

Surface water quality and ecosystem health with shale energy development

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Issue

The development of shale energy in Ohio and throughout the world has dramatically increased in recent years without full understanding of the impacts to surface water quality and, in turn, total ecosystem health. Shale energy exploration and extraction activities, including the construction and traffic use of access roads and well pads, can produce sediment that may runoff to surface waterbodies. Sediments carry metals, chemicals, and nutrients that when released may disrupt aquatic microbial communities and encourage the growth of toxic algae. Besides the disturbance to aquatic ecosystems, increased sediments and associated algae, especially blue green algae, may affect the health of terrestrial systems as humans and animals interact with surface waters.

Research Objectives

The overall goal of this research is to characterize the impacts of shale development to surface waters and ecosystem health. We will accomplish this with two main objectives:

- 1) Determine the changes to stream water quality with development of shale energy within a watershed in eastern Ohio, and
- 2) Evaluate the response of microbial communities to environmental stresses, such as changes in sediment loading and water quality.

Methodology

Beginning in June 2014, we installed equipment in perennial streams at the mouth of two small watersheds on property owned and managed by The Ohio State University, OARDC in Noble County Ohio. This equipment continuously measures and records water quality parameters including temperature, dissolved oxygen, conductivity, pH, and turbidity. We surveyed the stream channel at each gauging site and installed equipment that continuously measures flow velocity and depth and uses the channel dimensions to determine stream flow. All collected data are sent via cellular modem to a publically accessible website for viewing or download.

Beginning in March 2014 we took monthly grab samples at each site that were transported to an EPA certified lab and analyzed for total suspended solids (TSS). From June to October 2014, additional grab samples from the streams were analyzed for nutrients and microbes.

Findings and significance

This research was designed with the intent of comparing water quality measures and stream microbial communities in paired watersheds before, during and after shale energy development in one of the watersheds (treatment watershed). The timeline for drilling at the study site has been postponed. Although this development is a disappointment to the researchers, we feel confident that we have measured and recorded baseline data regarding water quality parameters and microbial communities in these watersheds and have the infrastructure ready to begin monitoring activities if or when shale energy development at the site begins. The work is summarized below.

ABSTRACT

Counties in Eastern Ohio within the Utica-Point Pleasant shale formation have experienced increased development of shale oil and gas. These operations have created areas of bare soil and dirt roads with high volumes of traffic. Soil erosion from these intensely used sites can lead to greatly increased sediment concentrations in waterways. The objective of this project was to examine how sediments in water affect the presence of microbes. The research also contributes baseline data to a larger project assessing the effects of shale development on water quality. Sedimentation has been recognized as the number one impairment of streams in the U.S., bringing with it negative environmental and human health consequences. Sediments may foster and transport bacteria, chemicals and other microbial organisms. The hypothesis of this project was that increased sediment in stream water would correlate with increased detection of *E. coli*. Baseline testing for cyanobacteria was also conducted. It was predicted that changing aquatic conditions including sediment levels caused by shale development would contribute to a shift in the aquatic cyanobacteria communities and be favorable to the species *M. aeruginosa*. The three adjacent watersheds for this study are located within The Ohio State University, Eastern Agricultural Research Station (EARS) in Noble County, Ohio. The site historically was mined for coal in the 1960s, but was reclaimed through the 1990s and is currently a site for livestock research. Most of the EARS property is contained within a larger basin that drains directly to the municipal water reservoir for the city of Caldwell, Ohio. Water samples were collected five times from each watershed and tested for turbidity, *E. coli*, phycocyanin and chlorophyll *a*. Additionally, real-time qPCR was used to detect genetic markers for human specific fecal contamination, ruminant specific fecal contamination, the phycocyanin intergenic spacer region (PC-IGS) of *Microcystis aeruginosa* and the microcystin synthetase gene A (*mcyA*) of

Microcystis aeruginosa. Finally, microcystin was tested using ELISA. Results show correlations between high turbidity and high *E.coli* counts ($R^2=0.50$, $p = 0.032$). A similar linear relationship between turbidity and chlorophyll *a* was also found ($R^2 = 0.59$, $p = .04$). *Microcystis aeruginosa* and microcystin were not detected. Further microbial profiling is recommended to gather more information on the overall microbial community structure in conjunction with their transport via sediments.

1. INTRODUCTION

1.1 Sediment

Sedimentation is a natural process that happens when particles settle to the bottom of a water body. Sediments are either suspended in the water column or deposited on the bed of the channel. The amount of suspended and bed load sediment is influenced by the energy and velocity of the water (FEM 2015). Most sediment in surface waters are soil particles (sand, silt and clay) and minerals from surface erosion processes, but may also include decomposing organic matter such as leaf litter or algae. Suspended solids refers to both minerals and organics while suspended sediments refers to just the mineral fraction of the solids load (Ongley 1996). Water bodies naturally contain sediments, but erosion and sedimentation rates have drastically increased due to modern agriculture practices, urban land-use changes, timber harvesting, construction and other anthropogenic disturbances. Sedimentation is the primary cause of stream impairment and the single greatest non-point source pollutant in the United States (EPA 2006a).

Suspended sediment concentration (SSC) is measured by weighing the dry mass of particulate in the water sample (Marquis 2005). This method of measurement requires lab equipment such as a vacuum filtration apparatus, filters and a drying oven. It is time consuming to transport, filter and weigh samples and is inconvenient for long term data. A more common and efficient way to estimate the amount of suspended sediment in water is with turbidity. Turbidity measures the degree to which light is scattered by particles suspended in a liquid in Nephelometric Turbidity Units (NTU) (USGS 2013). Different bodies of water such as streams, rivers or lakes will have different normal turbidity levels depending on the discharge, bedrock, and other conditions (CWT 2004). Dissolved organic materials such as decaying vegetation and humic matter can color the water and also contribute to turbidity (FEM 2015).

Sediments in water pose environmental, industrial and human health consequences. Suspended sediments can interfere with predator prey dynamics by reducing visibility in water (Vinyard et al. 1976) and can irritate fish gills (Marquis 2005). Increased sedimentation can divert traditional migration patterns and disrupt fish spawning by filling in the cracks of gravel beds where many organisms lay their eggs (Coen 1995). Contaminants attached to fine grained sediments are ingested by filter feeder species and biomagnified to higher trophic levels in the food chain (Schubel 1997). Suspended sediment also reduces the amount of sunlight that penetrates the water, allowing for less photosynthetic production of oxygen by underwater plants (Schubel 1997). Particles eroded from agricultural lands carry nutrients such as nitrogen and phosphorous that when released may encourage excessive algae growth. Subsequently, decomposition of organic material such as algae depletes oxygen levels and create hypoxic conditions detrimental to aquatic organisms (Burkholder 1992).

1.2 Sediments and Drinking Water

While it is possible to remove large sediments that cause physical damage to water treatment machinery, many small particles interfere with drinking water cleaning techniques. Sediments shield pathogens from disinfection processes like chlorination and ultraviolet irradiation (Marquis 2005). Turbidity in drinking water was once seen as harmless color, but studies have strongly linked suspended sediment to the presence of pathogens (WHO 2004). Sediments can promote the growth of pathogens by providing them food, shelter and transportation, and can lead to waterborne disease outbreaks (EPA 1999). Other studies have shown correlations between lowering turbidity and the removal of *Cryptosporidium* and *Giardia* as shown below in Figure 1 (LeChevallier et al. 1992).

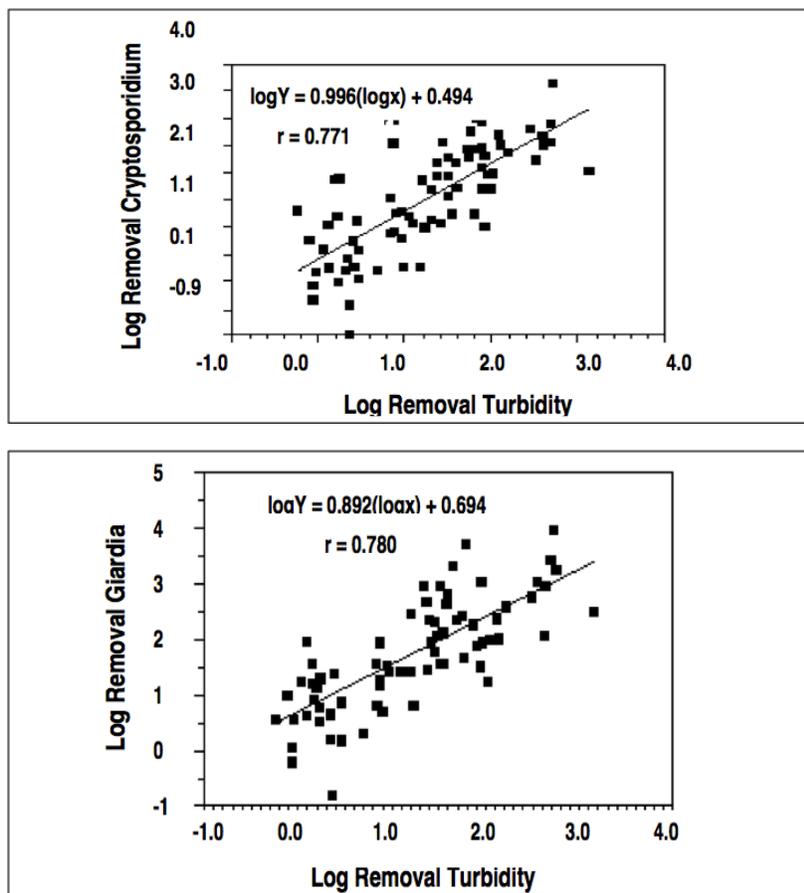


Figure 1. Relationship between removal of turbidity and removal of *Cryptosporidium*, and removal of turbidity and removal of *Giardia*. From LeChevallier et al. (1992).

1.3 *Escherichia coli*

During 2012, there were 831 foodborne disease outbreaks reported, responsible for 14,972 illnesses, 794 hospitalizations, and 23 deaths (CDC 2012). Some of the most common pathogens that cause outbreaks live in animal digestive tracts and are spread by the movement of water that has been contaminated with fecal matter. A safe and efficient way to test waters for fecal contamination is with an indicator organisms such as coliform bacteria or *Escherichia coli* (*E. coli*). The presence of these nonpathogenic indicators proposes that other disease-causing bacteria, viruses and protozoa that also live in animal intestines may be there as well (EPA 2012). Though most *E. coli* strains are harmless, there are Shiga-toxin producing strains (STEC)

that are pathogenic, such as O157:H7. These toxic strains originate in the guts of ruminant species, primarily cattle, and were responsible for 5% of the total etiological outbreaks in 2012 (CDC 2014; CDC 2012).

Total coliforms are a group of bacteria that are commonly found in nature and used as a broad indicator of bacterial contamination in drinking water sources. The EPA recommends that *E. coli* be used as an indicator in recreational waters because it is a more fecal-specific coliform bacterium (EPA 2012). Though *E. coli* is still commonly used across the United States, studies have shown that *E. coli* may be an active member of the microbial community and therefore not an effective indicator species. *E. coli* was thought to survive poorly exposed to stresses in the open environment, but has been found in soil, sediment and algal samples. One study repeatedly found isolated *E. coli* that had been outside of an animal host for longer than the bacteria has ever been expected to survive. This may provide evidence that *E. coli* have become “naturalized” members of the soil microbial community (Ishii & Sadowsky 2008).

Microbial source tracking methods have been designed to trace the origin of fecal contamination. There are genotypic, microbiological, phenotypic and chemical methods available. This project used the genotypic method of detecting host-specific genetic markers. This method is advantageous because it does not require culturing the organisms and can differentiate pathogenic properties of specific bacteria (Scott et al. 2002).

1.4 *Microcystis*

Another microbial concern in aquatic systems is cyanobacteria that, when grown in excess, can cause harmful effects (EPA 2015a). Cyanobacteria are generally referred to as ‘blue-green algae’ due to their coloring and appearance, but are not algae. The blue color comes from other accessory pigments such as phycocyanin, which work with the green pigment chlorophyll *a*

to contribute to photosynthesis. Cyanobacteria produce a suite of secondary metabolites including hormones, allelochemicals and toxins that can also cause human health concerns (Carmichael 1992).

The most common cyanobacteria species come from the genus *Microcystis* and are capable of producing microcystin toxins (MC). The toxins are released into water when a bacterial cell dies and ruptures. Ingestion or topical exposure to MCs can have serious health effects on humans, aquatic organisms, birds and land animals. MCs accumulate in fish and bird livers and can be taken up by fish embryos that interfere with development and hatching (California EPA 2009). The ability of *Microcystis* species to utilize nitrogen gas and photosynthesize light has allowed them form symbiotic relationships with plants, animals and other organisms. It also allows them to outcompete other aquatic phytoplankton species. *Microcystis* can be found in extreme aquatic and terrestrial environments as well as a wide range of latitudes, making them the most dominant phytoplankton group in eutrophic freshwater bodies throughout the world (Oberholster et al. 2004).

An increase in sediment load in a body of water can enact a dangerous feedback system of cyanobacterial and algal growth (Vahtera et al. 2007). Sediments carry nutrients (particularly phosphates) in water and under low dissolved oxygen conditions, sediments release the nutrients into the water column (Schubel 1997; NSW 2009). These large loads of what is usually the limiting nutrient allow algae to grow in excess. Cyanobacteria blooms are caused by a variety of factors including nutrient ratios, temperature, light attenuation and organic matter availability and are difficult to predict (EPA 2015c).

Excess algae growth eventually reaches a carrying capacity. Large quantities of decomposing algae absorb oxygen, create even worse hypoxic (low dissolved oxygen)

conditions, and allow for even more nutrients to be released from sediments. Hypoxic conditions have also been observed to cause decreased nitrogen levels. With nitrogen limited systems, nitrogen loads carried in urban and agricultural runoff water induce exponential algal growth (Vahtera et al. 2007). This feedback system can spiral to create large-scale algal blooms that have many devastating effects on aquatic ecosystems.

1.5 Quantification of Cyanobacteria and Cyanotoxins

Some species within the *Microcystis* genus produce toxins and some do not. Furthermore, not all *Microcystis* species produce the same levels of toxins, and toxicity can vary over time within a bloom. This means that the quantity of cyanobacteria cells does not directly correlate the quantity of toxin produced. *Microcystis* produces the most toxins of all cyanobacteria, and though there are various kinds of MCs from different strains of *Microcystis*, all have been found to be hepatotoxic with similar signs of poisoning (Sivonen et al. 1990). The most common and most studied freshwater cyanobacteria species is *Microcystis aeruginosa*. The MC that this species produces is a hepatotoxin peptide that causes liver failure and cancer in animals and humans (Carmichael 1992).

Directly measuring *Microcystis* and MC requires significant resources and time. Advanced methods have been developed over the years including a MC-specific enzyme-linked immunosorbent assay (ELISA) that directly detects MC (Chu et al. 1989). More recently, quantitative polymerase chain reaction (qPCR) methods have been used to amplify MC producing genes. The MC synthase complex that controls MC production can be found in a cluster of genes called the *mcy* operon, ranging from genes *mcyA* through *mcyJ* (Sevilla et al. 2008). Detection using qPCR analyses usually test for only one *mcy* gene, though not all *Microcystis* cells have them (Ouellette & Wilhelm 2003).

Advances in technology still require transport time and expensive lab equipment and are not practical for rapid screening of drinking or recreational waters. Indirect measures of MC such as bacteria biomass may be inaccurate since not all bacteria strains and cells produce toxins. Alternatively, non-molecular approaches have been developed such as quantification of phycocyanin pigment via fluorescence (Marion et al. 2012). Phycocyanin, a blue pigment that harvests light, is more telling of the presence of *Microcystis* in water than the green pigment chlorophyll *a*, which other algae also contain (Ahn et al. 2002).

1.6 Regulation of Cyanobacteria and Cyanotoxins

Algal blooms and toxicity levels are water quality issues affecting every continent, and HAB occurrence is predicted to increase as the climate warms (EPA 2015c). This poses a human health concern as well as an ecological hazard for wildlife. Cyanobacteria and toxins have been studied for decades, dating back to the early 1980s, but continue to reveal contradictory results. Modern technology has allowed for more accurate detection of *Microcystis* and MC, but their ecological role and rates of growth are still unclear. Environmental conditions such as temperature, light intensity, soluble metals, suspended sediments, available nutrients, nutrient ratios, precipitation events, water flow, pH and seasonal variability have all been studied with many contrary outcomes (Van der Westhuizen and Eloff 1985; Kromkamp et al. 1989; Sevilla et al. 2008; Harding and Wright 1999; Pimentel and Giani 2014; Zhu et al. 2014; Schatz et al. 2007).

The EPA published its latest guidelines and recommendations about cyanobacteria and cyanotoxins in June of 2015. The drinking water standard was set at 1.0 µg/L, a recreational low risk threshold of 6 µg/L, and a recreational moderate risk standard at 20 µg/L (EPA 2015). These

are low, short-term risk levels and exposure can cause skin irritation and acute gastrointestinal illness (EPA 2015b).

1.7 Sediments and Microbes

Microbes survive and are transported on sediment particles. The fluvial patterns and sediment type dictate the fate of microbes in alluvial systems. The literature reveals a general consensus that the majority of enteric bacteria in alluvial systems are associated with sediments (Wilkinson et al. 1995; Jamison et al. 2004; Costerson 1978; Auer and Niehaus 1993). There have been a number of field studies that have modeled the re-suspension of sediments during storm flow conditions and the subsequent degradation of water quality (McDonald et al. 1982; Sherer et al. 1988; Nagels et al. 2002). Most of the models that have been developed track indicator fecal bacteria such as *E. coli*, but are limited by the many factors that need to be mathematically represented.

1.8 Sediments and Shale Development

Natural gas extracted from deep shale reserves is increasingly in demand in the United States and around the world. Recent technological developments in horizontal drilling have made extraction faster and easier in unconventional oil reserves (Vidic et al. 2013). The Marcellus and the Utica-Point Pleasant formations are the main targets of the approximately 15,000 wells in Ohio that have used hydraulic fracturing since 1990. The number of permits for Utica-Point Pleasant wells in Ohio has skyrocketed from one single well in 2010, to 1,443 wells today (ODNR 2015).

The development of shale can be detrimental to the quality of surface water bodies. Sediments erode from the construction of well pads, installation of pipelines, and from heavily trafficked roads (Kiviat 2013). Erosion is a natural process, but the activities of hydraulic

fracturing can elevate levels of sediment in runoff water, thereby affecting surface water. Well pads, pipeline corridors, and gravel or dirt roads, especially used during wet weather, can be major sources of fine sediments in surface waters (Toman et al. 2011).

1.9 Objective and Hypothesis

The objective of this research was to identify a connection between environmental and public health concerns by correlating suspended sediment concentrations with microbial populations in three small streams in eastern Ohio. The hypothesis was that increased suspended sediment concentrations in stream runoff would cause an increased number of microbial cells found. This study measured turbidity at the mouth of three watersheds to see how strongly it correlated with *E. coli*, and how it would affect a shift in the cyanobacteria community. Results will reveal if other microbes associate with sediments in a way similar to *E. coli*. Furthermore, the DNA archived from the water samples will be saved as baseline data for further studies.

2. METHODS

2.1 Study Location

The research was located at The Ohio State University's Ohio Agriculture Research and Development Center's (OARDC) Eastern Agricultural Research Station (EARS) in Noble County, Ohio. Representative of the eastern Ohio landscape, the 2000-acre EARS site is comprised of agricultural land including grazing pastures for sheep and cattle, rolling hills, steep valleys and distinctive soils. The EARS property contains three watersheds that drain into Caldwell Lake and are surrounded by the cities of Caldwell and Belle Valley (Figure 2). This area has no history of cyanobacteria blooms, but the potential use of the site for hydraulic fracturing could create aquatic conditions favorable to their survival. The watershed on the eastern side of the property contains an intermittent headwater stream (Afton Creek) and is

characterized by a variety of land uses including agricultural pastures, forest, roads and some residential homes. Middle Creek watershed running through the center of EARS property has similar land use but is much larger (2.13 km² as compared to 0.86 km² in Afton Creek Watershed). Middle Creek is a 3rd order perennial stream with about half of the drainage area (48%) outside of the EARS property. The watershed on the western edge of EARS (Hickory Creek) has the smallest by area (0.27 km²) and is fed by springs that are located in an open cattle pasture. The watersheds differ in their size, road density and land use. These variables will be compared to the concentration of sediment measured.

Maps of the watersheds were made in ArcGIS using the NAD 1983 UTM Zone 17N projection, and the watersheds were defined using a USGS interactive map, Ohio StreamStats (USGS 2014). The EARS property boundary was found using Report All Real Estate Portal property parcel maps and confirmed by documents from the Nobel County Auditor, Mike Stritz.

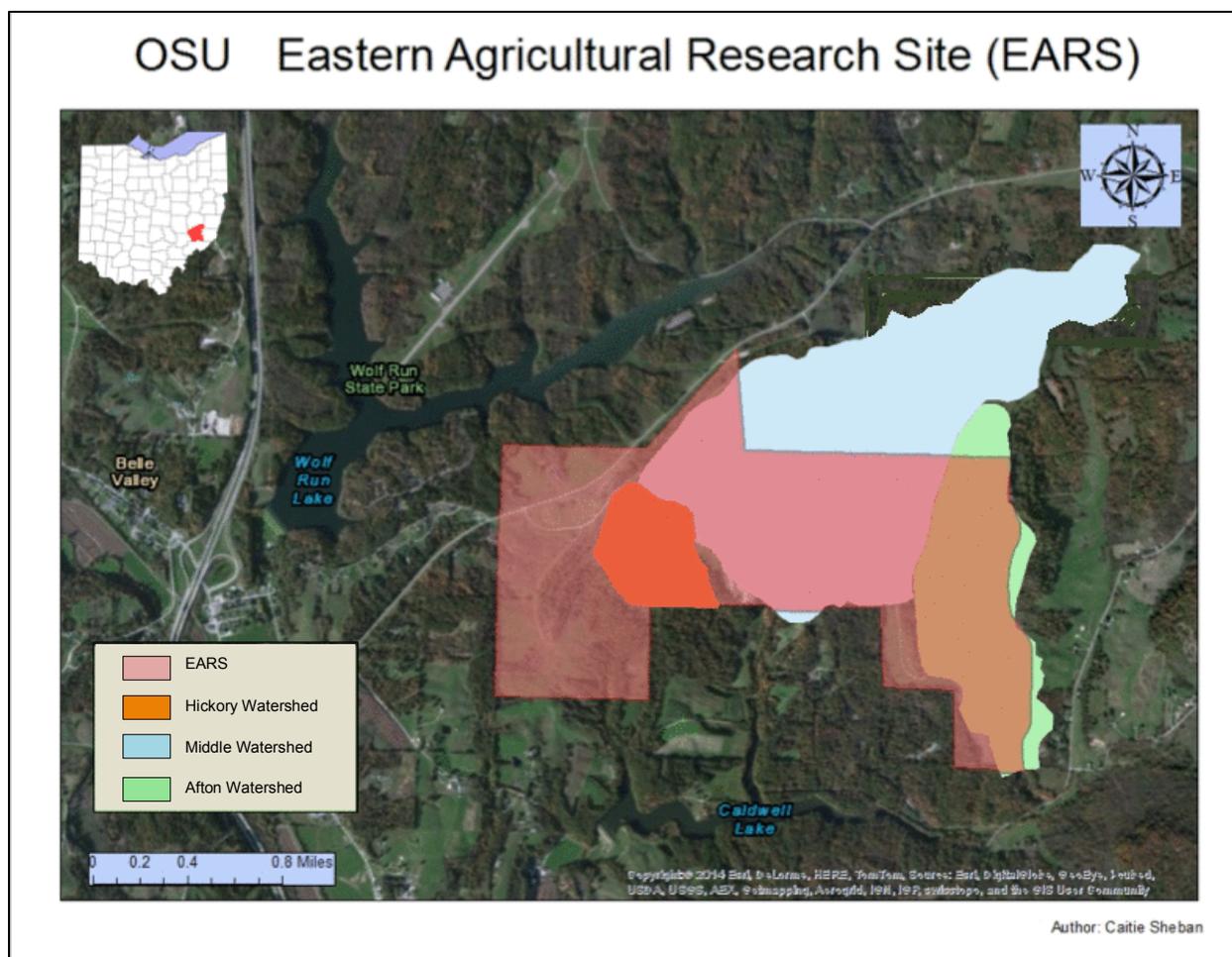


Figure 2. Boundaries of EARS research site and the three watersheds of interest with an inset of Noble County, Ohio.

Currently EARS is an active livestock operation with a research focus on management practices for beef cattle and sheep. The animal herds are rotated among pastures within all three watersheds. Service roads inside the property are constructed with unbound aggregate (sand, dirt and gravel with no binding agent) and are used by tractors, four-wheelers and trucks. Road location and land use for the watersheds are shown in Figure 3. Road length, watershed size, and land cover data are summarized in Table 1.

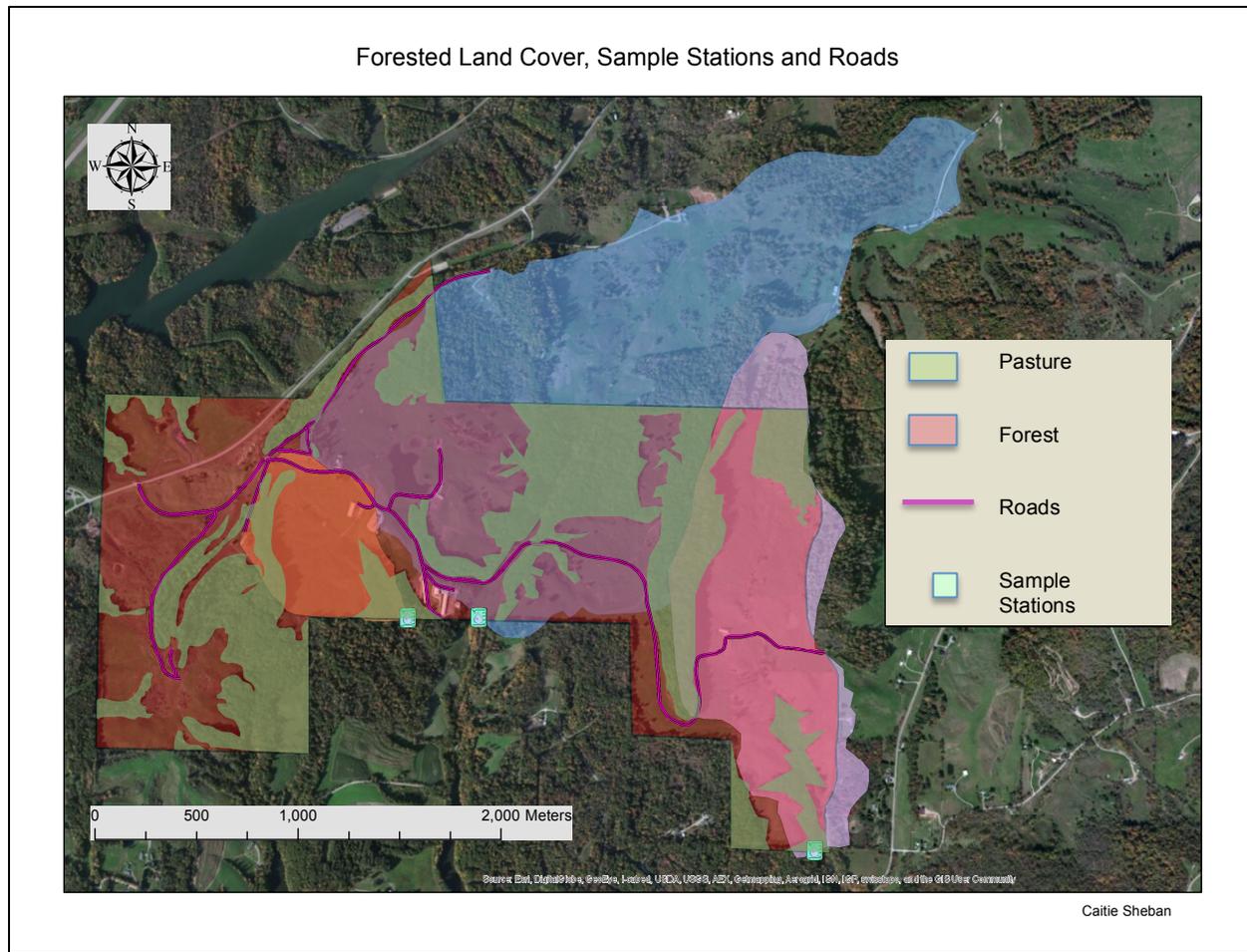


Figure 3. EARS property boundaries with red highlighting pasture land and green highlighting forest cover. The pink lines represent the service roads and sample stations are marked at the bottom of each watershed.

Table 1. Length of road, total area, area in EARS and proportions of pasture and forested land cover for each watershed.

Watershed	Length of Road (km)	Area (km²)	Area inside EARS property (sq km²)	% Forest	% Pasture
Afton	1.87	0.86	0.70	31	69
Middle	2.47	2.13	1.03	42	58
Hickory	0.68	0.27	0.27	24	76

While EARS is not currently being developed for horizontal hydraulic fracturing of shale oil and gas, there are many neighboring properties that have old and active wells. As of February 2015, there were 45 active wells in Noble County, 70 in neighboring Monroe County and 40 in Guernsey County. Figure 4 shows a map of the well sites (white squares) northeast of EARS that are: currently drilling (red), producing oil and gas (green), permitted (yellow) and drilled but not yet producing (gray). The lines represent underground horizontal well paths and the dots represent the bottom hole, or end of the well (uticashaleblog.com 2015).

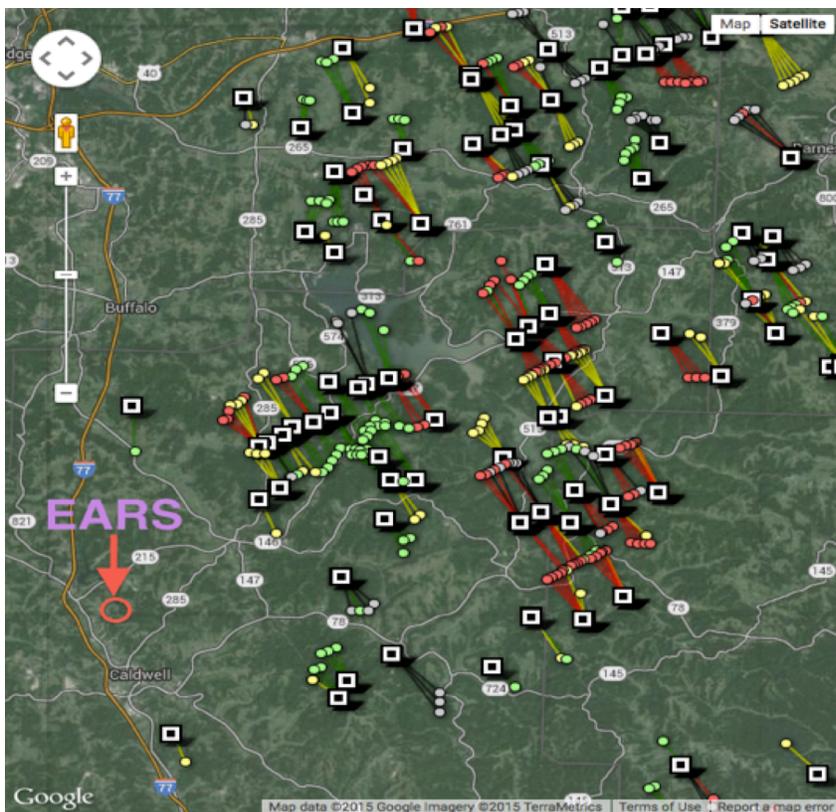


Figure 4. Current horizontal well development near EARS. White squares represent well pads, lines represent underground horizontal well paths and dots represent the bottom hole. Red means the well is drilling, green means it is producing, yellow means permitted only and gray shows drilling rigs that are drilled but not yet producing. Data updated on February 14, 2015 (uticashaleblog.com 2015).

2.2 Site Instrumentation and Sample Collection

A gauging station for each watershed was installed at the bottom of the watersheds and was contained by fencing to prevent disturbances from grazing animals. Each station contained a YSI Sonde that collected continuous turbidity, temperature, conductivity and pH of the stream that was used for a larger project. A weather station located on EARS provided precipitation information. Water samples at each gauging station were collected in the summer of 2014 between the months of May and September on six different dates, shown in Table 2. Each sample was collected between 10:00 AM and 2:00 PM.

There was a thunderstorm on sample date June 25, 2014, starting with a light rain in the morning while the samples from Afton and Middle Creeks were collected. It grew into a heavy downpour in the afternoon when the sample from Hickory Creek was collected.

The weather conditions towards the end of the summer were very dry. On the August 28 and September 28, 2014 sample dates, both Hickory and Afton Creeks were not flowing, but a sample was still collected from Middle Creek. The sample from Middle Creek on September 28, 2014 was taken from a stagnant pool near the sample site due to the lack of flowing water.

Table 2. *Precipitation at EARS on each sample date and for the three days before the sample date.*

Sample Date	72 hrs before (cm)	48 hrs before (cm)	24 hrs before (cm)	On Sample Date (cm)
6/18/14	0	0.69	0	0.76
6/25/14	0	0.30	0.05	3.28
7/10/14	0	0.36	1.14	0
7/22/14	1.91	0.03	0	0
8/29/14	0	1.70	0.03	0
9/28/14	0	0	0	0

2.3 Sample Analysis

Each grab sample was measured for turbidity in the field using a HACH turbidimeter. The samples from each watershed were then put on ice and transported to a lab at Ohio State for further testing. Grab samples were collected directly into autoclaved 1000 mL polypropylene bottles from the top of each stream. Due to the shallow depth of the streams, only one sample was collected as to not disrupt settled sediments from the streambed. The grab samples were taken back to the lab and examined within 24 hours using *in vivo* fluorometry for phycocyanin and chlorophyll *a* with a Turner Designs handheld AquaFluor fluorometer and filtered for *E. coli*. A 50 mL tube of each sample was also frozen at -40°C for future reference.

Fluorometers emit certain wavelengths of light and detect wavelengths that are emitted back from compounds. To detect chlorophyll *a*, fluorometers transmit a blue beam of light (460 nm) and detecting the amount of red light (685 nm) fluoresced by chlorophyll *a* in the sample. *In vivo* (meaning “within a living organism”) fluorometry measures chlorophyll that are in living algal cells compared to *in vitro* methods that measure chlorophyll after extracting it from the living cells (Turner Designs 1999). Phycocyanin, a blue pigment found in cyanobacteria, absorbs orange and red wavelengths (620 nm) and emits different red wavelengths (660 nm). Studies have shown that the level of fluorescence of the pigment phycocyanin is strongly correlated to cyanobacteria biomass and microcystin concentration (Kim 2013, Marion et al. 2012, McQuiad et al. 2010).

For *E. coli* measurements, 50 mL and 10 mL of water from each sample were vacuum filtered through a 0.45 µm pore size membrane. The membrane was then rolled onto an mTEC agar and traced with forceps to seal it and prevent air bubbles. The agar plates were inverted and incubated at 35°C for 2 hours and then at 44.5°C for 22 hours. The plates were then removed and

the magenta colored colony forming units (CFU) of *E. coli* that grew within the membrane boundaries were counted. The number of CFUs for each membrane was back calculated to units of CFU per 100 mL and results for each watershed were averaged. The mean *E. coli* value for each sample is referred to as the *E. coli* density in CFU/100 mL (Marion et al. 2012).

2.4 DNA Analysis

The water samples were stored on ice for approximately 24 hours before testing. The samples were vacuum filtered through a 0.4µm polycarbonate Isopore Membrane™ that was folded over and stuffed into a capped 2 mL test tube and frozen at -80°C to be used for DNA extractions. DNA extractions from the frozen filters were completed using a Qiagen QIAmp Fast DNA Stool mini kit.

The extracted DNA was tested with real-time qPCR (quantitative polymerase chain reaction) that amplifies, detects and quantifies fluorescence emitted from a reporter molecule that represents a targeted genetic sequence.

To determine if the DNA extractions had any inhibitors such as physical, chemical or biological compounds that may interfere with DNA amplification, a Sketa22 assay was performed. The control consisted of five microliters (µL) of salmon sperm DNA that were added to the water samples. If the expected amount of salmon sperm DNA was not detected with the qPCR, the DNA extraction was diluted and tested again.

The appropriately diluted DNA and a series of specific primers specific were pipetted into a well strips that were centrifuged, cleaned and placed into the real-time qPCR detection machine. The machine measured the number of amplification cycles it takes to reach a set threshold of fluorescence where significant and specific amplification occurs. The threshold

cycle number (C_t value) was compared to positive and negative controls to check for inhibition and recorded for each PCR run (Rulli 2012).

PCR analyses were run for DNA sequences of ruminant marker (Rum2Bac), human marker (HF183), a pathogenic toxin produced by a harmful *E. coli* strain (Shiga toxin 2), the phycocyanin pigment intergenic spacer (PC-IGS) of *Microcystis aeruginosa*, and a microcystin production gene (*mcyA*) of *Microcystis aeruginosa*. These procedures were conducted using the protocols found in Bernhard and Field (2000), Converse et al. (2009), Hauhland et al. (2010), Mieszkin et al. (2009), Kurmayer and Kutzenberger (2003), Tillett and Neilan (2000) and Yoshida et al. (2007). Table 3 summarizes the completed lab tests. Any positive data were compiled and analyzed in Excel.

To test for microcystin directly, an Abraxis Microcystin ADDA ELISA Test Kit was used following the procedure outlined in the kit. A series of three incubation periods using different solutions from the kit colored the detection wells if negative. The wells were then analyzed with a spectrophotometer.

Statistical analyses were run using IBM SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Macintosh, Version 22.0. Armonk, NY: IBM Corp) and Microsoft Excel.

Table 3. Summary of laboratory method used for each microbial marker in EARS water samples.

To Identify	Ruminant Feces	Human Feces	<i>E. coli</i> Toxin	Phycocyanin	<i>Microcystis aeruginosa</i>	microcystin production gene	microcystin
Gene Target	Rum2Bac	HF183	Stx 2	PC-IGS	NIES-843	<i>mcyA</i>	-
Method	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	ELISA

3. RESULTS

3.1 Turbidity

Turbidity measurements taken in the field ranged from 2.2 to 2,313 NTU and are summarized in Table 4. The Middle Creek sample from September 28, 2014 is included in Table 4 but was not used in the following statistical analyses. It was collected from a pool on a dry day during the end of the summer when the water in Middle Creek was not flowing. Afton and Hickory Creeks were dry on August 29 and September 28, 2014.

Table 4. Turbidity measurements in NTU for Afton, Middle and Hickory Creek watersheds at EARS, Noble County, Ohio between June 2014 and September 2014.

Sample Date	Watershed	Turbidity (NTU)
6/18/14	Afton	10.8
	Middle	3.1
	Hickory	8.5
6/25/14	Afton	71.2
	Middle	42.1
	Hickory	2313
7/10/14	Afton	11.9
	Middle	7.0
	Hickory	11.0
7/22/14	Afton	6.5
	Middle	3.9
	Hickory	11.3
8/29/14	Middle	2.2
9/28/14	Middle	22.0

3.2 Microbes

Only ruminant and human feces were detected in any of the samples and only ruminant feces could be quantified (Table 7). Results from the lab analyses are summarized in Tables 5 and 6. Human specific fecal contamination was detected in the June 18, 2014 Middle Creek sample and the August 29, 2014 Middle Creek sample, but in amounts below the quantifiable range ($1.1 \times 10^2 - 1.1 \times 10^6$ gene copies/mL) and are not used in the statistical analyses. Significant results were only found for ruminant specific fecal contamination (Rum2Bac). Ruminant specific fecal contamination was only detected in half of the samples (7 of 14) and two were also below

the range of quantification (“detected, not quantifiable” denoted DNQ). The June 18, 2014 sample from Afton Creek and the July 22, 2014 sample from Hickory Creek showed much higher gene copy counts (586.2 and 682.2 gene copies/mL, respectively) than the other three creeks on July 10, 2014 (average of 41.4 gene copies/mL). *E. coli* was detected from every sample except the August 29, 2014 sample from Middle Creek due to laboratory complications.

3.3 Pigments

Phycocyanin was detected using the *in vivo* fluorometer (mean = 1.5 µg/L, maximum = 12.4 µg/L, minimum = 0.1 µg/L). Chlorophyll *a* was detected in every water sample (average = 3.0 mg/L, maximum = 13.1 mg/L, minimum = 1.0 mg/L). The three highest values for phycocyanin and chlorophyll *a* were recorded on June 25, 2014.

Table 5. Summary of results for each microbial marker in EARS water samples.

To Identify	Ruminant Feces	Human Feces	<i>E. coli</i> Toxin	Phycocyanin	<i>Microcystis aeruginosa</i>	microcystin production gene	microcystin
Gene Target	Rum2Bac	HF183	Stx 2	PC-IGS	NIES-843	<i>mcyA</i>	-
Detection	Yes	Yes	No	No	No	No	No
Quantification	Yes	No	No	No	No	No	No

Table 6. Chlorophyll *a*, phycocyanin, *E. coli* and Rum2Bac counts for each sample. DNQ (detected, not quantifiable) for PCR analysis that detected gene copies below the quantifiable range.

Sample Date	Watershed	<i>in vivo</i> chlorophyll <i>a</i> (mg/L)	<i>in vivo</i> phycocyanin (µg/L)	<i>E. coli</i> (CFU/100mL)	Rum2Bac (gene copies/mL)
6/18/14	Afton	1.6	0.7	9.52E+02	586.2
	Middle	1	0.5	3.18E+02	-
	Hickory	1.8	0.6	8.12E+02	12.0 (DNQ)
6/25/14	Afton	4.9	1.6	2.77E+04	-
	Middle	3.3	1.0	4.20E+03	1.0 (DNQ)
	Hickory	13.1	12.4	2.50E+05	-
7/10/14	Afton	1.9	0.5	8.50E+02	25.2
	Middle	1.8	0.6	6.93E+02	59.9
	Hickory	2.4	0.7	1.14E+03	39.1
7/22/14	Afton	2.3	0.2	1.09E+03	-
	Middle	1.4	0.3	2.26E+02	-
	Hickory	2.8	0.4	2.06E+03	682.2
8/29/14	Middle	1.0	0.1	-	-
9/28/14	Middle	22.1	3.1	1.13E+02	-

3.4 Statistical Analysis

Turbidity and *E. coli* densities were compared and found to have a positive relationship ($R^2=0.50$, $p = 0.032$). Table 7 shows the summary statistics for turbidity and *E. coli* densities for each sample. The three data points from June 25, 2014 were analyzed separately as rain weather data, and the data from June 18, July 10, July 22 and August 29, 2014 represent dry weather conditions. The median turbidity value during the rainy weather (8.08×10^2 NTU) was over 100 times greater than the dry weather turbidity average (7.6 NTU). The mean *E. coli* density for rainy weather (9.40×10^4 CFU/100 mL) was over 100 times greater than dry weather (9.05×10^2 CFU/100 mL).

Hickory Creek exhibited the highest *E. coli* density but Afton Creek had the highest turbidity (see Table 8). Overall, Hickory Creek had the highest average turbidity and *E. coli*. Figure 5a-e shows the relationship between maximum, minimum and median values for wet and

dry data. The median values for the rain data are positively skewed. The box plots in Figure 5 show the different distributions of each data set.

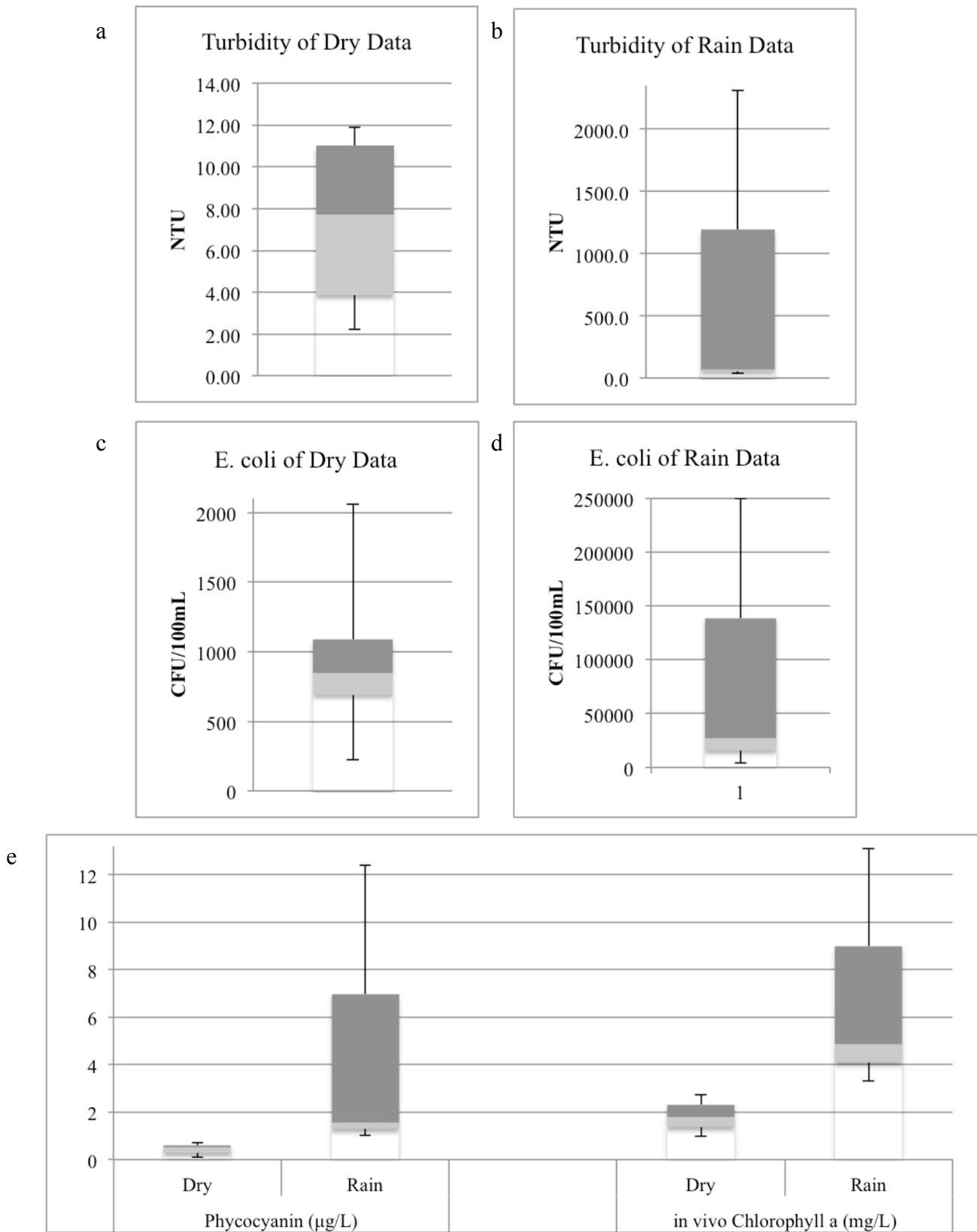


Figure 5. Box blots of dry and rainy weather data for turbidity, E. coli, phycocyanin and chlorophyll a. The maximum, 3rd quartile, median, 1st quartile and minimum are represented by the top error bar, top of box, middle of box, bottom of box and bottom error bar, respectively. Figures 5a and 5b as well as 5c and 5d are plotted separately due to the difference in the y-axis scale

Table 7. Summary turbidity measurements in NTU units for all watersheds at EARS on each sample date. Data used for this table exclude the grab sample from Middle Creek on 9/28/14.

Statistic	All Data		Weather Regime			
			Dry		Rain	
	Turbidity (NTU)	Mean <i>E. coli</i> density (CFU/100mL)	Turbidity (NTU)	Mean <i>E. coli</i> density (CFU/100mL)	Turbidity (NTU)	Mean <i>E. coli</i> density (CFU/100 mL)
Number of samples	13	12	10	9	3	3
Maximum	2313	2.50E+05	12	2.06E+03	2313	2.50E+05
Minimum	2.2	2.26E+02	2.2	2.26E+02	42	4.20E+03
Mean	192	2.42E+04	7.6	9.05E+02	809	9.40E+04
Median	10.8	1.02E+03	7.7	8.50E+02	71	2.77E+04
Standard deviation	637	7.15E+04	3.6	5.35E+02	1303	1.36E+05

Table 8. Dry weather turbidity and *E. coli* measurements summarized by watershed.

Statistic	Dry Weather Data					
	Afton Creek		Middle Creek		Hickory Creek	
	Turbidity (NTU)	Mean <i>E. coli</i> density (CFU/100mL)	Turbidity (NTU)	Mean <i>E. coli</i> density (CFU/100mL)	Turbidity (NTU)	Mean <i>E. coli</i> density (CFU/100mL)
Number of samples	3	3	4	3	3	3
Maximum	12	1.09E+03	7	6.93E+02	11	2.06E+03
Minimum	6.5	8.50E+02	1.8	3.18E+02	8.5	8.12E+02
Mean	9.7	9.64E+02	3.9	4.12E+02	10	1.34E+03
Median	11	9.52E+02	3.5	3.18E+02	11	1.14E+03
Standard deviation	2.8	1.20E+02	2.2	2.47E+02	1.6	6.47E+02

3.4.1 Dry Weather Data

Turbidity correlated with *E. coli* the strongest (Figure 6) with a significant positive linear relationship ($R^2=0.50$, $p = 0.032$). A similar relationship between turbidity and chlorophyll *a* can also be seen in Figure 7 ($R^2 = 0.59$, $p = 0.04$).

3.4.2 Rain Weather Data

The rain data from June 25, 2014 were considered on a logarithmic scale to account for their large variation. The linear relationship between turbidity and *E. coli* for the rain data was not significant ($p = 0.95$) and had a large R^2 value of 0.98 even after a logarithmic transformation (Figure 8).

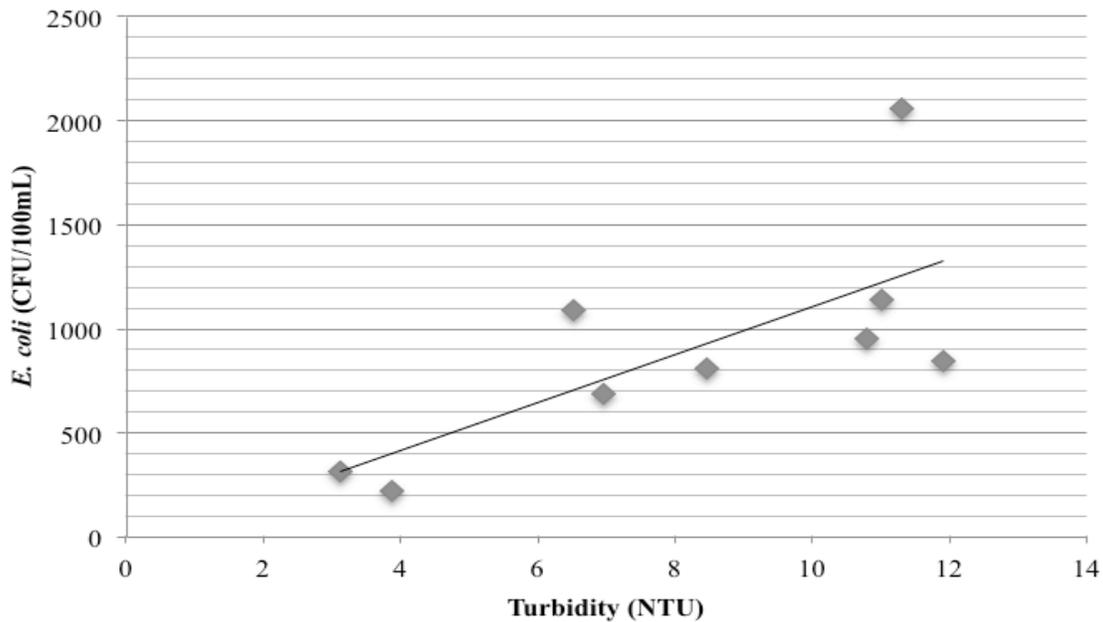


Figure 6. Dry weather data for turbidity from all three watersheds at EARS plotted against *E. coli* densities.

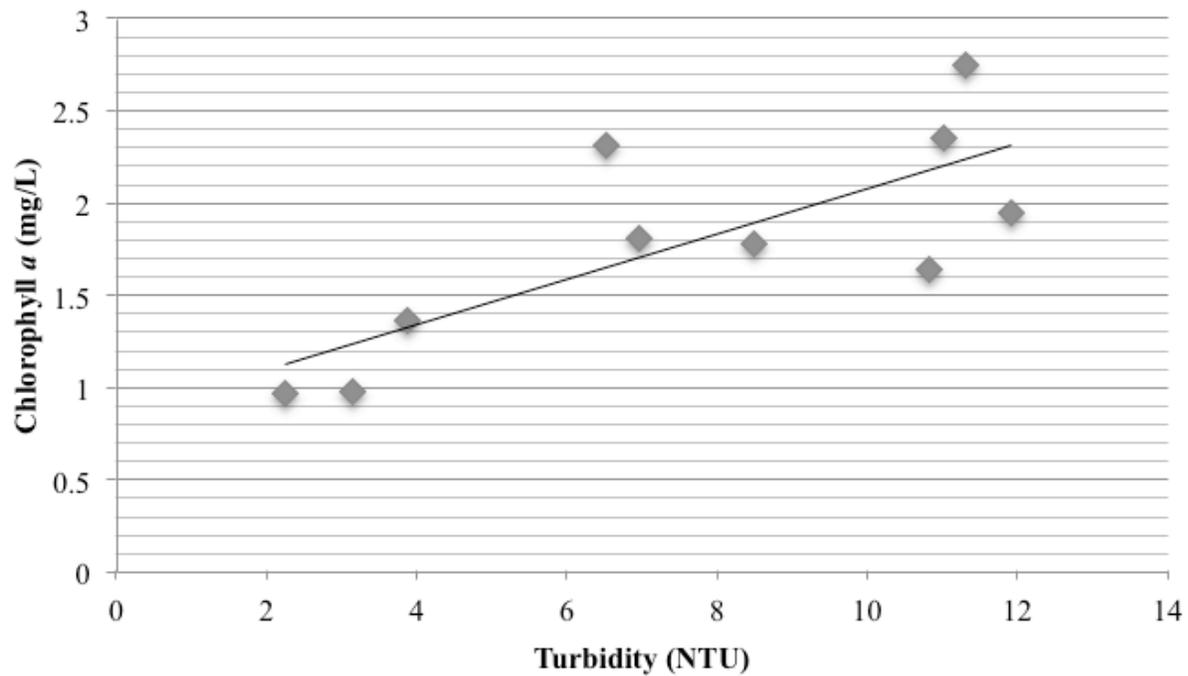


Figure 7. Dry weather data for turbidity from all three watersheds at EARS plotted against chlorophyll a.

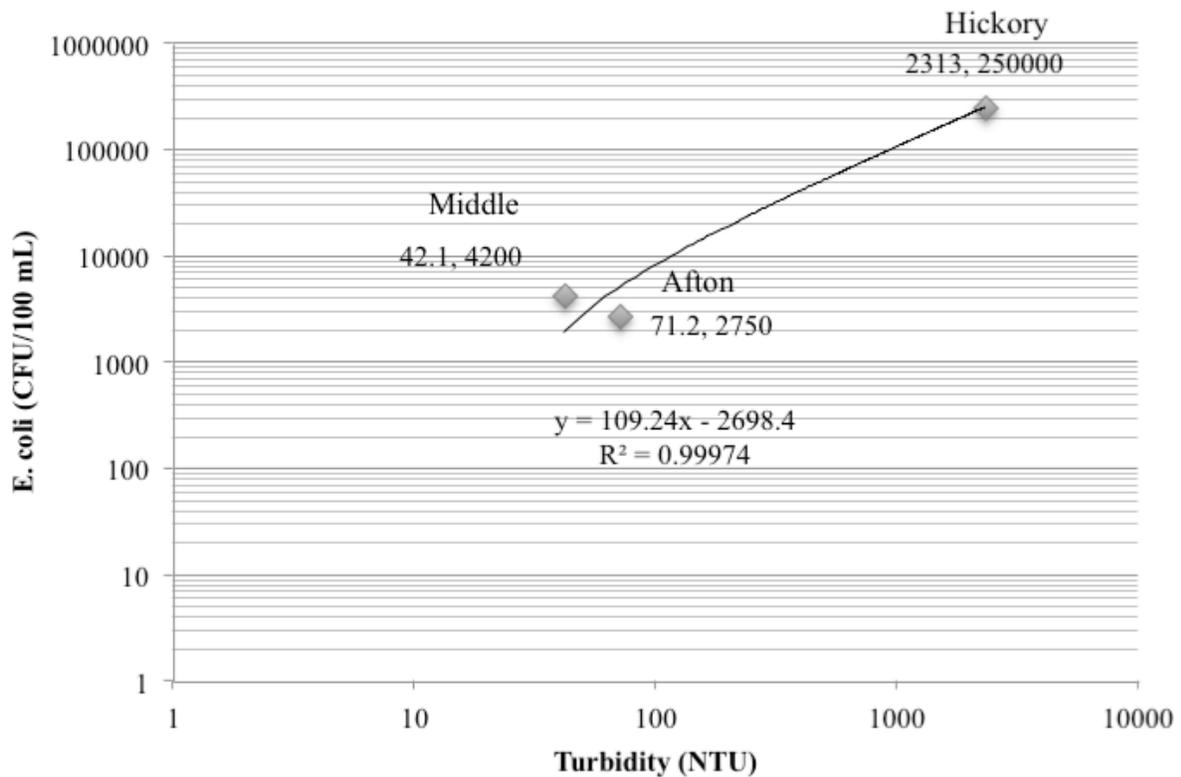


Figure 8. Three data points for water samples taken on June 25, 2014 from Afton, Middle and Hickory Creeks representing wet weather data. The Hickory Creek sample was collected after a heavy downpour and flash flood resulting in enormously increased E.coli and turbidity. The data are shown on a log-log scale to show the variability during rain events.

3.5 Land Cover

Middle Creek watershed has an area of 2.13 km² and is the largest of the three. Afton Creek watershed is 0.86 km² and Hickory Creek watershed is 0.27 km². Each watershed was split into pasture and forest land cover in ArcMap software using aerial photos. The percentage pasture of each watershed compared to average turbidity is shown in Figure 9.

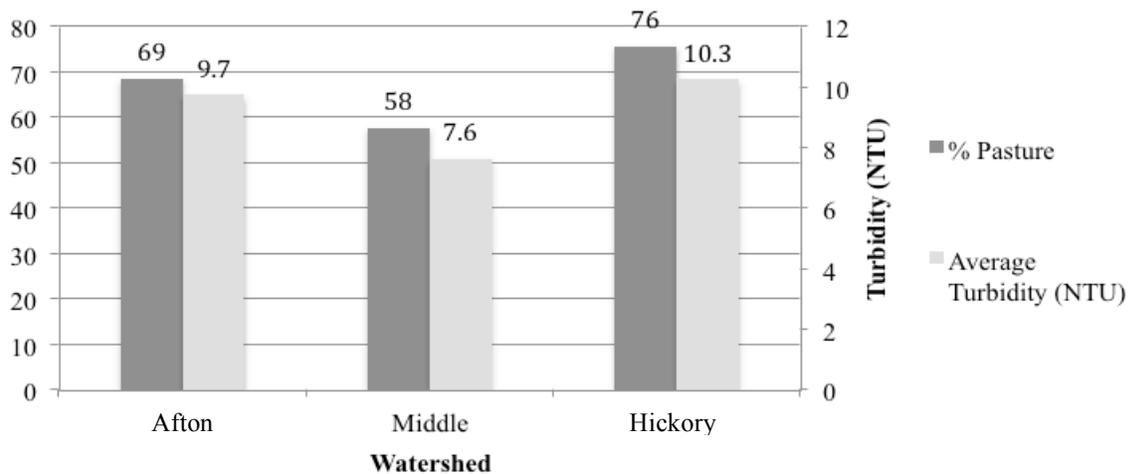


Figure 9. Average turbidity and percent pasture land cover for each watershed inside EARS. The land cover that is not pasture is predominately forested.

4. DISCUSSION

4.1 Turbidity, *E. coli*, and Ruminant Associated Fecal Contamination

The water samples collected from the three watersheds inside EARS shows a range of turbidity values from 2 to 2.31x10³ NTU, and *E. coli* ranged from 2.26x10² to 2.50x10⁵ CFU/100 mL. The vastly different conditions the creeks experienced during a rain event made the separation of the data into wet and dry weather conditions the most appropriate way to draw conclusions. Sediment concentration has been shown in many studies to be strongly correlated with fecal coliforms like *E. coli* and is divided into stormflow and dry flow (USGS 2012). Linear relationships between *E. coli* and turbidity are site specific, but may be developed so that

turbidity measures can be used to screen for elevated contamination. There are many other environmental factors that influence the survival of cyanobacteria and make it much more difficult to model and predict. While summary statistics were provided for the 14 grab samples, they are limited in their meaning. So few data points are not representative of all Ohio weather conditions or flow regimes. There is not enough data during rain events to confirm the relationship between wet and dry results, but due to the variability in precipitation intensities and occurrences in Ohio, much more variability in data for both weather regimes would be expected. Some studies have shown that turbidity and *E. coli* data during storm flow showed the most uniformity regardless of the rain event intensity. This is due to a mixing effect caused by high velocity water in the stream channel (USGS 2012).

Eastern Ohio gets an average of 11.6 cm (4.6 inches) of rain during the month of June, which is 0.39 cm a day (Current Results Nexus 2015). On June 25, 2014, 3.3 cm of rainfall was recorded in an event characteristic of an Ohio summer storm. Ohio experiences thunderstorms approximately 35-45 days a year, most commonly between April and September. Ohio thunderstorms can drop many inches of rain in a few hours producing flash floods that can cause serious damage in the southeastern Ohio hilly terrain (Schmidlim 1996). The elevated turbidity and *E. coli* data collected on June 25, 2014 give insight to the effects of a typical rain event at EARS on the amount of sediment discharged into the streams. More data during high flow rain events would help confirm what the relationship is between turbidity and *E. coli* at such extreme levels. Data for a variety of rainfall amounts is important to create a regression equation that can be used to predict *E. coli* densities.

Wet weather samples are expected to have highly elevated turbidity values caused by water that runs off over the landscape and carries soil particles. *E. coli* organisms living in

animal feces in the pastures attach to sediment particles that drain into the streams. High velocity flow in the stream channels may also re-suspended sediments and organisms along the streambeds and contribute to increased turbidity and *E. coli*.

E. coli is an indicator of general fecal contamination and was found in all of the tested samples, but the ruminant specific genetic marker was only detected in half of the samples. Ruminant specific fecal contamination was detected in all three watersheds on July 10, 2014, and the concentrations detected were within a range of 25 to 60 gene copies/mL (whereas other detected concentrations on June 18 and July 22, 2014 were 5.86×10^2 and 6.82×10^2 gene copies/mL, respectively). There is no information recorded about the animal rotations that certainly have an impact on fecal concentrations, but there was 1.14 cm of precipitation at EARS the day before that may have washed and settled over the landscape, contributing to the similar concentrations in each watershed.

4.2 Turbidity, Phycocyanin and Chlorophyll *a*

Chlorophyll *a* was also found to have a significant relationship with turbidity ($R^2 = 0.59$, $p = 0.04$), which supports the idea that floating photosynthetic organisms may also largely contribute to water cloudiness. It may also support the suggestions that sediments (represented by turbidity) carry nutrients that catalyze phytoplankton growth.

Chlorophyll *a* is the primary pigment for all photosynthetic organisms and can be used to represent total algal biomass (USGS 2008). Cyanobacteria exclusively produce the pigment phycocyanin in conjunction with chlorophyll *a*. For this reason, phycocyanin can be used in water monitoring indices as an alternative measure for microcystin levels (Marion et al. 2012). By measuring both chlorophyll *a* and phycocyanin, the relative amount of total algae and cyanobacteria could be compared to the direct measures of *M. aeruginosa* and microcystin. If a

stable relationship between the two could be confirmed, chlorophyll *a* and phycocyanin could be used as a rapid and practical index to screen for microcystin levels in the watersheds. These indices may be of interest to the City of Caldwell that uses Caldwell Lake as a municipal drinking water source, and Wolf Run State Park for recreational monitoring.

Turbidity and phycocyanin had a much weaker and insignificant correlation ($R^2=0.19$, $p = 0.24$). Turbidity may not be the sole indicator of cyanobacteria growth due to the sensitivity of cyanobacteria to other environmental conditions such as temperature, light intensity, nutrient ratios and pH, as discussed in the introduction. Chlorophyll *a* and phycocyanin exhibited a weak linear relationship ($R^2=0.06$, $p = 0.50$) and were likely occurring in separate species. Phycocyanin was measured at much lower quantities ($\mu\text{g/L}$) compared to chlorophyll (mg/L), and may have been degraded to quantities below the detection range.

4.3 Microbes and qPCR

The phycocyanin intergenic spacer gene (PC-IGS) was not detected. The PC-IGS sequence is specific to *M. aeruginosa*, but can be found in both toxic and non-toxic strains of the organism. The ELISA that tested for the toxin microcystin directly also showed no detection.

Inhibitors in the DNA extracts may explain the lack of detection of PC-IGS. There are many steps during the process of DNA extraction that are susceptible targets for inhibitors. Inhibition can be caused by the loss of sample nucleic acid via absorption to surfaces of tubes and pipets, degradation of primers by nucleases, or by contamination of ethanol used for cleaning. Some samples may also have naturally occurring inhibitors such as polyphenols (tannic acid) or humic substances (fulvic and humic acids) from decomposing plant matter (Pennington 2014). Table 8 below shows the dilutions of the DNA extractions that were necessary for qPCR analyses. The high dilutions needed for some of the samples may have hindered detection of the

desired DNA sequences, but the samples that were not diluted also showed no detection. The samples that needed to be diluted did not correlate with high turbidity values, which might imply the inhibitions were not physical. It is most likely that there were no *Microcystis aeruginosa* cells in the water.

Table 8. Turbidity values for each grab sample and the DNA extract dilution necessary to eliminate inhibitors for qPCR analysis. There is no obvious correlation between turbidity and inhibition.

Sample Date	Watershed	Turbidity (NTU)	Dilution
6/18/14	Afton	10.8	-
	Middle	3.1	1/500
	Hickory	8.5	-
6/25/14	Afton	71.2	-
	Middle	42.1	-
	Hickory	2313.0	-
7/10/14	Afton	11.9	-
	Middle	7.0	-
	Hickory	11.0	-
7/22/14	Afton	6.5	1/1000
	Middle	3.9	1/5000
	Hickory	11.3	-
8/29/14	Middle	2.2	1/5000
9/28/14	Middle	22.0	1/500

4.4 Land Cover

The highest average turbidity matches the highest relative pasture cover and the lowest average turbidity in the watershed with the lowest percent pasture. Highest and lowest average turbidity also match the smallest and largest watersheds, respectively. Watershed size and land cover type likely both contribute to turbidity.

Hickory Creek watershed had the highest average turbidity. This is likely because it was the smallest watershed with the shortest stream. The water deposited into the stream likely

experienced less natural filtration over the landscape and the sediments had less residence time in the water. It also had the highest percent pasture, which means the landscape has smaller, more uniform vegetative cover and likely more animal traffic.

4.5 Possibilities for Further Studies

Analysis of the general microbial community would have been a more appropriate starting point before testing for specific species. Microbial profiling is recommended going forth to better understand what genera are most prevalent in the EARS watersheds.

Further information about the materials being transported and suspended in the water could be obtained from a soil survey. This would help explain the naturally existing microbial community and give insight to the minerals and chemicals expected at EARS. It would also be helpful to include information about the rotations of animals grazing between watersheds. Currently, livestock movement is controlled by the farm managers at EARS who do not log such specific daily activity.

Finally, more samples over a variety of weather conditions would help to draw more concrete conclusions about the patterns and relationships of turbidity and *E. coli* at EARS. This study used turbidity to represent sediment, but it would be more accurate to measure total suspended solids (TSS). By measuring both parameters for each water sample, a relationship can be confirmed so that turbidity measures can be transformed into more accurate TSS measurements.

5. CONCLUSIONS

This study supports existing research that sediments in water foster microbial species. The linear relationship between turbidity and *E. coli* confirm the hypothesis that increased sediment concentrations in water correlate to an increased number of microbes and potentially

enteric pathogens. There are many different environmental factors that affect the natural microbial community in a watershed that could also be explored. The results of this study focused on *E. coli*, a common indicator species of fecal contamination. Fecal contamination is not only a problem in waterways near cattle farms, but in agricultural areas all around Ohio. Many disease-causing pathogens are transported via the fecal to oral route and travel far distances in waterways. Animal manure is commonly used as a soil fertilizer, but the nutrients and organic matter that are beneficial to crop growth also pollute the surrounding waterways. Nutrients can catalyze algal growth, ammonia is toxic to fish and the decomposition of organic matter reduces dissolved oxygen in the water necessary to support other aquatic life (Mancl & Veenhuizen 2015).

Though specific microbial species were not identified, this study demonstrated the patterns of sediment production due to precipitation and road use within a small watershed. This research was part of a larger project that focused on how road use produces sediments that wash into the creeks. This highlights the seriousness of road quality, material and structure. Construction projects in the area including shale development activities should be acutely aware that roads are a large source of stream contamination in a typical rural Ohio watershed.

There was no detection of *Microcystis* in the three watersheds, but harmful algae blooms are still a concern for many inland lakes in Ohio. Lakes in Eastern Ohio do not typically experience HABs, but the potential increase in salinity, sediment concentration and temperatures caused by shale activities may provide conditions favorable for HABs. The proximity of EARS to recreational water and drinking water sources make it an important study location to monitor for the growth of harmful species. EARS provides a unique opportunity for many studies to monitor baseline conditions of an Ohio watershed.

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