. A practical solution to this problem is to increase the volume of the reclaimed water samples analyzed and thereby increase the lower detection limit of the coliphage analysis. Increasing the sample volume from 100mL to 200 or 300 mL will likely overcome this problem in documenting 5 log<sub>10</sub> coliphage reduction performance. In analyses of future reclaimed water samples, we plan to increase the sample volume analyzed in an effort to overcome this lower detection limit problem in documenting 5 log<sub>10</sub> coliphage reduction performance. One of the 5 water reclamation plants studied, plant C, did not meet the performance targets of bacteria, viruses and the protozoan parasite surrogate because reclaimed water effluent concentrations had detectable levels of these microbes that exceeded the concentrations specified in the NC type 2 reclaimed water regulation and because log<sub>10</sub> reduction performance targets were not met consistently. It was determined that this water reclamation plant is actually not using dual

disinfection as required by the NC type 2 reclaimed water regulation but instead is using only a single disinfection treatment process. Hence, the single disinfection treatment barrier used at this water reclamation facility is unable to reduce target microorganisms to the low concentrations of the regulation or achieve the log<sub>10</sub> reduction targets of the regulation. Analysis of reclaimed water at this 5<sup>th</sup> treatment plant provides direct evidence that the dual disinfection barrier is critical to achieving the target low final microbial concentrations and target log<sub>10</sub> microbial reductions of the NC type 2 reclaimed water regulation.

### **Any deviations from original project plans:**

The deviation from the original project plan includes the incorporation of protozoan parasite analysis into the range of pathogens to address in NC Type 2 reclaimed water. This deviation was approved as an addendum to this project and all methodological changes, including the changes to the enteric virus concentration methods, are detailed in this addendum.

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### **Appendix 1: alphabetical list of abbreviations and symbols:**

*C. perfringens: Clostridium perfringens*

CFU: Colony forming unit(s)

*E. coli: Escherichia coli*

MPN: Most Probable Number

NC: North Carolina

NCT2RW: North Carolina Type 2 Reclaimed Water

PFU: Plaque Forming Unit(s)

QMRA: Quantitative microbial risk assessment

SMEWW: Standard Methods for the Examination of Water and Wastewater

US EPA: United States Environmental Protection Agency

WWTP: Wastewater Treatment Plant

WRRI: Water Resources Research Institute

### **Appendix 2a: list of publications or presentations resulting from the work to date:**

Publications:

Matthew Price's MSPH Thesis: "Comparison of the Single Agar Layer and Two Step Environment Spot Plate Methods in the Detection of Somatic and Male-Specific Coliphages in NC Type II Reclaimed Water Samples". December 2014.

Presentations:

4/19/15, Microbial Quality of Reclaimed Water to Meet NC Type 2 Performance for *Escherichia coli*, Coliphage Viruses, *Salmonella* spp., and *Clostridium perfringens*, The University of North Carolina at Chapel Hill Water Microbiology Conference, Chapel Hill, NC

Description: This session was a 15-minute PowerPoint presentation followed by a 5 minute question and answer period. The goal of this presentation was to describe NC Type 2 reclaimed water, the processes for producing it, the water quality requirements for it and then to present the log<sub>10</sub> reductions obtained by the NC Type 2 treatment processes at 5 wastewater reclamation utilities in the Research Triangle Area of Raleigh, NC.

4/20/15, Evaluation of a Candidate Bacteria Host for Simultaneous Detection and Quantification of Somatic and Male-specific/F+ Coliphages in Reclaimed Water, The University of North Carolina at Chapel Hill Water Microbiology Conference, Chapel Hill, NC

Description: This session was a 15-minute PowerPoint presentation followed by a 5-minute question and answer period. The goal of this presentation was to detail the evaluation of a combined somatic and male specific coliphage *E. coli* host, compared to the individual somatic and male-specific/F+ *E. coli* hosts for the detection of coliphage viruses in reclaimed water, raw sewage and tertiary treated wastewater.

4/21/15, Best methods for detecting *C. perfringens* in untreated and treated wastewater, The University of North Carolina at Chapel Hill Water Microbiology Conference, Chapel Hill, NC

Description: This session was a 15-minute PowerPoint presentation followed by a 5-minute question and answer period. The focus of this presentation was to present analysis on the simultaneous detection of *C. perfringens* on 3 candidate agar media in NC T2RW and raw sewage.

## **Appendix 2b. Technology Transfer Activities**

We have not completed any specific technology transfer activities to date. It is not clear that we are at a point where it is feasible or appropriate for technology transfer activities because we are continuing to evaluate the ability of water reclamation plants to meet microbial quality performance targets of NC Type 2 reclaimed water and the log<sub>10</sub> microbial reduction performance requirements of this regulation.

# Linkages of Mercury and Methane Cycles in Piedmont Streams and Rivers in North Carolina, and Implications for Mercury Bioaccumulation in Food Webs

## Basic Information

<b>Title:</b>	Linkages of Mercury and Methane Cycles in Piedmont Streams and Rivers in North Carolina, and Implications for Mercury Bioaccumulation in Food Webs
<b>Project Number:</b>	2014NC188B
<b>Start Date:</b>	5/1/2014
<b>End Date:</b>	4/30/2015
<b>Funding Source:</b>	104B
<b>Congressional District:</b>	NC12
<b>Research Category:</b>	Water Quality
<b>Focus Category:</b>	None, None, None
<b>Descriptors:</b>	None
<b>Principal Investigators:</b>	Martin Tsz-Ki Tsui, Craig J. Allan, Anne Hershey, Stephen C. Whalen

## Publications

There are no publications.

## **TITLE PAGE – FINAL REPORT (14-04-W)**

### **Project title**

Linkages of mercury and methane cycles in Piedmont streams and rivers in North Carolina, and implications for mercury bioaccumulation in food webs

### **Investigators**

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### **WRII project number**

14-04-W

### **Date of report**

February 25, 2016

## **ABSTRACT**

*Project title: Linkages of mercury and methane cycles in Piedmont streams and rivers in North Carolina, and implications for mercury bioaccumulation in food webs*

Mercury (Hg) is a global contaminant, and its inorganic form has been thought to be methylated by sulfate-reducing bacteria to become highly toxic methylmercury (MeHg). However, recent evidence showed that methanogens, which produces methane (CH<sub>4</sub>), can also methylate inorganic Hg. In twelve Piedmont streams, we examined the levels of total Hg, MeHg and CH<sub>4</sub> in different compartments (water, sediment, and/or biota). In general, we found very low levels of total Hg and MeHg, but high and variable levels of CH<sub>4</sub> in sediment porewater among sites. The direct relationship of Hg methylation and CH<sub>4</sub> production was not observed due to the fact that many stream samples had very low or undetectable levels of MeHg. In a control experiment using specific microbial inhibitors, our results demonstrated that Hg methylation in Piedmont stream sediment is mainly mediated by sulfate-reducing bacteria but there is a small but consistent role of methanogens in methylating Hg. Overall, this project is the first to conduct comprehensive sampling and analysis of Piedmont streams (within or near Greensboro, NC) for Hg and CH<sub>4</sub> cycling.

## **ACKNOWLEDGEMENTS**

Peter Blum (M.S. student) and Josh Brigham (Ph.D. student), both at UNC-Greensboro, completed the majority of the work outlined in this project, as part of their thesis/dissertation. We thank Kimber Corson and Matthew Monteverde (UNC-Greensboro) for assisting in the field/lab. We appreciate the assistance of Katherine Broadwater (UNC-CH) for methane analysis in Whalen lab. We also acknowledge the help from Dr. Chad Hammerschmidt and his group for performing mercury isotope analysis for our microcosm experiments. UNC-Greensboro Biology provided funding for P. Blum and J. Brigham to present parts of this project in regional and national conferences. Lastly, the funding from NC WRI for supporting this work is gratefully acknowledged.

# 1. INTRODUCTION

Atmospheric transport and deposition of mercury (Hg) contaminates the majority of natural ecosystems while a small fraction of systems are impaired by local point sources such as mining and industrial discharges (Morel et al., 1998). More importantly, the deposited Hg is mostly inorganic form [abbreviated as Hg(II)] and is not very bioavailable to organismal uptake (Tsui and Wang, 2004). However, Hg(II) can be efficiently methylated to become highly toxic methylmercury (MeHg), especially under anoxic conditions via anaerobic microbial communities (Gilmour et al., 1992), such as sulfate-reducing bacteria. MeHg can extensively bioaccumulate and biomagnify in aquatic food webs, leading to widespread fish consumption advisories (e.g., statewide fish consumption advisory in North Carolina) across the nation due to high Hg found in fish tissues (Mergler et al., 2007).

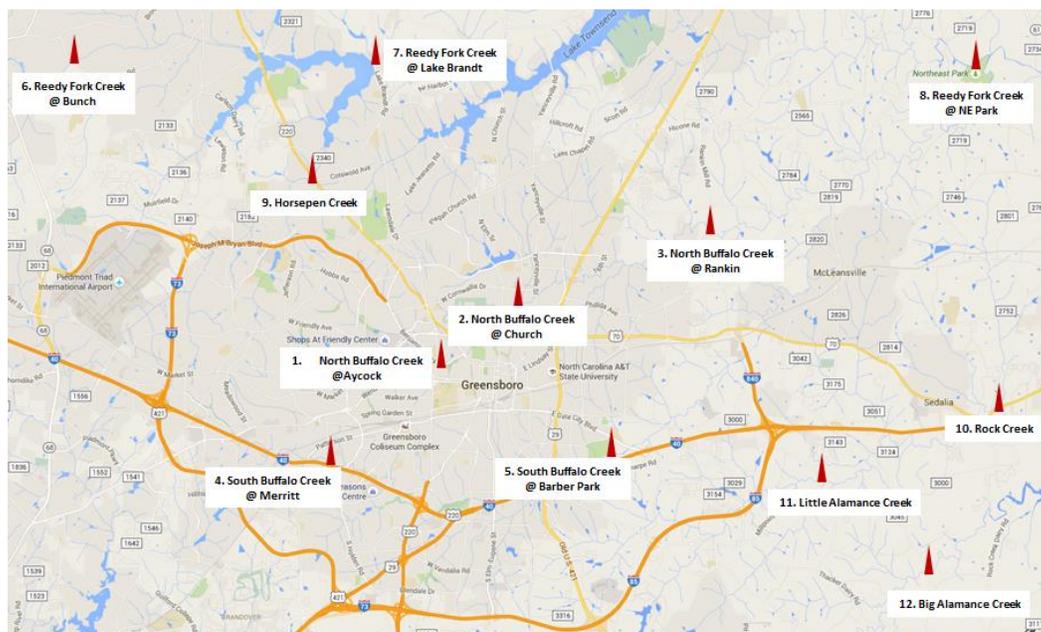
Methane (CH<sub>4</sub>), a potent greenhouse gas, is produced by methanogens through methanogenesis, which is an important pathway for organic matter decomposition that occurs universally in anoxic aquatic sediments (Whalen and Reeburgh, 2000). Streams typically are supersaturated in methane (CH<sub>4</sub>) and can result from allochthonous inputs as well as autochthonous methanogenesis. Studies have shown significant methanogenic potential and high abundance of methanogens on the streambed in Piedmont streams (Smith 2013), suggesting that zones of methanogenesis are widespread in stream ecosystems. Furthermore, methane concentrations may change radically from high to low gradient reaches, and with season (Jones and Mulholland 1998), reflecting in-stream conditions as well as lateral and upslope processes. Methanogen populations in streams are not well studied, but current studies in Piedmont streams indicate strong seasonal patterns and seasonal differences between streams in forested compared to urban landscapes (Smith 2013).

Recent experimental studies have shown the capability of certain groups of methanogens to methylate in both lab and field settings (Hamelin et al., 2011; Yu et al., 2013), suggesting that it is possible for the CH<sub>4</sub> and Hg cycles to be coupled in natural settings. In this work, we test the idea that the extensive methanogenesis in Piedmont streams may be coupled to Hg methylation. We hypothesized that: (1) streams and rivers with higher dissolved CH<sub>4</sub> will have higher levels of MeHg in water and sediment (porewater and solid phase), and high MeHg levels in consumers; (2) Hg methylation and methanogenesis are interrelated processes that affect bioavailability and bioaccumulation of MeHg. Our objectives are to: (i) examine spatial and temporal patterns of MeHg and CH<sub>4</sub> concentration in water, sediment and food webs; (ii) use experimental approaches to probe whether methanogens are important Hg methylators in controlled assays. We propose to use field surveys and experimental manipulation to assess the relationships between CH<sub>4</sub> and Hg methylation and demethylation in Piedmont streams.

## 2. METHODS

### 2.1. Field survey

A total of twelve Piedmont streams/sites were examined for mercury and methane distribution within or near the city of Greensboro, NC (**Fig. 1**). Study sites were chosen based on the variability in channel sizes and land use covers in the watershed. Intensive sampling was performed during low flow period in the summer of 2014.



**Fig. 1** Study sites for the field survey in the summer 2014.

At each site, a suite of abiotic and biotic samples were collected, including surface water, bulk sediment (< 1-mm sieved), porewater, seston, and dominant macroinvertebrates (including Asian clams (*Corbicula fluminea*) and hydropsychid caddisflies). **Table 1** summarized the specific sample types and analyses performed, in three different analytical laboratories.

**Table 1** Summary of sample type collected and parameters analyzed in this study.

Sample type/analytical methods	THg and MeHg (UNCG Tsui lab)	CH <sub>4</sub> (UNC-CH Whalen lab)	$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (CPSIL) <sup>a,b</sup>
Surface water	UF and FIL fractions	Dissolved gas	Seston only
Bulk sediment	< 1-mm sieved	-	< 1-mm sieved
Porewater	FIL fraction	Dissolved gas	-
Macroinvertebrates	Tissues	-	Tissues
<i>Analytical methods</i>	<i>Cold vapor atomic fluorescence spectroscopy</i>	<i>Gas chromatography flame ionization detector</i>	<i>Isotope ratio mass spectrometry</i>

<sup>a</sup> Colorado Plateau Stable Isotope Laboratory, Northern Arizona University (Flagstaff, AZ)

<sup>b</sup> Data for stable C/N isotopes are not shown in this report, as further interpretation will be required.

## 2.2. Specific microbial inhibitor experiments

In late January 2015, we set up control experiments (a total of 80 individual microcosms) with the addition of different microbial inhibitors for stream sediments from four different sites with contrasting land uses and sediment properties within or around Greensboro, NC (i.e., #1 North Buffalo Creek, #4 South Buffalo Creek, #8 Reedy Fork Creek, and #11 Little Alamance Creek). For each microcosm bottle, we use 200 ml glass serum bottle with a gas impermeable stopper, in which we added 100 g of wet and unsieved stream sediment with 100 ml of reconstituted soft freshwater (USEPA, 2002) (**Fig. 2**).



**Fig. 2** Picture of microcosm set up in this study (by P. Blum).

Specific microbial inhibitors were added according to **Table 2**, each treatment had a total of four replicates. The selection of microbial inhibitors and their final concentrations were based on the previously published studies on Hg(II) methylation (e.g., Fleming et al., 2006; Hamelin et al., 2011).

**Table 2** Summary of treatments of microbial inhibitor experiment.

Treatment	Inhibited Microbes	Unaffected Microbes
Control	None	All types
BESA <sup>a</sup>	Methanogens	Sulfate-reducing bacteria, other types <sup>b</sup>
Na <sub>2</sub> MoO <sub>4</sub>	Sulfate-reducing bacteria	Methanogens, others*
Na <sub>2</sub> MoO <sub>4</sub> + BESA	Sulfate-reducing bacteria, and methanogens	Other types
Chloramphenicol	All types	None

<sup>a</sup> (2-Bromoethanesulfonic acid)

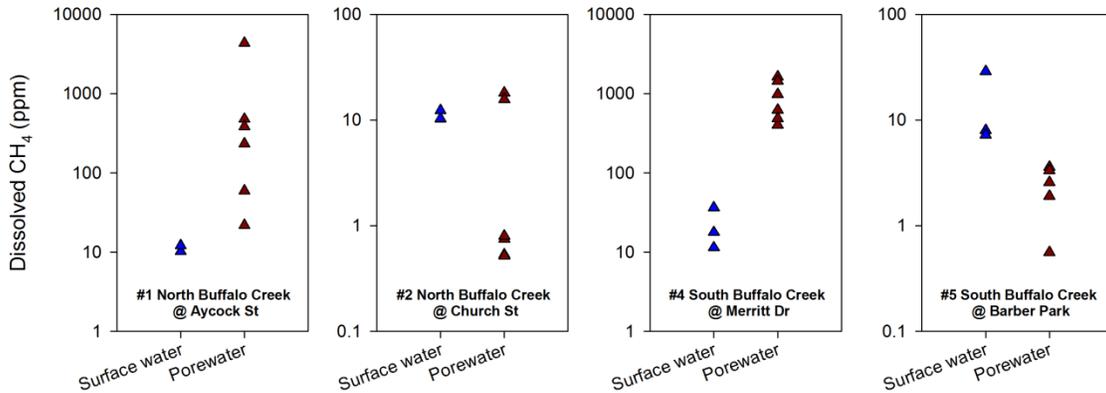
<sup>b</sup> Other types especially refer to iron-reducing bacteria that can also methylate Hg(II) (Fleming et al., 2006)

All microcosms were pre-incubated for 10 days to allow the development of the natural microbial communities, and on day 10, we spiked isotopically enriched <sup>200</sup>Hg(II) (96.4% purity; Oak Ridge National Laboratory) into each microcosm so as to double the total Hg in each microcosm, and microcosms were left to run for 8 more days before we sampled the sediment to analyze the fraction of <sup>200</sup>Hg(II) being methylated to become Me<sup>200</sup>Hg by CV-GC-ICP-MS at Chad Hammerschmidt's Lab at Wright State University (Dayton, OH). The Hg methylation potential would be calculated as %Me<sup>200</sup>Hg (i.e., percent of <sup>200</sup>Hg as Me<sup>200</sup>Hg). Meanwhile, we collected gas in the headspace from each microcosm over time (0, 0.5, 1, 1.5, 2, 3, 5, 7, 10, 13, 18 days) to measure CH<sub>4</sub> produced by the microbes.

### 3. RESULTS AND DISCUSSION

#### 3.1. Dissolved methane data in field survey

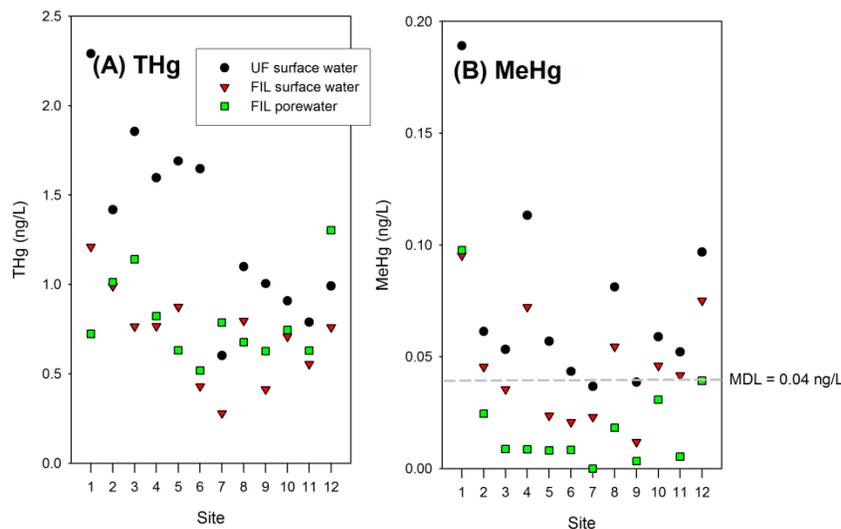
All surface water and porewater samples contain measurable concentrations of dissolved CH<sub>4</sub>. However, while the surface water represents a well-mixed pool of dissolved CH<sub>4</sub>, the porewater pool of CH<sub>4</sub> is very heterogeneous and the sample CH<sub>4</sub> concentration varied widely, over 2-3 orders of magnitude. **Fig. 3** shows CH<sub>4</sub> data from two urban streams at different locations within the city of Greensboro. As shown, porewater CH<sub>4</sub> can be highly variable, and can be higher or lower than that in surface (overlying) water.



**Fig. 3** Concentrations of dissolved CH<sub>4</sub> in surface water (collected in a fast-flowing portion within the channel) and porewater (5-10 cm in sediment) in two major urban streams in Greensboro.

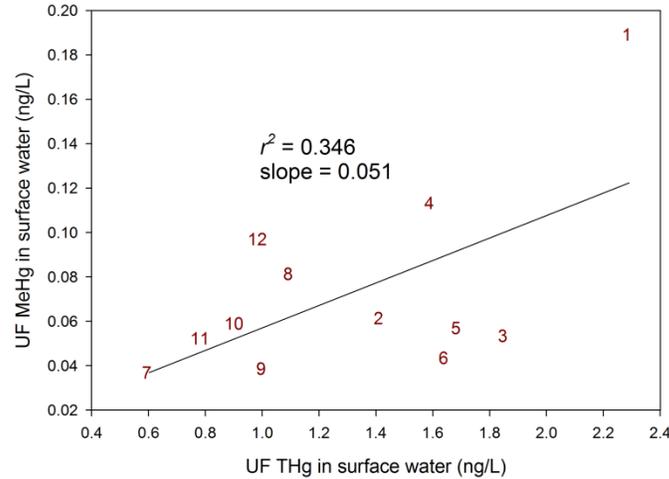
#### 3.2. Aqueous mercury data in field survey

All surface water (both UF and FIL fractions) and porewater samples have relatively low, but consistently above method detection limit (MDL, 0.2 ng/L), of THg concentrations (**Fig. 4**). FIL samples had about 50% of THg of UF samples. Porewater samples in most cases had lower THg than FIL samples. However, many sites had FIL surface water and FIL porewater with MeHg below our MDL of 0.04 ng/L (**Fig. 4**).



**Fig. 4** Aqueous THg and MeHg data.

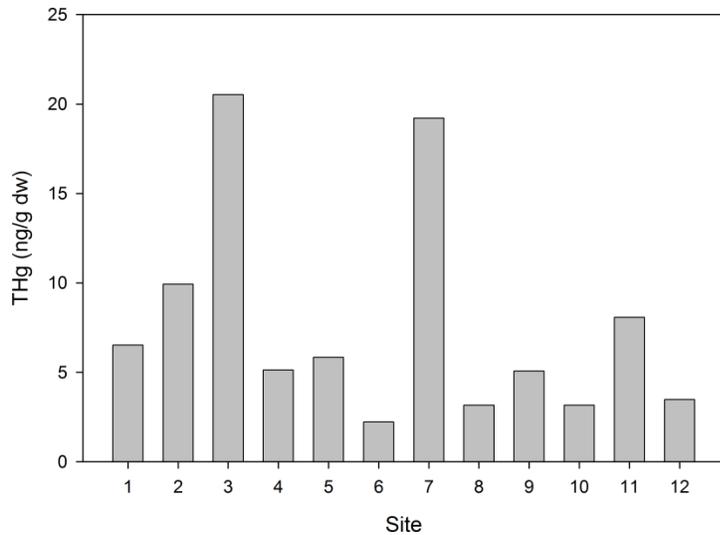
Interestingly, there was some relationship between THg and MeHg in UF surface water samples across sites (**Fig. 5**), being highest for both at an urban stream (#1 North Buffalo Creek @ Aycocock). The slope represents the mean fraction of THg as MeHg (or %MeHg) in these water samples, being 5.1%, and is at the low end of stream water %MeHg among other stream ecosystems (Brigham et al., 2009; Tsui et al., 2010).



**Fig. 5** Relationship between UF THg and UF MeHg across study streams.

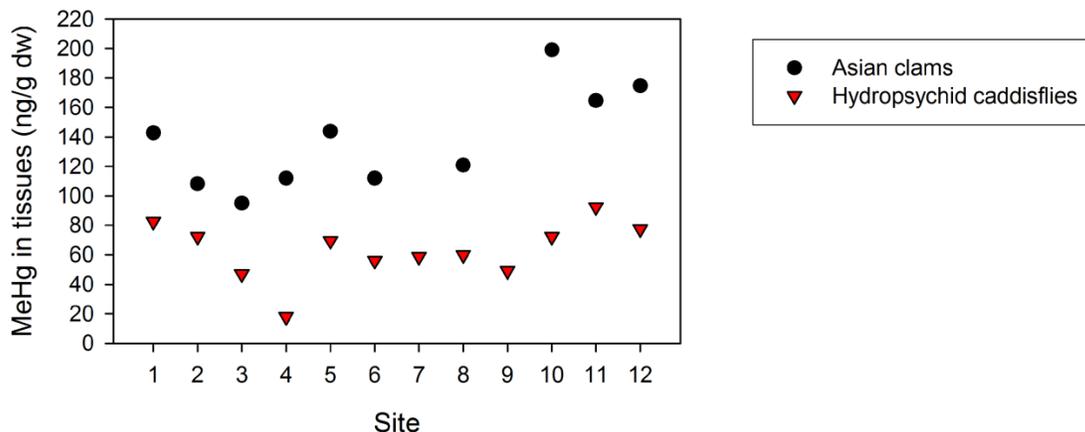
### 3.3. Sediment mercury data in field survey

In stream sediments (<1-mm sieved), THg concentrations are very low but variable among streams, however, the majority of samples had MeHg levels (*data not shown*) below our MDL (~0.1 ng/g dw). It should be noted that the stream sediments in our study sites are in general very sandy and coarse, and low in organic content, but Hg is often positively correlated with organic matter content in surface sediment (Chakraborty et al., 2015), thus it is not surprising to observe low Hg contents in sediment across sites.



**Fig. 5** Sediment (<1-mm) THg data.

In each site, we collected two major macroinvertebrates, Asian clams and hydropsychid caddisflies, when available (except two sites where we did not collect any clam samples). In general, these macroinvertebrates had %MeHg from 31-100%, and clams had variable but generally higher MeHg tissue concentrations than hydropsychids in the same site (**Fig. 6**).



**Fig. 6** MeHg in two macroinvertebrate tissues among study sites

### 3.4. Relationship between methylmercury and methane in field survey

Since a lot of FIL surface water, FIL porewater and bulk sediment had MeHg below our MDL, and thus we rely on the data of UF MeHg in surface water and MeHg in tissues of two macroinvertebrates to represent MeHg levels among study sites. For CH<sub>4</sub> data, we use the mean values of replicated analyses of surface water data but we only select the “highest” CH<sub>4</sub> data from porewater since they were very variable. As shown in **Fig 7A**, we did not observe significant relationship between CH<sub>4</sub> and MeHg in surface water, but we found that increasing CH<sub>4</sub> in surface water would have decreasing MeHg in tissue of biota (**Fig 7B**). As largely driven by a single data point, we found positive relationship between porewater CH<sub>4</sub> and UF MeHg in surface water among sites (**Fig 7C**). The relationship may be non-existing. However, increasing porewater CH<sub>4</sub> we found weak increases of MeHg in clams but not hydropsychids (**Fig 7D**).

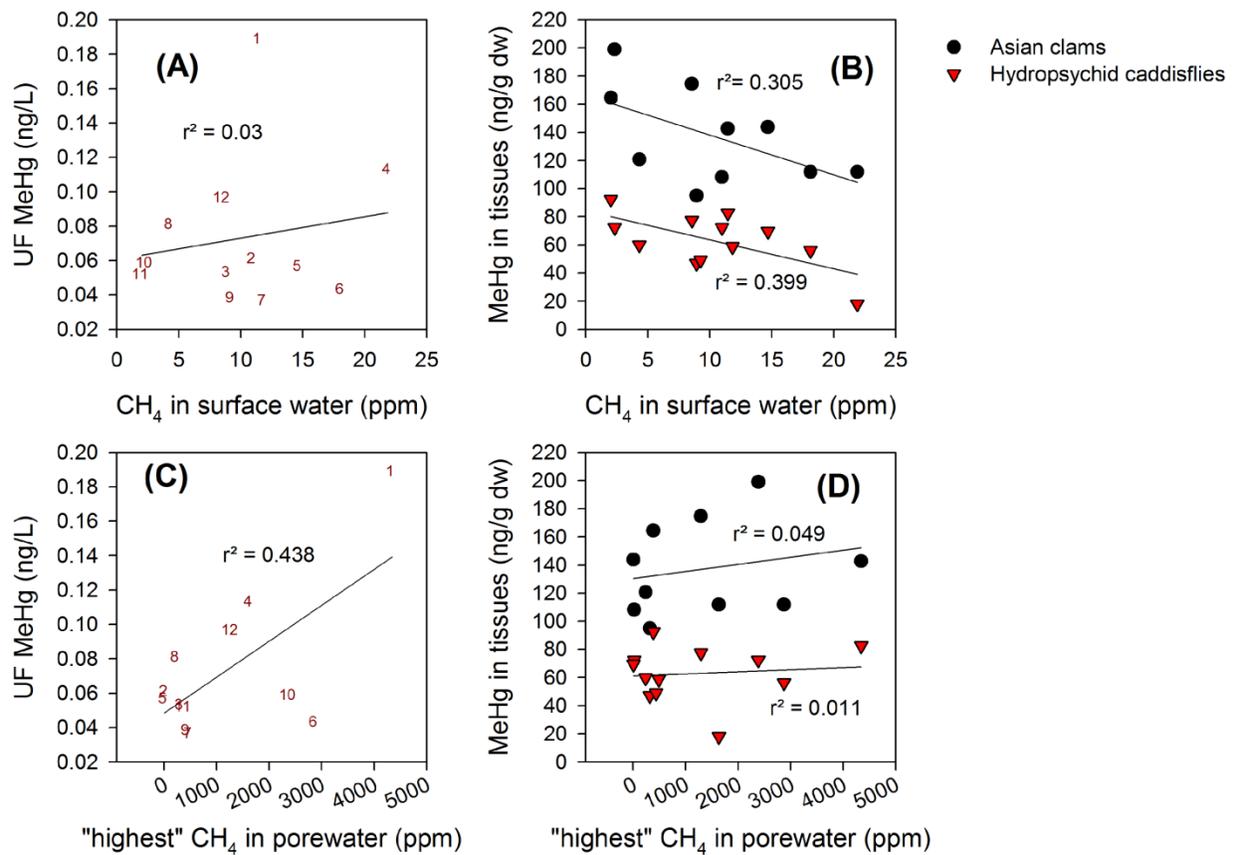
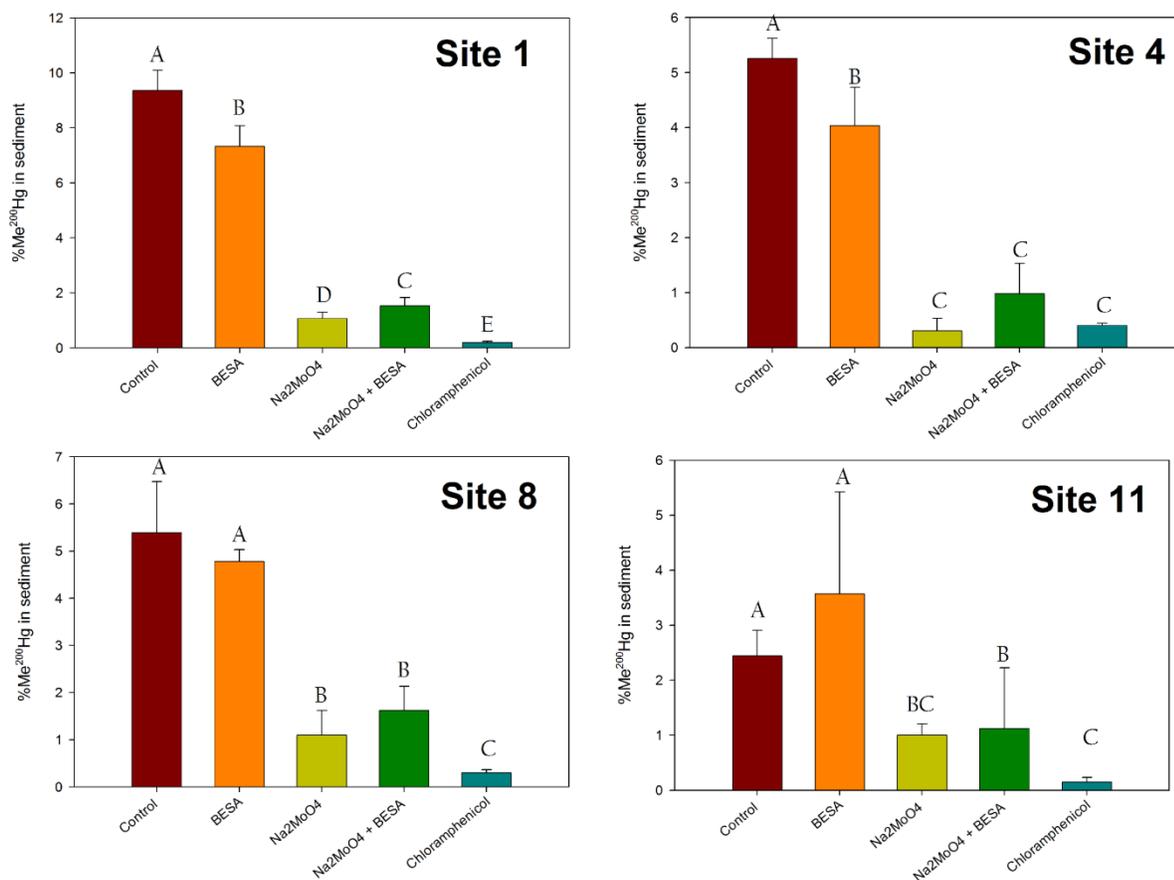


Fig. 7 Multiple relationships explored between CH<sub>4</sub> and MeHg among sites.

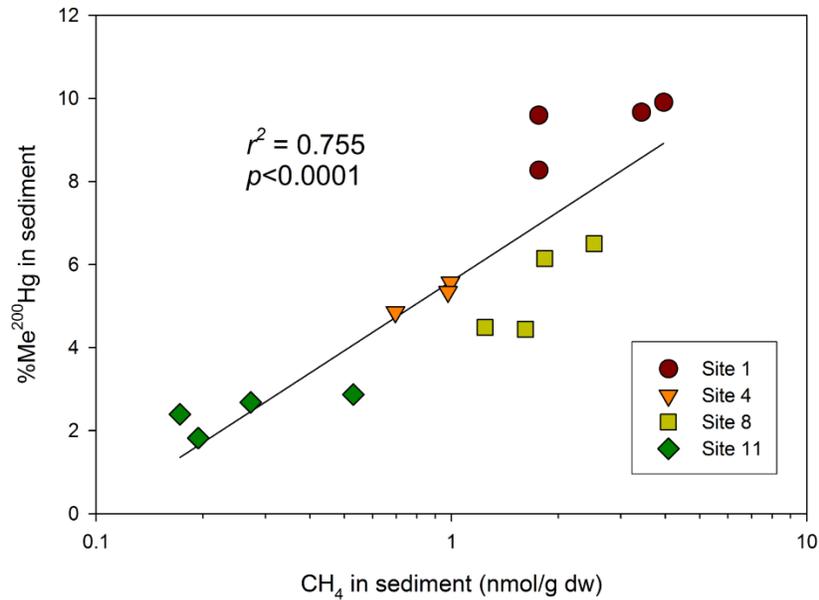
### 3.5. Microbial inhibition experiments

Our microcosm experiments have been analyzed for CH<sub>4</sub> samples over time and Me<sup>200</sup>Hg on the last day of incubation (i.e., day 18), and the data on %Me<sup>200</sup>Hg at the end of the incubation for the four stream sediments were shown in in **Fig. 8**. As shown, except site 11 sediments all sites had the highest mean %Me<sup>200</sup>Hg in the control without any microbial inhibitor, in most cases, by adding BESA (an inhibitor for methanogens) may decrease slightly %MeHg compared to the control. However, by adding Na<sub>2</sub>Mo<sub>4</sub> with or without BESA, or just chloramphenicol (general bacterial inhibitor) we observed significantly lower %MeHg ( $p < 0.05$ ). All these data strongly suggest that methanogens (inhibited by BESA) are not principal microbial groups in methylating Hg in these piedmont stream sediments, while we believe that sulfate-reducing bacteria (inhibited by Na<sub>2</sub>Mo<sub>4</sub> and/or chloramphenicol) are the main methylators of Hg, similar to the many other studies on freshwater wetland and lakes.



**Fig. 8** %Me<sup>200</sup>Hg in sediment microcosms of different stream sediments on day 18, without any microbial inhibitors (control) or with different types of microbial inhibitors .

Interestingly, we found that for all uninhibited microcosms (i.e., control) there are a positive and strong relationship between methane produced and %Me<sup>200</sup>Hg in sediment (**Fig. 9**), such results may contradict with our above findings that methanogens are not important in methylating Hg but instead should suggest that the higher microbial activities (i.e., higher methane production) would be associated also with higher Hg methylation activities (potentially mediated by sulfate-reducing bacteria, methanogens and other microbes).



**Fig. 9** Relationship between %Me<sup>200</sup>Hg and methane produced in sediment on day 18 of incubation without any microbial inhibitors (i.e., controls) among the four study sediments.

#### 4. SUMMARY, CONCLUSION AND RECOMMENDATIONS

To our knowledge, it is the first comprehensive study examining Hg distribution in a variety of streams in Piedmont of North Carolina, especially within or near the city of Greensboro. THg and MeHg concentrations are in general at the low end of stream ecosystems across the United States receiving mainly atmospheric deposition (Brigham et al., 2009; Chasar et al., 2009; Tsui et al., 2009). Due to the fact that MeHg levels are very low, it reduces our capability to explore the relationship between MeHg with CH<sub>4</sub> cycling. However, we did observe very variable levels of CH<sub>4</sub> concentrations in porewater samples, suggesting active methaogenesis within the stream sediment, result not previously reported in streams in the region.

Based on our control experiments, the research results suggest that methaogenesis plays a minor role in methylating Hg(II) while sulfate-reducing bacteria are the dominant groups in methylating Hg(II), consistent with reports on other lake and wetland ecosystems (Gilmour et al., 1992). Finally, we conclude that Hg contamination is not very serious in Piedmont streams but more future sampling would be needed to examine the temporal variability.

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## **APPENDIX 1 (ABBREVIATIONS AND SYMBOLS)**

THg = total mercury

MeHg = monomethylmercury

UF = unfiltered (water samples)

FIL = filtered (water samples)

CH<sub>4</sub> = methane

## **APPENDIX 2 (OUTPUTS FROM THE PROJECT)**

### **Presentations**

Blum PW, Hershey AE, Tsui MTK (2015) Methylmercury production from microbes in North Carolina piedmont streams. Society of Environmental Toxicology and Chemistry - Carolinas Chapter Annual Meeting. Raleigh, North Carolina.

Blum PW, Hershey AE, Tsui MTK (2015) Methylmercury production from microbes in North Carolina piedmont streams. Society of Freshwater Science Annual Meeting. Milwaukee, Wisconsin.

Brigham JS, Hershey AE, Tsui MTK (2015) Examining methane processes and methane derived carbon in food webs in North Carolina piedmont streams. Society of Freshwater Science Annual Meeting. Milwaukee, Wisconsin.

### **Theses/dissertations**

Peter Blum (M.S. thesis, in progress) Methylmercury production from microbes in North Carolina piedmont streams. Co-advised by MTK Tsui and AE Hershey

Josh Brigham (Ph.D. dissertation, in progress) Examining methane processes and methane derived carbon in food webs in North Carolina piedmont streams. Advised by AE Hershey

# Land Application of Aquaculture Effluents to Prevent Surface Water Eutrophication and Promote Groundwater Re-Infiltration in Coastal North Carolina

## Basic Information

<b>Title:</b>	Land Application of Aquaculture Effluents to Prevent Surface Water Eutrophication and Promote Groundwater Re-Infiltration in Coastal North Carolina
<b>Project Number:</b>	2014NC190B
<b>Start Date:</b>	7/1/2014
<b>End Date:</b>	6/30/2015
<b>Funding Source:</b>	104B
<b>Congressional District:</b>	NC-04
<b>Research Category:</b>	Water Quality
<b>Focus Category:</b>	Water Quality, Groundwater, Nutrients
<b>Descriptors:</b>	None
<b>Principal Investigators:</b>	Harry Daniels, Dennis William Hazel

## Publications

There are no publications.

## Project #2014-NCSG-03

Land Application of Aquaculture Effluents to Prevent Surface Water Eutrophication and Promote Groundwater Re-infiltration in Coastal North Carolina.

H. V. Daniels, E.G. Nichols, and D.W. Hazel, North Carolina State University

**Introduction:** Current production practices for hybrid striped bass require annual pond draining to harvest and move fish and to avoid infestations by the parasitic yellow or white grub. These practices create large volumes of effluent from April to October and have led to complaints from the general public about stream eutrophication and habitat degradation. Effluents released from the North Carolina fish farms are slow-moving and drain into wide, shallow coastal creeks that empty into waters such as the Pamlico Sound – part of the nutrient sensitive Tar-Pamlico River Basin (sub basin No. 7). In North Carolina, these streams are classified as zero-flow waters, which severely restricts their use for receiving effluents. The Albemarle Sound system and the Cape Fear River are also potentially impacted.

Although many studies have shown that the nutrient content of the effluents is low relative to other animal production systems, the high volume of water presents an engineering challenge that overwhelms surface water systems as well as traditional land application to row crops. Based on preliminary studies at the Tidewater Research Station, we developed a fish-water-tree system that has the potential to absorb the pond effluents and divert this wastewater to woody biomass production for carbon storage (managed forest plantations), sustainable wood products, or sustainable bioenergy such as wood pellets, a fast-growing renewable energy market in coastal North Carolina. This system re-infiltrates pond water back to groundwater systems allowing for groundwater recharge. Ideally, these systems could be designed to land-apply pond effluents on managed forest systems up-gradient of source wells to better manage groundwater resources and limit surface water impact.

This purpose of this study was to evaluate nutrient removal efficiency, water use and tree growth of this system during an entire production season. We found that the trees and soil microorganisms removed more than 80% of the water added to the site including precipitation and irrigation, which effectively doubled the amount received through precipitation. Approximately 90% of the chlorophyll-a, 25% of the Total Kjeldahl Nitrogen (TKN) and Total Organic Carbon (TOC) were removed and 50% of the Total Suspended Solids (TSS) were removed by this system. Overall, the reductions in these parameters meant that the water leaving the site, as subsurface export, was well below the regulatory limits required for discharge to Nutrient Sensitive Waters.

**Materials and Methods:** Clones of hybrid poplar (*Populus spp*) were planted (over 1,400 trees) on a 1.2-acre field site (Figure 1). Drainage tile was installed under one half of the field, while the other half (negative control) did not have additional drainage structures. Six shallow groundwater monitoring wells were installed in March 2014 on tiled (n=3) and non-tiled (n=3) areas. The entire field has perimeter drainage ditches typical of lower Coastal Plain agricultural fields. A 4-ft deep trench with a vertically-oriented polycarbonate sheet bisects the field and prevented the passage of water between the two halves. Water samples were collected according to the following schedule: (1) biweekly ground and surface water for nitrogen species, total suspended solids or chlorophyll a (Center for Applied Aquatic Ecology); (2) biweekly ground and surface water monitoring and gauging of physical

characteristics for hydrological modeling; and (3) end of growing season productivity and survival for Fall 2015. The site required weekly maintenance for irrigation and weed control. Aluminum irrigation pipe was placed throughout the field with sprinklers spaced at 100-ft intervals to evenly distribute water and minimize overlap. The ponds were stocked with hybrid striped bass (*Morone chrysops X M. saxatilis*) and were managed according to standard commercial practices. The trees were irrigated to field capacity (maximum water absorption of soils), which corresponded to approximately one-inch of water per week. Weekly water irrigation volumes along with related meteorological data (rainfall, evaporation, wind speed and relative humidity) were collected to calculate permissible water application rates and evapotranspiration.

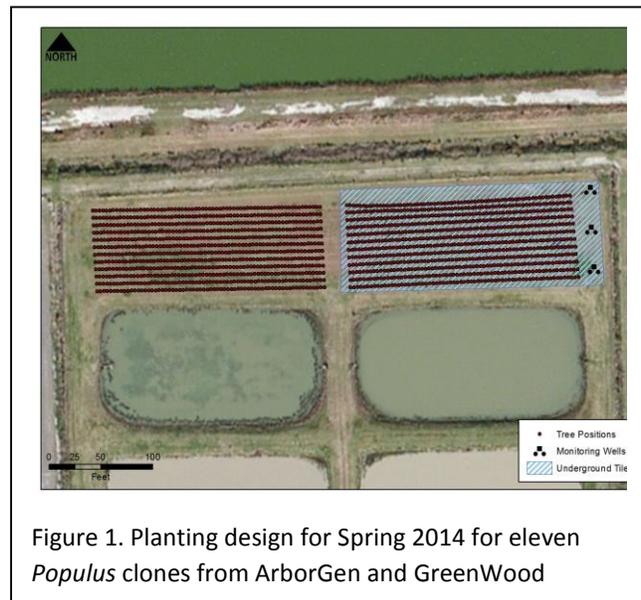


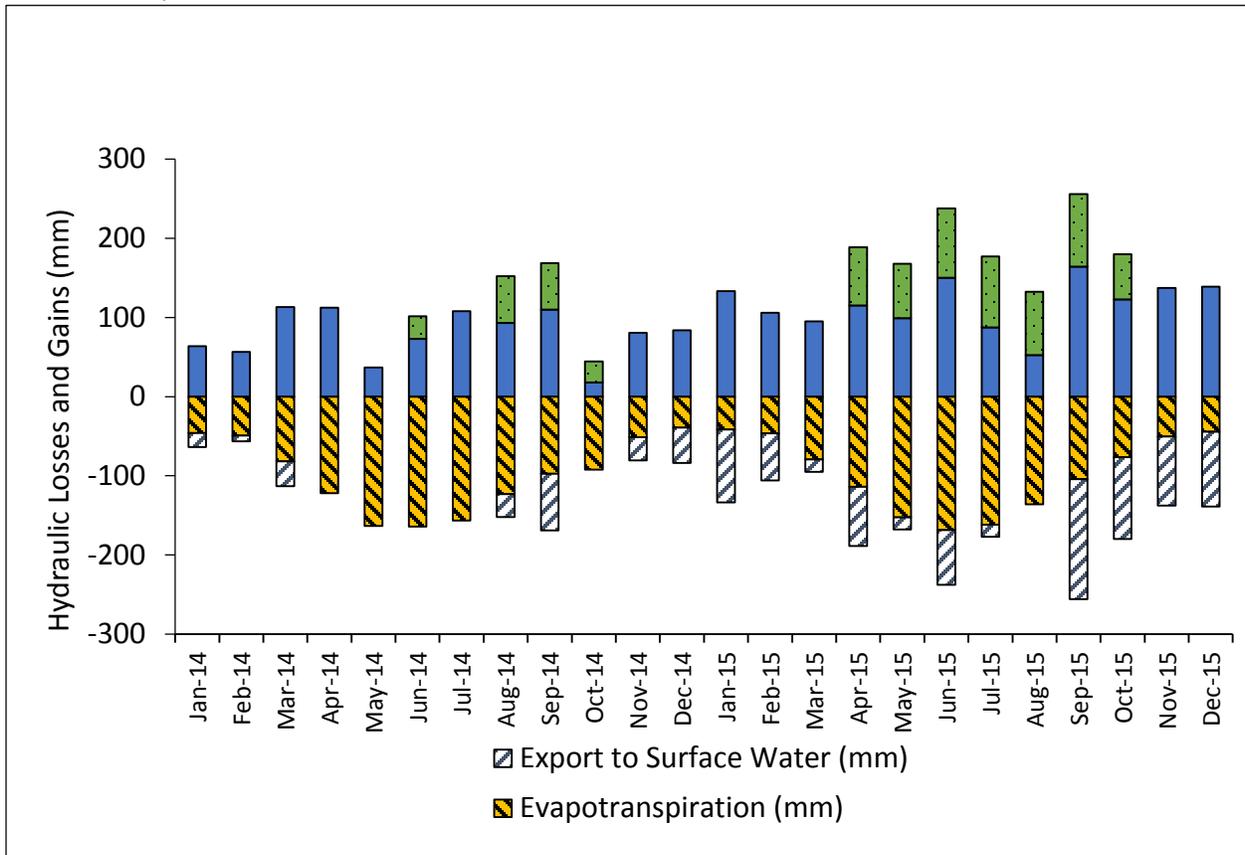
Figure 1. Planting design for Spring 2014 for eleven *Populus* clones from ArborGen and GreenWood

## Results and Discussion:

### Water budget

The majority of the effluent applied to the field was lost through evapotranspiration by the trees (Fig. 2). Percent removal through evapotranspiration was highest during the peak growing season in summer and declined beginning in early fall then remained low during the winter months. Water additions through irrigation nearly doubled the amount of water that the field received through precipitation during the summer months. Since the principal means of evapotranspiration is through leaves, we did not irrigate when leaves were not present on the tree limbs to avoid excessive water loss through subsurface export.

## Water Quality



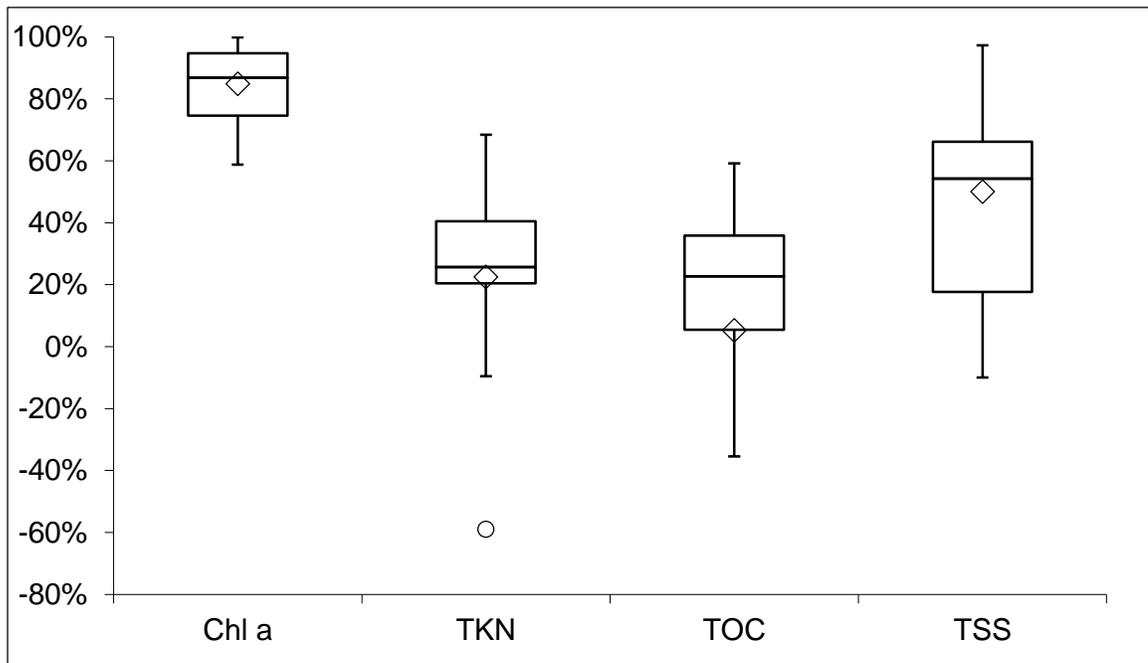
**Nutrient Removal:** Trees and soil microbiota removed approximately 20 – 92% of most water quality variables, such as chlorophyll-a, TSS, TOC and TKN (Table 1 and Figure 2). However, inorganic nitrogen levels either remained unchanged or increased markedly after water passed through the system. It is likely that the water added through irrigation was removing residual nitrogen that had accumulated through bacterial nitrification and was not bound to soil clays or organic matter.

Seasonal comparisons of nutrient export (Table 2) show that concentrations are markedly higher during the winter months versus the summer months when irrigation occurs. These values likely represent the normal water quality in subsurface waters after precipitation moves through soils when plants are inactive or dormant. The mean chlorophyll-a value during winter is particularly interesting because this value, 39.3  $\mu\text{g/L}$ , is very close to the EPA limit of 40  $\mu\text{g/L}$  for receiving streams in Nutrient Sensitive Waters.

**Table 1:** Mean water quality concentrations during irrigation

Parameter	Pond	Published Effluent Values*	Irrigation Ditch	Subsurface Export
Chl a ( $\mu\text{g/L}$ )	73.0 $\pm$ 3.0		73.3 $\pm$ 24.5	5.9 $\pm$ 1.4
TSS (mg/L)	42.3 $\pm$ 9.4	49*	44.5 $\pm$ 10.9	15.8 $\pm$ 2.0
TOC (mg/L)	12.4 $\pm$ 1.1		16.5 $\pm$ 1.3	12.6 $\pm$ 0.8
TKN (mg/L)	2.9 $\pm$ 0.4	7.1*	2.4 $\pm$ 0.2	1.6 $\pm$ 0.1
NO <sub>2</sub> -N&NO <sub>3</sub> -N (mg/L)	0.26 $\pm$ 0.11	0.43*	0.09 $\pm$ 0.02	0.75 $\pm$ 0.21
NH <sub>3</sub> -N (mg/L)	0.47 $\pm$ 0.2	0.95*	0.39 $\pm$ 0.11	0.33 $\pm$ 0.07

\*Values from Tucker et al. 1998 [33], a sampling of 20 HSB ponds in South Carolina.



**Fig 2.** Percent removal efficiency of hybrid poplar biofilter for chlorophyll a (Chl a), total Kjeldahl nitrogen (TKN), total suspended solids (TSS), and total organic carbon (TOC).

**Table 2:** Mean ( $\pm$  standard error) water quality values in subsurface export water during months without irrigation (winter) versus months with irrigation (summer).

<b>Subsurface Export</b>	<b>Winter</b>	<b>Summer</b>
<b>Chl a (<math>\mu\text{g/L}</math>)</b>	39.05 $\pm$ 1.76	5.9 $\pm$ 1.4
<b>TSS</b>	88.25 $\pm$ 2.88	15.8 $\pm$ 2.0
<b>TOC</b>	68.54 $\pm$ 0.79	12.6 $\pm$ 0.8
<b>TKN</b>	8.61 $\pm$ 0.16	1.6 $\pm$ 0.1
<b>NO3-N&amp;NO2-N</b>	9.23 $\pm$ 0.36	0.75 $\pm$ 0.21
<b>NH3-N</b>	0.86 $\pm$ 0.04	0.33 $\pm$ 0.07

**Tree Productivity:** Mean height, mean diameter at breast height (DBH), and mean volume all differed significantly between the twelve genotypes (Fig. 3). Four of the genotypes (356, 373, 140, 312) are currently exceeding the productivity level needed to meet the sustainability criteria (established by USDA) for carbon neutrality and woody biomass demand. These *Populus deltoides* genotypes had much greater productivity, although slightly average survival, and in some cases lower survival than the other clones. These findings suggest that not only the species, but the actual clone and genotype of that tree species is important to the success of a crop under this type of irrigation regime. It is clear that each clone responds very differently to the amount of water applied and the constituents of that effluent. This information is critical to the future success of land application research and/or demonstration studies because the economic feasibility of this system will be directly influenced by the specific tree genotype. Given these results, the productivity values, for these four clones of *P. deltoides*, is an encouraging outcome.

**Outreach:**

In addition to the presentations at State meetings to producer groups and scientific presentations at National and International meetings (shown below), we have taken several groups of undergraduate students (total of 12 Doris Duke Conservation Scholars from NC State University) to the study site for educational tours. Given the remote location of the study site, transportation of producers to the site is challenging. Instead, we have presented annual updates of our work for two consecutive years at the

North Carolina Aquaculture Development Conference, where the audience consists of a variety of producers from around the state.

Shifflett, S.D., **Culbreth, A.** Hazel, D. Daniels, H., Nichols, E.G. (2016). Integrating freshwater aquaculture and forest productivity for F.E.W. nexus resiliency in the mid-Atlantic Coastal Plains. North Carolina Sea Grant Graduate Student Workshop. New Bern, N.C. April 6-7, 2016. Poster.

**Culbreth, A.** (2016). Evaluation of the Treatment Efficiency of a Vegetative Filter on Hybrid Striped Bass (*Morone chrysops x Morone saxatilis*) Pond Effluents. Aquaculture America 2016, Las Vegas, NV. February 23-26, 2016. Presentation.

Shifflett, S.D., **Culbreth, A.** Hazel, D. Daniels, H., Nichols, E.G. (2016). Using land application systems to address the F.E.W. nexus: managing aquaculture effluents and growing woody biomass for bioenergy. The Food-Energy-Water Nexus: 16th National Conference and Global Forum on Science, Policy and the Environment. Washington, D.C. January 19-21, 2016. Poster.

**Culbreth, A.,** Shifflett, S.D., Hazel, D., Nichols, E., Daniels, H. 2015. The Food, Energy, Water Nexus: Using Wastewater from Aquaculture Operations to Fertilize Energy Crops. 2015 Stewards of the Future Conference. Raleigh, NC, November 2, 2015. Poster.

**Culbreth, A.,** Hazel, D., Nichols, E.G., Daniels, H. Land Application of Aquaculture Effluent. Water Resources Research Institute Annual Conference. Raleigh, NC. March 18-March 19, 2015. Poster.

Shifflett, S.D., **Culbreth, A.,** Begue, P., Hazel, D., Nichols, E.G., Daniels, H. Land Application of Aquaculture Effluents to Meet Dual Objectives: Grow Woody Biomass and Prevent Surface Water Eutrophication. Aquaculture America 2015. New Orleans, LA, February 18-February 22, 2015. Poster.

**Conclusion and Future Research:** The current fish-water-tree effluent treatment system continues to show promise as a viable method for effective water removal, nutrient reduction and economic potential for biomass production. The trees in this system will reach their peak maturity during the 2016 production season. We anticipate that water removal, through evapotranspiration, and nutrient removal will increase as a result of the greater leaf area and establishment of associated root systems and soil microbes. Combined with projected biomass production, these results should allow us to objectively evaluate the economic benefits of this system.

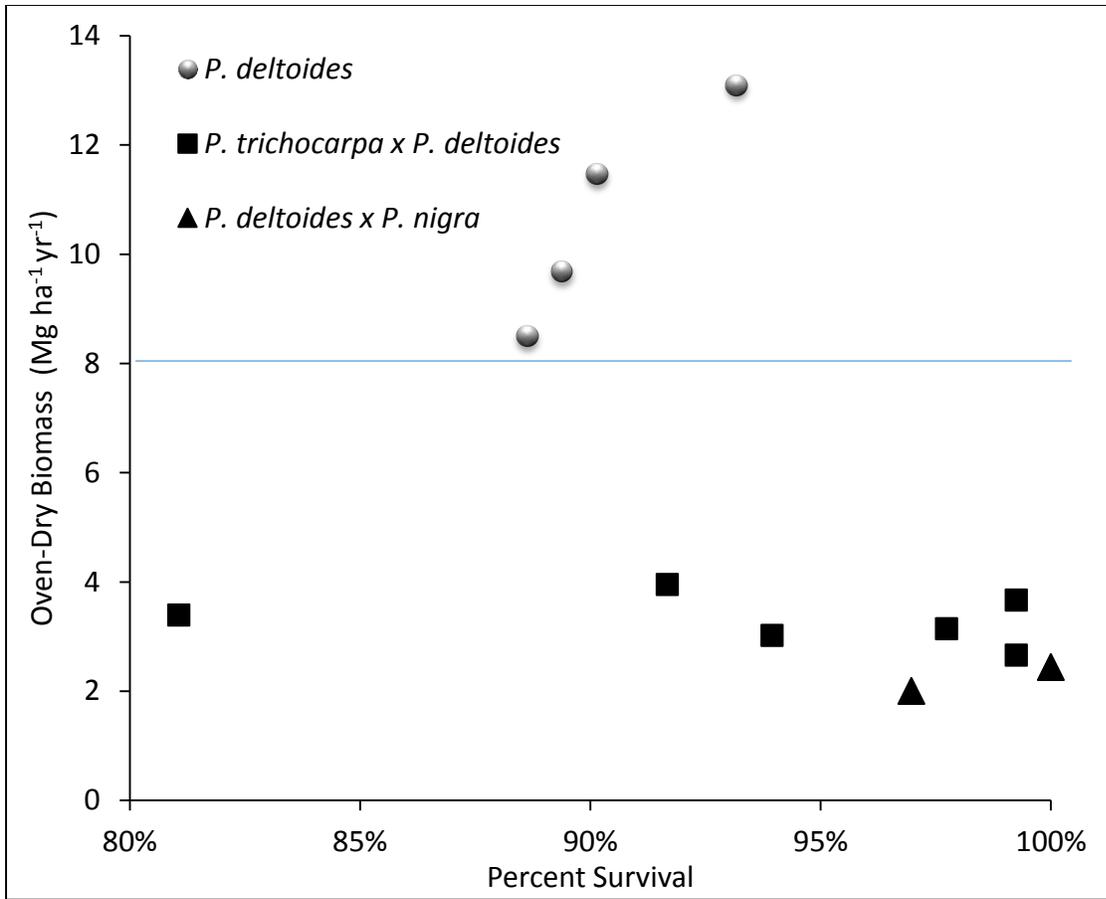


Fig 3. *Populus spp.* clone productivity measured in Mg/ha/yr of oven-dried biomass.

# Heavy metal analysis, gene proxies, and stable isotope tracers of coal ash contamination in the Dan River food web

## Basic Information

<b>Title:</b>	Heavy metal analysis, gene proxies, and stable isotope tracers of coal ash contamination in the Dan River food web
<b>Project Number:</b>	2015NC191B
<b>Start Date:</b>	3/1/2015
<b>End Date:</b>	2/29/2016
<b>Funding Source:</b>	104B
<b>Congressional District:</b>	12
<b>Research Category:</b>	Water Quality
<b>Focus Category:</b>	Toxic Substances, Ecology, Sediments
<b>Descriptors:</b>	None
<b>Principal Investigators:</b>	Anne Hershey, Parke Rublee, Martin Tsz-Ki Tsui

## Publications

There are no publications.

Final Report 15-02-W  
**Heavy metal analysis, gene proxies, and stable isotope tracers of coal ash  
contamination in the Dan River food web**

Water Resources Research Institute of The University of North Carolina  
Submitted March 31, 2016

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1. Abstract:

**Heavy metal analysis, gene proxies, and stable isotope tracers of coal ash contamination in the Dan River food web**

The goals of the project were to trace the extent of mercury (Hg) contamination in the Dan River food web and food web disruption derived from the coal ash spill, evaluate microbial pathways governing coal ash contaminant routing to the food web, and evaluate the extent to which stable isotope of sulfur could be used to trace coal ash contamination in food webs. Approaches for meeting the goals included analysis of coal ash Hg concentrations in river sediments and food web components at multiple sites upstream and downstream of the spill; using multiple stable isotopes to evaluate food web disruption and quantify the portion of the contamination that is attributable to the spill; and using gene proxies to assess the microbial mechanisms that route coal ash metals into the food web. Of four objectives, objectives (1) and (2) of the proposed work addressed the *research question*: How much metal contamination and food web disruption has occurred in the Dan River food web due to the February 2014 coal ash spill? Objectives (3) and (4) addressed the *research question*: How well do gene proxies for microbes that chelate metals or methylate Hg predict coal-ash derived heavy metals in the Dan River food web? The approaches used can readily be applied to long term Dan River monitoring or to other coal ash spill sites.

Our results are still preliminary and additional analyses are pending. We did observe some patterns that indicate negative effects of the coal ash spill: elevated Hg in downstream samples that was above the baseline attributable to organic matter content, and incorporation of coal ash derived sulfur into riparian spiders that feed on emerging insects. To date we do not see a signal of microbial response that can be conclusively attributable to coal ash spill, and further study will be needed to more fully evaluate those effects. Thus, although we have not seen an alarming coal ash effect to date on the microbial community, and impacts on invertebrate components of the food web were limited to spiders, further investigation is needed to evaluate Hg methylation over time as well as persistence of the coal ash Hg in river sediments.

Dan River, Coal ash, Mercury(Hg), Sulfur (S), Heavy metals, Stable isotope tracers, Gene proxies, Bioaccumulation, Food web

## **2. Acknowledgements**

We thank NC Water Resources Research institute for funding this work and the assistance of its staff in proposal development. The work would not have been possible without the participation of graduate students Kimber Corson and Ashley Williams, and the technical assistance of Matthew Monteverde. We also thank Mr. Brian Williams, Dan River Basin Association, for logistical assistance with the field work, including many long hours on the river.

# Heavy metal analysis, gene proxies, and stable isotope tracers of coal ash contamination in the Dan River food web

## 3. Introduction

A recent coal ash spill at the Dan River Steam Station near Eden, NC, generated public interest and raised policy and economic concerns, similar to those of a 2008 Tennessee spill (Ruhl, et al. 2009, 2012). Coal ash contains heavy metals that persist in the environment and are toxic to humans and wildlife. Some heavy metals, such as mercury (Hg), bioaccumulate in aquatic food webs (Mergler et al. 2007), leading to long-term environmental and human health risks even when water concentrations are not elevated. Aqueous Hg in the Dan River following the 2014 coal ash spill was barely detectable (Hesterberg et al. 2014). Although that result is important to immediate and short-term water use, it does not alleviate long-term concern over food web contamination from coal

ash because riverine sediments rather than the water are the primary vector for coal ash metals, including Hg, to enter the food web. Sediment Hg downstream of the spill site on 28 April 2014 was ~4-fold higher than upstream (Fig. 1a). Hg concentration in coal ash was greatly elevated compared to upstream sediments (Fig. 1a).

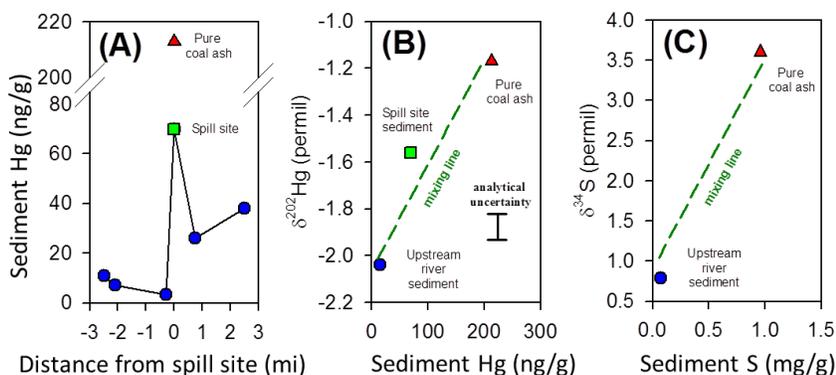


Fig. 1 (A) Total-Hg conc. in coal ash, spill site sediment (in Feb 14) and Dan River sediment (in Apr 14) both upstream and downstream of the spill site; (B) Relationship between stable Hg isotope ratios (as  $\delta^{202}\text{Hg}$ ) and total-Hg conc.; (C) Relationship between stable S isotope ratios (as  $\delta^{34}\text{S}$ ) and S conc.

The most toxic form of Hg, methylmercury (MeHg), is produced by anaerobic microbial methylation by various microbial groups, often dominated by sulfate reducing bacteria (SRB) (Parks et al. 2013). Some aquatic ecosystems have limiting sulfate availability (Muyzer and Stams 2008), but coal ash is a sulfur (S) source (Sheng et al. 2000, and see Fig 1c), potentially stimulating Hg methylation. Accordingly, research needs to investigate production of MeHg in the Dan River, and routing of MeHg and other coal ash contaminants to invertebrates and fish in the river food web. Furthermore, emerging aquatic insects have the potential to transport river contaminants into the terrestrial food web, impacting wildlife. Riparian spiders feed on emerging aquatic insects and terrestrial insects and can serve as sensitive monitors of changes in aquatic resources (Kelly et al. 2015). Contamination of fish and wildlife carries potential human health risks due to direct consumption, but also leads to economic losses related to recreational uses of the river (Fig. 2). Published studies of the TN spill noted accumulation of some metals in the food web (Otter et al. 2012) and the potential for food web risk due to elevated Hg and MeHg (Bartov et al. 2013, Deonarine et al. 2013).

The biological mechanisms underlying acute and long-term coal ash toxicity are not fully understood; coal ash contamination in the food web can be site specific and seasonally variable due to factors such as variation in temperature, flow, and sediment accumulation. For example microbial Hg methylation requires anaerobic conditions (e.g., Gilmour et al. 1992). Such conditions occur deep in the sediment profile of virtually all aquatic ecosystems (e.g., Whalen 2005), but are nearer to the sediment surface during warm, low flow conditions, when oxygen is lower and oxygen demand is greater. Bacterial metallothionein proteins also chelate metals, which, along with adsorption and methylation, can route metals into food webs (Gadd 2000, Haferburg and Kothe 2007, 2010, Blindauer 2011). Accordingly, evaluation of genes associated with sulfate reduction, Hg methylation, and metal chelation is needed to provide important, site-specific information on bioavailability of Hg as well as other metal contaminants from coal ash (Fig. 2), which is important to managing Dan River resource use.

Interpreting coal ash spill effects on the Dan River food web is hampered by the fact that the river was already impacted by coal combustion products from atmospheric inputs (Morel et al. 1998), and potentially from ash pond leaching at the Dan River Steam Station and upstream ash ponds in the watershed. Ratios of sulfur (S) stable isotopes,  $^{34}\text{S}/^{32}\text{S}$  (as  $\delta^{34}\text{S}$ ) are needed to distinguish anthropogenic from natural sulfur (S) sources in the environment (Derda et al. 2006). Because different coal combustion products from a given source (e.g., fly ash and bottom ash, dissolved  $\text{SO}_4^{2-}$  from leaching) have distinct S isotope signatures (Elswick et al. 2007), coal ash contamination derived from the ash pond can be traced downstream of the plant by measuring  $\delta^{34}\text{S}$  in the food web compared to  $\delta^{34}\text{S}$  in pond coal ash versus upstream, atmospheric, and pond leaching sources (Fig. 1c).

Coal ash spills can be a significant source of Hg to river ecosystems. Tennessee Valley Authority (TVA) studies of the 2008 coal ash spill showed that coal ash elevated total-Hg concentrations (133 ng/g) 3- to 4-fold compared to sediments upstream (Bartov et al., 2013). More importantly, the highly toxic and bioavailable MeHg was elevated up to 3-fold in downstream sediments near the spill (Deonarine et al., 2013). MeHg bioaccumulates and biomagnifies in food webs (e.g., Tsui et al., 2012), such that Hg methylation ultimately controls MeHg in river food webs. Further, coal ash also is a significant S source (Fig. 1c), which, as sulfate, can stimulate Hg methylation (Benoit et al., 2003).

Our synoptic sampling on 28 April 2014 of Dan River sediment (mainly collected near shore) included sites upstream and downstream of the ash spill. Through collaboration with Mr. Brian Williams of the Dan River Basin Association, we also obtained a coal ash sample. Overall, we

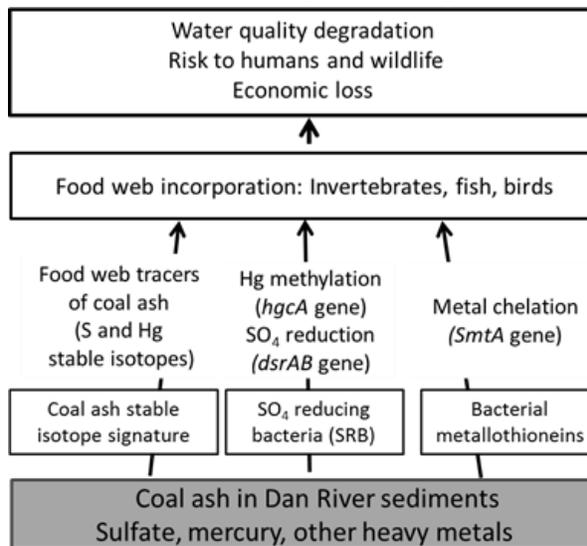


Figure 2. Conceptual diagram illustrating significance of proposed work to NC water resources. Work elements will provide site specific insight into processes leading to food web contamination and robust tools for quantifying coal ash contamination in food webs that are consistent through time and site independent.

found that the river sediment upstream of the ash spill had very low levels of total-Hg (3-11 ng/g or ng Hg/g dry wt) and S (0.07mg/g). However, the coal ash had about 213 ng/g which was 60% more Hg than that in coal ash in the TVA spill (i.e., 133 ng/g), and was also high in S (0.96 mg/g). The river sediment just below the spill had elevated Hg (70 ng/g) and downstream samples had ~4-fold higher Hg than upstream (Fig. 1a), a large fraction of which was coal ash (Fig. 1b).

Prior to the Dan River spill, Ruhl et al. (2012) noted the long-term risk from coal ash residues in groundwater and river sediments from the Dan River site and other ash ponds across NC, recommending continued monitoring. Since the spill, concern about the spill impact and threat of coal ash from ponds throughout the state has been a topic of legislative, media, and public debate. A fish consumption advisory is already in place for Hg in NC water bodies (<http://epi.publichealth.nc.gov/oeo/programs/fish.html>) due to human health risk of Hg contamination from coal burning (primarily from atmospheric deposition); the coal ash spill in the Dan River adds additional risk. Mitigation of the ash pond threat will not address the problem of atmospheric inputs, but it is essential that any added harm derived from ash pond spills be fully evaluated. Elevated sediment Hg levels below the spill (Fig. 1) point to a clear need to monitor Hg in fish, and estimate the fraction attributable to coal ash discharge. Quantification of coal ash-derived food web contamination in the Dan River below the discharge site due to the spill also is essential for interpreting effectiveness of clean-up efforts and providing information to managers. Such quantification can be accomplished using stable isotope ratios of S and Hg in samples below the site compared to those in reference sites (see Fig. 1). Once the relationship between coal ash and background S and Hg stable isotope ratios are established, the same relationships can be applied to evaluate coal ash contamination at any time and location downstream of a spill.

Microbial communities are a primary “gateway” for entry of dissolved materials, including pollutants, into foodwebs (Haferburg and Kothe 2010, Schaefer *et al.* 2014). Microbes may concentrate such materials by assimilation, sorption, or release of metal chelators, potentially altering their toxicity and availability to consumers. Hg is a well-known example; microbial metabolism, especially by SRB, methylates Hg, greatly increasing both toxicity and availability to the food web via invertebrate consumption of the microbes (Driscoll *et al.* 2012, Schaefer *et al.* 2014). Schaefer *et al.* (2013) utilized slightly degenerate primers to the Hg methylation gene *HgcA* and quantitative PCR (qPCR) to compare several sites for the abundance of known Hg methylating taxa including SRB and iron reducing bacteria (Gilmour et al., 2013; Yu et al., 2013). SRB are commonly found in anaerobic sediments, especially those high in organic carbon compounds and their activity is instrumental in Hg methylation (Gilmour *et al.* 2013). Fe-reducing bacteria are also found in anaerobic sediments, where they reduce iron from ferric ( $\text{Fe}^{+3}$ ) to ferrous ( $\text{Fe}^{+2}$ ) forms (Medihala, *et al.* 2012, Yu *et al.* 2012), and this reduction also promotes Hg methylation (Fig. 3). The detection of these key taxa in aquatic systems has also been accomplished recently by qPCR using primers designed to key structural or metabolic genes within the groups (Daly *et al.* 2000, Guan *et al.* 2013, Medihala *et al.* 2012, Yu *et al.* 2012).

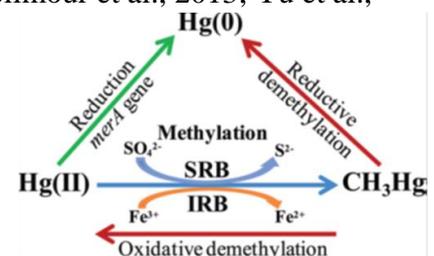


Figure 3. Interaction of sulfate and iron reducing bacteria with mercury methylation (from Yu *et al.* 2012).

The uptake or strong affinity for metal ions in microbes is thought to be through chelation by metallothionein proteins. While many chelators are known from eukaryotes, only a few have been identified in bacteria, along with a number of “metallothionein-like” proteins (Blindauer 2011). The best characterized bacterial metallothionein, *SmtA*, is known to bind Hg, among other metals, although metal binding is affected by environmental conditions (Robinson et al. 1990, Blindauer et al. 2002, Kelly *et al.* 2003, Blindauer 2011, Schaefer *et al.* 2014). Overall the understanding of regulation of Hg uptake, methylation, and export remains poorly known. PCR primers have been designed to the *SmtA* gene, and can be used to determine the abundance of the gene and its mRNA transcript. Since multiple heavy metals are typically elevated in coal ash, (200-1300 ppm, Golightly and Simon 1989), *SmtA* can be a proxy for enhanced metallothionein production by the microbial community due to coal ash contamination.

*3.1. Goals and Objectives.* The goals of this project were to trace the extent of mercury (Hg) and other heavy metal contamination in the food web and food web disruption in the Dan River that was derived directly from the coal ash spill, evaluate microbial pathways governing coal ash contaminant routing to the food web, and evaluate  $\delta^{34}\text{S}$  as a surrogate for coal ash food web contamination for use in future monitoring in the Dan River. Objectives (1) and (2) focused on answering the *research question*: How much metal contamination and food web disruption occurred in the Dan River due to the February 2014 coal ash spill? Objectives (3) and (4) address the *research question*: How well do gene proxies for microbes that chelate metals or methylate Hg predict coal-ash derived heavy metals in the Dan River food web?

*Objective (1) Evaluate persistence of coal ash-derived toxic metals in the Dan River food web 1.5 years following the February 2014 Dan River Steam Station spill.*

*Objective (2) Quantify food web contamination and disruption due to coal ash using stable isotopes of S ( $\delta^{34}\text{S}$ ), C ( $\delta^{13}\text{C}$ ), and N ( $\delta^{15}\text{N}$ ), and stable isotopes of Hg.*

*Objective (3) Evaluate population sizes and activity of SRB and other Hg methylating bacteria in sediments to assess the potential for transfer of MeHg to the food web.*

*Objective (4) Evaluate the abundance of the microbial metallothionein gene *SmtA*, the mercury methylating gene *hgcA*, and the sulfate reducing gene *dsrAB*, and their gene transcripts to assess potential for transfer of all metals to the food web.*

## 4. Methods

*4.1 Field Sampling.* A field survey was conducted to summer conditions upstream and downstream of the coal ash spill in the Dan River. We originally planned to conduct an abbreviated winter season sampling campaign as well, but extremely high discharge conditions precluded winter sampling. Samples were collected at 3 upstream reference sites, 1 site parallel to the ash ponds but upstream of the spill pipes (hereafter, leaching site), and 5 sites downstream (Fig. 4). The leaching reference site is needed to separate the impact of contaminant leaching from the ash ponds from that of the coal ash spill since any leaching effect will also be present below the spill. Site selection was constrained by river access, as there are very few boat ramps along the river, and two dams. Access to the leaching site was provided by Duke Energy.

4.2. *Food web sample collection.* Dredge collected at each site to obtain Asian clams for Hg and stable isotope analyses of S, C, and N, with repeated dredging until sufficient biomass of Asian clams was collected. We were unable to collect aquatic insects from the channel. Both insects and clams were collected from the shore at each site using a combination of dip nets and dredging. Riparian spiders were also collected at each of the sites. Subsamples of Asian clams and dominant insect groups were held overnight in filtered river water, dried, weighed and shipped to UC Davis or Northern Arizona Stable Isotope Facilities for  $\delta^{34}\text{S}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$  analyses.



Figure 4. River survey sampling sites. Sites 1-3 are upstream reference sites. Site 4 is a leaching reference site that is influenced by leaching from the ash ponds on the bluff along the north bank of the river, but upstream of the spill pipes. Sites 5-9 are downstream of the coal ash spill (5,8 dredged, 6,7,9 undredged).

4.3. *Hg analyses.* Sediment samples collected from upstream and downstream of spill site along Dan River were processed and analyzed for total-mercury concentrations. Briefly, sediment from the shore was homogenized using an acid-cleaned pestle and mortar while sediment from the mid-channel was sieved through 1-mm acid-cleaned polypropylene mesh to remove large and coarse particles. All samples were digested with concentrated trace-metal grade nitric acid and hydrogen peroxide (4:1, v:v) at 80°C in Teflon digestion vessels, and aliquots of acid digest were analyzed in duplicate by double-amalgamation technique cold vapor atomic fluorescence spectrometry at UNCG laboratory. Reagent blanks and reference standards (SRM Mess-3 marine sediment) were processed alongside all sample digestion and runs. Organic matter in each sediment sample was determined by loss-on-ignition at 500°C over 4 hours in a muffle furnace.

Work is in progress to determine the bioavailable, organic form of mercury, monomethylmercury, for all sediment samples, and the analyses are expected to finish by the early summer of 2016. Total-mercury and monomethylmercury analyses are in progress for all invertebrate samples collected upstream and downstream, and also riparian spiders along the river, and the analyses are expected to be completed by the early summer in 2016. Moreover, selected sediment samples will be processed and analyzed for stable mercury isotope ratios to examine the contributions of mercury from coal ash to these downstream sediment samples.

4.3. *Microbial analyses:* Sediment samples for microbial analyses were collected from dredge samples and homogenized and from cores that were sectioned. Sediment material was mixed and DNA and RNA from subsamples was extracted and purified. DNA samples were extracted by a CTAB technique (Stewart and Via 1997). RNA and DNA were extracted and purified using MoBio Power Soil RNA and DNA Extraction Kits. The RNA was subjected to rtPCR and both DNA and cDNA was probed by qPCR with primers to the *SmtA* gene sequence to provide quantitative data for comparisons among samples. Similarly, the same samples were probed for the Hg methylating gene *hgcA* and its transcripts as a measure of response to coal ash Hg (Schaefer et al. 2014). Finally, the SRB and iron reducing microbial communities were characterized using known primers (Daly et al. 2000, Guan et al. 2013) and the sulfate reducing gene *dsrAB*, and its transcripts (Guan et al. 2013, Yu et al. 2012). Overall, this was designed to characterize key microbial community responses to S and heavy metals from coal ash, and evaluate this route of uptake into higher trophic levels.

## 5. Results

Response variables were plotted against river distance (km) from the confluence of the Smith River with the Dan River. Our upstream sampling transect extended from 0.5 km downstream of the confluence to 2.53 km downstream of the confluence. The spill pipe was at approximately 5 km downstream; our downstream transect extended to Milton, NC, 64.7 km from the confluence.

### 5.1. Organic matter and Hg analyses

Organic matter % composition in sediments along the shore was similar between upstream and downstream sites, but channel organic matter % composition was higher downstream compared to upstream (Fig. 5), and higher in the channel compared to along shore.

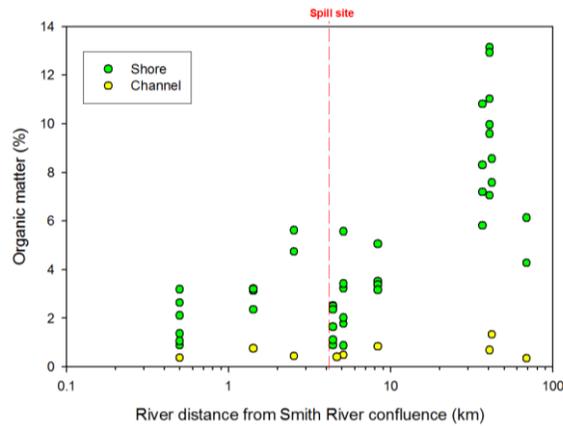


Fig. 5. Organic matter content in sediment samples plotted against the river distance along the Dan River starting from the confluence with Smith River (upstream of coal ash spill site, the approximate location of spill site is indicated by a vertical dashed red line).

Total Hg concentration in sediment showed a very similar pattern as organic matter, with higher concentration in channel compared to shore habitats, and higher concentration downstream compared to upstream of the spill (Fig. 6).

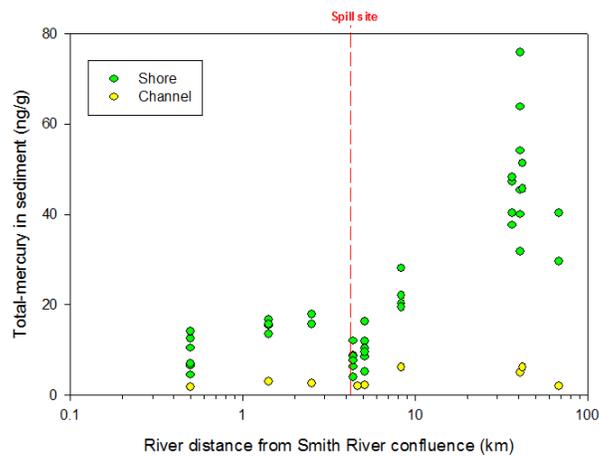


Fig. 6. Total-mercury concentrations in sediment samples from “shore” and “channel”

Since many previous studies have reported a tight relationship between organic matter and total-mercury, and Figs. 5 and 6 above also show a positive relationship between the two parameters, we normalized the total-mercury concentrations to actual organic matter in each sample, and the results are shown in Fig. 7.

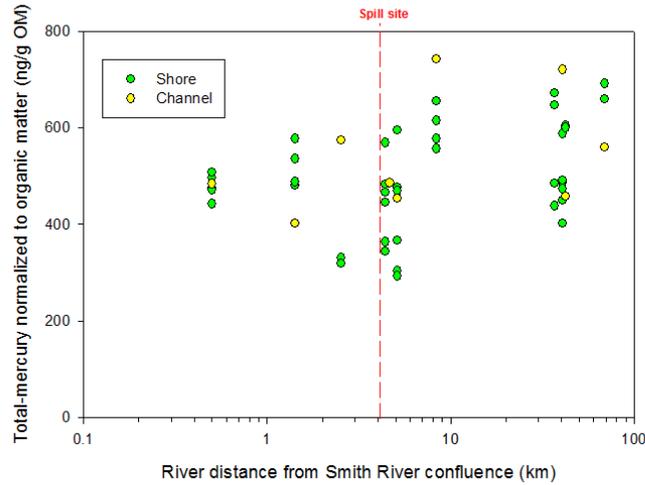


Fig. 7. Total-mercury in sediment normalized to organic matter content.

If we assume all sediment collected upstream is without any coal ash contamination due to the spill incident in 2014, we may infer that any sediment samples with total-mercury normalized to organic matter above the “blue line” in Fig. 8 is attributed to some additional mercury in downstream sections, likely derived from the coal ash spill.

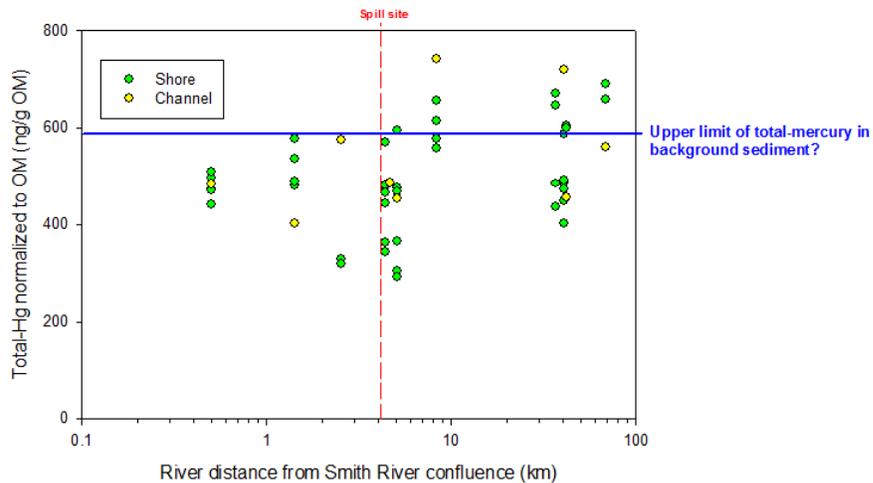


Fig. 8 “Blue line” indicating the potential limit of total-mercury in background sediment in Dan River.

## 5.2 Microbial DNA and genetic analyses

Results from microbial studies are preliminary and further analysis may clarify our understanding of patterns described in this report. DNA in sediments along the shore tended to decrease with distance downstream, while DNA in sediments within the channel appeared to increase. The increase along the channel was likely tied to the increase in organic matter (Fig. 5), as microbes colonize the organic matter.

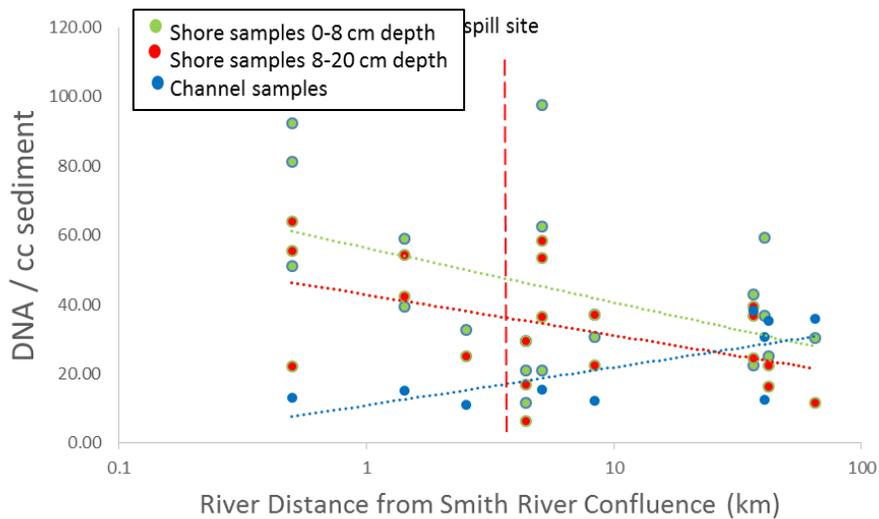


Fig 9. DNA abundance in sediments. Dotted lines represent trend lines. Vertical line indicates spill site.

We determined the relative potential for mercury methylation by running quantitative PCR reactions with published primers to a gene required for mercury methylation, *HgcA*. As a standard we used DNA extracted from a pure culture of *Desulfovibrio africanus* (ATCC #19997), an isolate known to contain the *HgcA* gene. As with the overall DNA, these results suggest decreasing mercury methylating potential downstream in sediments taken from cores along the shore and increasing potential in sediments recovered from the channel.

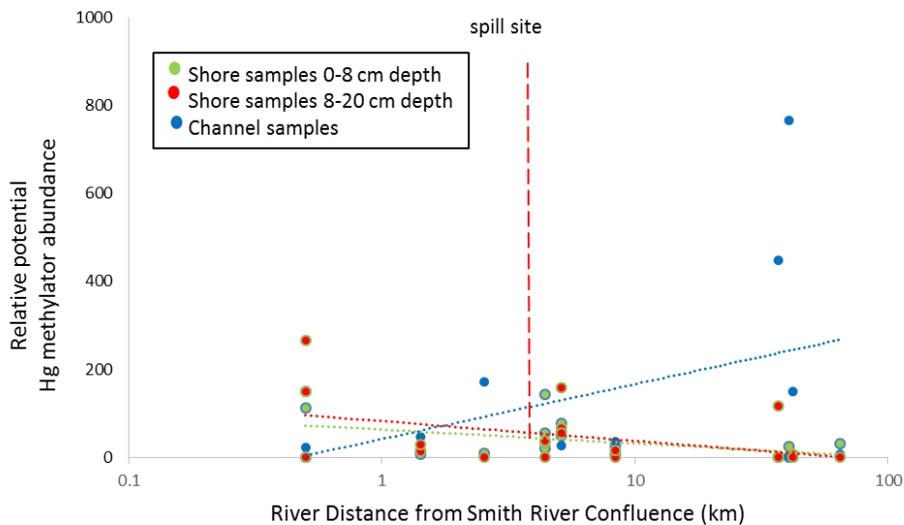


Fig. 10. Relative abundance of potential mercury methylating organisms. Dotted lines represent trend lines. Vertical line indicates spill site

Our results of the determination of the abundance of iron reducing bacteria also show the same pattern of apparent reduced abundance downstream for sediments taken from cores along the shore, contrasting with apparent increased abundance downstream for channel samples. We expect to obtain better insight as we complete assays for sulfate reducers and methanogens, along with assays of transcripts of *HgcA* genes which should give a better indication of mercury methylating activity.

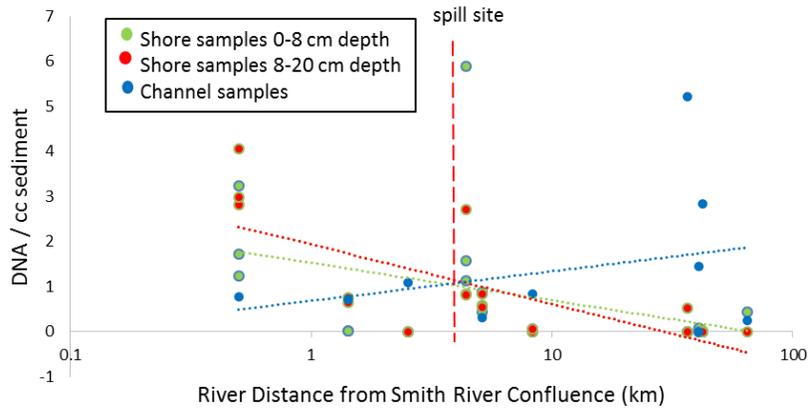


Fig. 11. Relative abundance of Fe reducing bacteria. Trend lines indicated by dotted lines. Vertical line indicates spill site.

### 5.2.1. Metal Tolerance among bacterial isolates

Our studies of metal tolerance of bacterial isolates from soil and coal ash typically show tolerance to a wide range of metal concentrations (Fig 12). These include concentrations that are well above environmental background levels.

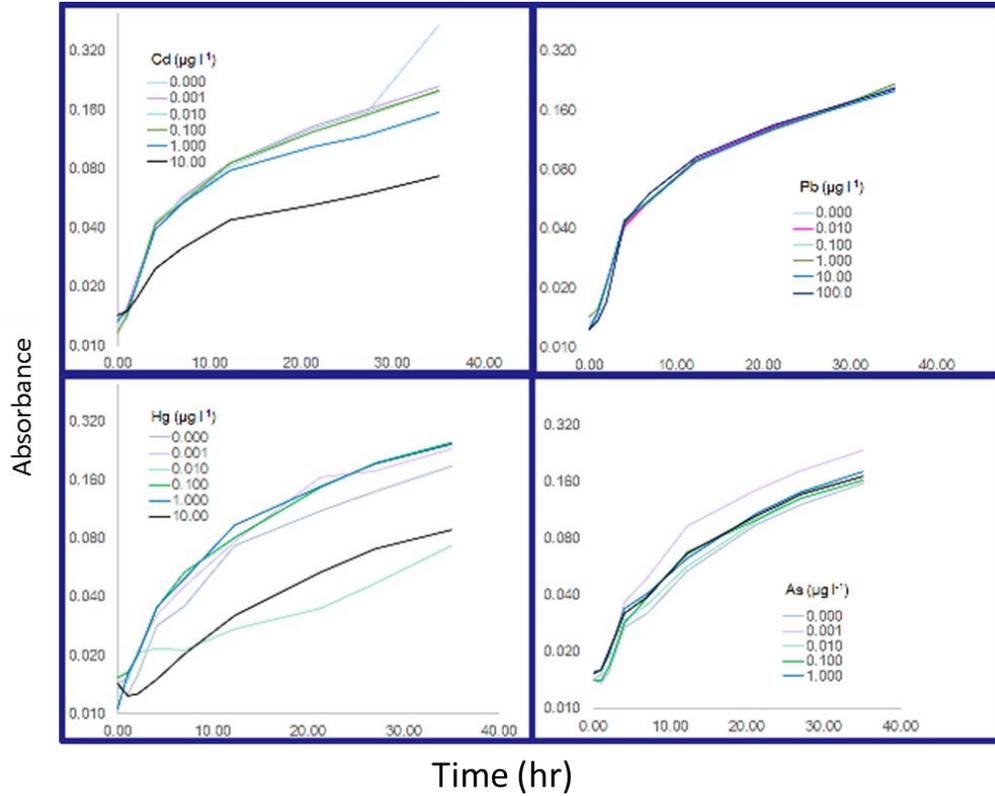


Fig. 12. Typical results of growth of coal ash isolated bacterial in culture when exposed to different additions of heavy metals.

To date we have run growth studies of 8 bacteria isolated from coal ash and 3 bacteria isolated from soils, which we presume to have had less exposure to heavy metals. There appears to be little difference among coal ash and soil isolates for most metals, *i.e.* both groups appear to be metal tolerant for those metals and concentration ranges that we tested. However, coal ash microbes were more tolerant of Cd than soil microbes (Fig. 13).

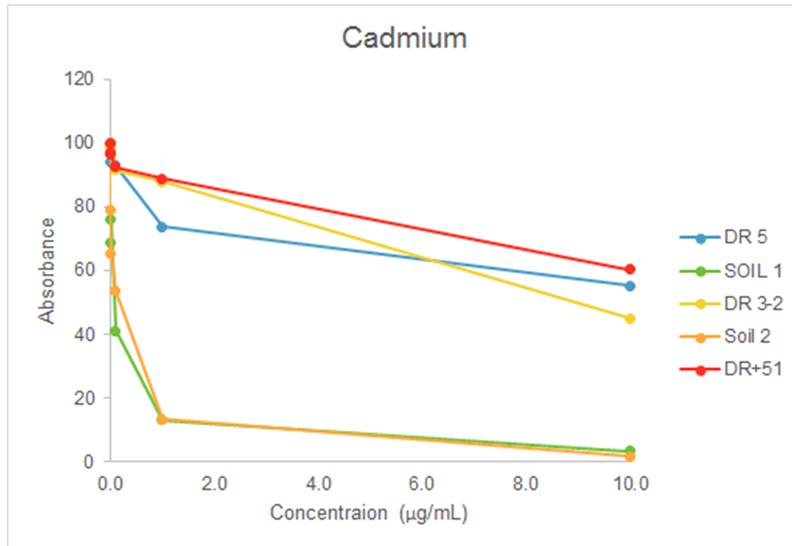


Fig. 13. Comparison of growth of coal ash isolates and soil isolates vs nominal Cd concentrations. Values are % growth compared to growth with no metal addition.

### 5.3. Invertebrate food web analyses

*Corbicula*, the invasive Asian clam, dominated the sediment macroinvertebrate community in the Dan River. *Corbicula*  $\delta^{34}\text{S}$  signature was significantly ( $t = 2.55$ ,  $p < 0.02$ ) lower overall in samples downstream ( $5.98 \pm 0.14$ ) of the spill pipe compared to upstream samples ( $6.56 \pm 0.18$ ), and declined approximately linearly with distance from the spill site (Fig. 14,  $p < 0.0001$ ,  $R^2 = 0.59$ ). This pattern was more pronounced in *Corbicula* collected from the channel than those collected from the shore, but both habitats show a similar and significant linear trend, thus are pooled in the regression analysis shown in Fig. 14. The  $\delta^{34}\text{S}$  pattern observed in *Corbicula* is not in the direction that would be expected from coal ash sulfur assimilation (see Fig. 1). *Corbicula*  $\delta^{13}\text{C}$  also shifted slightly downstream of the coal ash spill from  $-25.78 \pm 0.23$  to  $-26.72 \pm 0.18$  (data not shown), indicating a shift in the organic carbon resource that was used by the clams ( $t = 3.22$ ,  $p = 0.003$ ). The carbon shift was maintained at all downstream sites. There was no change in *Corbicula*  $\delta^{15}\text{N}$ , indicating no change in trophic position.

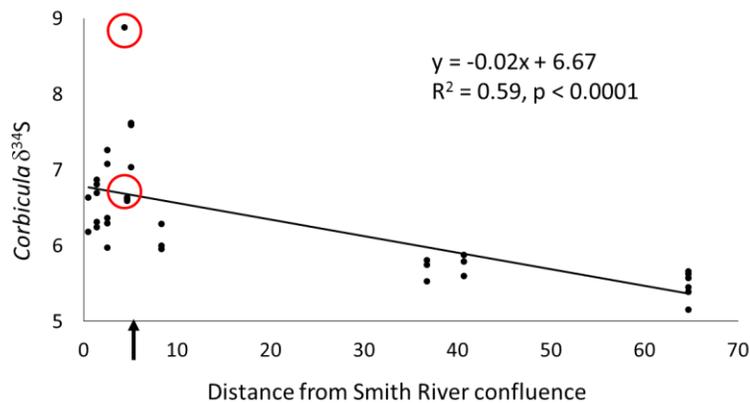


Fig. 14. Change in *Corbicula*  $\delta^{34}\text{S}$  in the Dan River with distance from the Smith River confluence. Arrow indicates the location of the spill pipe from the Duke Energy coal ash pond. Circled points ( $n = 4$ , including 2 hidden points) indicate samples taken laterally to the ash points that may have been influenced by leaching, and not included in the regression.

Predatory insects that could be collected in sufficient numbers at upstream and downstream sites included an aquatic beetle (Gyrinidae) and a dragonfly larva (Gomphidae). Gyrinidae displayed no isotopic shifts in  $\delta^{13}\text{C}$  or  $\delta^{34}\text{S}$ . Likely they were feeding on small invertebrates in the water column, which would be less likely to be influenced by coal ash components stored in the sediments. Gomphid dragonfly larvae live in riverine sediments, thus would have more direct exposure. However, similar to gyrinids, gomphids showed no difference in  $\delta^{34}\text{S}$  between upstream and downstream sites.

Riparian spiders, which were expected to reflect the degree to which coal ash contaminants were exported to avian and terrestrial consumers, also exhibited significant shifts in  $\delta^{34}\text{S}$  and  $\delta^{13}\text{C}$  (Fig. 15). Unlike *Corbicula*, spiders had significantly higher ( $t = 5.81, p < 0.0001$ )  $\delta^{34}\text{S}$  signature at downstream sites ( $6.58 \pm 0.55$ ) compared to upstream ( $5.00 \pm 0.2$ ), consistent with the expected pattern if they were assimilating coal ash derived sulfur (see Fig. 1). The spider  $\delta^{34}\text{S}$  signature remained fairly constant along the downstream transect (Fig. 15). Riparian spiders also had a significantly higher ( $t = 3.34, p < 0.007$ )  $\delta^{13}\text{C}$  signature at downstream sites ( $-26.11 \pm 0.30$ ) compared to upstream ( $-27.33 \pm 0.18$ ). However, regression analyses showed that although spider  $\delta^{13}\text{C}$  values were more enriched downstream of the spill,  $\delta^{13}\text{C}$  values declined significantly to return to upstream levels at the Milton site, approximately 60 river km downstream of the spill (Fig. 16). Spiders did not show and shift in  $\delta^{15}\text{N}$  between upstream and downstream sites, suggesting no change in their trophic position.

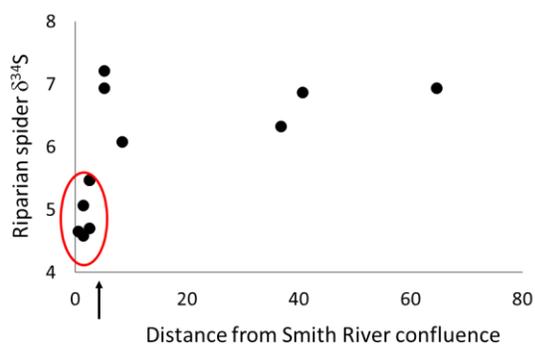


Fig. 15. Change in riparian spider  $\delta^{34}\text{S}$  in the Dan River with distance from the Smith River confluence. Arrow indicates the location of the spill pipe from the Duke Energy coal ash pond. Circled points ( $n = 6$ ) represent upstream samples. Note that all points downstream of the spill show  $^{34}\text{S}$  enrichment compared to upstream points, but there is no longitudinal trend with distance from the spill pipe.

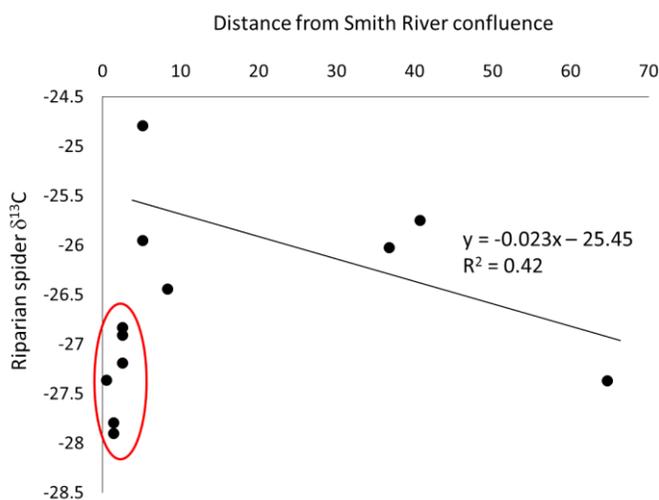


Fig. 16. Change in riparian spider  $\delta^{13}\text{C}$  in the Dan River with distance from the Smith River confluence. Arrow indicates the location of the spill pipe. Circled points ( $n = 6$ ) represent upstream samples. The line (which excludes upstream samples) shows that spider  $\delta^{13}\text{C}$  declines with distance from the spill to recover to upstream levels at Milton (65 km from Smith River confluence).

## 6. Discussion

Recent studies suggest that the published *HgcA* primers that we and others have used target a limited suite of Hg methylating organisms (Cynthia Gilmour, Smithsonian Institution, personal communication), thus interpretation of these results is a bit problematic and we are continuing to refine our approach in light of this new information. Results thus far indicate that Hg methylating genes increased in the river channel, consistent with the observed increase in Hg and higher concentration of organic matter. Even though Hg concentration is linked to organic matter, it was clear that some of the increase in Hg was clearly attributable to the coal ash spill. However, further analysis is needed to link the increase in Hg methylating genes to the coal-ash derived Hg.

The observed metal tolerance among coal ash isolates is consistent with previous studies (Klubek et al. 1992, Raja and Omine 2013, Stepanouskas et al. 2005). One caveat is that we did not directly measure metal ion concentrations in the laboratory experiment, so it is likely that some metal ions were bound to organics or other chemicals and exposure levels were lower than nominal concentrations. We are continuing to assay additional isolates. We have also extracted DNA from isolate cultures (Stewart and Via 1997) and will submit genomic DNA for sequencing in order to identify isolates (*e.g.* Bruce et al. 1992), if possible, by comparison to GenBank sequences for SSU rDNA.

Our studies of macroinvertebrate  $\delta^{34}\text{S}$  were designed to separate effects of longitudinal changes, such as increased % organic matter content, from effects that could be attributed directly to coal ash. Changes in *Corbicula*  $\delta^{13}\text{C}$  and  $\delta^{34}\text{S}$  occurred in association with the coal ash spill, but the observed  $\delta^{34}\text{S}$  pattern is not consistent with assimilation of coal ash-derived sulfur. Coal ash is enriched in  $^{34}\text{S}$  compared to natural sources (Fig. 1), and *Corbicula* were  $^{34}\text{S}$  depleted downstream of the spill compared to upstream. Accordingly, the shift in  $\delta^{34}\text{S}$  is more likely due to a shift in feeding mode, consistent with the concomitant shift in  $\delta^{13}\text{C}$ . *Corbicula* are known to function both as filter feeders and deposit feeders (Hakenkamp et al. 2001, Vaughn and Hakenkamp 2001), and shift between these modes of feeding as conditions change (Bullard and Hershey 2013). Accordingly, the *Corbicula* isotopic shifts observed may reflect a shift in the relative proportion of filter feeding versus deposit feeding behavior in response to the disturbance associated with the spill and/or altered conditions in the sediment.

Of the food web components that we studied, riparian spiders were the only component that showed a  $\delta^{34}\text{S}$  pattern consistent with incorporation of coal-ash derived sulfur. Riparian spiders feed on emerging aquatic insects and terrestrial insects in approximately equal proportions (Kelly et al. 2015) but have been observed to utilize up to 92% aquatic foods (Akamatsu et al. 2004). The  $\delta^{13}\text{C}$  shift observed in riparian spiders indicates a shift in some aspect of the spider diet; we have no basis for evaluating the nature of that shift. However, the pattern of declining spider  $\delta^{13}\text{C}$  with distance from the spill site to Milton in combination with the relatively constant spider  $\delta^{34}\text{S}$  along the same transect suggests that the observed  $\delta^{34}\text{S}$  shift observed in riparian spiders cannot be attributed simply to a dietary shift, and is more likely linked to utilization of coal ash derived sulfur. Any diet shift that occurred appeared to recover with distance, whereas use of coal-ash derived sulfur did not attenuate.

## 7. Summary

Hg was clearly elevated in sediments downstream of the coal ash spill, in a manner that was independent of longitudinal changes in organic matter content of the sediments, thus very likely derived from the coal ash spill itself. However, Hg stable isotope studies, which are pending, are needed to evaluate the coal ash source definitively. The patterns of total DNA, Hg methylating bacteria, and Fe reducing bacteria in the channel are consistent with a coal ash effect, but may also reflect the longitudinal increase in organic matter. The patterns of reduced total DNA abundance, potential mercury methylating bacteria, and iron reducing bacteria associated with nearshore sediments were unexpected, and would require further study to evaluate. They may reflect the variability associated with limited point sampling over a long reach rather than a consistent response to an environmental factor or perturbation. The appearance of metal tolerant bacteria isolated from coal ash was not surprising, but the apparent similar level of metal tolerance of soil bacteria not associated with coal ash for most metals suggests that ambient levels of the metals tested may be high enough to select for metal tolerance as a general rule. However, Cd was a clear exception. Further work on these isolates should provide more clarity.

With respect to entrainment of coal ash contaminant into the food web, our results were mixed. Although *Corbicula*  $\delta^{13}\text{C}$  and  $\delta^{34}\text{S}$  showed longitudinal changes, these patterns could not be attributed to assimilation of coal ash products, but rather indicated a shift in carbon source or carbon quality. However, the observed shift  $\delta^{34}\text{S}$  in riparian spiders was consistent with assimilation of coal ash sulfur.

## 8. Conclusions and Recommendations

Our results are still preliminary and additional analyses are pending. We did observe some patterns that indicate negative effects of the coal ash spill: elevated Hg in downstream samples that was above the baseline attributable to organic matter content, and incorporation of coal ash derived sulfur into riparian spiders that feed on emerging insects. To date we do not see a signal of microbial response that can be conclusively attributable to the coal ash spill, and further study will be needed to more fully evaluate such effects. Thus, although we have not seen an alarming coal ash effect on the microbial community to date, and impacts on invertebrate components of the food web were limited to spiders, further investigation is needed to evaluate Hg methylation over time as well as persistence of the coal ash Hg in river sediments.

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Appendix 1. Alphabetic index of abbreviations and symbols.

$\delta$  - ratio the concentration of heavy to light isotope of a given element in a sample relative to that of a standard, expressed in parts per thousand. Example  $\delta^{13}\text{C}$ : ratio of  $^{13}\text{C}$ : $^{12}\text{C}$  in a sample relative to the same ratio in the international standard for carbon (PeeDee limestone).

C – Carbon

Hg – Mercury

*HgcA* gene – microbial gene required for Hg methylation

N – Nitrogen

S – Sulfur

SRB – Sulfate reducing bacteria

## Appendix 2: List of project products to date

Corson, K. 2015. Mercury and other heavy metal analyses, gene proxies, and stable isotope tracers of coal ash contamination in the Dan River food web. Presentation to the Piedmont Bird Club, Greensboro, NC.

Cleary, J.A., A. Williams, S. Jones, D. Lewis, D. Stewart, N. Thomas, P.A. Rublee. 2016. Metal Tolerance of Bacteria Isolated from Coal Ash. NC Academy of Science Annual Meeting, April 1-2, 2016. Methodist Univ. Fayetteville, NC.

Hershey, A. E., P. A. Rublee, M. T-K. Tsui, A. Williams, and K Corson. 2015. Mercury and other heavy metal analyses, gene proxies, and stable isotope tracers of coal ash contamination in the Dan River food web. Poster presented at: Fostering advances in water resource protection and crisis communication, Lessons learned from recent disasters. May 27-29, 2015. WVU, Morgantown, WV.

Williams, A. S., P. A. Rublee, A. E. Hershey and M. T-K Tsui. 2015. Microbial response to the Dan River coal ash spill. Poster presented at: Fostering advances in water resource protection and crisis communication, Lessons learned from recent disasters. May 27-29, 2015. WVU, Morgantown, WV.

## Theses

Corson, K. Tracing coal ash derived contaminants derived from a coal ash spill into riparian spiders in the Dan River food web. MS Thesis, Biology Department, UNCG. In progress.

Williams, Ashley S. Microbial Response to the Dan River Coal Ash Spill. MS Thesis, Biology Department, UNCG. In progress.

# Legacy impacts of coal combustion residues on freshwater ecosystems in North Carolina

## Basic Information

<b>Title:</b>	Legacy impacts of coal combustion residues on freshwater ecosystems in North Carolina
<b>Project Number:</b>	2015NC192B
<b>Start Date:</b>	3/1/2015
<b>End Date:</b>	2/29/2016
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<b>Congressional District:</b>	NC-001
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<b>Descriptors:</b>	None
<b>Principal Investigators:</b>	Richard Di Giulio, Emily S Bernhardt, Jessica Brandt, Heileen Hsu-Kim, Avner Vengosh

## Publications

There are no publications.

**Project Title:**

Legacy impacts of coal combustion residues in freshwater ecosystems in North Carolina

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**WRI Project Number:** 15-03-W  
**Report Date:** March 31, 2016

## Project Abstract

Project Title: Legacy impacts of coal combustion residues in freshwater ecosystems in North Carolina

Coal combustion residuals (CCRs) released by permitted effluent streams partition to sediments and sediment pore waters where they accumulate to enriched levels in organisms at the base of the food chain. The objectives of this study were (1) to measure CCR uptake, trophic transfer, and toxicity in freshwater biota; (2) to identify CCR-specific signatures in receiving aquatic ecosystems; and (3) to measure Se in selected tissues of resident centrarchid fishes and determine differences in fish collected from CCR-impacted and reference sites as well as whether differences can be measured across sites belonging to different trophic statuses. Surface waters, sediment pore waters, three species of fish, and plankton were collected from six lakes in North Carolina; three lakes are CCR-impacted lakes due to their current or historical status as receiving waters for coal-fired power plant effluent streams and three lakes serve as reference lakes matched to each of the impacted-lakes on the basis of geographic proximity and trophic status. The research methods employed during this study include field collection of surface water, sediment pore water, and adult fish, contaminant analysis by ICP-MS and LA-ICP-MS, developmental toxicity assays, and fish feeding and breeding assays. While this study is ongoing, research results to date show that CCR contaminant signals are detectable in receiving freshwater reservoirs that both continue to and no longer receive effluent streams from associated coal-fired power plants. Contaminants including Se, As, Mn, Zn, Cu, and Sr are expected to persist in these systems for several years due to their continually elevated levels in surface waters, sediment pore waters, or fish tissues as measured in this study. Lake characteristics including surface area, depth, trophic status, and hydrological connectivity are important considerations because of their influence on contaminant retention. The results of this study suggest that ongoing monitoring of CCR receiving waters after effluent stream termination will be necessary to determine the duration of ecosystem impacts. Regular fish tissue monitoring of selenium will be especially useful for understanding persistence of selenium as a CCR contaminant in different aquatic ecosystems.

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## Acknowledgements

The authors of this report would like to thank the North Carolina Wildlife Resource Commission, especially Brian McRae, Michael Fisk, Jessica Baumann, Powell Wheeler, Kelsey Lincoln, Lawrence Dorsey, and Keith Hendrickson, for all of their assistance with the field sampling involved in this project. We would also like to thank Autumn Romanski, Danny Smith, and Debra Owen of the North Carolina Department of Environmental Quality (formerly Department of Environment and Natural Resources) for their input on project design.

## 1. Introduction:

In 2013, the most recent year for which complete data are currently available, the United States consumed 855,546 thousand tons of coal for electrical power production at electric utilities and independent power producer facilities (Electric Power Annual, 2013). In the same year, the operation of 300 coal-fired electric utilities collectively produced approximately 114.7 million tons of coal combustion residuals (CCRs) (Electric Power Annual, 2013 and 2013 Coal Combustion Product, 2013). In many cases, CCRs are released to aquatic environments via permitted waste streams to freshwaters where they pose a considerable ecological risk because they contain elevated concentrations of toxicologically relevant contaminants, including selenium, that have been shown to negatively impact the organisms found in these systems. In select case studies dating to the 1980s and 1990s, catastrophic consequences of CCR contamination in freshwater reservoirs were eventually traced to elevated selenium in aquatic food webs (Young et al., 2010 and Skorupa, 1998).

In recent years, proposed changes for CCR waste handling have been subject to legislative debate at the state and federal level. In December 2014, a new final rule was established by the Environmental Protection Agency regarding CCR disposal but stopped short of requiring solid waste management strategies in place of surface impoundments (US EPA Final Rule, 2015). Therefore, while some states or facilities may decide on an individual basis to convert their ash management systems, others will likely continue to discharge wastes to rivers and lakes.

Three separate cases of CCR contamination in the 1970s and 80s called attention to the ecological risks associated with aquatic discharges of coal-fired power plant (CFPP) wastes and spurred substantial research effort on the subject. In 1977, widespread reproductive failure of the fishery at Belews Lake in North Carolina occurred, resulting in the loss of all but three of the lake's 29 resident fish species. Sixty-five miles to the northeast, reductions of 38-75% of the adult fish population and up to 95% of the juvenile population were observed in Hyco Lake between the late 1970s and early 80s. And in 1978, Martin Reservoir in Texas witnessed an approximate 90% decline in planktivorous fish biomass after receiving 8 months of unauthorized coal ash pond discharges from two ponds at the nearby CFPP (Young et al., 2010 and Skorupa, 1998).

Each of these reservoirs was constructed or impounded in order to supply cooling water for its associated CFPP. The observed effects on fish populations came shortly after CFPP wastes were first introduced or substantially increased and a majority of species was affected at each site (Young et al., 2010 and Skorupa, 1998). Although CCRs are characterized by elevated concentrations of several contaminants (e.g. Mn, Cr, As, V, Li, Mo) (Ruhl et al., 2012), only that of selenium were notably elevated in both water columns and fish tissues. Subsequent to CFPP technological modifications or installations of dry fly ash handling systems, measurable effects on fish populations and community structures persisted at each site for as many as 10 years. That Se levels in sediment and fish tissues remained elevated relative to reference systems while water column concentrations declined highlighted that these lentic reservoirs were retaining Se for prolonged periods of time. Data from the Belews Lake case study were used for revising the U.S. EPA's aquatic life chronic dissolved Se criterion from 35 to 5  $\mu\text{g/L}$  in 1987, but relatively severe biotic responses at or below this level supported the argument that it was insufficiently low (Young et al., 2010, Skorupa, 1998, Lemly, 1997, and Young et al., 2010).

In the years since, considerable research on selenium contamination, biogeochemistry, and toxicity in the context of these cases as well as those involving CCR discharge to lotic systems, open-pit and mountain-top coal mining, oil refining, agricultural irrigation and drainage, phosphate and sulfide ore mining, uranium milling, and mercury remediation has progressed our understanding of the

associated ecological risks (Young et al., 2010, Skorupa, 1998, and Janz et al., 2014). However, important gaps in understanding remain and continue to be relevant as natural resource extraction activities expand and new causes for concern (e.g. nanotechnology and livestock diet supplementation) emerge (Young et al., 2010, Janz et al., 2014, and Janz, 2011).

The overarching aim of this project was to address legacy-specific consequences of CCR contaminants in lentic waters that receive, or have historically received, coal-fired energy facility effluents. The work was approached via three main objectives each of which served to specifically further the understanding of CCR-related contamination issues in aquatic systems:

1. To examine the extent to which CCR-specific chemical signatures are apparent in receiving aquatic ecosystems; and
2. To measure concentrations of CCRs in selected tissues of resident centrarchid fishes and determine differences in fish collected from CCR-impacted and reference sites as well as whether differences can be measured across sites belonging to different trophic statuses; and
3. To measure CCR uptake and trophic transfer in freshwater biota (e.g. periphyton, fish species) and toxicity in fish species in laboratory-based studies.

The work emphasizes selenium among the many major and trace elements under analysis because of the great potential to lend further understanding to an active and critically important sub-field of environmental research.

## **2. Methods:**

**2.1 Field site selection:** As of July 2012, Duke Energy owns and operates all coal-fired energy facilities in the state of North Carolina (Duke Energy/Progress Energy, 2012). The associated wastes generated by these facilities are stored in coal ash ponds that eventually discharge to public receiving waters via permitted waste streams regulated by the U.S. Environmental Protection Agency (U.S. EPA) under the National Pollutant Discharge Elimination System (NPDES). North Carolina's Department of Environment and Natural Resources (N.C. DENR), and specifically the Division of Water Resources (DWR), administers the permitting and compliance program for NC-specific NPDES permits (NCDENR, NPDES Wastewater). Of the fourteen NPDES-associated receiving waters in N.C., four are lakes (i.e. lentic systems) – Hyco Lake, Mayo Lake, Mountain Island Lake, and Lake Sutton. The original design of the field study included each of these lakes as impacted study sites. Lakes were paired with reference lakes on the basis of three primary criteria: (1) regional proximity to respective study site, (2) similar lake productivity classification as determined by the North Carolina Trophic State Index (NCTSI) (personal communication with Debra Owen, NC Lakes Monitoring Program Coordinator, Division of Water Resources, N.C. DENR), and (3) the condition that the lake had not been historically impacted by coal-fired energy facility waste streams (NCDENR, Ambient Lakes Monitoring). NCTSI scores are calculated from chemical and physical parameters with the following equation:

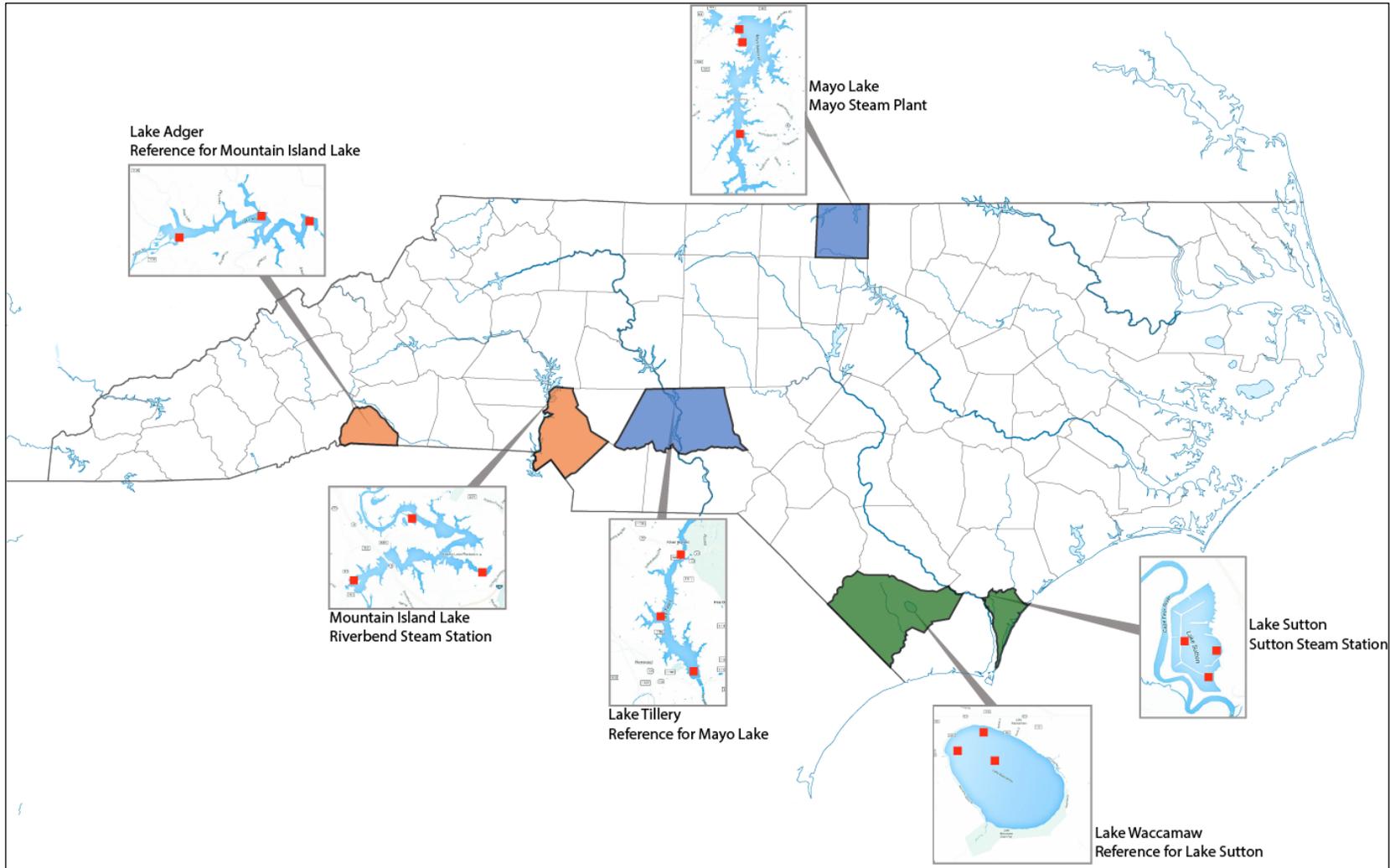
$$\text{NCTSI} = \text{TONScore} + \text{TPScore} + \text{SDScore} + \text{CHLScore}$$

where TON = total organic nitrogen (mg/L), TP = total phosphorus (mg/L), SD = secchi depth (inches), and CHL = chlorophyll a ( $\mu\text{g/L}$ ). Trophic status is determined according to the following scale: oligotrophic, <-2.0; mesotrophic, -2.0 – 0.0; eutrophic, 0.0 – 5.0 (Dwyer and Vengosh, 2008).

Lake Tillery, Lookout Shoals Lake, Lake Adger, and Lake Waccamaw were included as respective reference sites for the impacted study sites listed above. Due to alterations in the N.C. Wildlife Resources Commission’s (N.C. WRC) annual sampling plan, Hyco Lake was not included in the 2015 sampling schedule and Lake Tillery replaced Lookout Shoals Lake as the reference site for Mayo Lake. Incorporation of these changes to the study design maintained the inclusion of sites representing the three primary trophic classifications. Sampling details and dates are summarized in Table 1.

Table 1: Field sites, justification, and sampling dates

<b>Lake</b>	<b>Justification</b>	<b>Trophic classification</b>	<b>Sampling dates</b>
Sutton Lake	Sutton Plant cooling reservoir	eutrophic	3/17 & 3/31/2015
Lake Waccamaw	L. Sutton reference site	eutrophic	4/14/2015
Mayo Lake	Mayo Plant receiving water	mesotrophic	4/22/2015
Lake Tillery	Mayo L. reference site	mesotrophic	5/6/2015
Mountain Island Lake	Riverbend Steam Station cooling reservoir	oligotrophic	5/13/2015
Lake Adger	Mountain Island Lake reference site	oligotrophic	4/28/2015



**Figure 1:** Map of North Carolina showing location of lakes included in this study within their respective counties. Green counties contain the paired eutrophic lakes (Lake Sutton and Lake Waccamaw), blue counties contain the paired mesotrophic lakes (Mayo Lake and Lake Tillery), and orange counties contain the paired oligotrophic lakes (Mountain Island Lake and Lake Adger). Individual lakes are blown up to show approximate location of sites within the lake where water and sediment samples were collected (red squares).

2.2 Surface water, sediment, and pore water sampling: Surface water samples were collected from three selected locations within each study and reference site by surface grab sampling. Sub-samples were processed according to associated analytical protocol, transported to Duke University on ice, and stored at 4°C. Water samples collected for trace elements and cation analysis were filtered in the field (syringe filters, 0.45 µm). Sediment samples were collected from the same three locations per lake as the surface water samples with a Wildco box corer (≤ depth of 25 cm). Sediment samples were stored in 2 gallon acid-washed plastic buckets for transportation to Duke University where they were stored at 4° C. Within 48 hours of collection, sediment samples were homogenized, then aliquoted and centrifuged in 50 mL metal-free sterile polypropylene centrifuge tubes at 3000 x g for 25 minutes. Supernatant pore waters were subsequently filtered by vacuum filtration (0.45 µm, polyethersulfone (PES) membrane) and stored at 4°C prior to ICP-MS analysis or frozen for use in developmental toxicity assays.

2.3 Fish collection and dissection: Fish collections were conducted in accordance with Duke University IACUC protocol #A184-13-07 and NC Collection Permit #15-SFC00163. Fish were collected from the six lakes included in this study by electroshocking in collaboration with N.C. WRC district biologists over the course of the spring 2015 sampling period. Targeted species of the Centrarchidae family included largemouth bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*), redear sunfish (*Lepomis microlophus*), and redbreast sunfish (*Lepomis auritus*). Fish were retrieved by net, maintained in an oxygenated live well during sorting, euthanized tagged, and transported on ice to Duke University where they were stored at 4° C overnight. Within 24 hours of collection, fish were dissected for otoliths, vertebral column, liver, skinless fillet (i.e. muscle), and gonadal tissues. Otoliths were stored in 1.5 mL eppendorf tubes while all other tissue types were weighed, bagged, and frozen for future processing prior to analysis. Table 2 provides the number of fish collected from each lake, organized by species and sex.

Table 2: Collected fish by species and sex:

Lake	Species	Total #	#Female	#Male
Sutton Lake	Largemouth Bass	32	13	19
	Bluegill	7	2	5
	Redear Sunfish	19	12	7
Lake Waccamaw	Largemouth Bass	20	7	13
	Bluegill	16	11	5
	Redear Sunfish	21	17	4
Mayo Lake	Largemouth Bass	19	6	13
	Bluegill	16	1	15
	Redear sunfish	21	10	11
Lake Tillery	Largemouth Bass	22	6	16
	Bluegill	20	3	17
	Redear sunfish	20	9	11
Lake Adger	Largemouth Bass	22	10	12
	Bluegill	24	9	15
	Redbreast sunfish	7	2	5
Mountain Island Lake	Largemouth Bass	22	14	8
	Bluegill	8	2	6
	Redear sunfish	12	2	10
	Redbreast sunfish	11	3	8
Totals		339	139	200

2.4 Chemical analysis of surface and pore waters: Dissolved trace elements were measured by inductively coupled plasma mass spectrometry (ICP-MS, VG PlasmaQuad-3 – Thermo Fisher Scientific Inc.), major elements by direct current plasma optical emission spectrometry (DCP- OES, ARL Fisons SpectraSpan 7 – Thermo Fisher Scientific Inc.), and anions by ion chromatography (IC) in the Vengosh Laboratory at Duke University. Nitrate, orthophosphate, and TOC-TN analyses of surface and pore water samples were conducted at Duke University’s River Center (Lachat QuikChem 8500 autoanalyzer, Shimadzu TOC-VCPH Analyzer with TNM-1 module and ASI-V autosampler).

2.5 Fish tissues: Fish liver, muscle, and ovary + egg samples were microwave digested (CEM Discover SP-D closed vessel microwave digester) in omnitrace nitric acid (HNO<sub>3</sub>, EMD Millipore, CAS 7697-37-2) at a ratio of 1g tissue:10 mL acid (EMD Millipore, CAS 7697-37-2). 300 mL of each digested samples were diluted with 9.7 mL of a 2% HNO<sub>3</sub>/0.5 % HCL mixture in 15 mL metal free centrifuge tubes. Trace element concentrations were measured using ICP- MS (Agilent 770X ICP-MS equipped with an Octopole Reaction System, Hsu-Kim Lab). Method blanks and standard reference material (SRM) (NRC DORM-4, fish protein for trace metals) were processed and analyzed alongside tissue samples (n=33).

2.6 Isotope analyses: Strontium isotopes in surface and sediment pore waters were analyzed as previously described in Ruhl et al. (2014) in the Vengosh Lab at Duke University. Strontium isotopes in surface and sediment pore waters were be pre-concentrated by evaporation and re-digested in 3.5N HNO<sub>3</sub> prior to strontium separation with an Eichrom Sr-specific ion-exchange resin. Samples were loaded to the Triton TIMS at Duke University on rhenium filaments with

tantalum oxide activator solution and heated to obtain an  $^{88}\text{Sr}$  beam intensity of  $\sim 3$  V. NIST SRMs 987 was run alongside field collected samples (Ruhl et al., 2014).

2.7 Otolith analyses: Whole fish otoliths were dissected, rinsed in deionized water, and stored in 1.5 mL eppendorf tubes. Samples were shipped to Stantec Consulting, Ltd. in Winnipeg, MB, Canada. Prior to analysis, otoliths were embedded in epoxy resin and cut along the dorso-ventral cross section from the otolith nucleus in order to expose annuli. Samples were mounted, ground, polished, and washed in an ultra-sonic cleaner and analyzed by a Thermo Finnigan Element 2 ICP-MS coupled to a Merchantek LUV 213 Nd:YAG laser according to a method previously described (Friedrich et al., 2011, Friedrich et al., 2008, and Friedrich et al., 2010). Samples will also be analyzed for determination of fish age.

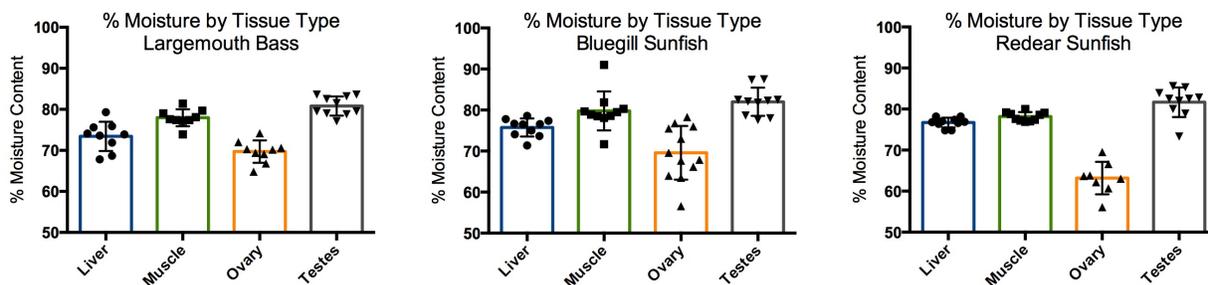
2.8 Developmental toxicity assays: Within one hour of fertilization, zebrafish embryos were plated in 25 mL glass petri dishes at a density of 10 embryos / 10mL of exposure material. Each experiment evaluated developmental progress at 24 hours and 120 hours in a method adapted from Truong et al. (2011). At 24 hours post fertilization (hpf), embryos were evaluated for viability, developmental progression (i.e. organogenesis), and spontaneous movements. At 120 hpf, larvae are evaluated for the full suite of larval morphology endpoints as well as motility and tactile responses. Each study replicate compares embryo development in pore waters from study and respective reference lakes (100% concentrated) as well as in 30% concentrated Danieau solution as a negative control (Nasevicius and Ekker, 2000). Four petri dishes per treatment group comprise a single replicate. Statistically, observed effects are averaged within the treatment group providing an n=1 per replicate.

2.9 Plankton collection and trophic/maternal transfer study: The biofilm collection and feeding study originally proposed as part of this project has been replaced with a plankton collection and feeding study (see description below). Insufficient biofilm accumulation, sedimentation of biofilm collection rig, and missing rig at Mayo Lake in the summer 2015 field plan were the primary factors contributing to this decision.

Plankton for use in a trophic and maternal transfer study will be collected from Lakes Sutton and Waccamaw in Summer 2016. Collected samples will be freeze-dried and prepared into a dry food. Sample subsets will be digested and analyzed by ICP-MS for selenium concentration in the Vengosh Laboratory at Duke University. After adequate acclimation, 10 female adult fathead minnows will be placed in 10 gallon tanks, where they will remain for the duration of the feeding study. Fish will be fed twice daily with the prepared flake food at  $\sim 5\%$  bw/day ration for at least 30 days to allow for Se concentrations in tissues to reach steady state (Janz et al., 2014 and Phibbs et al., 2011). At the conclusion of the exposure period, female fish will be combined with male fish for breeding. Resulting embryos will be collected for bulk selenium analysis. A subset of embryos will also be collected for developmental effects analysis. Adult fish will be humanely euthanized with an overdose of tricaine mesylate (MS-222, Sigma-Aldrich CAS 886-86-2) and dissected for selected tissues. Tissues will be frozen until time of analysis. Concentrations in fish tissues (liver, muscle, and gonad) will be assessed by ICP-MS according to the same method described above (methods section, research aim 1) and compared to those measured in field collected plankton. This method serves to study the transfer of CCR contaminants from a field-collected exposure source to a lab-reared model fish species that is

also native to NC lakes, thereby representing environmentally relevant exposures under controlled laboratory conditions where confounding variables (e.g. pH, temperature) can be controlled (Young et al., 2010).

**2.10 Analysis of fish tissue moisture content:** Species-specific moisture content was determined for each of the tissues and fish included in this study (Figure 2). This allowed for the determination of accurate conversion factors (Table 3) of contaminant concentrations in tissues from wet weight to dry weight following ICP-MS analysis.



**Figure 2:** Percent moisture content by tissue type for the three target fish species collected for this study. From left to right, largemouth bass (*Micropterus salmoides*), bluegill sunfish (*Lepomis macrochirus*), and redear sunfish (*Lepomis microlophus*).

**Table 3:** Wet weight to dry weight conversion factors applied to fish tissue trace element concentrations following ICP-MS analysis

	Liver	Muscle	Ovary	Testes
Largemouth Bass	0.27	0.22	0.30	0.19
Bluegill Sunfish	0.24	0.20	0.30	0.18
Redear Sunfish	0.23	0.22	0.37	0.18

### 3. Results:

**3.1 Ongoing work:** Due to the scope of this project, the following sample analyses are currently ongoing with data expected to be available in the coming months.

- A. Bluegill sunfish (*Lepomis macrochirus*) and redear sunfish (*Lepomis microlophus*) tissues (liver, muscle, and gonad) are currently being analyzed by ICP-MS in the Vengosh Lab at Duke University with data expected in early April. These data will be added to the dataset of largemouth bass fish tissues (included in this report). With several species worth of data, species to species comparisons within and between lakes will be possible. It will also be possible to compare how fish of each species distribute contaminants among their tissues and whether differences in fish from different lakes are associated with species or lake trophic status.
- B. A subset of 142 fish otoliths representing each fish species and each lake included in the study are currently undergoing laser ablation ICP-MS (LA-ICP-MS) at Stantec Consulting in Winnipeg, Canada. Otoliths are being analyzed for Se, As, Mn, Cd, Zn, and the isotope ratio  $^{87}\text{Sr}/^{86}\text{Sr}$  as well as age analysis. Data are expected in early May and will provide information about exposure to those contaminants over the time course corresponding to otolith layers.  $^{87}\text{Sr}/^{86}\text{Sr}$  isotope ratios in fish otoliths will be compared to

those measured in the lakes from which the fish were collected to determine whether Sr isotope signals correspond between abiotic and biotic field samples as tracers of CCR input.

- C. Fish toxicity analyses will be conducted in summer 2016 following plankton collection at Sutton Lake and Lake Waccamaw. A single pair of study and reference lakes was selected to study uptake of Se from plankton as a food source, maternal transfer of Se from adult to F1 generation fathead minnows, and developmental toxicity effects in F1 generation fish. Analysis of trace element concentrations in plankton will complement those of surface waters, pore waters, and fish tissues to provide a more complete understanding of element distribution among abiotic and biotic compartments in the lakes included in this study.

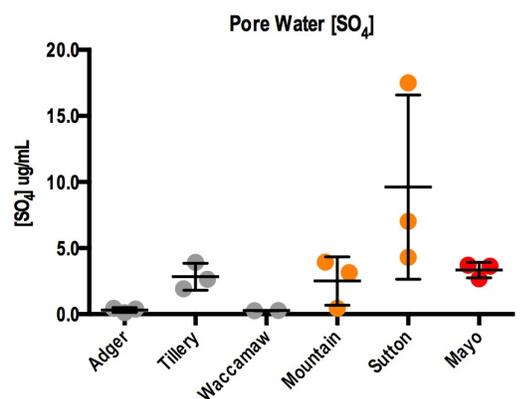
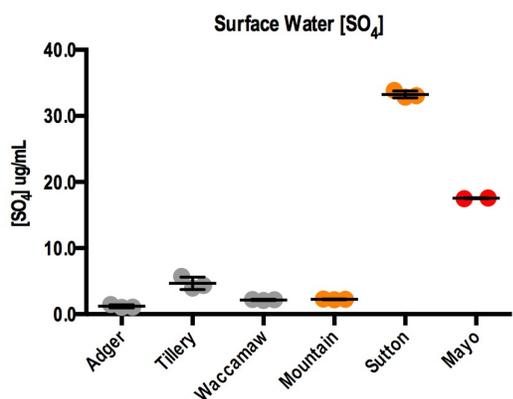
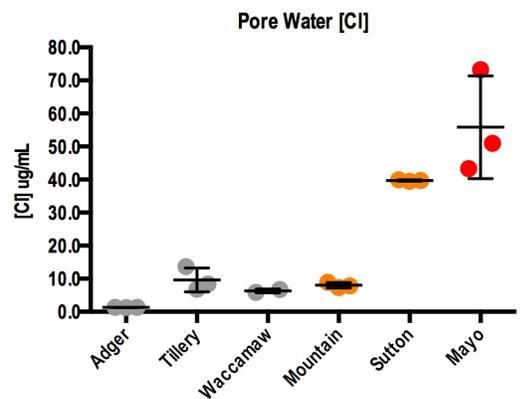
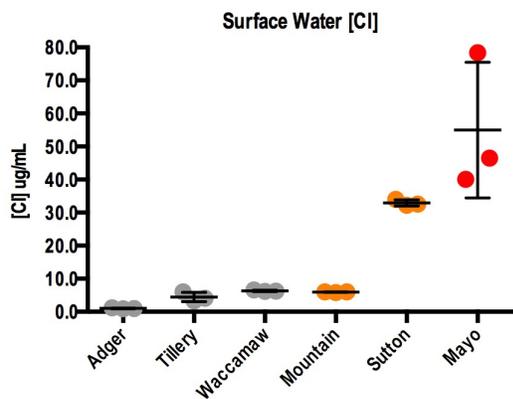
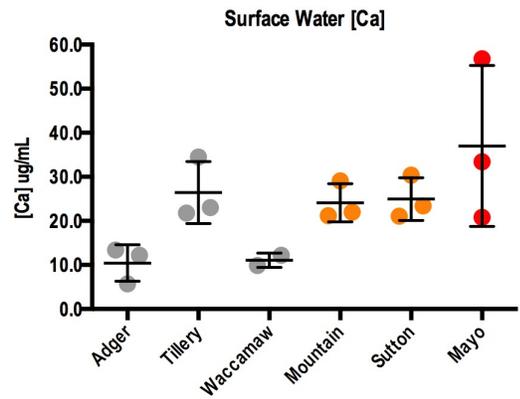
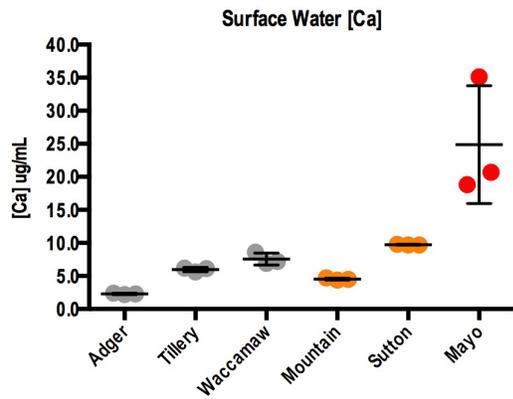
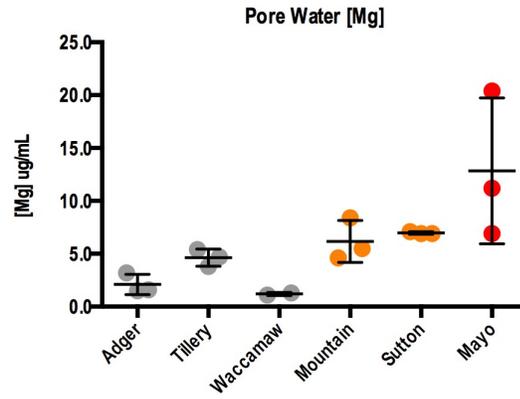
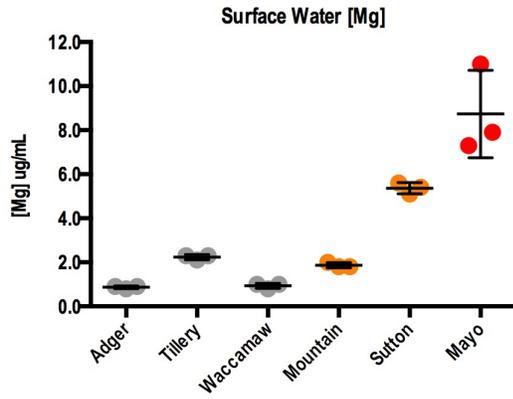
### 3.2 Water Chemistry

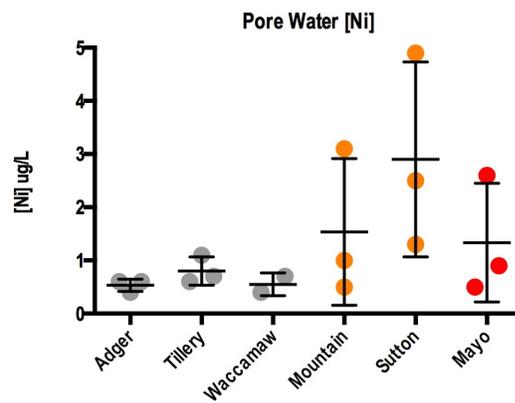
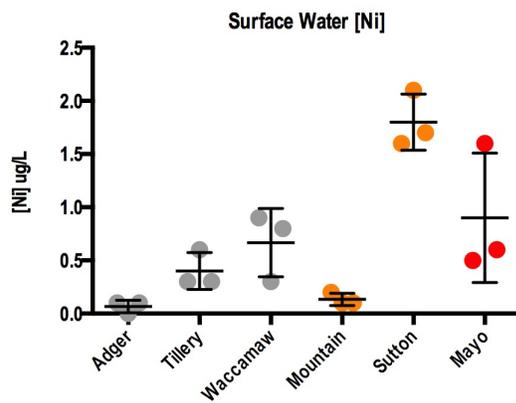
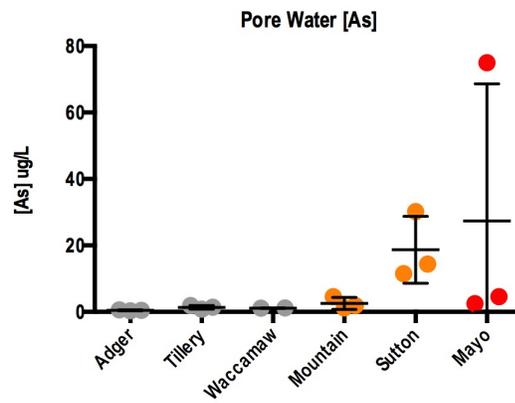
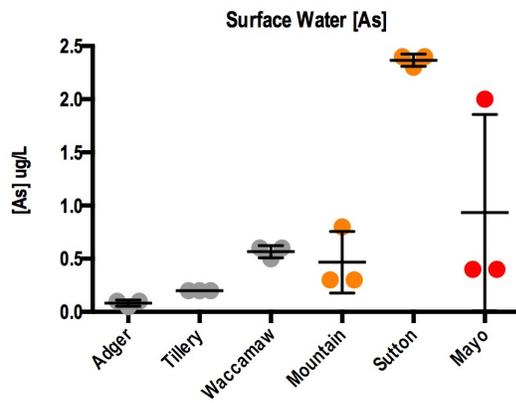
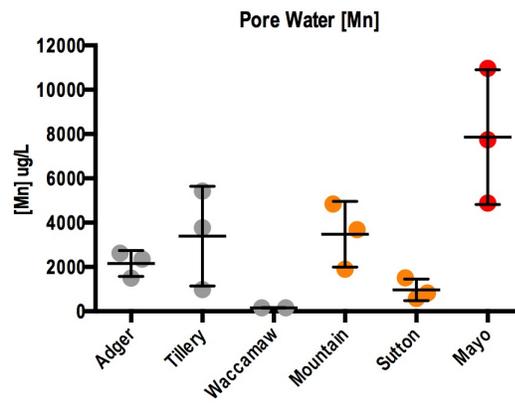
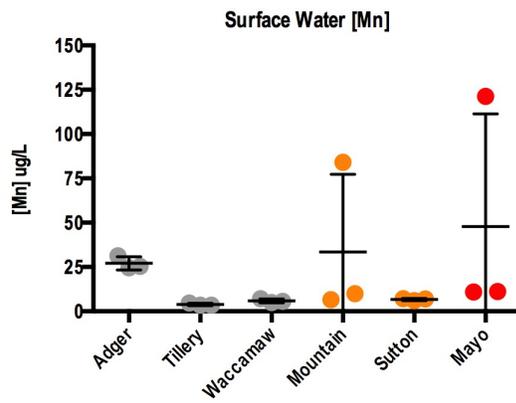
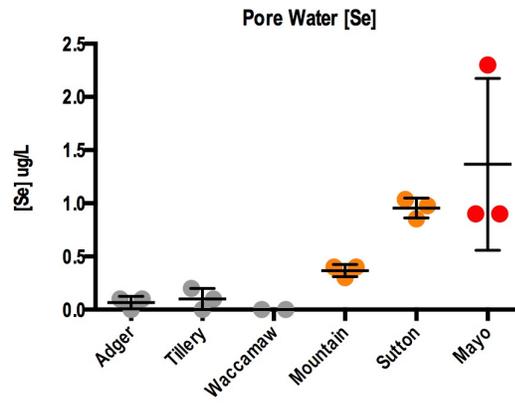
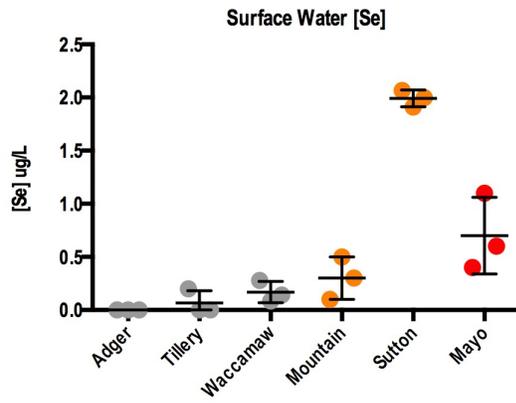
3.2.1 General water parameters: Table 4 provides averaged values of general water parameters for each of the studied lakes at both the water surface and just above the sediment surface.

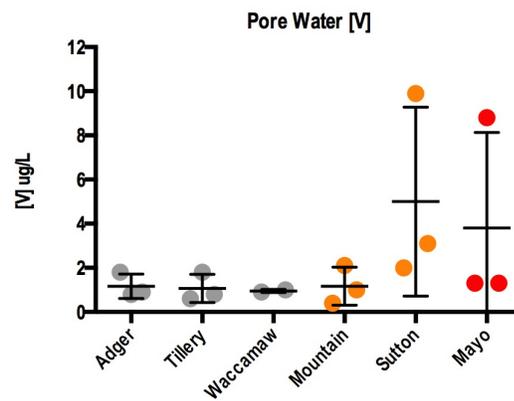
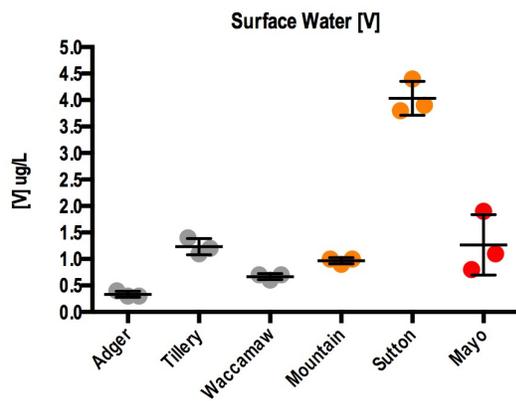
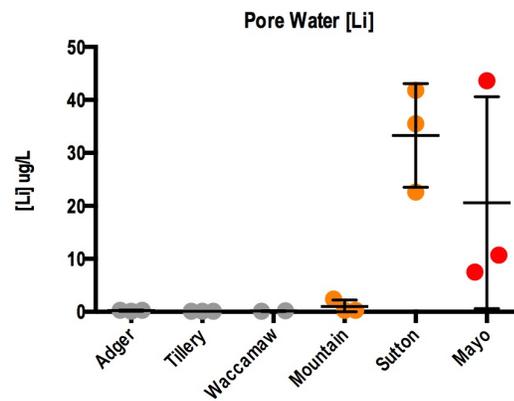
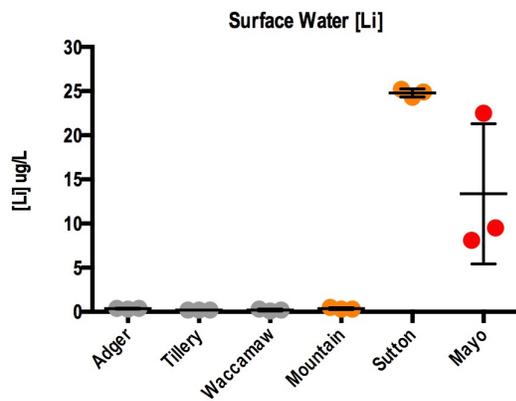
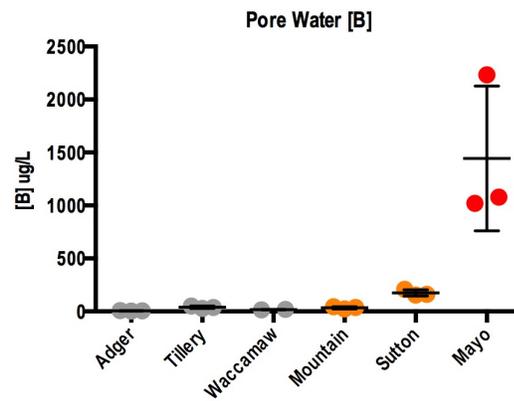
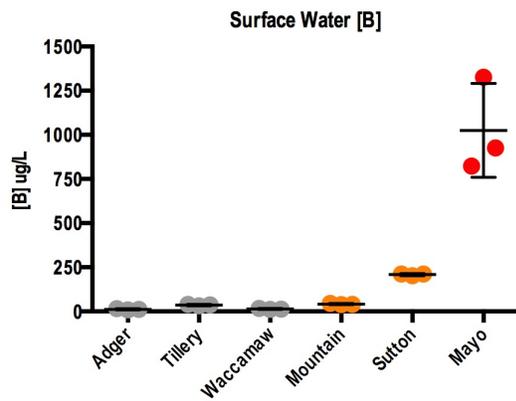
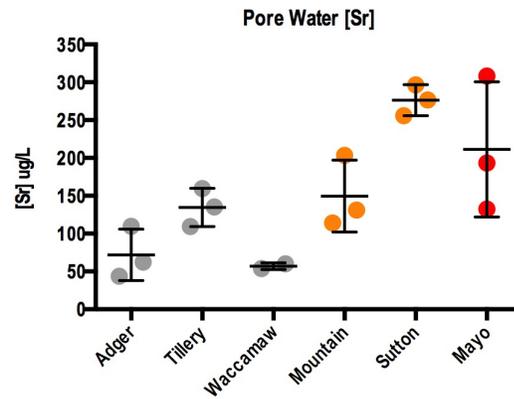
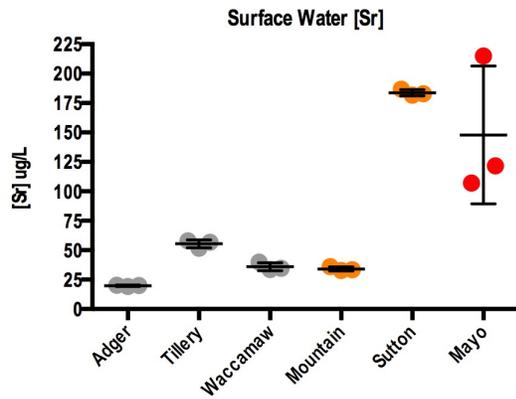
Table 4: Mean water quality measurements

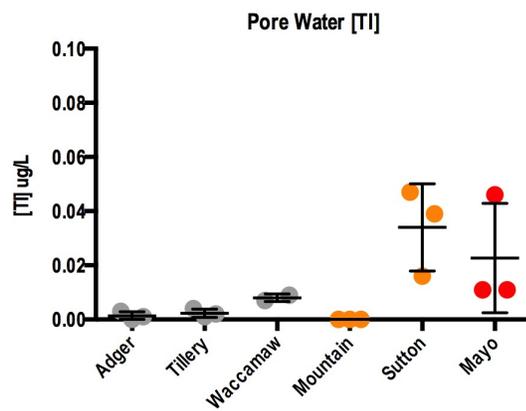
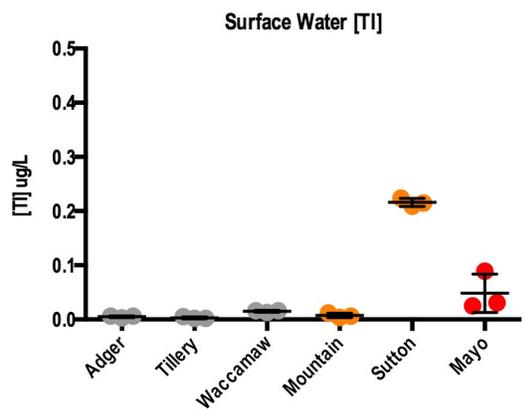
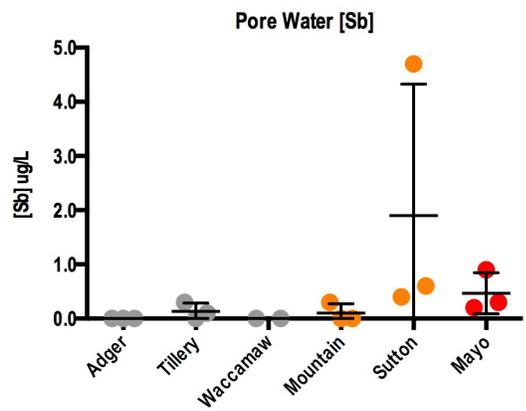
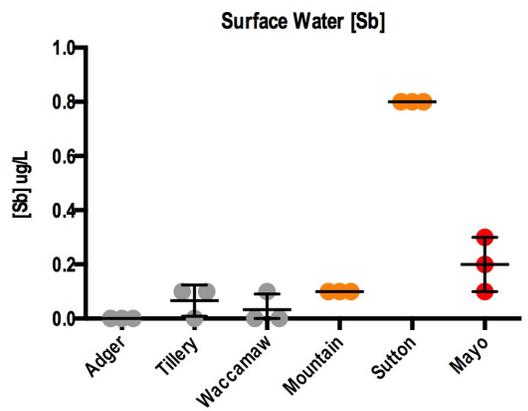
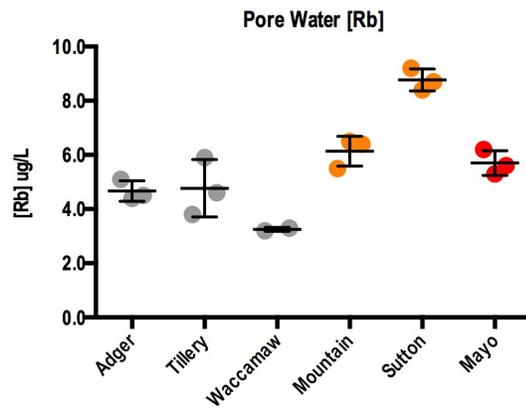
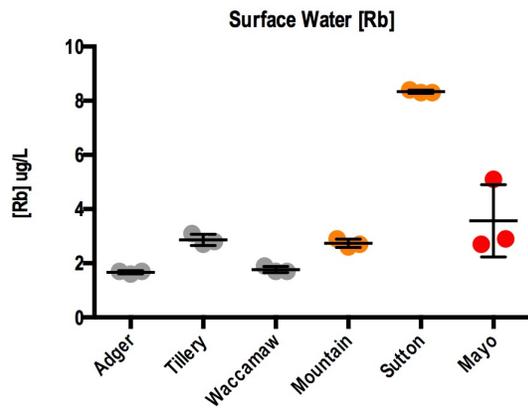
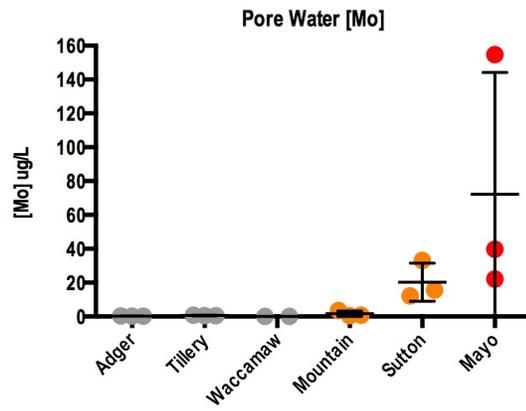
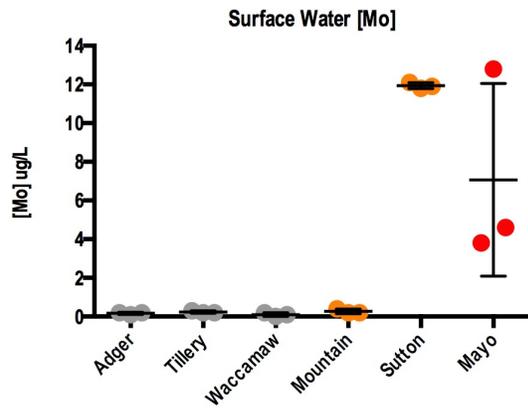
Lake		pH	Conductivity ( $\times$ s/cm)	DO (mg/L & %)		Temp. ( $^{\circ}$ C)
Sutton	Surface	7.45	210.97	11.91	90.63	21.5
	Bottom	7.45	208.26	8.14	91.13	20.97
Waccamaw	Surface	7.04	51.76	8.02	90.23	21.1
	Bottom	7.03	51.76	7.95	89.3	21.03
Mayo	Surface	7.51	0.287	8.97	98.5	19.8
	Bottom	7.39	0.264	9.17	96.4	17.9
Tillery	Surface	7.76	0.093	10.6	120.3	21.76
	Bottom	7.51	0.094	9.96	112.5	21.33
Mountain Island	Surface	7.19	0.068	8.58	102.6	24.28
	Bottom	7.12	0.068	8.58	102.3	24.37
Adger	Surface	7.03	0.034	9.90	101.0	16.2
	Bottom	6.84	0.034	9.38	92.77	14.9

3.2.2 Surface and pore water trace element concentrations: A subset of the major elements, anions, and trace elements analyzed by DCP-OES, IC, and ICP-MS for this study are shown in Figure 3. Of those analyzed, Mg, Ca, Cl, SO<sub>4</sub>, Rb, V, B, Se, Mn, Ni, Sb, Tl, Li, As, Sr, and Mo are elevated in study lake surface and pore waters relative to reference lakes. The concentrations of several elements (e.g. Li, Ni, SO<sub>4</sub>, Rb, Tl, Sb) are higher in the surface and pore waters of lakes that used to (but no longer) receive CCR inputs from the associated coal facility than in the surface and pore waters of Mayo Lake, a lake that continues to receive CCR inputs from the Mayo Steam Station effluent stream. These results indicate that there is a legacy effect of many CCR contaminants in which lake characteristics (e.g. surface area, depth, and hydrological connectivity to a lotic water system) influence the retention of these contaminants and their recycling to the water column.



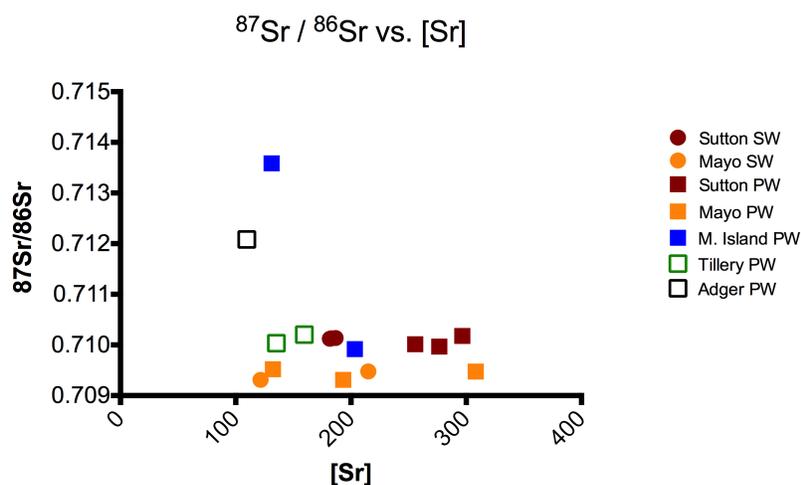






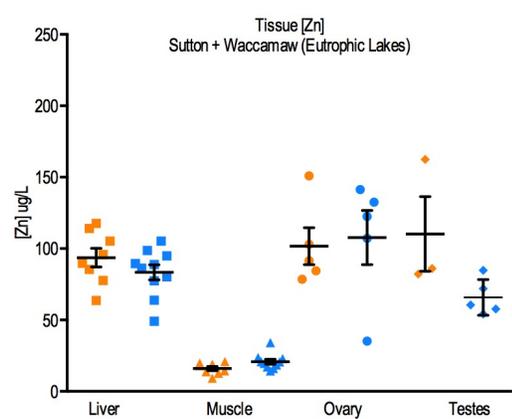
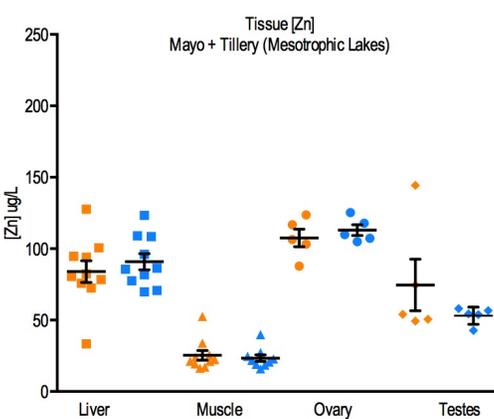
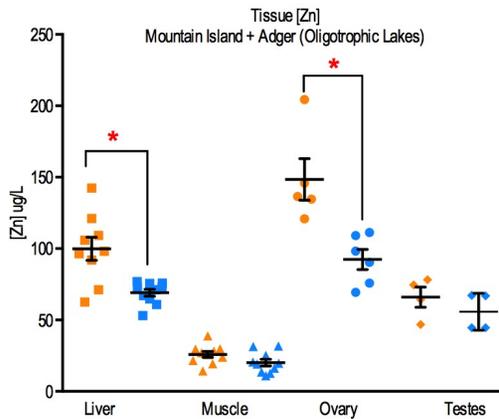
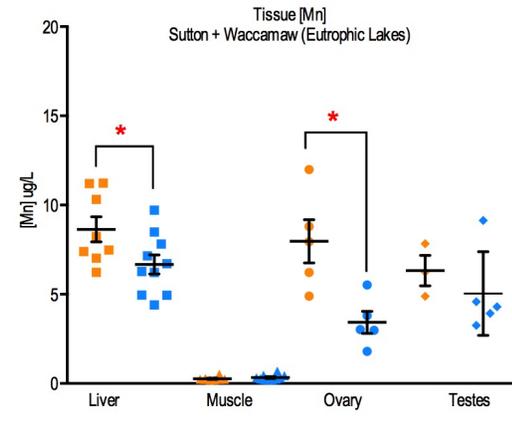
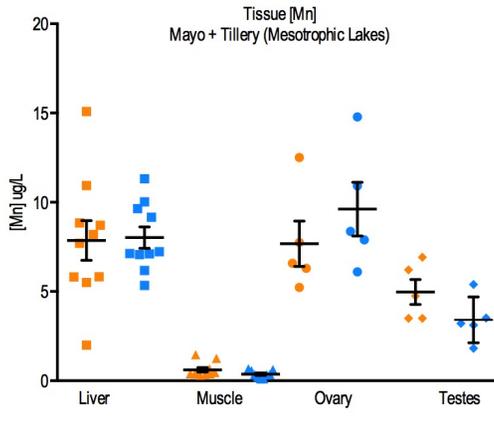
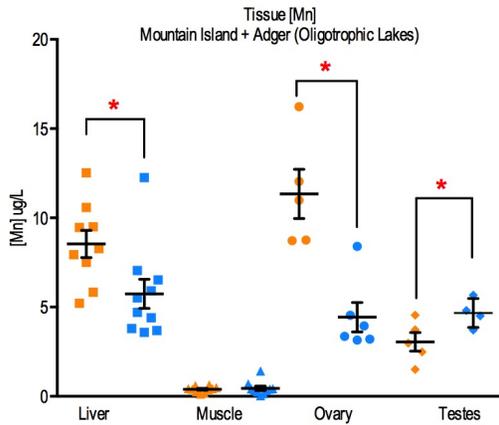
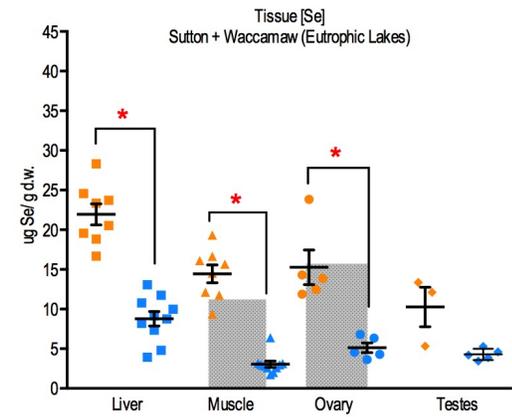
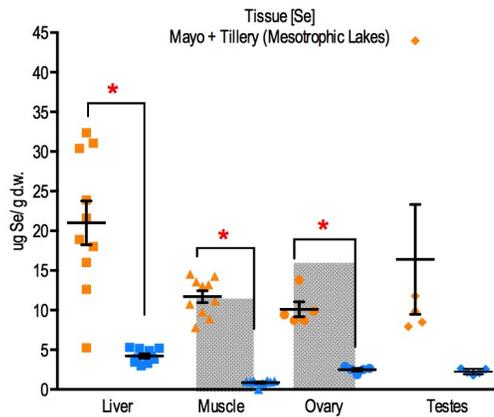
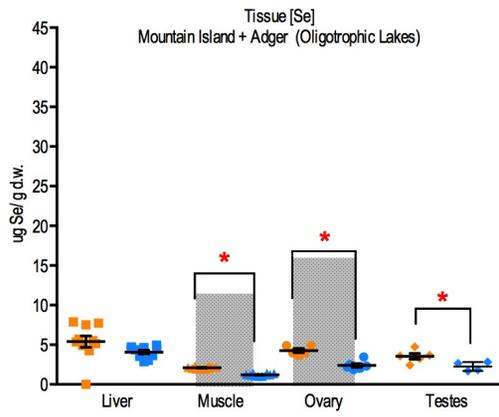
**Figure 3:** Trace element concentrations in surface and sediment pore waters from each study and reference lake as measured by ICP-MS. Samples taken from different locations in each lake are individually represented by the circles shown in each graph with mean and standard deviation bars overlaid. Grey circles represent samples taken from reference lakes. Study lakes are divided into two categories; samples taken from lakes with legacy, but not active CCR inputs from the associated coal facility, are represented by orange circles and samples taken from lakes with active CCR input are represented by red circles.

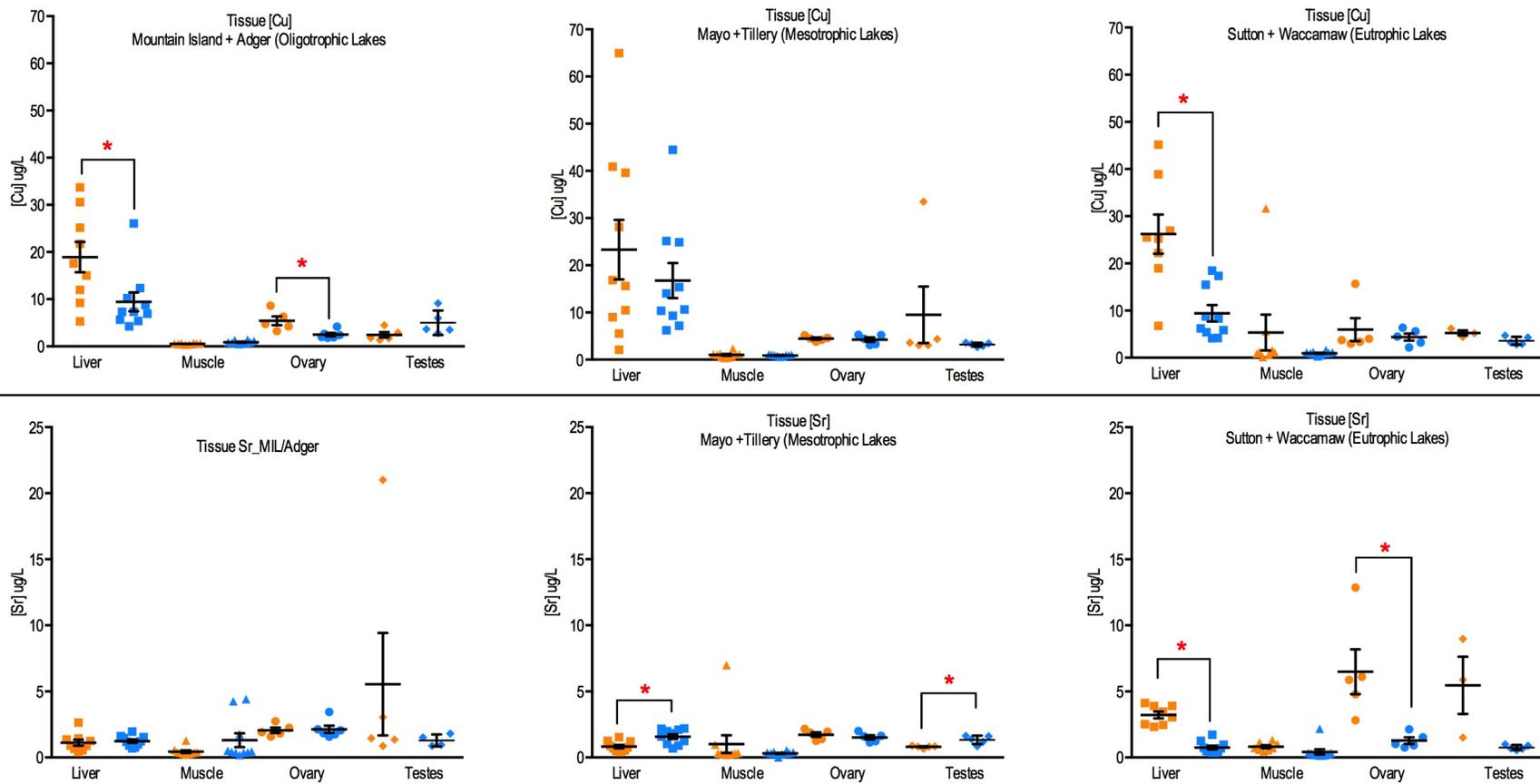
**3.2.3 Strontium isotope ratios in lake surface and pore waters:** Strontium isotope ratios and concentrations are shown in Figure 4. Samples of the same shape and color are taken from the same lake and same position in the water column (i.e. Sutton Lake surface water). The surface water Sr isotope ratios in CCR impacted lakes have a narrow range of 0.7093 to 0.7102. Pore water Sr isotope ratios in CCR impacted lakes have a wider range from 0.7093 to 0.7136 that overlaps with Sr isotope ratios in pore waters from reference lakes (0.7100 to 0.7121). These data are consistent with data from a previous study (Ruhl et al., 2014) and will be compared with strontium isotopes in fish otoliths.



**Figure 4:** Strontium isotope ratios ( $^{87}\text{Sr}/^{86}\text{Sr}$ ) are shown as a function of strontium concentration. Each symbol represents an individual sample measurement. Circles denote surface water samples and squares denote pore water samples. Solid symbols represent samples taken from impacted study sites and outlined symbols represent samples taken from reference lakes. This data set remains incomplete.

**3.3 Trace element concentrations in fish tissues:** Figure 5 shows tissue specific profiles of select CCR contaminants analyzed in largemouth bass tissues by ICP-MS. Selenium levels are consistently and significantly elevated in the tissues of fish from impacted lakes relative to those from paired reference lakes. Selenium levels in the muscle and ovary tissues of fish collected from Mayo Lake and Sutton Lake exceed the US EPA’s proposed chronic aquatic life criteria of 11.3 and 15.8  $\mu\text{g Se/g d.w.}$ , respectively. Concentrations of Mn, Zn, Cu, and Sr are also elevated in study lake fish tissues relative to reference lake fish tissues but the patterns are less consistent between lake pairs and tissue types. Bluegill and redear fish tissues are currently undergoing analysis. With that data in hand, clearer data patterns may emerge and it will be possible to make species-specific comparisons of contaminant accumulation and tissue distribution.





**Figure 5:** Concentrations of select CCR contaminants in largemouth bass by tissue type. Each graph compares fish collected from an impacted study lake (orange values) with those from its paired reference lake (blue values). Grey bars in the selenium graphs represent the proposed chronic aquatic life criteria for selenium in muscle (11.3  $\mu\text{g Se/g d.w.}$ ) and ovary tissues (15.8  $\mu\text{g Se/g d.w.}$ ). Red stars denote significant differences between corresponding tissue samples in fish from impacted study lakes and matched reference lakes ( $p$ -value  $< 0.05$ ).

#### 4. Discussion:

4.1 CCR chemical signatures in receiving aquatic systems: This study reports elevated concentrations of known CCR constituents in the surface and pore waters of lakes that serve as receiving waters for coal-fired power plant effluents under the conditions of NPDES permits in the state of North Carolina. At the time of sampling (March-May 2015), two of the impacted study lakes (Sutton Lake and Mountain Island Lake) could be classified as legacy CCR lakes because the effluent source of CCRs to the lakes had been terminated. Mayo Lake, in contrast, continues to receive effluent discharges from the Mayo Steam Station and is classified as an active lake. Selenium levels in these surface and pore waters are of particular interest to this study due to selenium's status as an essential nutrient in most living organisms with a narrow margin between essentiality and toxicity in oviparous vertebrates such as fish, reptiles, and birds (Janz, 2011). For this reason, the U.S. EPA is currently revising the Criterion Continuous Concentration (CCC) for selenium in freshwaters from 5 µg/L to 1.2 µg/L (USEPA, 2015, *Draft Aquatic Life*). In surface waters, selenium levels were only found to exceed the proposed threshold in all sampled locations of Sutton Lake (mean [Se] = 2.0 µg/L). Samples taken in Mayo Lake near the effluent outfall approached this value ([Se] = 1.1 µg/L) but were much higher in the corresponding sediment pore water ([Se] = 2.3 µg/L). The pore water samples taken from Sutton Lake, however, followed the opposite pattern and were lower than their corresponding surface water samples (mean [Se] = 0.96 µg/L). In Mountain Island Lake, selenium concentrations in surface and pore waters were much lower than those measured in Sutton and Mayo (mean [Se] = 0.3 µg/L and 0.37 µg/L, respectively). While effluent status of the associated coal-fired power plant does not readily explain selenium concentrations in surface and pore water of impacted lakes, several other factors such as lake surface area, average depth, bathymetry, and hydrological connectivity are important considerations. Sutton Lake, for example, has the smallest surface area of the lakes included in this study. It is also the shallowest and the least hydrologically connected to its source water system. In contrast, Mayo Lake has a greater average depth and drains into Mayo Creek. Mountain Island Lake has the largest surface area and is the most hydrologically connected to its source water system (The Catawba River).

Several other trace elements were analyzed alongside selenium for this study. Aluminum levels in Sutton Lake pore waters exceed the EPA's CALC of 87 µg/L but this is not considered a CCR-specific contaminant and was also found to be substantially elevated in Lakes Adger and Waccamaw, included as reference lakes for Mountain Island Lake and Sutton Lake, respectively. Levels of arsenic, lead, and nickel were all measured well below their respective CCC thresholds though arsenic and nickel were elevated relative to levels in surface and pore waters of their matched reference lakes. Mean arsenic levels in pore waters from Sutton Lake (mean [As] = 18.7 µg/L) and Mayo Lake (mean [As] = 27.4 µg/L) also exceed the U.S. EPA's drinking water maximum contaminant level (MCL) of 10.0 µg/L, an enforceable standard for the protection of public human health (USEPA, 2016).

4.2 CCR concentrations in resident fish species: The primary motivation of this study was to determine consequences of CCR inputs to lakes for the health of resident aquatic species, specifically fish species due to their established sensitivity to elevated selenium concentrations in the water and aquatic food web (Lemly, 1997 and Skorupa, 1998). Despite fairly low levels in surface and pore waters, selenium levels in the tissues of largemouth bass collected from Mayo Lake and Sutton Lake were significantly elevated relative to those of fish collected from

reference lakes of the same trophic status. Due to selenium's biogeochemical cycling patterns and substantial bioaccumulative enrichment in primary producers, impacted and reference lakes were matched on the basis of trophic status (Maher et al., 2010, Stewart et al. 2010.) Several fish from these lakes had muscle and ovary selenium levels exceeding the proposed regulatory thresholds of 11.3 mg Se/ kg d.w. and 15.8 mg Se/kg d.w., respectively, intended to protect fish reproductive health and population abundance. Levels in fish collected from Mountain Island Lake are much lower and this is expected to be due to the lake's nutrient poor status as well as the size, depth, and hydrological connectivity of the lake to the Catawba River.

Levels of manganese, zinc, copper, and strontium are also elevated in some tissues of fish collected from CCR-impacted lakes relative to reference lakes but none of these trends are as clear or consistent as those of selenium. Surprisingly, fish from reference Lake Tillery have higher levels of manganese, zinc, and strontium than those in corresponding tissues in fish from Mayo Lake. This suggests that Lake Tillery is impacted by underlying geochemical factors or receives a unique, non-CCR, source of contamination.

Data from ongoing analyses of fish otoliths and bluegill and redear sunfish fish tissues are forthcoming. These data will allow for: (1) consideration of strontium isotopes in fish otoliths as biotic environmental CCR tracers; (2) comparisons of interspecies tissue CCR concentrations; and (3) analysis of species-specific selenium distribution among tissues and how fish size, age, and tissue contaminant concentrations are associated with lake trophic status (i.e resource richness).

#### 5. Summary/ Conclusions:

This study shows that coal-fired power plant effluent streams significantly affect receiving reservoir water quality and ecosystems. Surface water, sediment pore water, and fish tissue samples were sampled from three CCR-impacted lakes in the state of North Carolina and compared with those collected from non-CCR-impacted reference lakes matched on the basis of geographic proximity and NCTSI trophic status. Trace element analysis of these samples revealed elevated levels of CCR-associated contaminants in samples collected from lakes that either currently or historically received CCRs via permitted effluent streams from coal-fired power plants. These signals are not only measureable in water and fish sampled from these systems while effluent streams are actively discharged to receiving waters (e.g. Mayo Lake water quality and fish tissue data) but also following effluent stream termination (e.g. Sutton Lake and Mountain Island Lake water quality and fish tissue data). Therefore, effluent stream termination is not expected to be reflected by decreases in CCR concentrations in abiotic or biotic environmental compartments in the near term, especially in lentic water systems due to greater contaminant retention times. Factors including lake surface area, depth, trophic status, and hydrological connectivity to a lotic water system are expected to influence CCR persistence and impact on receiving ecosystems. The results of this study suggest that tissue selenium measurements may provide the most consistent indication of CCR persistence in lentic freshwater ecosystems.

#### 6. Recommendations:

The authors of this report recommend that state regulatory agencies continue to monitor these lakes among other receiving waters of CCR effluents even after CCR releases via effluent

streams are terminated. Regular interval fish tissue monitoring of selenium especially will provide data necessary for determining the persistence of selenium as a CCR contaminant in lentic freshwater ecosystems.

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## Appendix 1: Abbreviations and symbols

Al	Aluminum
As	Arsenic
B	Boron
Ca	Calcium
CCC	Criterion Continuous Concentration
CCR	Coal combustion residual
Cd	Cadmium
Cl	Chloride
Cr	Chromium
Cu	Copper
DCP-OES	Direct current plasma optical emission spectrometry
Hpf	Hours post fertilization
IC	Ion chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
LA-ICP-MS	Laser ablation ICP-MS
Li	Lithium
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
NC	North Carolina
NCTSI	North Carolina Trophic State Index
Ni	Nickel
NPDES	National Pollutant Discharge Elimination System
Pb	Lead
Rb	Rubidium
Sb	Antimony
Se	Selenium
SO <sub>4</sub>	Sulfate
Sr	Strontium
Tl	Thallium
U.S. EPA	United States Environmental Protection Agency
V	Vanadium

## Appendix 2: Presentations and publications

### Presentations:

1. October 2015: *Legacy impacts of coal combustion residuals in freshwater ecosystems in North Carolina*. Canadian Ecotoxicity Workshop, Saskatchewan, Canada. Invited Platform Presentation.

### Expected publications:

1. Brandt, JE et al. Legacy impacts of coal combustion residuals in receiving freshwater ecosystems in North Carolina. *In preparation*. Expected 2016.
2. Brandt, JE et al. Assessment of selenium persistence and toxicity in freshwater lakes: A North Carolina Example. *In preparation*. Expected 2016.

### Expected dissertations:

1. JE Brandt, PhD Dissertation (expected 2017). *Persistence of coal combustion residuals in freshwater ecosystems in North Carolina and mechanisms of selenium toxicity in freshwater fish*. Duke University, Nicholas School of the Environment

### Efforts at technology transfer or communication of results:

1. At least two scientific research publications are expected to result from this work. The data included in this report will be published in peer-reviewed journals where they will be available to the greater scientific community.
2. Following publication of expected manuscripts, data from this project will be distributed to state regulatory and environmental agencies including the Department of Environmental Quality and the Wildlife Resource Commission to inform (1) ongoing regulatory decisions concerning CCR disposal and effluent streams to receiving waters, and (2) monitoring and research efforts for the protection of ecosystem health and fish population abundance.
3. Jessica Brandt presented preliminary research results from this project during a platform presentation at the annual Canadian Ecotoxicity Workshop on October 5, 2015 in Saskatchewan, Canada.
4. Final results are expected to be presented in Fall 2016 at the Society of Toxicology and Chemistry (SETAC) annual meeting in Orlando, Florida.
5. The authors have been working with Duke University's Center for Research Communication and Superfund Research Center's Research Translation Core (RTC) in concert with University of Carolina at Chapel Hill's RTC to organize community information sessions on the coal ash topic.
6. Jessica Brandt, Richard Di Giulio, and Avner Vengosh are members of Duke University's Environmental Health Scholars Program. This program aims to coordinate communication among medical, environmental, and legal researchers about research needs and data transfer for informing policy decisions. The Program organizes annual symposia in which environmental lawyers and scientists convene to discuss pressing research and policy needs. The first of these symposia was held in November 2015 with another expected in fall 2016.

## Coal ash constituents at the base of aquatic food webs: Processes affecting bioaccumulation and trophic transfer of arsenic

### Basic Information

<b>Title:</b>	Coal ash constituents at the base of aquatic food webs: Processes affecting bioaccumulation and trophic transfer of arsenic
<b>Project Number:</b>	2015NC193B
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There are no publications.

# Coal ash constituents at the base of aquatic food webs: Processes affecting bioaccumulation and trophic transfer

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1 **ABSTRACT.**

2 Project Title: Coal ash constituents at the base of aquatic food webs: Processes affecting  
3 bioaccumulation and trophic transfer.

4 The goal of this research was to investigate arsenic bioaccumulation at the base of aquatic food  
5 webs, including uptake of arsenic from solution and depuration kinetics by benthic invertebrates,  
6 uptake and bioconcentration of arsenic by periphyton, and potential trophic transfer to primary  
7 consumers. To better understand arsenate bioaccumulation dynamics in lotic food webs we used  
8 a radiotracer approach to characterize accumulation in periphyton and subsequent trophic  
9 transfer to benthic grazers. Flux rates from solution for a variety of benthic invertebrates are also  
10 described. Our results show that over an 8 day period periphyton concentrated As from  
11 environmentally realistic exposures 3,200–9,700-fold on a dry weight basis without reaching  
12 steady state. These As-enriched diets resulted in negligible accumulation of As in *Neocloeon*  
13 *triangulifer* relative to the concentration in periphyton after a full lifecycle exposure. Other  
14 dietary studies with invertebrate grazers showed that the assimilation efficiency of As from  
15 periphyton is generally quite low, ranging from 22% in the mayfly *N. triangulifer* to 75% in the  
16 mayfly *Isonychia sp.*, suggesting factors controlling bioavailability limit the amount of As that is  
17 transferred to grazers. We propose that two such mechanisms may be the role of As adsorption to  
18 iron oxides in periphyton, and biotransformation of As by periphyton. Data showing relatively  
19 low uptake rate constants ( $K_u$ ) from solution in benthic invertebrates ranging from  $0.063 \pm 0.04 \text{ L}$   
20  $\text{g}^{-1}\text{d}^{-1}$  in *Psephenus herricki*, to  $0.001 \pm 0.003 \text{ L g}^{-1}\text{d}^{-1}$  in *M. pudicum*. Efflux ( $K_e$ ) was generally  
21 high ranging from  $0.15 \pm 0.03 \text{ d}^{-1}$  in *Maccaffertium sp.* to  $0.03 \pm 0.03 \text{ d}^{-1}$  in *Pleurocera sp.* Together  
22 these results have broad implications for monitoring programs by highlighting the role of  
23 periphyton as a sink for arsenate as well as interspecies differences in As bioaccumulation.

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## 36 **1.0 Introduction:**

37 A growing body of literature highlights the importance of bioaccumulation of potentially toxic  
38 trace elements at the base of freshwater food webs (e.g.,(Patrick 1978; Farag et al. 1998; Ancion  
39 et al. 2010; Cain et al. 2011)) and the importance of dietary exposure routes in dictating  
40 accumulation.(Luoma and Rainbow 2005) Periphytic biofilms comprise different types of  
41 diatoms, algae, bacteria, fungi, and detritus that are often the predominant food resource at the  
42 base of aquatic food webs. Periphyton can significantly bioconcentrate trace elements and act as  
43 a dietary vector for metal exposures to grazing fauna. For example, cadmium,(Bradac et al. 2009;  
44 Xie et al. 2010) zinc,(Kim et al. 2012) copper,(Cain et al. 2011) and selenium(Conley et al. 2009;  
45 Conley et al. 2013) have all been shown to accumulate in periphytic biofilms and are trophically  
46 transferred to invertebrate grazers. In contrast, less is known about periphytic uptake,  
47 bioconcentration, and trophic transfer of arsenic.

48 Arsenic is the 20<sup>th</sup> most abundant element in the Earth's crust,(Woolson 1975) and is a common  
49 contaminant in aquatic ecosystems as well as an EPA priority pollutant.(U.S. EPA 2014) The  
50 mineral co-localization of As with geologic resources such as metal rich ores and coal often  
51 result in As contamination associated with the extraction and use of these natural resources (e.g.  
52 mining, smelting, and coal combustion). Background concentrations of As in rivers are reported  
53 to range from 0.02  $\mu\text{g L}^{-1}$  to 2  $\mu\text{g L}^{-1}$ , while contaminated rivers typically range from 1-280  $\mu\text{g L}^{-1}$   
54 but have been reported as high as 79,000  $\mu\text{g L}^{-1}$ .(Smedley and Kinniburgh 2002) While  
55 arsenate is expected to be the dominant chemical species of As in lotic systems,(Smedley and  
56 Kinniburgh 2002) the biogeochemistry of As is complex. As exists in different oxidation states  
57 in the environment (-3, 0, +3, and +5) and can be converted biologically to several organic  
58 forms(Smedley and Kinniburgh 2002; Rahman et al. 2012) or converted between inorganic  
59 oxidation states (e.g.,(Kulp et al. 2004; Levy et al. 2005)). These chemical forms dictate how As  
60 behaves in the environment and its potential to cause toxicity.(Akter et al. 2005; Sharma and  
61 Sohn 2009) Less is understood about the dynamics of As at the base of freshwater food webs,  
62 particularly with respect to accumulation into periphytic biofilms and its availability to  
63 invertebrate grazers.

64 Field studies indicate that As accumulation in periphytic biofilms is potentially important since  
65 measured concentrations have been shown to exceed those for water or sediment (e.g.,(Ramelow  
66 et al. 1987; Drndarski et al. 1993; Koch et al. 1999)). Similar observations have been reported  
67 for algae,(Koch et al. 1999; Schaeffer et al. 2006) bryophytes,(Culioli et al. 2009) and aquatic  
68 plants,(Favas et al. 2012) though the complexity and variability in natural systems complicates  
69 quantifying accumulation dynamics for As. Laboratory studies similarly demonstrate that As is  
70 accumulative in a variety of aquatic plants (e.g.,(N.-X. Wang et al. 2013; Y. Wang et al. 2013;  
71 Sibi 2014)), algae (e.g.,(N.-X. Wang et al. 2013; Y. Wang et al. 2013; Sibi 2014)), and  
72 bacteria.(Y. Wang et al. 2013; Z. Wang et al. 2013) While this accumulation is highly  
73 variable(Jasrotia et al. 2014; Sibi 2014; Srivastava et al. 2014) several species are such strong As  
74 accumulators that they have been proposed for use in As bioremediation.(Yin et al. 2012;  
75 Jasrotia et al. 2014; Srivastava et al. 2014; Islam et al. 2015) In comparison to these single-  
76 species evaluations, much less is known about the accumulation dynamics of As in  
77 environmentally realistic and complex assemblages of periphyton.

78 Accumulation of As by primary producers at the base of the food web may have important  
79 implications for trophic transfer, though there is conflicting evidence in the literature regarding  
80 which route of exposure drives As accumulation in primary consumers. For example, field  
81 studies report that tissue concentrations of As in organisms are better correlated with the  
82 concentration in their food than with water.(Aida M Farag et al., 2007) Dietary exposure has  
83 also been suggested to drive As accumulation in several laboratory studies. For example  
84 Maeda(Maeda et al., 1990) found that benthic grazers accumulated an order of magnitude greater  
85 As from food than from water, and Williams et al.(Williams, Dutton, Chen, & Fisher, 2010)  
86 reported that ingested microalgae could be responsible for more than 80% of accumulated As in  
87 suspension/deposit feeding amphipods. Slightly lower dietary contributions of 30–60% were  
88 reported by Casado-Martinez et al.,(Casado-Martinez, Smith, Luoma, & Rainbow, 2010) but this  
89 was still an important pathway of accumulation. Contradictory findings have been reported by  
90 Kalman et al.(Kalman, Smith, Bury, & Rainbow, 2014) who used a biokinetic approach to  
91 determine that dissolved exposure was responsible for 50–90% of acquired As in an estuarine  
92 bivalve. Similarly, a field study conducted by Hare et al.(Hare, Tessier, & Campbell, 1991)  
93 reported that 95% of measured As was associated with the exoskeleton of invertebrates rather  
94 than the gut (3%), and Spehar et al.(Spehar, Fiandt, Anderson, & DeFoe, 1980) reported between  
95 100–200 fold increase in As concentration relative to dissolved concentrations for several aquatic  
96 invertebrates. Interspecies variability in accumulation of As from solution has also been  
97 reported; Canivet et al.(Canivet, Chambon, & Gibert, 2001) noted that two thirds of investigated  
98 species accumulated As from solution while the other third did not. Together these  
99 inconsistencies point to the need for a broader fundamental understanding of the dynamics and  
100 behavior of As at the base of aquatic food webs as well as the factors driving accumulation and  
101 trophic transfer.

102 In this study we used a radiotracer approach to quantitate the bioconcentration of arsenate by  
103 natural periphyton assemblages at environmentally realistic concentrations. Lab-reared  
104 parthenogenetic *Neocloeon triangulifer* larvae were then raised on these differentially  
105 contaminated diets for a full life cycle experiment to investigate trophic transfer. These dietary  
106 bioaccumulation studies were combined with assays that examined As assimilation efficiency  
107 from food, uptake from solution, and efflux for a variety of benthic invertebrates to better  
108 understand As accumulation dynamics at the base of the aquatic food web. Finally, XANES and  
109 XRF microprobe analyses of As in periphyton were conducted to better understand As  
110 accumulation dynamics at the base of the aquatic food web.

## 111 **2.0 Methods:**

### 112 *2.1 Reagents:*

113 Arsenate ( $\text{HAsNa}_2\text{O}_4 \cdot 7\text{H}_2\text{O}$ ) was obtained from Alfa Aesar (MA, USA).  $^{73}\text{As}$  was obtained  
114 from the National Isotope Development Center (U.S. Dept. of Energy) as As(V) in 0.1 M HCl.  
115 Working secondary stock solutions were prepared in 0.1 N Omnitrace™ nitric acid (EMD  
116 Chemicals, Darmstadt, Germany). American Society for Testing and Materials (ASTM) artificial  
117 soft water (ASW) (mM: 0.57  $\text{NaHCO}_3$ , 0.17  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.25  $\text{MgSO}_4$ , and 0.03 KCl) was  
118 also used for all experiments.

### 119 *2.2 Test animals:*

120 *N. triangulifer* (WCC-2 clone originally obtained from culture at Stroud Water Research Center  
121 [SWRC], Avondale, PA) were reared in the lab at room temperature with ambient light. Other  
122 larval insects and benthic invertebrates were field collected from the Eno River (Efland, NC and  
123 Durham, NC) and Basin Creek (Sparta, NC), and allowed to acclimate without food for at least  
124 48 hours to the laboratory cold room (approximately 15°C).

### 125 2.3 Natural periphyton communities:

126 Natural periphyton assemblages were obtained from SWRC, where they were cultivated by  
127 allowing fresh water from White Clay Creek, PA to flow continuously over acrylic plates (6.5 x  
128 23 x 0.15 cm; see Appendix 3 for historical taxonomic data). Periphyton plates were shipped  
129 overnight on ice and were subsequently aerated and held at room temperature until experimental  
130 use. Background concentrations of As in periphyton ( $4.5 \pm 1.2$  mg kg<sup>-1</sup> dry wt) were determined  
131 using nitric acid digestion and ICP-MS at the Environmental and Analytical Testing Services lab  
132 at North Carolina State University. All experiments measuring As in periphyton characterize  
133 newly acquired As only.

### 134 2.4 Radioactivity measurement:

135 All measurements of radioactivity in water, periphyton, and invertebrates were performed with a  
136 Perkin-Elmer Wallac Wizard 1480 automatic gamma counter. All samples were programmed to  
137 be counted for three minutes to achieve counting errors generally <5% (errors >10% were not  
138 included in analysis). All As concentrations are reported accounting for radioactive decay (half  
139 life = 80.5 days), counting efficiency, and mass specific activity.

### 140 2.5 Experimental design:

#### 141 2.5.1 pH experiment:

142 Arsenic uptake rates in periphyton were studied across a range of environmentally relevant pHs  
143 by collecting small scrapings of similar wet weight ( $0.1165 \pm 0.0077$  g) and transferring them  
144 into individual exposure cups with 25 mL of pH-adjusted (6.5, 7.0, 7.5, 8.0, and 8.5) ASW  
145 (5mM bis-tris propane used as a buffer) at a nominal concentration of 10 µg/L arsenate along  
146 with <sup>73</sup>As as a radiotracer. Three replicates were prepared for each time point. To ensure proper  
147 aeration, exposure cups were held on mixer tables. Uptake was measured at 3, 6, and 9 hours. At  
148 each time point, samples were rinsed with 300 mL of concentration-matched stable arsenate (no  
149 radioisotope) to remove superficially adsorbed arsenic, vacuum filtered on to dried and pre-  
150 weighed filter paper, and dried overnight at 65°C. Dried samples were weighed and assayed for  
151 radioactivity.

#### 152 2.5.2 Periphyton enrichment and food preparation for the full life cycle experiment with 153 *Neocloeon triangulifer*:

154 Two separate batches of periphyton plates (referred to as trial 1 and trial 2) were used to assess  
155 arsenate bioconcentration. These labeled periphyton plates were then used as a food source for  
156 developing mayfly larvae. The experiments were staggered 16 days apart to supply sufficient  
157 food for developing larvae and were conducted in the same manner. Immediately upon arrival,  
158 individual periphyton plates were placed in aerated exposure jars at room temperature with 1.8 L

159 of pH-adjusted ( $7.6 \pm 0.04$ ) ASW at nominal exposure concentrations of 0, 1, 5, 10, and  $20 \mu\text{g L}^{-1}$   
160 total As in addition to As-73 as a radiotracer ( $0.002 \mu\text{Ci mL}^{-1}$ ). Three replicates per  
161 concentration were allowed to accumulate As for 8 days for the first round of plates and 10 days  
162 for the second round of plates. Nominal exposure concentrations corresponded to measured  
163 concentrations of stable arsenate (ICP-MS) of  $<0.1$ , 1.09, 4.97, 10.4, and  $20.7 \mu\text{g L}^{-1}$  in trial 1  
164 and  $<0.1$ , 1.28, 6.85, 16.0, and  $24.7 \mu\text{g L}^{-1}$  in trial 2.

165 During the initial 8 day loading period (trial 1) 1 mL water samples were collected daily from  
166 each replicate for radioactivity measurement and periphyton scrapings of similar weight ( $\sim 0.05$ –  
167  $0.07 \text{ g}$ ) were collected daily from each replicate onto dried, pre-weighted filter paper. Scrapings  
168 were dried at  $65^\circ\text{C}$ , weighed, and measured for radioactivity to determine As content. Sample  
169 collection from this trial was discontinued on day 8 when newly hatched mayfly larvae were  
170 introduced to the chambers (see below). In trial 2, periphyton sampling was conducted less  
171 frequently to maintain a high food level for larvae (samples collected on days 4, 5, 6, 7, and 10).  
172 These trial 2 periphyton plates were added to the chambers containing the trial 1 plates and  
173 mayfly larvae (day 11 of trial 2, day 18-19 of larval development).

174 After hatching (1–2 days), 15 *N. triangulifer* individuals were randomly placed in each  
175 experimental replicate with As-enriched periphyton as described above. Larvae were reared on  
176 arsenate-enriched periphyton plates along with residual aqueous concentrations (see SI for  
177 detailed exposure characterization) until adult emergence. Periphyton plates from trial 1 were the  
178 food source for mayfly rearing days 1–18, though these plates remained in the experimental  
179 chambers throughout the study. Beginning day 18 the periphyton plates for trial 2 were also  
180 available as a food source for mayfly rearing through study termination. Larvae were assayed  
181 for radioactivity on days 25–26 prior emergence as subimagos (days 26–34) to determine the  
182 average As content in larvae for each exposure concentration. Larvae were not weighed to  
183 minimize handling stress and were returned to their exposure chambers to allow them to  
184 complete development to adulthood. Subimagos were assayed for radioactivity beginning on day  
185 26 and were then transferred to molting jars with moist paper towels overnight until final  
186 molting. Adults were placed in individual microcentrifuge tubes, which were first frozen at -  
187  $20^\circ\text{C}$ , then dried at  $65^\circ\text{C}$  for 48 hours before being weighed to the nearest 0.001 mg.

### 188 2.5.3 Assimilation efficiency (AE%):

189 Periphyton samples were labeled with  $^{73}\text{As}$  for 3–4 days in 150 mL ASW ( $0.004 \mu\text{Ci mL}^{-1}$  for all  
190 experiments). No stable As was used in the labeling process. Labeled periphyton was then rinsed  
191 twice with ASW and added to exposure cups containing ASW only. Field collected benthic  
192 grazers ( $n=10$ – $20$ ) were allowed to consume radio-labeled periphyton ad libitum for 4–6 hours  
193 before being transferred to individual containers with ASW and clean food for 15 hours. Animals  
194 were assayed for radioactivity immediately following consumption of radiolabeled periphyton,  
195 and again following consumption of clean food. AE was calculated as the percent of  
196 radioactivity remaining after consumption of clean food compared to initial radioactivity  
197 measured after consumption of radio labeled periphyton.

### 198 2.5.4 Microscale elemental associations and biotransformation of As:

199 Periphyton plates were exposed to nominal concentration of 20  $\mu\text{g L}^{-1}$  stable arsenate in pH-  
200 adjusted (6.5 $\pm$ 0.02) ASW for 4 days. To maximize As uptake, solutions were refreshed daily. On  
201 the final day of exposure, periphyton samples (~0.2 g wet weight) were rinsed and suspended in  
202 10 mL ASW and vacuum filtered onto a 0.2  $\mu\text{m}$  Millipore Isopore polycarbonate filter  
203 membrane. The filter was immediately mounted on a 4  $\times$  2.5 cm acrylic window using Kapton  
204 tape. The periphyton mount was then quickly placed in an air-tight glove box covered in  
205 aluminum foil to eliminate light exposure and dried with  $\text{N}_2$  gas for 5 hours before being  
206 packaged for overnight shipment to Stanford Synchrotron Radiation Lightsource (SSRL).  
207 Microscale spatial distributions of As, Si, P, S, K, Ca, Ti, Mn, Fe, Cu, and Zn were mapped on  
208 an approximately 4800  $\times$  1100  $\mu\text{m}$  region of the periphyton sample using micro X-ray  
209 fluorescence ( $\mu\text{XRF}$ ) at Beamline 2-3. The beam spot size was nominally 1  $\times$  1  $\mu\text{m}^2$ , and images  
210 were collected with a step size of 0.01  $\mu\text{m}$  and dwell time of 50 milliseconds. XANES spectra  
211 were additionally collected on regions of interest.

#### 212 2.5.5 Dissolved uptake and efflux:

213 For aqueous uptake and efflux experiments, field collected invertebrates were transferred to  
214 individual acid-washed exposure cups (n=5 per exposure concentration; see Appendix 3 for full  
215 taxonomic classification) with a small square PTFE substrate, filled with 25 mL pH-adjusted  
216 (7.2 $\pm$ 0.2) ASW at nominal concentration of 10  $\mu\text{g L}^{-1}$  arsenate along with As-73 as a radiotracer  
217 (volume of isotope adjusted to account for decay and achieve final specific activity in exposure  
218 chambers of 0.003–0.005  $\mu\text{Ci mL}^{-1}$  for all experiments). To obtain initial uptake rates from  
219 solutions, animals were analyzed *in vivo* for radioactivity at 3, 6, and 9 hours following a rinse  
220 with concentration-matched stable As (no radioisotope) solution to remove any superficially  
221 adsorbed radiotracer. After the 9 hour time point, animals were returned to their exposure  
222 solutions for an additional 4-5 days of loading before being transferred to clean water to measure  
223 efflux. Efflux was measured daily for 5-10 days by assaying individuals for radioactivity. Clean  
224 ASW was provided each day to reduce re-uptake of radiotracer. Rough estimates of  
225 bioconcentration factors (BCFs) were obtained by dividing the average  $K_u$  by the average  $K_e$  for  
226 a given species.

#### 227 2.6 Data Analysis:

228 Periphyton bioconcentration of As from water was calculated by dividing the mean measured As  
229 in periphyton on the final day of loading by the average measured As concentration in water (on  
230 a mass basis where 1 L water = 1 kg) across all days of the periphyton loading phase.  
231 Comparisons of As accumulated in larvae were calculated by averaging the measured mass of As  
232 in all individuals in each replicate and all replicates per exposure, which was then compared to  
233 the average final mass of As accumulated per gram of dry weight of periphyton.

234 Uptake rate constants ( $K_u$ ) were estimated as the slope of the measured As concentration over  
235 time (linear regression) divided by the exposure concentration. Efflux rate ( $K_e$ ) was estimated as:

$$236 \quad C_t = C_i \times e^{K_e \times t}$$

237 where  $C_t$  = tissue concentration at time t,  $C_i$  = tissue concentration at time 0 d,  $K_e$  = efflux rate  
238 constant, and t = time in days.

239 X-ray microprobe images of As and other element spatial distributions were processed using  
240 Sam's Microprobe Analysis Toolkit (SMAK, developed by Samuel Webb, Stanford Synchrotron  
241 Radiation Lightsource, Palo Alto, CA).(Webb 2011) A blur filter of 5 (Stdev = 0.85) was  
242 applied to images before plotting spatial correlations of As with the other elements imaged.  
243 Pearson correlation coefficients were derived by taking the square root of the  $R^2$  value reported  
244 in SMAK. XANES data were analyzed using SixPack.

245 Data are expressed as mean  $\pm$  standard error unless otherwise specified and analyzed using  
246 GraphPad Prism (V6).

### 247 3.0 Results

248 Uptake rate constants ( $K_{it}$ ) in periphyton at pH of 6.5, 7.0, 7.5, 8.0, and 8.5 were  $1.147 \pm 0.1114$ ,  
249  $0.6626 \pm 0.08733$ ,  $0.7897 \pm 0.08332$ ,  $0.4934 \pm 0.1071$ , and  $0.1573 \pm 0.06517 \mu\text{g As g}^{-1} \text{ ww day}^{-1}$ ,  
250 respectively (Fig 1). Uptake rates were statistically significantly different ( $p < 0.05$ ) at all pH  
251 levels except for 7 and 7.5 ( $p = 0.3104$ ). At the same concentration of arsenic, the periphyton at  
252 pH 6.5 concentrated almost 3 times more arsenic from solution than the periphyton at pH of 8.5.

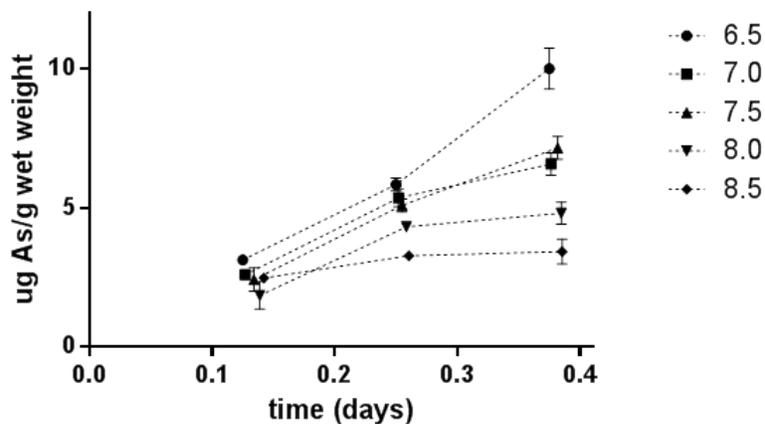


Figure 1. Uptake of arsenate in periphyton across different pH levels over 3, 6, and 9 hours of exposure

253 Two independent trials of periphyton exposed to a single pulse of 1, 5, 10 and 20  $\mu\text{g L}^{-1}$  arsenate  
254 resulted in consistent decreases in dissolved As concentrations. Dissolved As concentrations  
255 dropped rapidly over the first few days of exposure and stabilized at roughly 50% of their initial  
256 concentrations (Fig 2A,C) by days 2–4. Uptake of As into periphyton was less consistent and  
257 did not always mirror dissolved As concentrations. For example in trial 1, periphyton As  
258 concentrations generally increased over time but not in a monotonic fashion (Fig 2B).  
259 Periphyton As appeared to decrease briefly at days 4–5 before increasing again thereafter, most  
260 notably at the highest exposure level. Similarly, in trial 2, periphyton appeared to decrease in  
261 concentration after an initial rapid uptake at the higher exposure concentration; in this trial As in  
262 periphyton continued to decrease over days 4–10 (Fig 2D).

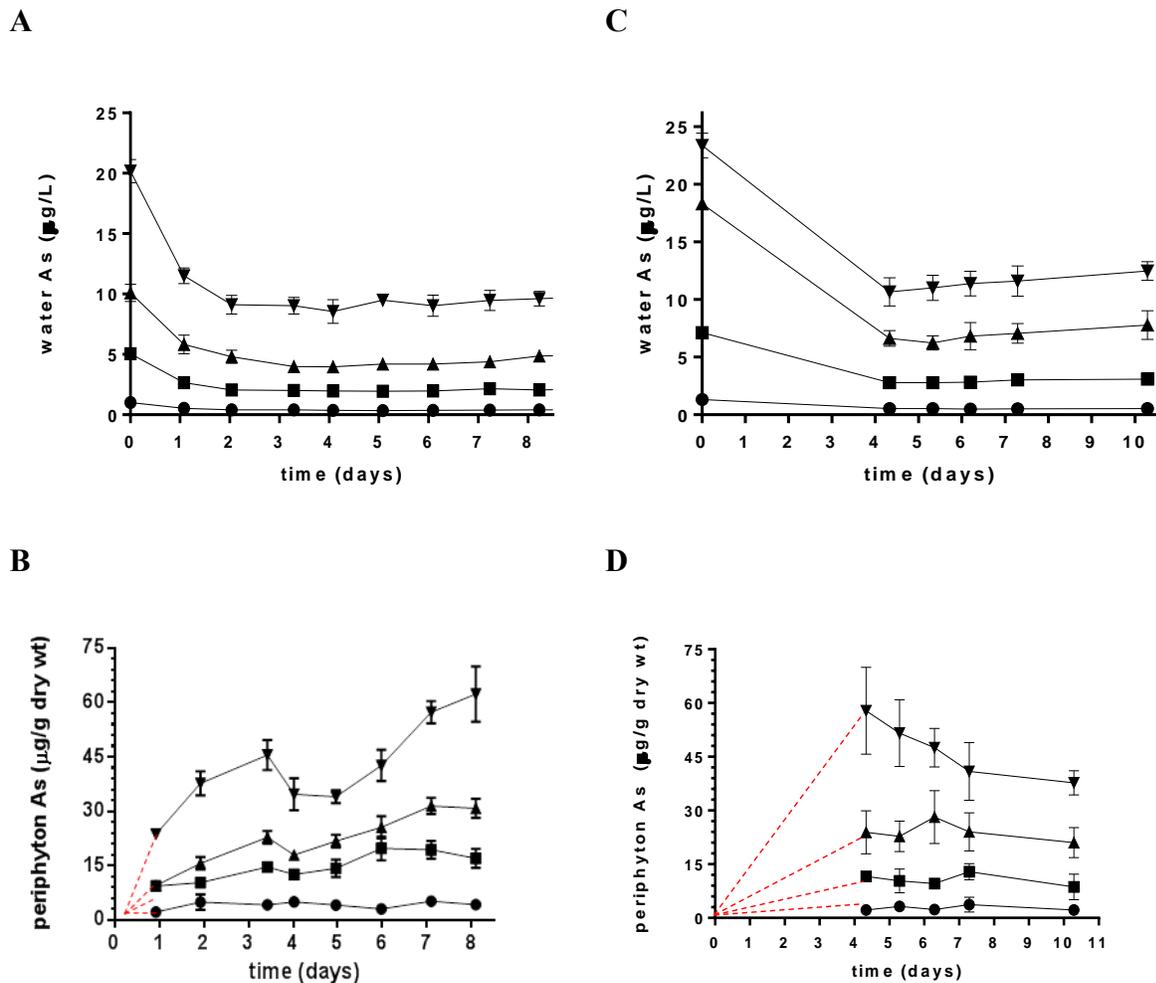
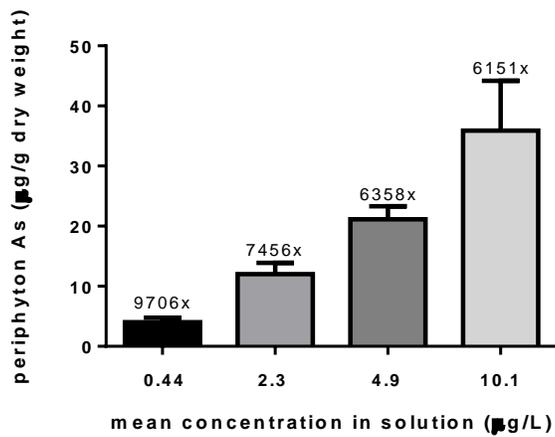


Figure 2. Temporal trends in dissolved As concentrations (A) and newly acquired As in periphyton (B) during 8 day loading period (trial 1). Measured concentration of As in solution (C) and newly acquired As in periphyton (D) during the 10 day loading period (trial 2). Red dashed lines (B,D) indicate intervals where samples were not taken. N=3 for each treatment at each time point. Symbols (low to high) represent initial nominal dissolved As concentrations of 1, 5, 10, and 20 µg L<sup>-1</sup>arsenate, respectively. Values plotted are mean ± SEM.

263 After 8 days of exposure to a single pulse of arsenate (1, 5, 10, or 20 µg L<sup>-1</sup>), the concentration of  
 264 As in periphyton (dry weight basis) was compared to the average dissolved As concentration  
 265 over the 8 day exposure period to quantify As bioconcentration in periphyton. In trial 1,  
 266 periphyton bioconcentrated As 6,000-9,000-fold (Fig 3A). Ratios of periphyton As to mean  
 267 dissolved As decreased with increasing dissolved As concentrations. In trial 2, periphyton  
 268 bioconcentrated As 3,200-4,200-fold (Figure 3B) after 10 days of exposure to a single pulse of  
 269 arsenate. Ratios of periphyton As to mean dissolved As were more consistent in this trial and did  
 270 not trend with dissolved concentrations.

**A**



**B**

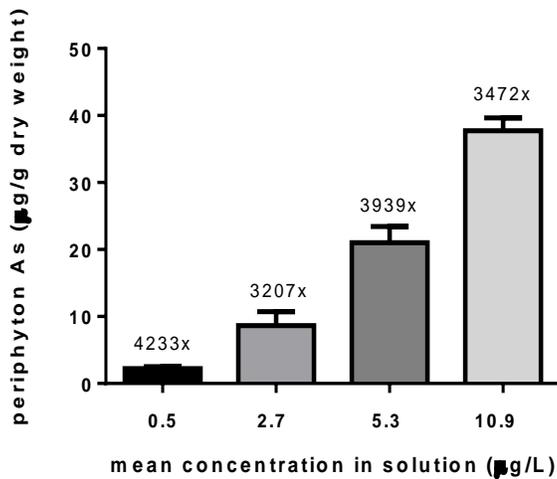


Figure 3. Periphyton bioaccumulation of arsenate after 8 days of loading for the first round of plates (A) and after 10 days of loading in the second round of plates (B). Values plotted are mean  $\pm$  SEM; n=3 for final measured arsenic concentration in each exposure group. Numbers above each bar represent fold increase of As concentrations in periphyton compared to the average concentration in solution. Initial nominal concentrations in solution were 1, 5, 10, or 20  $\mu\text{g L}^{-1}$  As.

271 To test whether periphyton-bioconcentrated As was trophically available to an invertebrate  
272 grazer, we reared the mayfly *N. triangulifer* on the periphyton diets described above (see SI for  
273 full exposure characterization). Very low radioactivity was measured in the larvae corresponding  
274 to 0.0006–0.005  $\mu\text{g}$  As per individual (Fig. 4). While these individuals were not weighed to  
275 avoid handling stress, if we assume approximately 1 mg dry weight (average for developmental  
276 stage) we estimate that tissue As concentrations were 18–35% lower than As concentrations in  
277 periphyton, suggesting significant biodilution. When assayed again as subimagos radioactivity  
278 could not be detected.

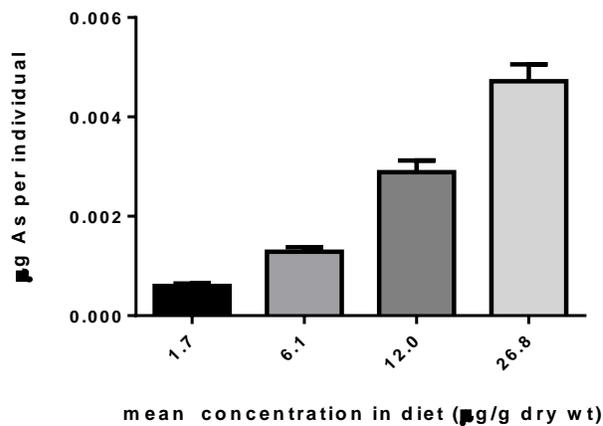


Figure 4. Bioaccumulation of As in *N. triangulifer* larvae fed on differentially contaminated periphyton plates in full lifecycle exposures. Values plotted are mean  $\pm$  SEM; n=26–34.

279 Assimilation efficiencies (AE) of As from periphyton in *N. triangulifer* and other invertebrate  
 280 grazers varied among species tested (Fig. 4). *N. triangulifer* had the lowest assimilation  
 281 efficiency (22±8.5%) followed by *Pleurocera sp.* (28±10%), *Corbicula fluminea* (57±12.7%),  
 282 *Maccaffertium sp.* (60±13.2%), and *Isonychia sp.* (75±8.5%). *Hydropsyche betteni* did not  
 283 acquire enough radioactivity from labeled periphyton until 15 hours of exposure, and therefore  
 284 only had 8 hours on clean food for excretion. Thus our estimate of 71±6% as an AE for this  
 285 species may be an over-estimate and is not included in Fig. 5.

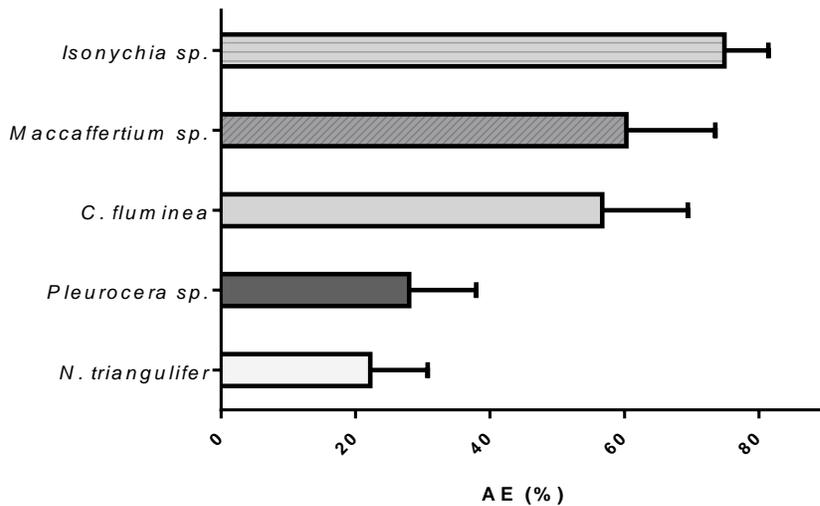


Figure 5. Assimilation efficiencies (AE) from dietary arsenic exposure in periphyton for several species of aquatic invertebrates. Data shown are mean ± SD; n=6–15.

286 Periphyton treated with arsenate by static-renewal for 5 days and analyzed using X-ray  
 287 fluorescence mapping (XRF) revealed that As was not strongly correlated with Si, P, S, K, Ca,  
 288 Ti, Mn, Cu, or Zn (data not shown). Conversely, As and Fe were largely co-localized across the  
 289 sample area analyzed (Fig. 6A) and showed a strong correlation (R=0.92). This sample also  
 290 showed evidence of bioreduction of arsenate to arsenite using XANES (Fig. 6B). Arsenic was  
 291 not adequately measured by XRF in mayflies that had eaten As-enriched periphyton for 10 days,  
 292 therefore no speciation or elemental associations could be evaluated (data not shown).

A



B

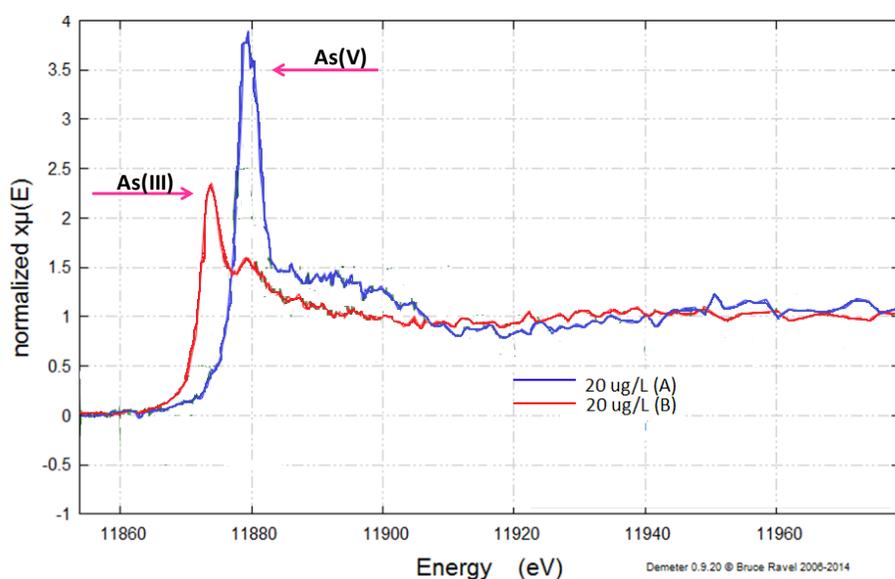


Figure 6. (A) X-ray fluorescence microprobe image showing co-localization of arsenic (red) and iron (blue) in a periphyton sample. The scale bar represents 500  $\mu\text{m}$ , and areas of purple and magenta represent As-Fe associations. Correlation of As and Fe in periphyton  $R=0.92$ ;  $n=54,432$  from the image. (B) XANES spectra showing distinct peaks at the arsenate energy and at the arsenite energy.

293 To explore aqueous As bioaccumulation pathways, we estimated uptake rate constants ( $K_u$ ) and  
 294 efflux rate constants ( $K_e$ ) in several aquatic invertebrates (see SI for taxonomic characterization  
 295 and raw data). Mean  $K_u$  varied across multiple benthic invertebrate species, but were generally  
 296 low (Fig. 7). *Talpoerla sp.* did not absorb enough measureable As during the experiment to be  
 297 included. *Ephemerella sp.*, *Maccaffertium sp.*, *N. triangulifer*, and *M. pudicum* all had  $K_u$  values  
 298 of  $\sim 0.001 \text{ L g}^{-1} \text{ d}^{-1}$ . *C. fluminea*, *Pleurocera sp.*, and *P. immarginata* all had  $K_u$  values of  $\sim 0.01 \text{ L}$   
 299  $\text{g}^{-1} \text{ d}^{-1}$ . *A. abnormis* and *H. betteni* were  $0.02\text{--}0.03 \text{ L g}^{-1} \text{ d}^{-1}$ , and *Corydalus sp.*, *Isonychia sp.*, and  
 300 *P. herricki* had  $K_u$  values of  $\sim 0.05\text{--}0.06 \text{ L g}^{-1} \text{ d}^{-1}$ .

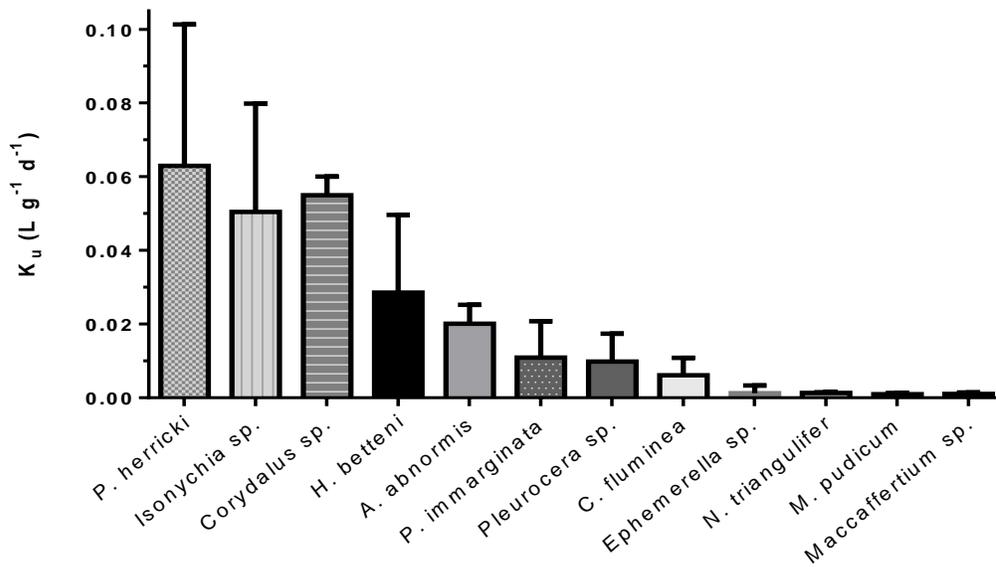


Figure 7. Dissolved arsenic uptake rate constants ( $K_u$ ) in several species of aquatic invertebrates;  $n=5$ . Error bars represent the standard errors of the slope for each regression line (uptake measured at 3, 6, and 9 hours).

301 In contrast to the relatively low uptake rate constants we observed, mean  $K_e$  tended to be  
 302 relatively high (Fig 8). Some of the tested species eliminated As too rapidly (24–48 hours) to be  
 303 included in this study (*N. triangulifer*, *M. pudicum*). On the slower end, *C. fluminea*, *Pleurocera*  
 304 *sp.*, and *P. herricki* had  $K_e$  values of  $\sim 0.03$  d<sup>-1</sup>. *H. betteni*, *Ephemerella sp.*, and *A. abnormis* had  
 305  $K_e$  values between 0.06 and 0.09 d<sup>-1</sup>. The highest  $K_e$  values reported were for *Maccaffertium sp.*  
 306 and *Isonychia sp.*, which were  $\sim 0.15$  d<sup>-1</sup> and 0.29 d<sup>-1</sup>, respectively.

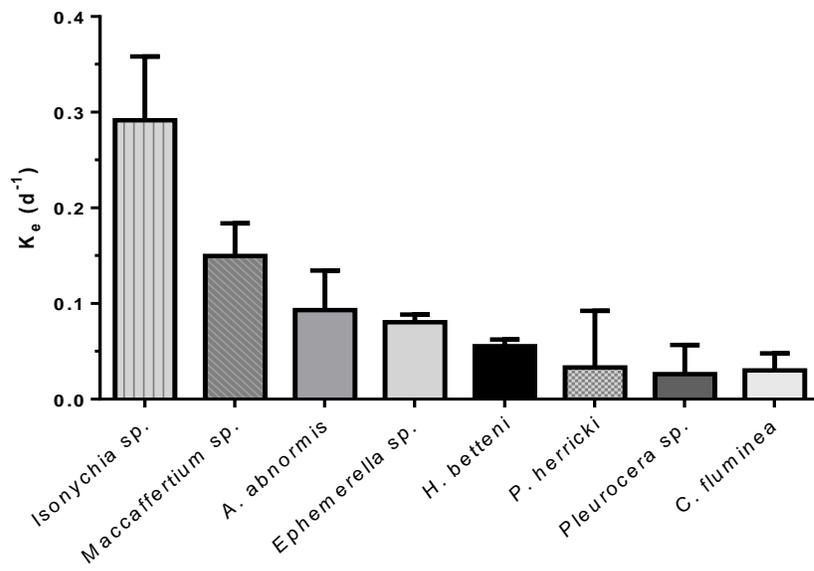


Figure 8. Efflux rate constant ( $K_e$ ) in several species of aquatic invertebrates after 4-5 days of aqueous exposure;  $n=5$ . Error bars represent the standard errors of the slope for each regression line (efflux measured daily for up to 10 days).

307 For the subset of organisms for which both  $K_e$  and  $K_u$  could be derived, BCF estimates were  
 308 similar (200–250) for *Isonychia sp.*, *C. fluminea*, *A. abnormis*, and *Pleurocera sp.* The highest  
 309 estimated BCF was approximately 960 for *P. herricki*, while the lowest estimated BCF was  
 310 approximately 7 for *Maccaffertium sp.* (data not shown).

## 311 4.0 Discussion:

### 312 4.1 Periphyton uptake of As across pH

313 The influence of protonation state on uptake of AsV in primary producers has been characterized  
 314 in laboratory studies for a few species. Generally there is good agreement between studies that  
 315 lower pH facilitates greater concentration of AsV into primary producers including algae  
 316 (Pawlik-Skowrońska et al. 2004; Sibi 2014), and aquatic plants (Tu & Ma 2003). There is some  
 317 variability however; Favas et al. (2012) reported that of several aquatic plant species  
 318 investigated, only two had highly significant negative correlations between tissue arsenic and pH  
 319 while one species had a significant positive correlation. Chen et al. (Chen et al. 2014) similarly  
 320 report a positive correlation between pH and total arsenic accumulation in an aquatic plant  
 321 exposed to AsV, which was proposed to be due to the fact that phosphate transporters, which are  
 322 tricked into transporting As due to structural similarities (Zhao et al. 2009), have a higher affinity  
 323 for the more electronegative AsO<sub>4</sub><sup>3-</sup> species than for the more protonated forms dominating

324 lower pHs. In this study, pH had an inverse relationship on As uptake in periphyton, which  
325 could result in higher or lower As bioconcentration by periphyton under different site specific  
326 environmental conditions. Our results suggest that AsV uptake in periphyton as a function of pH  
327 is more similar to what is reported for other aquatic algae species.

#### 328 4.2 Periphyton loading and enrichment:

329 Arsenic concentrations in lotic ecosystems vary widely ( $\leq 2 \mu\text{g L}^{-1}$  in reference streams to  $>300$   
330  $\mu\text{g L}^{-1}$  in contaminated streams)(Smedley and Kinniburgh 2002). Thus the range of dissolved As  
331 concentrations used in our experiments ( $1\text{--}20 \mu\text{g L}^{-1}$ ) represent environmentally common  
332 exposures. After a single pulse of such concentrations, periphyton rapidly removed and  
333 concentrated As from solution, suggesting that periphyton communities are an important sink for  
334 arsenate. For example, in this experiment periphyton exposed to  $20 \mu\text{g L}^{-1}$  As for 8–10 days  
335 accumulated approximately  $30\text{--}60 \text{ mg kg}^{-1}$  As on a dry weight basis. This observation is  
336 supported by several field studies showing accumulation of As by periphyton (e.g.,(Ramelow et  
337 al. 1987; Drndarski et al. 1993; Koch et al. 1999)). When measured water samples from  
338 differentially impacted streams averaged  $\sim 20\text{--}40 \mu\text{g L}^{-1}$  As, periphyton was found to have As  
339 concentrations ranging from  $0.6\text{--}50 \text{ mg kg}^{-1}$  on a dry weight basis.(Ramelow et al. 1987) When  
340 industrially impacted water concentrations were approximately  $32 \mu\text{g L}^{-1}$  As, periphyton were  
341 reported to range from 46 to  $57 \text{ mg kg}^{-1}$  dry weight.(Drndarski et al. 1993) Similarly, naturally  
342 elevated water concentrations ranging from approximately  $250\text{--}300 \mu\text{g L}^{-1}$  resulted in microbial  
343 mats with  $82\text{--}290 \text{ mg kg}^{-1}$  As on a dry weight basis.(Koch et al. 1999) Thus the measured As in  
344 periphyton reported here is approximately representative of sites with mild to moderate  
345 contamination from field studies, though variations in environmental conditions limit broader  
346 generalizations. Similar findings of As accumulation by other primary producers are also  
347 reported from laboratory investigations (e.g.,(N.-X. Wang et al. 2013; Sibi 2014; Islam et al.  
348 2015)). For example, after exposure to  $1000 \mu\text{g L}^{-1}$  As, the aquatic plant *Micranthemum*  
349 *umbrosum* was found to accumulate  $1219 \text{ mg kg}^{-1}$  dry weight As,(Islam et al. 2015) and different  
350 species of microalgae accumulated  $3,000\text{--}17,000 \text{ mg kg}^{-1}$  As on a dry weight basis when  
351 exposed to a range of concentrations from  $10,000\text{--}50,000 \mu\text{g L}^{-1}$  As;(Sibi 2014) however, these  
352 exposure concentrations are representative of only the most extreme As-impacted  
353 waters.(Smedley and Kinniburgh 2002) To our knowledge this is the first study to characterize  
354 accumulation of As by ecologically realistic periphyton assemblages at environmentally relevant  
355 exposure concentrations in a laboratory setting.

356 We have conducted several iterations of time-course investigations that have shown uptake into  
357 periphyton is non-monotonic. This is most apparent from the first trial of periphyton loading at  
358 the highest initial concentration of  $20 \mu\text{g L}^{-1}$ , but is also demonstrated by the 5 and  $10 \mu\text{g L}^{-1}$   
359 treatments in this study as well as in two smaller scale pilot studies (data not shown). In all  
360 instances periphyton generally increase in As concentration over time, but appear to have a  
361 notable decrease in As content between the third and fifth days of exposure before continuing to  
362 increase. Similarly, we observed a slight reduction in As periphyton concentration on days 4-10  
363 in trial 2. One possible explanation is that certain species within the periphyton may be  
364 detoxifying and excreting As,(Wang et al. 2015) or that surficial cells accumulate As, die and  
365 slough off the periphyton surface.

366 In laboratory studies, BCFs have been reported as 527–4,000 in macrophyte shoots and  
367 roots,(Xue and Yan 2011) and 220–360 for several submerged plant species.(Chen et al. 2015)  
368 In the field, As bioconcentration by primary producers is highly variable (BCF of 152 reported  
369 for bryophytes in a contaminated tributary,(Culioli et al. 2009) and a range of values from 107 to  
370 52,000 across several species of aquatic plants)(Favas et al. 2012). We could not find any  
371 laboratory studies of periphyton to compare our results to. While our periphyton values are not  
372 BCFs (steady state not reached in trial 1 for example) they appear to be on the higher end of  
373 values reported from the field, which vary from 30–1,250 (along a pollution gradient),(Ramelow  
374 et al. 1987) 300–1,062.(Koch et al. 1999), and 1,438–1,781.(Drndarski et al. 1993) One possible  
375 explanation for this discrepancy is that the periphyton we worked with did not come from a  
376 contaminated setting and may contain highly bioaccumulative taxa that may be extirpated from  
377 more highly contaminated settings. It is difficult, however to directly compare BCF results from  
378 both field data and laboratory studies since As accumulation can vary with different  
379 environmental factors,(Sibi 2014) seasonality,(Ramelow et al. 1987) exposure  
380 concentrations,(Sibi 2014) and exposure durations(Chen et al. 2015) in addition to the broad  
381 variability observed between species.(Sibi 2014; Chen et al. 2015) Differences in the thickness  
382 or density(Rosemond 1994; Alam et al. 1997; Kanavillil and Kurissery 2013) of periphyton  
383 growth may also play a significant role in differential accumulation and may have contributed to  
384 the differences observed in our two trials.

#### 385 4.3 As content in *N. triangulifer* larvae and adults

386 *N. triangulifer* has been a useful laboratory species to study trace element bioaccumulation for  
387 zinc,(Kim et al. 2012) cadmium,(Xie et al. 2010) and selenium.(Conley et al. 2009) Here, after  
388 being reared for a full lifecycle with both As-enriched diet (and residual aqueous exposure), *N.*  
389 *triangulifer* larvae were found to have minimal measureable As, and As was not detectable in  
390 adults. Larvae of this species had very low uptake from solution and after several trials, we were  
391 unable to quantify efflux due to rapid elimination (24-48 hours) of any radiolabel obtained (data  
392 not shown). In the current experiment larvae were rinsed in concentration-matched solution to  
393 reduce the contribution of externally adsorbed radiotracer, however it is possible that not all  
394 superficially adsorbed As was removed in this process. In addition, gut contents of larvae were  
395 not purged prior to analysis. Thus, As adsorption to the exoskeleton(Hare et al. 1991; Cain et al.  
396 1992; Mason et al. 2000; Lavilla et al. 2010) and As associated with gut contents(Smith et al.  
397 2015 Sep 21) in the larvae could have contributed greatly to measured radioactivity in larvae.

398 Intraspecific variation in As content across different life stages appears common for insects. For  
399 example, 72% of As was found to be eliminated between the fourth instar and adult stages in *C.*  
400 *riparius*,(Mogren et al. 2012) which was proposed to be accomplished through the  
401 meconium.(Mogren et al. 2013) Similar findings are reported for a terrestrial moth *Agrotis*  
402 *infusa*,(Andrahennadi and Pickering 2008) and aquatic mayfly *Ephoron virgo*,(Cid et al. 2010)  
403 however a specific removal mechanism was not proposed in either case. These observations are  
404 in good agreement with our data where virtually no measureable As was detected in emerged *N.*  
405 *triangulifer* adults, although it is important to note that assayed larvae were not purged overnight  
406 and therefore the small amount of radioactivity detected could also be contributed solely from  
407 gut content. Assimilation efficiency and bioavailability

#### 408 4.4 Assimilation efficiency:

409 While several studies emphasize dietary exposure as the driver of As accumulation,(Maeda et al.  
410 1990; Suhendrayatna and Maeda 2001; Williams et al. 2010) there appears to be a great deal of  
411 variation in AE among and between aquatic invertebrate taxa. While our results indicate a broad  
412 range from 22–75%, these values are on the upper end of what is reported in the literature. For  
413 example, AEs for primary consumers are reported as 7.8% in *Arenicola marina*,(Casado-  
414 Martinez et al. 2010) 11% in *Leptocheirus plumulosus*,(Williams et al. 2010) 25.3% in  
415 *Scrobicularia plana*,(Kalman et al. 2014) 29% in *Nereis diversicolor*,(Rainbow 2011) and 72%  
416 in *Alitta succinea*.(Baumann et al. 2012) For secondary consumers, AEs are similarly low, for  
417 example 9.4% in killifish fed amphipods,(Dutton and Fisher 2011) and 46–61% in two species of  
418 *Hydropsyche*.(Awrahman et al. 2015) Our AE estimates may be biased high because we did not  
419 use stable As in the preparation of labeled periphyton for these experiments. Taken together,  
420 these results are in agreement with our findings of generally low AE for As in benthic  
421 invertebrates as well as the observed inter-and intra-species variability in assimilation.

#### 422 4.5 Elemental associations and speciation

423 Arsenate is known to have strong associations with iron oxides in soils (e.g.,(Maji et al. 2007;  
424 Miretzky and Cirelli 2010 Jan 28)) as well as in aquatic environments (e.g.,(Meng et al. 2002)).  
425 This association has been leveraged in treatment of As-contaminated water as a removal  
426 mechanism (e.g.,(Driehaus et al. 1998; Guan et al. 2008)). Iron (Fe) is an essential trace element  
427 for primary producers that can be involved in photosynthesis, chlorophyll biosynthesis, and  
428 respiratory electron transport.(Street and Paytan 2005; Raven et al.) In some cases Fe may be a  
429 limiting factor much like nitrogen or phosphorus, and Fe limitation has been linked to decreased  
430 primary production.(Vrede and Tranvik 2006) Although Fe is typically associated with small  
431 colloids or organic ligands in freshwater, it can be taken up directly by plant cells if it is in  
432 dissolved form or it can be solubilized from particles and colloids.(Street and Paytan 2005)  
433 Excess Fe can also form plaques externally (e.g.,(Robinson et al. 2006a; Rahman et al. 2008;  
434 Taggart et al. 2009a)). Together these observations indicate that As uptake by plants is complex  
435 with direct uptake through phosphate transporters(Oremland and Stolz 2003; Robinson et al.  
436 2006b; Zhao et al. 2009; Rahman and Hasegawa 2011) (shown to be positively correlated to Fe  
437 uptake),(Rahman et al. 2008) indirect uptake through solubilization of Fe colloids and therefore  
438 any As associated with those colloids,(Street and Paytan 2005) or through external sorption of  
439 As on Fe plaques.(Rahman et al. 2008; Letovsky et al. 2011)

440 Several studies have noted As associations with Fe in aquatic plants (e.g.,(Zhao et al. 2009;  
441 Taggart et al. 2009b; Xing 2011)), terrestrial plants (e.g.,(Zhao et al. 2009)), and  
442 fungi,(González-Chávez et al. 2014) however no studies were identified that investigated co-  
443 localization of As and Fe in periphyton. Here we observed a strong correlation between arsenate  
444 and Fe distributions in As enriched periphyton using XRF. The implications of As-Fe  
445 associations for trophic transfer are not fully understood. There are conflicting views on the  
446 bioavailability of metals associated with Fe oxides. Newman and McIntosh(Newman and  
447 McIntosh 1989) suggest that Fe association reduces bioavailability, which is supported by data  
448 reported by Baumann et al.(Baumann et al. 2012) where the highest AE for As was reported  
449 from radiolabeled pure diatoms (72%) while hardly any As associated with Fe oxide was  
450 assimilated (2%). Conversely, others show evidence that Fe content of sediments(Sharma and  
451 Sohn 2009) and biofilms(Farag et al. 2007) drives As accumulation in deposit feeders and  
452 benthic grazers, respectively. In fact, Farag et al.(Farag et al. 2007) suggest this association is a

453 critical link in trophic transfer. More work should be done to characterize the role of As-Fe  
454 associations in dictating arsenate bioavailability from freshwater periphyton to benthic grazers.

455 Laboratory studies have demonstrated that a variety of aquatic microalgae and bacteria species  
456 are capable of oxidizing AsIII to AsV (e.g., (Levy *et al.* 2005; Qin *et al.* 2009; Zhang B, Wang  
457 LH 2011)), reducing AsV to AsIII (e.g., (Hasegawa *et al.* 2001; Hellweger *et al.* 2003)),  
458 biomethylating As (e.g., (Hasegawa *et al.* 2001; Ye *et al.* 2012), or synthesizing complex  
459 arsenosugars or arsenolipids (e.g., (Murray *et al.* 2003; Levy *et al.* 2005; Xue *et al.* 2014)).  
460 While there is general consensus on the biotransformation capabilities of primary producers,  
461 there is a great deal of variability in what is reported as the dominant arsenic species in tissues  
462 compared to which As species primary producers were exposed to in solution. For example,  
463 there is some evidence that when exposed to AsV or AsIII solutions the predominant arsenic  
464 species in plant tissues is AsIII in submerged macrophytes (Xue *et al.* 2012) and duckweed  
465 (Zhang *et al.* 2009). Others have reported that AsV is the dominant species in tissues after  
466 exposure to either AsV or AsIII in submerged macrophytes (Zheng *et al.* 2003), cyanobacteria  
467 (Wang *et al.* 2013) and blue-green algae (Yin *et al.* 2012). Interestingly, (Wang *et al.* 2013)  
468 reported that cyanobacteria accumulated more AsV from AsIII treatment than from AsV  
469 treatment. In light of this conflicting evidence, our results of distinct AsIII regions in the AsV-  
470 treated periphyton from the first experiment is not surprising, but does not fully answer the  
471 question of which arsenic species would dominate in natural conditions.

472 The results presented here along with those reported by others support the idea that the most  
473 significant step in As accumulation occurs from water to primary producers with a much smaller  
474 step, or even biodilution occurring from primary producers to invertebrate grazers (Fig. 9). Most  
475 accumulation of As therefore occurs at the base of the aquatic food web and then is  
476 biodiminished through subsequent trophic transfer to primary and secondary consumers, as  
477 supported by laboratory (e.g.,(Maeda *et al.* 1990; Cheng *et al.* 2013)) and field studies  
478 (e.g.,(Chen *et al.* 2000; Chen and Folt 2000; Farag *et al.* 2007; Culioli *et al.* 2009; Dovick *et al.*  
479 2016)).

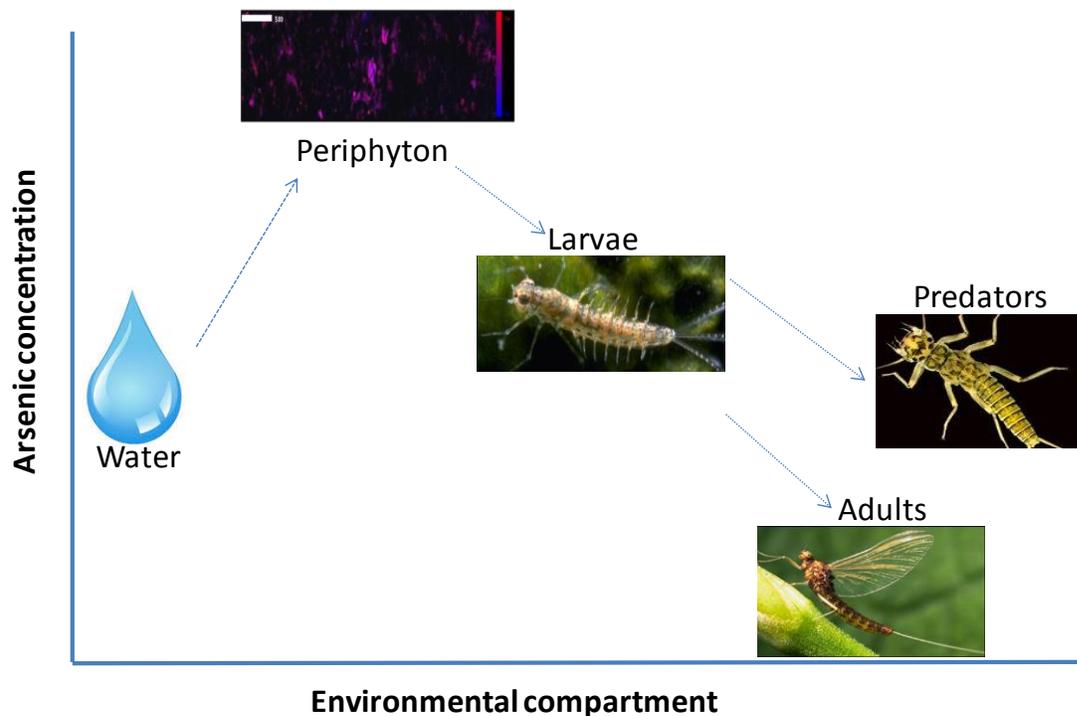


Figure 9. Food web conceptual model of arsenic bioaccumulation dynamics in riverine ecosystems

#### 480 4.6 Dissolved uptake/efflux

481 Our results show a great deal of variation in uptake from solution ( $K_u$ ) between species (ranging  
 482 from  $0.001 \pm 0.003$  to  $0.063 \pm 0.04$   $L\ g^{-1}d^{-1}$ ; see Appendix 3 for full taxonomic information and raw  
 483 data). Efflux of As acquired from solution ( $K_e$ ) was also variable (ranging from  $0.03 \pm 0.03$  to  
 484  $0.15 \pm 0.03$   $d^{-1}$ ). In addition, several species tested lost measurable As too quickly (24–48 hours)  
 485 to be included in analysis (*N. trianfulifer*, *M. pudicum*, data not shown). Our findings of  
 486 relatively low uptake from solution and relatively fast efflux rates are supported by other studies  
 487 in the literature. For example, Williams et al. (Williams et al., 2010) reported  $K_u$  and  $K_e$   
 488 constants for estuarine amphipods of  $0.028$   $L\ g^{-1}d^{-1}$  and  $0.091$   $d^{-1}$ , respectively.  $K_u$  and  $K_e$  values  
 489 were reported as  $0.057$   $L\ g^{-1}d^{-1}$  and  $0.049$   $d^{-1}$ ) respectively for *Nereis diversicolor* (PS Rainbow,  
 490 2011) and  $0.165$   $L\ g^{-1}d^{-1}$  and  $0.045$   $d^{-1}$ , respectively for *Arenicola marina*. (Casado-Martinez et  
 491 al., 2010) Higher  $K_u$  values were reported for two different *Hydropsyche* sp. ( $0.350 \pm 0.049$  and  
 492  $0.435 \pm 0.054$   $L\ g^{-1}d^{-1}$ ), though  $K_e$  were comparable to what is reported here for *H. betteni*. (mean  
 493  $0.0731$  and  $0.0532$   $d^{-1}$ ). (Awrahaman et al., 2015) Interestingly, estuarine bivalves were reported  
 494 to have rapid  $K_u$  of  $0.807 \pm 0.129$   $L\ g^{-1}d^{-1}$ , but the efflux rate for As was the highest reported  
 495 ( $0.06 \pm 0.001$   $d^{-1}$ ) among the trace metals investigated (As, Ag, Zn). (Kalman et al., 2014) In  
 496 addition, some species tested in our experiment did not acquire enough measurable As after 2–5  
 497 days to quantitate uptake (*Peltoperla* sp., *Chironomus dilutus*, data not shown). This is in  
 498 agreement with other laboratory observations where some organisms did not acquire As from  
 499 solution (e.g., (Canivet et al., 2001; Dutton & Fisher, 2011)). These results from the literature are  
 500 generally consistent with the degree of interspecies variability reported here, pointing to the need

501 for better characterization of As flux rates in benthic invertebrates, particularly for species or  
502 taxa that are commonly used for biomonitoring.

503 Rough estimates of bioconcentration factors (BCFs) can be obtained by dividing the average  $K_u$   
504 by the average  $K_e$  for a given species. In the small subset of organisms for which both  $K_e$  and  $K_u$   
505 could be derived in this study, BCFs were generally consistent across different taxa (~250) with  
506 the exception of *Maccaffertium sp.* which was much lower (~7). Several other studies also  
507 support minimal accumulation from solution. For example Spehar et al.(Spehar et al., 1980)  
508 found that fish and amphipods exposed to As for 28 days had the same tissue concentration as  
509 controls, and stoneflies and snails had tissue concentrations that resulted in generally low BCFs  
510 ranging from 16—131. EPA(U.S. EPA, 2003) reports BCFs from the literature ranging from  
511 0.048 in the common carp to 14 in stoneflies. Culioli et al.(Culioli et al., 2009) derived BAFs for  
512 different trophic linkages for field collected biota starting from 0.713 for primary producers to  
513 primary consumers and decreasing with each trophic level to 0.005 for invertebrates to trout.  
514 Similarly, data reported by Canivet et al.(Canivet et al., 2001) can be used to estimate 10 day  
515 exposure concentration factors for several aquatic invertebrates ranging from 1.2 in larval  
516 mayflies to 1094 in larval caddisflies, though half of the species tested were on the order of 200–  
517 300-fold above the average concentration in solution. Generally BCFs reported for benthic  
518 invertebrates are lower than those reported for primary producers and estimated here for  
519 periphyton. While there is a great deal of variability across benthic invertebrate species for  $K_u$   
520 and  $K_e$ , and generally modest BCFs, there is still uncertainty and conflicting evidence  
521 (e.g.,(Kalman et al., 2014)) regarding the importance of aqueous exposure in As accumulation.

## 522 **5.0 Summary:**

523 Aquatic invertebrates have been widely used for assessing and monitoring environmental  
524 disturbances,(Hodkinson and Jackson 2005) particularly contamination of aquatic ecosystems  
525 with trace metals and metalloids.(Hare et al. 1991; Cain et al. 1992; Rainbow 2002) In many  
526 cases, only a single species or a handful of species are used for assessment. Our data along with  
527 other research shows there is a great deal of variability in flux rates and assimilation efficiency  
528 not only between species, but among closely related taxa or species with similar feeding  
529 strategies, making it particularly important to identify which species may be best suited for As  
530 monitoring. Our data has also identified periphyton, which has been proposed for biomonitoring  
531 efforts (e.g.,(Ramelow et al. 1987; Rhea et al. 2006)), as an important sink for arsenate. Unlike  
532 for other trace elements that are trophically available from natural periphytic biofilms, As  
533 bioremediation by periphyton may be a viable strategy since there is only modest apparent  
534 trophic transfer and evidence in the literature of biodiminution.(Spehar et al. 1980; Maeda et al.  
535 1990; Chen and Folt 2000; Mason et al. 2000; Dutton and Fisher 2011; Rahman et al. 2012) The  
536 results presented here provide data for the accumulation dynamics of As in periphyton and  
537 invertebrate grazers, which is critical to understanding the behavior of As at the base of aquatic  
538 food webs and potential impacts at higher trophic levels.

## 539 **Recommendations:**

540 We suggest that field biomonitoring studies should carefully consider interspecific differences in  
541 arsenic accumulation dynamics when selecting monitoring species. Further, site-specific  
542 environmental variables should be consistently measured and reported, including concentrations

543 of other important minerals (i.e., Fe) and pH that influence As mobility and bioavailability.  
544 Consistent reporting of these variables and continued efforts to characterize accumulation  
545 dynamics in a broader range of benthic invertebrates will continue to shed more light on  
546 interspecies variability, potential contributions to body burden from food and water, and other  
547 environmental factors that have not yet been investigated thoroughly. This knowledge is critical  
548 to interpreting existing biomonitoring data, as well as understanding the behavior of As at the  
549 base of aquatic food webs and potential impacts at higher trophic levels.

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821

822

**Appendix 1: abbreviations, symbols**

AE	Assimilation efficiency
As	Arsenic
Fe	Iron
Ke	Efflux rate (proportional daily loss)
Ku	Uptake rate constant
XANES	X-ray absorption near edge structure
XRF	X-ray fluorescence

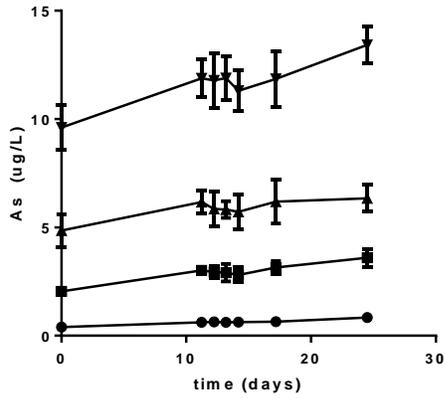
## **Appendix 2: List of presentations and publications (including thesis)**

All presentations and publications resulting from this research are listed below.

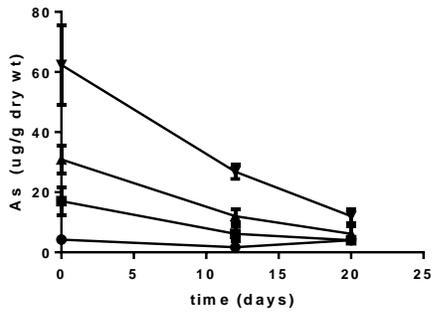
- 825 Harris, A; Buchwalter, D. Coal fly ash constituents at the base of aquatic food webs: Uptake and  
826 efflux of arsenic. Carolina Area Biologists Workgroup. April 1 - 3, 2015. Hot Springs, North  
827 Carolina.
- 828 Harris, A; Buchwalter, D; Hesterberg, D. Dynamic behavior and speciation of arsenic at the base  
829 of aquatic food webs. Society for Environmental Toxicology and Chemistry Annual Meeting.  
830 November 1 - 5, 2015. Salt Lake City, Utah
- 831 Lopez, A; Buchwalter, D; Hesterberg, D.; Webb, S. Trace elements from coal ash at the base of  
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833 North Carolina.
- 834 Lopez, A; Buchwalter, D. Periphyton Bioconcentration and Biotransformation of Arsenic:  
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838 10<sup>th</sup>, 2016. Raleigh, NC.
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840 Freshwater Science, Annual Meeting, May 21-25 2016. Sacramento, CA.
- 841

### Appendix 3: Supplemental Information

A



B



Supplemental Figure 1. Aqueous (A) and dietary (B) exposure conditions for *N. triangulifer* larvae reared for a full lifecycle. Values plotted are mean  $\pm$ SEM; n=3 for each treatment at each time point. Symbols represent

initial nominal dissolved As concentrations of 1, 5, 10, and 20  $\mu\text{g L}^{-1}$  arsenate, respectively.

Supplemental Table 1. Species composition of periphyton plates evaluated June 2009 – December 2009

	Month		
	June–July	October	December
<b>Diatoms<sup>1</sup></b>	<i>Melosira varians</i>	<i>Melosira varians</i>	<i>Melosira varians</i>
	<i>Diatoma vulgare</i>	<i>Cymbella sp.</i>	<i>Gomphonema sp.</i>
	<i>Synedra sp.</i>	<i>Synedra sp.</i>	<i>Nitzschia sp.</i>
	<i>Nitzschia sp.</i>	<i>Nitzschia sp.</i>	<i>Synedra sp.</i>
	<i>Cymbella sp.</i>	<i>Navicula sp.</i>	<i>Fragilaria sp.</i>
	<i>Gomphonema sp.</i>	<i>Achnanthydium sp.</i>	<i>Cymbella sp.</i>
	<i>Fragilaria sp.</i>	<i>Planothydium sp.</i>	<i>Navicula sp.</i>
	<i>Navicula sp.</i>	<i>Frustulia sp.</i>	<i>Diatoma sp.</i>
	<i>Achnanthydium sp.</i>	<i>Cocconeis sp.</i>	<i>Achnanthydium sp.</i>
	<i>Diademesmis sp.</i>	<i>Fragilaria sp.</i>	<i>Asterionella sp.</i>
	<i>Diatoma sp.</i>	<i>Diatoma sp.</i>	<i>Meridion sp.</i>
	<i>Brachysira sp.</i>	<i>Gomphonema sp.</i>	<i>Cyclotella sp.</i>
	<i>Rhoicosphenia sp.</i>	<i>Rhoicosphenia sp.</i>	<i>Planothydium sp.</i>
	<i>Nedium sp.</i>		<i>Cocconeis sp.</i>
	<i>Cyclotella sp.</i>		
<b>Green Algae</b>	<i>Gongrosira</i> or <i>Apatococcus sp.</i>	<i>Spirogyra</i>	<i>Stigeoclonium</i>
	<i>Scenedesmus sp.</i>		
	<i>Monoraphidium sp.</i>		
	<i>Ankistrodesmus sp.</i>		
	<i>Unidentified colonial sp.</i>		
<b>Blue-green Algae</b>	<i>Oscillatoria sp.</i>	<i>Oscillatoria sp.</i>	<i>Oscillatoria sp.</i>
	<i>Pseudanabaena sp.</i>		<i>Pseudanabaena sp.</i>

	<i>Leptolyngbya sp.</i>		
	<i>Phormidium sp.</i>		
	<i>Merismopedia sp.</i>		
	<i>Small unidentified colonial sp.</i>		
<b>Desmids</b>	<i>Staurastrum sp.</i>	<i>Cosmarium sp.</i>	<i>Cosmarium sp.</i>
		<i>Closterium sp.</i>	<i>Closterium sp.</i>
			<i>Staurastrum sp.</i>

<sup>1</sup>diatoms listed generally from most abundant to least abundant

Note: Species composition included in this table is for reference only. Taxonomy was not conducted on periphyton plates used in the experiments presented here, however these compositions are fairly stable by season when periphyton plates of are similar gestation period.

Supplemental Table 2. Taxonomic classification of aquatic invertebrates used to measure uptake from solution ( $K_u$ ), and efflux ( $K_e$ ) along with average body weights

Class	Order	Scientific Name	Average Wet Weight (g)	$K_u$ ( $Lg^{-1}d^{-1}$ )	$K_e$ ( $d^{-1}$ )
Insecta	Ephemeroptera	<i>Isonychia sp.</i>	0.02±0.004	0.05±0.03	0.29±0.07
		<i>Maccaffertium pudicum</i>	0.009±0.003	0.001±0.003	NA
		<i>Maccaffertium sp.</i>	0.06±0.03	0.001±0.004	0.15±0.03
		<i>Ephemerella sp.</i>	0.05±0.007	0.001±0.002	0.08±0.01
		<i>Neocloeon triangulifer</i>	0.003±0.001	0.001±0.0002	NA
	Trichoptera	<i>Hydropsyche betteni</i>	0.035±0.003	0.03±0.2	0.055±0.01
	Coleoptera	<i>Psephenus herricki</i>	0.016±0.008	0.06±0.04	0.03±0.06
	Megaloptera	<i>Corydalus sp.</i>	0.18±0.05	0.06±0.01	-
	Plecoptera	<i>Acroneuria abnormis</i>	0.16±0.05	0.02±0.01	0.09±0.04
		<i>Paragnetina immarginata</i>	0.08±0.02	0.01±0.01	-
Gastropoda	Neotaenioglossa	<i>Pleurocera sp.</i>	0.3±0.08	0.009± 0.01	0.026±0.03
Bivalvia	Veneroidea	<i>Corbicula fluminea</i>	0.9±0.3	0.006±0.004	0.03±0.02

NA = data could not be determined; “-“= not evaluated

## Information Transfer Program Introduction

The Water Resources Research Institute (WRRI) is designed to provide water resources information to a range of stakeholders including private industry, academics, non-profit groups, and governmental entities. WRRI maintains a strong information transfer program by cooperating with various state agencies, municipalities, and professional organizations to sponsor conferences, workshops and other educational events, as well as seeking grants for relevant activities and publishing and distributing research results.

WRRI's newsletter editor resigned to take another position out-of-state during this reporting period; however, WRRI continues to deliver news items as possible on the news section of the website and through updates on social media. A new website, [wri.ncsu.edu](http://wri.ncsu.edu), was developed and launched in August 2015. Six research publications were published during this reporting period. Listservs managed by WRRI reach over 2000 water professionals and students statewide.

WRRI's long-term grant from the Department of Environmental Quality's Division of Energy, Mineral and Land Resources, ended during this reporting year. Through this funding, WRRI annually assisted the Division in meeting its obligations for educating erosion and sediment control (ESC) professionals under the state's Sedimentation Pollution Control Act. WRRI continues to explore opportunities to provide education and training in this topic area but no longer receives the financial support from the Division. During this reporting cycle, WRRI hosted three ESC-themed workshops that reached 286 professionals from around the state.

WRRI continues to administer the NC Urban Water Consortium (UWC) and the UWC-Stormwater Group (SWG), which comprise drinking/wastewater utilities and municipal stormwater programs, respectively. WRRI plays an active role in developing agendas for quarterly meetings for each group (a total of 8 held during this reporting period) that highlight emerging priority research projects in the state, exploring topics of concern for each group, and pursuing opportunities to educate and engage group members to better enable their management activities.

WRRI continues to sponsor continuing education credits by the NC Board of Examiners of Engineers and Surveyors as an Approved Sponsor of Continuing Professional Competency activity for Professional Engineers and Surveyors licensed by the State of North Carolina. In addition, WRRI submits information for approval to the N.C. Board of Landscape Architects to offer contact hours to landscape architects. This allows WRRI to offer Professional Development Hours (PDHs) to engineers and surveyors, and Continuing Education Units (CEUs) to landscape architects for attendance at the WRRI Annual Conference and other workshops, seminars and forums that WRRI sponsors.

During this reporting year, WRRI provided 46.5 PDHs and 32.5 CEUs to 996 people at 11 workshops, seminars, and other events described in the following pages. WRRI is also expanding its reach by offering webinar options for many of its events.

WRRI continues to expand its activities under the umbrella of the Center of Excellence for Watershed Management (CEWM). Through the CEWM, WRRI's Sustainable Waters and Communities Coordinator helps communities identify local opportunities and implement sustainable practices for managing their waters. Community leadership and participation in watershed efforts are paramount to protecting waters, and the CEWM provides services and support for these efforts. The CEWM aids communities by supporting the NC Watershed Stewardship Network (NCWSN), providing tools and training opportunities, and coordinating local watershed specific projects. The NCWSN continues to grow in size, scope and network-sponsored activities. The NCWSN is guided by a Steering Committee of twenty four people from watershed organizations across the state, and is coordinated in partnership with the UNC Institute for the Environment.

# WRRRI Informaiton Transfer Program

## Basic Information

<b>Title:</b>	WRRRI Informaiton Transfer Program
<b>Project Number:</b>	2015NC194B
<b>Start Date:</b>	3/1/2015
<b>End Date:</b>	2/29/2016
<b>Funding Source:</b>	104B
<b>Congressional District:</b>	NC-04
<b>Research Category:</b>	Not Applicable
<b>Focus Category:</b>	None, None, None
<b>Descriptors:</b>	None
<b>Principal Investigators:</b>	Nicole Wilkinson

## Publications

1. Report UNC-WRRRI-447 by Sara McMillan “Nutrient Retention and Floodplain Connectivity in Restored Piedmont Streams” available at [go.ncsu.edu/12-03-W](http://go.ncsu.edu/12-03-W)
2. Report UNC-WRRRI-457 by William Hunt “Performance of Permeable Pavement over a Tight, Clay Soil in Durham, North Carolina: Hydrology, Water Quality and the Calibration and Validation of DRAINMOD” available at [go.ncsu.edu/12-10-SE](http://go.ncsu.edu/12-10-SE)
3. Report UNC-WRRRI-455 by David Buchwalter “Experimental Approaches to Understanding Temperature Responses of Select North Carolina Macroinvertebrates” available at [go.ncsu.edu/13-02-SW](http://go.ncsu.edu/13-02-SW)
4. Report UNC-WRRRI-459 by Matthew Polizzotto “Surface and Subsurface Properties Regulating Manganese Contamination of Groundwater in the North Carolina Piedmont” available at [go.ncsu.edu/13-05-W](http://go.ncsu.edu/13-05-W)
5. Report UNC-WRRRI-453 by Jeffrey Hughes “Aligning Revenue Stability and Water Conservation Goals with New Business Models in Four North Carolina Water Utilities” available at [go.ncsu.edu/13-08-U](http://go.ncsu.edu/13-08-U)
6. Report UNC-WRRRI-454 by Jeffrey Hughes “Why and How to Better Understand Non-Residential Water Customers” available at [go.ncsu.edu/13-08-U](http://go.ncsu.edu/13-08-U)
7. Messier, K. et al. 2015. Estimation of Groundwater Radon in North Carolina Using Land Use Regression and Bayesian Maximum Entropy Environmental Science and Technology. 49(16):9817-9825
8. Down, A., Schreglmann, K., Plata, D., Elsner, M., Warner, N., Vengosh, A., Moore, K., Coleman, D., Jackson, R.B. (2015) Pre-drilling background groundwater quality in the Deep River Triassic Basin of central North Carolina, USA. Applied Geochemistry, 60, 3-13.

## FY 2015 Information Transfer Program Progress & Achievements

### WRRI-SPONSORED WORKSHOPS, FORUMS AND SEMINARS

Below is a list of the educational and training events WRRI sponsored during the project year, along with a description of each and the number of attendees. In total, WRRI provided 46.5 PDHs and 32.5 CEUs to 996 people at 11 education, training and outreach events.

#### ***MULTIPLE DATES - Erosion and Sedimentation Control Planning and Design Workshop***

*Description:* These workshops are structured to educate and familiarize design professionals with the NC Sedimentation Pollution Control Act (SPCA), the rules implementing the Act, design standards for erosion and sedimentation control BMPs, and elements that are necessary to submit an erosion control plan. This comes directly from the source—the NC Division of Energy, Mineral and Land Resources Land Quality Section – and its partners to provide professionals with the information they need to submit an erosion control plan and prevent pollution by sedimentation. Early FY workshops were conducted with funding from the Division, while later FY workshops were conducted by WRRI alone due to cuts in state funding which did not permit the Division to renew its contracts with WRRI to offer these trainings.

*Attendance:*

March 10, 2015 – 81

April 14, 2015 – 67

December 10, 2015 – 138

#### ***March 18-19, 2015 WRRI Annual Conference and NCWRA Symposium***

*Description:* The 17th Annual WRRI Conference highlighted diverse topics in water research, management, and policy in North Carolina. Stantec consulting, whose staff have been long-term partners of WRRI on numerous efforts, sponsored the conference. The event featured concurrent oral presentations, poster presentations, themed panel discussions, and ample networking opportunities. This year's conference also featured new, hands-on interactive sessions for more in-depth discussions and problem solving related to water resources. The conference was again held in conjunction with the North Carolina Water Resources Association (NCWRA) Annual Symposium. This year's symposium title was "Resiliency in Water Resources: What Does it Mean to You?" This conference highlighted WRRI's 50-year history in North Carolina with keynote presentations that reflected on its effective history of contributions to research and engagement that have led to changes in water resources management in NC, and that highlighted the incredible diversity of NC's natural resources that are intimately tied to the quality and quantity of water resources our state enjoys.

*Attendance:* 280 (including 47 student attendees)

#### ***March 31-April 1, 2015 DREAMS Field Trip and Service Activity "From your creek to the coast"***

*Description:* Middle and high school students from DREAMS participated in a field trip to see how stormwater impacts their community, and downstream wildlife. Stops on the trip included planting flowers at Gregory Elementary School's rain garden, and seining (dragging a net in the water) for fish at the coast with NC Coastal Federation. DREAMS is a nonprofit that provides youth in need with high-quality, free-of-charge programming in the literary, visual, multimedia and performing arts.

*Attendance:* 15 youth participants

#### ***April 16, 2015 NC Watershed Stewardship Network Steering Committee Forum on Environmental Justice***

*Description:* The NCWSN Steering Committee invited guests from Partners for Environmental Justice and EPA Region 4 Environmental Justice to participate in a forum about engaging minority and low income audiences in local watershed efforts. The forum was highlighted in a NCWSN blog story afterwards.

*Attendance:* 20

#### ***May 12, 2015 Water Resources Thought Leaders Workshop***

*Description:* WRRRI invited people from private and public organizations to meet at the NC Botanical Garden in Chapel Hill to tap their creative sides and share ideas for innovative alternative strategies to protect water that integrate social, economic, and environmental objectives. In particular they considered, "How can we protect drinking water supply while supporting a prosperous economy and high quality of life for people in the Jordan Lake Watershed?" Participants heard a keynote presentation, *Transforming Our Approach to Water Resources Management*, Trevor Clements, Tetra Tech. Trevor discussed benefits and principles for integrating multiple values into water resource management, and examples of the principles in action. Participants then brainstormed and discussed examples, local successes and resources for advancing innovative water approaches. Three big ideas arose simultaneously across several discussion groups, along with many supporting activities and resources noted.

*Attendance:* 50

***September 21, 2015 NCWRA Seminar, "The Effect of the New Definition of Waters of the US from the U.S.EPA and Army COE in NC"***

*Description:* On June 29, 2015, the US EPA and COE published the final rule for the modified definition of Waters of the US in the Federal Register which will become effective on August 28, 2015 (Federal Register 80(124): 37054-37127). We have conducted an in-depth review of the rule to determine its implications in North Carolina specifically for wetlands and stormwater permitting programs. In general, we expect that there will be minimal change in North Carolina from this new rule since the Corps and EPA have long followed the general approach outlined in the rule. The changes may result in some confusion in North Carolina as well as, potentially, some simplification of the process. Details on the new rule and its effect on the permit application process in NC were presented by John Dorney of Moffatt and Nichol during this talk.

*Attendance:* 89

***December 7, 2015 NCWRA Seminar "Emerging and On-Going Challenges for Water and Wastewater Systems"***

*Description:* This presentation covered an array of emerging and on-going challenges facing water and wastewater systems, the background to those challenges and how the systems need to be addressing them. Challenges discussed included aging infrastructure, climate change, declining rate bases, cyanotoxins, cyber security, disaster preparedness, illegal dumping into wastewater systems, and others.

*Attendance:* 36

***December 15-16, 2015 Tools of Watershed Management: Achieving Your Goals Through Strategy, Action and Engagement***

*Description:* This free two-day workshop was open to anyone who works at the local watershed level and is ready to plan and implement watershed improvement projects in their community. Content included the overall steps for conducting a watershed plan and focused on building the leadership capacity to move from planning to action.

*Attendance:* 22

***February 8, 2016 NCWRA Seminar and Webinar "SCITS, WRAPS and FOATS - New Tools to Satisfy Regulatory Needs and Track Water Restoration and Protection Efforts"***

*Description:* This presentation focused on workflows and tools being developed within the Department of Environmental Quality Division of Water Resources to address 303(d) listed (Impaired) waters in North Carolina. The tools are being developed to streamline the watershed restoration and protection planning process and develop a robust tracking and communications framework. Tools include mobile applications for identifying pollution sources and conveyances and identifying solutions, a calculator for evaluating costs and benefits and return on investment for small and large scale restoration projects. Workflows include online plan status and implementation tracking, connecting projects with funding and interested participants, and importantly tracking the success of projects in the Clean Water Act framework to ease reporting and tell the story of water quality/quantity improvement efforts in North Carolina.

*Attendance:* 53

### ***February 16, 2016 Freshwater in the North Carolina Coastal Plain: Understanding and Preparing for 21st Century Challenges***

*Description:* NC's coastal plain comprises over 40 counties and over 40% of the state's land area. The geography, hydrology and economy of this region create some unique challenges related to access to fresh water supply, drinking water treatment and wastewater management. A lack of centralized information about freshwater and the entities responsible for managing this resource underscores the need for education and communication about issues and solutions. This conference was designed for participants to learn from and interact with some of the state's leading experts in water treatment, field research, utility management and more to help identify solutions for the 21st century challenges.

*Attendance:* 117

## **CENTER OF EXCELLENCE FOR WATERSHED MANAGEMENT**

### ***NC Watershed Stewardship Network***

In FY15, WRRRI continued its commitment to the NC Watershed Stewardship Network (WSN), which was formed through a collaboration in which WRRRI was highly active and engaged, with continued funding for its Sustainable Waters and Communities Coordinator to serve part-time as co-coordinator of the network. During this time period, the WSN launched a website, developed an online mapping and database tool that will help to connect watershed stakeholders from around the state with each other as well as provide access to local watershed data, continued to host steering committee meetings at regular intervals around the state, and finalized a strategic plan and logic model to help guide the network's efforts into the future. In FY 2015, the NCWSN engaged 58 participants in training and networking forums.

### ***Community watershed restoration efforts***

The Sustainable Waters and Communities Coordinator continues to manage two community watershed restoration efforts funded and supplemented by EPA 319 grants and cost-sharing contributed by partnering organizations. These include the Black Creek Watershed Association in the Neuse River Basin and the town of Cary; the Burnt Mill Creek Watershed Initiative in the Cape Fear River Basin and the city of Wilmington; and the Walnut Creek Wetland Community Partnership in southeast Raleigh. These projects involve engaging local municipal and citizen partners in education, installing stormwater control measures to reduce urban runoff, and monitoring impacts. In FY 15, the CEWM engaged 73 people, including eighteen K-12 students, in community projects to protect and restore watersheds. Black Creek watershed volunteers donated 58 hours of time in 2015, for a value of \$1,245. Local contractors were employed to install stormwater control measures in Black Creek.

An additional grant was received in February 2016 for \$143,870, provided by US EPA through the NC Department of Environmental Quality, to partner with a school community, the Town of Cary and homeowners to continue improvements in Black Creek for an additional 2.5 years. An additional proposal to the NC Attorney General Environmental Enhancement Grant program was submitted in an effort to fund activities with the Walnut Creek Wetland Partnership.

## **PUBLICATIONS**

Two peer-reviewed publications from previous WRRRI projects were published during this reporting period.

FY 2012 project "Establishing a Pre-drilling Baseline of Water-Quality Measurements in North Carolina Before Shale-gas Extraction" by PI Robert Jackson: Down, A., Schreglmann, K., Plata, D., Elsner, M., Warner, N., Vengosh, A., Moore, K., Coleman, D., Jackson, R.B. (2015) Pre-drilling background groundwater quality in the Deep River Triassic Basin of central North Carolina, USA. *Applied Geochemistry*, 60, 3-13.

FY2011 project "Space/time geostatistical estimation of nitrate and radon groundwater contaminants" by Marc Serre published: Messier, K. et al. 2015. Estimation of Groundwater Radon in North Carolina Using Land Use Regression and Bayesian Maximum Entropy *Environmental Science and Technology*. 49(16):9817-9825

WRRRI published six internal research reports during this reporting period.

- Report UNC-WRRRI-447 by Sara McMillan “Nutrient Retention and Floodplain Connectivity in Restored Piedmont Streams” available at [go.ncsu.edu/12-03-W](http://go.ncsu.edu/12-03-W)
- Report UNC-WRRRI-457 by William Hunt “Performance of Permeable Pavement over a Tight, Clay Soil in Durham, North Carolina: Hydrology, Water Quality and the Calibration and Validation of DRAINMOD” available at [go.ncsu.edu/12-10-SE](http://go.ncsu.edu/12-10-SE)
- Report UNC-WRRRI-455 by David Buchwalter “Experimental Approaches to Understanding Temperature Responses of Select North Carolina Macroinvertebrates” available at [go.ncsu.edu/13-02-SW](http://go.ncsu.edu/13-02-SW)
- Report UNC-WRRRI-459 by Matthew Polizzotto “Surface and Subsurface Properties Regulating Manganese Contamination of Groundwater in the North Carolina Piedmont” available at [go.ncsu.edu/13-05-W](http://go.ncsu.edu/13-05-W)
- Report UNC-WRRRI-453 by Jeffrey Hughes “Aligning Revenue Stability and Water Conservation Goals with New Business Models in Four North Carolina Water Utilities” available at [go.ncsu.edu/13-08-U](http://go.ncsu.edu/13-08-U)
- Report UNC-WRRRI-454 by Jeffrey Hughes “Why and How to Better Understand Non-Residential Water Customers” available at [go.ncsu.edu/13-08-U](http://go.ncsu.edu/13-08-U)

### ONLINE RESOURCES

WRRRI overhauled its website and launched a new version, [wrrri.ncsu.edu](http://wrrri.ncsu.edu), in August 2015. This revision brought WRRRI’s site into alignment with NC State University’s branding efforts, reflects current trends in website appearance and functionality, and is a great improvement in how WRRRI showcases its impacts and achievements. WRRRI also expanded its online presence through the creation of a twitter account (@NC\_WRRRI), through which it shares WRRRI-generated research results, news items, and other relevant water-related information.

### WRRRI ELECTRONIC LISTS

WRRRI maintains the following electronic mail lists (listservs) for information transfer purposes, which reach a combined total of almost 2000 people statewide:

- **Water-Research list** — informs water researchers from NC universities about calls for papers, grants, upcoming conferences, student internships, etc.;
- **WRRRI-News list** - informs researchers, local governments, municipalities, interest groups etc. about calls for papers, grants, upcoming conferences and events, etc.;
- **NCWRA-info list** - provides information of the North Carolina Water Resources Association sponsored events;
- **Sediments list** - used to disseminate erosion and sedimentation control information in North Carolina;
- **Watershed Stewardship Network (WSN) list** – provides watershed professionals, volunteers and stakeholders throughout the state with a mechanism to contact, network, and learn from each other as well as to learn about the WSN and its offerings;
- **Urban Water Consortium (UWC) list** for Urban Water Consortium member communications;
- and **UWC-Stormwater Group list** for the UWC Stormwater Group member communications.

### NC URBAN WATER CONSORTIUM

WRRRI administers the NC Urban Water Consortium (UWC) and meets with the members quarterly. The consortium was established in 1985 by the Institute, in cooperation with several of North Carolina's larger cities to provide a program of research and development, and technology transfer on water problems that urban areas share. Through this partnership, WRRRI and the State of North Carolina help individual facilities and regions solve problems related to local environmental or regulatory circumstances. Participants support the program through annual dues and enhancement funds and guide the program through

representation on an advisory board, selection of research topics, participation in design of requests for proposals, and review of proposals. There are 12 member cities/special districts in North Carolina, and members hosted four quarterly meetings throughout the state in FY15.

The UWC also provided financial support to two research projects, which increased WRRRI's ability to fund other high quality research with 104(b) funds. The two projects funded by the UWC in FY 15 were:

- “Microbial Quality and Health Risks of Alternative Surface Sources of Drinking Water Impacted by Waste Water” by PI Mark Sobsey of UNC-Chapel Hill
- “Improving Startup and Operation of Anaerobic Co-Digestion of Grease Interceptor Waste” by PI Tarek Aziz of NC State University

#### **NC URBAN WATER CONSORTIUM - STORMWATER GROUP**

In 1998, several members of the NC UWC partnership formed a special group to sponsor research and technology transfer on issues related to urban stormwater and management. The Urban Water Consortium (UWC) Stormwater Group is administered by WRRRI. Participants support the program through annual dues and enhancement funds. They guide the program through selective representation on the WRRRI advisory board, determining stormwater-related research priorities, participation in the design of requests for proposals and review of proposals submitted to WRRRI directly or to the SWG. Four meetings were hosted by rotating SWG members throughout the state during the reporting year.

The SWG also provided continued financial support for the second phase of a research project that was chosen out of the special RFP that NC WRRRI helped the group create and evaluate. The project is entitled:

- “Biological Condition in NC Urban Streams Phase II: Predictors of biological condition in urban NC streams and development of management options for urban stream aquatic life uses” by PI Michael Paul of Tetra Tech

WRRRI has worked directly with the PI and the SWG member municipalities on this project to ensure that the results are actively being translated and transferred to state agency staff with a high interest and need for these results to inform future stream monitoring and management efforts. WRRRI facilitated a meeting with staff from across divisions and units within the NC Department of Environmental Quality, as well as a co-developed a participatory session at the 2016 WRRRI conference aimed at further developing this project to maximize the usefulness of the results.

# USGS Summer Intern Program

None.

<b>Student Support</b>					
<b>Category</b>	<b>Section 104 Base Grant</b>	<b>Section 104 NCGP Award</b>	<b>NIWR-USGS Internship</b>	<b>Supplemental Awards</b>	<b>Total</b>
<b>Undergraduate</b>	2	0	0	4	6
<b>Masters</b>	3	0	0	3	6
<b>Ph.D.</b>	2	0	0	2	4
<b>Post-Doc.</b>	1	0	0	3	4
<b>Total</b>	8	0	0	12	20

# Notable Awards and Achievements

## WRI PARTICIPATES IN DUKE UNIVERSITY CAREER FAIR

WRI's Coordinator for Research and Outreach was invited to participate in a career fair at Duke University's Nicholas School of the Environment in September 2015. Through tabling at this event, WRI interacted with approximately 30 graduate students, primarily those seeking a master's degree in environmental management. WRI helped students understand the range of water-related careers and advised on courses and skills to pursue that can enhance career success. Following the tabling portion, WRI then participated in a panel with two other water professionals in a roundtable setting with 15 students for more intimate discussion about the water sector and mentoring about interviews, job searches, professional interactions, and more.

## STUDENT AWARDS AND ACHIEVEMENTS

Several students supported through WRI-funded research received awards for their efforts. These include: - 1st place winner at the NCSU Latin American Research Symposium awarded to Catalina Lopez-Velandia for her poster entitled "1,4-Dioxane: Occurrence, sources, and treatment options for an emerging surface water contaminant," February 19, 2016 (issued by NC State University). - 2nd Prize in the Best Poster Award Competition at the North Carolina AWWA-WEA Annual Conference awarded to Catalina Lopez-Velandia for her poster entitled "1,4-Dioxane: Occurrence and treatment options for an emerging surface water contaminant," November 16, 2015 (issued by NCAWWA-WEA) - Master's student, Peter Blum, received the best poster presentation award at a meeting of Carolinas Chapter of Society of Environmental Toxicology and Chemistry held at NC State University in April, 2015. Mr. Blum recently graduated from the UNC-Greensboro master's program and will continue on to pursue his PhD in the UNC-G Department of Biology.

## WRI-SPONSORED RESEARCH LEADS TO ADDITIONAL FUNDING AWARDS

Dr. Detlef Knappe's research on 1,4-Dioxane lead to an additional \$50,000 RAPID/GOALI grant from the National Science Foundation entitled "Sources of 1,4-Dioxane in the Cape Fear River Watershed of North Carolina and Treatment Options for 1,4-Dioxane Control"

Jessica Brandt, a PhD student working on Dr. Rich Di Giulio's research project entitled "Legacy impacts of coal combustion residues on freshwater ecosystems in North Carolina," received a Duke University Health Scholars Award of \$5,000. Ms. Brandt was also awarded the U.S. Environmental Protection Agency's Science to Achieve Results (EPA STAR) Fellowship, with this project described as dissertation research for fellowship proposal. The fellowship is in the amount of \$132,000 for 3 years of tuition, fees, stipend, and travel/supply allowance.

## WRI-SPONSORED RESEARCH LEADS TO CHANGES IN CONTAMINANT INPUTS TO NC WATERWAYS

As a result of funding awarded to Dr. Detlef Knappe by WRI, a working group was formed that includes representatives from the North Carolina Department of Environmental Quality (DEQ), drinking water providers impacted by 1,4-dioxane, wastewater managers from communities with elevated 1,4-dioxane levels in wastewater, and NC State University researchers. Results of this research informed the working group about the location of 1,4-dioxane discharges and led to the initiation of voluntary source reduction efforts in communities from where 1,4-dioxane originates. In addition, DEQ has begun to revise National Pollutant Discharge Elimination System (NPDES) discharge permits for municipal wastewater treatment plants in

municipalities in which wastewater contains high levels of 1,4-dioxane.

#### WRRRI CONTRIBUTES TO NC'S NUMERIC NUTRIENT CRITERIA DEVELOPMENT EFFORTS

The North Carolina Department of Environmental Quality (NCDEQ) is currently working with the U.S. Environmental Protection Agency, Albemarle-Pamlico Estuary Partnership (APNEP), and stakeholders to develop numeric nutrient criteria in all state waters to further reduce nutrient pollution. In 2015-16, APNEP partnered with WRRRI's Law, Policy, and Community Development Specialist to conduct a study on the potential legal issues with developing numeric nutrient criteria and barriers to implementation. This study included an analysis of numeric nutrient criteria efforts in other states, focusing on their policy challenges and successes. A final report was submitted to NCDEQ in January 2016. After submitting the final report, the Law, Policy, and Community Development Specialist and her research assistant presented the study's results to the NCDEQ nutrients workgroup at its January 2016 meeting. The Specialist and her research assistant also were invited to submit an article on this topic for a special issue of the Duke University School of Law Environmental Law and Policy Journal. A draft version of the article is currently in review, and the Specialist presented on the topic at Duke's Environmental Law and Policy Symposium on February 6, 2016. Publication of the special issue of the journal is expected in 2016. WRRRI's Deputy Director also serves on the Nutrient Criteria Implementation Committee, where he is able to represent both NC WRRRI and NC Sea Grant and share perspectives and priorities regarding available and needed research.

#### SERVICE ON BOARDS AND COMMITTEES

WRRRI team members are actively engaged in board and committee activities around the state where they bring expertise and perspective to efforts to address NC's water issues. WRRRI is represented on the following:

- NC Water Resources Association Board of Directors
- NC Sedimentation Control Commission
- NC Nutrient Criteria Implementation Committee
- NC Defense Coastal/Estuarine Research Program Regional Coordinating Committee
- National NIWR-USGS Partnership Committee
- Greater Triangle Stewardship Development Association Board of Directors