Ecological constraints of antibiotic resistant gram-negative coliforms in an urban riverine ecosystem

Angela M. Klock

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Committee:

Daniel Vogt, Committee Chair

Marilyn Roberts, Committee Member

Christian Torgersen, Committee Member

Robert Edmonds, Committee Member

Program Authorized to Offer Degree:

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University of Washington

ABSTRACT

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gram-negative coliforms in an urban riverine ecosystem

Angela M. Klock

Chair of the Supervisory Committee:
Associate Professor Daniel Vogt
School of Environmental and Forest Sciences

The distribution of antibiotic resistant bacteria in surface water is largely unknown but nowhere is this information more critical to understanding ecosystem function and protecting public health than in urban streams. The environmental factors that drive the distribution of antibiotic resistant gram-negative coliforms were evaluated along the main corridor and tributaries of the Green-Duwamish River watershed of the Puget Sound. I collected 65 samples of water, biofilms and sediments, and used culture-based methods to evaluate the distribution of total levels of resistance to ampicillin, chloramphenicol, and tetracycline from a mixed population of organisms. Antibiotic resistance was spatially-explicit and largely partitioned between lactose fermenting and non-lactose fermenting coliforms at each site and Analysis of Similarity indicated that resistance was more dissimilar between stream compartment and functional process zone than longitudinal distance along the river. Antibiotic resistance was negatively correlated with river power and channel sinuosity at the reach scale and Distance-based Redundancy Analysis determined 59.1% of the explainable variance was due to the environment and 4.6% due purely to geographic distance at the watershed scale. Resistance to more than one

antibiotic was most common in water samples from middle watershed tributaries and the middle watershed main stem, with gradients of 1-2%. These results indicate that antibiotic resistance among gram-negative coliforms is controlled primarily by niche ecology at the microbial scale, stream morphometry at the reach scale, and environmental forcing at the landscape scale.

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Angela M. Klock
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INTRODUCTION

Urban waterways are a common landscape feature in Washington State where 14 major rivers and over ten thousand streams empty into the Puget Sound. The Seattle-Tacoma region is the state's largest metropolitan area home to 3.6 million people, and the fastest growing area in the country (US Census Bureau 2010). Near Seattle, the shoreline of Elliot Bay and the banks of the Duwamish River have been extensively hardened from industrialization over the last one hundred years. The river has undergone several major diversions and been extensively channelized for urban development. In spite of these impacts King County has some of the best municipal water supplies in the country requiring little pre-treatment. This clean water supply is due to the foresight of previous generations to purchase and preserve thousands of acres of the forested headwaters of two major basins, the Cedar River and the Green-Duwamish River watersheds (Figure 1). Most of the headwater forests of these watersheds have been protected since the early 1900s. The major difference between the two watersheds is how the forests are managed. In the Green-Duwamish watershed, water resource inventory area (WRIA 9) logging is conducted but road building is limited and riparian buffer zones are mandated. In the Cedar River watershed (WRIA 8) no logging currently occurs.

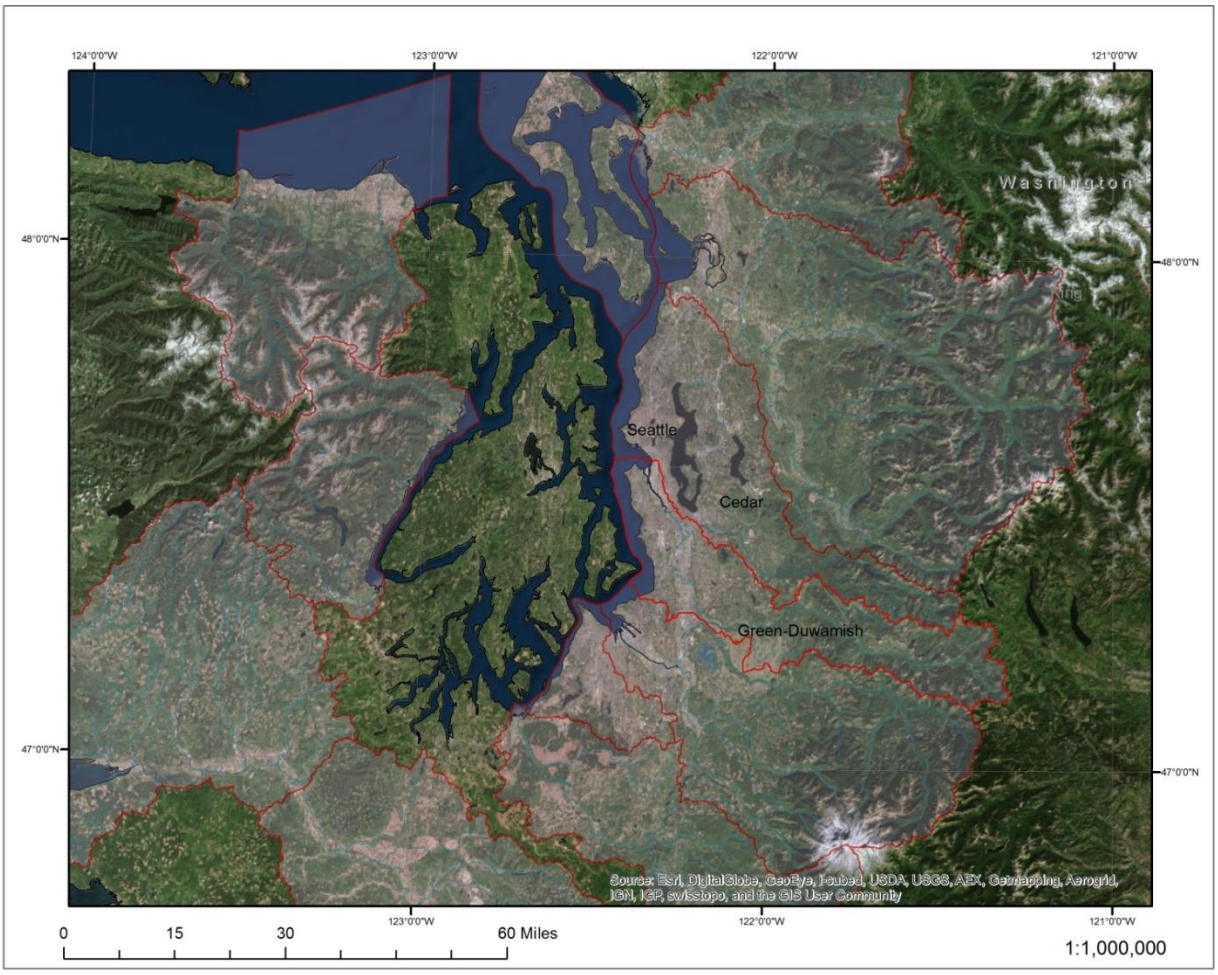


Figure 1. The Green-Duwamish River and Cedar River watersheds of the Puget Sound.

WRIA 9 encompasses a total of 127,427 hectares, and 57,342 hectares (45%) of the watershed which lies above the Howard Hansen dam is closed to public access. The headwaters of the Green River originate in the relatively pristine forests of the Cascade Mountain range and flow westward through gradually increasing levels of residential and commercial development until becoming the Duwamish River approximately 19 km upriver of Elliot Bay. The last eight km of the Duwamish River were named an EPA Superfund site in 2001. In the

downstream direction the riverine habitats become more homogeneous as the slope becomes gentler and the river widens until emptying into the bay. Being an urban watershed, the river is significantly impacted by land use and development, and runoff is the major cause of degraded water quality in the middle and lower watershed. These sections of the watershed are also where subsistence fishing and recreational use is most common. The upper watershed is predominately covered by forests and largely un-used by people and is home to many species of wildlife.

Urban rivers are primarily contaminated by both point and non-point sources of pollution including; break through from septic systems, runoff from farms, industrial agriculture, and stormwater, and outflow from combined sewage overflow systems. Among the many pollutants from these sources are biologically active antibiotics, antibiotic metabolites, enteric bacteria, and antibiotic resistant "determinates." Antibiotic resistance determinates include resistant bacteria, free DNA or resistant genes released from lysed bacterial cells, and mobile genetic elements containing resistant genes. Although this is a widespread phenomenon the Green River has never been surveyed for antibiotic resistance. Water is the perfect habitat for bacterial mixing, and an urban waterway is the perfect vector for the distribution of antibiotic resistant determinants into the wider environment. Environmental hot spots form where the ecology of gene exchange between bacteria is fostered (Ash et al. 2002). Along a river,

hot spots for gene exchange between resistant enterics and sensitive environmental species occur where large amounts of terrestrial inputs mix with natural riverine bacteria in high densities.

Knowing the distribution of antibiotic resistance in an urban river may answer many socio-ecological questions such as; "does the environment play a role in the distribution of environmental resistance"? "can we identify the environmental constraints of resistance in natural and impacted river segments"? and "is there a nexus between environmental resistance and community acquired resistant infections"? In this study I seek to understand if and how riverine ecosystems influence the distribution of antibiotic resistance along physical and ecological gradients as the river flows through land-use gradients from pristine forests to heavily industrialized development.

LITERATURE REVIEW OF THE PROBLEM

The abundance and distribution of enteric bacteria in urban rivers and the levels and types of antibiotic resistance (ABR) present in them is a particularly relevant and urgent concern for public health (WHO 2014), and in light of the significant ecological roles bacteria play, for ecosystem function (G. Falkowski 2008). Antibiotics in sufficient concentrations exert selective pressure on bacterial communities and result in population shifts to more resistant strains (Kümmerer 2009a). This shift in community

structure alters the fundamental river ecology for all higher trophic levels (Pace et al. 1999), and could diminish the ecological resilience of the river when subjected to other disturbances including climate change (Allison and Martiny 2008). If resistant enterics transfer their genes to nonpathogenic environmental species or are able to persist in urban rivers they may become genetic reservoirs of resistance for human and animal pathogens (Baquero et al. 2008).

In the major watersheds of the Pacific Northwest animal manure from agriculture is the primary source of excess nutrients and enteric bacteria. Approximately 9,979,032 kg of inorganic nitrogen enters the Puget Sound each year from agricultural manure (Inkpen and Embrey 1998). This manure contains enteric bacteria (antibiotic resistant and sensitive), antibiotics, antibiotic metabolites, and antibiotic resistant determinants such as naked DNA or mobile genetic elements (Martinez 2009).

In urban rivers metal contamination also increases with development (Fechner et al. 2012) and bacteria frequently exhibit co-resistance to metals and antibiotics (Vishnivetskaya et al. 2011). The propensity for co-selection of resistance represents a widespread and persistent problem as metal pollution in rivers is common and may result in ABR even in the absence of antibiotic contamination (McArthur and Tuckfield 2000). In addition to metals and antibiotics, many bacterial species also exhibit co-selection for

resistance to other industrial pollutants (Sabater et al. 2007) which are common in urban waterways.

Bacteria are suggested to be the most important groups of organisms on earth as they are universally distributed and drive all biogeochemical cycles (O'Malley 2008). Bacteria could easily be the most perfectly adapted species (Mcarthur et al. 1992); as their abundance (Whitman et al. 1998), fast generation times (Boto and Martínez 2011), and small size assure their continued evolutionary success.

Bacteria demonstrate seemingly endless species diversity (Torsvik and Øvreås 2002, Falkowski et al. 2008), metabolic uniqueness (Preston-Mafham et al. 2002), and genetic plasticity (Kirk et al. 2004) allowing them to exploit every available habitat and respond rapidly to changing environmental conditions.

Riverine bacteria are found in the water column, in sediments, in-and-on plants and animals, and as components of complex biofilms on surfaces. Bacteria are the foundation of the food web in aquatic systems and are grazed upon by the macro-invertebrates required for a robust fishery (Dopheide et al. 2009). Bacteria are also the metabolic engine of the river ecosystem, processing and recycling nutrients from autochthonous and allochthonous sources and supporting primary productivity (Gaedke 2006). As nutrient gradients wax and wane the bacterial community structure

changes in-step which is reflected in the overall trophic status of the lotic ecosystem (Vinten et al. 2011). Bacterial communities also interact and compete with each other which adds another complex dynamic to understanding these systems (Liu et al. 2013).

Riverine habitats are hierarchal in arrangement and vary by several orders of magnitude in size (Pringle et al. 1988). The distribution and structure of each habitat offers a unique set of conditions that in turn prompts unique responses from the resident bacterial populations. Bacteria vary genetically more due to habitat than distance (Mcarthur et al. 1992) indicating that local conditions may have more selective power than the local gene pool.

The geographic distribution of antibiotic resistant bacteria is a rapidly expanding field of research but there remains a divide between our understanding of macro- and micro-organismal ecology (Bent and Forney 2008). The distribution of any organism is an inherent property of the ecological system in which it is embedded and bacteria are key players in our understanding of larger ecological questions. Asking ecological questions through a microbiological lens may offer a unique perspective in understanding macro-organism conservation (Smith 2007) including the impacts of antibiotic resistance on wildlife (Kümmerer 2009c). This perspective is being incorporated into reintroductions of salmonids above dams in the Pacific Northwest. Knowledge of microbial functional groups enhances ecological models that are driven by a bottom-up processes or

that support trophic cascades allowing managers to evaluate the feasibility of these reintroductions more effectively (Beauchamp 2011).

SOURCES OF ANTIBIOTIC RESISTANCE IN RIVERS

Water quality is the most important ecological issue today as runoff and effluent from urban, agricultural, and industrial development add immeasurable amounts of chemicals, toxins, metals, and antibiotics to the water cycle each year (Viau et al. 2011). Antibiotics are considered an emerging contaminant (Pruden et al. 2006, Zhang et al. 2009, Proia et al. 2013) and resistant determinants are currently found in virtually every global environment (Marti et al. 2013).

Bacterial ABR is a pressing medical issue as over 23,000 people die in the US from resistant infections (US Dept of Health and Human Services 2013), and the World Health Organization estimates that 150,000 deaths occur from multi-drug resistant tuberculosis alone, every year.

Antibiotics arrive in streams from many point and non-point sources; stormwater runoff (Ancion et al. 2014), waste-water treatment plants (Port et al. 2012), septic systems (Baquero et al. 2008), agriculture (Angulo 2013), aquaculture (Pruden et al. 2012), agricultural soils when they have been irrigated with wastewater or surface water that receives runoff, or soils fertilized with contaminated manure (Marshall and Levy 2011). Veterinary antibiotics are also released into the environment through wastewater, or

from the waste of pets or farm animals receiving antibiotics (Kümmerer 2009c).

In addition to antibiotics and their metabolites, antibiotic resistant bacteria and antibiotic resistant genes are expelled from the gastrointestinal tracts of humans and animals (Sommer et al. 2010). These antibiotic resistant determinants enter river systems by the same means as antibiotics. Even the construction of roads increases the levels of bacterial ABR in the environment as roads are directly related to the presence of people and their use of antibiotics (Eisenberg et al. 2012).

Water is the perfect medium for the mixing of environmental and exogenous bacteria (ASTM 2000, Baquero et al. 2008), and urban streams are the distribution pathways of resistant determinants and terrestrial bacteria into the wider environment. Some common urban hot spots are waste water treatment plants (Rizzo et al. 2013) and terrestrial facilities that generate large amounts of manure or other biological waste which eventually end up in the water cycle (Cantas 2013). What is less clear are the effects environmental transport have on the stability of antibiotics in rivers. For example tetracycline transport is negatively correlated with sulfonamide transport (Pruden et al. 2012). This may represent differing ecologies for either the conjugative mobile element or the bacterial host.

There are three primary bacterial genetic transfer mechanisms to acquiring resistance; transformation, transduction, and conjugation. Bacteria are able to acquire accessory genes as mobile genetic elements which confer adaptive advantage and allow rapid proliferation under selective pressure in the environment (Levin and Bergstrom 2000, Malachowa and DeLeo 2010, Rankin et al. 2011). Most commonly environmental bacteria acquire resistance from horizontal gene transfer (HGT) during conjugation with resistant strains (Aminov 2009). Although bacteria evolve rapidly under selective pressure, antibiotic resistance always comes with a physiological cost in the environment (Malachowa and DeLeo 2010, Boto and Martínez 2011), indicating that microbial ecology will constrain the frequency and efficiency of the uptake and retention of new genes in the organism. The impact to environmental bacterial populations from HGT with exogenous strains is not clear, but what is coming into focus is that environmental conditions determine the rate of HGT among bacteria in natural systems (van Elsas and Bailey 2002). The identification of HGT hot spots would increase our understanding of microbial and conjugation ecology and could provide insight into the biogeographic modeling of antibiotic resistant species in the environment.

While hot spots provide the opportunity for environmental and exogenous bacteria to share genetic resources we do not see antibiotic resistance everywhere and the patterns are variable (Eisenberg et al. 2012). For

example, we find antibiotic resistant bacteria in pristine habitats with no legacy of antibiotic contamination (Leff et al. 1993), but we also see sensitive strains in areas with substantial antibiotic pollution (Garcia-Armisen et al. 2011), and there is also a background level of naturally occurring resistance (Singer et al. 2006).

Becking's bacterial distribution idiom "everything is everywhere, but the environment selects" (Baas-Becking 1934) remains attractive in light of the difficulty in identifying how antibiotic resistance is distributed and the physical and ecological factors most important to environmental resistance. Identifying bacterial ecological traits may help decipher their distributions under natural conditions in a spatially explicit manner.

As expected, antibiotic resistance occurs in highly polluted waters (West et al. 2010), but also in pristine streams (Pei et al. 2006) where its adaptive advantage is unclear. Although resistance is an ancient trait that has been evolving for hundreds of millions of years (Garau et al. 2005), our understanding of its ecological role and significance in natural populations is just emerging. But it seems to clear that natural levels of antibiotics in the environment are usually low (Aminov 2009).

Environmental antibiotic resistance most likely results from many environmental conditions, stochastic events and natural processes that are currently obscure. But what role does microbial ecology play? We are

beginning to see how ecology operates within the human microbiome as HGT was found to be shaped more by chemical gradients and symbiosis than phylogeny or habitat niche (Smillie et al. 2011), and the patterns were consistent across spatial scales and functional class.

The large amount of anthropogenic antibiotic inputs into rivers may not be the only selective pressure for environmental bacteria to acquire resistance (Salyers 1997), as antibiotics are naturally produced compounds whose complete range of functions remain unknown (Aminov 2009). Several likely antibiotic functions include defense, competition and quorum sensing (Martinez 2009), sole-carbon energy sources for specific metabolic pathways (Baquero et al. 2008), regulatory or signaling compounds in response to changing environmental conditions (Martı and Fajardo 2008), or regulators of bacterial gene expression (Davies et al. 2006).

Antibiotic-producing bacteria occur naturally in fresh-water and resistance occurs in samples prior to the production of commercial antibiotics (Singer et al. 2006). The natural background concentrations of antibiotics is an important factor as antibiotics such as ß-lactams, streptomycins, and aminoglycosides are produced by soil bacteria (Kümmerer 2009b) and these inputs will vary by runoff quantity and soil percolation rates. This indicates that levels of resistance in rivers probably change seasonally and depend if the host is an animal, plant, or soil (Baquero et al. 2008). Most antibiotics don't persist in the environment and are readily degraded or eliminated by

natural processes including biodegradation by bacteria or fungi, elimination by sorption, hydrolysis, oxidation/reduction, or photo-degradation (Engemann et al. 2008). To better understand the ecology of antibiotic resistance in rivers we should identify the environmental constraints that regulate its distribution (Horner-Devine et al. 2004).

The source of resistant determinants is beyond the scope of this study but it is well established that land use plays a major role in stream contamination and there is no debate that urban streams have undergone significant degradation and are particularly impacted from stormwater runoff. What are unknown are the ecological conditions that determine where resistance occurs and persists and few studies have focused on antibiotic resistance in rivers in a spatial-explicit manner. By considering landscape ecology and microbial ecology we may be able to determine patterns of habitat heterogeneity at multiple spatial scales that will allow us to predict the locations most likely to harbor resistance reservoirs in rivers.

The inputs of sewage or runoff containing animal waste is a critical factor for river ecology as this mixes large numbers of enteric bacteria, antibiotics, and resistance determinants with naturally occurring bacterial strains. Most (97%) of the resistant isolates in oligotrophic lakes were found to be gram-negative bacteria (Pontes et al. 2009), and many enterics including

Escherichia coli (E. coli) easily survive in the environment and readily transfer resistance genes to environmental species (Galvin et al. 2010).

Non-continuous selective pressure along a river may result in a spatially-distinct resistant niche architecture where the physiological cost of maintaining antibiotic resistant genes is balanced by the environmental constraints on fitness (Elliott et al. 2010).

Identifying the distribution and resistance profiles of aquatic bacteria should be done in a scale-dependent manner; 1) bacterial abundance in the stream compartment; water, sediment, and biofilm where genetic exchange is density-dependent (Baquero et al. 2008), 2) in the stream reach where water physicochemistry and hydrology (shear and transport) drive successional changes (Clifford et al. 2002), 3) by the river segment or "functional process zone" where top-down controls of geomorphology determine microbial ecology (Callow and Boggs 2013), and 4) across the watershed where anthropogenic activity dominates the landscape and gradients of resistance correlate with development.

Antibiotic resistance confers a competitive advantage when there are antibiotics in the environment but sometimes resistance persists without selective pressure when it is linked to genes for metal resistance or other adaptive traits (Mcarthur and Tuckfield 2000). Urban rivers are commonly polluted with metals (Fechner et al. 2012) and antibiotics (Lupo et al. 2012)

making the physiological cost of maintaining multiple resistant genes a fitness advantage.

MEASUREMENT FRAMEWORKS TO ADDRESS THE PROBLEM

Rivers are heterogeneous and dynamic ecosystems that exhibit habitat zones along physical, chemical and biological gradients (Mcguire et al. 2014). These habitat zones are driven first by physical forcing, and second by ecology (Besemer et al. 2009). As the physical landscape changes so does the microbial community structure which in turn alters the functional status of the stream in very fundamental ways (Reynolds and Elliott 2012). Patterns of dissolved organic matter and water chemistry are influenced by the geological variation of the river bedrock which is correlated with bacterial composition (Mosher and Findlay 2011).

A widely held perception of river ecology is the "river continuum concept" (RCC) from Vannote (Vannote and Cummins 1980). This concept was novel at the time and represented rivers as gradual gradients of conditions from headwaters to the sea driven largely by basin geomorphology. The RCC was good at describing the distributions of macro-invertebrates which is widely utilized for monitoring water quality, but the concept may not describe how bacteria distribute themselves across a watershed where land use, pollution and stochastic events disrupt ecological gradients at various scales. There is also debate about the continuum's usefulness in describing microbial communities from a purely scalar perspective. In order to predict

microbial distributions in urban rivers we must understand the ecological constraints both in the headwater's where the stream is minimally impacted to downstream as the ecosystem is transformed by development.

Rivers are currently classified in a number of ways; by seasonal flow dynamics (Villeneuve et al. 2011), channel morphology and bedform (Besemer et al. 2009), stream power (Barker et al. 2008), and riparian vegetation (Lowell et al. 2009) so a complete measurement framework that represents rivers as hydrologically connected but with spatially-stratified habitats may be meaningful.

RIVERINE SYNTHESIS MODEL

A more integrative approach to describing riverine habitats has been written about in several papers and books known as the "riverine ecosystem synthesis framework" (RES) by James Thorp. This framework couples large-scale discontinuous patterns with small-scale ecological conditions (Thorp et al. 2006). In order to apply this model to antibiotic resistance in rivers we must consider landscape ecology (the human impact on the land), microbial ecology, and the ecology and geography of antibiotic "hot spots" in the environment. This approach may help to explain patterns of antibiotic resistance we observe in rivers.

River ecosystems are inherently hierarchal and compartmentalized but are also networks with a mostly unidirectional flow (Isaak et al. 2014). Stream

order increases as elevation decreases, the main corridor is broken up into segments between confluences and sinuosity changes with topography and drainage density (Som et al. 2014). Rivers are composed of alternating pool-riffle-run sequences which demonstrate unique substrate composition, size, embeddedness, and roughness (Palmer et al. 2010). Pool-riffle-run sequences increase in number and decrease in size with increasing river sinuosity (Frissell et al. 1986). River water chemistry and energy change across multiple spatial scales but all river biota are constrained by top-down physical forcing, predation or grazing; and by bottom-up biogeochemical cycles; all within the context of stochasticity at all scales and magnitudes (Beauchamp 2011); from landslides that divert entire rivers to a cow crossing a stream and breaking down the bank.

FUNCTIONAL PROCESS ZONES AND THEIR COMPONENTS

The foundation of the RES framework is the functional process zone (FPZ). These zones are unique sections in the river determined by hydrogeomorphic and physiochemical patches from which ecosystem structure and function flow (Thorp et al. 2006). By decoupling the components of an ecosystem we can account for the influence of each and fairly evaluate differences between and among patches at any spatial scale (Pickett and White, 1985). It is at the patch level that people have contact with the water and swimming and fishing are localized activities along a

river. Parks, boat launches, and other river access points are places where many people and their pets frequent in an urban setting.

The linkages between pattern and process are also scale-dependent. A typical river *pattern* is the alternating pool-riffle-run sequence along a river bend with an associated *process* the rate of oxygen diffusion into the water column due to the rugosity of the substrate and depth of the water. These unique conditions are then evaluated by gravid salmon for nesting suitability based on the oxygen profile. Bacteria also show distinct distributions with regards to microhabitat conditions such as pH (Rousk et al. 2010), temperature (Maal-Bared et al. 2013), and habitat heterogeneity (Lowell et al. 2009, Singer et al. 2010).

The RES model comprises several tenets for predicting patterns: i) recurring hydrogeomorphic patterns or FPZs, that are independent of longitudinal river position, ii) high diversity transition zones between FPZs, iii) hyporheic zones within FPZs that provide connectivity between microhabitats that vary with water flow and substrate size and are important sites for nutrient fluxes and organismal transport, and iv) areas of water intermittency such as gravel bars and side channels where successional changes are driven by disturbances of water flow.

The physical boundaries between FPZs can be identified by channel morphology, slope, and flow, and each FPZ can be characterized by geology,

drainage basin size, water chemistry, substrate type and size, stream power, and soil percolation rate. As FPZs change the biotic community adjusts and areas such as confluences and reservoirs may reset environmental conditions or act as reservoirs of certain traits including antibiotic resistance.

FPZ transition zones are ecotones that are inherently more diverse, and reaches with intermediate levels of disturbance are the most diverse (Townsend et al. 1997). Bacterial abundance is positively correlated with substrate size and some fresh-water biofilm communities generate their own diversity to maintain ecological resilience (Boles et al. 2005). Bacteria diversity and abundance changes rapidly in response to change in water temperature and flow (Febria et al. 2011) indicating that the scale of microbial ecotones will be dynamic and resist simple classification.

The location of ecotones in natural river segments are influenced by geomorphology, hydrology and land cover, but the most common natural river ecotones are at tributary confluences, surrounding vegetated islands, and between side channels (Thorp et al. 2006). As rivers flow into urban areas ecotone frequency declines as habitats become more homogeneous and are driven mostly by land use, anthropogenic inputs, and bank stabilization and channelization activities.

There are five common microbial compartments in rivers: (1) the water column, (2) on the surfaces of suspended matter, (3) as biofilms on stones or other large surfaces (epilithon), (4) in sediments, and (5) on or in aquatic animals (van Elsas and Bailey 2002). Biofilms and sediments have been found to contain higher numbers of antibiotic resistant bacteria (Maal-Bared et al. 2013) so they may sequester and maintain resistant reservoirs in the river. When biofilms or sediments are disturbed by storm events they may release resistant determinants that then become mobilized in the environment. Bacterial distribution patterns are usually partitioned along environmental or biotic controls (Borcard et al. 2014), but incorporating spatial dimensionality and environmental processes into a modeling framework may better explain resistance bacterial distributions.

PURPOSE

The goals of this study are to survey the distribution of antibiotic resistant gram-negative coliforms from the headwaters to the mouth of the Green-Duwamish watershed. The objectives of this study are to evaluate if any water quality or environmental variable, land use or geographic measure is correlated with the distribution of antibiotic resistance.

HYPOTHESES

H_{O1}: Total coliform abundance will increase along an urban development gradient across the watershed.

> H_{a1}: Total coliform abundance will show non-linear spatial distribution along the longitudinal reaches of the watershed.

H_{O2}: Antibiotic resistance profiles will increase with land-use development along the longitudinal reaches of the watershed.

> Ha_2: Antibiotic resistance profiles will be more similar in like habitats than either is to adjacent assemblages in unlike habitats.

To test these hypotheses, I used culture-based methods to compare the MPN of coliforms and antibiotic resistance to the physical features and forces along the river. To my knowledge no previous study has compared the distribution of gram-negative antibiotic resistance with environmental characteristics and constraints across a mixed-land use watershed. Antibiotic resistance is a natural component of microbial ecology but is also a product of anthropogenic inputs and landscape ecology manifested at different spatial scales.

MATERIALS AND METHODS

STUDY AREA

The Green-Duwamish river watershed lies in southern King County Washington (Figure 2). The Green River is the main corridor that originates in the Cascade Mountains near Stampede Pass and flows north-west for over 144 km until becoming the Duwamish River 19.3 km before emptying into Eliot Bay at Seattle.

The watershed can be divided into three sub-watersheds; the lower sub-watershed is mostly residential, industrial, and commercial land use; the middle sub-watershed extends up to the Howard Hanson Dam and is a mix of residential, managed forests, and agriculture, and the upper sub-watershed extending from the dam to the crest of the Cascade Mountains is entirely forested.

The Green River serves as the City of Tacoma's primary water supply and the city owns about 14% of the upper watershed and partners with private land owners to protect and manage the forested headwaters. Snow melt and rainfall are the major sources of the river water. The upper watershed is surrounded by mountains with limited roads and all access roads are gated and guarded. About five km below the Howard Hanson reservoir at the Headwaters Dam 246 million liters of fresh water are diverted each day to provide 300,000 people with drinking water.

On the western end of the watershed the Duwamish River empties into Elliot Bay and has served as a major cargo transportation and industrial corridor for over a hundred years. The lower Duwamish is flanked by industrial and manufacturing facilities, public parks, and the Seattle neighborhoods of South Park and Georgetown.

The lower Duwamish River is extremely polluted due to the historical legacy of industrialization that contaminated the waterway with manufacturing waste including heavy metals, polychlorinated biphenyls (PCBs), dioxins, and carcinogenic polycyclic aromatic hydrocarbons (PAHs). The contamination is extensive in the sediments and the lower eight km of the river was designated by the Environmental Protection Agency (EPA) a Superfund Site in 2001.

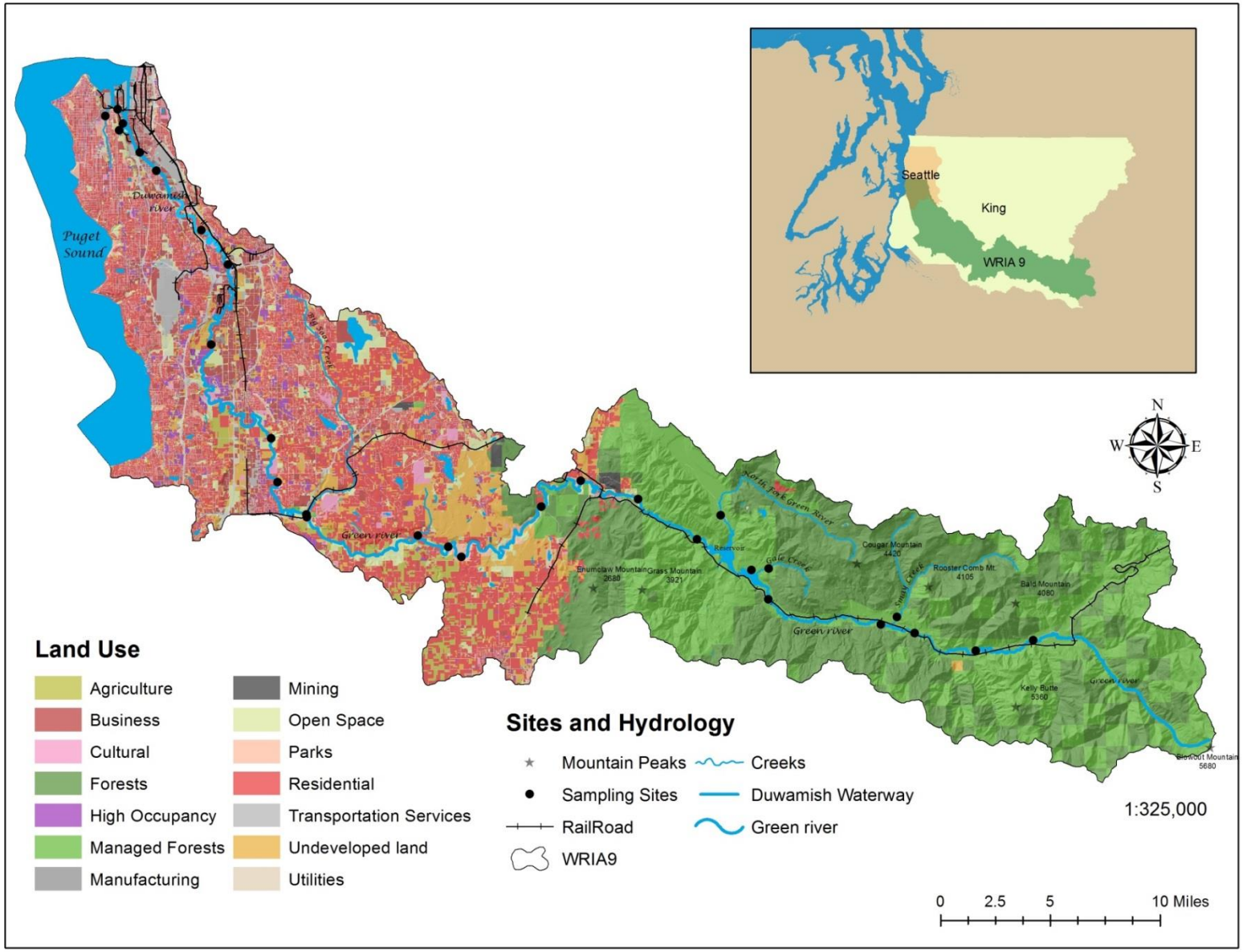


Figure 2. Map of study sites and land use categories in the Green-Duwamish River Watershed in Washington.

In spite of this ominous designation recreational use of the river is common. There are several parks, boat launches and walking trails in the area and sport and subsistence fishing are common activities for many members of the community including the Muckelshoot Indian Tribe. Many of the toxic compounds have reached unsafe levels in resident fish and shellfish and advisories are placed near parks and other access points to inform the public (Figure 3).

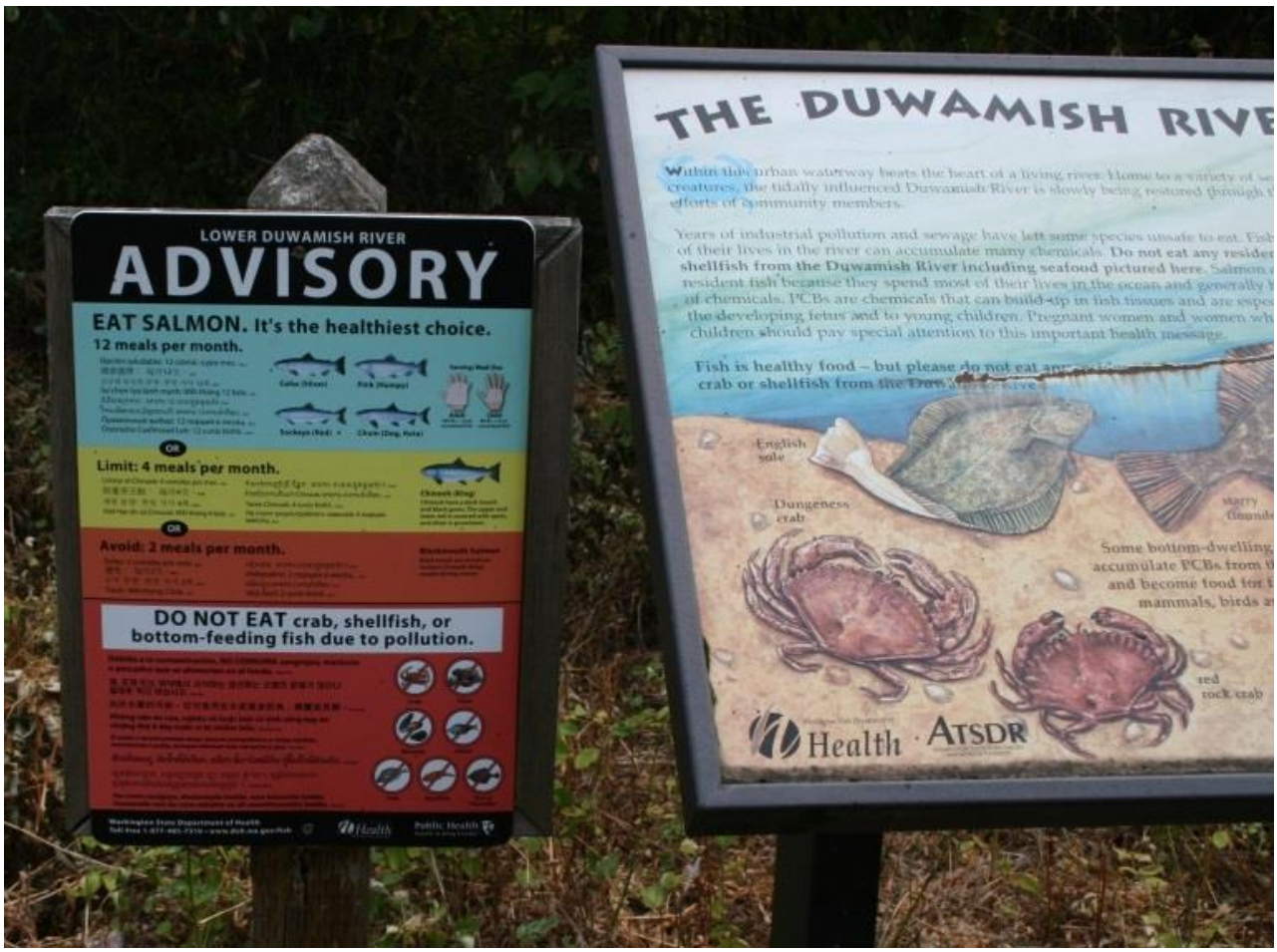


Figure 3. Public signs for contamination of fish and shellfish along the Duwamish River. (Photo A. Klock)

In stark contrast, the upper sub-watershed has generally good water quality and provides extensive recreational opportunities and wildlife habitat. The Green River is a salmon spawning and rearing habitat for Chinook, chum, coho and winter steelhead, and the upper watershed is home to a large herd of elk, as well as black bear, cougar and many species of native birds.

STUDY DESIGN

The study is a nested design at three scales; i) watershed-wide, ii) site (29 fixed) within the watershed; and iii) stream compartment with three levels (water, biofilm, sediment) nested within site.

SAMPLING AND FIELD DATA COLLECTION

Approximate sampling site locations were mapped prior to the survey. Many sites were in highly developed and inaccessible areas such as industrial parks or flanked by private property. Sites were selected so as to be spaced approximately eight km apart from the estuary to the headwaters. Permission was acquired to sample above the Howard Hansen dam with the requirement of having an official escort from the City of Tacoma during that portion of the sampling (Sites 19-29) (Figure 4).

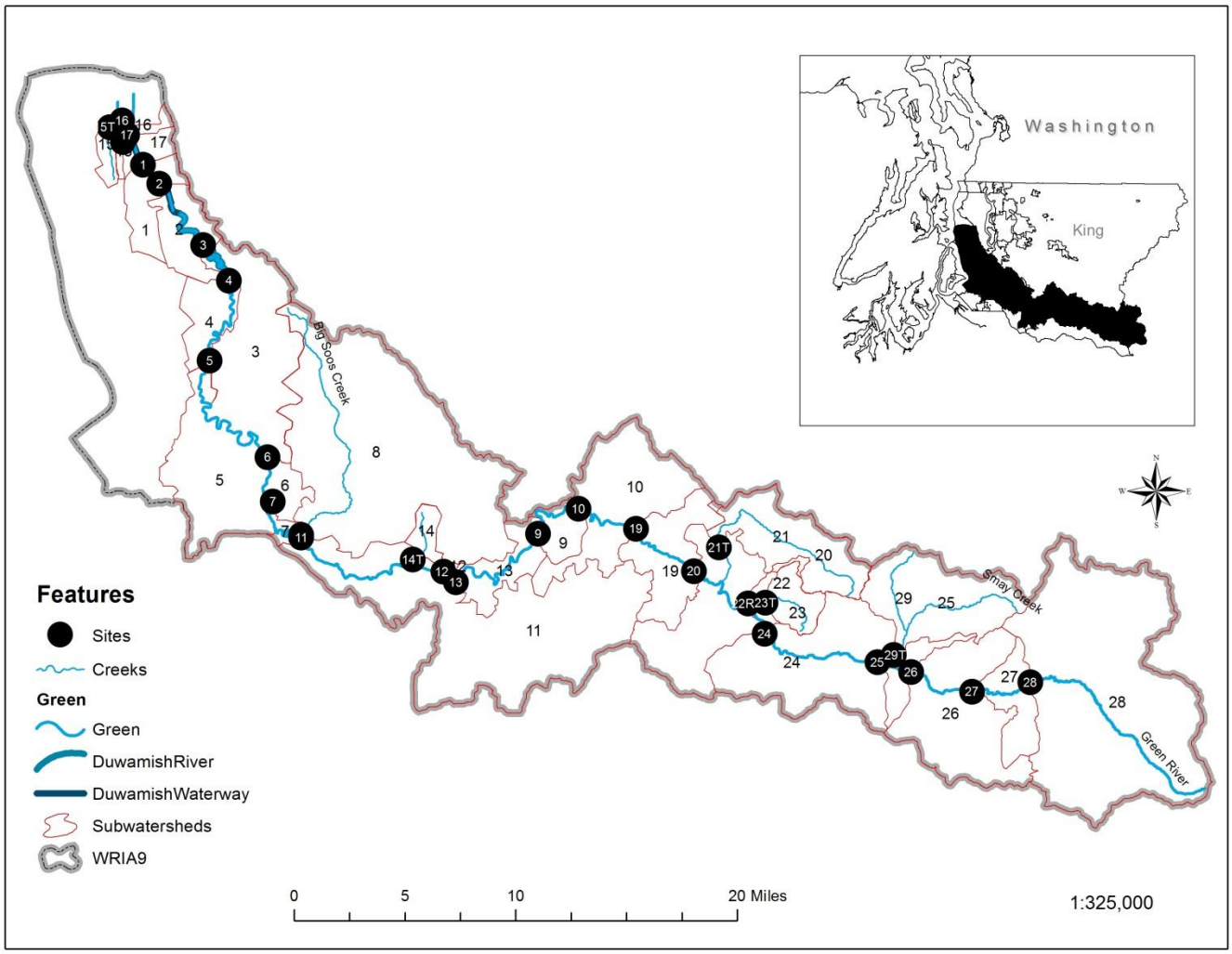


Figure 4. Overview map of sampling locations and associated sub-watersheds.

Twenty nine sampling sites were visited between July 1 to September 3, 2014 (Table 1). Sampling was conducted during the summer months of June through the middle of September during dry times with no appreciable rain for more than a week. The summer is also the most nutrient poor time of the year.

Upon arriving at each site a GPS position was recorded and the area was photographed.

Table 1. Summary of sample sites.

Site No.	*Date*	*River Mile*	*Stream Name*
15	8/12	1.28	Longfellow creek
16	8/12	1.63	Duwamish waterway
17	8/12	2.33	Duwamish waterway
18	8/12	2.53	Puget creek
1	7/1	3.62	Duwamish river
2	7/1	5.10	Duwamish river
3	7/1	9.45	Green river
4	7/1	12.75	Green river
5	7/1	18.42	Green river
6	7/1	28.20	Green river
7	7/1	30.60	Green river
8	8/8	34.25	Big Soos creek
11	8/8	34.40	Green river
14	8/8	40.40	Crisp creek
12	8/8	43.27	Green river
13	8/8	44.55	Green river
9	7/6	51.50	Green river
10	7/6	56.30	Green river
19	9/3	60.00	Green river
20	9/3	63.90	Green river
21	9/3	65.60	Northfork Green river
22	9/3	68.10	Reservoir
23	9/3	68.30	Gale creek
24	9/3	69.70	Green river
25	9/3	76.04	Green river
29	9/3	76.54	Smay creek
26	9/3	77.70	Green river
27	9/3	81.22	Green river
28	9/3	85.24	Green river

A total of 65 samples were taken in this study (29 water, 22 biofilm, 14 sediment). Eighteen sites were on the Green river main corridor and five sites on Green river tributaries; four sites were along the Duwamish River or waterway, and two were taken from Duwamish waterway tributaries. At each site a water sample was taken, and a biofilm or sediment or both depending on the substrate size on the stream bed at each site.

Water samples were collected in three sterile 60mL Nalgene bottles that were submerged and opened approximately 10cm above the substrate, allowed to fill with stream water and recapped underwater. Biofilms were collected by wiping a cobble or boulder surface with a sterile Speci-sponge(™) several times and replacing the sponge into its sterile zip pouch. Sediment samples were scooped into a sterile 100mL specimen container and capped under water. Biofilm and sediment samples were taken from approximately 20cm depths. All samples were stored on ice and processed within 24-hours as per standard methods for the examination of water and wastewater (APHA et al., 1998).

Environmental and water quality data were collected to evaluate any association between environmental variables with the presence and abundance of coliforms and levels of antibiotic resistance.

Water parameters were measured just prior to sample collection including temperature (°C), pH, conductivity (µS/cm), specific conductivity (µS/cm), and salinity (ppm) using a YSI-63 sensor (Yellow Springs, OH). Several river channel

characteristics were measured; wetted stream width, bank full width, inclination, azimuth and canopy height with a laser Range Finder (Redfield). The stream substrate was classified by diameter size and the riparian vegetation along the bank characterized by estimating percent canopy cover and tree phenology. The reach was photographed upstream and downstream of each sampling site and bank structure, bank confinement, and elevation were recorded.

COLIFORM MOST PROBABLE NUMBER

Viable coliform counts were calculated as the most probable number (MPN) with the Colilert™ Method with the Quanti-Tray/2000 system (IDEXX Laboratories) according to the manufacturer's instructions. The Colilert system simultaneously detects both total coliform and "presumptive" *E. coli* in water. It is based on a defined substrate technology utilizing *o*-nitrophenyl-β-dgalactopyranoside (ONPG, and the sample turns yellow), and 4-methyl-umbellfieryl-b-d-glucuronide (MUG, and the sample fluoresces) to identify β-glucuronidase, an enzyme present in >95% of all *E. coli* isolates.

The Colilert method has a minimal detectance of 1 colony forming unit (CFU) per 100mL and a maximum cell density of >2914 CFU/100mL. One hundred milliliter water samples were processed without dilution unless a high coliform count was suspected, then a 10 to 100 fold dilution was processed so as to not exceed the optimal sensitivity range of the test. The samples were incubated for 24-hours at 36.5°C. The tray cells where then enumerated for yellow-colored

cells. The trays were then viewed with a 6-watt, 365-nm ultra-violet light placed within 10cm of the sample in a dark environment and all cells that fluoresced were enumerated and marked (Figure 5).

Figure 5. Quanti-Tray 2000 under UV light indicating florescent cells, some with gas formation, that are positive for MUG metabolism.

BACTERIAL EXTRACTION FROM BIOFILMS

In the laboratory 25mL of sterile water were added to the sponge packet and the sponges were massaged by hand for several minutes to detach all of the material and then squeezed to remove the entire sample from the sponge. A 25mL suspension was pipetted from the pouch into a sterile 125mL bottle and

vigorously vortexed with sterile glass beads for five minutes to break-up the biofilm polysaccharide complex. The Colilert substrate was then added to the bottle and sufficient sterile water to total 100mL and was gently mixed to dissolve and then processed as a water sample.

BACTERIAL EXTRACTION FROM SEDIMENT

Twenty-five grams of sediment with all of the river water removed were added to a 125mL glass bottle to which 25mL of sterile water were added. The mixture was then vigorously vortexed with sterile glass beads for five minutes. The resulting slurry was filtered through 12cm filter paper into a sterile 125mL glass bottle with the Colilert substrate and enough sterile water to total 100mL. The suspension was swirled until dissolved and then processed as a water sample.

The MPN for each tray was calculated with the IDEXX MPN Generator Version 3.2 for the Quanti-Tray 2000. The total number of large and small cells are entered and MPN and 95% confidence intervals are returned. MPN ranges are 1.0 to >2419.6 CFU/mL per each 100 mL sample processed which was extended by diluting samples prior to testing.

ESCHERICHIA COLI ANTIBIOTIC RESISTANCE DETERMINATION

To test for antibiotic resistance two Colilert tray cells positive for presumptive *E. coli* were haphazardly selected and marked. The back of the tray was sprayed with 70% ethanol and the cell sliced open with an alcohol-flamed scalpel. A

1.5mL sample was extracted from each cell and placed into a sterile 2mL Eppendorf tube. From each sample four 10µL were sequentially withdrawn and pipetted into the center of each of four 100mm MacConkey plates. One prepared with no added antibiotics (control), and three prepared with 25µg/mL of ampicillin, chloramphenicol, and tetracycline, respectively, in duplicate. Each sample was spread plated with an alcohol-flamed L-shaped glass rod and the plates incubated at 36.5°C for 24 hours and then observed for growth.

Plates with colony growth in the countable range (20-300) were enumerated and the CFU/ml calculated as the colony count divided by the amount pipetted on the plate times the dilution factor. Counts were identified for their ability to ferment lactose; either lactose positive (Lac+) *E. coli* (pink colonies), or lactose negative (Lac-) coliforms (clear colonies) (Figure 6).

Figure 6. MacConkey control plate with growth of lactose positive (on left) and lactose negative (on right) colonies. (Photo A. Klock)

All MacConkey plates were prepared with 25μg/mL of either ampicillin, chloramphenicol or tetracycline which is similar to other studies (Lee 1978). Each of these antibiotics has different modes of action within the bacterial cell and represents different classes of antibiotic.

Ampicillin is a beta-lactam antibiotic with therapeutic uses against both gram-negative and gram-positive bacteria. Ampicillin is listed as one of the most important beta-lactam medications by the WHO. Ampicillin has been used extensively since 1961 and acts as an irreversible inhibitor of the enzyme *transpeptidase* which is an enzyme required for cell wall synthesis. Ampicillin resistance is due to *Beta*-lactamases which break the bonds of the inner four-atom ring of the antibiotic. Beta-lactamases are commonly secreted by gram negative bacteria when antibiotics are present in the environment.

Chloramphenicol is a broad-spectrum antibiotic that first became available in 1949 and was commonly used to treat typhoid fever. It is no longer used in the U.S. due to its toxicity but remains common in developing countries. Chloramphenicol is also effective against gram negative and gram positive bacteria and it disrupts protein synthesis by inhibiting *peptidyl transferase* activity of the ribosome by interfering with substrate binding. There are several mechanisms of resistance to chloramphenicol; reduced membrane permeability, mutation of the 50S subunit, and changes to chloramphenicol *acetyltransferase.* Chloramphenicol resistance may be carried on a plasmid that also codes for

resistance to other drugs. One example, the ACCoT plasmid carries resistance for ampicillin, chloramphenicol, and tetracycline. Chloramphenicol was rarely found in fresh water systems during the 1970s according to Lee (1978), but its presence in fresh-water systems has been increasing (Leff et al. 1993, Pontes et al. 2009).

Tetracycline is a broad-spectrum antibiotic produced by the *Streptomyces* Actinobacteria and is a protein synthesis inhibitor. *E. coli* is susceptible to as little as 1ug/ml concentration and resistance is usually acquired by HGT of an efflux pump gene. Efflux pumps actively eject tetracycline from the cell, preventing the buildup of antibiotic concentrations in the cytoplasm.

STATISTICAL ANALYSES

Statistical analyses were conducted using R (ver. 3.1.0, R Foundation for Statistical Computing, RStudio (ver. 0.98.97, RStudio, Inc), and ArcGIS 10.2 (ESRI, Inc.). I used multivariate statistical methods to illustrate the relationships among coliform abundance sampled from different locations, and a geospatial statistical analysis to model and predict geographic locations of antibiotic resistance along the river network. A distance-based redundancy analysis and ordination was used to identify and visualize the environmental drivers that best explained the observed antibiotic resistance patterns among coliforms and *E. coli*.

The MPN data for coliforms and *E. coli*, and the antibiotic resistant colony counts for each antibiotic of both were processed as eight "species" representing bacterial community structure.

Rarely are ecological count data and proportions normally distributed, so all continuous explanatory variables were plotted in a correlation matrix in R (Appendix A). To remove the possible influence of autocorrelation highly correlated variables were deleted and the remaining variables checked for collinearity with histograms and Shapiro-Wilks Test of Normality. If any variable distributions were highly skewed they were log transformed, with the exception of salinity and pH. The normality of the response data was also checked using Shapiro–Wilks and variables were log(x+1) transformed to improve normality and validate the assumptions of subsequent statistical analysis. Response data resistant to transformation were tested with non-parametric methods. Nonparametric tests are distribution-free and have fewer assumptions, but are less powerful (i.e., when the alternative is true, they may be less likely to reject H_0).

Homogeneity of variance was tested with Fligner-Killeen to meet the assumptions of equal variance among groups. The Fligner-Killeen test has been determined to be robust against departures from normality with non-parametric data sets (Conover et al. 1981).

To avoid artificially increasing the variance and reducing the statistical power of the analyses from multicollinearity all variables were evaluated with a variance inflation factor analysis (VIF). Excessive correlation among explanatory variables can also obscure the identification of an optimal set of explanatory variables for the model. VIF is a powerful diagnostic tool and is easily interpreted; higher VIF score = higher collinearity. The VIF for a single explanatory variable is obtained using the R^2 value of the regression of that variable against all other explanatory variables. The VIF for variable $_j$ is the reciprocal of the inverse of R^2 from the regression.

$$(VIFj = \frac{1}{1 - R_j^2})$$

A step wise R script was used to test and remove variables scoring higher than 7.5 (Table 2). Two known control variables with higher scores were retained; temperature and conductivity. In this study there was high collinearity between many variables including longitude, river mile, and elevation due to topography, basin shape, and flow direction of river. I wanted the models to be as parsimonious as possible and sought to balance complexity and precision.

Table 2. Continuous variables after VIF Analysis

Variable	*Est.*	*Std. Error*	*t-val*	*Pr(>|t|)*
Metazoa	3.97	4.34	8.74	***
StrOrder	-2.78	1.35	-2.06	***
StrLenTot	4.63^{-05}	6.69^{-6}	9.85	***
DrainDen	-6.70^{-2}	1.98^{-2}	-3.46	**
Sinuosity	4.32	3.26	1.32	
Power	4.83^{-5}	2.19^{-5}	2.18	*
SubSmall	8.40	5.56^{-1}	1.51	
SubLarge	1.89	5.06^{-1}	3.74	***
PercAvg	2.11^{+1}	2.78	7.59	***

Variable	*Est.*	*Std. Error*	*t-val*	*Pr(>|t|)*
NearRoad	7.68^{+1}	1.39^{+1}	5.51	***
NearBridge	1.96	2.01	9.72	***
NearRR	1.96	1.01	1.92	.
Refuse	-2.91	4.9	-0.59	
CanCov	1.63^{-1}	4.31^{-2}	3.86	***
Agriculture	-9.98^{-1}	3.67^{-1}	-2.72	**
Commercial	-7.07^{-3}	1.53^{-1}	-0.04	
pH	1.21	1.02	1.18	
Salinity	-1.54^{-1}	1.97^{-1}	-0.77	

GEOGRAPHIC INFORMATION SYSTEM ANALYSIS

Multivariate regression was conducted in a geographic information system (GIS) using ArcMap Ver. 10.2. An exploratory ordinary least square (OLS) regression was run to select the best predictive variables for six antibiotic resistant datasets (three antibiotics x coliform + *E. coli*). The best fitting models were then used to populate a geographically weighted regression (GWR) to make predictions of antibiotic resistance along the stream network.

All field sampling data and laboratory results were recorded in an Excel spreadsheet with latitude and longitude coordinates in the geographic coordinate system WGS 1984 which was the datum native to the GPS unit used in the field. The data sheet treated rows as observations and columns as attributes.

A file geodatabase was created and feature datasets for boundaries, hydrology, land features, infrastructure, raster datasets, and geoprocessing results were created. As data was derived or gathered it was imported and projected to the NAD1983 (HARN State Plane Washington North FIPS 4601 Feet) to ensure alignment and spatial statistical processing accuracy. The data spreadsheet was imported as a table and added to maps as *x, y* data and the sampling sites plotted as a point features.

Ten-meter digital elevation model (DEM) tiles were obtained from the Washington State GIS Data site (WAGDA) and a raster mosaic processed to smooth boundaries. The mosaic was then clipped to the WRIA 9 boundary and the raster processed to derive a hillshade layer, slope, aspect, and 5-meter contour lines for the watershed. Reach slope was calculated from the DEM by standard process in GIS by dividing the range of the 5-meter contours by the reach length through at least two intersections of the river with a contour.

$$slope = \frac{(y2 - y1)}{(x2 - x1)}$$

A sub-basin was calculated for each sampling site with Arc Hydro Tools by treating the sampling site as a pour point. Land use and land cover were spatially joined to the new sub-basin that contained them and summary statistics entered into the sampling site attribute table.

Hydrology layers were obtained from the National Hydrology Dataset (NHD) Geospatial Portal from the USDA-US Forest Service website. The sub-basin drainage density was calculated as sub-basin size divided by the total length of all streams in that sub-basin.

Stream sinuosity or the sinuosity coefficient is the ratio of the curvilinear length (along the stream) divided by the straight line valley distance in the downstream direction between a minimum of two stream inflection points.

$$Sinuosity = \frac{StreamLength}{ValleyLength}$$

Stream sinuosity was calculated for each sampling site by measuring upstream and downstream with the sampling site centered in the area measured.

Stream power is a measure of energy dissipation on the bedforms and banks of a river and can be a meaningful index of flow, sediment transport, channel pattern, migration potential and erosion. Stream power (Ω) was estimated per unit area of the reach by the equation:

$$\Omega = \gamma Q s$$

Where (Ω) is the stream power (watts/m^2), γ the specific weight of water (density 1000kg/m^3) at 10°C times the force of gravity (9.8m/s^2), times Q the water discharge rate (m^3/s), times s the slope of the channel at the sampling site. Stream power was calculated from course resolution discharge data collected

from five gauging stations monitored by the US Army Corps of Engineers in the Green River basin according to the standard method.

Data layers for GIS were obtained from many public domain sources. The county and WRIA boundary polygons were obtained from the King County GIS depot and the Washington Department of Ecology. Geology and soils data were obtained from the National Map Viewer Data Download site (USGS).

Landsat 7 imagery at 30m resolution was collected for land cover, canopy cover and impervious surface from the Multi-Resolution Land Characteristics Consortium (MRLC) 2011 database which is the most recent national land cover product by the MRLC Consortium. The land cover layer includes a 16-class land cover classification scheme at a spatial resolution of 30m. Data transformations were made with conversion tools as needed for spatial analysis.

The watershed geological layer was obtained from the Washington Department of Natural Resources Geoscience Data Center. This layer is at 1:100,000 scale and represents the geologic age of the bedrock and the lithology of the surface features. The geological features for each site were obtained by vector overlay of the sub-basins at 1:10,000 map scale and joined to the site layer.

Stream channel confinement was noted in the field and verified at the reach scale with aerial imagery. Classification were according to standard methods from the Washington Department of Ecology in six categories; (0) hardened (rip

rap), (1) entrenched, (2) confined, (3) frequently confined, (4) occasionally confined, and (5) unconfined, (Figure 7).

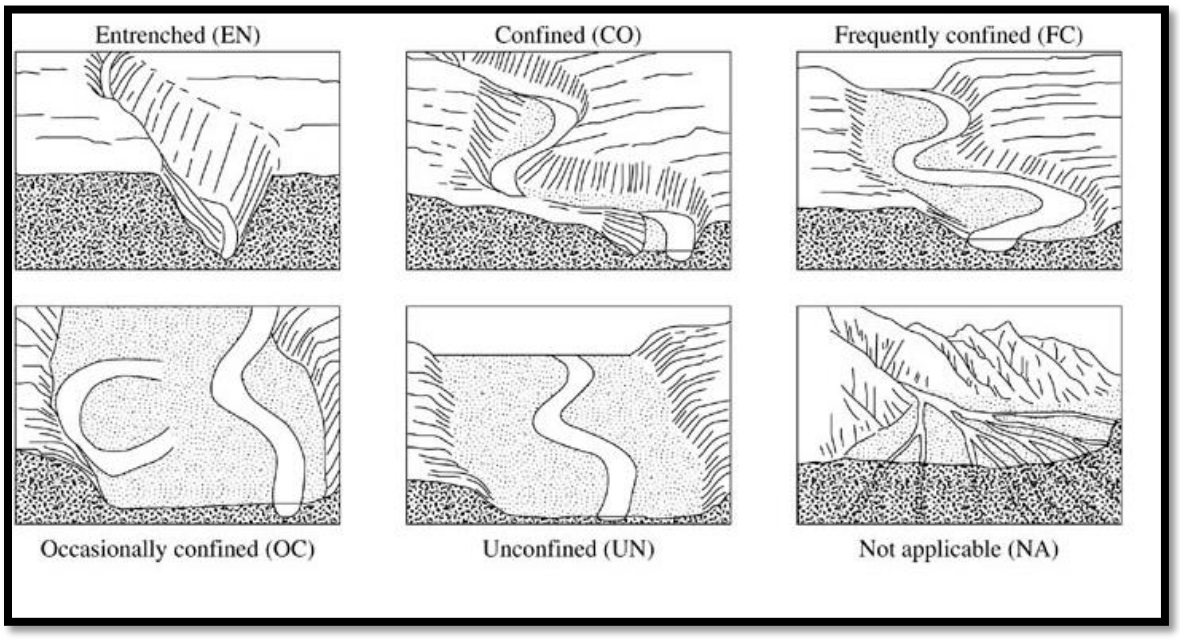


Figure 7. Schematic of stream channel confinement (BC Fisheries 2001).

Stream channel patterns were categorized and ranked from onsite documentation and ortho-imagery into six categories; (1) straight, (2) sinuous, (3) irregular, (4) regular, (5) tortuous and (6) braided, (Figure 8).

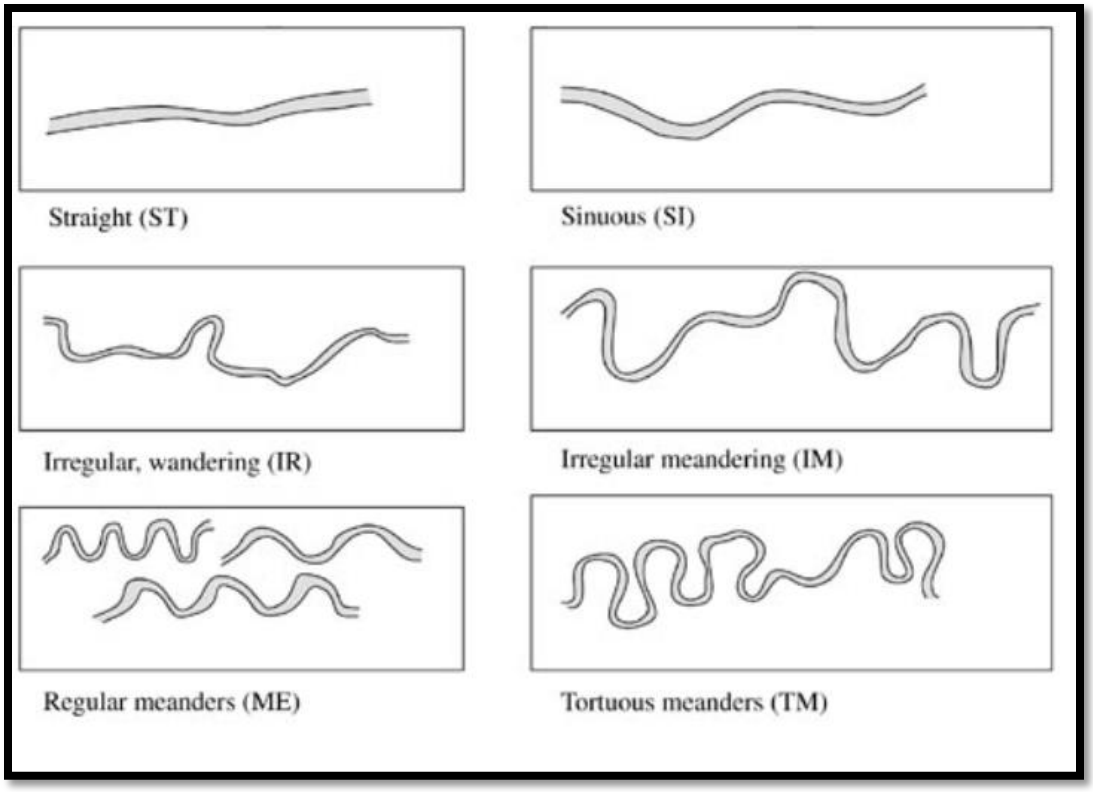


Figure 8. Schematic of stream channel patterns (BC Fisheries 2001).

A near analysis was conducted in GIS to calculate the distance from each sampling site to roads, railroads and bridges and entered into the master data sheet.

The MPN data for coliforms and *E. coli*, and the antibiotic resistant colony counts for each antibiotic, and continuous explanatory variables were interrogated with the OLS tool in ArcMap. The regressions were processed at the watershed and sub-basin scale. The OLS tool iterates through all possible combinations of predictor variables to look for models that best explain the dependent variable. The tool thresholds were set at a minimum adjusted R^2 value that would explain

20% of the variance, a maximum coefficient $p \leq 0.05$, the VIF cutoff of 7.5, a minimum acceptable Jarque-Bera $p \leq 0.10$, and a minimum acceptable spatial autocorrelation $p \leq 0.10$. We selected the OLS model that performed the best and that passed all or most of the diagnostic checks (Table 3).

Table 3. Parameters identified by OLS exploratory regression in GIS.

	AdjR²	*AICc*	*Model: DV~*	*Sig Variables >10%*		*(% neg / % pos)*	
FC	0.38	119.46	-SINUOSITY***	PH	99.09	0.00	100.00
			+NEARBRIDGE***	SINUOSITY	92.85	100.00	0.00
			+NEARRR**	SALINITY	68.69	0.12	99.88
			+HUMANUSE***	STRLENTOT	59.03	99.76	0.24
			+PH***	NEARBRIDGE	35.60	0.69	99.31
				PERCAVG	33.26	90.49	9.51
				NEARROAD	28.02	0.00	100.00
				FCWIDTH	19.52	15.05	84.95
				METAZOA	17.69	0.36	99.64
				HUMANUSE	16.48	59.85	40.15
				REFUSE	13.84	99.88	0.12
				POWER	11.18	81.71	18.29
EC	0.24	144.32	+METAZOA**	SINUOSITY	71.24	100.00	0.00
			+STREAMSLOPE***	IMP ERVIOUS	41.56	0.32	99.68
			+NEARBRIDGE***	PERCAVG	40.99	99.90	0.10
			+IMPERVIOUS***	PH	34.91	0.24	99.76
			-SALINITY**	STOR	29.34	99.64	0.36
				SALINITY	25.10	93.85	6.15
				POWER	17.01	93.24	6.76
				STREAMSLOPE	15.57	8.76	91.24
				WCWIDTH	15.48	86.72	13.28
				NEARRR	13.73	98.45	1.55
Amp	0.20	267.54	+SINUOSITY**	FCLOG	49.76	0.00	100.00
			-POWER**	NEARBRIDGE	42.04	100.00	0.00
			-NRBR*	AGURE	16.28	100.00	0.00
			+COMMERCIAL	COMMERCIAL	15.83	0.00	100.00
			+FCLOG***	SINUOSITY	14.16	0.00	100.00
				HUMANUSE	13.09	2.10	97.90
				PH	10.80	99.44	0.56
Chlor	0.41	1633.20	+STREAMSLOPE +PERCAVG -NRBR -NEARRR+FCLOG	STRLENTOT	25.28	0.37	99.63
Tet	0.17	1663.04	+SINUOSITY** -NRBR*** -SALINITY +IMP -POWER	SINUOSITY	37.19	0.00	100.00
				NEARBRIDGE	32.87	100.00	0.00

(Pr(>\|t\|) 0.05, 0.001**, 0.001***).*

There are six basic steps to good model fitness in ArcGIS (ESRI 2014).

1) Only use explanatory variables that are meaningful to the model? The OLS tool calculates a coefficient for each explanatory variable and tests to determine whether that variable is important.

2) Check for nonstationary variables with a Koenker test.

3) Make sure the variables are behaving logically and as expected?

4) Remove explanatory variables that are redundant with VIF.

5) Check the model standard residuals or local R^2 for over or under predictions and a non-significant Jarque-Bera test.

6) How good is the adjusted R^2 value?

After fitting the OLS model I used a GWR to improve the model for non-stationary variables and to construct predictive maps. GWR is a form of linear regression that can model spatially varying relationships and works by evaluating each response/explanatory variable and constructing a separate equation for every feature in the layer. The output is a feature class with standard residuals and local R^2 values that can be mapped and observed for deviations in predictive power. If there are over or under predictions in some areas the model can then be modified and re-run if necessary. All antibiotic resistance data and the best explanatory OLS models were entered into the GWR and mapped. Total coliform and *E. coli* MPN were included as additional

explanatory variables for antibiotic resistance. The final predictive model was then joined to the hydrology feature dataset and a kernel density plot run on the regression predicted values for the stream network. Distance thresholds were left at the default settings. The kernal density tool was used to produce a raster of feature densities along the streams reaches based on the regression model predicted values. The symbology of the raster was set as high as possible (32 classes) and a color ramp selected. All values of zero were coded white.

DISTANCE-BASED REDUNDANCY ANALYSIS (DB-RDA)

Distance based redundancy analysis (db-RDA) is a linear method with environmental variables represented by arrows. This method is particularly appropriate if you have environmental gradients where most of the species are positively correlated. A db-RDA can measure "species" in different units so the ordination with MPN and resistance as either a binary or count response could be plotted together. Db-RDA is more useful when gradients are short (<3.0), indicating that the majority of species exhibit linear responses to the environmental conditions.

The Partial Constrained Analysis of Principal Coordinates (CAP) ordination method was used with Bray–Curtis distance. Bray-Curtis is a dissimilarity matrix that compares the degree to which sites share the same species (Hawkins and Norris 2000), resulting in an interpretable measure of ecological distance. The distance matrix was corrected for missing rows by adding a tiny value to

each site, and negative eigenvalues were adjusted by adding a constant. A backward removal of the GWR explanatory variables was used until only significantly correlated variables remained. To evaluate the significance of the conditional effects, Monte Carlo permutation of the full model was applied with 9999 unrestricted permutations. Db-RDA is effective with small sample sizes when the explanatory variables are the treatment. Since I was only interested in the environmental context of antibiotic resistance, ordination was used to visualize the gradients of the environmental drivers after partitioning out the variance attributable to purely geographic distance. All significance testing was assessed by permutational analysis of variance (PERMANOVA) with 500-9999 resampling permutations. All multivariate analyses were performed using the R add-on package "vegan" (Oksanen et al. 2013).

Analysis of Similarity

Antibiotic resistance and resistance count data were analyzed with analysis of similarity (ANOSIM) to evaluate the differences between sites and continuous and categorical factors. Again, 9999 randomized permutations were standard for resampling. The ANOSIM statistic R is calculated by the difference of the between-group and within-group mean rank similarities indicating the degree of separation between groups. An R value of one indicates complete separation and a value of zero indicates no separation. A p value is also assigned for each R value.

RESULTS

COLIFORM BACTERIA MOST PROBABLE NUMBER

All sites had measurable counts of total coliforms and *E. coli (n=65),* except three sites (i.e., 6, 10, 14) where *E. coli* were not detected in the biofilm. Fifteen sites had less than 0.5 CFU/mL of *E. coli*. The average MPN for Lac- coliforms was 20.8 (range 0.5-151.2) CFU/mL, and Lac+ coliforms was 0.9 (range 0.0-15.1) CFU/mL across the watershed. The highest levels of total coliforms were from the lower Duwamish (151 CFU/mL) in the sediment and biofilm, and at the Howard Hansen Dam reservoir (121 CFU/mL) in the water. The lowest concentrations were (i.e., 6.3, 15.8, 5.1 CFU/mL) at sites 5, 8, and 10, respectively (Figure 9).

The highest *E. coli* MPN were 7.4 and 15.7 CFU/ml at Longfellow Creek (RM 1.2) in water and biofilm, and 4.9 and 5.2 CFU/ml from the Duwamish River in water and biofilm, respectively (Figure 10).

The MPN of *E. coli* ranged from 20% of total coliforms in a first order tributary on the lower Duwamish River, to less than 0.01% of total coliforms at the Howard Hansen dam reservoir.

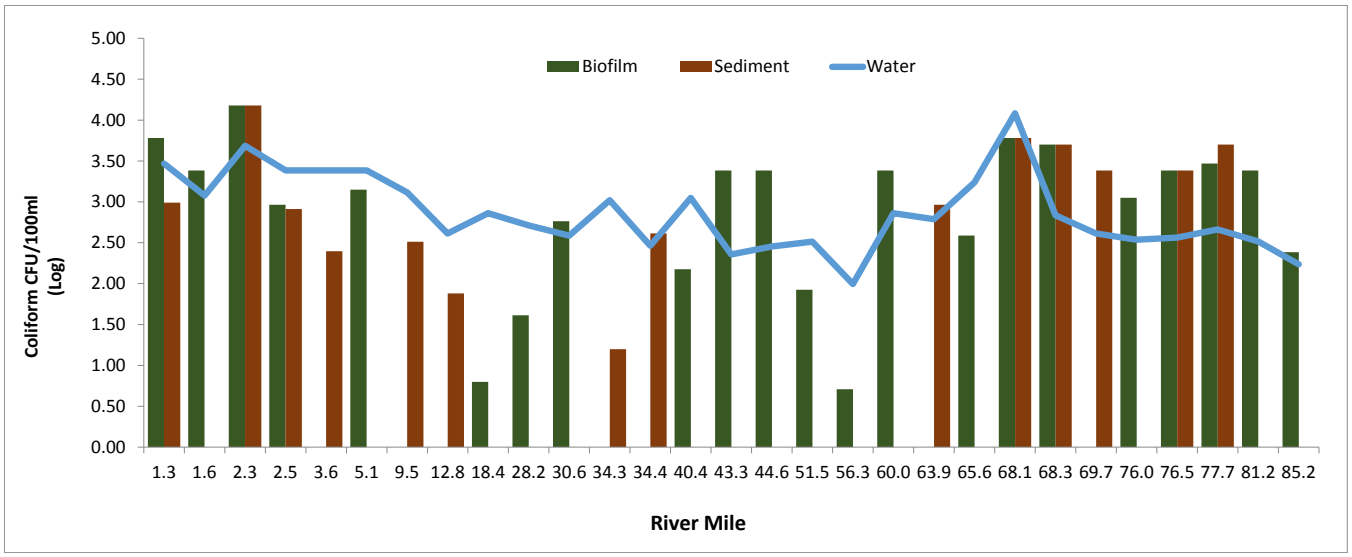


Figure 9. MPN by stream compartment for coliform and river mile.

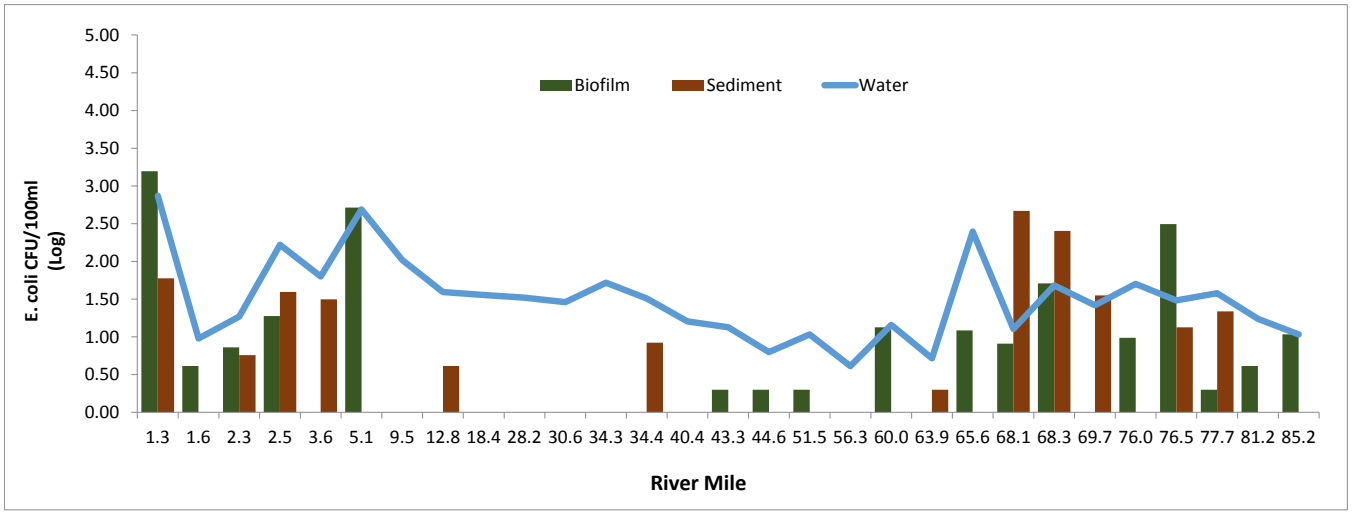


Figure 10. MPN by stream compartment for *E. coli* and river mile.

Generally, pooled MPN data from each site for *E. coli* showed weak correlations with environmental variables including, residential development *(r=0.225, p=0.005)*, being near bridges *(r=0.142, p=0.02)*, agricultural land use *(r=0.116,p=0.03)*, and temperature *(r=0.168, p=0.01)*. When tested by stream

compartment, new correlations appeared; samples from water became significant with stream power *(r=0.273, p=0.002)* and impervious surface *(r=0.141, p=0.02),* while samples from sediments and biofilms did not. Sediments near transportation infrastructure and water near residential development showed significant relationships as well. In general coliform MPN in sediments and biofilms were more highly correlated with environmental conditions than those from water samples (Table 4).

Table 4. Pearson product-moment correlation coefficients (r) for variables and MPN data.

Parameter	***Pooled Mean***		***Sediment***		***Biofilm***		***Water***	
	TC	*EC*	*TC*	*EC*	*TC*	*EC*	*TC*	*EC*
pH	***0.389****	***0.254****	*0.288*	*0.332*	***0.445****	*0.266*	***0.406****	*0.296*
Salinity	***0.497****	*-0.074*	***0.802****	*-0.155*	***0.574****	*-0.073*	*0.207*	*-0.067*
Drainage	***-0.263****	*-0.172*	*-0.357*	*-0.335*	*-0.255*	*-0.138*	*-0.234*	*-0.184*
WCWidth	***0.642****	*-0.090*	***0.790****	*-0.070*	***0.773****	*-0.105*	*0.353*	*-0.100*
Sinuosity	***-0.267****	***-0.277****	*-0.263*	*-0.348*	*-0.217*	*-0.294*	*-0.339*	*-0.306*
Substrate	***-0.339****	*0.026*	*-0.235*	*-0.187*	***-0.468****	*-0.026*	*-0.313*	*0.130*
Percolation	***-0.399****	*-0.260*	*-0.664*	*-0.523*	***-0.526****	*-0.158*	*-0.244*	*-0.192*
Near Road	***0.485****	*0.013*	*0.378*	***0.780****	*0.324*	*-0.158*	***0.778****	*-0.171*
Near RR	*-0.157*	*-0.082*	*0.121*	***0.583****	*-0.230*	*-0.159*	*-0.154*	*-0.090*
Canopy Cov	***-0.41****	*-0.013*	***-0.557****	*-0.272*	*-0.344*	*0.051*	***-0.383****	*-0.020*
Residential	***-0.282****	*0.249*	***-0.589****	*-0.350*	*-0.244*	*0.397*	*-0.076*	***0.360****

(*Pr(>|t|) 0.05*, 0.001**, 0.001****).

Antibiotic resistance along the Green River was common but spatially explicit. Eight sites had resistance in every sample compartment and half were tributaries (T); site 15T, 16, 17, 18T, 1, 3, 14T, and 21T. Twenty one sites had at least one sample that was antibiotic sensitive and these were evenly distributed among stream compartments; water 48% sensitive, biofilms 50% sensitive, and sediments 50% sensitive. There were five sites with no resistance above site 10. The distribution of antibiotic resistance appeared to be non-random (Figure 11).

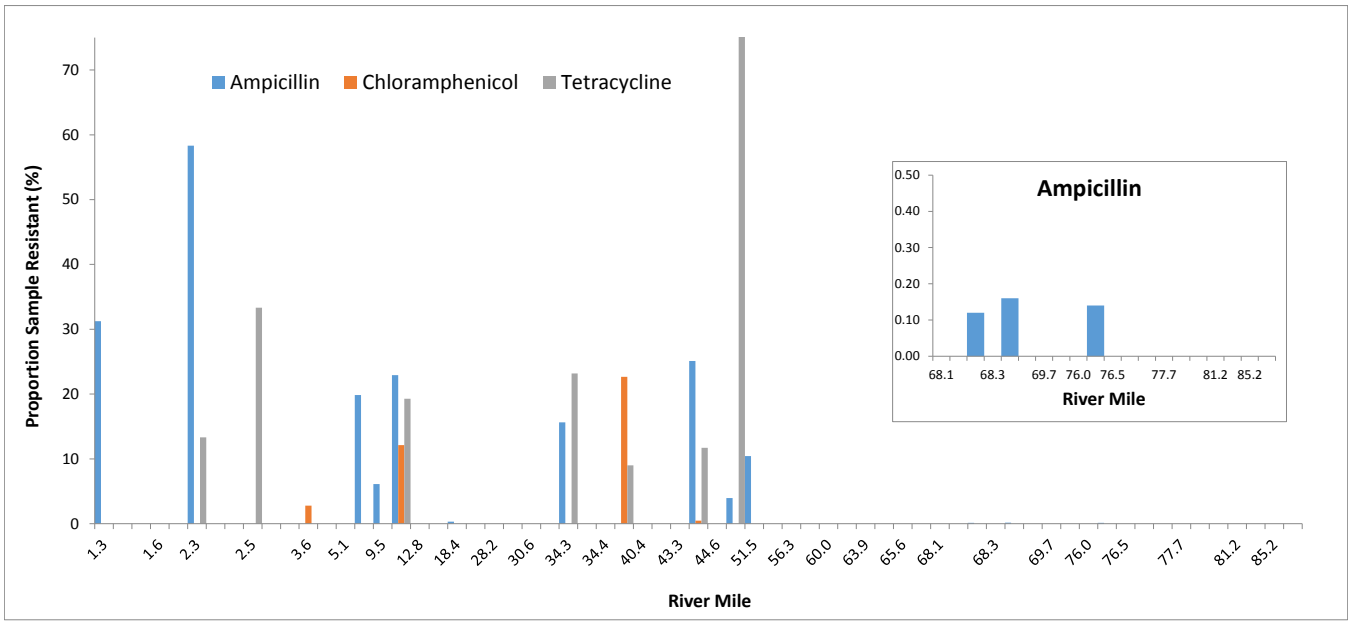


Figure 11. Profile of antibiotic resistance of *E. coli* across the watershed by river mile.

The resistant colony counts on MacConkey agar plates from all sites ranged from 1.2×10^1 CFU/mL to 7.5×10^5 CFU/mL. The largest proportion of resistance was ampicillin (55.4%), followed by tetracycline (29.2%), and chloramphenicol (15.4%) (Table 5).

Table 5. Resistance as a percent of samples by antibiotic among stream compartments.

Compartments	***Amp TC***	***Amp EC***	***Chlor TC***	***Chlor EC***	***Tet TC***	***Tet EC***	***Total %***
Water *n=29*	10 *(34.5)*	6 *(20.7)*	6 *(20.7)*	1 *(3.5)*	6 *(20.7)*	3 *(10.3)*	***49.2***
Biofilm *n=22*	8 *(36.4)*	5 *(22.7)*	0 *(0.0)*	1 *(4.5)*	2 *(9.1)*	2 *(9.1)*	***27.7***
Sediment *n=14*	5 *(35.7)*	2 *(14.3)*	1 *(7.1)*	1 *(7.13)*	4 *(28.6)*	2 *(14.3)*	***23.1***
Samples (n=65)	***35.4***	***20.0***	***10.8***	***4.6***	***18.5***	***10.7***	***100%***

Ampicillin resistance in *E. coli* changed proportions from main channel to tributary (Figures 12-14), and was only found at three sites above the Howard Hansen dam in very small proportions (~0.15%) in sediments and biofilms mostly among environmental species (Appendix B).

There was no correlation found between *E. coli* MPN and resistance among environmental coliforms, which has been noted by others (Garcia-Armisen et al. 2011), but in the current study there were weak relationships of the converse, total coliform MPN to *E. coli* MPN and *E. coli* resistance (Table 6), and resistant Lac- coliforms were significantly different by site (ANOSIM *R=0.343, p=0.012*).

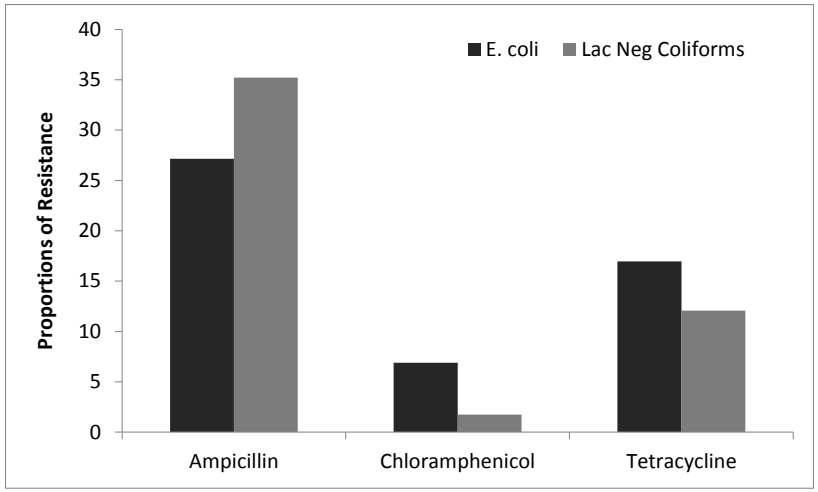


Figure 12. Relative proportions of resistant coliforms from all samples tested.

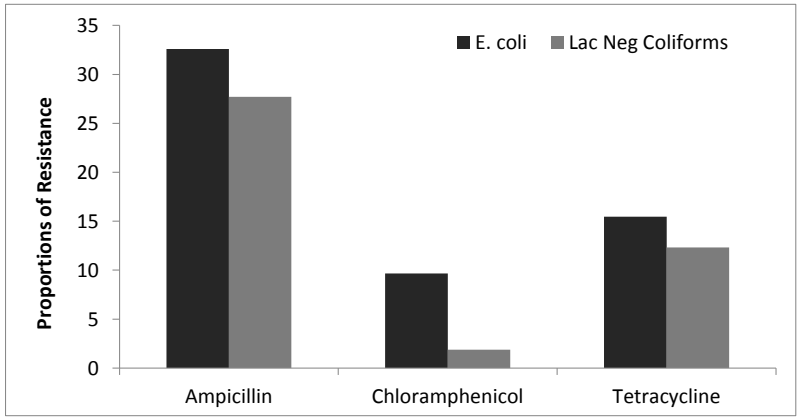


Figure 13. Relative proportions of resistant coliforms in main channels.

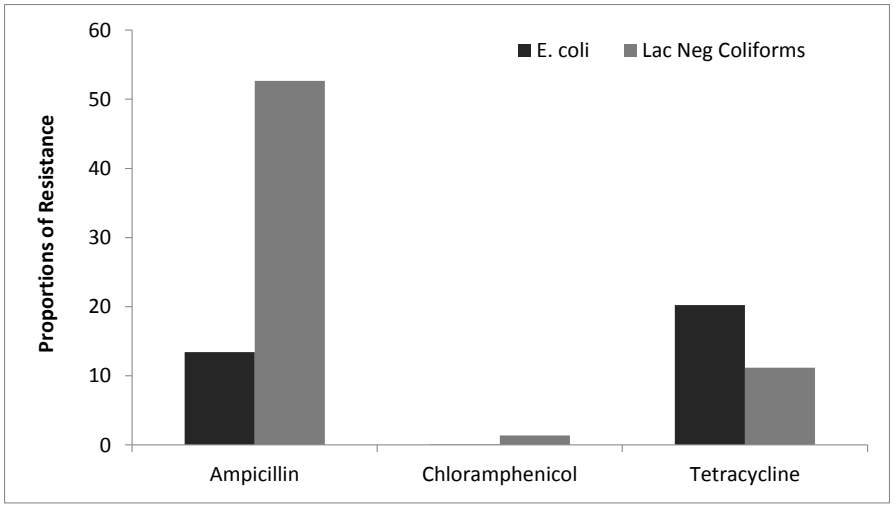


Figure 14. Relative proportions of resistant coliforms in tributaries.

Table 6. Simple regression coefficients of MPN as an explanatory variable for *E. coli* resistance among stream compartments (Chloramphenicol was not significant).

Ampicillin		***Beta***	***p value***	***Adj. R^2***
MPN	Total*	0.776	*0.001*	*0.202*
Coliform	Water	0.897	*0.007*	*0.204*
	Biofilm	0.848	*0.012*	*0.229*
	Sediment		**	
E. coli	Total*	1.476	*<0.001*	*0.172*
	Water	1.660	*0.005*	*0.213*
	Biofilm	1.752	*0.032*	*0.161*
	Sediment		**	
Tetracycline		***Beta***	***P value***	***Adj. R^2***
MPN	Total*	0.587	*<0.001*	*0.147*
Coliform	Water	0.597	*0.020*	*0.147*
	Biofilm	0.635	*0.036*	*0.155*
	Sediment		**	
E. coli	Total*	0.757	*0.040*	*0.049*
	Water	1.036	*0.028*	*0.131*
	Biofilm		**	
	Sediment		**	

**Includes all observations. **Not significant at p<0.05.*

The proportion of antibiotic resistant to control plate counts varied by stream compartment and antibiotic. The proportion of ampicillin resistance ranged from 0.12% to 58.3%, chloramphenicol from 0.5-22.6%, and tetracycline from 9.0-95.0% (Appendix C). Proportions under 1% were most common in biofilms. Although all resistance occurred more frequently in the water, ampicillin and tetracycline resistance was completely partitioned between Lac+ and Lac- coliforms at each site (i.e., no sites had Lac+ and Lac- organisms resistant to these antibiotics), and this pattern persisted in sediments and biofilms except for two sites which had ampicillin resistance in both organisms in biofilms (i.e., Site 13, Site 23), and Site 11 which was the only site with resistance across multiple stream compartments and species. Chloramphenicol resistance, although less common, behaved differently and was mostly found in both Lac+ and Lac- coliforms and often at the same site. Most chloramphenicol resistance was in low levels (200-100,000 CFU/mL) except for site 11 which had 6.5×10^5 Lac+ CFU/mL in the sediment (Figure 15).

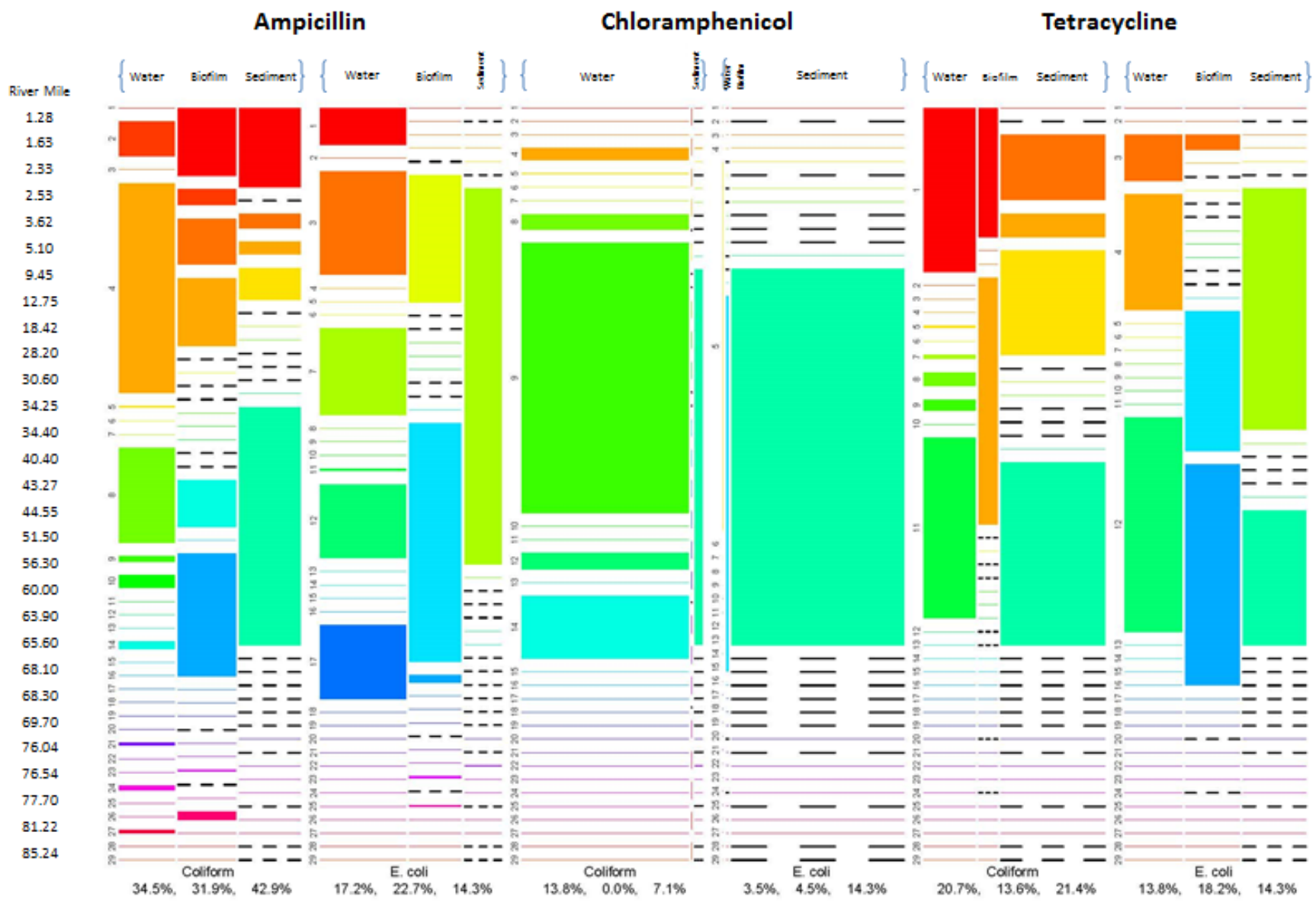


Figure 15. Mosaic plot of probabilities for resistance by antibiotic, stream compartment, and coliform group. Percentages are the proportion of samples resistant in each stream compartment.

Pearson correlation coefficients between antibiotic resistant CFUs were calculated for each antibiotic for Lac- and Lac+ coliforms. There were not enough observations of Lac+ chloramphenicol resistance *(n=3)* (Table 7).

Table 7. Antibiotic resistant CFU Pearson correlation matrix.

Variables	*Amp Lac+*	*Amp Lac-*	*Chlor Lac-*	*Tet Lac+*	*Tet Lac-*
Amp (+)	1.00				
Amp (-)	0.201	1.00			
Chlor (-)	0.061	-0.181	1.00		
Tet (+)	0.145	0.007	-0.434	1.00	
Tet (-)	-0.049	0.685	0.065	-0.255	1.00

The relative proportions of resistance to more than one antibiotic varied between Lac+ and Lac- coliforms but was not statistically significant among antibiotic classes; Ampicillin *t(1)=-1.0195, p=0.4939;* Tetracycline *t(2)=-1.0085, p=0.4194*.

There were six sites where Lac+ bacteria were resistant to more than one antibiotic and eight where Lac- coliforms exhibited multiple resistances, and four sites overlapped among them (i.e., 8, 11, 17, 18) (Tables 8-9).

Table 8. Summary of sites with resistance to more than one antibiotic in Lac + cultures.

Site	*Compartment*	*Resistance*	*Antibiotics*	*FPZ*
3	Water	2	Ampicillin, Tetracycline	Lower main stem
8T	Water	2	Ampicillin, Tetracycline	Middle tributary
11	Biofilm	2	Chloramphenicol, Tetracycline	Middle main stem
12	Biofilm	3	Ampicillin, Chloramphenicol, Tetracycline	Middle main stem
17	Water	2	Ampicillin, Tetracycline	Lower main stem
18T	Water	2	Chloramphenicol, Tetracycline	Lower tributary

Table 9. Summary of sites with resistance to more than one antibiotic in Lac- cultures.

Site	*Sample*	*Resistance*	*Antibiotic*	*FPZ*
1	Water	3	Ampicillin, Chloramphenicol, Tetracycline	Lower main stem
1	Sediment	2	Ampicillin, Tetracycline	Lower main stem
4	Water	3	Ampicillin, Chloramphenicol, Tetracycline	Lower main stem
8T	Water	3	Ampicillin, Chloramphenicol, Tetracycline	Middle tributary
11	Sediment	3	Ampicillin, Chloramphenicol, Tetracycline	Middle main stem
14T	Water	2	Ampicillin, Tetracycline	Middle tributary
15T	Biofilm	2	Ampicillin, Tetracycline	Lower tributary
17	Sediment	2	Ampicillin, Tetracycline	Lower main stem
18T	Water	2	Ampicillin, Chloramphenicol	Lower tributary
18T	Biofilm	2	Ampicillin, Tetracycline	Lower tributary
18T	Sediment	2	Ampicillin, Tetracycline	Lower tributary

Among common sites with multi-resistance, two (i.e., 11, 17) had resistance that was partitioned by stream compartment. Lac+ and Lac- coliforms were resistant

to all antibiotics at one site and four sites, respectively. The proportion of resistance was very similar between Lac+ and Lac- organisms 54.6% to 45.4% (Figure 16).

Site 11 demonstrated the highest CFU counts and exhibited resistance to all antibiotics in both organisms in the sediment sample except for Lac+ resistance to ampicillin. This site is just above the confluence of Soos Creek on the Green River and is surrounded by agricultural fields and residential neighborhoods.

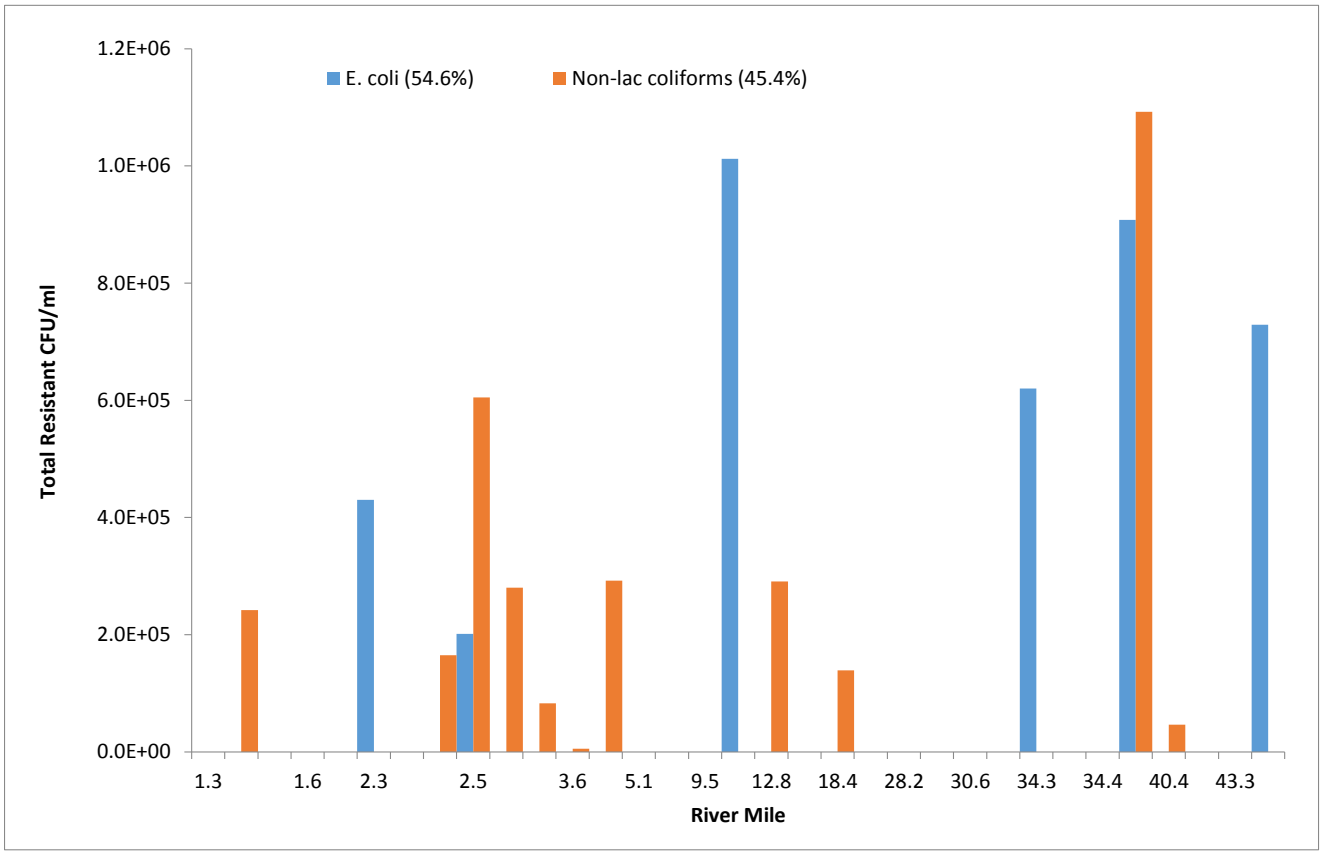


Figure 16. Site 11 exhibited the total highest CFU counts of resistance to three antibiotics in Lac+ (*E. coli*) and Lac- bacteria (Non-lac coliforms).

Lac+ MPN was consistently negatively correlated with stream power *(Beta =-3.800, p<0.01) adj. R^2=0.08* and sinuosity *(Beta =-0.726, p<0.005) adj. R^2=0.103*; as were Lac- coliforms (*Beta =-3.22, p<0.01) adj. R^2=0.07* and *(Beta =-0.823, p<0.001) adj. R^2=0.165*, respectively. Salinity and pH were significant for Lac- coliforms but temperature and conductivity were not. Lac+ coliforms were sensitive to pH *(Beta =0.215, p<0.008) adj. R^2=0.09* but not temperature, conductivity or salinity. Stream slope was positively correlated upstream *(Beta =0.03, p<0.05) adj. R^2=0.04* but reversed after the Howard Hansen dam at river mile 63. In the water samples, these correlations were stronger but all compartments showed similar responses along the watershed (Figures 17-18).

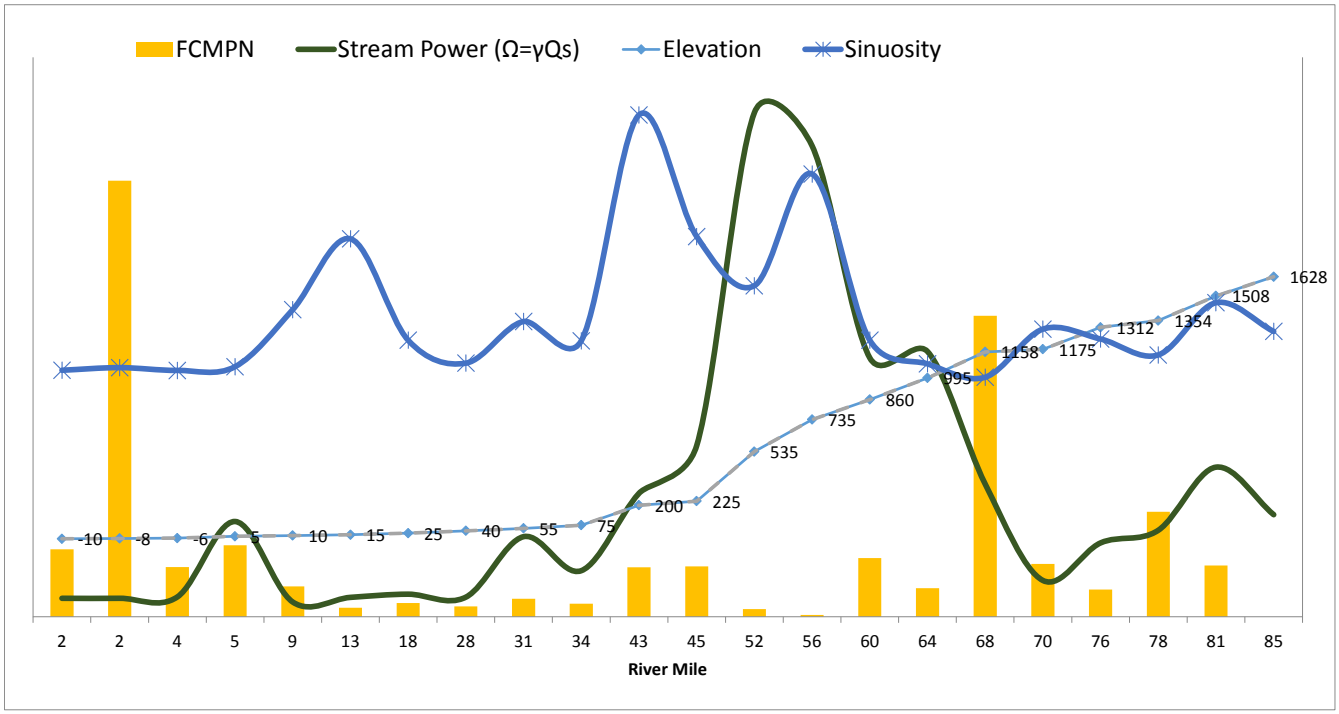


Figure 17. Relationship between coliform MPN (FCMPN) and stream conditions at the reach scale.

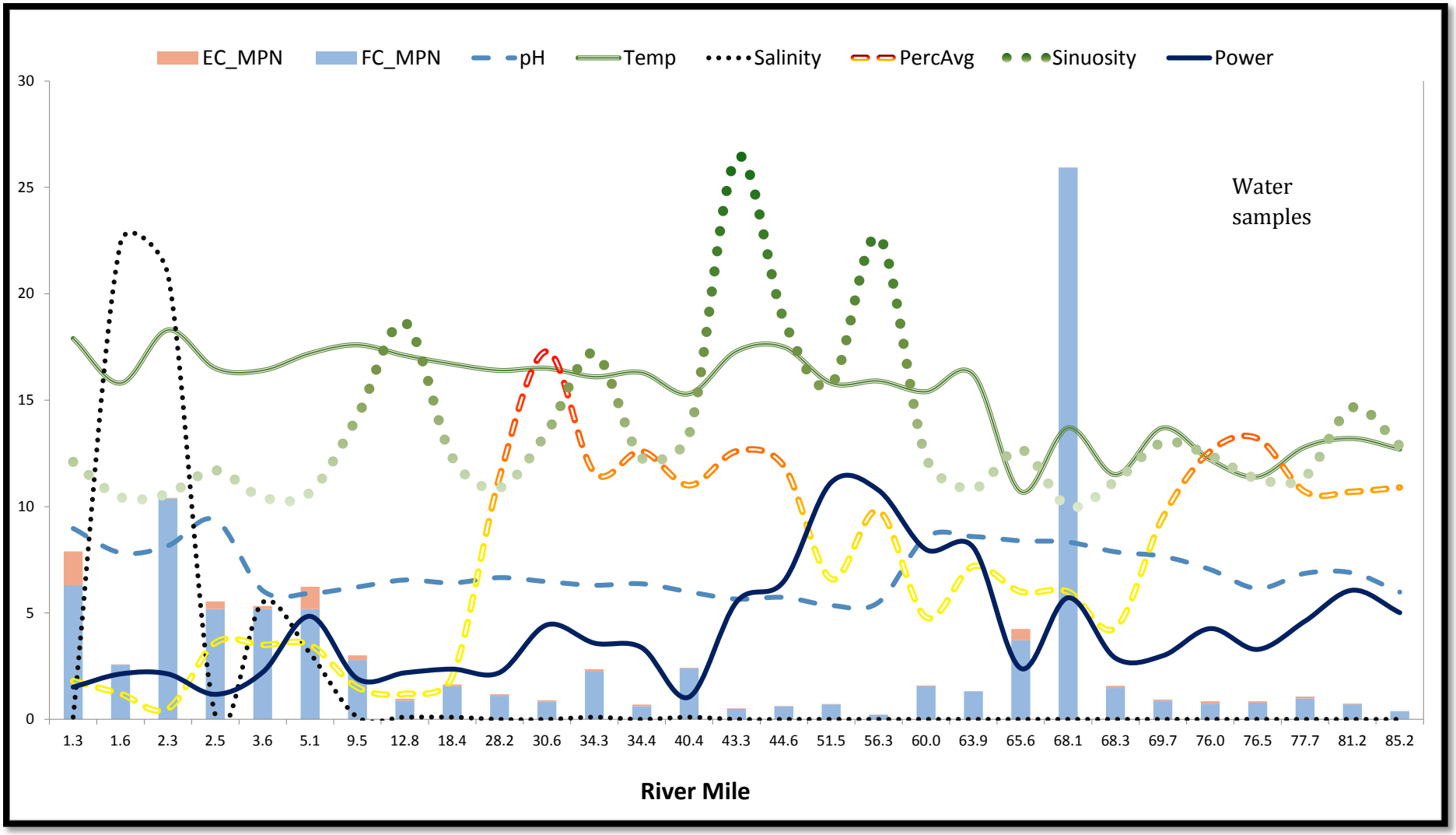


Figure 18. MPN of *E. coli* (EC_MPN) and coliforms (FC_MPN) to water physicochemistry, soil percolation, stream channel sinuosity and stream power in water samples by river mile.

DISTANCE-BASED REDUNDANCY ANALYSIS FOR VARIANCE PARTITIONING

The distanced-based redundancy analysis (db-RDA) partitions the total explainable variance into two categories of explanatory variables; environmental parameters and spatial coordinates. The unique contribution of each was determined by removing the influence of one and using the remaining group as the explanatory variable. These partial ordinations were then compared to the total variance explainable with all variables in the model. The environmental variables explained more of the variation in MPN and antibiotic resistant distribution than geography. The environment explained 59% of the variance for total coliforms in any stream compartment, and more interestingly explained 76.6% of the variance for the distribution of antibiotic resistant Lac+ bacteria. The variance of biofilm samples were consistently explained; 72.9% Lac+ and 78.4% in Lac- coliforms. The highest adjusted R^2 value was in the sediment samples; $R^2=0.515$ and $R^2=0.853$ for Lac- and Lac+, respectively.

Antibiotic resistant abundance prediction was stronger in Lac- coliforms which may be indicative of a more transient occurrence for Lac+ bacteria. Partitioning of the variance allows us to reject the NULL hypothesis that environmental variables do not explain the variation of the dependent variable (Appendix D).

The first two axes of the db-RDA biplot explained 49.1% of the total variability and effectively captured the main patterns of variation in the original variables.

A distance biplot of the eigenvectors was scaled to site and species. The db-RDA of the MPN dataset produced three significant variables. Axis 1 accounted for 36.1% and Axis 2 accounted for 13.0% of the variance, respectively (Figure 19).

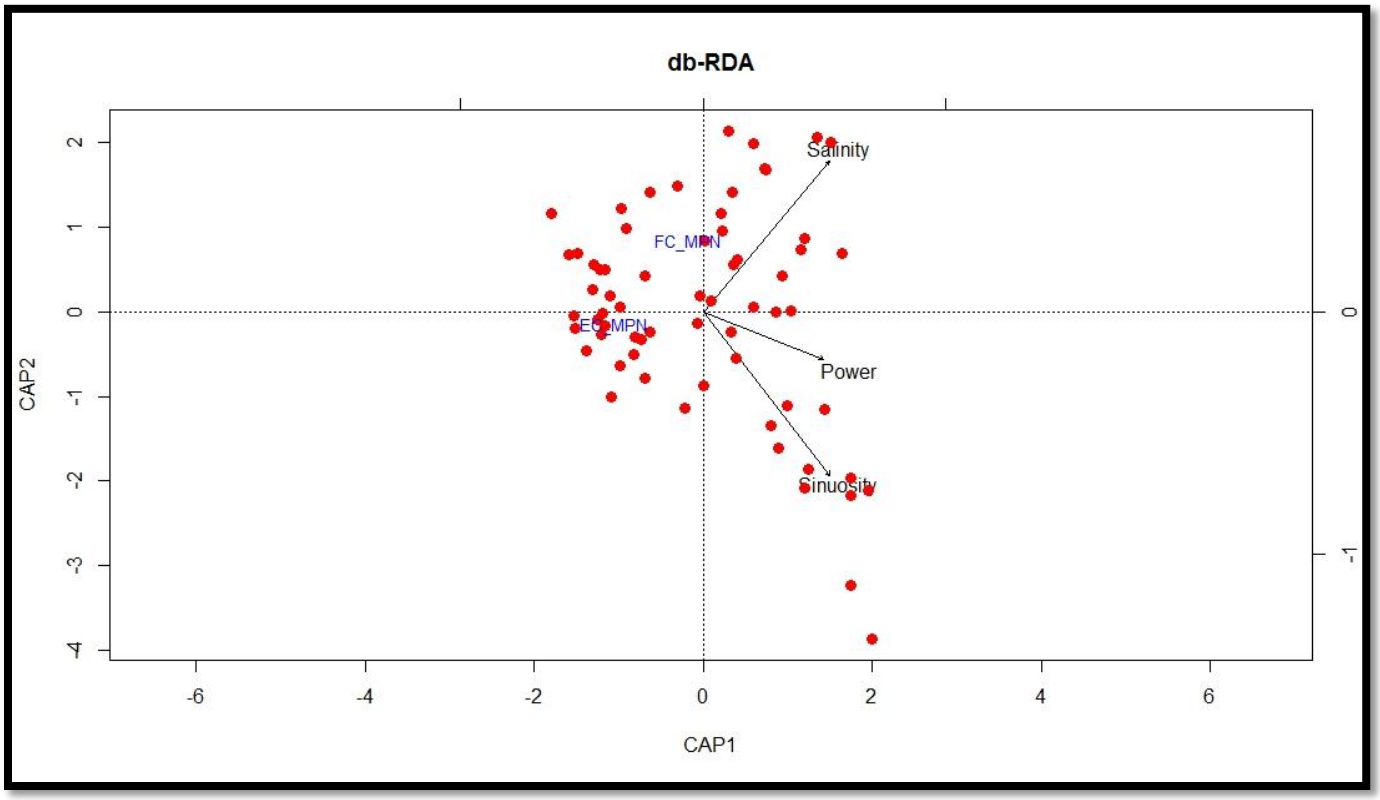


Figure 19. Distance-based RDA ordination showing the coliform MPN composition in relation to significant environmental variables. The environmental variables were significantly related to the variation of MPN ($p < 0.05$).

The db-RDA for antibiotic resistance produced four significant variables. Axis 1 accounted for 18.5% and Axis 2 accounted for 7.5% of the variance, respectively, or 26.0% of the total (Figure 20). Points represent samples, arrows represent environmental variables and the direction of increase, and the length of each arrow indicates the degree of correlation with the ordination axes.

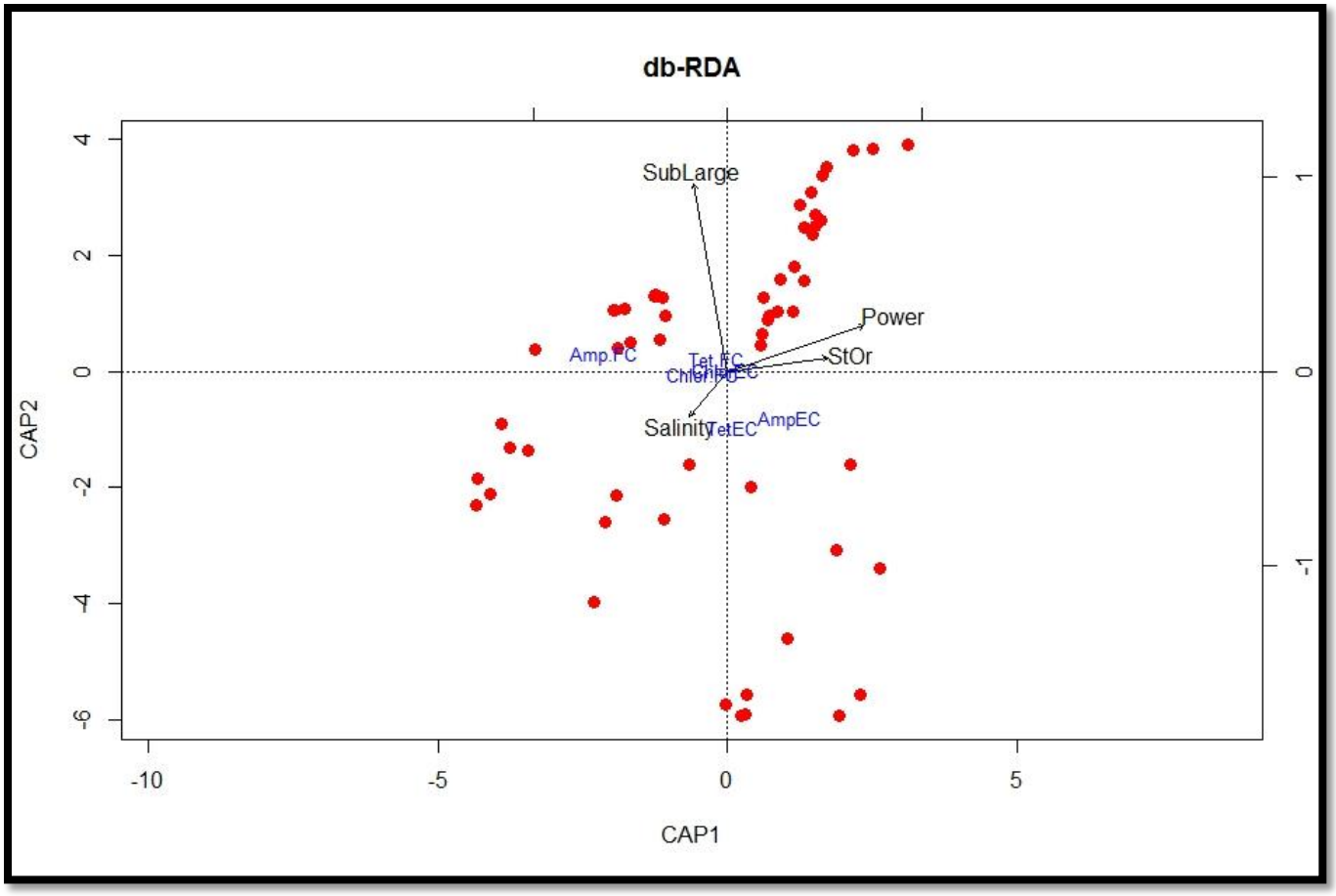


Figure 20. Distance-based RDA ordination showing the antibiotic resistance in relation to significant environmental variables. The environmental variables were significantly related to the variation of resistance ($p < 0.05$).

The spatial distribution of ABR in the Green-Duwamish watershed demonstrated a higher proportion of resistance in heavily developed and industrial areas in the lower Duwamish River as expected, but there were areas along the main corridor and in several tributaries also with high levels of resistance.

Total coliform MPN to ampicillin resistance in Lac+ cells was not significant *(Beta = 1.476, n.s.),* but Lac+ MPN to ampicillin resistance overall was *(Beta = 21606, p<0.001) adj.* R^2*=0.172*, and Lac- coliform MPN to ampicillin resistance also was significant *(Beta = 0.7761, p<0.001) adj.* R^2*=0.202.*

By the stream compartment water and biofilm explained most of the variance of resistance Water: *(Beta = 0.897, p < 0.007) adj* R^2*=0.204*, and Biofilm: *(Beta = 0.8484, p < 0.01)* R^2*=0.224*, but sediment was not a significant predictor *(Beta = 75.75, n.s)*.

The results of the GWR standard residuals and predicted values mapping indicate clear spatially-explicit antibiotic resistance distributions that are independent of both Lac- and Lac+ MPN distributions and land-use gradients (Figures 21-23).

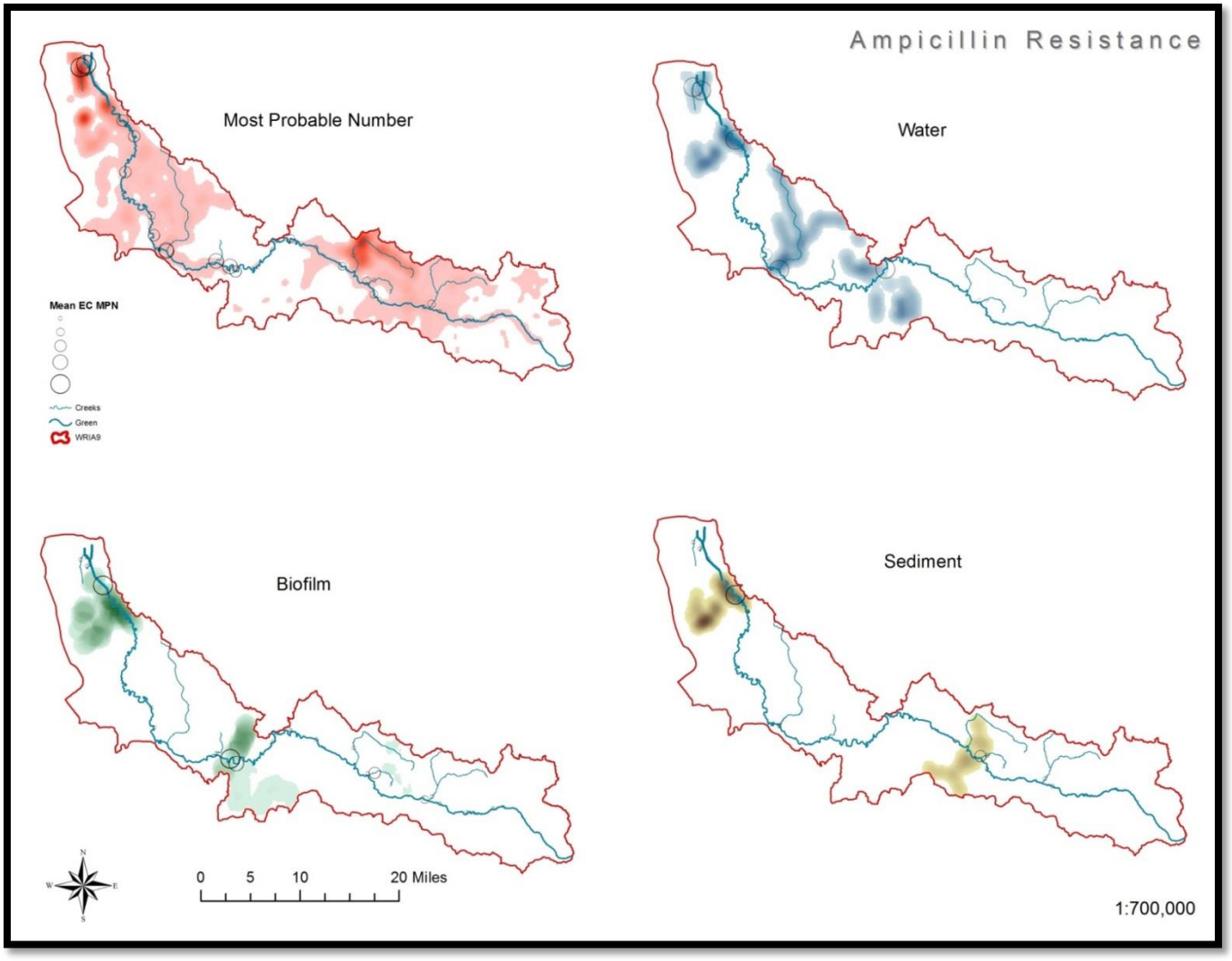


Figure 21. Geographic weighted regression predicted values mapped for ampicillin.

In each stream compartment for Lac+ CFU counts. Graduated symbols ◯ indicate the observed pooled sample mean for each site for comparison to the predicted distributions. Color ramps are standardized for comparison only and are not scaled to proportions of organisms between maps.

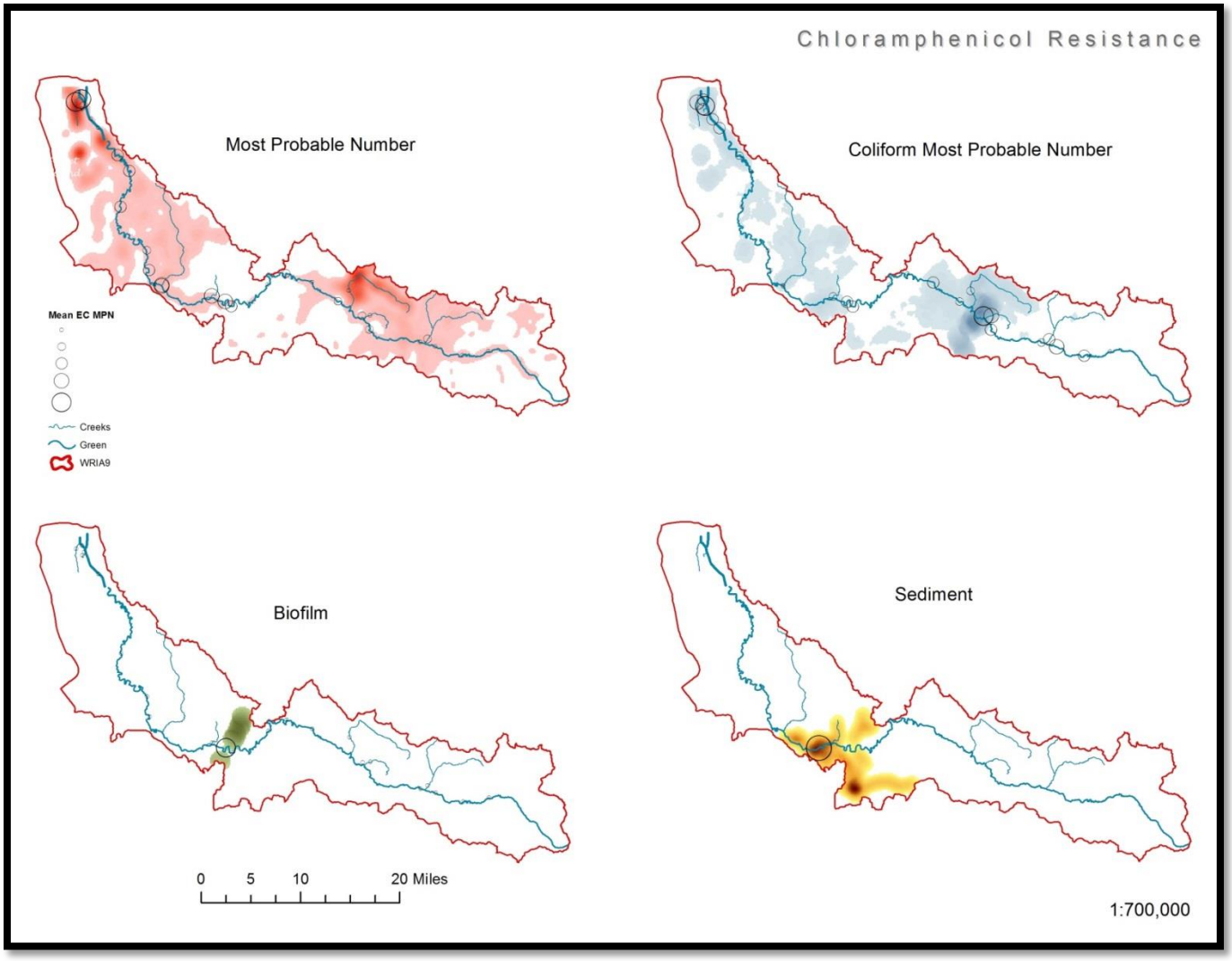


Figure 22. Geographic weighted regression predicted values mapped for chloramphenicol.

In each stream compartment for Lac+ CFU counts. Graduated symbols◯ indicate the observed pooled sample mean for each site for comparison to the predicted distributions. MPN for Lac+ and Lac- are shown. There was only one sample in the water which is not shown. For sediment and biofilm samples were pooled from Lac+ and Lac- coliform observations at each site. Color ramps are standardized for comparison only and are not scaled to proportions of organisms between maps. Some antibiotic resistance abundances were very small.

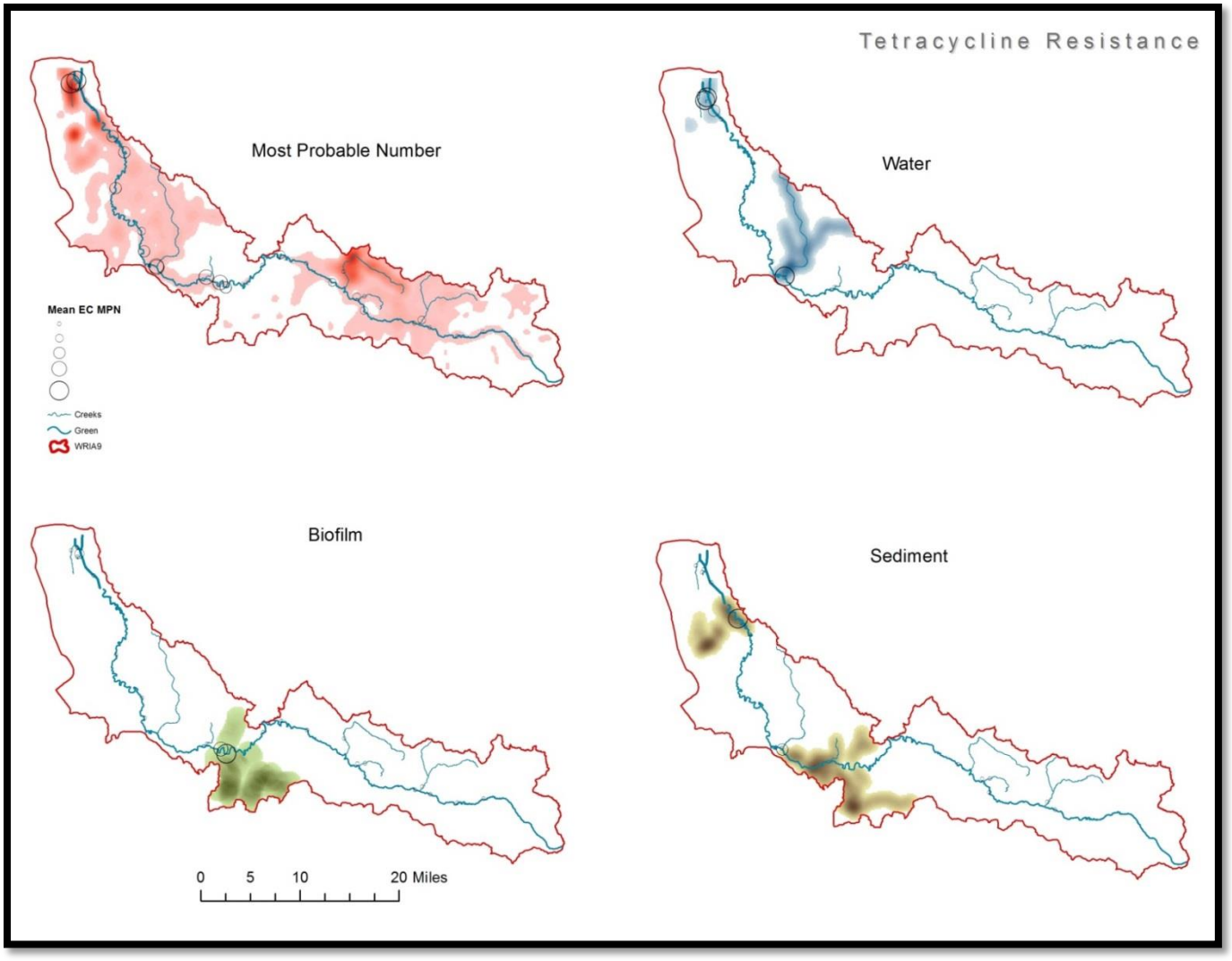


Figure 23. Geographic weighted regression predicted values mapped for tetracycline.

In each stream compartment for Lac+ CFU counts. Graduated symbols◯ indicate the observed pooled sample mean for each site for comparison to the predicted distributions. Color ramps are standardized for comparison only and are not scaled to proportions of organisms between maps.

The ANOSIM between sites were meaningful; MPN (*R=0.166, p=0.022)* and ABR (R*=0.242, p=0.0024).* Lac+ bacteria were different among stream compartments (*R=0.109, p=0.004).* Most of the site differences were related to stream power (*R=0.71, p=0.001),* sinuosity (*R=0.62, p=0.001*, and functional process zone (*R=0.447, p=0.001)* (Figure 24).

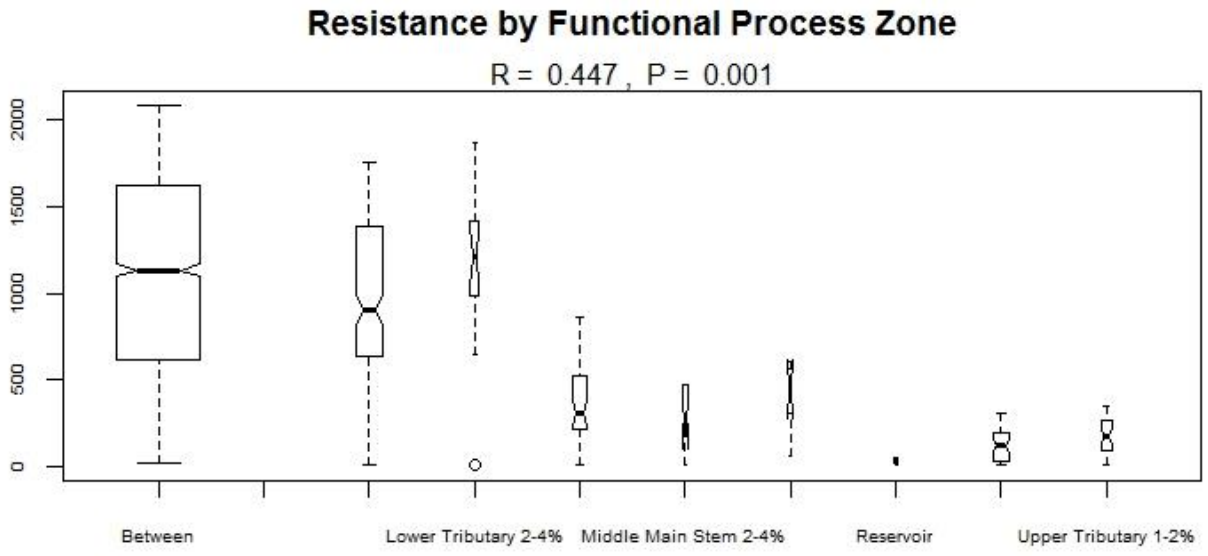


Figure 24. Significant dissimilarities of ABR by functional process zones of the river.

ANOSIM revealed that the dissimilarities between samples and the R statistic increased as we considered individual antibiotic resistance responses to environmental conditions (Table 10). Part of this is a function of the smaller sample sizes of tetracycline and chloramphenicol where local conditions will have more of an influence and tend to increase the dissimilarity ranks between classes. But taking that into account there is an increase in dissimilarity in

several river morphometric features; bank confinement (*R=0.492, p=0.001),* basin lithology (*R=0.257, p=0.001),* and stream order (*R=0.137, p=0.04)* were significant. Between the main stem reaches and tributaries there was a weak difference in resistance (*R=0.076, p=0.0725)*.

Table 10. ANOSIM dissimilarity matrix.

Interaction	***Lac+MPN***	***p***	***Tet Lac+***	***P***	***Chlor Lac+***	***P***	***Amp Lac+***	***p***
FPZ	0.194	0.002	0.301	0.001	0.539	0.001	0.194	0.002
Site	0.217	0.005	0.575	0.001	0.911	0.001	0.428	0.001
Land Cover	0.396	0.001	0.581	0.001	0.752	0.001	0.415	0.001
Basin Lithology	0.257	0.001	0.481	0.001	*	*	*	*
Channel Width	0.142	0.037	0.382	0.001	0.547	0.001	*	*
DrainDen			0.466	0.001	0.592	0.001	*	*
Sinuosity	0.192	0.011	0.406	0.001	0.639	0.001	0.44	0.001
Stream Slope	0.168	0.011	0.447	0.001	0.775	0.001	0.313	0.001
Power	0.197	0.008	0.47	0.001	0.866	0.001	0.39	0.001
Perc Average	0.182	0.003	0.496	0.001	0.636	0.001	0.363	0.001
Near Road	0.113	0.034	0.191	0.012	0.134	0.043	0.453	0.001
Near Bridge	0.199	0.006	0.52	0.001	0.818	0.001	0.453	0.001
Human Use	0.346	0.001	0.518	0.001	0.59	0.001	0.338	0.001
Refuse	0.353	0.001	0.476	0.001	0.63	0.001	*	*
Agriculture	0.16	0.018	0.242	0.016	*	*	0.226	0.011
Residential	0.356	0.001	0.561	0.001	0.578	0.001	*	*
Commercial	0.235	0.003	0.381	0.001	0.407	0.001	0.272	0.007
Impervious	0.395	0.001	0.529	0.001	0.645	0.001	*	*
pH	0.217	0.002	0.575	0.001	0.911	0.001	0.428	0.001
Salinity	0.444	0.001	0.601	0.001	*	*	*	*
StrLenTot	0.217	0.004	0.575	0.001	*	*	*	*
Metazoa	0.261	0.001	0.475	0.001	*	*	*	*

*p>0.05.

DISCUSSION

Bacterial communities regulate the flux of nutrients and therefore the chemical energy in streams, but little is known about the environmental constraints that determine the distribution of resistant strains in a river ecosystem and how those populations impact ecosystem function. By restricting my sampling to one watershed (a single biome) I was able to constrain many environmental factors including climate, geology, inorganic water chemistry, and terrestrial vegetation which allowed me to focus my analysis on other features of the river at multiple scales.

Quantifying the influence of riverine habitat features by focusing on natural stream processes in the context of the microbial community, and then on anthropogenic inputs as a watershed-level driver, is essential to capturing the natural bacterial community structure as well as how environmental species respond to resistance determinants as they enter the river. Environmental enterics often show weak positive correlations with each other in the environment (Drozd et al. 2013) and I found this to be true for resistance at the watershed scale but observed partitioning of resistance among stream compartments and by species at the reach and site scale. I observed the expected correlations between water physicochemical and MPN, and an overall increase of resistance with downstream distance, but there were localized conditions that controlled both MPN and antibiotic resistance among Lac+ and Lac- cultures.

It is known that physical processes and water quality play an important role in metazoan community structure in river ecosystems; fish diversity (Shields et al., 1994), and macro-invertebrate diversity (Maul et al. 2004) are positively correlated with river segments with the most heterogeneous habitats, stable bank structure, and nutrient retention as a result of the lower energy associated with more sinuous fluvial processes (Shields et al. 2008). Bacterial community structure is also tightly coupled with habitat heterogeneity (Singer et al. 2010), and chlorophyll *a* concentrations which tend to show gradual downstream increases unless there is an abrupt change in nutrient inputs (Winter et al. 2007) such as a confluence or a slack-water pool. Bacterial denitrification rates also track spatially with sediment character (Tatariw et al. 2013) which in-turn influences primary productivity and this varies seasonally with flow that creates denitrification hotspots. More specifically, *gama*-proteobacteria community structure has been found to be positively correlated with heavy metal fluvial deposition in the hyporheic zone of a river (Feris et al. 2003) indicating that fluvial processes also have direct impacts on species sensitive to metal concentrations below surface water flow. While watershed-wide drivers act as constraints over the dynamics of smaller scale processes, the interactions within these smaller hierarchies will vary by channel morphometry due to the direct influence channel shape has on the attenuation of stream power, and indirect affects which increase habitat heterogeneity and therefore metazoan diversity.

Each of these factors indirectly constrains nutrient delivery and thus will exert top-down control on microbial activity that may be unique at each river reach.

Focusing on river segment physical forcing and the powerful influences of local ecological conditions may reveal the spatial heterogeneity of bacterial resistance along river courses throughout the watershed. Many resistant populations occur distant from substantial anthropogenic inputs and these populations do not appear to be random (Singer et al. 2006). Along watershed gradients; lakes, reservoirs, and tributaries may function as natural resistance reservoirs (Czekalski et al. 2012), and confluences may perturb stream processes as I noted with MPN samples at the reservoir and near major confluences. This process may be especially relevant to lower watershed segments with first order tributaries that could function as resistance refugia from the extremes of salinity, pH, temperature, or stream power. Near Elliot Bay two first order tributaries measured very high coliform and *E. coli* levels and demonstrated multi-antibiotic resistance that did not align with the resistance profile in the main corridor.

My results indicate that river morphometry may be strongly related to the variation of bacterial antibiotic resistance and although water physicochemistry plays an important role in microbial ecology, stream power may better encapsulate the sum of the physical forces tied to nutrient fluxes and sediment transport. Stream power was the first or second most significant factor in the redundancy analysis and was statistically significant or present in all geographically weighted regression models. In high flow areas the ability to

withstand flow stress results in low diversity as only species that tolerate current stress persist and are usually represented by fewer, but closely-related species (Larson and Passy 2013). In areas with the highest stream power and low habitat heterogeneity there was no antibiotic resistance and the lowest levels of MPN. This may indicate that species diversity was low and did not contain species that could acquire resistance, there was no selective pressure for resistance for the species that were present, maintaining resistance did not increase fitness, or bacterial abundance was below the sensitivity of my tests.

Sinuosity is related to stream power but is not a part of the power equation so was utilized as a stand-alone explanatory variable. Sinuosity explained a large portion of the variability in the distribution of bacterial resistance. Total habitat area and total habitat heterogeneity increase with sinuosity where slopes are gentler and the river channel is free to migrate around the landscape as flow regimes change over time. This continual, but moderate, change will maintain high species diversity according to the intermediate disturbance hypothesis (IDH) (Connell 1978). Biofilm architecture has been found to become more rugose and thicker, with increased surface area in low velocity, nutrient-limited flows (Battin et al. 2003). This growth pattern reduces surface resistance to solute mass transfer and facilitates nutrient uptake. As nutrients become non-limiting new species are able to accumulate into the biofilm when flow stress is low, but species diversity increases when flow conditions vary (Larson and Passy 2013). In accordance with IDH, equilibrium conditions in rivers are probably short-lived

if they occur at all and high species diversity will be maintained most of the time by seasonal flow and nutrient regime shifts, and random stochastic events in natural river systems. This model also explains the maintenance of high habitat heterogeneity and niche architecture as channels migrate seasonally and with storm events which will tend to maintain a moderate disturbance flux.

Conversely, under slow flow rates and high nutrient loads there tends to be lower diversity which has been explained as finer resource partitioning among related species (Larson and Passy 2013). The Lower Duwamish River is characterized by slow flow and high nutrient loads from runoff and resident pollution so it can be expected that microbial diversity would be lower in this sub-watershed due to these constraints. Microbial diversity has been found to be higher in some polluted systems (Li et al. 2010), but in general diversity increases upstream from development until reaching above the tree line where autochthonous inputs diminish such as in glacial headwaters (Wilhelm et al. 2013). In addition, disturbance irregularity tends to promote species richness. Therefore, management practices that preserve spatial and temporal habitat heterogeneity should have positive effects on biodiversity. The water column is usually more diverse than biofilms in streams (Besemer et al. 2012), but biofilms and sediments are more stable matrices and are less likely to have pronounced physicochemical shifts under normal flow conditions. The predominance of bacterial functional redundancy in riverine systems maintains ecological resilience during disturbance events and lower diversity depresses ecosystem

functionality (Peter 2011). Microbial community resilience is also a function of temporal-nutrient interactions as allochthonous bacteria will drive shifts in community composition that rebound to the original species composition when the system is non-nutrient limited, but community shifts after a disturbance in nutrient poor areas tend to persist until nutrient availability improves (García-Armisen et al. 2014). In the context of other environmental constraints, this process will differ spatially and temporally among sub-watersheds.

It is clear that development and runoff significantly influence antibiotic resistance in rivers, and there is no doubt that there are strong linkages between water quality and land use, but the spatial distribution of resistant bacteria seem to be less responsive to these influences at smaller scales. The amount of variance explained by purely geographic distance was small indicating that environmental conditions are the predominant drivers of antibiotic resistance in this watershed. But consideration must be given to the nutrient poor conditions during the summer months when bacterial communities are less diverse and less abundant under nutrient stress. There is also less selective pressure due to the lack of runoff during dry spells and coliforms may exhibit less antibiotic resistance due to a lack of sufficient abundances for HGT and less selective pressure from fewer resistant determinates in the water column. Seasonal sampling would probably tell the tale of this dynamic. Other important variables may be the amount of water released from the dam, the number of returning salmonids and their reproductive cycles, and leaf drop from riparian vegetation.

Each of these variables will influence nutrient flux and coliform abundances, as well as the level of resistant determinates.

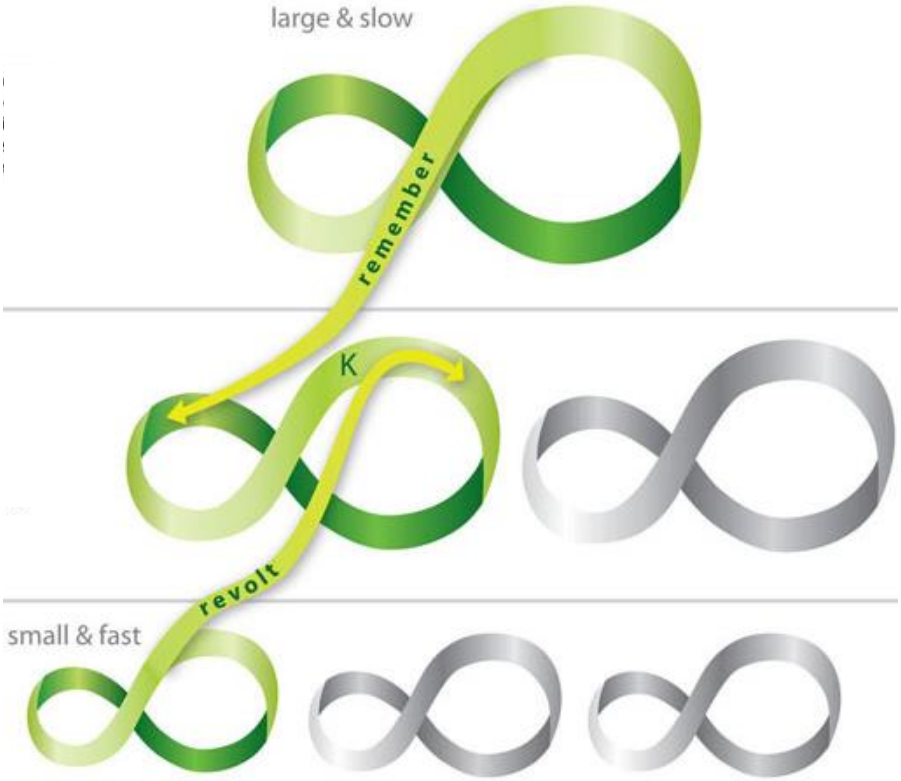
CONCLUSION

The distribution of antibiotic resistant coliforms in the Green River watershed is panarchial in nature. Metacommunity theory states that ecosystems are embedded within larger cycles of change processes in response to environmental disturbances at varying scales (Leibold et al. 2004), and "panarchy" (Holling and Gunderson 2002) describes a framework for coupling socio-ecological systems (Figure 25). Taken together these two conceptual frameworks can unite environmental structure with ecological functions as they manifest over ever increasing temporal and spatial scales.

Antibiotic resistance could be the defining human health issue and ecological problem of our time. Understanding how resistance functions and behaves in the environment is critical. It appears that resistance is under-dispersed and correlated to predictable variables in river ecosystems. There is short-cycle partitioning of antibiotic resistance at smaller scales; between Lac+ and Lac- coliforms in each stream compartment, and between stream compartments at each site, and a longer-cycle of antibiotic resistance as a gradient across the longitudinal distance of the watershed.

Most of the spatial variation in antibiotic resistance was explained by stream morphometry and power at the segment scale, which was reset at the reservoir and near confluences.

Figure 25. Nested structure of watershed ecosystems and processes that account for resilience and disturbance.

Large and slow system: Climate, watershed size, regional topography, geology, development, land-use, human population density, antibiotic use in humans and animals, waste-water treatment, runoff Middle and moderate system: river diversion, channelization, water removal, land cover, riparian structure, stream morphometry & power, human use, biodiversity, HGT hot spots Small and fast system: microbial abundance and diversity, HGT, nutrients, water chemistry, pollutants	

Adapted from Panarchy, 2002 page 34.

Interestingly, the correlation of sinuosity was reversed from 100% negative for MPN to 100% positive for resistant samples. A reversal of an important variable could mean one of two things, either there is an important variable that is missing that would account for nonstationarity, or the ecology of resistant strains is very different from sensitive populations at those sites. Since this trend was

consistent it is an intriguing question as increasing sinuosity generally dissipates more hydrologic energy within the system but increases habitat heterogeneity as channel shape becomes more complex. Stream power was negatively correlated across all groups and at all sites. It may be that this variable is being captured in the energy budgets of resistant strains or is simply a function of less nutrient mass transfer due to flow stress or the populations were too small for my measurement framework.

Although the environment played the most important role in the distribution of antibiotic resistance, geographic patterns and distance may have the potential to overwhelm these effects during storm events and changing flow regimes, or with increasing inputs from anthropogenic sources. Therefore, repeated sampling immediately after these events would be enlightening.

The inundation of antibiotics into the environment and the geographic patterns of antibiotic resistance among enteric and environmental bacteria in fresh-water systems has not been adequately modeled or mapped although these types of representations are readily producible.

Other studies have represented resistant *E. coli* by choropleth mapping based on demographic units (Galvin et al. 2013) but waterways are not constrained by political constructs and the use of GIS is an incredibly powerful tool that has not been adequately utilized for this purpose. Mapping is a routine procedure but spatial statistical analysis and modeling is less common and we could find no

studies that have statistically modeled the distribution of antibiotic resistance in rivers with GIS.

This work shows that GIS statistical analysis is an accessible tool for studying the distribution of resistance in watersheds and is applicable to any spatial or temporal scale for developing probabilistic models. The use of GIS to examine the spatial heterogeneity of resistance in urban waterways could have significant public health benefits if it is used to locate HGT hot spots that overlap with sites where the public has frequent contact with the water. There are large amounts of demographic and spatial data available that is easily incorporated into field sampling and remote sensing data and there are many new high resolution remote sensing technologies that exploit the native fluorescence of bacterial molecules (Dartnell et al. 2013). These new techniques offer many opportunities for monitoring waterways in very specific ways, such as looking for a particular pathogen during times of the year when outbreaks are anticipated. Remote sensing of environmental variables is already an active area of research which has been successfully implemented to predict *vibrio* based on sea surface temperatures (Lobitz et al. 2000), blooms of cyanobacteria in the ocean from chlorophyll *a*, and *E. coli* from florescence in large lakes to an accuracy of 91 CFU/ml by processing LANDSAT thematic data (Vincent et al. 2014). Prior work in this area has demonstrated that these fluorescence-based methods of detection are sensitive and are able to distinguish between bacterial species in the environment.

Scale matters when considering bacterial antibiotic resistant distributions. Although some sampling sites may overestimate the relative importance of small-scale conditions and underestimate landscape level forcing, this can be improved with increased sampling density. These results suggest that the biogeography of bacterial resistance can be observed and predicted in *E. coli* and other coliforms and is fundamentally different from evaluating correlations with land use or measuring distance upstream or downstream from point sources.

The dissimilarity between resistant bacterial groups is largely unrelated to geographic distance and indicates that this metric is not the best predictor of resistance along a river. Comparisons can be made to other river systems with similar functional process zones or environmental characteristics. My results provide some evidence that physical processes and channel characteristics are important explanations for the spatial distribution of antibiotic resistance in coliforms. In the river environment, the distribution and structure of resistant communities can largely be understood in terms of habitat properties alone and is non-random at each scale examined, and resistance at the watershed scale can largely be predicted with a single variable, stream power.

Although the biogeography of antibiotic resistance remains poorly understood, and hard to predict, a thorough integration of stream morphometry and hydrological models with landscape and bacterial ecology could improve predictions of resistance distributions; and modeling the complete environmental pathway through a watershed network is not far behind.

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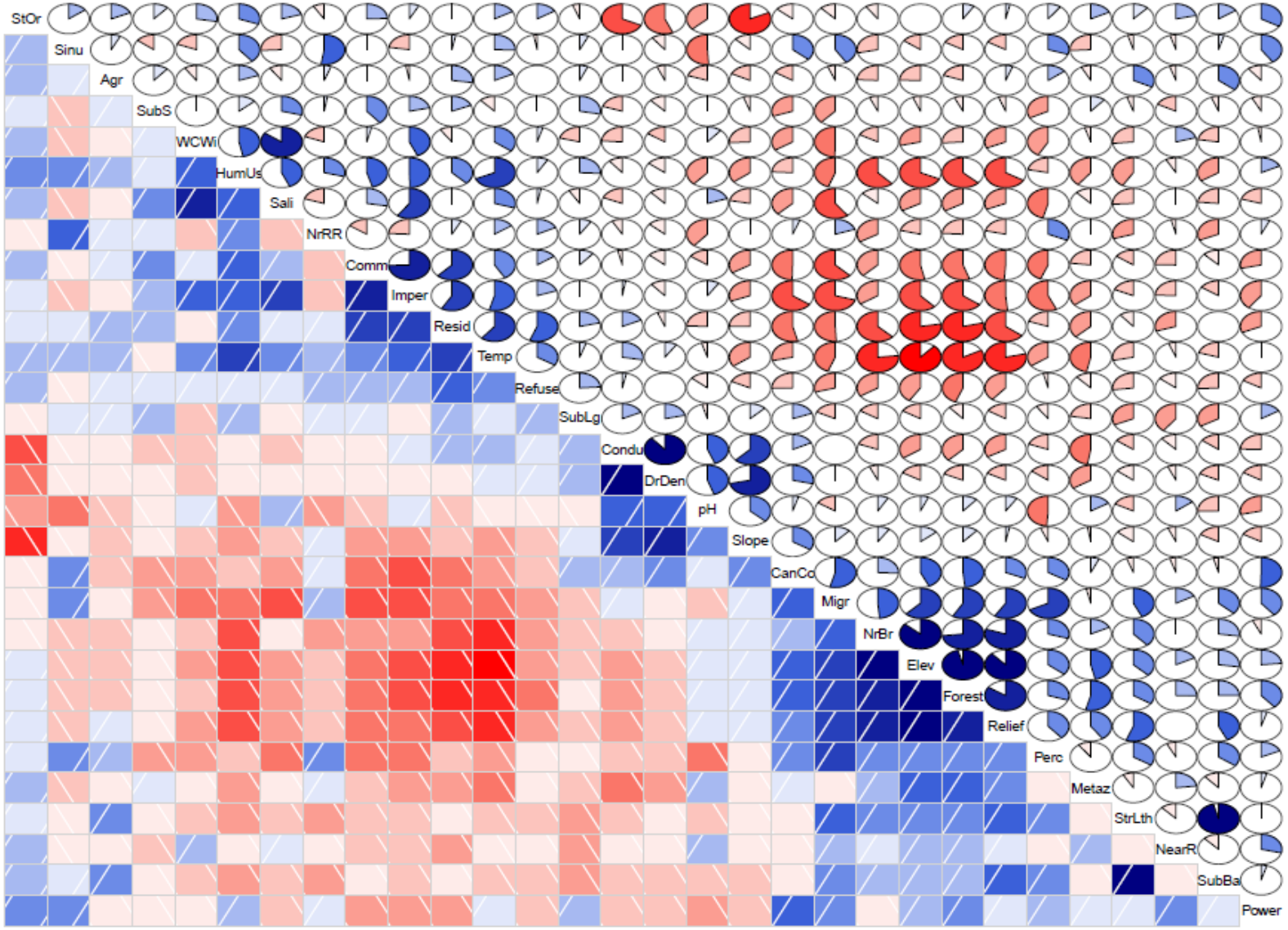
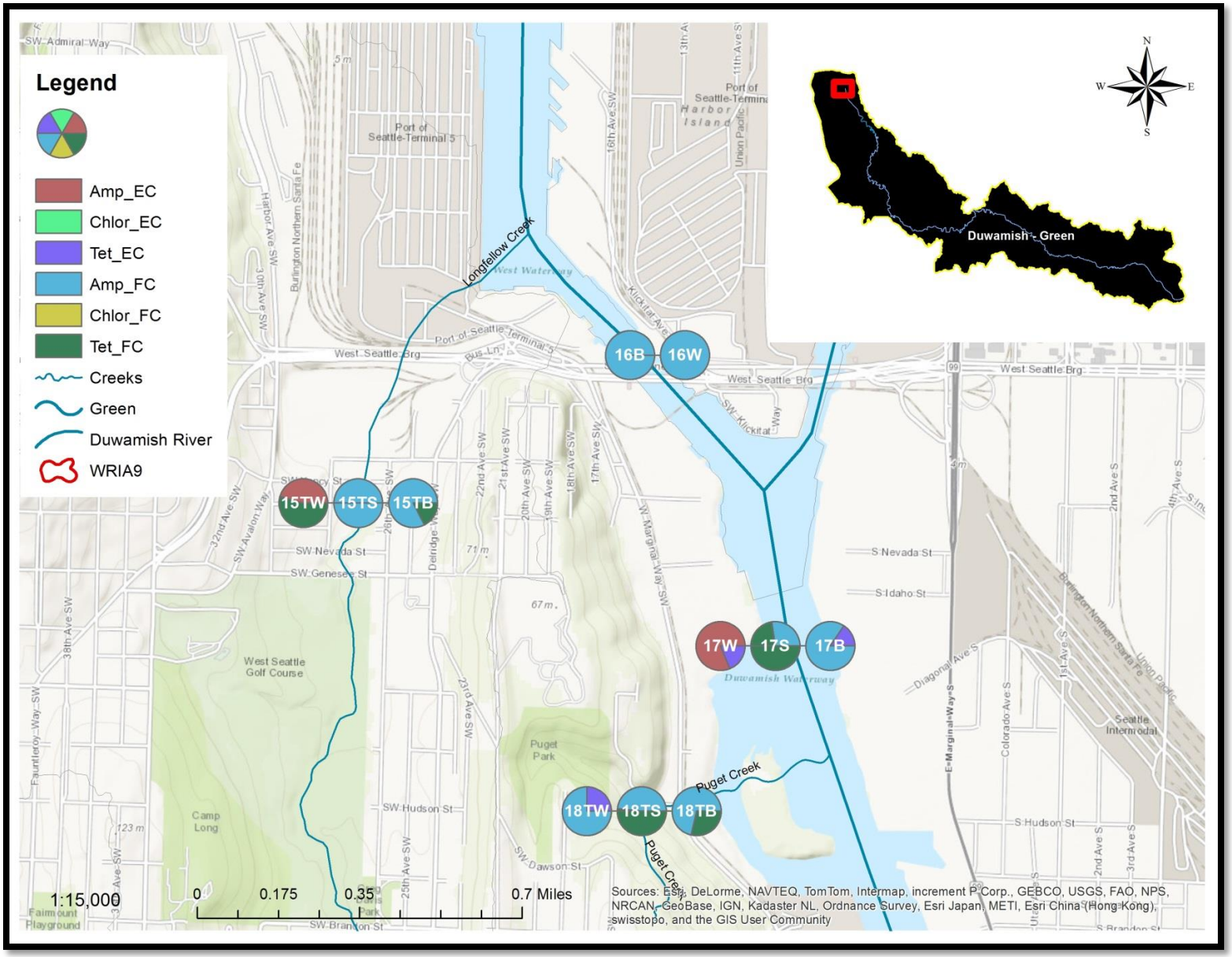
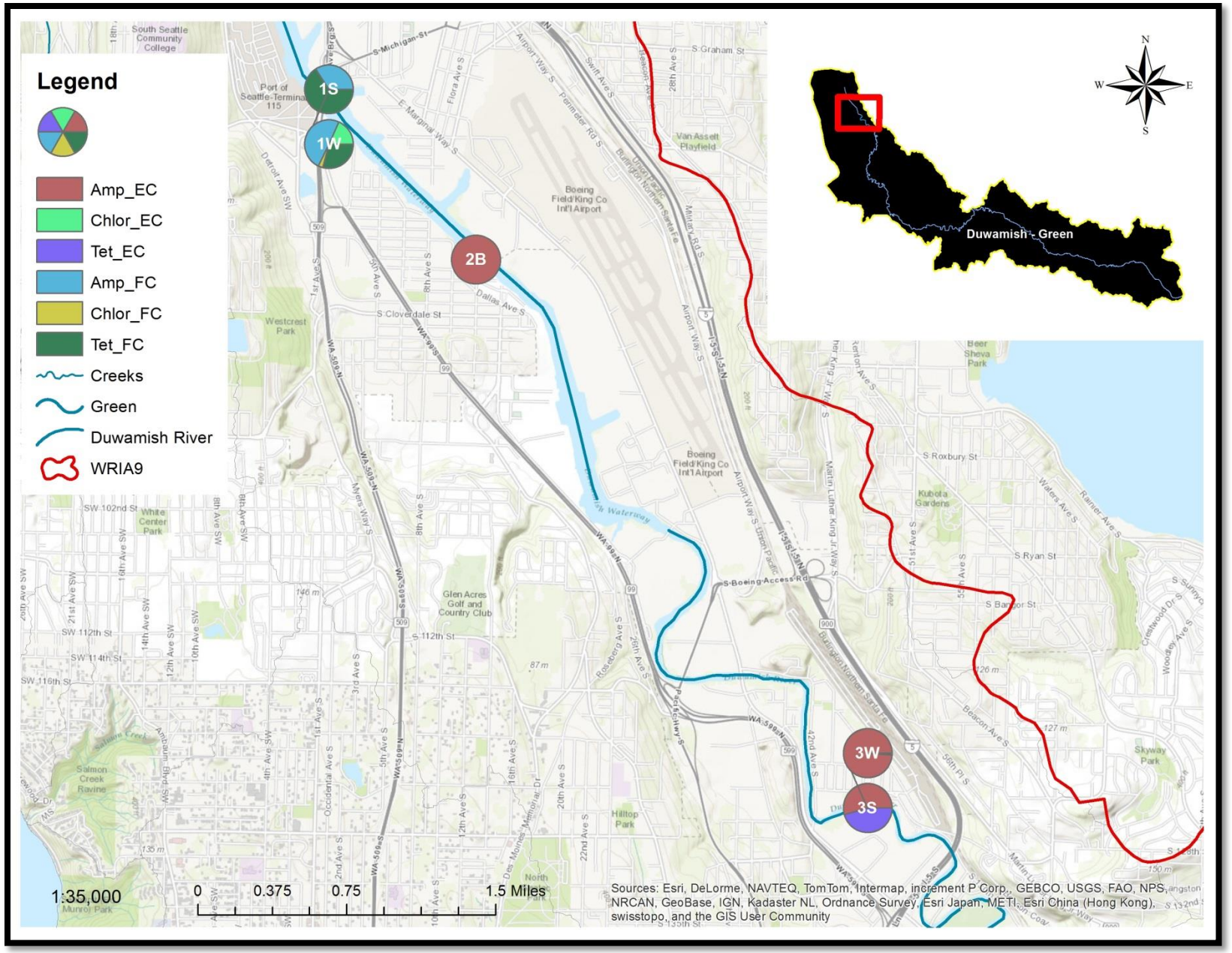
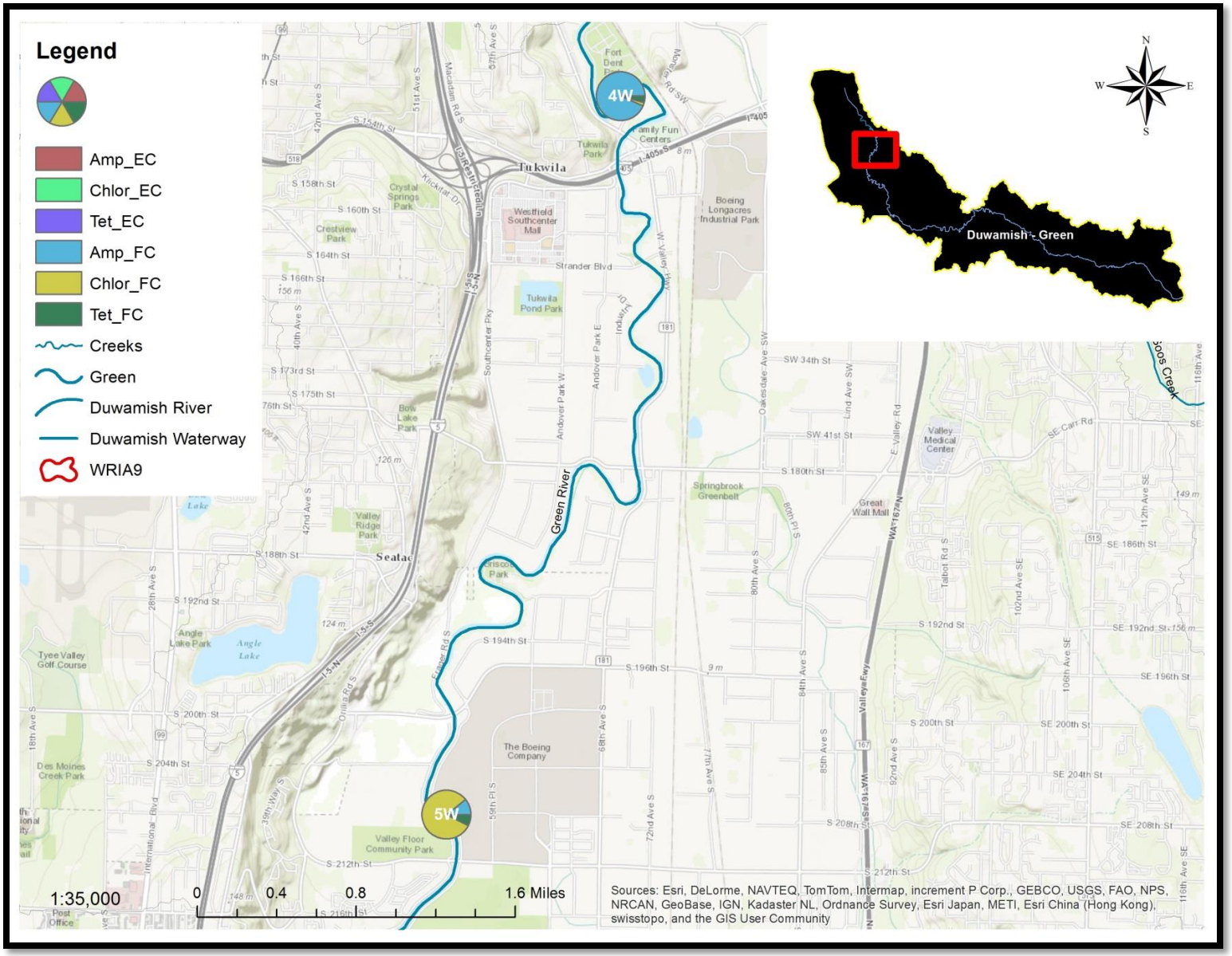
APPENDIX A. CORRELATION MATRIX OF ALL EXPLANATORY VARIABLES

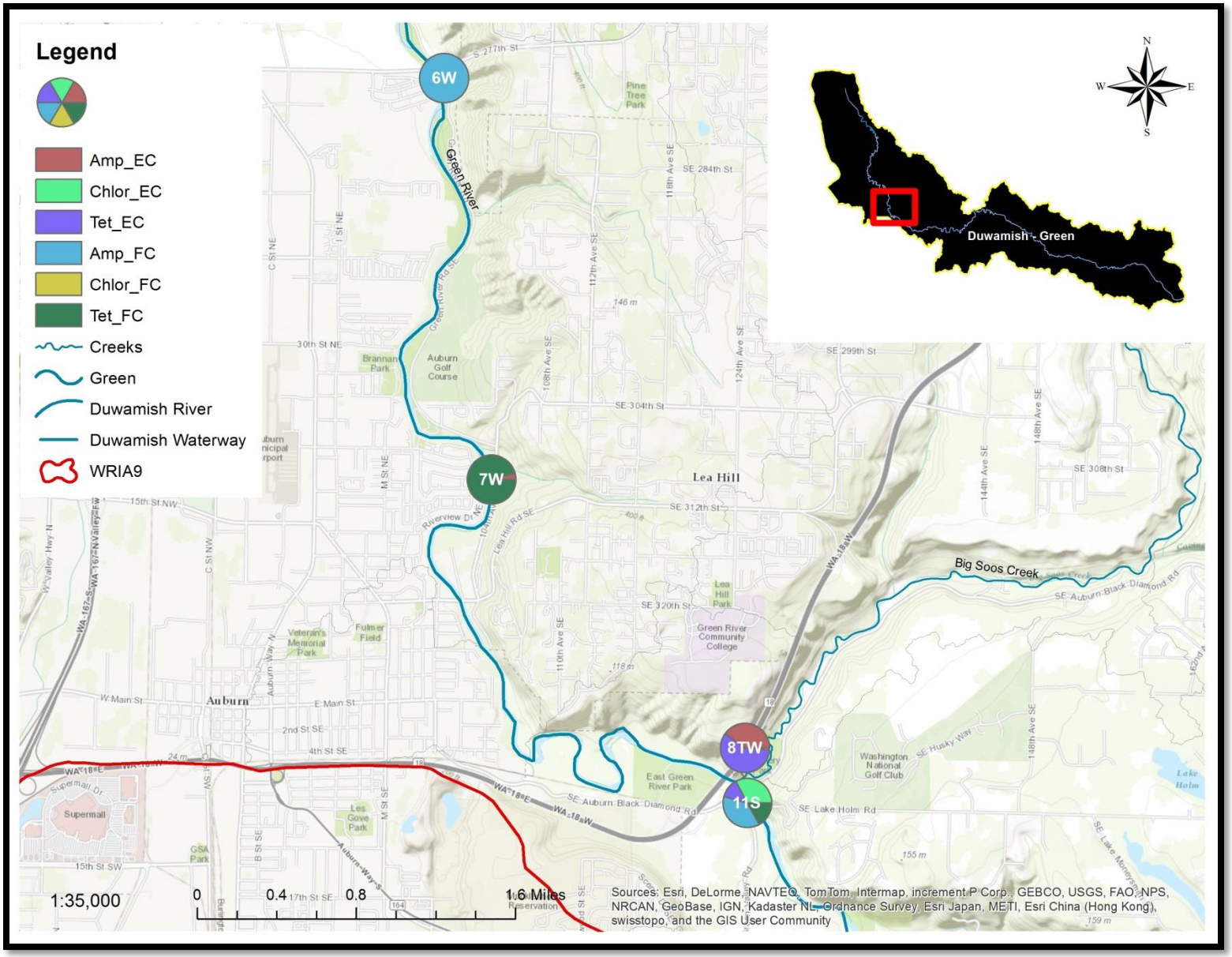


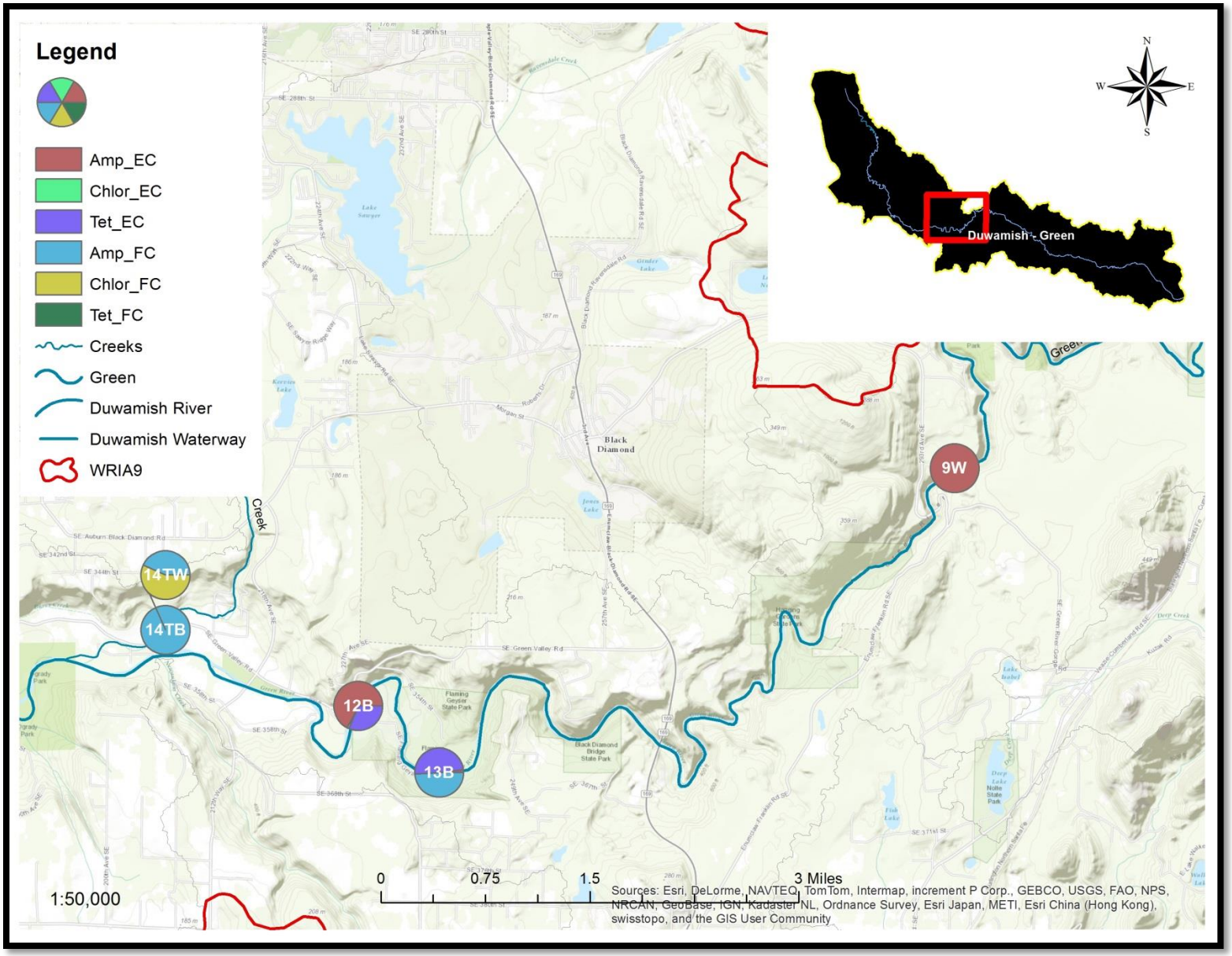
APPENDIX B. LAC+ AND LAC- ANTIBIOTIC RESISTANCE DISTRIBUTION ACROSS WRIA 9

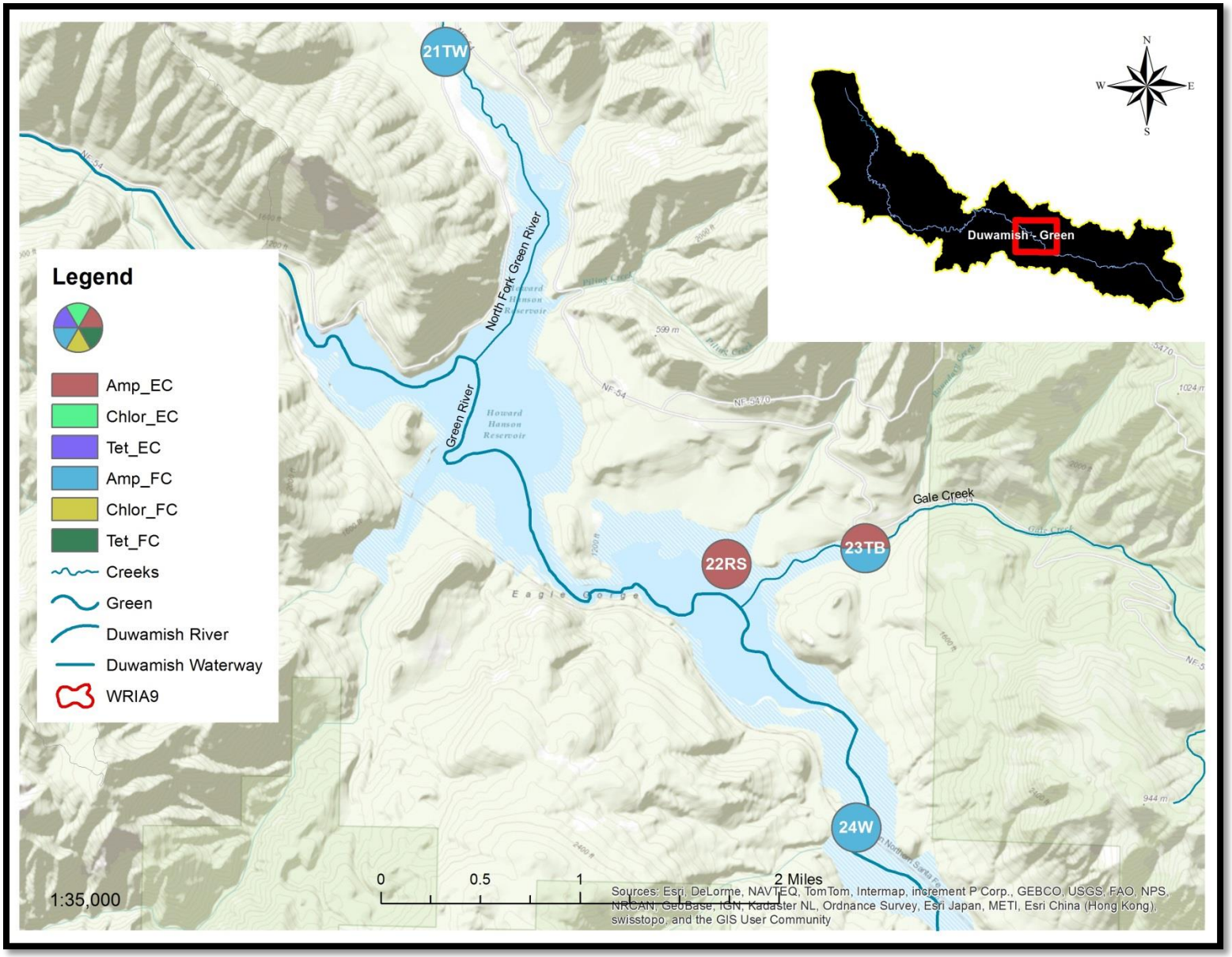

Legend
Amp_EC
Chlor_EC
Tet_EC
Amp_FC
Chlor_FC
Tet_FC
Creeks
Green
Duwamish River
WRIA9
Duwamish - Green
N
W
E
S
16B
16W
15TW
15TS
15TB
17W
17S
17B
18TW
18TS
18TB
Longfellow Creek
Puget Creek
West Waterway
Duwamish Waterway
Port of Seattle-Terminal 5
Harbor Island
West Seattle Brg
SW Admiral Way
SW Nevada St
SW Genesee St
S Nevada St
S Idaho St
S Hudson St
SW Hudson St
SW Dawson St
SW Brandon St
West Seattle Golf Course
Puget Park
Camp Long
Seattle Intermodal
1:15,000
0
0.175
0.35
0.7 Miles
Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community

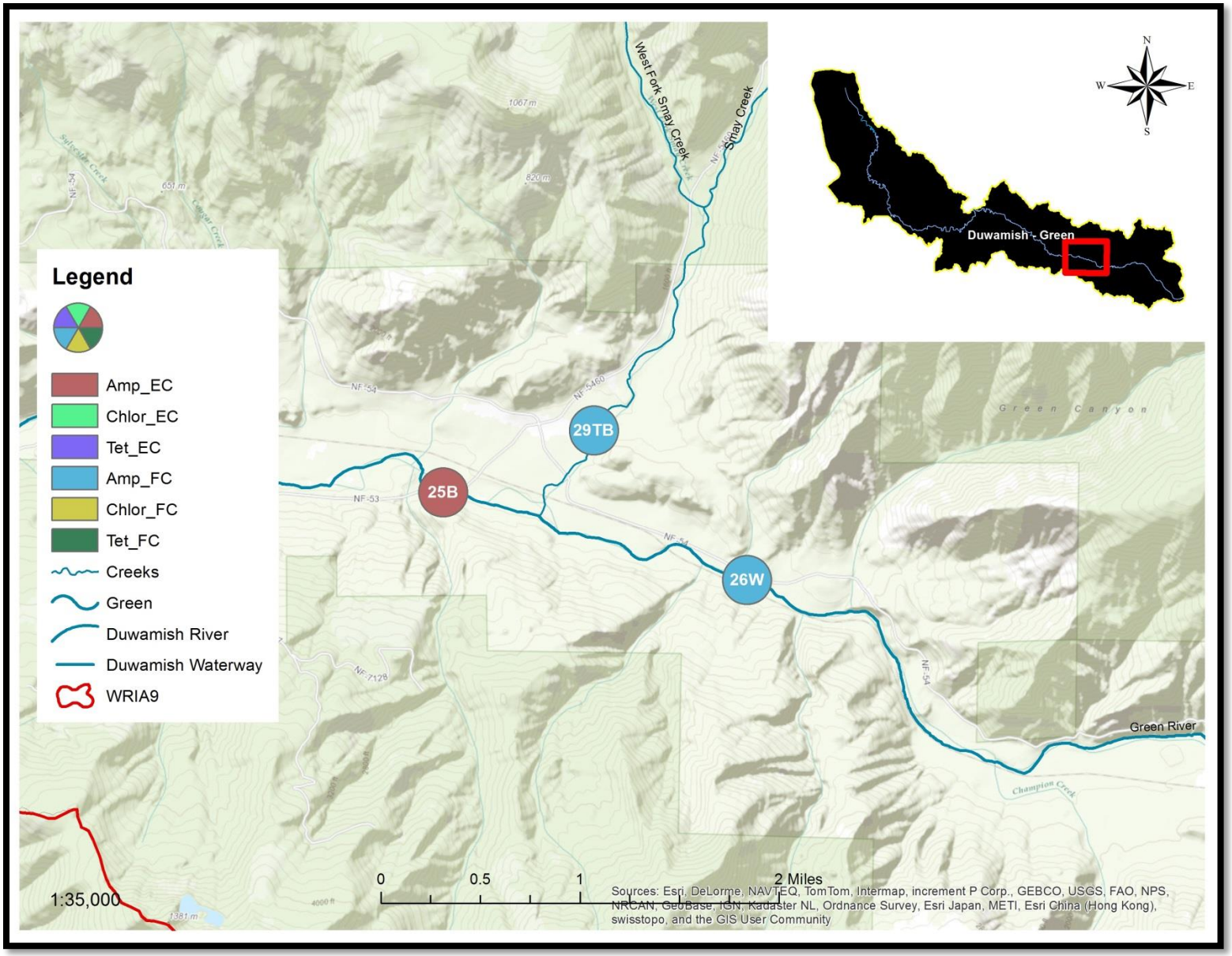

Legend
Amp_EC
Chlor_EC
Tet_EC
Amp_FC
Chlor_FC
Tet_FC
Creeks
Green
Duwamish River
WRIA9
1S
1W
2B
3W
3S
Duwamish - Green
N
W
E
S
1:35,000
0
0.375
0.75
1.5 Miles
Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community


Legend
Amp_EC
Chlor_EC
Tet_EC
Amp_FC
Chlor_FC
Tet_FC
Creeks
Green
Duwamish River
Duwamish Waterway
WRIA9
4W
5W
Green River
Duwamish - Green
N
S
E
W
1:35,000
0
0.4
0.8
1.6 Miles
Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community


Legend
Amp_EC
Chlor_EC
Tet_EC
Amp_FC
Chlor_FC
Tet_FC
Creeks
Green
Duwamish River
Duwamish Waterway
WRIA9
6W
7W
8TW
11S
Green River
Big Soos Creek
Lea Hill
Auburn
Duwamish - Green
N
S
E
W
1:35,000
0
0.4
0.8
1.6 Miles
Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community


Legend
Amp_EC
Chlor_EC
Tet_EC
Amp_FC
Chlor_FC
Tet_FC
Creeks
Green
Duwamish River
Duwamish Waterway
WRIA9
N
W
E
S
Duwamish - Green
14TW
14TB
12B
13B
9W
Black Diamond
1:50,000
0
0.75
1.5
3 Miles
Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community


21TW
North Fork Green River
Green River
Howard Hanson Reservoir
Gale Creek
22RS
23TB
24W
Duwamish - Green
N
W
E
S
Legend
Amp_EC
Chlor_EC
Tet_EC
Amp_FC
Chlor_FC
Tet_FC
Creeks
Green
Duwamish River
Duwamish Waterway
WRIA9
1:35,000
0
0.5
1
2 Miles
Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community


Legend
Amp_EC
Chlor_EC
Tet_EC
Amp_FC
Chlor_FC
Tet_FC
Creeks
Green
Duwamish River
Duwamish Waterway
WRIA9
Duwamish - Green
N
W
E
S
West Fork Smay Creek
Smay Creek
29TB
25B
26W
Green Canyon
Green River
Champion Creek
NF-54
NF-53
NF-5460
NF-7128
1:35,000
0
0.5
1
2 Miles
Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community

APPENDIX C. ANTIBIOTIC RESISTANCE BY STREAM AND COMPARTMENT

River Mile	*River Name*	***Water***	*Biofilm*	*Sediment*	*Total % E. coli resistance*
1.28	Longfellow creek	**Amp (+), Tet (-)**	Amp (-), Tet (-)	Amp (-)	Amp 31.3%
1.63	W Duwamish wway	**Amp (-)**	Amp(-)	Ns	
2.33	W Duwamish wway	**Amp (+), Tet (+)**	Amp(-), Tet(+,-)	Amp (-), Tet (-)	Amp 58.2%, Tet 13.3% water
2.53	Puget creek	**Amp (-), Chlor (-), Tet (+)**	Amp (-), Tet (-)	Amp (-), Tet (-)	Tet 33.3%
3.62	Duwamish river	**Amp(-), Chlor (+), Tet (-)**	Ns	Amp (-), Tet (-)	Chlor 2.8%
5.10	Duwamish river	**Sensitive**	Amp (+)	Ns	Amp 19.9%
9.45	Green river	**Amp (+), Tet (-)**	Ns	Amp (+), Chlor (+), Tet (+)	Amp 6.1% water; Amp 22.9%, Chlor 12.1%, Tet 19.3% sed
12.75	Green river	**Amp(-),Chlor (-), Tet (-)**	Ns	Sensitive	
18.42	Green river	**Amp (+,-), Chlor (-), Tet (-)**	Sensitive	Ns	0.31%
28.20	Green river	**Amp (-)**	Sensitive	Ns	
30.60	Green river	**Sensitive**	Sensitive	Ns	
34.25	Big Soos creek	**Amp (+), Chlor (-), Tet (+)**	Ns	Sensitive	Amp 15.6%, Tet 23.2%
34.40	Green river	**Sensitive**	ns	Amp (-), Chlor (+), Tet (+)	Chlor 22.7%, Tet 9.0%
40.40	Crisp creek	**Amp (-), Chlor (-)**	Amp (-)	Ns	
43.27	Green river	**Sensitive**	Amp (+), Chlor (+), Tet (+)	Ns	Amp 24.1%, Chlor 0.5%, Tet 11.7%
44.55	Green river	**Sensitive**	Amp (+,-), Tet (+)	Ns	Amp 3.9%, Tet 95.3%
51.50	Green river	**Amp (+)**	Sensitive	Ns	Amp 10.4%
56.30	Green river	**Sensitive**	Sensitive	Ns	
60.00	Green river	**Sensitive**	Sensitive	Ns	
63.90	Green river	**Sensitive**	Ns	Sensitive	
65.60	NF Green river	**Amp (-)**	Amp (-)	Ns	
68.10	Reservoir	**Sensitive**	Sensitive	Amp (+)	Amp 0.12%
68.30	Gale creek	**Sensitive**	Amp (+,-)	Sensitive	Amp 0.16%
69.70	Green river	**Amp (-)**	Ns	Sensitive	
76.04	Green river	**Sensitive**	Amp (+)	Ns	Amp 0.14%
76.54	Smay creek	**Sensitive**	Sensitive	Amp (-)	
77.70	Green river	**Amp (-)**	Sensitive	Sensitive	
81.22	Green river	**Sensitive**	Sensitive	Ns	
85.24	Green river	**Sensitive**	Sensitive	Ns	
Total % Samples w/ABR		***15/29 =51.7%***	***11/22 =50.0%***	***8/14 = 57.1%***	

APPENDIX D. DB-RDA VARIANCE PARTITIONING

1) Total Coliforms (n=65) p<0.005	*Variance*	*Proportion*
Explainable	0.411	*Adj R^2 = 0.322*
Environmental	0.2429	59.09
Geography	0.01927	4.688
Env/Geo	0.1488	36.204

--Total Coliforms-water p<0.005	*Variance*	*Proportion*
Explainable	0.330	*Adj R^2 = 0.235*
Environmental	0.1882	57.03
Geography	0.03794	11.496
Env/Geo	0.10386	31.472

-- Total Coliforms – biofilms p=0.14	*Variance*	*Proportion*
Explainable	1.0163	*Adj R^2 = 0.128*
Environmental	0.5661	55.70
Geography	0.1606	15.80
Env/Geo	0.2896	28.49

-- Total Coliforms – sediment p=0.23	*Variance*	*Proportion*
Explainable	1.1763	*Adj R^2 = 0.515*
Environmental	0.6686	56.83
Geography	0.1922	16.33
Env/Geo	0.3155	23.82

2) E. coli ABR (n=65) p=0.01	*Variance*	*Proportion*
Explainable	2.689	*Adj R^2 = 0.117*
Environmental	2.0877	77.63
Geography	0.1151	4.28
Env/Geo	0.4862	18.08

-- E. coli ABR – water p=0.005	*Variance*	*Proportion*
Explainable	4.488	*Adj R^2 = -0.133*
Environmental	2.365	52.69
Geography	0.2165	4.82
Env/Geo	1.90	42.33

-- E. coli ABR – biofilm p=0.0225	*Variance*	*Proportion*
Explainable	6.379	*Adj R^2 = -0.080*
Environmental	4.654	72.95
Geography	0.2229	3.49
Env/Geo	1.502	23.54

-- E. coli ABR sediment p=0.0225	*Variance*	*Proportion*
Explainable	5.706	*Adj R^2 = 0.853*
Environmental	4.015	46.11
Geography	0.3668	6.42
Env/Geo	1.324	23.002

3) Lac Neg Coliforms ABR (n=65) p=0.005	*Variance*	*Proportion*
Explainable	7.177	*Adj R^2 = 0.096*
Environmental	4.452	62.03
Geography	0.3317	4.62
Env/Geo	2.393	33.34

-- Lac Neg Coliforms ABR – water p=0.02	*Variance*	*Proportion*
Explainable	10.12	*Adj R^2 = 0.040*
Environmental	7.213	71.27
Geography	0.817	8.07
Env/Geo	2.09	20.65

-- Lac Neg Coliforms ABR biofilm p=0.016	*Variance*	*Proportion*
Explainable	11.799	*Adj R^2 = 0.0960*
Environmental	9.254	78.43
Geography	0.7538	6.38
Env/Geo	1.791	15.18

APPENDIX E. MAPS OF TOTAL ANTIBIOTIC RESISTANCE

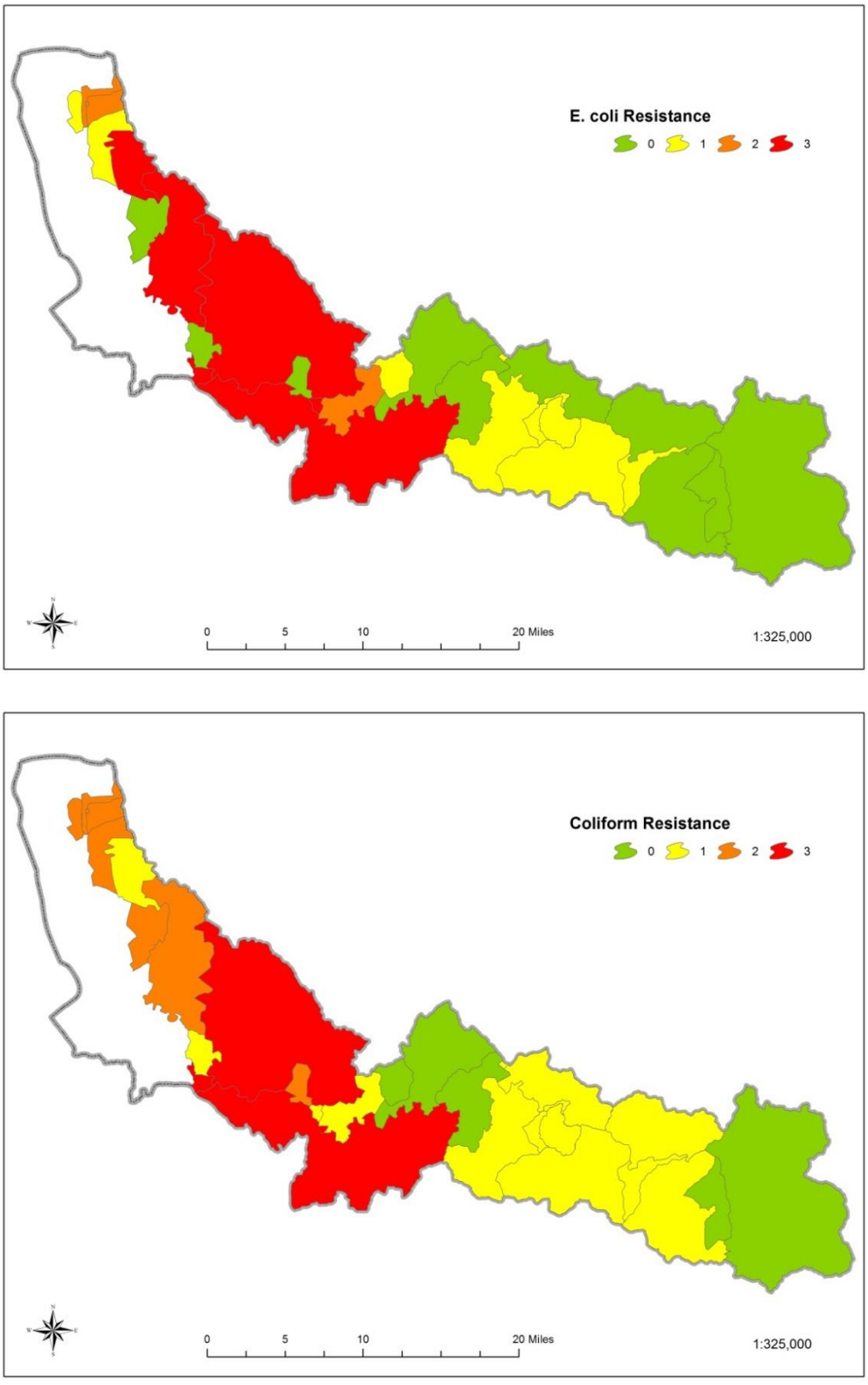


Figure 26. Antibiotic resistance profiles in the sub-watershed of each sample site. The values are the number of antibiotic classes that were represented by resistant cultures.

Ampicillin Proportion Resistant to Controls

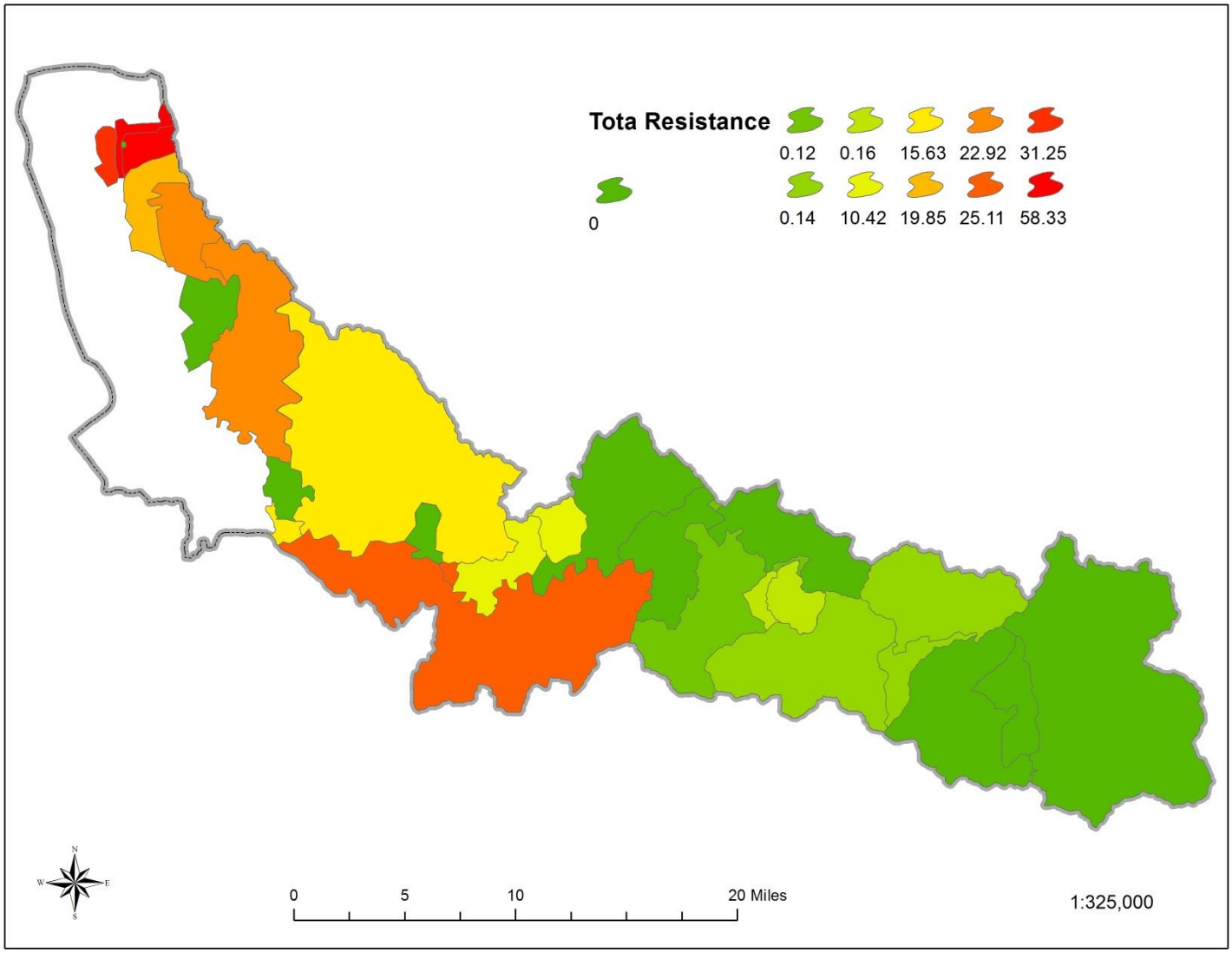


Figure 27. Proportions of ampicillin resistant *E. coli* colony forming units in the sub-watershed of each sample site. The values are the total percentage of CFUs to controls by Jenks natural breaks classification method.

Chloramphenicol Proportion Resistant to Controls

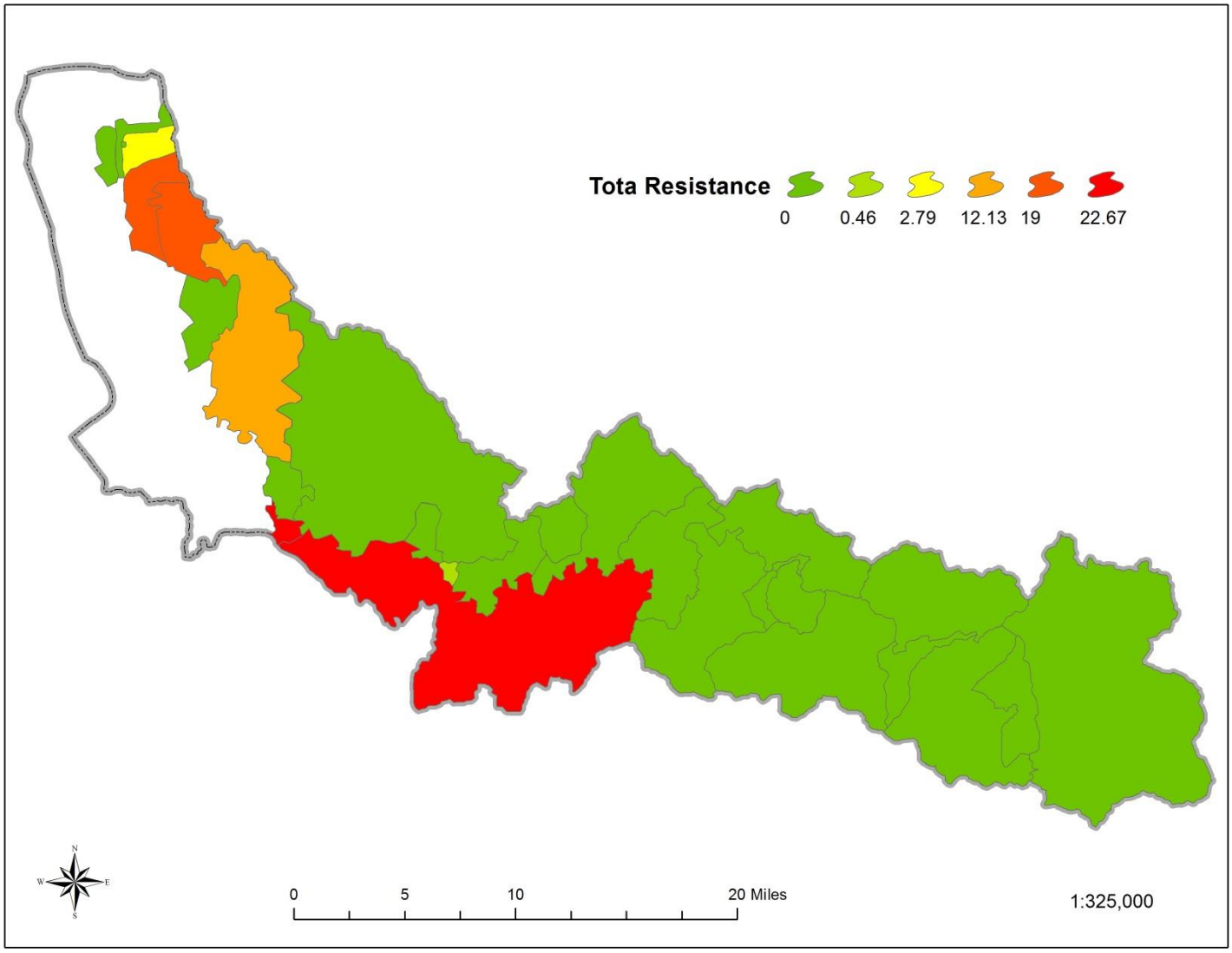


Figure 28. Proportions of chloramphenicol resistant *E. coli* colony forming units in the sub-watershed of each sample site. The values are the total percentage of CFUs to controls by Jenks natural breaks classification method.

Tetracycline Proportion Resistant to Controls

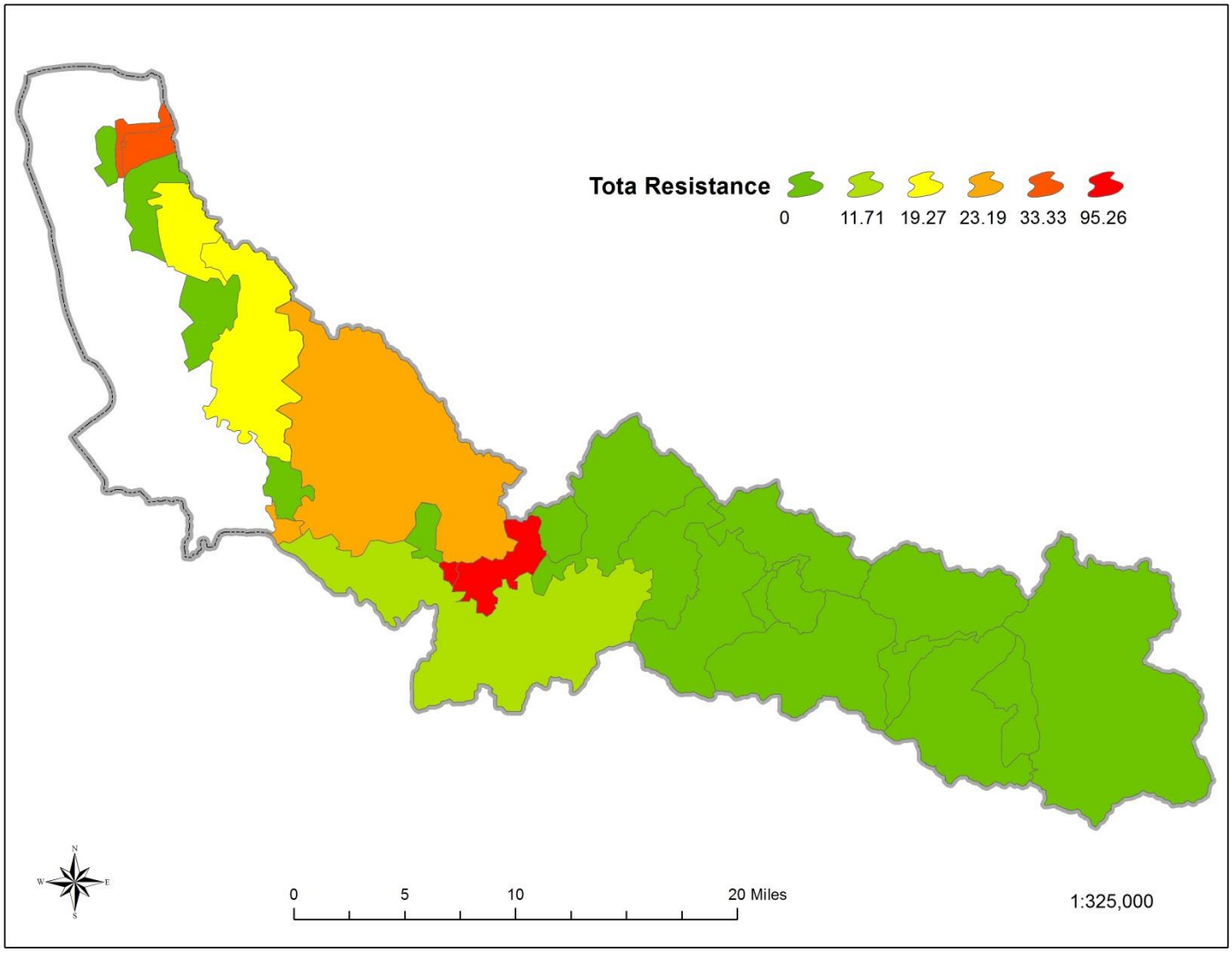


Figure 29. Proportions of tetracycline resistant *E. coli* colony forming units in the sub-watershed of each sample site. The values are the total percentage of CFUs to controls by Jenks natural breaks classification method.