FACTORS AFFECTING DENITRIFICATION POTENTIAL AND THE MICROBIAL ECOLOGY OF ESTABLISHED BIORETENTION CELLS ACROSS THE EASTERN MID-ATLANTIC REGION

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ABSTRACT

Increases in impervious surfaces caused by urbanization has led to higher volumes and rates of stormwater runoff that transports urban pollutants directly into natural waterways. Bioretention cells (BRCs) are vegetated soil systems designed to intercept stormwater runoff and reduce loads of water and contaminants discharged to surface waters. Nitrogen removal efficiency is highly variable and improvements are constrained by a poor understanding of the physical, biological, and chemical processes that occur within a BRC. The objectives of this study are to characterize and quantify the microbial communities in a range of existing BRCs, and determine which design factors have the greatest impact on denitrification, a microbial process responsible for removing nitrogen from stormwater. We sampled 23 BRCs throughout MD, VA, and NC, and quantified patterns in populations of denitrifying bacteria, denitrification potential, and microbial community structure within the soil medium. We found the greatest denitrifier populations and denitrification potential in the upper layer of the soil medium, which does not coincide with the internal water storage zone that is engineered to harbor anaerobic conditions favorable to denitrifying bacteria at the bottom of recent BRC designs. Results indicate that BRC vegetative cover, soil media nitrogen, and organic carbon concentrations are among the variables that facilitate nitrifying and denitrifying bacteria populations in BRCs. Bacterial community composition was most different between the top and bottom samples of the BRCs while fungal community composition differed most by BRC vegetative cover. Both fungal and bacterial community compositions were influenced by nitrogen and carbon concentrations.

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Literature Review

1.1 Stormwater Impacts

Surface waters act as a source and supplement for ecological, cultural, and economic services for the United States and worldwide. An estimate of the average global value of ecosystem services provided by coastal estuaries, lakes, and rivers is nearly $31,000/ha/yr (de Groot et al., 2012) and locally, Mid-Atlantic finfish and shellfish landings totaled over $435 million in 2009 (NMFS, 2010). However, the byproducts of a growing human population and urbanization have created an increasingly severe threat to the health of water resources in urban watersheds. As population densities have increased, so has the infrastructure needed to maintain the populace. Construction of roadways, sidewalks, and buildings fundamentally alter the natural hydrology of the watershed, particularly during storm events. The consequences of urban development include decreased groundwater infiltration, increased peak flows and rates of stormwater runoff, and transport of sediments and urban pollutants to surface waters (Niemczynowicz, 1999). Common urban pollutants include metals, hydrocarbons, nitrogen (N), phosphorus (P), carbon (C), suspended solids, bacteria, and thermal shocks to draining water bodies (EPA, 2005).

Nutrient additions by stormwater runoff pose a particularly serious threat to the health of both fresh and coastal waters. In the past several decades, N has emerged as a pollutant of concern due to its increasing use in agriculture and its transformation into a reactive and mobile species via fossil fuel combustion (Galloway et al., 2004). During storm events, these nutrients are transported to freshwater systems and can cause cyanobacterial blooms that reduce sunlight penetration to underlying vegetation and hypoxic conditions that kill fish species and alter food webs (Conley et al., 2009). Estuarine systems face an even greater threat due to development of urban areas around coastal waterways and the fact that N is often a rate limiting nutrient in marine systems (Howarth, 1988). Estuarine systems have similar symptoms of eutrophication as in freshwater, including diminished water clarity, reduction of seagrasses, hypoxic conditions, and species composition changes that have a reverberating effect on the surrounding ecosystems (Kemp et al., 2005).

The effect of N inputs on eutrophication was realized in the early 1970s (Ryther & Dunstan, 1971; Vince & Valiela, 1973) but still remains a problem as the need for agricultural systems to feed a growing population and urbanization continues to increase. Deadzones caused by hypoxia have increased dramatically in the past several decades. Diaz and Rosenburg (2008) have documented over 400 cases of hypoxia caused by eutrophication that collectively span an area of nearly 95,000 square miles around the globe. In the United States specifically, a national inventory of assessed surface waters indicated 44% of U.S. streams, 64% of lakes, and 30% of bays and estuaries are considered impaired, with urban stormwater runoff being among the top 10 sources of pollution within each water system (EPA, 2009). There is growing evidence that non-point source pollution reduction strategies and stormwater treatment measures may be having an impact on the pollutant reductions. Data from USGS monitoring stations indicate that N loads delivered to the Chesapeake Bay via river inputs are decreasing over the long-term and while this is certainly a positive trend, there is still much progress to be made (Moyer & Blomquist, 2016).

1.2 A Brief Background of Stormwater Management

Urban stormwater management practices were introduced in the United States in the 1950s, with the goal of redirecting stormwater from sidewalks and roadways as quickly and efficiently as possible to prevent flooding (NRC, 2008). This was accomplished through the use of simple diversion structures such as catch basins and piping systems that directly channel runoff into the nearest body of water. As a result, flooding and erosion issues quickly emerged and the focus of stormwater management shifted towards retention and detention basins (i.e., wet and dry stormwater storage structures, respectively) as improved means to manage stormwater through reductions in surface water peak flows. The use of these basins was bolstered with findings by the Nationwide Urban Runoff Program conducted by the Environmental Protection Agency (EPA) in 1983, which concluded that these structures not only regulated runoff volumes but also significantly decreased urban runoff pollutants. However, despite their ability to redirect flows and reduce urban pollutants, detention and retention basins increased channel erosion, discouraged localized infiltration, and failed to reduce overall flow volumes (Holman-Dodds et al., 2003).

In an attempt to solve the unsolved issues left by wet and dry basins and to incorporate stormwater management practices into more densely populated urban areas, there has been a push towards the development of on-site, small-scale, stormwater management practices (EPA, 2000). Prince George's County, Maryland officially branded the idea of on-site stormwater treatment as low impact development (LID) in the late 1990s (Prince Geroge's County, 1999). The goal of LID is to preserve or restore the pre-development hydrology of the landscape, which is accomplished through a variety of best management practices (BMPs) such as pervious pavement, green roofs, and bioretention cells (BRCs) (Dietz, 2007). The objective of each of these practices is to encourage infiltration of stormwater runoff into engineered systems that are designed to intercept and treat stormwater runoff through a variety of physical, chemical, and biological processes before infiltration to the underlying soil or discharge to storm sewer systems (Lehner, 1999). Bioretention cells specifically have become one of the most widely used best management practices in the United States and globally due to their small size, low construction and maintenance costs, aesthetic values, and pollutant removal capabilities (Davis et al., 2009; LeFevre et al., 2015).

1.3 Bioretention Design

As illustrated in Figure 1.1 below, bioretention relies on a variety of mechanisms such as vegetative uptake, filtration, adsorption, and microbial transformation to intercept and treat stormwater before it infiltrates the soil or is discharged to surface waters, creating a solution for both water quantity and quality effects (Davis et al., 2003). The vegetation in these systems typically consists of grasses, shrubs, or woody plants to decrease incoming stormwater velocities, facilitate biological activity, increase soil porosity, and allow for evapotranspiration (Davis, 2008; Davis et al., 2009). Cells that are not strictly grassed typically contain a mulch layer that also acts to decrease stormwater flow, as well as adsorb pollutants and maintain soil moisture (Davis et al., 2001). Beneath the mulch layer is a 0.7 – 1.2 m media mix layer, typically consisting of sand, silt, clays, and organic matter that act to facilitate infiltration and bind incoming pollutants (Davis et al., 2009; Hunt et al., 2006). Depending on the depth of the water table and the infiltration capacities of the underlying soil, BRCs are optionally equipped with an underdrain and/or a liner (Davis et al., 2009; DeBusk & Wynn, 2011). The underdrain is generally a perforated pipe integrated into a bed of gravel and sometimes preceded by a

permeable geofilter fabric to prevent clogging from sand and fines from the media mix layer (Hunt & Lord, 2006; Liu et al., 2014). As described in detail below, recent BRC design has attempted to increase N removal with the inclusion of carbon sources and permanently saturated zones by increasing stormwater hydraulic retention times.

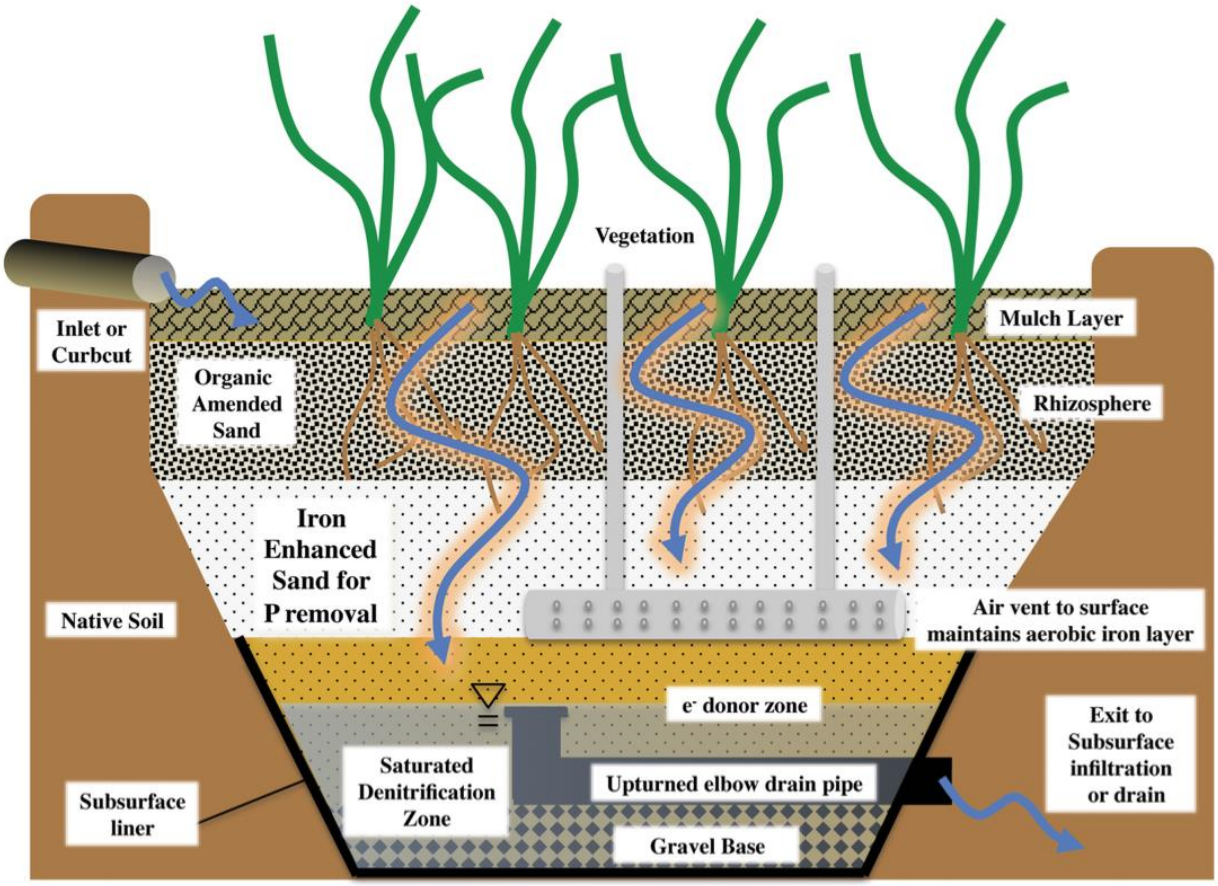

Figure 1.1. Bioretention cell illustrating various pollutant removal mechanisms associated with each component of the cell. Used with permission from ASCE (LeFevre et al., 2015).

1.4 BRC Pollutant Removal Mechanisms

Along with reducing flow volumes, a primary goal of bioretention is to improve stormwater quality. Due to their pollutant removal abilities, BRCs are often used to assist in achieving total maximum daily load (TMDL) goals for watersheds (Hunt et al., 2012). As stormwater passes through each successive layer of the bioretention cell, it is exposed to a multitude of physical, chemical, and biological processes that can all play a role in the removal

of particular pollutants. Because pollutant removal efficiency is a function of the cell properties, the contact time between stormwater and the bioretention medium, termed hydraulic retention time, becomes a governing factor for removing pollutants of almost every kind (Hunt et al., 2012). Commonly targeted urban pollutants include total suspended solids (TSS), pathogens, hydrocarbons, metals, P, N, C, and thermal pollution. While hydraulic retention time is to some degree a master variable, the design and selection of materials for BRCs also play a crucial role in the removal of urban stormwater contaminants. Multiple lab and field scale studies have evaluated the pollutant removal capabilities of BRCs and identified the mechanisms responsible for the reduction of these common pollutants.

BRCs are typically vegetated with sod, herbaceous cover, woody plant species, or a combination thereof. The physical structure of vegetation helps to reduce incoming stormwater velocity, while vegetation also biologically immobilizes nutrients such as N and P, potentially phytoaccumulates heavy metals, and facilitates a symbiotic relationship with the underlying microbial community. For example, Lucas and Greenway (2008) found a 15% increase in P retention and a 37% increase in N retention in vegetated bioretention mesocosms compared to unvegetated controls. Phytoextraction research has shown the ability of some plant species to hyperaccumulate metals of up to 10% of their dry weight in metals (Kumar et al., 1995), but recent studies on bioretention plant species demonstrate metal accumulations ranging from 1% - 3.3% (Dietz & Clausen, 2006; Sun & Davis, 2007). Furthermore, numerous studies have documented positive feedback mechanisms between plants and microbial activity (Grayston et al., 1997; Hamilton & Frank, 2001; Kuiper et al., 2004) that could increase decomposition, pollutant degradation, and nutrient cycling. However, it may be important to consider that if vegetation is incorporated into the BRC design as a continuous pollutant removal mechanism, nutrient uptake could plateau and eventually pollutants could return to the system if vegetative biomass is not harvested (Davis et al., 2003; Payne et al., 2014).

The upper layers of most bioretention cells typically include a mulch layer and top soil cover that play a crucial role in reducing incoming stormwater velocity, sedimentation filtration, and binding of heavy metals and hydrocarbons. Studies on the reduction efficiency of total suspended solids (TSS) have demonstrated that capture of TSS is facilitated by the upper 8 cm of the BRC (Li & Davis, 2008), with mass removal rates ranging from 50% to > 99% (DeBusk &

Wynn, 2011; Hatt et al., 2009a; Li & Davis, 2009). Polycyclic aromatic hydrocarbons (PAHs) from sources such as vehicular combustion, road tar, and vehicle fluids, are also primarily confined to the top several centimeters of the BRC with average removal rates as high as 90% (Diblasi et al., 2009). Heavy metals from vehicular and infrastructure deterioration are also of concern and multiple studies have shown confinement of these compounds to the mulch layer and upper 10 cm of the bioretention surface with capture efficiencies ranging from 88 to > 99% (Davis et al., 2003; Sun & Davis, 2007). As a result of the capture and binding efficiency of the upper layers of the BRC, accumulation of sediments and metals may warrant the removal and replacement of the top several inches of the BRC due to clogging or metal toxicity (Davis et al., 2009).

Beneath the mulch and topsoil layer lies the media mix. This 0.3 – 1.2 m layer is normally composed of a combination of sand, silt, fines, and organic matter (Davis et al., 2009). The purpose of the media mix is to assist in the removal of stormwater pollutants while facilitating adequate infiltration rates. Low infiltration rates can cause excessive ponding to the degree that stormwater is diverted to the overflow structure, essentially short-circuiting and removing any stormwater quantity or quality potentials of the BRC, while high infiltration rates reduce contact time and limit the ability of the BRC to capture or remove pollutants. Numerous studies have investigated media mix blends that optimize infiltration and pollutant removal capabilities, yet findings have been variable and opinions have changed over time, as reflected by changing design guidelines. BRCs were initially conceptualized to act as simple groundwater infiltration structures, using high infiltrating native soils (Clar & Green, 1993). However, due to high loam contents causing failures and areas with inadequately infiltrating soils, media mixes with 30-80% sand have become the current standard for BRC design (Davis et al., 2009). Additionally, several studies have begun to focus on the incorporation of water treatment residuals (WTRs) into the media mix and have shown their ability to be effective in the long-term removal of P (Lee et al., 2015; Liu et al., 2014).

1.5 Nitrogen Removal Issues in BRCs

Since its inception, bioretention technology has struggled to consistently achieve adequate N removal efficiencies. Since nutrient reduction for water quality is a primary objective for BRCs, design enhancement for N removal has received considerable attention in the past

several decades. BRCs constructed with a conventional design (i.e., freely draining and lacking structures that increase hydraulic retention times or induced saturated zones) have consistently demonstrated poor N removal efficiencies, and even N exports (Hatt et al., 2009b; Hsieh & Davis, 2005; Hunt et al., 2008). Some BRCs equipped with internally saturated zones to provide anoxic conditions for the denitrifying microbial community have demonstrated increased N reduction efficiencies (Brown & Hunt, 2011; Passeport et al., 2009), but others have failed to show differences in reductions in BRCs with and without ISZs (Davis, 2007; Dietz & Clausen, 2006; Hunt et al., 2006). Furthermore, different types of added carbon sources (Kim et al., 2003) and vegetation types (Gautam & Greenway, 2014) have demonstrated varying contributions to N removal efficiencies in bioretention mesocosms-scale studies.

Stormwater N inputs to BRCs include dissolved organic nitrogen (DON), particulate organic nitrogen (PON), ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) (Taylor et al., 2005). The partitioning of each of these species within a particular flush of stormwater runoff is dependent on the storm intensity and the source of the runoff. Li and Davis (2014) evaluated several storm events from a roadway and parking lot runoff source and found that the stormwater was primarily made up of PON (57%) and dissolved organic and inorganic N (43%). As described by Collins et al. (2010) and mentioned by Li and Davis (2014), the methods by which N species are contained or removed in bioretention systems are specific to the N species. These mechanisms include sedimentation and filtration of PON, NH_3 fixation, plant uptake, microbial assimilation, nitrification, and denitrification.

Since close to half of parking lot runoff is PON, and this fraction is contained in the cell via sedimentation and infiltration, the other dissolved N forms are subject to capture through sorption mechanisms, plant immobilization, or removal through microbial conversion. Collins et al (2010) further notes that plant uptake and denitrification are the dominant forms of N removal within BRCs and similar environments (Vymazal, 2007) and recent research using a stable isotope tracer in bioretention mesocosms found that nearly 99% of incoming stormwater nitrate was plant assimilated (Payne et al., 2014). While plant uptake may be a major pathway for N removal, it is limited to the biomass accumulation of the plant, and as previously mentioned, potentially returns to the cell once the plant matter dies and N is mineralized. Meanwhile,

denitrification requires little to no maintenance, is constrained only by the rates of N conversion by the microbial community, and potentially allows for complete N removal from the BRC.

Denitrification is the biologically mediated, stepwise process by which oxidized N species are reduced to nitrogen gas (N_2) under anaerobic conditions and potentially provides for the complete conversion of mobile polluting forms of N to inert nitrogen gas. If denitrification is the desired primary pathway for N removal, the BRC must not only foster the anoxic conditions for denitrification, but it must also supply an environment for the transformation of all N species which require contrasting redox conditions. Therefore, both an oxidizing environment for nitrification and a reducing environment with incorporated carbon sources for denitrification is a necessary component of BRC design. As a further complication, N in the NO_x (NO_2^- or NO_3^-) phases carry a negative charge that is repelled by the similarly negatively charged soil matrix, allowing these forms of N to quickly pass through the cell before being denitrified. Despite the many challenges involved in the removal of N from stormwater, BRC research shows several promising design approaches that may allow for efficient removal of a wide range of N species.

1.6 Factors Affecting Denitrification

To understand the design approaches engineers have taken to increase N removal efficiency in BRCs, it is first important to have a fundamental understanding of the factors that affect and can potentially promote denitrification. There are several underlying conditions that must be present for denitrification to occur. These include: 1) the presence of denitrifying organisms; 2) an organic carbon source to act as an electron donor; 3) an oxygen deficient environment; and 4) the presence of oxidized N species to act as terminal electron acceptors (Philippot et al., 2007). Denitrification has been shown to occur in a wide range of organisms including bacteria (Heylen et al., 2006; Song et al., 2000; Zumft, 1997), fungi (Kobayashi et al., 1996; Laughlin & Stevens, 2002; Shoun & Tanimoto, 1991), and archaea (Bartossek et al., 2010; Cabello et al., 2004; Philippot, 2002), but it is generally believed that bacteria are the dominant denitrifiers in most environments (Wallenstein et al., 2006). Denitrification is carried out through several steps controlled by four groups of enzymes that can be identified by their functional genes (illustrated in Figure 1.2): nitrate reductase (*narG*, *napA*, *euk-nr*, and *nas*), nitrite reductase (*nirS & nirK*), nitric oxide reductase (*norB*), and nitrous oxide reductase (*nosZ*) (Chen et al. 2013). Because denitrification is a microbially mediated process, the rate at which denitrification

occurs and the concentration and distribution of bacterial denitrifiers is reliant upon many factors, including the native environmental conditions, resource availability, and the establishment and persistence of the microbial community. Wallenstein et al. (2006) further characterize these controlling factors of denitrification rates and denitrifier populations as "proximal" and "distal". Proximal controls are described as the factors that have an effect on denitrification rates at any given moment, while distal controls are factors that control the denitrifying community over the long term.

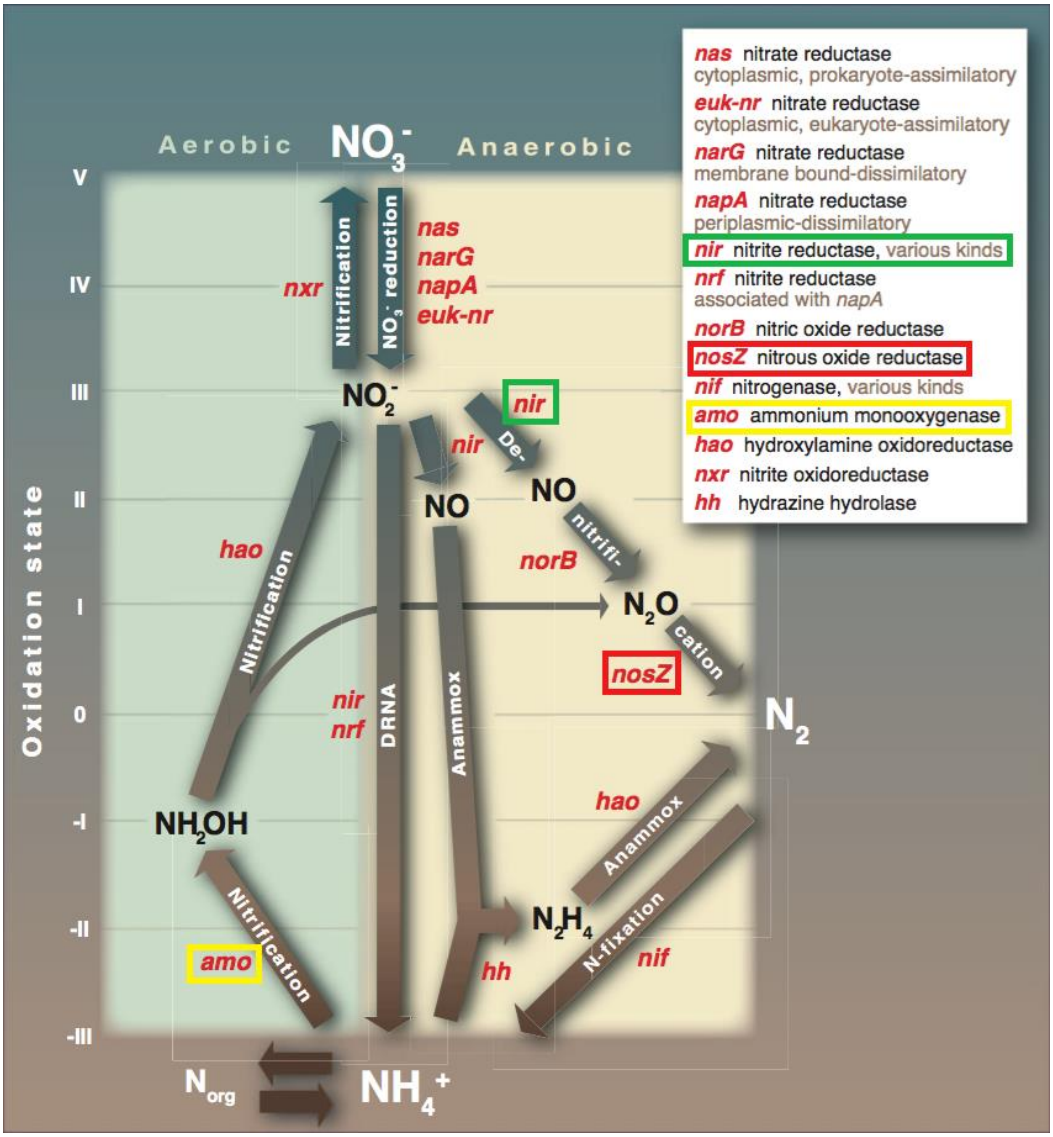

Figure 1.2 Illustration of the nitrification/denitrification cycle and the genes associated with each transformation. Boxed genes were included in this study. Used with permission from AAAS (Canfield, 2010).

As with most ectothermic organisms, metabolic activity is a function of temperature, and thus the transformation of N species via microbial enzyme activity is strongly influenced by climate as well as local weather. Day to day fluctuations in temperature that affect instantaneous denitrification rates can be classified as a proximal control, while climatic, long-term averages in temperature can be understood as distal controls which shape the formation and persistence of the denitrifying community. Denitrification rates generally increase with temperature, peaking at 65°C, significantly decreasing at 15°C, and nearly ceasing at 5°C under laboratory conditions (Bailey & Beauchamp, 1973; Bremner & Shaw, 1958; Sirivedhin & Gray, 2006). Although it is difficult or impossible to manage climatic factors in any outdoor BMPs, it is reasonable to expect that denitrifier populations and denitrification rates are highly variable across regions, seasons, and daily fluctuations.

Although there exists a wide variety of denitrifying organisms, it is understood that the majority of denitrifiers are heterotrophic, meaning these organisms are reliant upon an organic carbon source to serve as an energy supply and as a source for cell synthesis (Zumft, 1997). Therefore, the persistence of heterotrophic denitrifiers is strongly influenced by the quantity and quality of organic carbon sources. Water soluble carbon, specifically, has shown to be a major factor controlling denitrification rates (Blowes et al., 1994; Burford & Bremner, 1975; Decatanzaro & Beauchamp, 1985). Generally speaking, the more labile a carbon source is, the quicker it is utilized by the bacterial community. However, natural degradation of a carbon source in excess of the needs of the microbial community must be avoided or nutrient additions will exceed microbial consumption and cause net exports of mineralized N, negating the N reduction benefits of denitrification.

The majority of denitrifiers are facultative anaerobes, which produce ATP via aerobic respiration under oxygenated conditions, but are capable of anaerobic respiration using electron acceptors other than oxygen under anaerobic conditions. For denitrifiers, these electron acceptors are the oxidized N species that are ultimately reduced to N_2 in the process of denitrification. Low oxygen concentrations and increased moisture content have been shown to increase denitrification rates across multiple lab and field scale experiments (Grundmann & Rolston, 1987; Maag & Vinther, 1996). Lab-based research has shown that denitrification occurs only in soil samples with less than 5% oxygen content, and that it is the primary means of microbial

respiration under these conditions (Hochstein et al., 1984; Hwang & Hanaki, 2000). It is also interesting to note that pure culture studies have shown that varying levels of oxygen concentration have an effect on the expression of individual denitrification enzymes that are responsible for the conversion of N species at different steps within the denitrification pathway (Bonin et al., 1989; Korner & Zumft, 1989). Such expression varies across bacterial species and oxygen contents, which implies that the rate and proportion of denitrification products may vary with inundation time and denitrifier community composition.

As previously noted, nitrate and the further reduced species (NO_2, NO, and N_2O) are the substrates that denitrifying bacteria use as terminal electron acceptors to generate energy during the process of denitrification. Many studies have examined the relationship between denitrification rate and N supply and found increased denitrification rate with increased inorganic N input (Dandie et al., 2008; Firestone et al., 1980). However, it is important to consider that organic N and ammonium can be just as important to denitrification rates and denitrifier populations due to the natural wetting and drying cycles to which BRCs are subject. These N species have been shown to make up between 65 - 82% of N species in urban stormwater (Li & Davis, 2014; Taylor et al., 2005) are mineralized and nitrified during extended dry periods, and accumulate in the media mix between saturation events (Brown et al., 2013). The fact that a large proportion of incoming N is not immediately available for denitrification holds important implications for denitrification rates and populations. Much of the N transformation occurs in the upper layers of the BRC and throughout extended dry periods during conditions that are unfavorable to denitrification.

1.7 BRC Design to Enhance Nitrogen Removal

As bioretention has become an increasingly popular method for stormwater treatment, research has focused on design methods that maximize N removal efficiency. Although most studies are confined to single cell or mesocosm studies, they are nonetheless essential to understanding the mechanisms and progress of bioretention systems, and how various design options have an impact on denitrification and the native microbial population.

1.7.1 Internally Saturated Zones

The upper layer of the BRC remains oxygenated, which promotes aerobic mineralization and nitrification and provides products for denitrification. Anoxic environments conducive to denitrification can exist in microsites in the upper layers of the cell, ephemerally during cell saturation following a storm event, or they can be induced by the addition of an internal saturated zone (ISZ). ISZs have been created by controlling outflow volumes (Lucas & Greenway, 2008), using low permeable bottom layers within the media mix (Hsieh et al., 2007), and more commonly, by inserting a vertical elbow at the outflow of the underlying drainage pipe (Chen et al., 2013; Hunt et al., 2006; Luell et al., 2011). In BRCs without an ISZ, extended dry periods can accumulate nitrified products and actually cause net exports of nitrites and nitrates (Hunt et al., 2006). Various studies have achieved nitrite and nitrate removal rates up to 80% (Kim et al., 2003; Zinger et al., 2007) while other studies report insignificant differences between cells with and without ISZs (Davis, 2007; Dietz & Clausen, 2006; Hunt et al., 2006). It is also important to note that while nitrite and nitrate exports may be lower in a cell with an added carbon source, total nitrogen (TN) levels may be higher due to the decomposition of the added organic matter (Randall & Bradford, 2013). Furthermore, most studies of BRC media mixes do not target N due to a lack of adsorption or physiochemical interaction and because of the highly mobile nature of nitrite and nitrate species (Davis et al., 2006).

1.7.2 Vegetation

Vegetation plays an important role in N removal efficiency in BRCs through biotic assimilation and stimulation of the microbial community responsible for nutrient cycling. Many studies have shown enhanced N removal with the presence of vegetation (Bratieres et al., 2008; Henderson et al., 2007; Lucas & Greenway, 2008; Lucas & Greenway, 2011). Henderson et al. (2007) showed that on average, vegetated bioretention soil columns removed twice the amount of N than non-vegetated columns. Plants stimulate the microbial community by the addition of carbon through root exudates and by creating a uniform redox potential via root oxygen release or heterotrophic breakdown (Payne et al., 2014). Research by Payne et al. (2014) is the only known study to use a nitrate isotope tracer to determine the fate of nitrate in bioretention soil columns, and found that that nearly all incoming nitrate was almost immediately plant assimilated, leaving less than 10% of incoming nitrate to be microbially denitrified. Although

these types of results would vary in the field and across different types of vegetation, it certainly highlights the importance and potential dominance of vegetative assimilation within bioretention cells.

1.7.3 BRC Sizing

Field scale studies that compare N removal efficiencies of similar BRCs with varying sizing specifications have received little attention in comparison to other BRC design attributes. Although The Virginia Stormwater Handbook (1999) recommends BRC surface area to be 2.5% of their runoff surface area, BRCs are often constructed with surface area ratios of 5-7% (Diblasi et al., 2009) and can even exceed that range in cases where retrofitting in an urban landscape is necessary. Luell et al. (2011) investigated the pollutant removal capabilities of two differently sized BRCs receiving the same runoff. The "small" BRC was considerably undersized by North Carolina design standards with 28% of the bowl volume storage of the (correctly designed) large cell and approximately 50% of the bioretention cell SA/runoff SA ratio as the larger cell. By analyzing the concentration of the effluent, the study found that the small cell reduced 84% as much TN as the larger cell, indicating that a smaller cell size does impact N removal efficiency, but may be sufficient in circumstances where retrofitting in an urban setting is necessary. However, it is difficult to say whether this trend can be generalized beyond a single study focused on these two BRCs.

1.7.4 Added Carbon Sources

As previously mentioned, due to the heterotrophic nature of denitrifying bacteria, an organic carbon source is necessary as an electron donor and for cell synthesis for the bacterial community. Typically, the surface layer of the BRC accumulates organic matter via the planting mixes that are added for the incorporated vegetation, the formation of an organic topsoil cover, and incoming organic carbon additions to the cell. Without additional added carbon sources, most organic carbon is filtered out or captured in the upper layers of the cell, leaving insignificant contributions of carbon to the bottom layers of the bioretention cell. This becomes a rate limiting substrate for denitrification within the lower layers of BRCs designed with an internally saturated zone to facilitate denitrification. Carbon additions can be tricky, however, because they must be essentially maintenance free and balanced with the needs of the microbial community to avoid export of nutrients or carbon. Labile organic sources in media mixes have

shown to cause net nutrient exports (Clark & Pitt, 2009; Hunt et al., 2006). Multiple studies have been carried out on various carbon sources (e.g., compost, newspaper, sawdust, wheat straw, leaf litter, and woodchips) and their relation to denitrification (Blowes et al., 1994; Kim et al., 2003; Volokita et al., 1996). Current research shows finely shredded woodchips to be a promising source for the use in bioretention cells as they are generally maintenance free, affordable, and provide the microbial community with carbon over the course of decades (Moorman et al., 2010; Peterson et al., 2015; Robertson, 2010). Nonetheless, many bioretention cells with a variety of added carbon sources are still in operation and their resident denitrifying microbial community has yet to be evaluated.

1.7.5 Media Composition

As detailed in previous sections, due to the fact that the majority of denitrifying bacteria are heterotrophic, facultative anaerobes, these microorganisms require anoxic conditions and a carbon source for anaerobic respiration. Therefore, media mixes with decreased hydraulic conductivities and carbon amendments may favor denitrification. Research on BRC media mix composition has been a large facet of research with opinions and practices changing over time. For example, early research by Hsieh and Davis (2005) showed that a sandy loam media mix with a 20 – 70% soil composition (percentage based on vegetation needs) was most effective in providing adequate infiltration and pollutant removal rates for a wide range of contaminants, but these "loamy" mixes were prone to clogging (Davis et al., 2009). While modern BRC design has turned towards media mixes with higher sand contents (e.g., 85-88% sand, 8-12% fines, and 3-5% organic matter) as described by Hunt and Lord (2006) to facilitate infiltration, many states and municipalities still recommend mixes with 30-50% sand and higher topsoil and organic matter additions (Prince George's County, 2002; Lucas, 2005). Due to BRC design guidelines with variable sand contents and organic matter compositions, it is expected that denitrifier populations that rely on these conditions would vary across BRCs with differing media mixes.

1.7.6 Quantifying Denitrifiers in BRCs

Chen et al. (2013) conducted one of the few studies that quantified denitrifying bacteria within a bioretention cell. They quantified total bacteria as well as the nitrifying gene *amoA,* and four denitrifying genes - *nirS*, *nirK*, *norB,* and *nosZ* - at varying locations and depths within a BRC that receives runoff from a four lane highway in Lenexa, Kansas. Concentrations of 16S

rDNA were highest in cores near the effluent (denoted by overflow structure) and were inversely correlated with depth in the cell. Concentrations of *amoA* and all denitrifying genes were significantly correlated with pH and the *amoA*, *nirK*, and *nirS* genes were directly correlated with nitrate and organic matter concentrations. Sites within the cell that exhibited longer inundation times also had the highest densities of gene copies for ammonia oxidizers and denitrifiers. The research suggested that a threshold inundation time may be necessary for significant denitrification to occur and an optimal pH and sufficient carbon additions may allow for greater nitrifying and denitrifying bacterial populations in the bottom layers of the cell.

More recently, Willard et al. (2014) quantified the denitrification genes *nirK* and *nosZ* in a functioning bioretention cell in Blacksburg Virginia, receiving runoff from a nearby parking lot. The results showed that *nirK* and *nosZ* populations were positively correlated with each other and both genes were negatively correlated with depth. Furthermore, *nirK* showed positive correlations with TN, total carbon (TC), total phosphorus (TP), silt, and clay but negative correlation with sand content. *nosZ* populations were also positively correlated with TN, TC, TP, and silt. These results indicate that nutrient concentrations and design parameters, such as carbon content, media mix composition, and BRC depth cause varying denitrifier populations and can potentially be altered to increase N removal efficiencies.

1.8 Microbial Community Structure

A single gram of soil is estimated to contain $10^3 – 10^6$ individual "species" (Gans et al., 2005; Tringe et al., 2005) of bacteria that interact with the environment in which they exist. Patterns of abundance, diversity, and the structure of microbial communities and how these metrics relate to ecosystem function have interested scientists for decades, but scientific methods and financial constraints have hampered the ability and practicality of answering related questions. Historically, the classification and characterization of soil microbes have been limited to the physiological attributes of culturable laboratory strains. These techniques are particularly restrictive and frequently referred to as the "great plate count anomaly" because over 99% of bacteria are unculturable (Staley & Konopka, 1985). However, in the past decade, non-culture based approaches to microbial ecology, such as next generation sequencing methods, have become the gold standard for classifying bacterial and fungal diversity due to their ability to fully characterize both culturable and unculturable taxa (Claesson et al., 2010) and the decreasing cost

of high-throughput molecular tools to generate sequencing data. These methods allow for the classification of soil microbial communities into taxonomic, phylogenetic, and functional traits. Although it is generally understood that a myriad of factors regulate the composition of soil bacterial communities, several phyla appear to dominate the greater proportion of soil communities across continents, including *Proteobacteria, Acidobacteria, Actinobacteria, Bacteriodes, Firmicutes, Verrucomicrobia, Chloroflexi, Planctomycetes,* and *Gemmatimonadetes (*Janssen, 2006; Lauber et al., 2009). Recent research has attempted to understand microbial community composition within the light of environmental gradients and how community structure affects biogeochemical cycling (Barberan et al., 2014; Strickland et al., 2009), but a detailed understanding of how genomic diversity relates to these processes is still lacking.

Since the advent of affordable high-throughput sequencing tools, data processing methods, and publically accessible rRNA databases in the past decade, researchers have put forth a great effort to describe microbial communities within the context of their native environmental conditions. Immeasurable numbers of variables or combinations of variables likely interact to influence the abundance, structure, and diversity of the microbial community. Of these variables, pH and moisture content have long been referred to as the "master variables" that control microbial activity as well as community structure and diversity. Recent molecular research has bolstered that belief, as both soil moisture (Baker et al., 2009; Brockett et al., 2012) and pH (Fierer & Jackson, 2006; Lauber et al., 2009; Rousk et al., 2010) have shown to be good predictors of microbial community structure across a wide geographic distribution and land use types. Bacterial diversity and abundance have shown to decrease with soil depth, most likely due to decreasing resource availability and carbon sources (Fierer et al., 2003). Both field and lab scale studies have demonstrated phylum level predictability in relation to carbon availability (Fierer et al., 2007), shifts in the bacterial community in response to additions of carbon substrates (Cleveland et al., 2007; Eilers et al., 2010), and N gradients in soils (Fierer et al., 2012; Ramirez et al., 2012). Furthermore, vegetation has shown to have a particularly strong effect on the abundance of microorganisms and microbial community composition (Carney & Matson, 2006; Mitchell et al., 2010).

Generally speaking, fewer fungal species have been identified and the overall dynamics of fungal community structure is largely unknown in comparison to bacteria. Many factors that

induce responses in bacterial communities also affect soil fungal communities. Studies have noted changes in community structure due to pH, although not to the degree of bacterial communities (Rousk et al., 2010). Also similar to bacteria, fungi demonstrate reduced abundance and diversity with increasing soil depths (Fierer et al., 2003; O'Brien et al., 2005). Research has reported strong changes in fungal diversity and taxonomic changes in systems with varying nutrient contents (Allison et al., 2007; Lauber et al., 2008) and broad scale changes in vegetation and plant traits (Crowther et al., 2014; de Vries et al., 2012).

To our knowledge, no research has attempted to characterize the bacterial or fungal communities within bioretention cells. However, based on the existing research of microbial communities within biomes and across environmental conditions, it is reasonable to infer that bacterial and fungal communities would differ and, potentially, follow a predictable gradient in response to bioretention cell design. For example, soil nutrient levels attributed to the source of runoff or the total volume of runoff treated by an individual BRC could influence bacterial and fungal community composition. The different types of vegetation planted in BRCs may have an effect on diversity or community structure that could potentially mirror ecosystems with similar vegetation types and climate schemes. The incorporation of an organic carbon source in the lower layers of the cell or organic matter formation in the upper layers of the cell as a function of age may cause for shifts in microbial community composition.

This research attempts to open the “black box” of N removal in BRCs by quantifying bacterial denitrifying populations and denitrification potential across bioretention cells with varying design parameters. By identifying several key design components that most influence denitrification, future research may determine how these can be manipulated to increase N removal in BRCs. Additionally, by determining microbial community composition in BRCs, we can begin to investigate how these structures function in relation to similar ecosystems. This information can then be used in combination with BRC design to select for communities that increase denitrification and broader pollutant degradation.

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Chapter 2: Factors Affecting Denitrification Potential and Nitrogen Cycling Bacteria of Existing Bioretention Cells Across the Mid-Atlantic Region

Abstract

Rapid urbanization has led to increases in impervious surfaces in many watersheds, causing higher volumes and rates of stormwater runoff that transports urban pollutants directly into natural waterways. Bioretention cells (BRCs) are small, vegetated soil systems engineered to intercept stormwater runoff and reduce loads of water and contaminants discharged to surface waters. However, their efficiency in reducing these contaminants is variable, particularly for nitrogen (N) removal. Design improvements to assure more consistent performance are hampered by a lack of knowledge of the physical, biological, and chemical processes that occur within a BRC. The objective of this work was to characterize the diversity and function of microbial denitrifying populations in a range of functioning BRCs and determine which design factors have the greatest impact on denitrification, an important biological process for removing N from stormwater. We sampled 23 BRCs throughout Maryland, Virginia, and North Carolina and quantified populations of one bacterial nitrifying gene, three bacterial denitrifying genes, and denitrification potential within the soil medium. Our results show that the top layers of the cell harbor greater concentrations of denitrifying bacteria compared to the bottom layers of the cell, despite the presence of environments thought to be preferential to denitrifiers at this depth. Out of the 12 predictor variables used in the analysis, our results indicate media mix composition, vegetation type, nitrogen concentrations, and total organic carbon (TOC) to be the primary drivers of nitrifying and denitrifying bacteria in BRCs.

1. Introduction

Urbanization has dramatically increased since the early 1900s, both worldwide and in the United States. The global urban population has grown from 746 million in 1950 to 3.9 billion in 2014 (United Nations, 2014) and currently, the United States' urban population accounts for approximately 81% of the nation's total population (Bureau, 2012). Corresponding decreases in natural ponding areas, increased vegetation removal, and large-scale formations of impervious surfaces resulting from urbanization have altered the natural hydrology (Misra, 2010). The consequences of urbanization and increased impervious surfaces are not only larger peak flows and volumes of stormwater runoff, but also increased loads of nutrients, sediments, heavy metals, and thermal shocks to draining water bodies (Moglen, 2009). Perhaps the greatest threat posed by untreated stormwater runoff is eutrophication – the process by which dissolved oxygen levels are depleted due to algal blooms caused by nutrient additions to surface waters. Eutrophication results in finfish and shellfish kills, species composition changes, and a loss of natural habitat that has devastating effects on the environment and economies worldwide (Smith, 2003).

In an attempt to reduce urban stormwater volumes and pollutants into surface waters, stormwater management techniques have progressively focused on on-site treatment of urban runoff. One of the most popular mechanisms used for urban stormwater treatment is bioretention. Bioretention cells (BRCs) typically have a vegetated and mulched surface layer, followed by several feet of media mix, and are optionally equipped with a saturated layer as well as an underdrain to either export treated runoff to the stormwater system or to facilitate infiltration. The overall design objective is to intercept and treat stormwater runoff through a variety of physical, chemical, and biological processes as it passes through the cell. Bioretention offers a viable solution for the treatment of stormwater runoff and many studies have recorded high removal rates for common urban stormwater pollutants such as heavy metals, suspended solids, and bacteria. In contrast, however, observed N removal rates have been highly variable.

Denitrification is the process by which nitrate (NO_3^-) is microbially reduced to nitrogen gas (N_2) and potentially provides for complete N removal from stormwater entering a BRC. Recent studies have suggested that permanently saturated zones and added carbon sources promote denitrification in BRCs (Kim et al., 2003; Passeport et al., 2009; Zinger et al., 2007),

but results have been variable and most research is confined to single cell or lab-based studies. While some states and counties have BRC design protocols or recommendations, many of these are outdated and there has yet to be an agreed upon standard for many design parameters for the Mid-Atlantic region. A comprehensive field-based study of the microbial fraction within a variety of functional bioretention cells has yet to be conducted, and only two published studies have quantified densities of bacterial denitrifying genes within a bioretention cell to date. An understanding of BRC design factors that affect bacterial denitrifier populations could lay the foundation for more controlled experiments, with the ultimate goal of informing future BRC design. Therefore, the goal of this research is to determine the effect of BRC design parameters on denitrification by quantifying bacterial denitrifying genes and denitrification potential in BRCs across the mid-Atlantic region with varying design components.

2. Methods

2.1 Data Collection and Site Locations

Design specifications were acquired for approximately 50 BRCs in the Eastern Mid-Atlantic region (MD, VA, & NC) from published journals and public resources. We selected 23 of these for sampling (Figure 2.1 and Table 2.1) to ensure sufficient variability of design characteristics or features that have shown to be influential on N removal efficiencies (e.g. presence of saturated zone, vegetation, media mix composition, etc.) or have yet to be tested (e.g. age, geographic region, temperature, precipitation, etc.). Due to possible construction inaccuracies and compaction from the time of construction to the sampling date, media mix depth was determined by the distance from the bottom of the BRC to the cell surface during the collection of the bottom 10 cm samples. Average cell depths were measured for all but three BRCs, in which case the reported design depths were used in the analysis.

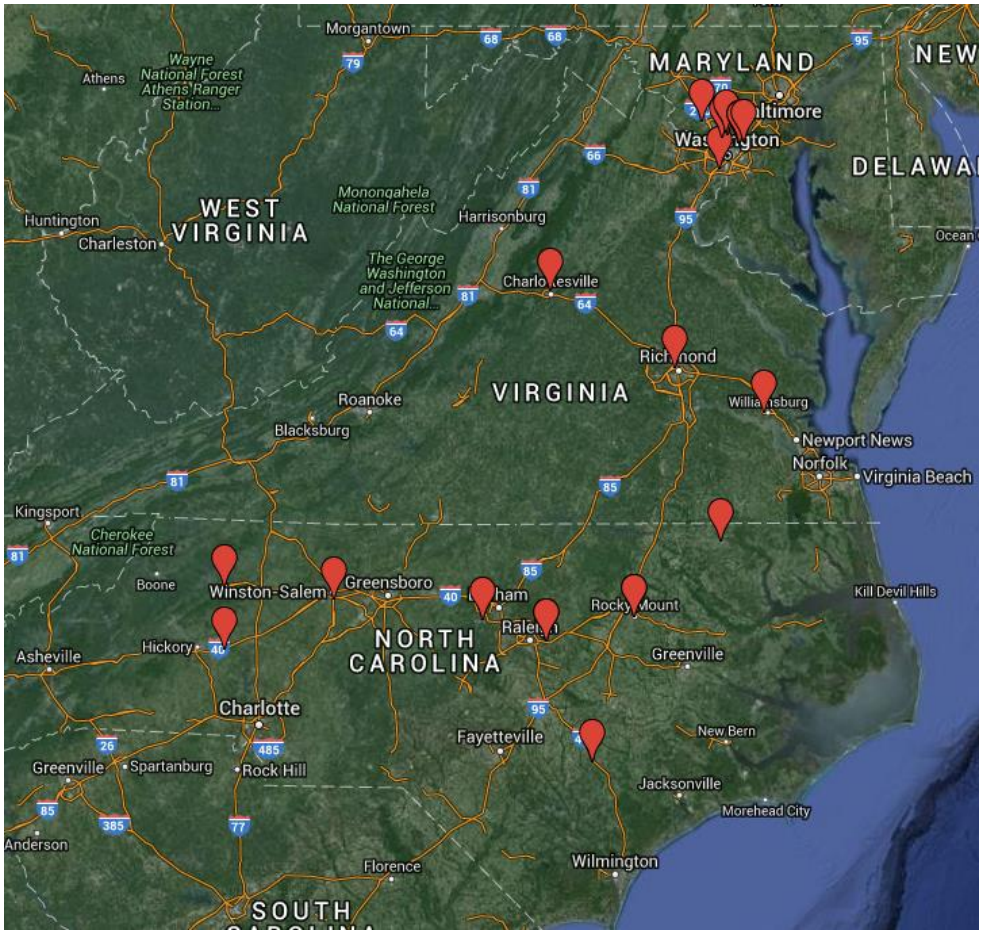

Figure 2.1. BRC sampling locations across MD, VA, and NC.

Site Description	Lat/Long	Source
St. Stephens & St. Agnes Middle School	38.830089, -77.104652	US Army Corps of Engineers Demonstration Project
Kensington Park Library	39.029356, -77.082125	Montgomery County Planning Specialist
Saint Andrew's Church	39.046696, -77.031627	Montgomery County Planning Specialist
Capital One Bank	39.058719, -77.049047	Montgomery County Planning Specialist
Ridgeview Middle School	39.126951, -77.248917	Montgomery County Planning Specialist
University of Maryland	38.993308, -76.944311	Dr. Allen Davis, UMD
University of Maryland	38.993136, -76.935422	(Davis, 2007, 2008)
University of Maryland	38.993136, -76.935422	(Davis, 2007, 2008)
University of Maryland	38.993558, -76.939155	(Diblasi et al., 2009; Li & Davis, 2009)
Beltway Plaza Mall	39.000841, -76.907212	(Davis et al., 2003)
Interstate 40 Exit Ramp	36.061978, -80.230905	NCDOT
Chapel Hill University Mall	35.927484, -79.024913	(Hunt III, 2003; Hunt et al., 2006)
Dublin I-40 Rest Area	34.989116, -78.133184	NCDOT
U.S. 258 Exit	36.442810 -77.087402	NCDOT
Rocky Mount Science Center (Grassed)	35.946197, -77.796184	(R. Brown & Hunt, 2008; R. A. Brown & Hunt, 2011)
Rocky Mount Science Center (Shrubbed)	35.946497, -77.796579	(R. Brown & Hunt, 2008; R. A. Brown & Hunt, 2011)
Knightdale I-540 (Large)	35.784256, -78.513500	(Luell et al., 2011)
Knightdale I-540 (Small)	35.784320, -78.513391	(Luell et al., 2011)
Catawba County Rest Stop	35.726094, -81.127780	NCDOT
NW NC Visitor Center**	36.135016, -81.121886	NCDOT
CHS Biofilter	38.052951, -78.477273	(Yancey, 2011)
Science Museum of Virginia	37.561193, -77.467587	Science Museum of Virginia
Williamsburg-James City Courthouse	37.275880, -76.740852	(Sample et al., 2014)

Table 2.1 Sampling descriptions, locations, and sources.

2.2 Sampling

All BRCs were sampled within approximately a one month period and only after at least one week without rainfall during November and December 2014. As illustrated in *Figure 2.2*, soil media samples were collected from both the front (identified by the inlet) and the rear of the cell (identified by the overflow structure). The top 10cm and bottom 10cm of soil from each core was captured aseptically and three subsamples from each location were homogenized to provide a total of 4 samples per cell. The samples were stored on ice for travel back to the laboratory and stored at – 80° C until further analysis. A portion of the sample was refrigerated at 4° C for no longer than seven days for use in the denitrification potential analysis.

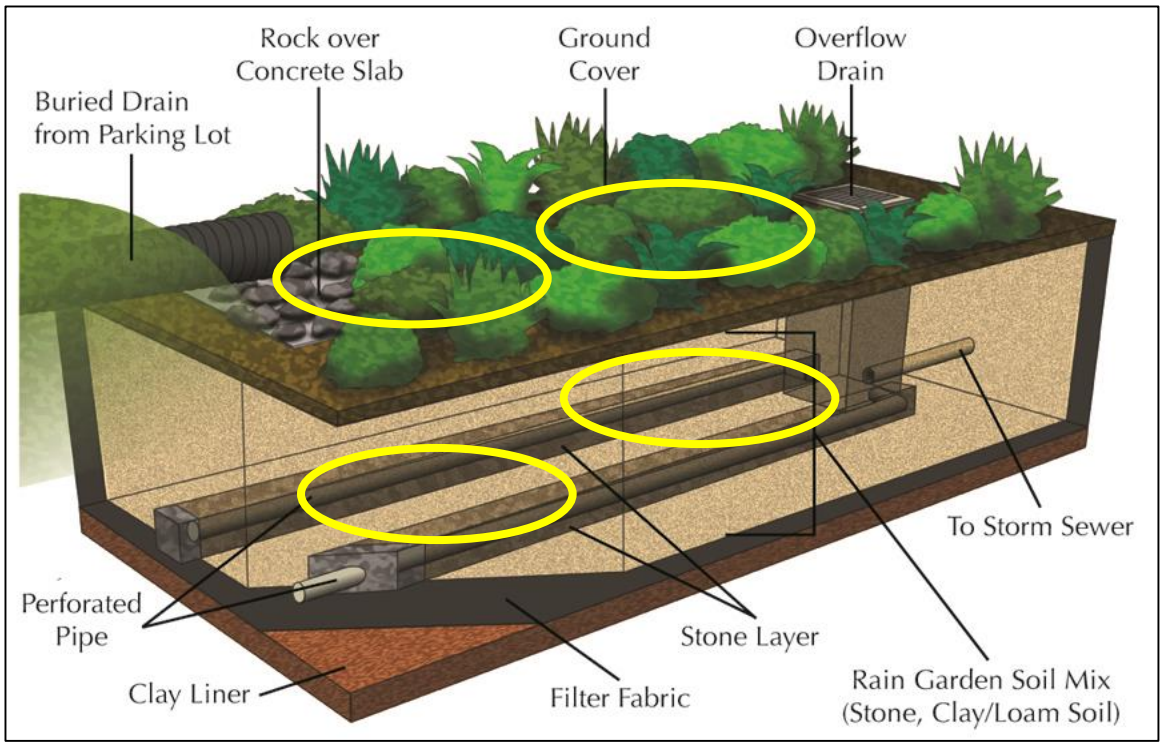

Figure 2.2. Sampling locations within each BRC. Yellow circles represent separate locations, each location to be subsampled three times and homogenized allowing for a total of 4 samples/cell.

2.3 Quantifying denitrifier populations

For each composite soil medium sample, DNA was extracted using the PowerSoil DNA Isolation-Kit (MOBIO Laboratories INC, CA, USA) following the recommended protocols. Mass of extracted DNA was determined using the Qubit 2.0 fluorometer (Invitrogen, USA) and stored at -20° C prior to analysis. Bacterial populations were analyzed via quantitative polymerase chain reaction (qPCR). To allow for a comprehensive view of the N cycle with an emphasis on denitrification, one bacterial nitrification gene *amoA* (Rotthauwe et al., 1997), and a suite of bacterial denitrification genes were quantified including: *nirK* (Henry et al., 2004), *nirS* (Throback et al., 2004), and *nosZ* (Rosch et al., 2002). Nitrification is carried out through the ammonium monoxygenase enzyme (*amoA),* which is responsible for the transformation of ammonia (NH_3) to hydroxylamine (NH_2OH) and ultimately nitrite (NO_2^-) , the first step in nitrification (Rotthauwe et al., 1997). The *nirK* and *nirS* genes are functionally equivalent but structurally different genes that encode for the enzyme nitrite reductase, responsible for the conversion of nitrite (NO_2^-) to nitric oxide (NO) (Braker et al., 2000). Finally, *nosZ* is responsible for the final step in the denitrification pathway; the conversion of nitrous oxide (N_2O) to inert dinitrogen gas (N_2) (Canfield et al., 2010). qPCR assays were carried out by comparing cycle threshold values to known standards of plasmids containing the target gene using an Eppendorf Mastercycler RealPlex^2 thermocycler. Thermal profiles are outlined in *Table 2.2* and were modelled after Harter et al. (2013), which were modified after Towe et al. (2010) for *amoA*, Oliver et al. (2010) for *nirS* and *nirK,* and Babic et al. (2008) for *nosZ.* Each sample was run in triplicate along with a triplicate no template control for the identification of any possible contamination. qPCR standard curve R^2 values for all genes were 99% or greater. Standard curve efficiencies varied among genes, but were similar to or higher than previous studies. Efficiencies ranged from 92.7 – 97.6% for the *amoA* gene (Bannert et al., 2011; Regan et al., 2011), 90.9 – 106.4% for *nirK* (Henry et al., 2006; Regan et al., 2011), 86.6 – 94.2% for *nirS* (Harter et al., 2014; Towe et al., 2010), and 83.9 – 96.5% for *nosZ* (Harter et al., 2014; Towe et al., 2010).

Target Gene	Primer Sequences and References	Reaction Mixture	Volume (μL)	Thermal Profile
amoA	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC (Rotthauwe et al., 1997)	SsoAdvanced™Universal SYBR® Green Supermix *amoA*1F (10 μM) *nosZ*2R (10 μM) PCR water Template (~3 ng/μL)	12.5 1.25 1.25 5 5	98°C – 3 min 1 cycle 98°C – 60s 60°C – 60s 72°C – 60s 40 cycles
nirK	ATYGGCGGVCAYGGCGA GCCTCGATCAGRTTRTGG Modified after Henry et al. (2004)	SsoAdvanced™Universal SYBR® Green Supermix *nirK*876C (10 μM) *nirK*1040 (10 μM) PCR water Template (~3 ng/μL)	12.5 0.5 0.5 6.5 5	98°C – 15 s 63-58°C – 30 s 72°C – 30 s 80°C – 30 s 6 cycles 98°C – 15 s 58°C – 30 s 72°C – 30 s 80°C – 30 s 40 cycles
nirS	GTNAAYGTNAARGARACNGG GASTTCGGRTGSGTCTTGA (Michotey et al., 2000) (Throback et al., 2004)	SsoAdvanced™Universal SYBR® Green Supermix cd3af (10 μM) R3cd (10 μM) PCR water Template (~3 ng/μL)	12.5 1.25 1.25 5 5	98°C – 3 min 1 cycle 98°C – 60s 56°C – 60s 72°C – 60s 40 cycles
nosZ	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA (Henry et al., 2006)	SsoAdvanced™Universal SYBR® Green Supermix *nosZ*2F (10 μM) *nosZ*2R (10 μM) PCR water Template (~3 ng/μL)	12.5 0.5 0.5 6.5 5	98°C – 30 s 65-60°C – 30 s 72°C – 30 s 6 cycles 98°C – 15 s 60°C – 15 s 72°C – 30 s 40 cycles

Table 2.2. Reaction mixtures, primers, references, volumes, and thermal profiles used for the targeted genes in this study.

2.4 Denitrification Potential

Denitrification potential, used as a direct measurement of denitrification enzyme activity, was measured by the acetylene blockage technique originally described by Smith and Tiedje (1979) and more recently adapted by Carter and Gregorich (2007). Because dinitrogen gas is abundant in the atmosphere and production is difficult to detect, acetylene is used to block the final transformation and force production of nitrous oxide instead. Soil samples were incubated in an anoxic environment after adding glucose, nitrate, and acetylene, and the amount of nitrous oxide was measured regularly over a 5 h period to determine the rate of N_2O production and estimate the rate at which denitrification could potentially occur within a soil sample. Gas samples were stored no longer than two weeks and analyzed on a gas chromatogram. Due to instrument difficulties, some samples were not able to be run quickly enough and had to be discarded. Because of this, denitrification potential data is only presented for 48 of the 86 BRC media samples.

2.5 Microbial Biomass, Nitrogen, & Carbon

BRC soil media total organic carbon (TOC) and N concentrations were a product of the microbial biomass measurements explained in the *Materials and Methods* section and presented in the *Results* sections of *Chapter 3*. TOC, ammonium (NH_4^+-N), and nitrate-nitrite (NO_2^--NO_3^--N) concentrations were determined from the non-fumigated blanks. Each sample was analyzed in duplicate, using 0.5 M potassium sulfate (K_2SO_4) and a four-hour extraction using a side-arm shaker. TOC was analyzed on an OI Model 1010 total organic carbon analyzer using the standard method 5301c (APHA, 2005). Ammonium and nitrate concentrations were determined using a Lachat QuikChem 8500 Flow Injection Analyzer following the QuikChem Method 10-107-04-1-L and APHA Method 4500-NO3- I (APHA, 2005; Lachat Instruments, 2007).

2.6 Temperature and Precipitation Data

Average temperature and precipitation values for each sampling site were based on the averages of temperature and precipitation data from 1981 - 2010 using the United States Department of Agriculture's (USDA) Geospatial Data Gateway (https://gdg.sc.egov.usda.gov/) (NRCS, 2008). These data were integrated into a GIS layer and values for each sampling location were determined by the interpolation of known temperature and precipitation sites. For

this reason, sites in very close proximity were assigned very similar and in some cases, identical climatic values.

2.7 Data Analysis

Given the field-based nature of this study, the sampled BRCs varied greatly in terms of design attributes, making an ANOVA type of analysis impractical (i.e. there were no a priori levels of treatment defined). Instead, a multivariate model selection approach that identified the most influential design characteristics for a particular response variable (i.e. gene type, microbial biomass, etc.) was used. Specifically, Akaike's Information Criterion (AIC) (Akaike, 1998; Burnham & Anderson, 2002) was used to address the complex variability inherent in site variables among these samples. This approach has become increasingly popular in understanding ecological processes and dynamics in datasets in which variables cannot be isolated (Johnson & Omland, 2004; Richards et al., 2011; Symonds & Moussalli, 2011), a common problem with field samples in ecological surveys (Richards, 2005). Akaike's Information Criterion allows for the quantitative ranking of variables or combinations of variables (models) that reduce the amount of information lost when used to predict an individual dependent variable, while at the same time correcting for increasing model complexity (Burnham et al., 2011). Essentially, this multivariate statistical model selection tool determines factors and design parameters, or combinations thereof, that best predict the distribution of a response variable such as denitrification potential or the concentration of a denitrifying gene.

Akaike's Information Criterion first works by gathering data within the ecosystem model on an *a priori* basis to discourage the formulation of models that coincidentally fit a response variable (i.e., data dredging). These data are used to create models, or combinations of data, that are compared against a predictor variable to formulate AIC values. The difference between AIC values (ΔAIC) are calculated to determine models with the most confidence. The formula for AIC is noted below, with k denoting the number of parameters in the model and L representing the Kullback-Leibler divergence value:

Equation 2.1. $$AIC = 2k - 2\ln(L)$$

The Kullback-Leibler (K-L) divergence formula (Equation 2.2 shown below) is a likelihood formula used to calculate the information lost when the entire dataset (*f(x)*) is compared to a modeled, or candidate data set (*g(x)*):

Equation 2.2. $$D_{KL} = \int f(x) \log\left(\frac{f(x)}{g(x)}\right) dx$$

By integrating the log ratios of a candidate models to the entire dataset, the Kullback-Leibler formula calculates a "distance" metric. Models with minimal distances, or those with the least amount of information lost, are considered to be better predictors of the variables that drive change within a modeled system. AIC also includes a penalty for increasing the number of predictive variables within a model with the incorporation of the 2*k* value in the AIC formula. This multiplicative term increases the value by which the Kullback-Leibler distance is subtracted from and models with a greater number of variables receive higher AIC values. Because "top" models are those with the lowest ΔAIC value, models with high numbers of variables would be ranked lower compared to models with a similar Kullback-Leibler distance but fewer variables.

For the use of AIC in this project we used a variation of the AIC formula, AIC_c, a second order formula recommended for use when the number of parameters is not large in comparison to the number of samples, specifically when sample number divided by the number of parameters is less than 40 (Burnham & Anderson, 2002) (the ratio for this data set was 5.7). The formula, included below, corrects for smaller samples sizes, with *k* representing the number of parameters and *n* denoting the sample size.

Equation 2.3. $$\mathrm{AIC_C} = \mathrm{AIC} + \frac{2k(k+1)}{n-k-1}$$

ΔAIC_c values can then be used to calculate likelihood values and further transformed into what are called "Akaike weights." These weighted values give insight into the relative importance of that model in relation to the other candidate models and were used to average the top models for a specific predictor variable. Model averaging produces "relative variable importance" (RVI) values, which (on a scale 0 – 1) represent their importance in relation to the specific predictor variable. Values closer to one are considered most important, while values closer to 0 are comparatively less important.

Burnham and Anderson (2002) suggest that models with $\Delta AIC_c < 2$ are highly indicative of a primary model, values from 4-7 are less important, and values > 10 are least likely to be a top model. Using these guidelines, all models with < 10 ΔAIC_c were averaged to identify the most influential predictor variables for each response variable. Variables that were purely environmental and those that could be manipulated through design were initially analyzed separately to reduce complexity. After identifying the top models for each category (environmental and design), the variables in these categories were combined and analyzed together. The most important variables were determined by those with the greatest RVI value and variables that had an RVI of ≥ 80% of the top RVI value. The intent of this approach was to eliminate elements which indicated a marginal effect on the predictor variable and ultimately determine if the most influential variables were environmental in nature or could be manipulated by design. Due to the environmental conditions unique to the top and bottom layers of a BRC, this analysis was conducted separately on samples originating from the top 10 cm of the cell and on samples originating only from the bottom 10 cm of the cell; and all samples were analyzed together. Predictor variables were transformed using a log or square root transformation to reduce lop-sided and long-tailed distributions in the dataset. This both provided a better representation of the gradients within our data and allowed for the identification of fewer top models with higher confidences.

Due to the potential of covariance among response variables that could mask model selection differences, variance inflation factors (VIFs) were calculated to identify collinearity issues. The variance inflation factor is a metric calculated to determine mulitcollinearity between continuous variables in a least squares regression analysis. If the calculated VIF is greater than 10, the two variables are considered to be severely mutlicollinear. The highest VIFs were produced for temperature & precipitation, ammonium & nitrite-nitrate, ammonium & TOC, and nitrite-nitrate & TOC. The respective VIF values for these combinations of variables were calculated to be 2.5, 1.9, 1.9, and 2.4, concluding these variables were not highly collinear. Due to the similarities between variables and, in an attempt to decrease model complexity, temperature and precipitation and ammonium and nitrite-nitrate were combined separately, and values were replaced by their principle component values from a principle component analysis (PCA). To briefly summarize PCA, the approach uses an eigenvector, or essentially a straight line through the data points that explains the most amount of variance through those points to

create eigenvalues that can be plotted. The associated PCA scores can then be used to represent the original dataset and reduce dimensionality. The principle component analysis explained 89% of the variance between temperature and precipitation and 83% of the variation between ammonium and nitrite-nitrate.

3. Results and Discussion

3.1 Environmental and Design Trends

Although differences among the nitrifying gene and denitrifying response variables existed, there were some clear and consistent trends within the data. The data presented in the figures for BRC variables were used for model selection.

3.1.1 BRC Age

The sampled BRCs ranged in age from just over 1 year to 22 years in operation, with a mean age of 9 years, and a median age of 11 years. Due to difficulties in obtaining timely gas sample analysis, only 48 of the 86 denitrification gas samples were analyzed, resulting in the decreased number of sampling points for denitrification potential. Linear regressions for the nitrifying gene ammonium monoxygenase (*amoA*), denitrifying genes (*nirk, nirS,* and *nosZ*), and denitrifying potential produced coefficients of determination (R^2) ranging from 0.01 to 0.09 and analysis of variance (ANOVA) p-values ranging from 0.147 to 0.004 (Figure 2.3). Although the linear regression parameters do not demonstrate strong relationships, denitrification tended to increase with BRC age. These trends could be attributed to the growth of bacterial populations over time or clogging of the BRC media mix. Media mixes with high organic matter contents, silts, and clays tend to clog as the cell accumulates suspended solids from stormwater runoff (Gulliver et al., 2008), which increases periods of time that the media remain saturated, and potentially increases anaerobic periods that promote denitrification.

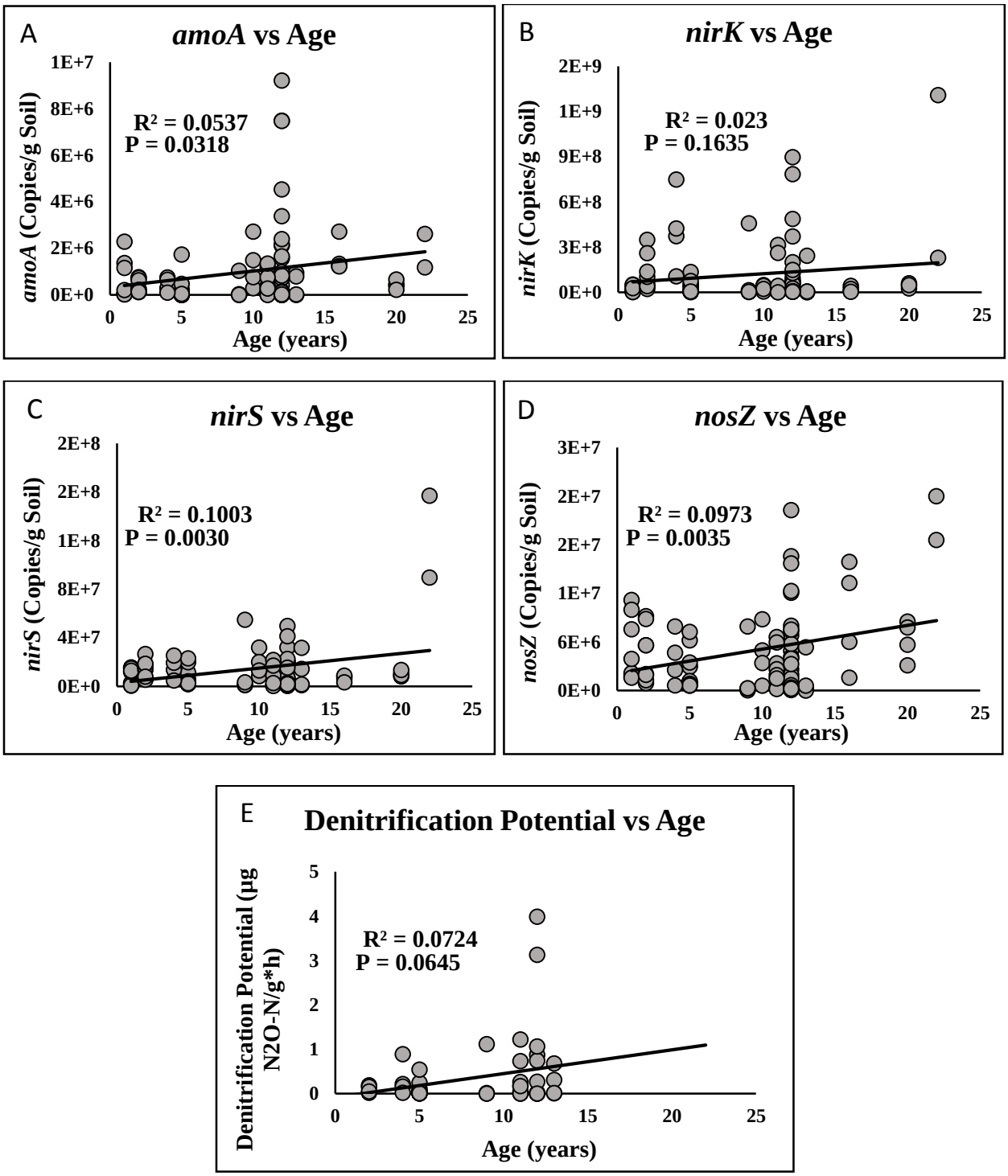

Figure 2.3. Linear regressions of concentrations of nitrogen cycling genes in the soil medium for all 4 sampling locations from each of the 23 BRCs sampled in this study (n = 86): *amoA* (A), *nirK* (B), *nirS* (C), and *nosZ* (D). Denitrification potential (E) is plotted from all locations (n = 48) from the 13 available BRCs.

3.1.2 BRC Heterogeneity

The number of top and bottom samples from all BRCs combined were nearly equally distributed, with 44 of the 86 samples originating from the top 10 cm and 42 samples originating from the bottom 10 cm samples. Bar plots represent the mean value for each response variable within each BRC location (Figure 2.4). The copies of nitrifying and denitrifying genes and denitrification potential were different between soil depth ($p = < 0.0001$). The strongest effect of depth occurred in denitrification potential, which was near zero in the bottom 10 cm. The *nirK* gene was nearly an order of magnitude higher in the top than bottom 10 cm depth. Although the lower BRC layers stay saturated at least as long as the upper layers of the cell, denitrifying gene copies and potential denitrification rates were consistently lower in the bottom 10 cm samples, in agreement with the findings of both Willard et al. (2014) and Chen at al. (2013). Higher denitrification gene copies and denitrification potential in the top layers of the BRCs, where aerobic conditions are expected to persist, support the contention of Hsieh et al. (2007) that substantial microsite denitrification may contribute a significant proportion of N removal in BRCs.

Our results showing that the N cycling genes and denitrification potential are higher in bottom 10 cm of conventionally drained BRCs compared to those with an ISZ cast doubt on the validity of designing BRCs with internally saturated zones to promote denitrification in the lower layers. Although the *nirK* gene was the only denitrification gene for which the difference was statistically significant, the mean values for *nirS*, *nosZ*, and denitrification potential were consistently higher in conventionally drained BRCs (Figure 2.5). These findings are contrary to the design goals of some states, which encourage the inclusion of an ISZ (NCDENR, 2007). It should be noted that only one of the 5 BRCs with an ISZ documented the addition of a carbon source to the saturated zone. The lack of organic carbon and anaerobic conditions may have suppressed both nitrifier and denitrifier populations, by restricting denitrifier growth and limiting the conversion of N species in the lower layers of the BRC. Further research on denitrifier populations in BRCs with both a carbon amendment and an ISZ could be helpful in determining if this design alteration is beneficial to the microbial community and N removal.

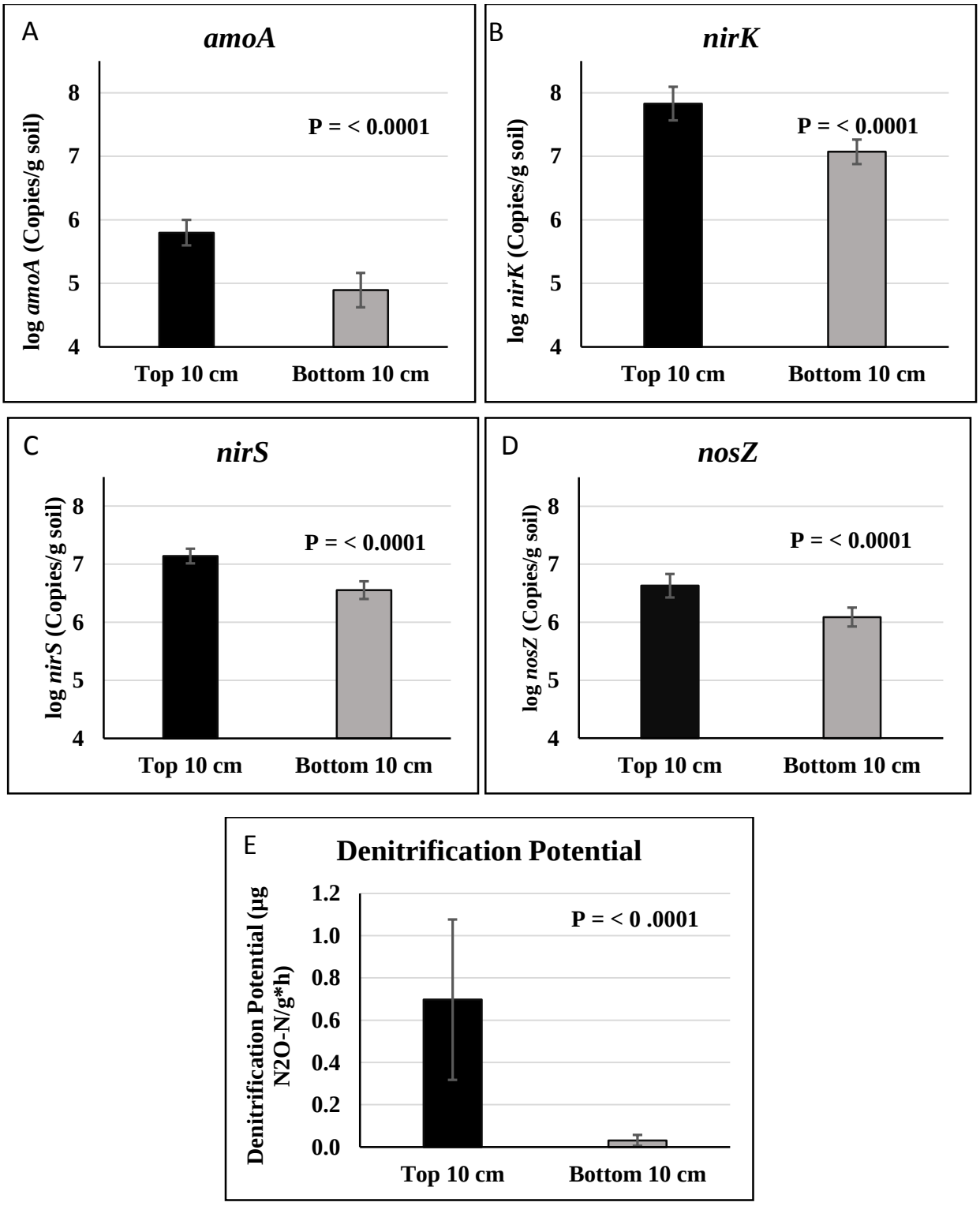

Figure 2.4: Mean values for the nitrifying and denitrifying genes for all 23 BRCs in the top 10 cm (n = 44) and bottom 10 cm (n = 42) samples of the soil media *amoA* (A), *nirK* (B), *nirS* (C), *nosZ* (D). Mean denitrification potential (E) is plotted from a total of 13 BRCs in the top (n=24) and bottom 10 cm (n=24) samples. Error bars represent the 95% confidence interval of the mean.

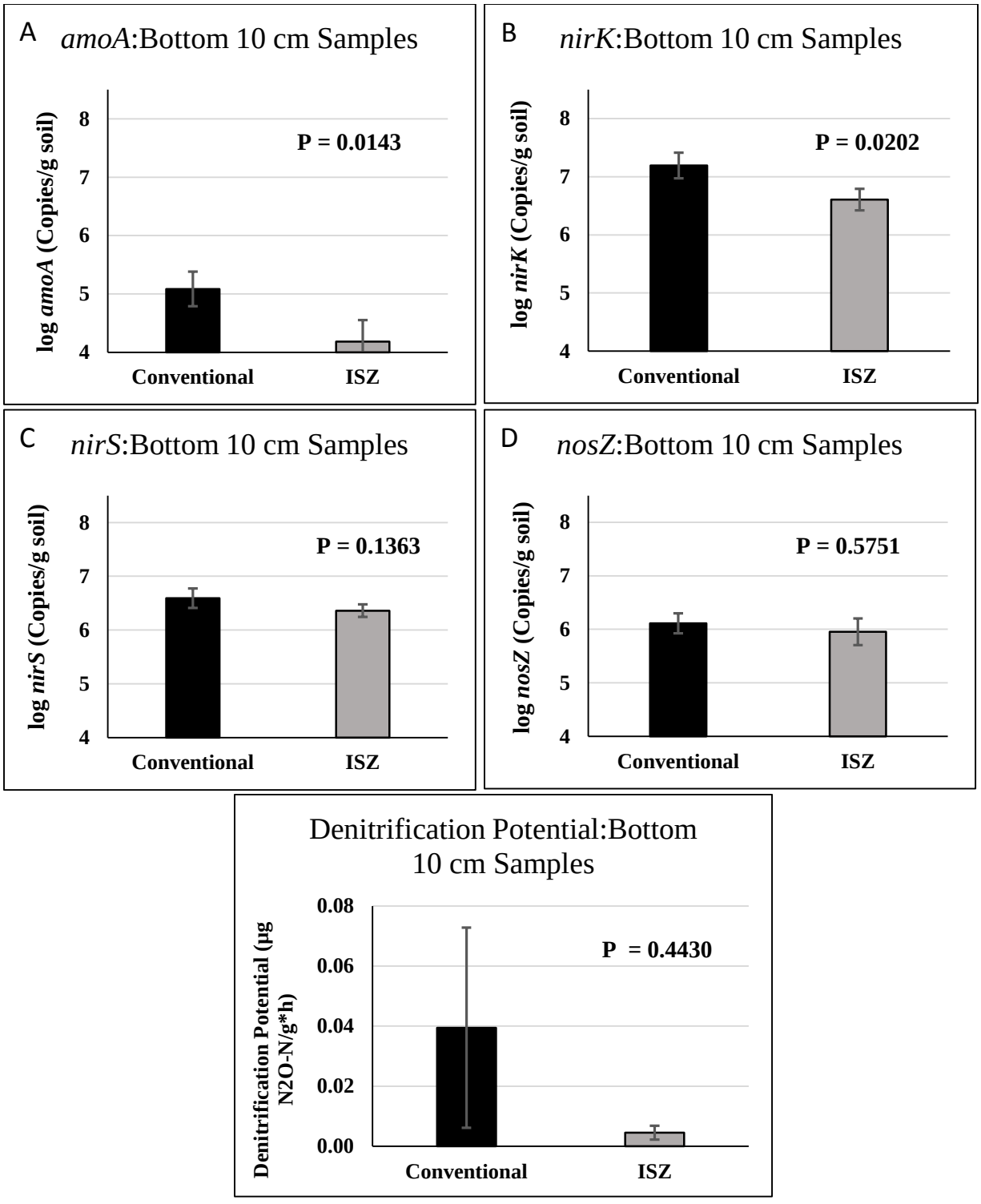

Figure 2.5: Mean values for *amoA* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) gene abundances for the 18 conventionally drained BRCs in the bottom 10 cm samples (n=34) and the 5 BRCs with an ISZ in the bottom 10 cm (n=8) samples in the soil media. Mean denitrification potential (E) is plotted for the bottom cm samples of 4 BRCs with an ISZ (n=18) and 9 conventionally drained BRCs (n=6). Error bars represent the 95% confidence interval of the mean.

3.1.3 Media Composition

Due to the large variations among the compositions of the sampled BRCs, media mix was broadly classified into two categories: media mixes comprised of ≤ 50% sand and those with ≥ 80% sand. The BRCs with ≤ 50% sand comprised approximately 40% (8 of 19) of the sampled BRCs with known media mix composition, and BRCs with ≥ 80% sand made up the remaining 11 BRCs with known media mix composition. Given that denitrification potential values are only available for NC and VA cells, only 1 known BRC in this data set had a media mix composition ≤ 50% sand; however, all data are presented. Higher denitrification values were obtained for media mixes with a lower sand content, with *amoA* and *nosZ* having significant differences in gene copy numbers between media mix categories (Figure 2.6). Denitrification potential was marginally significant ($p = 0.06$), but this interpretation should be approached with caution due to the low sample size.

Media composition can have a pronounced effect on urban pollutant capture due to the physical, chemical, and biological transformations associated with the constituents of a particular media mix composition. While it is assumed that higher organic matter contents can be beneficial to ammonia oxidizers and denitrifier populations as a source of mineralizable N, terminal electron acceptors, and energy substrates, excessive organic matter also increases N and carbon export. Media containing greater organic matter, silt, and clay contents may clog and cause water to pond as the cell increases in age (Gulliver et al., 2008; Li & Davis, 2008; Mikkelsen et al., 1997). And while ponding may be desired for larger storm events as a means of reducing peak flows, storing runoff in the media layers is preferred, as this contact time allows for nutrient removal mechanisms within the media to act on the stormwater (Hsieh & Davis, 2005). A media mix that balances the carbon needs of the microbial community while facilitating infiltration should be a key consideration in BRC design that achieves both flow reduction and N removal goals as BRCs age.

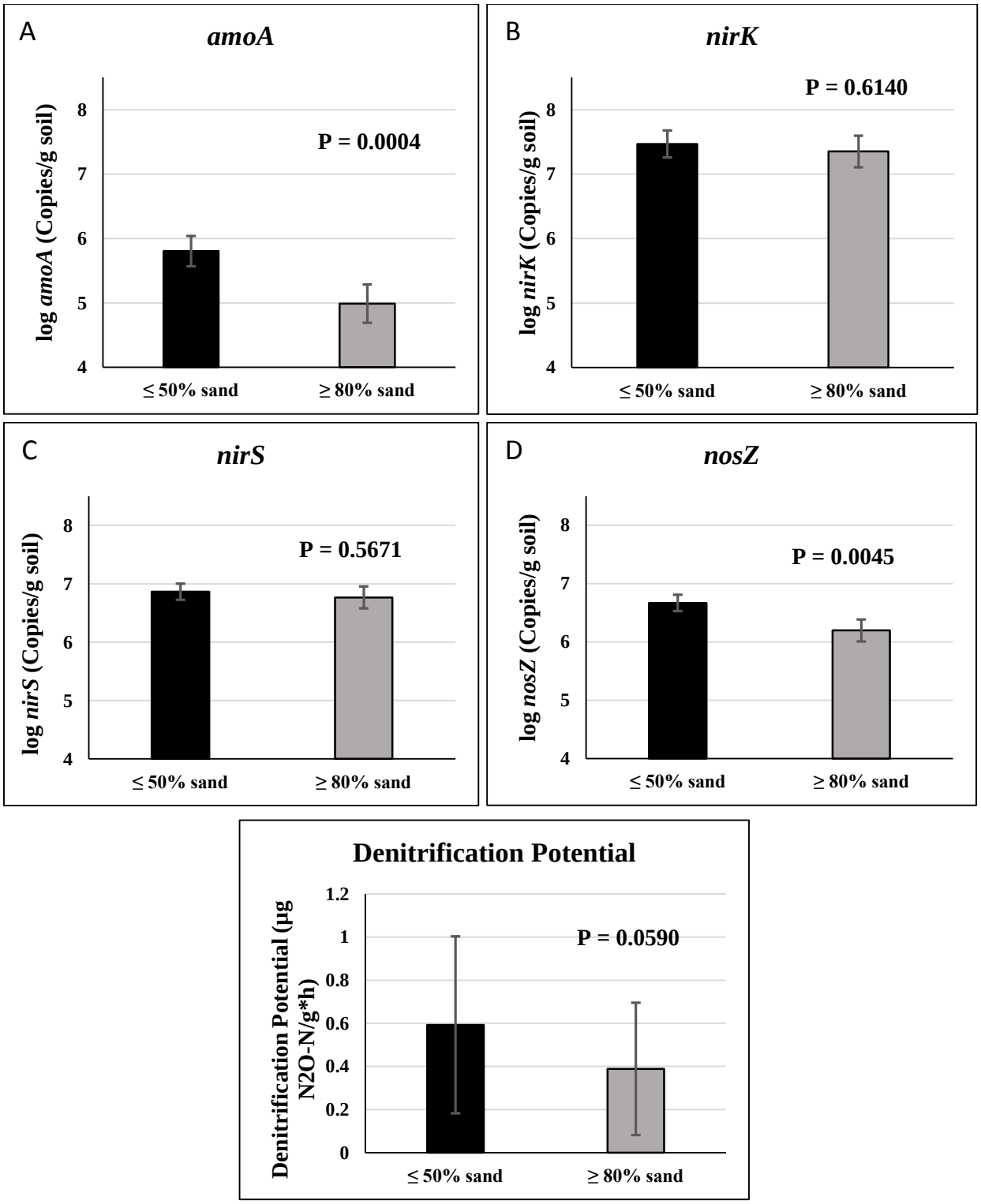

Figure 2.6. Mean values for *amoA* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) in all sampling locations of the 19 BRCs with known media mixes categorized by ≤ 50% sand (n=32) and ≥ 80% sand (n=40). Denitrification potential vs BRC media mix composition (E) of ≤ 50% sand (n=4) and ≥ 80% sand (n=32) for all samples within BRCs with known media mix compositions (n = 10). Error bars represent the 95% confidence interval of the mean.

3.1.4 Vegetation Type

Vegetation type was broadly classified into three categories: grassed, landscaped, and overgrown. The "grassed" category included 4 of the sampled BRCs and was characterized by cells that were sodded, did not contain a mulch layer, and did not contain any type of herbaceous or woody cover. "Landscaped" BRCs included 11 of the sampled cells and were categorized by cells that appeared to have a pre-defined planting scheme, were typically mulched, and appeared to be maintained so that native species did not overtake the planted vegetation. "Overgrown" BRCs included 7 of the sampled cells which did not appear to be maintained, and contained crowded and dense native vegetation that grew among the previously planted species or completely overtook the cell. Missing denitrification potential data resulted in differences in sample numbers for vegetation categories: 3 BRCs were overgrown, 3 "grassed," and 7 "landscaped."

The copy numbers for the nitrifying gene *amoA*, the denitrification genes, and denitrification potential were consistently lowest in grassed BRCs (Figure 2.7). Differences among vegetation types occurred for all quantified genes but not for denitrification potential. Grassed cells made up only 14 of the 86 samples, whereas overgrown BRCs made up 24 of the total samples and landscaped cells comprised more than half (48 of the 86) of the BRCs sampled. Differences in sampling numbers certainly decrease the statistical confidence, although the plotted error bars represent the 95% confidence intervals of the means and take the reduced sample sizes into consideration.

The greatest differences in gene copy number and denitrification potential occurred between grassed BRCs and the landscaped and overgrown BRCs. We hypothesize that these differences were due to the dense rooting systems in the grassed BRCs that likely outcompeted microbes for available N. It is also possible these differences exacerbated differential depth effects, as rooting density is greatest in the top 10 cm samples of grassed cells. In planted or overgrown BRCs, rooting structures for weeds, shrubs, and woody plants probably extend into the intermediate or deeper layers and thus differences in the abundance of nitrifiers and denitrifiers may be different if integrated over the entire depth of the cell. Competition for nutrients between plants and microbes in natural systems is a widely recognized phenomenon (Kuzyakov & Xu, 2013; Templer et al., 2003; Zak et al., 1990) and Payne et al. (2014), estimated

that plant N uptake in bioretention mesocosms accounted for the fate of > 97% of added nitrate. Although these findings are the product of only one study, we hypothesize that similar effects occur in field scale BRCs with dense vegetation.

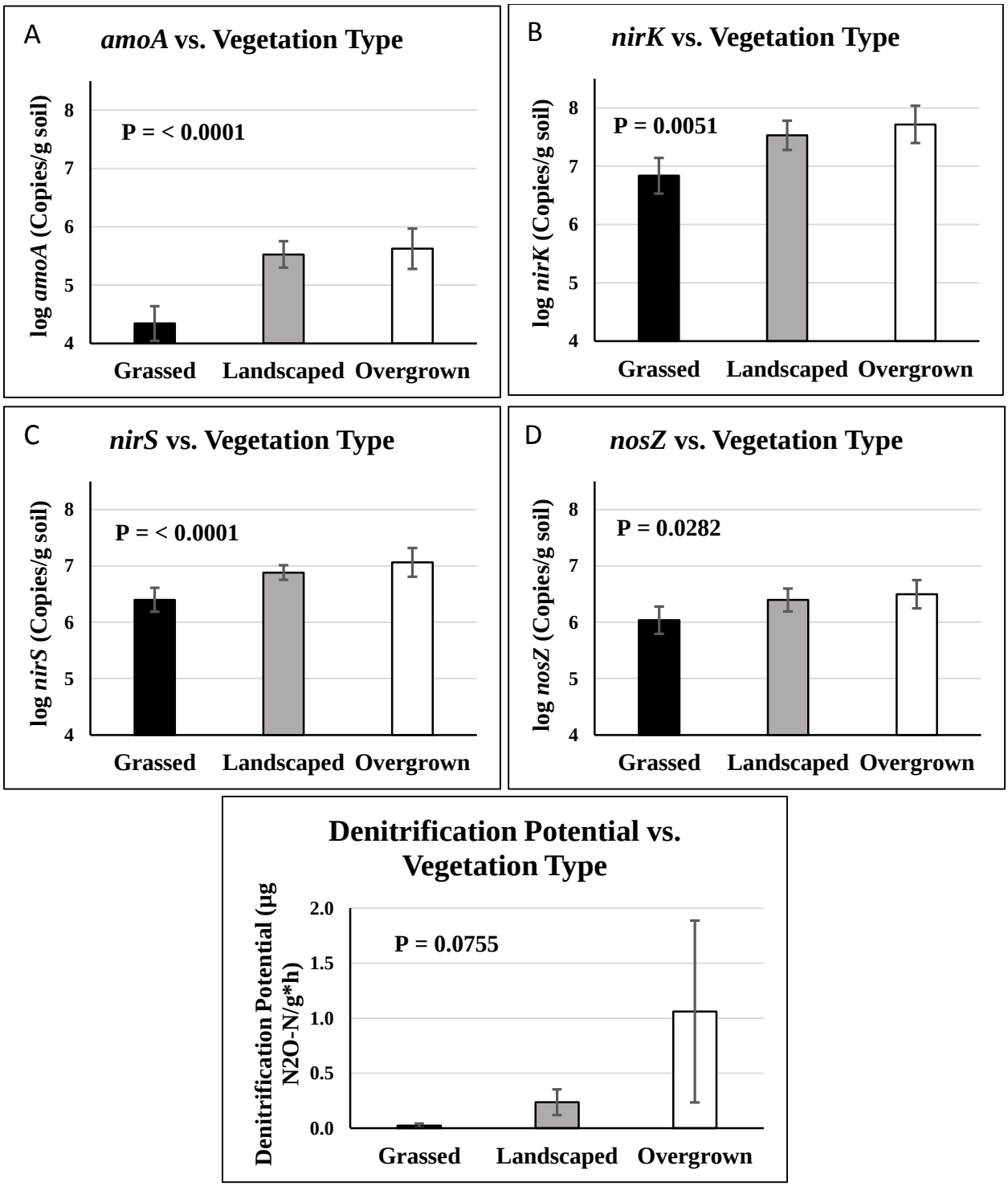

Figure 2.7. Mean values for *amoA* (A), *nirK* (B), *nirS* (C), *nosZ* (D), and denitrification potential for all sampling locations in BRCs (n=86) with grassed (n=15), landscaped (n=48), and overgrown (n=24) vegetation schemes. Mean denitrification vs vegetation is plotted for 13 of the BRCs, comprising of 10 grassed, 28 landscaped, and 10 overgrown cells. Error bars represent the 95% confidence interval of the mean.

3.1.5 Total Organic Carbon

Total organic carbon (TOC) concentrations ranged from below detection to 1.02 mg/g dry soil with a mean value of 0.160 mg/g dry soil and a median of 0.114 mg/g dry soil. Missing values for denitrification potential resulted in only 48 of the 86 samples plotted. Linear trends for all of the response variables with the exception of *amoA* and *nirS* show a strong and statistically significant relationship with total organic carbon (Figure 2.8). These findings are different of Chen et al. (2013), who found significant ANOVA p-values for *amoA* and *nirS* with organic matter but did not show significant values for *nirK* and *nosZ*. Our results are in agreement with results in natural environments that found correlations between *nirK* and *nosZ* with TOC (Barta et al., 2010; Kandeler et al., 2006). Willard et al. (2014) also found significant correlations between *nirK* and *nosZ* and organic carbon in the sampled bioretention cell. The insignificant linear relationship between the *amoA* gene and TOC might be expected since nitrifiers are primarily chemotrophic, and use inorganic substances rather than carbon as an energy source. To our knowledge, no potential denitrification measurements have been conducted on functioning bioretention cells, but our results are in agreement with both lab and field scale measurements which indicate increasing potential denitrification rates with increasing organic carbon concentrations (Bijaysingh et al., 1988; Pfenning & McMahon, 1997; Philippot et al., 2009).

The relationship between TOC and denitrifiers is biologically sensible considering that most denitrifiers are heterotrophs and utilize organic carbon as an energy source. However, it is interesting to note the differences between the *nirS* gene and its structurally different but functionally equivalent counterpart, *nirK.* These two genes are differentiated by the cytochrome cd_1 enzyme present in the *nirS* gene and the copper-containing enzyme in the *nirK* gene (Zumft, 1997). Factors suggested to explain niche preferences between the two genes include salinity (Jones & Hallin, 2010), nutrient availabilities (Yi et al., 2015), soil structure (Enwall et al., 2010), and available carbon sources (Hallin et al., 2006). However, the two genes can be more broadly classified as terrestrial (*nirK)* and aquatic (*nirS*) since the *nirS* gene has shown to favor lower dissolved oxygen concentrations and lower redox potentials (Graham et al., 2010; Knapp et al., 2009; Tatariw et al., 2013) .*nirK/nirS* gene ratios are generally > 1 in soil systems (Barta et al., 2010; Enwall et al., 2010; Jones et al., 2014) and < 1 in saturated systems such as wetlands and estuaries (Beman, 2014; Ligi et al., 2014; Lindemann et al., 2016). Regardless of niche and

saturation preferences, both genes are native to heterotrophic bacteria and thus it would be expected that both genes would correlate significantly correlate with TOC.

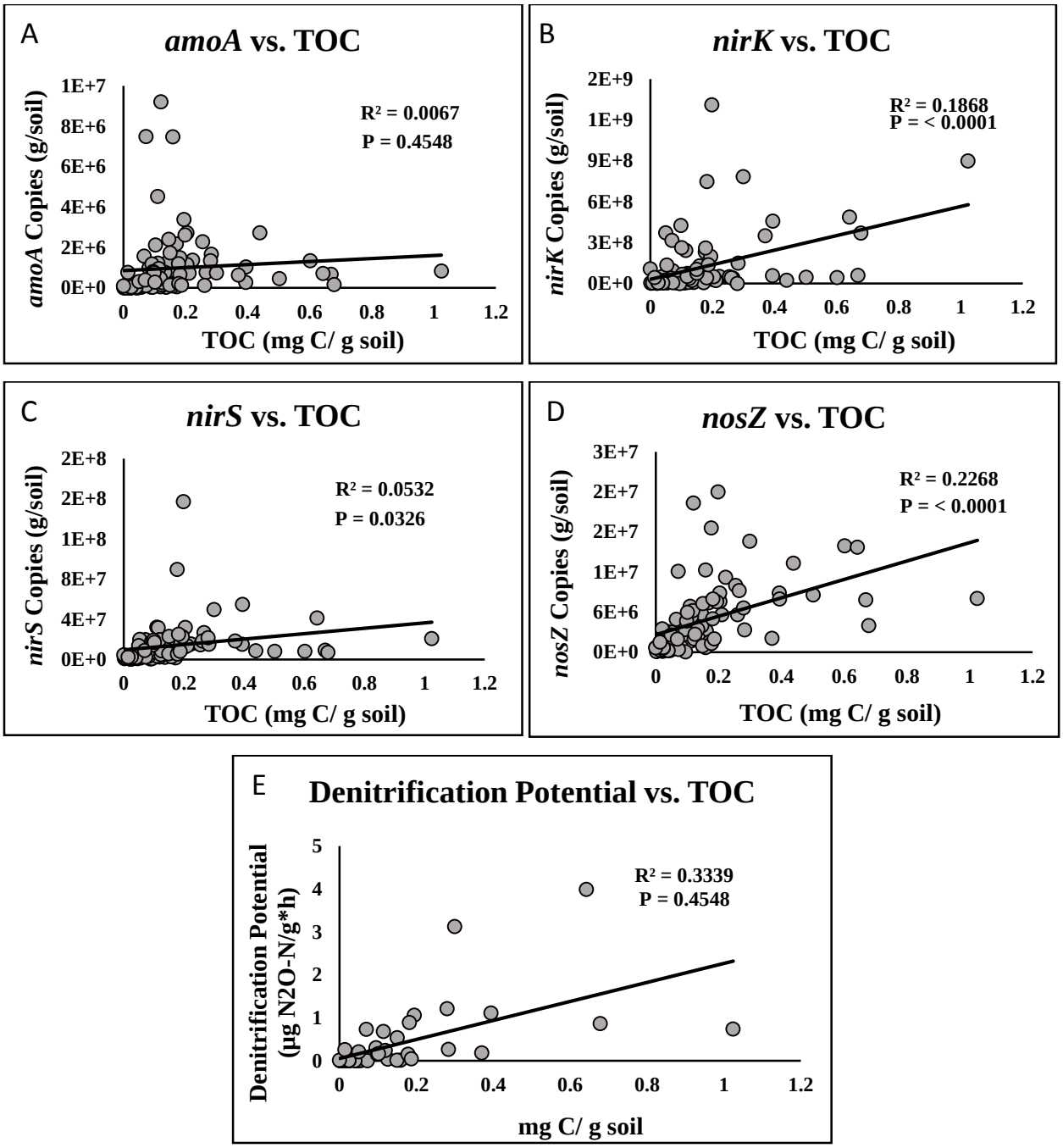

Figure 2.8. Linear regressions for soil media total organic carbon (TOC) vs. gene concentrations of *amoA* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) for all locations in the 23 BRCs sampled (n=86). Denitrification potential vs TOC (E) in 13 of the sampled BRCs in all sampling locations (n=48).

3.1.6 Ammonium

Extractable ammonium concentrations ranged from below detection to 136 μg/g dry soil with a mean of 12.9 μg/g dry soil and a median value of 6.95 μg/g dry soil. Missing data points for denitrification potential measurements resulted in only 48 of the 86 sample points plotted. Statistically significant linear relationships were observed for all response variables except *amoA* (Figure 2.9). Denitrification has been shown to increase with ammonium concentrations (Avrahami et al., 2002; Philippot et al., 2009), which makes sense considering ammonium is nitrified to nitrite and nitrate that can then be used by the denitrifying community. Although Willard et al. (2014) quantified total nitrogen (TN) rather than individual N species, significant correlations were found between *nirK* and *nosZ* and TN, suggesting agreement with our results.

The *amoA* gene, which encodes for the enzyme that converts ammonia to hydroxylamine was not significantly correlated with ammonium, which is contrary to what was expected. Furthermore, although the *amoA* populations are often interchangeably referred to as ammonium and/or ammonia oxidizing bacteria, it is generally understood that ammonia (NH_3) rather than ammonium (NH_4^+) is utilized by these bacteria, so the proportions of the NH_x constituents in the soil may affect the growth of *amoA* populations (Kowalchuk & Stephen, 2001; Suzuki et al., 1974; Wood, 1986). A study by Verhamme et al. (2011) examined ammonium oxidizing bacteria (AOB) and ammonium oxidizing archaea (AOA) in sandy loam soil microcosms with no amendment (<0.5 μg NH_4^+ -N/ g soil), an intermediate amendment (20 μg NH_4^+ -N/ g soil), and a high amendment (200 μg NH_4^+ -N/ g soil). The study found that AOB gene copies grew only in the high amendment, while AOA grew at all concentrations. Considering our samples had a mean ammonium content of 13.6 μg NH_4^+ -N/g, it is possible that our observed *amoA* correlations are weak due to ammonium concentrations that allowed AOB populations to persist but did not cause significant gradients in AOB populations vs. ammonium to be ovserved. Further, multiple studies have shown that AOA dominate ammonium oxidizer populations under low ammonium concentrations (Hofferle et al., 2010; Offre et al., 2009). These findings indicate that AOA may potentially contribute to a significant proportion of ammonium transformations in BRCs and could be an important variable to focus on in future studies.

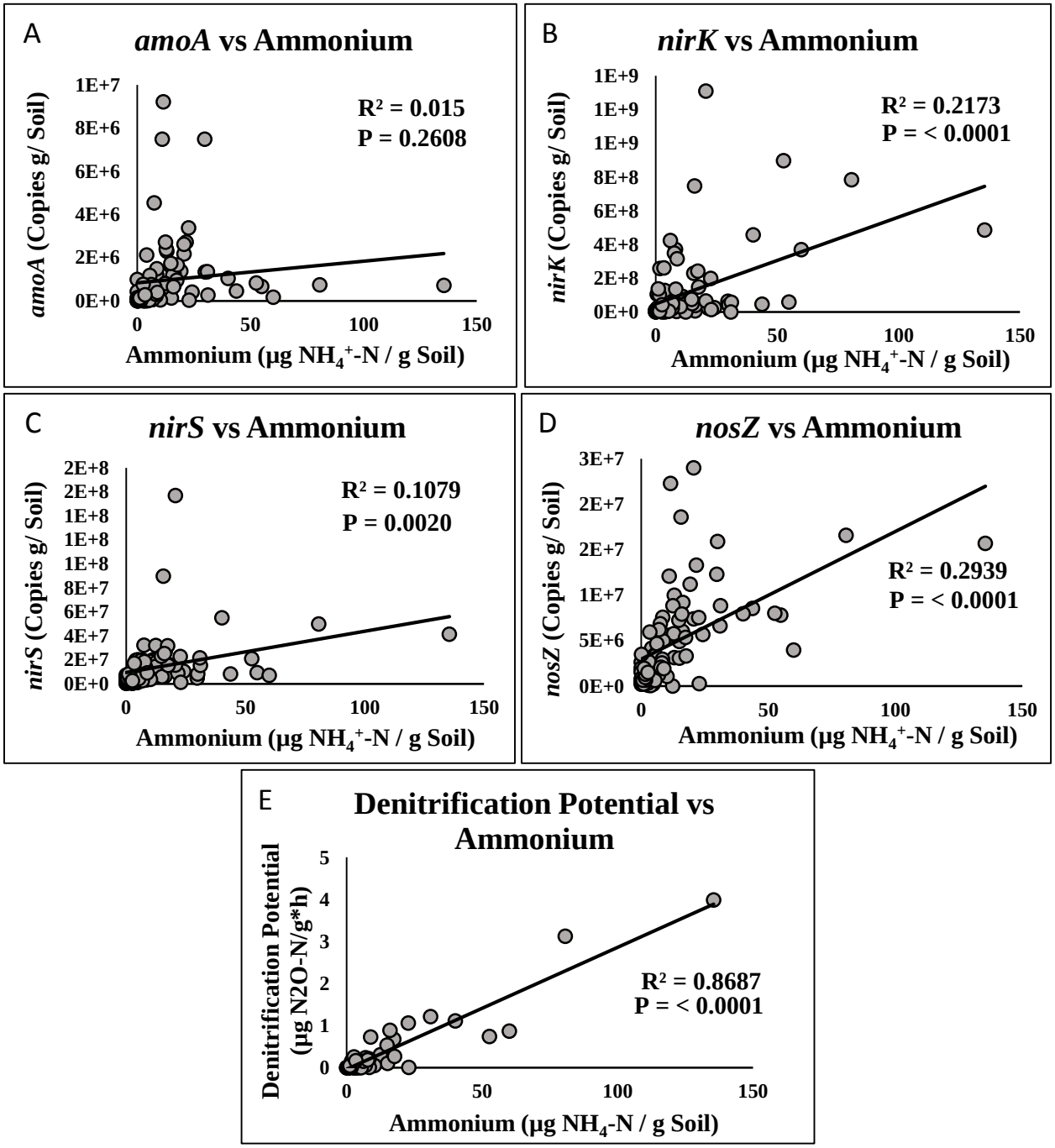

Figure 2.9. Linear regressions for *amoA* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) vs extractable ammonium (NH_4-N) in the soil medium for all BRCs (n=23) in all sampling locations (n=86). Denitrification potential is plotted for 13 BRCs in all sampling locations (n=48)

3.1.7 Nitrite – Nitrate

Extractable nitrite – nitrate (NO_2^--NO_3^-) concentrations ranged from below detection to 38.2 μg/g dry soil with a mean of 6.27 μg/g dry soil and a median of 1.18 μg/g dry soil. The R^2 values ranged from 0.11 to 0.48 with significant relationships for all response variables (Figure 2.10). These findings agree with previous observations that more nitrate can increase denitrification rates in soils (Bowman & Focht, 1974; Firestone et al., 1980). Nitrification ultimately leads to NO_2^- and NO_3^- production, and bacterial denitrifiers rely upon these as a terminal electron acceptor in the first steps of denitrification. These results agree with the study conducted by Chen et al. (2013), who showed significant relationships between *nirS* and *amoA* and NO_3-N. Bacteria with the *nir* genes directly use NO_2 as a terminal electron acceptor and thus we expected these genes to respond strongest to nitrite-nitrate concentrations. However, the strongest relationship observed was for the *nosZ* gene and denitrification potential, which are further down the denitrification pathway and rely on nitrous oxide (N_2O) and nitric oxide (NO) as terminal electron acceptors. Nonetheless, these results highlight the importance of nitrite-nitrate concentrations as a primary control for denitrification in BRCs.

The presence of *nosZ* populations that correlate with nitrite-nitrate is important environmentally. This relationship indicates that the products produced during nitrification are being completely converted to N_2. This is particularly important considering that N_2O, which is a denitrification intermediate, has ~300 times the global warming potential of carbon dioxide (CO_2). Despite the linear relationship with nitrite-nitrate, there are still fewer *nosZ* genes compared to the *nirS* and *nirK* genes (Figure 2.10 B, C, and D), meaning that nitrous oxide is potentially still being emitted from most BRCs. Higher abundances of nitrite reducing genes compared to the nitrous oxide reductase (*nosZ)* genes have been found in many soils, indicating that this phenomenon is not restricted to BRCs (Henry et al., 2006; Liu et al., 2013; Morales et al., 2010). One recent explanation for lower *nosZ* abundances hypothesizes that there are simply fewer denitrifiers that have the ability to synthesize the nitrous oxide reductase gene (Philippot et al., 2011; Regan et al., 2011). Future studies aimed at increasing *nosZ* genes in BRCs could be important in ensuring that a water quality problem is not inadvertently converted into a climate change problem.

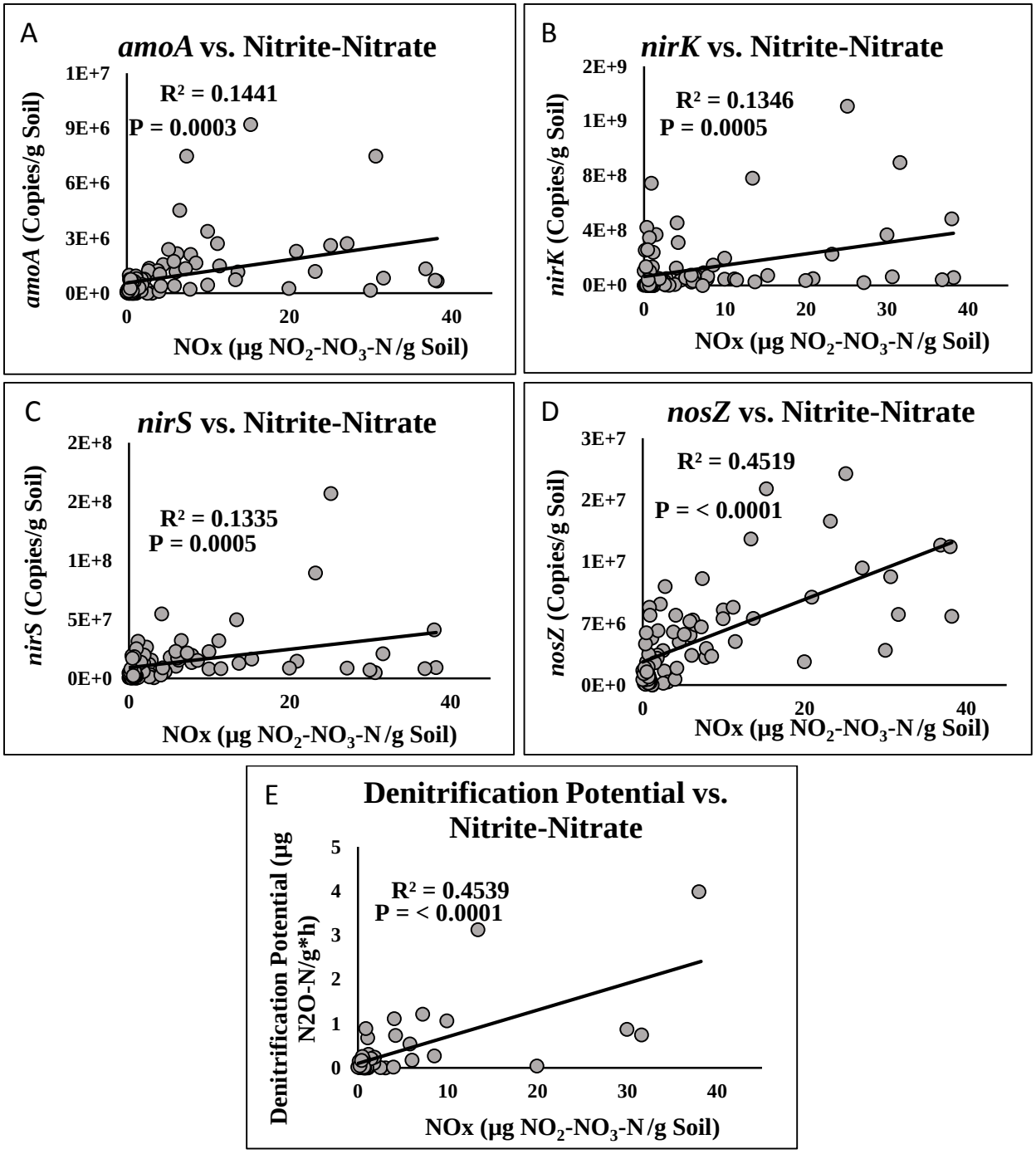

Figure 2.10. Linear regressions for *amoA* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) vs extractable nitrite-nitrate (NO_2-NO_3-N) in the soil medium for all BRCs (n=23) in all sampling locations (n=86). Denitrification potential is plotted for 13 BRCs in all sampling locations (n=48)

3.1.8 Temperature and Precipitation

Mean temperature values for the BRC locations ranged from 12.8 – 16.5 °C with a mean value of 14.2 °C and a median of 14.2 °C. Precipitation values ranged from 1041 – 1244 mm/year with a mean value of 1117 mm/year and a median of 1117 mm/year. For the nitrifying and denitrifying response variables, R^2 values for temperature ranged from <0.0001 to 0.762, with *amoA, nosZ,* and denitrification producing p-values < 0.05, indicating a significant linear correlation (Figure 2.11). R^2 values for precipitation ranged from 0.003 – 0.135, with *amoA* and *nosZ* p-values < 0.05, indicating significant linear correlations. The relationship between the response variables and mean annual temperature and precipitation values indicate a weak inverse relationship for *amoA, nirS,* and *nosZ,* while *nirK* shows no relationship and denitrification potential is weakly positively correlated (Figure 2.11).

Because our sampling range was limited to a relatively small geographic region of the eastern Mid-Atlantic, the limited gradients in temperature and precipitation may explain the conflicting relationships between temperature and precipitation values across the response variables. Additionally, there is a slight confounding effect between temperature, precipitation, and media composition. Nearly all of the sampled BRCs in NC, where average temperature and precipitation is highest in our dataset, were comprised of ≥ 80% sand. While it may appear that there is a significant negative relationship between temperature and precipitation and the response variables, this was probably due to lower gene abundances in the higher sand media mixes, particularly in *amoA* and *nosZ,* where the difference in gene abundances across media mixes is greatest (Figure 2.6 A and E). Both nitrification (Breuer et al., 2002; Myers, 1975; Shammas, 1986; Szukics et al., 2010) and denitrification (Bailey & Beauchamp, 1973; Bremner & Shaw, 1958; Sirivedhin & Gray, 2006) rates increase with temperature until a critically high threshold temperature is met. Because the denitrification measurements were only available for ≥ 80% sand media, the confounding effect was removed, and there is a slight increase with temperature and precipitation that might be expected over a small climatic range (Figure 2.11 E). This would agree with previous research of natural systems which indicate increases in denitrification rates with increases in temperature and anaerobic conditions (Davidson & Swank, 1986; Firestone et al., 1980; Pfenning & McMahon, 1997).

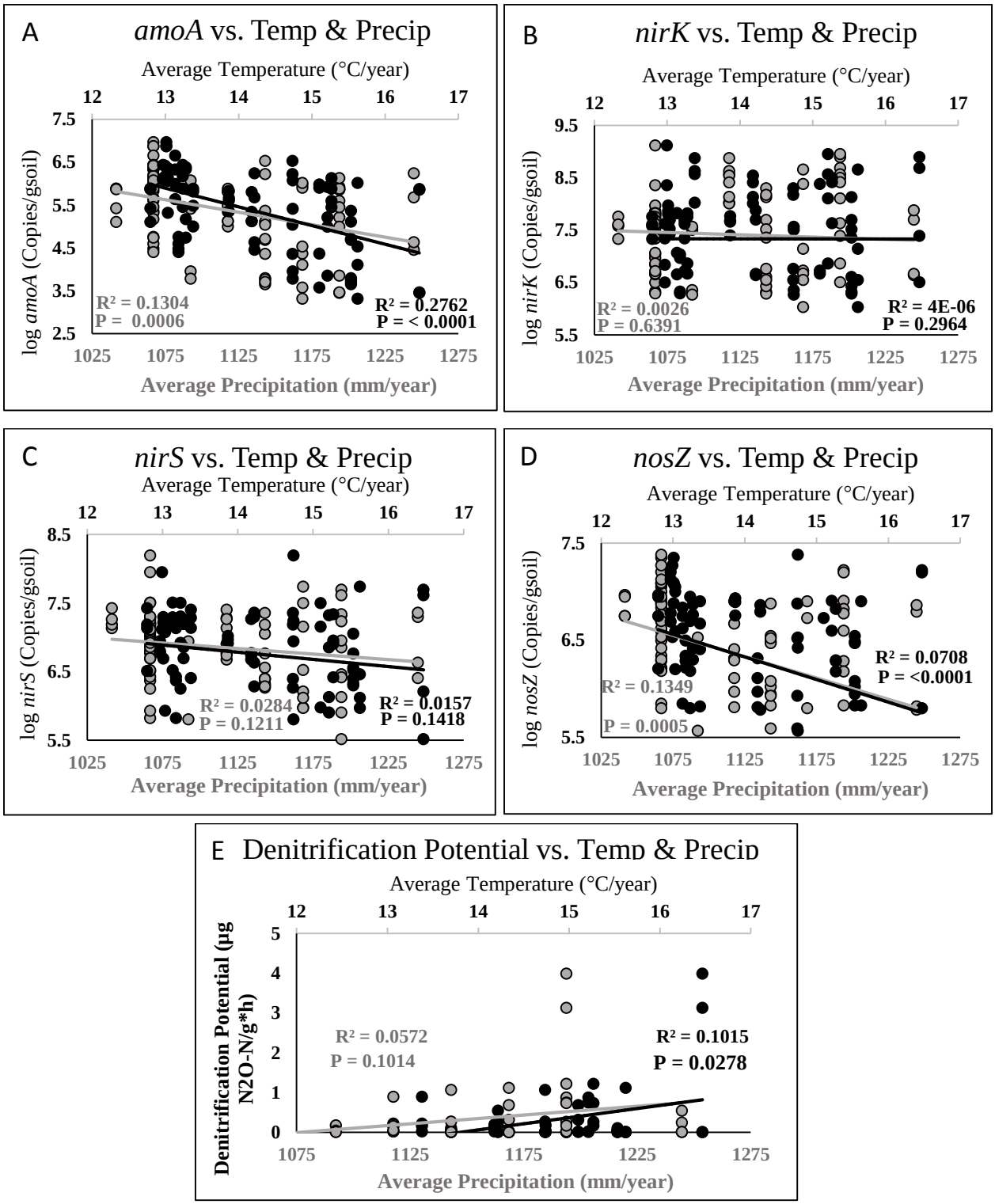

Figure 2.11. Linear regressions for *amoa* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) vs mean temperature and precipitation values for all BRC samples (86). Denitrification potential is plotted for 13 BRCs, totaling 48 samples. Colors of statistical values and axes correspond to the data points. Gray represents temperature while black represents precipitation.

3.2 Model Selection

Due to the large number of variables analyzed in this study and the many correlations between potential predictors and N cycling response variables, a method to determine the most important predictors of individual response variables was necessary. Akaike's Information Criterion, a model selection approach, was used to identify the combination of design and/or environmental response variables most important in predicting the nitrifying and denitrifying response variables. The following tables present the averaged top models that include combinations of response variables that had a ΔAIC_c of < 10, which was proposed by Burnham and Anderson (2002) to identify the most plausible models for a particular response variable. The reported values represent "relative variable importance" and are calculated by summing the AIC weights across the top models. These values are on a scale of 0 to 1 with values closer to one deemed more plausible among competing predictor variables in relation to the response variable. Higher values are those that have a greater model weight, appear more often in the top models, or both. Therefore, it is possible that a variable that is included only within the very best models (ΔAIC_c of < 2) can be handicapped by less important variables that appear more consistently in the lower valued models (ΔAIC_c between 4-10). However, given the exploratory nature of this project, and the goal of identifying the important variables for future research, this approach provides the best integrative analysis of these data.

3.2.1 *amoA*

Abundances of the nitrifying gene, *amoA*, ranged from $2.07x10^3$ to $9.21x10^6$ copies/g field moist soil with a mean of $9.72x10^5$ copies/g field moist soil and median of $4.00x10^5$ copies/g field moist soil. The model selection results for *amoA* indicated that vegetation type, the combined temperature and precipitation variable, and N concentrations are the most important in driving populations of nitrifiers in both the top and bottom layers of the sampled BRCs (Table 2.3). Nitrite-nitrate and media mix were identified as important variables for nitrifying populations in the bottom 10 cm samples and when all sample data was run together. The location of the sample (top vs. bottom) was also an important factor for the total sample analysis which is not surprising considering nearly an order of magnitude difference in concentrations of *amoA* gene copies between the top and bottom samples (Figure 2.4.A). The combined temperature and precipitation variable consistently appeared as a variable affecting nitrifying gene abundances within all location analyses; however, this relationship is most likely caused by the confounding climatic factors and media compositions previously discussed. Not only are higher temperatures associated with increasing *amoA* and nitrification activity, but decreases in soil moisture have demonstrated reductions in *amoA* genes and transcript abundances (Vasileiadis et al., 2012).

Differences in nitrifying populations caused by vegetation categories may be attributed to a variety of factors that cannot be verified due to the limited data on vegetation types collected for the sampled BRCs. We hypothesize that nutrient competition potentially decreases ammonium availability in the grassed plots and restricts the growth of nitrifier populations. Given the generally shallow, dense rooting systems of grasses, intense competition for N in the upper 10 cm likely exists. Schimel and Bennett (2004) detail this phenomenon and hypothesized that in areas with moderate N availability and high competition for N, N mineralization primarily occurs in microsites. As NH_4 percolates into the soil and diffuses across high N microsite gradients, plant roots and their associated mycorrhizae have the ability to outcompete microbes, particularly in areas with dense rooting structures. The deeper rooting systems of the landscaped and overgrown BRCs potentially increases porosity, oxygen diffusion, and exhibits limited competition for ammonium in the sampled upper 10 cm layers that allows for the proliferation of nitrifying bacteria (Figure 2.12).

Although the model selection results indicate that media mix was not an important variable for *amoA* gene concentrations in the top 10 cm samples and is least important among the variables in the bottom 10 cm, our results support the effects of media mix composition on nitrifying populations. While nitrifiers should persist in higher populations in the presumably more aerobic, coarser textured media, N mineralization is dependent upon the decomposition and moisture holding capacities of mixes with lower sand content mixes (Accoe et al., 2004; Barrett & Burke, 2000; Burke, 1989). The supply of organic matter necessary for mineralization might be satisfied by accumulation in the upper layers of BRCs regardless of media mix composition, while nitrifier populations in the bottom layers may be limited by the organic matter sourced from the media (Figure 2.13). A lack of organic carbon in the lower layers could restrict both nitrifiers and denitrifiers, since denitrifying bacteria rely on organic carbon and the nitrified species produced by nitrifying bacteria.

amoA

	Vegetation	Temp & Precip	Ammonium & Nitrate	Media Mix	Cell Depth	Age	Location
Top 10 cm	1.00	0.67			0.41	0.36	
Bottom 10 cm		0.96	0.87	0.85			
Total Samples	1.00	0.93	0.41	0.88	0.35		1.00

Table 2.3: Model averaging results for concentrations of the nitrifying gene, *amoA*, in the BRC soil media for the top 10 cm (n=42), bottom 10 cm (n=36), and all samples analyzed together (n=72).

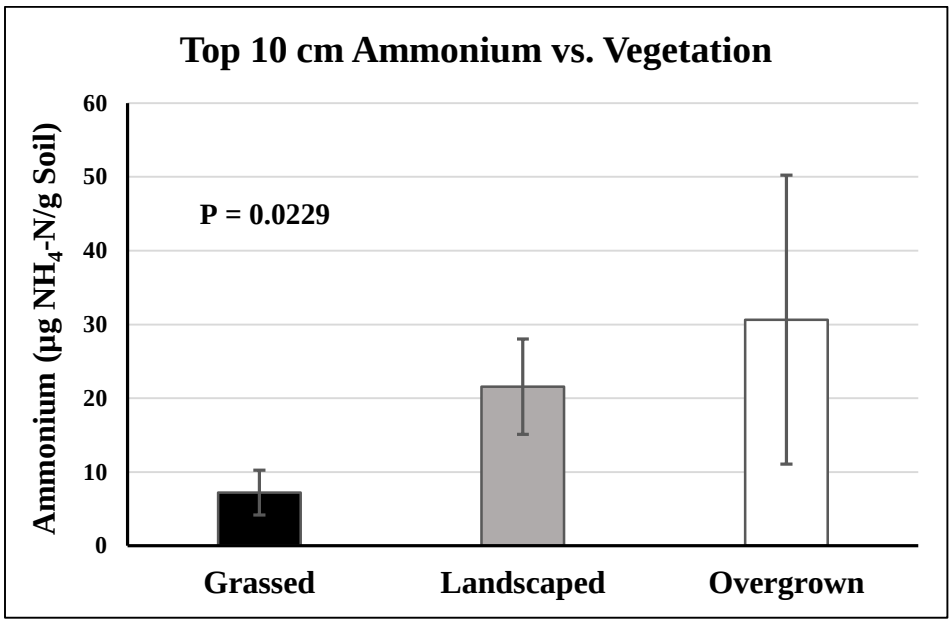

Figure 2.12: Mean extractable ammonium concentrations across BRC vegetation types within top 10cm samples (n=44). Error bars represent the 95% confidence interval of the mean.

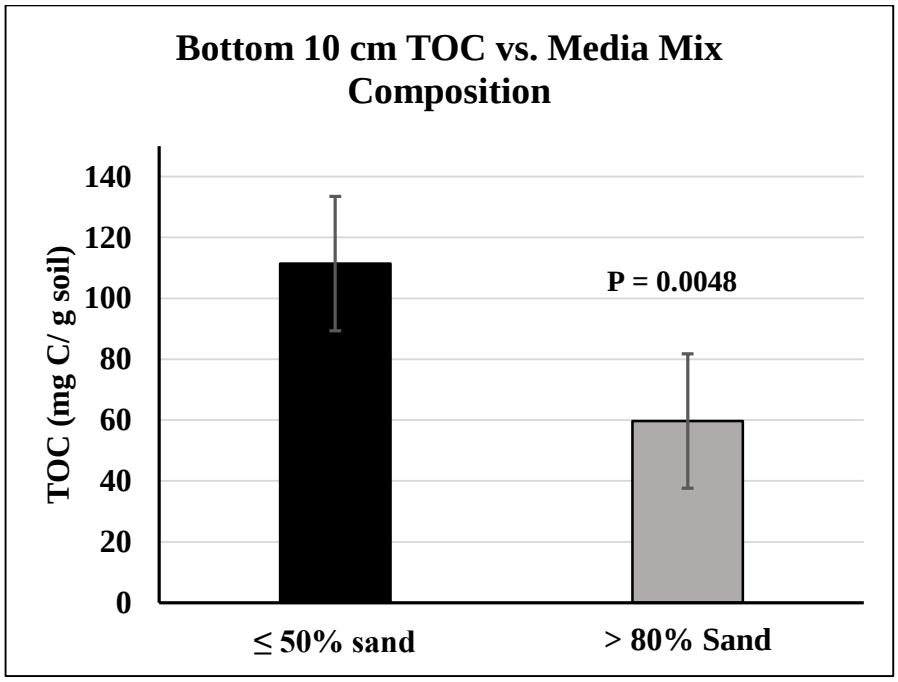

Figure 2.13: Mean extractable TOC vs media mix compositions for bottom 10 cm samples (n=42). Error bars represent the 95% confidence interval of the mean.

3.2.2 *nirK* and *nirS*

The *nirK* gene abundances ranged from 2.79×10^3 to 1.31×10^9 copies/g field moist soil with a mean of 1.18×10^8 copies/g field moist soil and a median of 4.00×10^7 copies/g field moist soil. *nirS* gene abundances ranged from 2.59×10^5 to 1.57×10^8 copies/g field moist soil with a mean of 1.41×10^7 copies/g field moist soil and a median of 8.55×10^6 copies/g field moist soil. The model selection results for the nitrite reductase genes, *nirK* and *nirS*, indicated some similarities between the two gene types but also highlighted several differences (Tables 2.4 and 2.5). The *nirS* gene was generally controlled by similar variables in both the top and bottom samples, while controls of *nirK* seemed to vary considerably across the sampling locations. Differences among variables affecting the genes may be due to niche preferences, as proposed by previous observations of the *nirK* and *nirS* genes across environment types (Enwall et al., 2010; Hallin et al., 2009; Philippot et al., 2009). The analysis indicated TOC and media composition as an important variable for both genes. In addition, vegetation was also a strong predictor of *nirS* gene abundances, while N concentrations were more important predictors for *nirK* populations. Similar to the nitrifying gene, the sampling location was an important predictor in the top models when all samples were analyzed.

As discussed in previous sections, the importance of TOC in the model selection results for *nirK* agrees with the heterotrophic nature of denitrifiers and, despite the weak linear relationship between organic carbon and *nirS*, our results indicate TOC is important for denitrifiers in the bottom 10 cm samples of the sampled BRCs. If TOC is considered within the context of BRC design, it is particularly important that the type and amount of carbon source is carefully chosen. Although our results indicate that denitrifiers increase with organic matter, the benefits of denitrification could be negated if the organic matter is a source of leaching carbon and nutrients. Therefore, a crucial balance must be met between an organic carbon source that can fulfill the needs of the microbial community but does not contribute to nutrient leaching. A study by Peterson et al. (2015) addresses this problem with the use of woodchips and found optimal N removal rates using 5 mm woodchips at a quantity of 4.5% by mass. The high C:N ratio of woodchips promoted a slow degradation rate had the potential to continuously supply the microbial community over time while preventing nutrient leaching.

nirK

	TOC	Media Mix	Ammonium & Nitrite-Nitrate	Vegetation	ISZ	Location
Top 10 cm	0.38	0.56	0.57			
Bottom 10 cm	0.86	0.31		0.27	0.42	
Total Samples	0.61		0.56			0.67

Table 2.4: Model averaging results for the nitrite reductase gene, *nirK* in the top 10 cm samples (n=36), bottom 10 cm samples (n=36) and all samples analyzed together (n=86).

nirS

	Vegetation	TOC	Media Mix	Age	Location
Top 10 cm	1.00		0.85	0.61	
Bottom 10 cm		1.00	0.28		
Total Samples	1.00	0.39			1.00

Table 2.5: Model averaging results for the nitrite reductase gene, *nirS,* in the top 10 cm samples (n=36), bottom 10 cm samples (n=36), and all samples (n=86).

To our knowledge, research has not yet been conducted that relates carbon sources, N reduction efficiency, and the microbial community, which could be a key piece in optimizing N removal efficiency in BRCs. Additionally, it is worth considering that the surface layers of BRCs also naturally accumulate organic matter over time, so there may be a need to remove this layer periodically (Li & Davis, 2014).

Similar to the results for *amoA* (Table 2.3), the model selection results indicate vegetation is the most important variable controlling *nirS* populations in the top 10 cm sampling locations (Table 2.5). We hypothesize that the importance of vegetation in denitrifying populations is due to the differences in N availability across the vegetation schemes in the sampled BRCs. Similar to ammonium concentrations (Figure 2.12), there is significantly less nitrite-nitrate concentrations in the top 10 cm samples of grassed BRCs compared to those that are landscaped or overgrown (Figure 2.14). Again, we hypothesize that rooting densities in grasses compared to landscaped or overgrown BRCs cause differences in N availabilities that ultimately influence the populations of N cycling bacteria.

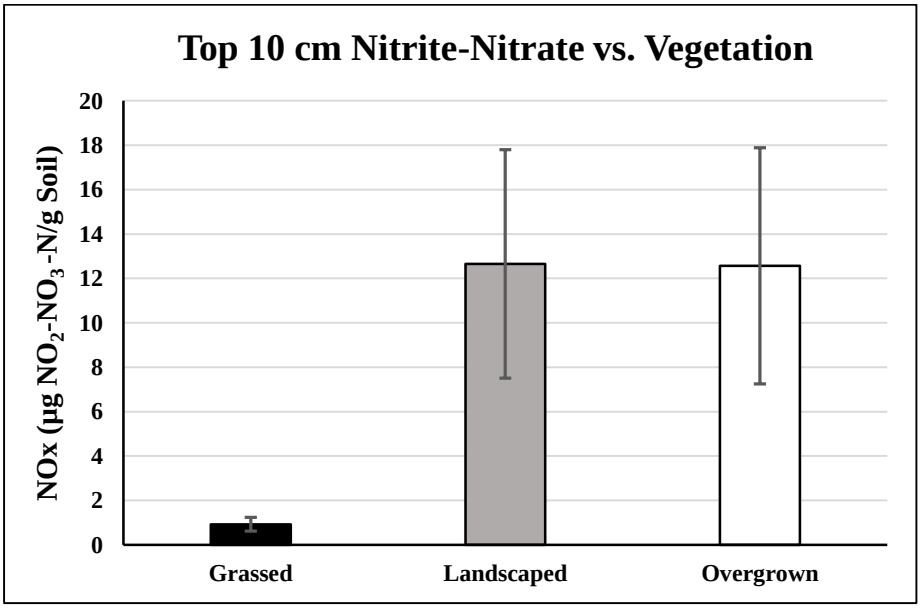

Figure 2.14: Mean nitrite-nitrate concentrations vs vegetation type within the top 10 cm samples. Error bars represent the 95% confidence interval of the mean.

The effect of vegetation elicits an interesting question about BRC design and management. Considering that grasses are a hardy species with constant growth rates, and a moderate to high N demand, should grass be the BRC vegetation of choice? A study by Passeport et al. (2009) reported pollutant removal rates in grass BRCs equal to and, in some instances, comparatively better than BRCs that contained trees, shrubs, and mulch. If grass clippings can be consistently bagged after mowing, grassing BRCs may be an effective strategy if used in combination with an ISZ and a stable carbon source that can supplement microbial denitrification. Li et al. (2014) speculate that nitrification of trapped particulate organic carbon (DON) and NH_3 during dry periods contributes to N leaching following precipitation events. Considering nitrification most likely occurs in the shallow aerobic zones of BRCs, a plant species with high nutrient accumulation potential and dense rooting systems in these layers may capture and reduce the export of these nitrified products. However, if grassed BRCs are simply mowed and clippings are returned to the cell, N removal is only temporary and will return to the system via mineralization (Davis et al., 2006; Li & Davis, 2014), which could be a source of net N export.

Media composition was an important variable for both the top and bottom samples in regards to *nirS* and *nirK* gene abundances (Tables 2.4 and 2.5). We speculate this was due to the increased saturation time of the slower infiltrating media mix with (≤ 50% sand) and a greater amount of nitrite-nitrate available from the resulting mineralization and nitrification from the degradation of higher organic matter contents. Without a more detailed study it is difficult to determine to what degree these factors played a role in structuring *nirS* and *nirK* populations. Organic carbon and nitrite-nitrate concentrations were, on average, higher in the media mix with ≤ 50% sand, but these differences were not significant (Figures 2.15 and 2.16). Considering that media mixes with higher organic matter contents are prone to clogging (Gulliver et al., 2008), are typically more expensive (Davis et al., 2009), and have been shown to leach DON (Hatt et al., 2009), the issues associated with these media mixes may negate the benefits of increased denitrifier populations.

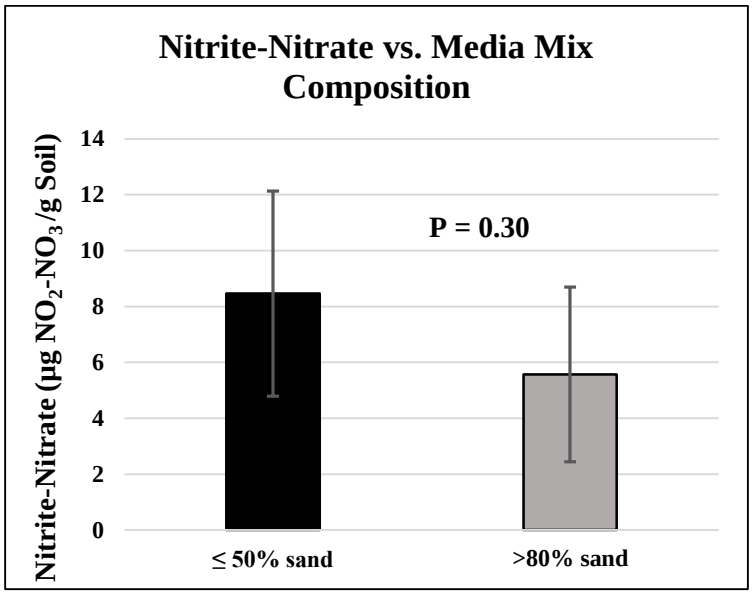

Figure 2.15: Mean extractable nitrite-nitrate concentrations vs. media mix composition for all samples. Error bars represent the 95% confidence interval of the mean.

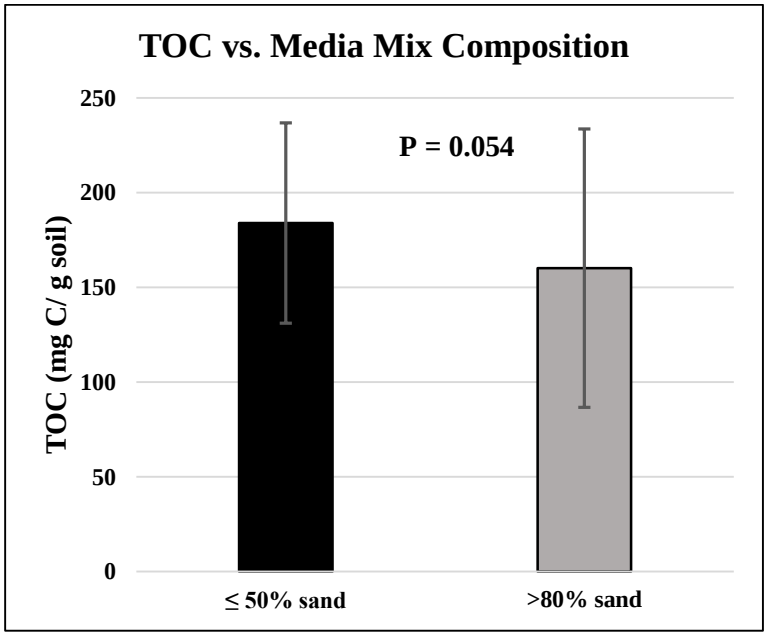

Figure 2.16: Mean extractable TOC concentrations vs. media mix composition for all samples. Error bars represent the 95% confidence interval of the mean.

Although age appeared only once and was the least important variable for the top 10 cm samples, the linear regression for *nirS* copies versus age is significant (Figure 2.3.C) and shows *nirS* populations increase as BRCs age. This trend could be caused by increased anaerobic conditions due to clogging, increased organic matter and its subsequent nitrification, or a general growth of the microbial community over time. Finally, the model selection results indicate the presence of an ISZ to be the least important variable among those variables that were within the top models for the bottom 10 cm analysis for *nirK*. While BRCs with an ISZ may affect denitrifier populations, our findings demonstrate that BRCs with saturated zones actually have lower abundances of denitrifier genes (Figure 2.5). This may be due to carbon limitations in the studied BRCs with saturated zones; however, it is important to consider that if BRCs are designed with saturated zones and omit a crucial component such as carbon, it can actually have a negative effect on the denitrifying microbial community.

3.2.3 Denitrification Potential

Denitrification potential ranged from below detection to 3.99 μg N_2O-N/g dry soil/h, with a mean of 0.364 μg N_2O-N/g dry soil/h and a median of 0.0484 μg N_2O-N/g dry soil/h. Denitrification measurements were only available for the NC and VA samples that had more homogenous media mix compositions, unlike the gene abundance variables. The BRCs containing ≤ 50% sand accounted for 4 of the 47 total samples included in the denitrification potential model selection analysis and had nearly double the mean denitrification rate of the BRCs with ≥ 80% sand. Due to a lack of variation in media mix compositions in the dataset and because media composition dominated the model selection results, media composition was removed and the dataset was re-analyzed (Tables 2.6 and 2.7).

Among all of the measured variables, denitrification potential is influenced by vegetation type, inorganic N, BRC surface area ratio, TOC concentrations, and region (Tale 2.7). The analysis indicated that the top 10 cm samples were most influenced by vegetation and inorganic N, while the bottom 10 cm samples were most influenced by the BRC runoff surface area ratio. Due to a large portion of near zero values for denitrification potential measurements in the bottom 10 cm samples, a lack of variation among denitrification rates could have reduced the ability of the model selection analysis to identify the most important variables. When all of the samples were analyzed together, inorganic N, vegetation type, and TOC were the top variables. Location was not the most important variable affecting potential denitrification rates when all samples were analyzed. This was surprising considering the large differences between denitrification potential rates in the top and bottom samples.

The denitrification potential analysis was conducted using the acetylene blockage method, which inhibits the nitrous oxide reductase enzyme (*nosZ*). Therefore, the N_2O quantified in this assay is a function of the genes in the earlier portions of the denitrification pathway, but ultimately relies upon the transformation from NO to N_2O. This conversion is carried out by the nitric oxide reductase gene, *norB,* which was not quantified in this study. It would be expected that similarities between the nitrite reductase genes (*nirk* and *nirS*) would exist. These similarities are evident in the relative importance of the vegetation, inorganic N, and TOC concentrations that similarly appeared as important variables for the *nirS* and *nirK* genes (Tables

2.4 and 2.5). Thus, it is likely that increases in inorganic N, organic carbon, and nutrient competition in grasses affect potential denitrification rates similarly to the nitrite reductase genes.

It is interesting that region appeared as an important variable for denitrification potential rates. If we consider region as representative of a climate gradient, we would have expected that the precipitation and temperature would also have been important for denitrification. We would not have expected that the geologic differences between the Piedmont and Coastal Plain would affect denitrification since BRCs are excavated and re-filled with an engineered soil mixture. While pH appeared to be a less important variable within our top models, all of the sampled BRCs had pH values between 5.6 and 8.3. Such a pH range would not be expected to cause significant differences within denitrifier populations, since this is within the optimal range for the denitrification enzymes (Nömmik, 1956; Van Cleemput & Patrick, 1974).

Denitrification Potential

	Media Mix	Vegetation	Ammonium & Nitrite-Nitrate	ISZ	TOC	Location	Region
Top 10 cm	0.97	1.00	0.87	0.96	0.13		
Bottom 10 cm	1.00						0.16
Total Samples	0.21	0.95	1.00		1.00	0.39	

Table 2.6. Model averaging results for denitrification potential for the top 10 cm samples (n=17), bottom 10 cm samples (n=18), and total sample analysis (n=35) when media mix composition was included.

Denitrification Potential

	Ammonium & Nitrite-Nitrate	Vegetation	SA Ratio	TOC	pH	Location	Region
Top 10 cm	1.00	0.93		0.83	0.30		0.19
Bottom 10 cm		0.28	0.47				0.30
Total Samples	1.00	1.00		1.00		0.82	

Table 2.7. Model averaging results for denitrification potential for the top 10 cm samples (n=23), bottom 10 cm samples (n=24), and total sample analysis (n=47) when media mix composition was omitted from the analysis.

3.2.4 *nosZ*

The *nosZ* gene abundances ranged from $1.53x10^3$ to $2.40x10^7$ copies/g field moist soil. The mean and median gene abundances were $4.88x10^6$ and $3.22x10^6$ copies/g field moist soil, respectively. Model selection results for *nosZ* show inorganic N, cell depth, and climatic factors to be primary drivers for both the top 10 cm and bottom 10 cm samples (Table 2.8). The top 10 cm sample results suggest inorganic N to be the most important response variable followed by vegetation, cell depth, and climate. Results from the bottom 10 cm samples also indicate inorganic N is the most important, with climatic factors second most important, followed by BRC depth and media mix. If the confounding effect of media mix and climatic factors are considered, it is difficult to distinguish the degree to which these variables affect *nosZ* populations; however, there was a significant difference in *nosZ* population across media mix compositions (Figure 2.6 D). When all samples were analyzed together, location and inorganic N were most important followed by cell depth and vegetation.

The *nosZ* populations are particularly important because these bacteria are responsible for converting N_2O, a potent greenhouse gas, into inert dinitrogen. Similarities between the *nosZ* and *nir* genes were evident by the importance of inorganic N, vegetation, and media composition as controls. This may be due to the fact that *nosZ* populations must rely upon on the *nir* populations, at least during anaerobic conditions where inorganic N would otherwise be limiting. Considering the similar physiological characteristics between the *nir* and *nosZ* bacteria, we hypothesize that similar factors affect *nosZ* populations. These include N availability, vegetative nutrient competition, and longer saturation times and the organic matter content in media compositions may also affect *nosZ* populations.

Unlike the *nir* population results, the *nosZ* results indicate media mix depth, the depth from the surface of the cell to the gravel layer, to be an important variable affecting *nosZ* populations in both the top and bottom 10 cm samples (Table 2.8). Upon comparing N removal rates in two BRCs of varying media depths (0.6 and 0.9 m) and similar runoff ratios and sources, Brown and Hunt (2011) found similar reductions in TN and increases in NO_2 and NO_3. Although deeper media depths have the ability to hold larger volumes of stormwater, shallower BRCs may enable longer saturation periods to promote anaerobic conditions. The linear relationship between *nosZ* and media depth is insignificant (Figure 2.17).

nosZ

	Ammonium & Nitrite-Nitrate	Cell Depth	Vegetation	Temp & Precip	Media Mix	Location
Top 10 cm	1.00	0.76	0.79	0.81		
Bottom 10 cm	0.99	0.83		0.57	0.84	
Total Samples	1.00	0.97	0.91	0.66	0.85	1.00

Table 2.8: Model selection results for the nitrous oxide reductase gene, *nosZ* for only top 10 cm samples (n=42), only bottom 10 cm samples (n=36), and for samples from both locations analyzed together (n=72).

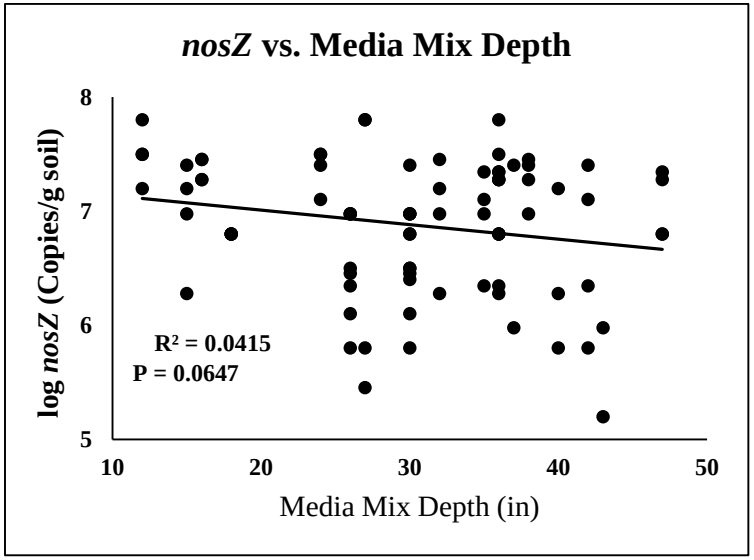

Figure 2.17: Linear regression for *nosZ* gene abundances in all samples (n=86) vs. average media mix depth.

4. Conclusions and Future Research

Multiple trends in the data point towards important relationships between BRC design and N cycling. Despite the considerable amount of design information and environmental variables that were collected and included in the analysis, several of these consistently appeared to affect nitrifier and denitrifier populations. These included vegetation, inorganic N, organic carbon, and media composition; all of which can be manipulated by BRC design.

Despite the fact that increases in organic carbon tended to increase nitrifying and denitrifying bacteria, the source and quantity of an organic carbon amendment that supplements the bacterial community but does not leach nutrients is an important balance that must be determined. Current research has pointed towards wood chips as a promising substrate, but research on the denitrifying community in relation to woodchips or other amendments with high C:N is needed. Despite the fact that our results indicate that nitrifiers and denitrification would be increased with media mixes containing higher organic matter contents, previous research has indicated that these mixes likely do more harm than good. Design recommendations with higher sand contents in conjunction with future research in identifying an optimal carbon source should be highly considered if BRCs are used as a tool for the reduction of N and carbon leading to urban streams.

Inorganic N is an important control of both the nitrifying and denitrifying bacterial communities. The source of inorganic N is not only a function of the runoff the BRC receives, but is also a product of the nitrification processes that occur from the breakdown of intercepted organic matter and N species. Considering that the runoff source largely can not be controlled, the primary mechanisms of N removal in BRCs are microbial transformations and vegetative uptake. Future research should focus on how these processes can co-exist in a fashion that maximizes functions that increase N removal efficiency. Our data suggest that N uptake from grassed BRCs results in lower levels of inorganic N in upper 10 cm compared to other planting schemes or neglected and overgrown BRCs. Previous research has determined the effectiveness of grassed BRCs to be better than other planting schemes, but collection and removal of grass clippings should be studied as a management requirement. The relationship and competition dynamics between grasses and various types of vegetative cover commonly used in BRCs could

be an important research area to promote high N plant plant assimilation and microbial transformation.

Finally, despite the increasing trend towards constructing BRCs with permanently saturated zones, our research failed to detect increases in denitrifier populations in the bottom samples in BRCs with an ISZ. Our data actually suggest that BRCs with ISZs contained fewer denitrifiers in the bottom layers than conventionally drained BRCs. As only 5 of the 22 sampled BRCs contained a saturated zone, these samples may have been statistically under-represented. Either way, only one of the BRCs with a saturated zone reported the addition of an organic substrate to this layer and a lack of an organic carbon source in the majority of the sampled BRCs with an ISZ probably restricted denitrifier populations despite the anaerobic conditions. Constructing BRCs with both a saturated zone and a carbon source may be necessary for denitrifier populations but future research is needed to verify this.

Overall, these results represent the largest field-scale study of bioretention design factors in existing BRCs and one of very few that directly quantify denitrifier populations. In fact, only two other known studies have quantified denitrifiers in these BMPs and each within only a single bioretention cell. The results represent a unique and valuable data set quantifying a nitrifying gene, a suite of denitrifier genes, and denitrification potential in multiple BRCs with varying design parameters across a latitudinal gradient. In general, our findings generally agree with the understanding of microbial community dynamics and the general functioning of bioretention cells and highlights several design features of BRCs that show the most promise for further design enhancements that promote denitrifiers. These results also highlight the value of examining bioretention systems from a microbial perspective to better understand important biological processes. Future studies should continue to focus on microbial responses to BRC design and should include further consideration of these variables in controlled experiments.

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Chapter 3: Bacterial and Fungal Ecology of Existing Bioretention Cells across the Eastern Mid-Atlantic Region

Abstract

Increases in impervious surface from the development of urban infrastructure causes higher volumes and peak flows of stormwater that transports urban pollutants directly into surface waters. Bioretention cells (BRCs) are engineered soil systems designed to capture and treat stormwater before it infiltrates into the underlying soil or is discharged to the stormwater conveyance system. These systems offer a viable solution for the reduction of stormwater volumes and the capture and/or transformation of urban pollutants, but observed nitrogen (N) removal efficiencies have been highly variable due to a poor understanding of the factors that control N cycling within the microbial community. To our knowledge, there have been no studies conducted on the total bacterial or fungal communities within BRCs in regard to diversity or community structure. It is, however, generally recognized that the structure of microbial communities potentially has significant effects on biogeochemical cycling in most ecosystem types. The objective of the study was to gain an understanding of the effects of bioretention design and environmental factors on microbial community structure in BRCs that have the potential to influence N removal efficiency in BRCs. We quantified total bacterial and fungal populations using qPCR and chloroform fumigation extraction, as well as taxonomically characterized the microbial community using next generation sequencing in 23 BRCs across the Eastern Mid-Atlantic region. The dominant phyla in both the fungal and bacterial communities in the sampled BRCs were similar to studies in natural soils, but differed in their relative proportions. The bacterial community composition between the top and bottom samples of the BRCs indicate influences of N and carbon (C) concentrations. Fungal community composition differed most across BRC vegetation type and was similarly influenced by N and C concentrations. Total microbial biomass was most influenced by C and N concentrations, similar to the individual analysis of total fungal and bacterial concentrations measured by real-time PCR.

1. Introduction

Since the onset of the agricultural and industrial revolution in the 18th century, urbanization has rapidly increased in both the United States and across the globe. The world's urban population currently accounts for over 3.9 billion people and is expected to increase by an additional 2.5 billion people by 2050 (United Nations, 2014). The development of infrastructure such as roadways, parking lots, and buildings needed to maintain the urban population has drastically altered the natural hydrology of the landscape. This impervious cover replaces the natural vegetation that formerly reduced stormwater flows and facilitated infiltration. As a result, surface waters are receiving unprecedented volumes of stormwater that contain urban pollutants such as heavy metals, suspended solids, nutrients, and hydrocarbons that can have a devastating effect on the health of downstream aquatic ecosystems and the economies that rely on them (EPA, 2009; Konrad & Booth, 2005; Mallin et al., 2009). Nutrient additions to surface waters are particularly threatening; phosphorus and N additions stimulate algal blooms that decompose and reduce dissolved oxygen concentrations. Anoxic conditions caused by eutrophication are responsible for the formation of over 400 dead zones globally that have a cascading effect on ecosystem health and community structure (Diaz & Rosenberg, 2008).

Bioretention cells (BRCs) offer a practical and cost effective way to mitigate urban stormwater flows and pollutants. These engineered systems are part of the low impact development (LID) paradigm, a movement towards the integration of localized stormwater reduction methods that aim to maintain or restore the original hydrology of the landscape. These systems are typically mulched and vegetated above several feet of engineered fill mixture designed to capture and transform urban pollutants while facilitating infiltration. Filtered stormwater is discharged to the sewage/stormwater system or is infiltrated through the underlying soil. Since their implementation in the late 1990s, BRCs have demonstrated impressive reduction efficiencies for most urban pollutants (R. Brown & Hunt, 2008; Davis, 2007; Trowsdale & Simcock, 2011). Nitrogen reduction, however, has been highly variable due to a limited understanding of the biological mechanisms that are responsible for N transformation. Research in the past century has focused on design alterations aimed at increasing N removal efficiency in BRCs, but results have been variable and a design standard has yet to be established (R. A. Brown & Hunt, 2011; Davis, 2007; Hunt et al., 2006).

Aside from the studies conducted by Chen et al. (2013) and Willard et al. (2014), which quantified bacterial denitrifiers in a single BRC, there has been no published attempt to our knowledge to characterize the microbial community within a bioretention cell. This represents an important knowledge gap in the function and design of these systems, given that microbial diversity and community composition can have a significant effect on biogeochemical cycling (Falkowski et al., 2008; Reed & Martiny, 2007; Strickland et al., 2009) and denitrification, in particular (Fierer et al., 2012; Peralta et al., 2010; Philippot et al., 2013). BRC design attributes such as the volume of runoff treated per unit area of the cell, organic matter inputs, vegetation, and the inclusion of a saturated zone alter the environmental conditions and potentially have a profound effect on microbial community diversity and structure. Recent advances in high throughput sequencing technologies have allowed for the phylogenetic characterization of entire environmental samples. By analyzing these data across a broad range of functioning BRCs, it is possible to gain insight into the relationships between BRC design and microbial community composition and diversity that may allow for a better understanding of nitrogen cycling in BRC's. This study aims to determine the contributions of design parameters on the microbial community in a range of existing BRCs across the Eastern Mid-Atlantic in an attempt to understand and potentially manipulate these factors to increase N removal efficiency in future BRC design.

2. Materials and Methods

2.1 BRC Selection and Sample Collection

Design information on approximately 50 BRCs across MD, VA, and NC was collected using published journal articles and public resources. Twenty-three BRCs having the greatest variability in design parameters were selected for sampling. Exact sampling locations, descriptions, and sources are provided in *Chapter 2*. All sites were sampled within ~1 month and at least one-week after rainfall during the months of November and December of 2014. Samples were aseptically collected from the top 10 cm and bottom 10 cm from front of the cell (denoted by the inflow) and back of the cell (denoted by the overflow structure). Three samples within each location were collected and homogenized for a total of four samples per BRC.

2.2 Microbial Biomass, Nitrogen, & Carbon

Microbial biomass measurements as well as extractable carbon and nitrogen concentrations were determined using simultaneous chloroform fumigation-extraction (sCFE) based on the method described in Fierer & Schimel (2002, 2003). All measurements were carried out in duplicate, including duplicate fumigated and non-fumigated blanks. Microbial biomass measurements give insight into the relative biomass of microbes in a given sample based on the difference of carbon and nitrogen measurements between a lysed (via chloroform fumigation) and non-lysed sample. As an added benefit, this method also allows for the quantification of extractable total nitrogen, total organic carbon, nitrite-nitrate, and ammonium concentrations for each sample. Total organic carbon was analyzed on an OI Model 1010 total organic carbon analyzer using standard method 5301c (APHA, 2005). Ammonium and nitrite-nitrate concentrations were determined using a Lachat QuikChem 8500 Flow Injection Analyzer following the QuikChem Method 10-107-04-1-L and APHA Method 4500-NO3- I APHA, 2005; Lachat Instruments, 2007). Total nitrogen and total carbon was determined using a FlashEA 1112 Series Elemental Analyzer based on the method from Zimmerman et al. (1997).

2.3 DNA Extraction, Quantification, and Sequencing

Soil media was stored at -80 °C until extraction. Sample DNA was extracted using the PowerSoil DNA Isolation-Kit (MOBIO Laboratories INC, CA, USA) following the manufacturer's protocol. Sample DNA concentrations were determined using Qubit 2.0

fluorometer (Invitrogen, USA) and stored at -20° C until analyzed. Total bacterial and fungal populations for use in the determination of fungal/bacteria ratios were quantified by targeting the 16S rRNA and ITS regions, respectively. Thermal profiles, reaction mixtures, and primers are outlined in *Table 2.1*. Bacterial 16S rRNA and fungal ITS genes were PCR-amplified in triplicate using barcoded 515F and 806R (Caporaso et al., 2012) and 5.8s and ITS1f (Fierer et al., 2005) primers, respectively. Thermal profiles for PCR amplification for sequencing are shown in *Table 2.2* below. Amplification was verified by visualization on agarose gels. Triplicate PCR-amplified samples were combined and concentrations of amplicon DNA were determined using a Qubit fluorometer (Invitrogen, USA).

Target Gene	Primer Sequences and References	Reaction Mixture	Volume (μL)	Thermal Profile
16S	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG (Muyzer et al., 1993) (Lane, 1991)	SsoAdvanced™Universal SYBR® Green Supermix Eub338 (10 μM) Eub518 (10 μM) PCR water Template (~3 ng/μL)	12.5 1.25 1.25 5 5	96°C – 3 min 1 cycle 95°C – 30 s 55°C – 30 s 72°C – 30 s 40 cycles
ITS	TCCGTAGGTGAACCTGCGG CGCTGCGTTCTTCATCG (Gardes & Bruns, 1993) (Vilgalys & Hester, 1990)	SsoAdvanced™Universal SYBR® Green Supermix ITS1F (10 μM) 5.8S (10 μM) PCR water Template (~3 ng/μL)	12.5 1.25 1.25 5 5	98°C – 3 min 1 cycle 98°C – 60s 53°C – 60s 72°C – 60s 40 cycles

Table 3.1. Reaction mixtures, primers, references, volumes, and thermal profiles used for the quantification of fungi and bacteria.

Target Gene	Primer Sequences and References	Reaction Mixture	Volume (μL)	Thermal Profile
16S	AATGATACGGCGACCACCGAGA TCTACACTATGGTAATTGTGTGC CAGCMGCCGCGGTAA CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAGC CGGACTACHVGGGTWTCTAAT	2.5X 5 Prime HotMaster mix	12.5	94°C – 5 min 1 cycle
		515F (10 μM)	0.5	
		806R (10 μM)	0.5	94°C – 45 s
		PCR water	13	50°C – 45 s
		Template (~3 ng/μL)	1	72°C – 90 s 35 cycles
	(Caporaso et al., 2012)			72°C – 10 min 1 cycle
ITS	AATGATACGGCGACCACCGAGA TCTACACTATGGTAATTCTCTTG GTCATTTAGAGGAAGTAA CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGT CAGATGCTGCGTTCTTCATCGATGC	2.5X 5 Prime HotMaster mix	12.5	94°C – 4 min 1 cycle
		ITS1 (10 μM)	0.5	
		ITS2 (10 μM)	0.5	94°C – 30s
		PCR water	13	50°C – 60s
		Template (~3 ng/μL)	5	72°C – 90s 40 cycles
	(Bellemain et al., 2010)			

Table 3.2. Reaction mixtures, volumes, and thermal profiles used for amplification of the 16S and ITS genes. X'd portion of sequence represents variable barcoded region.

Amplicons were pooled in equimolar ratios and bi-directionally sequenced (PE 250 bp) using the Illumina MiSeq platform at Virginia Tech Biocomlexity Institute. Forward and reverse reads were merged and filtered based on minimum length and expected errors as outlined in the USEARCH pipeline (Edgar, 2010). After filtering, 11,912,617 high quality 16S bacterial sequences and 2,136,290 high quality ITS fungal sequences were obtained in total. These sequences were then clustered into 37,282 bacterial and 6,580 fungal operational taxonomic units (OTUs) with a 97% threshold similarity. Chimeric sequences were identified and removed via UCHIME (Edgar, 2013; Edgar et al., 2011) and the RDP classifier and SILVA databases were further used for taxonomic assignment (Koljalg et al., 2013; Quast et al., 2013; Wang et al., 2007).

2.4 Data Analysis

2.4.1 Model Selection

As detailed in the *Materials and Method*s section of *Chapter 2*, a model selection method, specifically Akaike's Information Criterion, was used to determine the most important environmental and design parameters effecting total fungi, total bacteria, fungal/bacterial ratios, and microbial biomass. Environmental and design parameters were first analyzed separately. After the identification of the most important variables for each category (environmental and design), these variables were combined and analyzed together to reduce complexity and to determine if the identified factors were strictly a product of the environment or if they could be potentially manipulated through BRC re-design. Separate analyses were conducted on the top 10 cm and bottom 10 cm samples, as well as all samples together to evaluate the BRC as a whole. Predictor variables were transformed using a log or square root transformation to reduce lop-sided and long-tailed distributions within our dataset. Not only was this a better representation of the gradients within our data, but it allowed for the identification of fewer top models with higher confidences.

2.4.2 Centroid Plotting

Using the 'vegdist' function found in the 'Vegan' R package (Oksanen et al., 2015; R Core Team, 2015), a Bray-Curtis dissimilarity index was calculated for each sample using the OTU counts from the sequencing data. The Bray-Curtis dissimilarity index formula is detailed below, and uses a simple ratio of the sums of the absolute differences of similar species counts across sites divided by the sums of all species counts across those sites, essentially allowing for a distance metric, or a normalized comparison across samples.

Equation 3.1.
$$BC_{ij} = 1 - \frac{\sum_{j=1}^{J} |n_{ij} - n_{ik}|}{\sum_{j=1}^{J} (n_{ij} + n_{ik})}$$

Using the Bray-Curtis dissimilarity matrix as a distance metric, the "betadisper" function in R was used to calculate and plot the sample distances to the centroid. The centroid represents the average community similarity within a group, which, in combination with individual samples, allows for a visual comparison of diversity differences among and within categorical data types.

Permutational ANOVA (9,999 permutations) was performed using the ‘permutest’ function to determine statistical differences within categories and the ‘adonis’ function was used to determine statistical differences among categories.

2.4.3 Distance-based Redundancy Analysis (db-DRDA)

Using the ‘capscale’ function in the ‘Vegan’ R Package (Oksanen et al., 2015; R Core Team, 2015), distance-based redundancy analysis ordination (db-RDA) plots were created for both bacterial and fungal sequencing data based on the Bray-Curtis similarity index. A db-RDA uses a similarity index to create an ordination plot of samples overlaid with environmental vectors. The spatial distribution of samples in relation to each other and to the environmental vectors enables a visual interpretation of community similarity and the influence of the environmental variables on community structure. Due to the skewed distributions and outliers that we assumed to be true values in our data, predictor variables were transformed using either log or fourth root transformations. These transformations produced better representations of the overall gradients in our data, and allowed for patterns between community structure and the measured environmental parameters to be revealed.

2.4.4 Diversity Calculations & Taxonomy Plots

The ‘Vegan’ R package was used to calculate Shannon diversity indexes for categorical data types. Taxonomy plots were made using the sample species distribution output from QIIME (Caporosa et al., 2010).

3. Results and Discussion

3.1 Environmental and Design Trends

3.1.1 BRC Age

The sampled BRCs ranged in age from 1 year to 22 years with a mean age of 9 years and a median of 11 years. The R^2 values ranged from < 0.001 to 0.20 and significance values (alpha = 0.05) ranged from < 0.0001 to 0.9646 (Figure 3.1). Microbial biomass was the only variable that showed a statistically significant relationship with age (Figure 3.1), which suggests that the microbial fraction in BRCs generally increase as the cell increases in age. Fungi and bacteria when analyzed separately showed a weak, but consistent increasing linear relationship with age. The fungal/bacterial (F/B) ratio best fit line was near zero, indicating essentially no relationship with the age of the sampled BRCs.

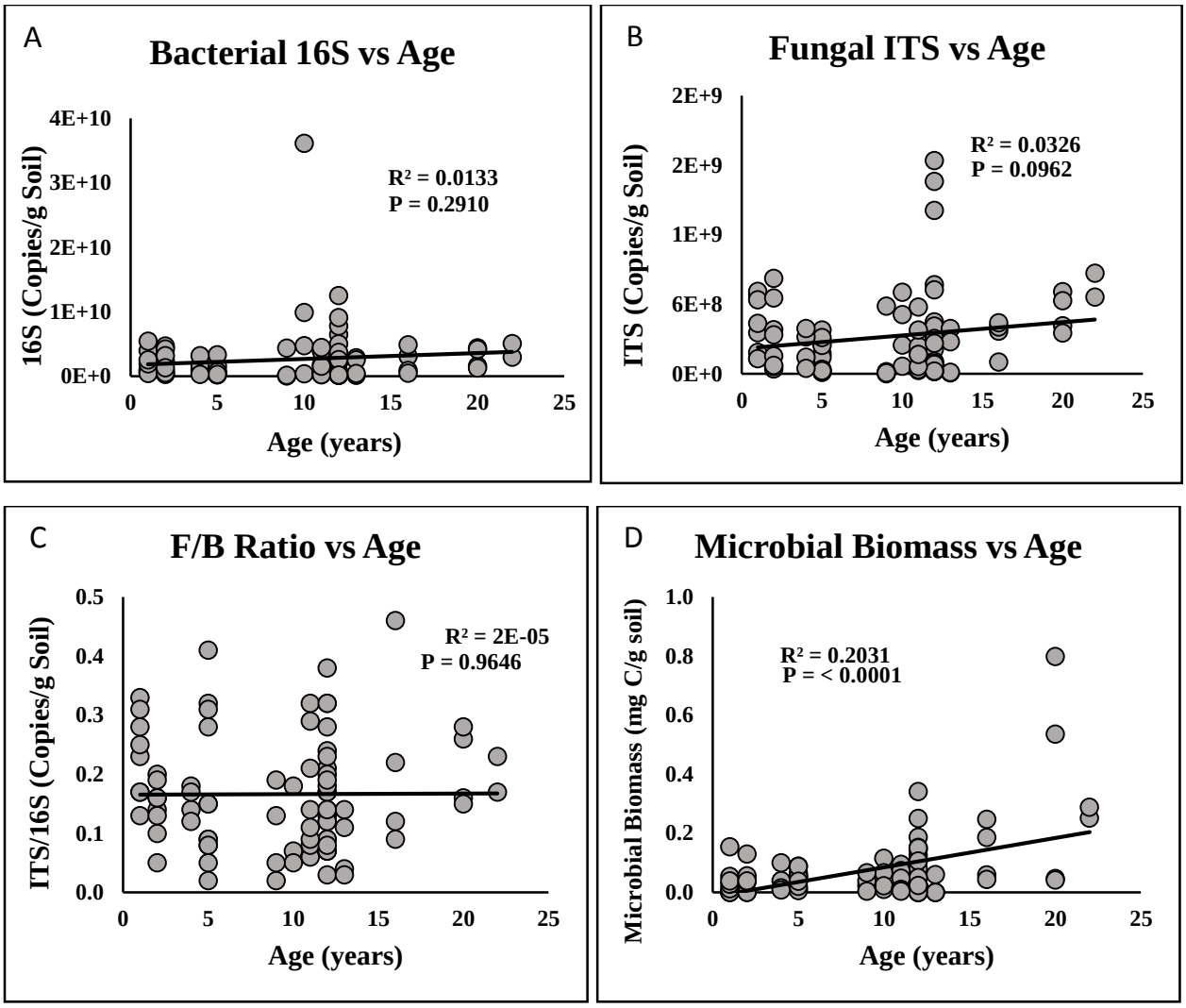

Figure 3.1. Linear regressions for all 4 sampling locations from each of the 23 BRCs sampled in this study (n = 86) for Bacteria (A), Fungi (B), Fungal/Bacterial Ratio (C), and Microbial Biomass (D) vs age.

3.1.2 BRC Heterogeneity

The number of top and bottom samples from all BRCs were almost equally distributed, with 44 samples originating from the top 10 cm and 42 samples originating from the bottom 10 cm. All of the measured response variables indicate a consistent and significant trend of higher abundances in the top of the BRCs compared to the bottom, with the exception of the F/B ratio, which did not show a difference (Figure 3.2). Differences in microbial biomass values were most evident, with mean concentrations nearly 5 times higher in the top 10 cm compared to the bottom 10 cm. Both bacterial and fungal populations were nearly an order of magnitude higher in the top 10 cm samples compared to the bottom 10 cm. Mean F/B ratios are ~0.5 in both sampling locations, indicating bacterial dominance does not change across the vertical profile of the sampled BRCs.

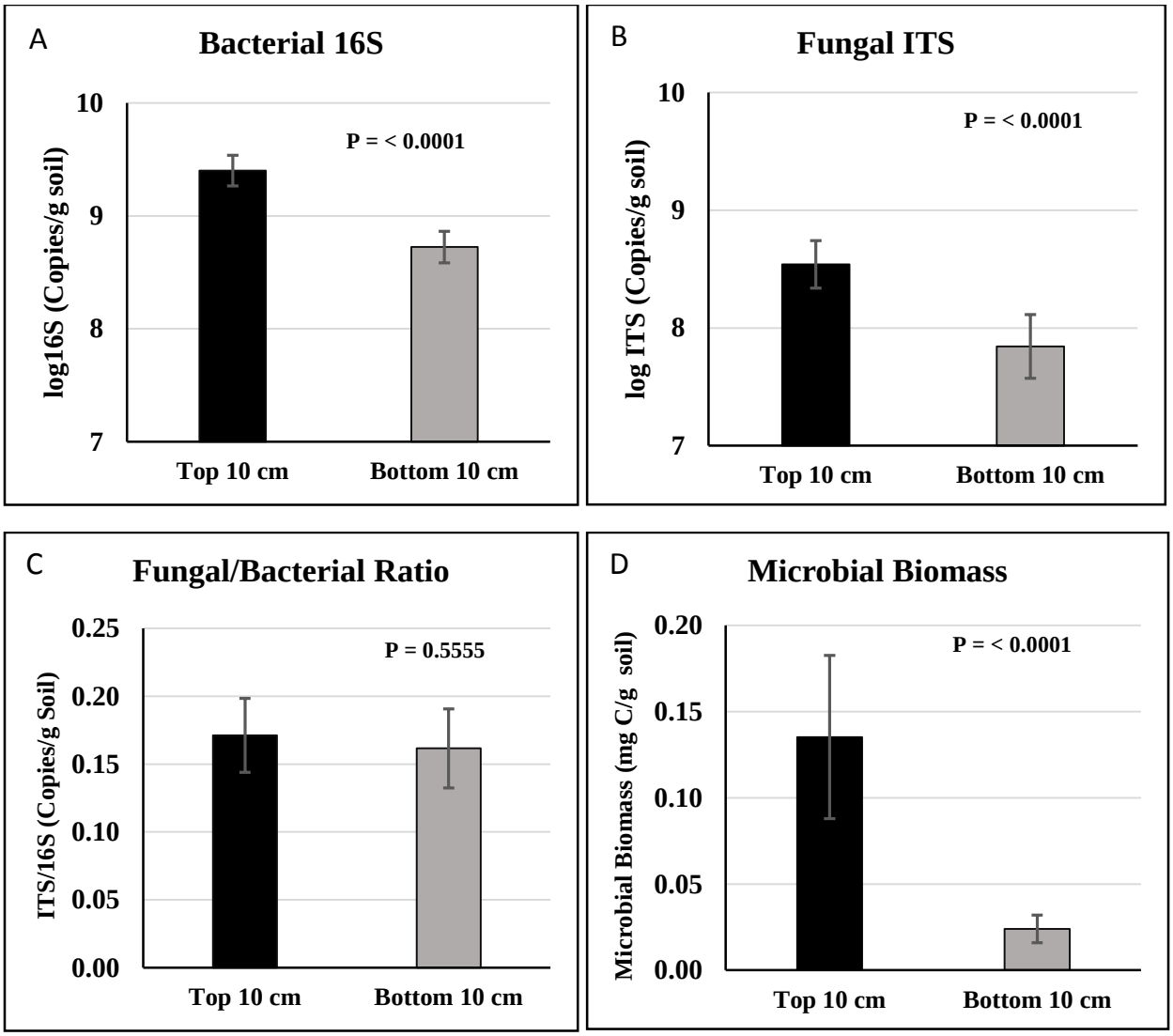

Figure 3.2. Mean values for the microbial response variables for all 23 BRCs in the top 10 cm (n = 44) and bottom 10 cm (n = 42) samples of the soil media for total bacteria (A), total fungi (B), fungal/bacterial ratio (C), and microbial biomass (D). Error bars represent the 95% confidence interval of the mean.

3.1.3 Media Composition

Sampled BRCs were broadly classified into two media mix categories: those that were comprised of ≤ 50% sand and those with ≥ 80% sand. BRCs with ≤ 50% sand made up of 8 of the 19 BRCs with known media mix compositions. BRCs with media compositions of ≥ 80% sand made up the remaining 11 sampled BRCs with known media mix compositions. Among the measured predictors, total bacterial and fungal populations showed the greatest differences between media compositions (Figure 3.3). Mean populations were higher in mixes with ≤ 50% sand, although these differences were only marginally significant. F/B ratios and microbial biomass values elicited the same pattern of higher populations in lower sand content mixes, however, these differences were not significant.

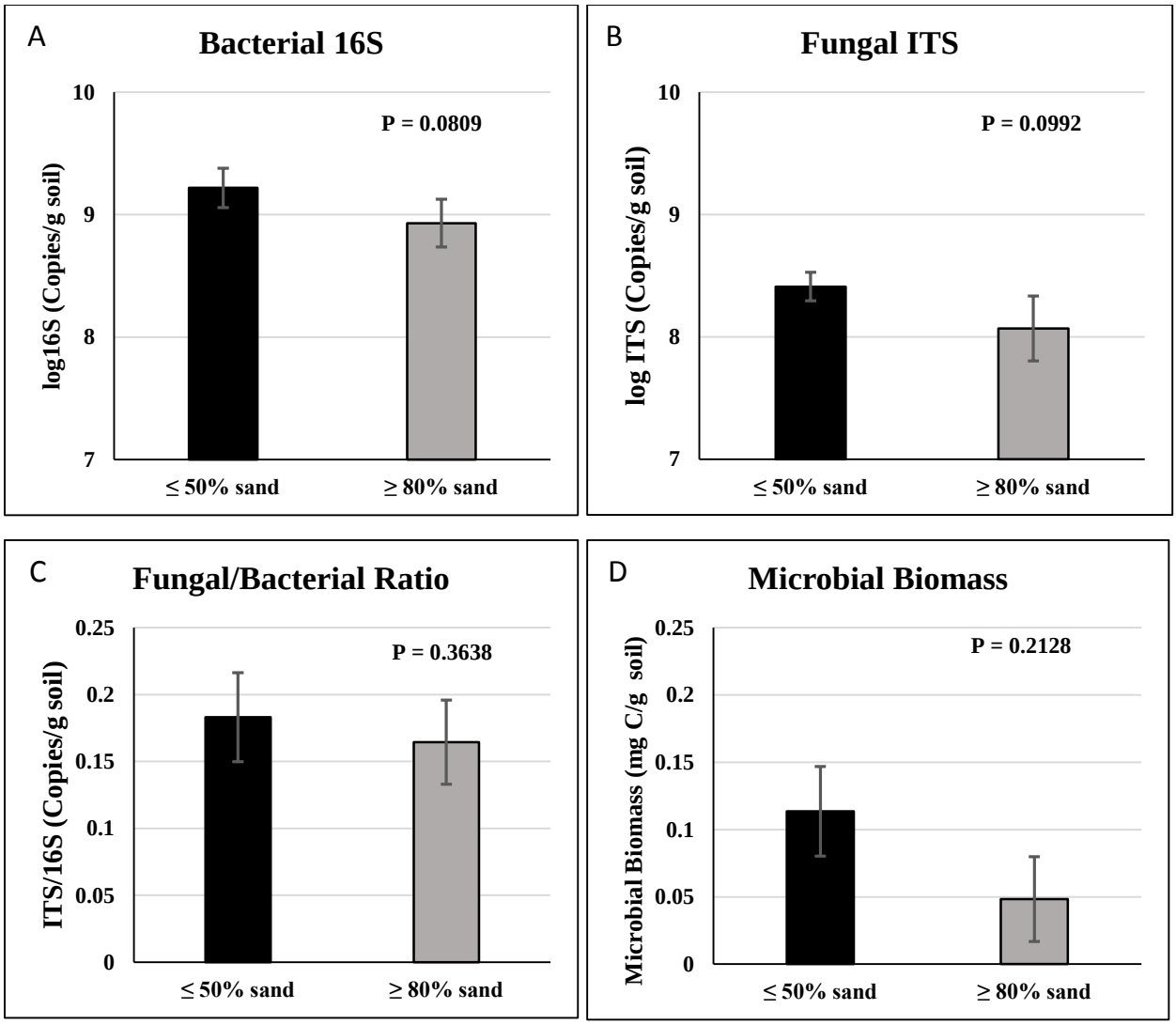

Figure 3.3 Mean values for total bacteria (A), total fungi (B), fungal/bacterial ratio (C), and microbial biomass (D) in all sampling locations of the 19 BRCs with known media compositions categorized by ≤ 50% sand (n=32) and ≥ 80% sand (n=40). Error bars represent the 95% confidence interval of the mean.

3.1.4 Vegetation Type

Grassed BRCs made up 4 of the 23 sampled cells and were characterized by those that only contained a grass species and did not contain a mulch layer or any type of herbaceous or woody cover. Landscaped BRCs made up the majority (11) of the sampled BRCs and were classified by those that appeared to have a defined planting scheme consisting of shrubs, herbaceous cover, and/or woody plant species. Overgrown BRCs made up the remaining 7 sampled BRCs and were classified by those in which native species grew among the previously planted vegetation or completely overtook the cell. Mean bacterial and fungal populations were significantly lower in the grassed BRCs, with indistinguishable differences between landscaped and overgrown cells (Figure 3.4). Mean microbial biomass values were also lower in the grassed BRCs, although this difference was not significant. Mean F/B ratios were higher in the grassed BRCs, although F/B ratios among vegetative types were not significant.

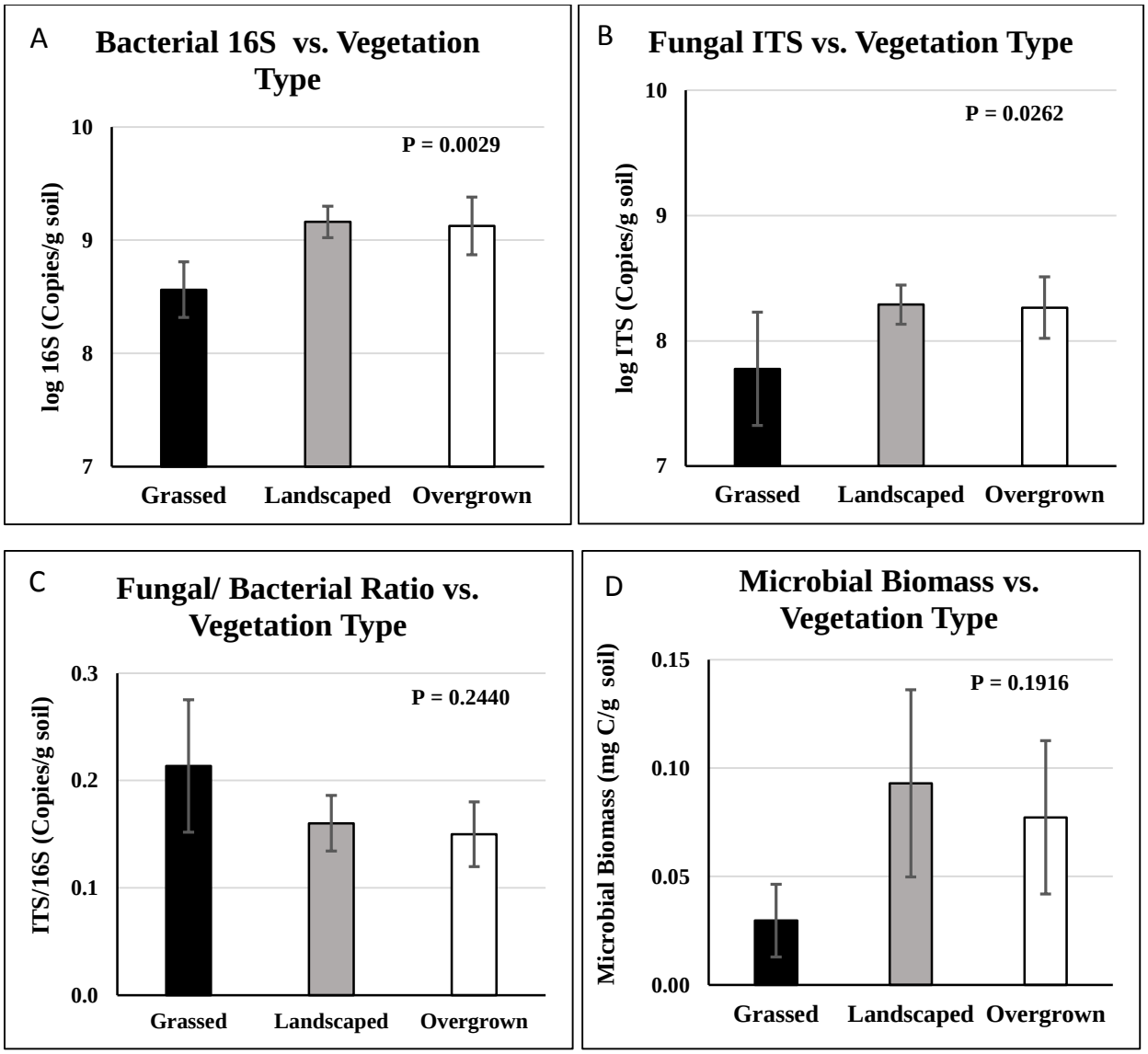

Figure 3.4 Mean values for total bacteria (A), total fungi (B), fungal/bacterial ratio (C), and microbial biomass (D) for all sampling locations in 23 BRCs with grassed (n=15), landscaped (n=48), and overgrown (n=24) vegetation schemes. Error bars represent the 95% confidence interval of the mean.

3.1.5 Total Organic Carbon

Extractable TOC concentrations ranged from 1.02 mg/g dry soil with a mean of 0.160 mg/g dry soil and a median of 0.114 mg/g dry soil. With the exception of F/B ratios, all of the response variables showed strong and statistically significant relationships with TOC (Figure 3.5). R^2 values ranged from 0.212 to 0.402 with the exception of the F/B ratio near zero. The relationship between microbial biomass and TOC was strongest followed by fungi and bacteria, respectively.

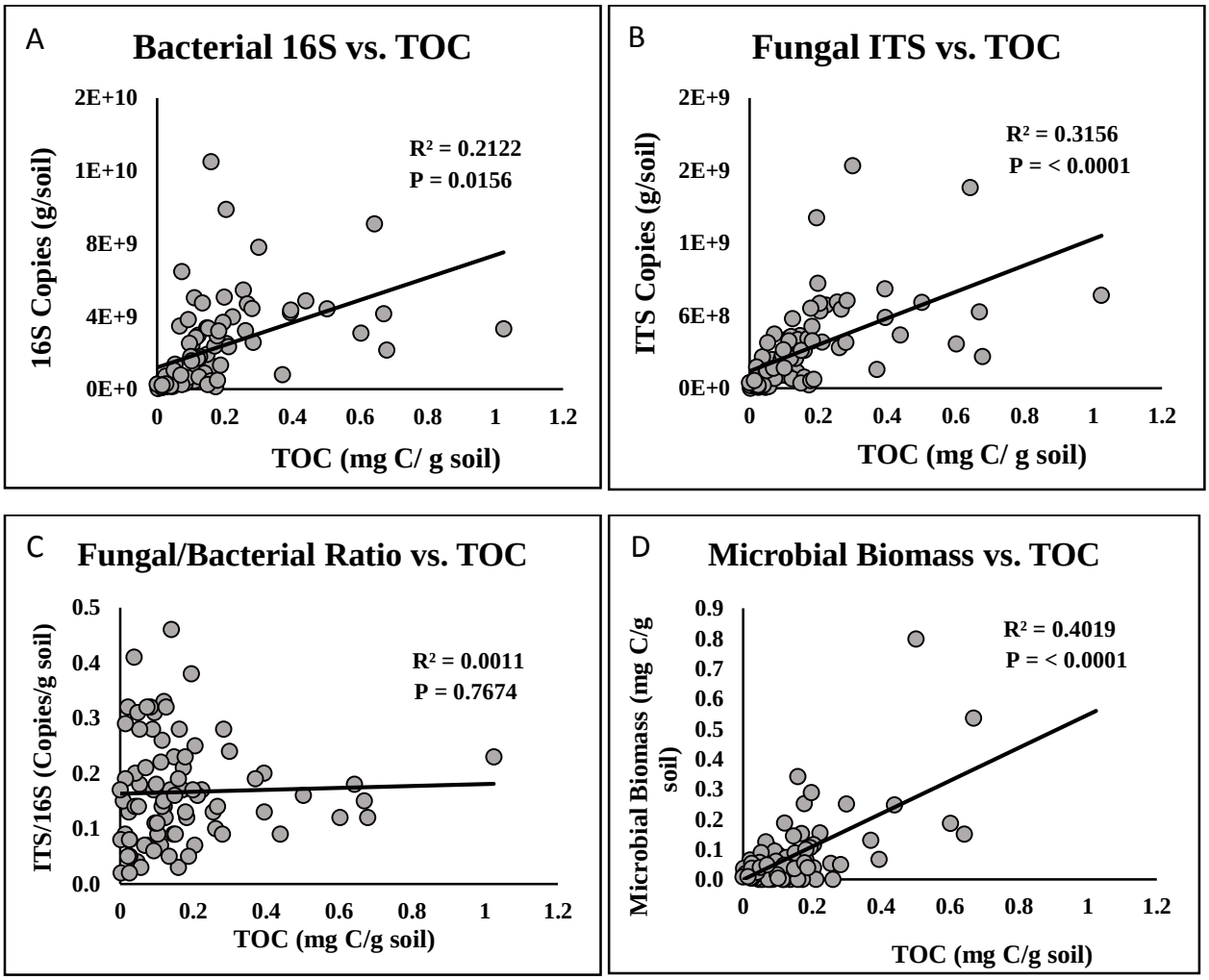

Figure 3.5. Linear regressions for all 4 sampling locations from each of the 23 BRCs sampled in this study (n = 86) for Bacteria (A), Fungi *(*B), Fungal/Bacterial Ratio (C), and Microbial Biomass (D) vs extractable total organic carbon.

3.1.6 Ammonium

Extractable ammonium (NH_4^+-N) concentrations ranged from below detection to 136 μg/g dry soil with a mean of 12.9 μg/g dry soil and a median of 6.95 μg/g dry soil. The R^2 values ranged from 0.00381 to 0.572 and all of the response variables indicated a significant linear relationship with the exception of the fungal/bacterial ratio (Figure 3.6). The regressions indicate that fungal populations seem to respond more strongly than bacteria to ammonium concentrations. Generally speaking, bacteria have lower C:N ratios compared to fungi, and thus the bacterial N demand is greater than that of fungi. Because of this, we expected to see a stronger response from bacteria, however, our results indicate the opposite effect. The relationship between ammonium and microbial biomass was weaker than the independent regressions for fungi and bacteria. Nonetheless, the R^2 value of 0.279 explains a significant amount of variation for a microbial population. Finally, the fungal/bacterial ratio best fine line is near zero and seems to be unaffected by soil media ammonium concentrations.

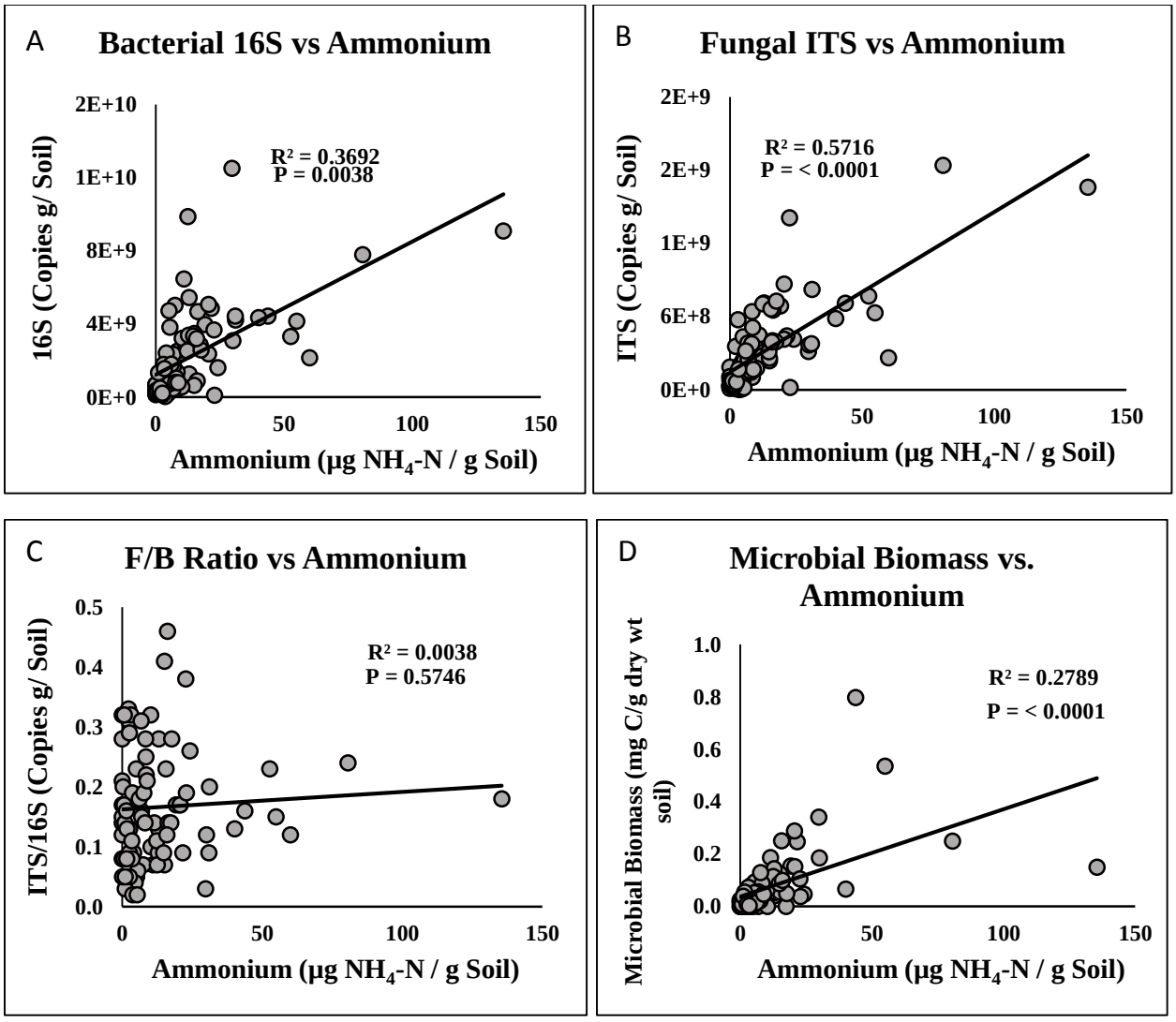

Figure 3.6. Linear regressions for all 4 sampling locations from each of the 23 BRCs sampled in this study (n = 86) for Bacteria (A), Fungi *(*B), Fungal/Bacterial Ratio (C), and Microbial Biomass (D) vs extractable ammonium.

3.1.7 Nitrite-Nitrate

Extractable nitrite-nitrate (NO_2^--NO_3^-) concentrations ranged from below detection to 38.2 μg/g dry soil with a mean of 6.27 μg/g dry soil and a median of 1.18 μg/g dry soil. Similar to ammonium concentrations, all of the predictors with the exception of the fungal/bacterial ratio elicited a strong response to soil media nitrite-nitrate concentrations (Figure 3.7). R^2 values ranged from 0.317 to 0.381 with the exception of the F/B ratio which was near zero. Opposite of ammonium concentrations, the R^2 value for the regression of bacteria and nitrite-nitrate was higher than that of fungi, which we hypothesized would occur due to the higher N demands of bacteria. Additionally, microbial biomass, which is an indicator of combined fungi and bacteria, populations was highest. The F/B ratio was not affected by nitrite-nitrate concentrations with the slope of the best fit line near zero.

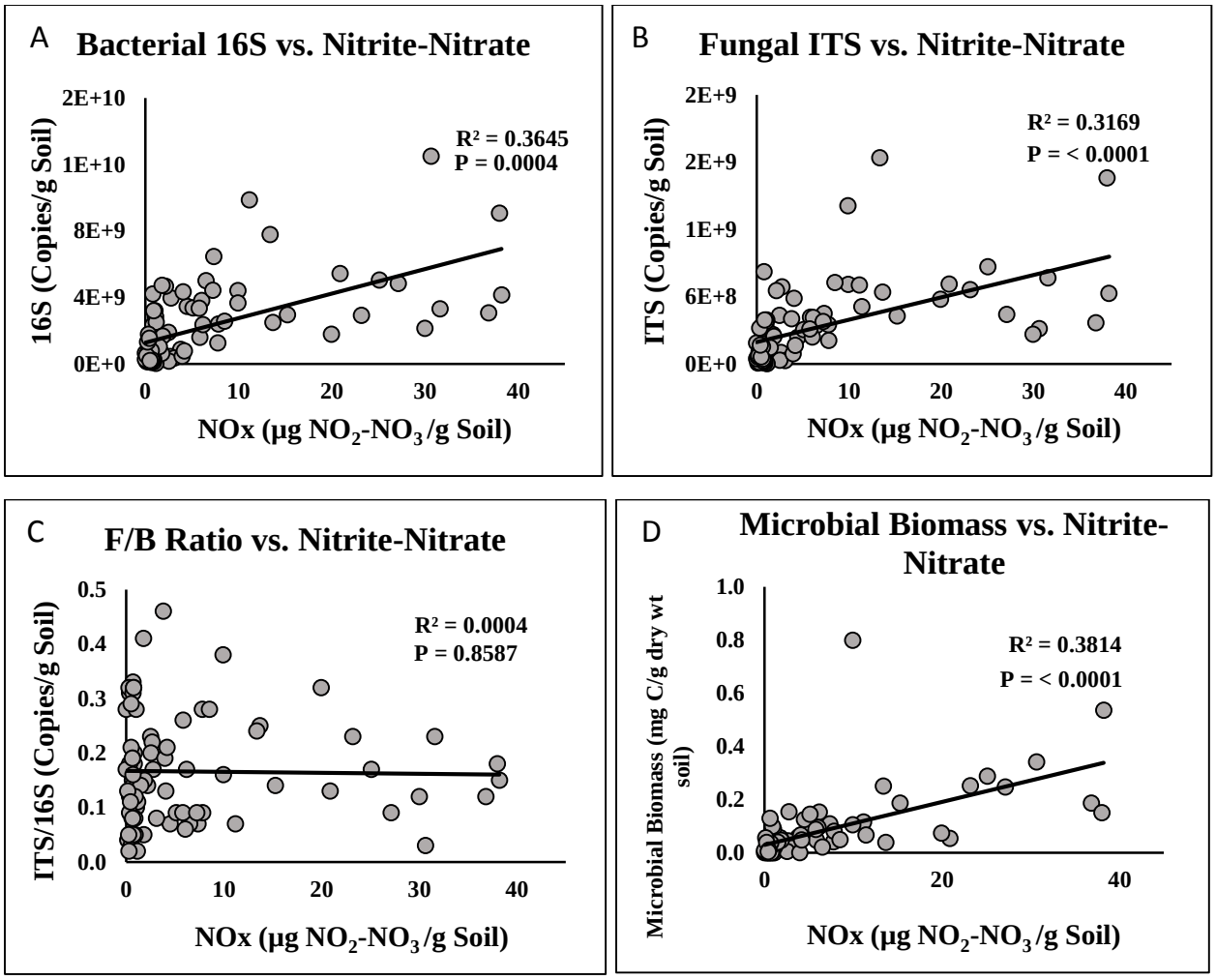

Figure 3.7. Linear regressions for all 4 sampling locations from each of the 23 BRCs sampled in this study (n = 86) for Bacteria (A), Fungi *(*B), Fungal/Bacterial Ratio (C), and Microbial Biomass (D) vs extractable nitrite-nitrate.

3.1.8 Temperature and Precipitation

Mean precipitation values for the locations of the sampled BRCs ranged from 1041 – 1244 mm/year with a mean value of 1117 mm/year and a median of 1117 mm/year. Mean temperature values ranged from 12.8 – 16.5 °C with a mean value of 14.2 °C and a median of 14.2 °C. Total bacteria and fungi demonstrated a significant negative linear relationship for both temperature and precipitation, suggesting that bacterial and fungal populations generally decrease as temperature and precipitation increase (Figure 3.8). However, this relationship was probably caused by the confounding effect of media mix and latitude, as discussed previously. Lower bacterial and fungal populations were found in media mixes with higher sand contents (Figure 3.3); BRCs with these media mixes were almost exclusively sampled from NC where mean precipitation and temperature are higher. Microbial biomass also slightly decreased with temperature and precipitation, while F/B ratios showed no effect of temperature or precipitation.

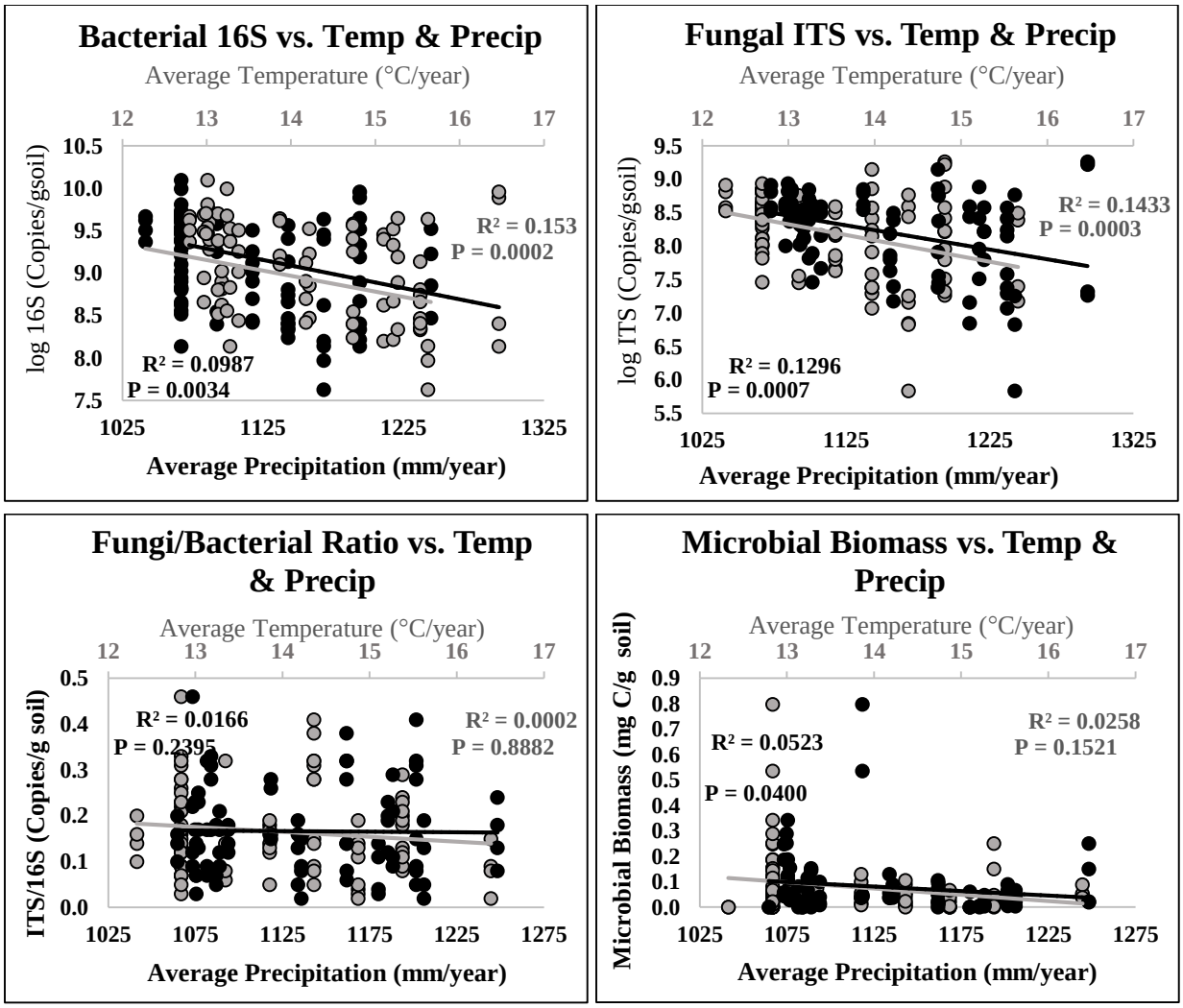

Figure 3.8. Linear regressions of average yearly temperature and precipitation vs 16S (n=85) (A), ITS (n=86) (B), Microbial Biomass (n=79) (C), and Fungi/Bacterial ratios (n=85) (D) in all BRC sampling locations. Data points correlate with colors of statistical values and axes. Gray represents temperature while black represents precipitation.

3.2 Microbial Biomass

Microbial biomass C values ranged from 0 to 0.534 mg microbial C/g dry soil with an average of 0.068 mg microbial C/g dry soil and a median of 0.039 mg microbial C/g dry soil. The model selection results indicate that microbial biomass C in the top 10 cm layer of the sampled BRCs was most influenced by the age of the BRC, followed by ammonium and nitrite-nitrate concentrations, and finally vegetative cover as the least important (Table 3.3). The results for the bottom 10 cm layers of the sampled BRCs identify organic C and N concentrations as most important, followed by media mix composition and the BRC surface area/runoff surface area ratio for microbial biomass C. When all of the samples were analyzed together, N concentrations were most important, followed by vegetation, which had a relative importance value (RVI) of only 0.32. It is important to note that the chloroform fumigation extraction method used for the determination of microbial biomass C is the most widely used but is not without error (Fierer et al., 2009). Extraction of non-microbial C in high organic matter soils can cause for over-estimation of microbial biomass measurements (Badalucco et al., 1997; Jenkinson et al., 2004). Therefore, microbial biomass C measurements in top 10 cm samples with high organic matter contents could be over-stated and the interpretation of the results should be approached with caution.

Linear regressions for ammonium, nitrite-nitrate, and TOC (Figure 3.6.D, Figure 3.7.D, and Figure 3.5.D) illustrate the strong relationships between nutrient and C sources for microbial biomass. R^2 values for TOC, ammonium, and nitrite-nitrate were 0.40, 0.28, and 0.38, respectively, which account for a large portion of variance in microbial biomass from an ecological perspective. Although the model selection results only identify TOC as the most important variable within the bottom 10 cm samples, linear regressions show a strong relationship ($R^2 = 0.40$) across all samples originating from both top and bottom samples (Figure 3.2.D). These results agree with research conducted on soil from a range of biomes, which also found strong relationships with increasing soil organic C and N with microbial biomass (Cleveland & Liptzin, 2007; Diazravina et al., 1993; Fierer et al., 2009; Wardle, 1998). Phosphorus was not quantified and may account for some of the variability in our microbial biomass measurements, as research has shown phosphorus to have a significant impact on microbial biomass (Cleveland & Townsend, 2006; Reed et al., 2007). However, due to the nutrient rich nature of stormwater, phosphorus limitations would not be expected in most BRCs.

Microbial Biomass Carbon

	Ammonium & Nitrite-Nitrate	TOC	Age	Media Mix	Vegetation	Surface Area Ratio	Location
Top 10 cm	0.86		0.97		0.30		
Bottom 10 cm	1.00	1.00		0.68		0.21	
Total Samples	1.00				0.32		

Table 3.3. Model averaging results for microbial biomass carbon in the soil media for the top 10 cm (n = 38), bottom 10 cm (n = 36), and total sample analysis (n=80).

3.3 Total Bacteria

Bacterial 16S gene abundances ranged from $4.24x10^7$ copies/g field moist soil to $3.61x10^{10}$ copies/g field moist soil. The mean and median gene copies were $2.99x10^9$ gene copies/g of soil and $1.33x10^9$ gene copies/g of soil, respectively. The model selection results shown in *Table 3.4* for the bacterial 16S gene indicate N concentrations, temperature and precipitation, and cell depth to be the most important variables controlling total bacterial populations. Vegetation type, followed by N concentration, were the most important variables for the top 10 cm sampling location. Relative variable importance (RVI) values for the bottom 10 cm samples were very close to one (>0.90) for cell depth, the temperature and precipitation variable, and N concentration, indicating these variables were most important. When all samples were analyzed together, the model selection results were less clear. All of the variables were assigned relative variable importance (RVI) values very close to one with the exception of pH and media mix. Although the results indicate that all of the variables with values of one are most important, their similarity in value decreases the ability to distinguish if one these variables was more influential to bacterial populations than another.

Although one of our samples had total 16S gene copies as high as $3.61x10^{10}$, the qPCR triplicate sample results were consistent and these findings are in agreement with 16S gene copy numbers in low sand, high organic matter soils, similar to the BRC media mix from which the sample originated (33% compost, 33% perlite, 33% soil) (He et al., 2007; Lopez-Gutierrez et al., 2004). Our results are similar to the findings of Chen et al. (2013), who found total bacterial gene copies in the sampled BRC to range from $2x10^7$ to $7x10^9$. The 16S gene copy numbers were seven times higher in the top 10 cm samples compared to the bottom 10 cm across the sampled BRCs (Figure 3.2.A). Chen et al. (2013) reported that total bacteria were 13 times higher. Similarly, research by Willard et al. (2014) also found 16S gene copy numbers in higher abundances in the upper than lower layers of the BRC. Chen et al. (2013) found significant inverse relationships between 16S concentrations with both depth and nitrite-nitrate concentrations, similar to our results in the model selection analysis (Table 3.4 and Figure 3.7.A).

The model selection results identified BRC media mix depth as one of the most important variables for the bottom 10 cm samples and for the total sample analysis. The total sample

regression for 16S abundance vs cell depth produced a weak but significant inverse relationship (Figure 3.9.A). When the bottom 10 cm samples were individually regressed, the relationship was substantially stronger (Figure 3.9.B), suggesting that bacteria in the bottom layers of BRCs are more affected by depth than bacteria residing in the top layers. Additionally, none of the measured predictor variables varied across media mix depths in the bottom 10 cm samples; regressions for ammonium, nitrite-nitrate, pH, and TOC vs cell depth in the bottom 10 cm samples were statistically insignificant. We hypothesize that oxygen penetration to BRCs with increasingly deep media mixes limit the growth of bacteria. Although a survey of bacterial populations across BRCs has yet to be carried out, these results agree with studies of soil systems that find decreasing bacterial populations with increasing depth (Hansel et al., 2008; Kandeler et al., 2009; Marhan et al., 2011).

Total Bacteria:16S

	Ammonium & Nitrite-Nitrate	Media Mix Depth	Temp & Precip	Vegetation	pH	Media Mix	Location
Top 10 cm	0.98	0.48	0.59	1.00	0.76		
Bottom 10 cm	0.96	0.90	0.96			0.24	
Total Samples	1.00	0.98	1.00	1.00			1.00

Table 3.4. Bacterial 16S model selection results for the top 10 cm samples (n=37), bottom 10 cm samples (n=36), and total sample analysis (n=83).

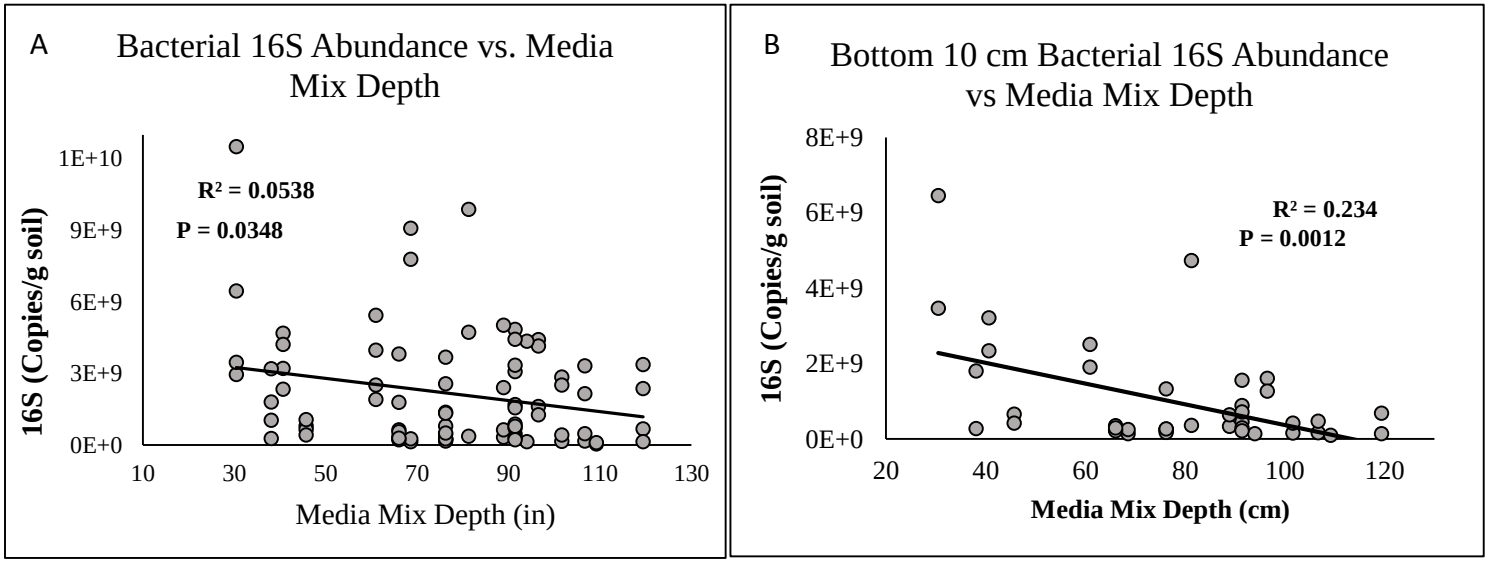

Figure 3.9. A: Linear regression of media mix depth vs16S gene abundance for all samples (n=83) in all sampled BRCs (n = 23). B: Total fungi vs. media mix depth in bottom 10 cm samples (n=43).

Figure 3.10 illustrates a principle coordinates analysis (PCoA) plot of similarity in bacterial community structure with samples divided into groups by location within the cell and the centroids plotted for each group. Top 10 cm samples were similar in diversity and species composition and significantly different from the bottom 10 cm samples, which were also similar to each other in diversity and community composition. No significant differences in dispersion or centroid location were detected among samples in the top 10 cm when analyzed tougher or the samples in the bottom 10 cm when analyzed together. However, significant values for both location and dispersion, which are metrics of community similarity and diversity, were significant when the bottom samples were compared to the top. While were significant differences in community structure when comparing among other categorical data types, we focused on cell location for futher analysis and discussion because it had the strongest effect.

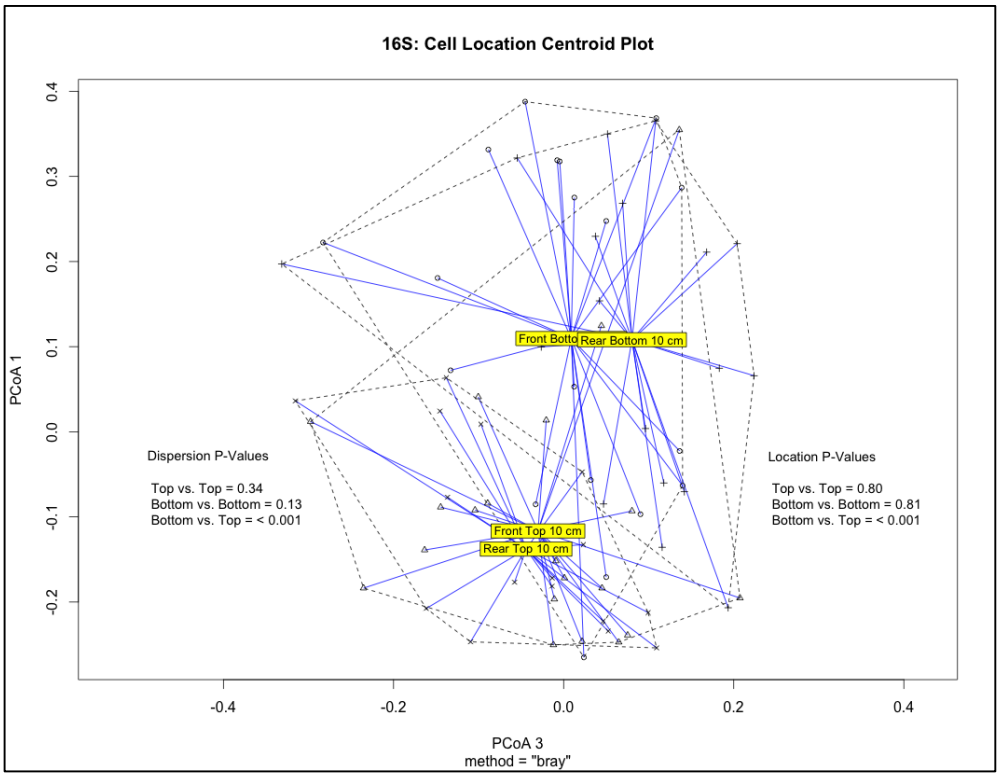

Figure 3.10. Bacterial community centroids and permutational ANOVA significance values by sampling location.

The distance based redundancy analysis plot for the bacterial community sequences salso eparated most noticeably by sampling location (Figure 3.11). Similar to the centroid plots, the separation of the points indicates that the the community composition between the top and

bottom sampling locations are different from each other. There were not differences between the front (BRC inlet) and rear (BRC overflow structure) and, thus, all top and bottom samples were combined. Similar to the centroid plot for sample location (Figure 3.9), the top and bottom 10 cm samples distinctly separate on a diagonal plain running from the top left hand corner to the bottom right hand corner of the plot. A large proportion of top 10 cm samples are clustered around the ammonium and nitrite-nitrate variable, while a large proportion of bottom 10 cm samples are clustered opposite of the TOC vector. The orientation of these points in relation to the environmental vectors indicate that bacterial community structure in the top 10 cm is influenced by an abundance of N while community structure is effected by a lack of TOC in the bottom layers of the BRCs. A large amount of variability also correlated with other factors, such as BRC age, temperature, precipitation, and cell depth. The clustering of samples around the BRC age vector suggests that bacterial communities may be expected to change in composition over time, but note that temperature and precipitation vectors closely parallel the age vector, making it difficult to tease out which factors may cause for the variability.

Several studies have observed shifts in bacterial community composition and diversity with increasing N inputs (Allison et al., 2008; Campbell et al., 2010; Ramirez et al., 2010). The differences in community structure between the top and bottom 10 cm samples may be explained by the *copiotrophic* hypothesis. The hypothesis suggests that under relatively high resource availability, similar to the conditions in the upper layers of BRCs, community structure shifts towards bacterial taxa that can quickly break down labile C sources. In conditions with relatively low nutrient and resource abundance, such as the lower layer of BRCs, slower growing taxa that the breakdown of more recalcitrant organic C tend to dominate (Fierer et al., 2007; Fontaine et al., 2003; Ramirez et al., 2010). The change in community structure may also have contributed to total bacterial populations, potentially explaining the model selection results in Table 3.4. Additionally, the larger percentage of the *Proteobacteria*, *Actinobacteria,* and *Bacteriodetes* phylum that make up the bacterial community in the top 10 cm compared to the bottom 10 cm samples (Figures 3.12 and 3.13) are in agreement with previous studies that documented similar phylum change in N amended soils (Fierer et al., 2007; Nemergut et al., 2008; Ramirez et al., 2010).

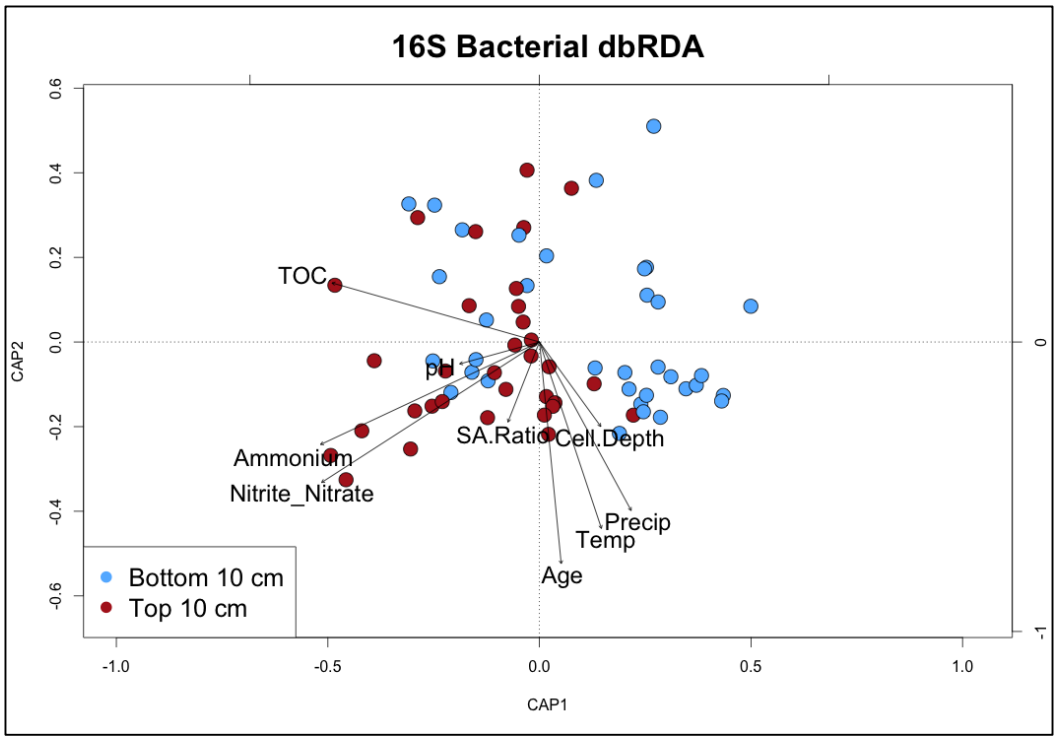

Figure 3.11. Bacterial distance based redundancy analysis plot by sample location.

The bacterial taxonomic structure of the BRC soil samples show *Proteobacteria, Acidobacteria, Actinobacteria* and *Bacteriodes* dominate the structure of the bacterial communities, making up 80% of the top 10 cm samples and 73% of the bottom 10 cm samples. *Chloroflexi, Verrucomicrobia, Firmicutes, Gemmatimonadetes,* and *Planctomycetes* made up the majority of the remaining phyla, comprising 13 and 16% of the top and bottom 10 cm samples, respectively. For ease of interpretation, phyla that made up less than 1% of the sequences were not included in the bar graph; hence, the totals are less than 100%. In general, *Proteobacteria, Actinobacteria,* and *Bacteriodes* made up greater proportions of the upper 10 cm samples, while *Acidobacteria* and *Chloroflexi* made up a greater proportion of the bottom 10 cm samples. Shannon diversity values, which is a metric commonly used to describe diversity differences based upon the presence and abundance of species in each community, calculated to be 7.3 for the top 10 cm samples and 7.1 for the bottom 10 cm samples.

Research relating microbial taxonomy, diversity, and functional traits to ecosystem processes has become a topic of interest for microbial ecologists. Insight into these relationships could greatly contribute to our understanding and potential manipulation of ecosystem function

in the future. This is made difficult, however, due to number of functional traits that are shared across species and the changing interactions across community assemblages (Philippot et al., 2010). Recent approaches have attempted to determine relationships between microbial classifications and their associated environments in an attempt to infer on ecosystem function (Koch, 2001). Although strictly associating species composition and environment types is likely an over-simplification of ecosystem function, our data does agree with some broad classifications of soil taxa and their typically associated environmental conditions.

Our species composition findings agree with previous investigations into soil bacterial community structure which also found *Acidobacteria, Actinobacteria, Proteobacteria,, Verrimicrobia*, and *Bacteriodetes* to be the dominant phyla in soils across multiple biomes (Fierer, Leff, et al., 2012; Fierer et al., 2009; Lauber et al., 2009). *Acidobacteria* are commonly associated with and have been found to persist in environments with low pH values (Jones et al., 2009; Lauber et al., 2009; Nacke et al., 2011). And although we found higher proportions of these bacteria in the bottom 10 cm samples (shown in Figure 3.10), where nutrients and organic matter are more scarce, the mean pH values for these locations were neutral and nearly identical (6.92 in the top 10 cm and 6.86 in the bottom 10 cm). Fierer et al. (2012) using cross biome bacterial sequencing, found *Bacteroides* to be more prevalent in desert soils, suggesting these bacteria are potentially more resistant to desiccation. *Bacteroides* were higher in abundance in the top 10 cm of BRCs, where the media would be expected to experience more frequent dry periods than the lower layers (Figure 3.10). *Proteobacteria* populations, specifically *β-Proteobacteria* and *γ-Proteobacteria,* have demonstrated considerable population shifts in response to the additions of C substrates (Eilers et al., 2010). *Proteobacteria* made up 5% more of the phyla in the top 10 cm samples (Figures 3.10 and 3.11), where C concentrations are higher, although the subphyla *β-Proteobacteria* and *γ-Proteobacteria* are only slightly higher (1%). Additionally, abundances of *Actinobacteria, Bacteroidetes, Gemmatimonadetes* and *γ-Proteobacteria* have all been observed to increase with N inputs (Fierer, Lauber, et al., 2012; Nemergut et al., 2008; Ramirez et al., 2010). Our results show higher average abundances of all these phyla with the exception of *Gemmatimondaetes* in the upper 10 cm samples where mean inorganic N concentrations were 5 times higher than the bottom 10 cm samples. Finally, higher abundances of *Firmicutes* have been found in the early stages of forest succession and are thought to persist due to the ability of some members such as *Bacilli* and *Clostridia* to sporulate.

The higher abundances of both of these members (Figure 3.12) may explain why these species are in relatively higher abundances in the bottom of BRCs, where substrate and nutrient concentrations are lower.

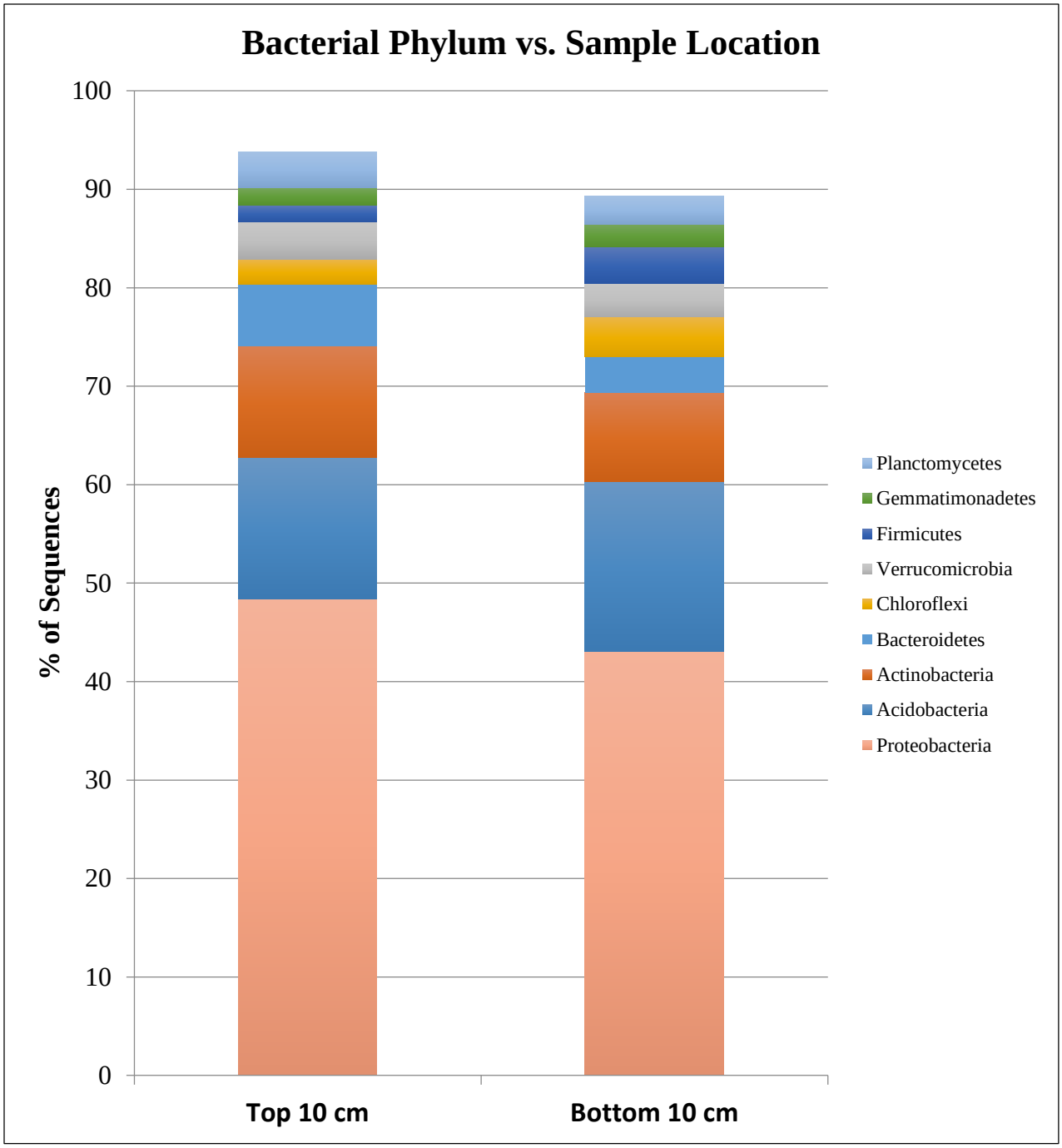

Figure 3.12. Averaged bacterial community composition by phylum within the top (n = 38) and bottom 10 cm (n = 37) samples.

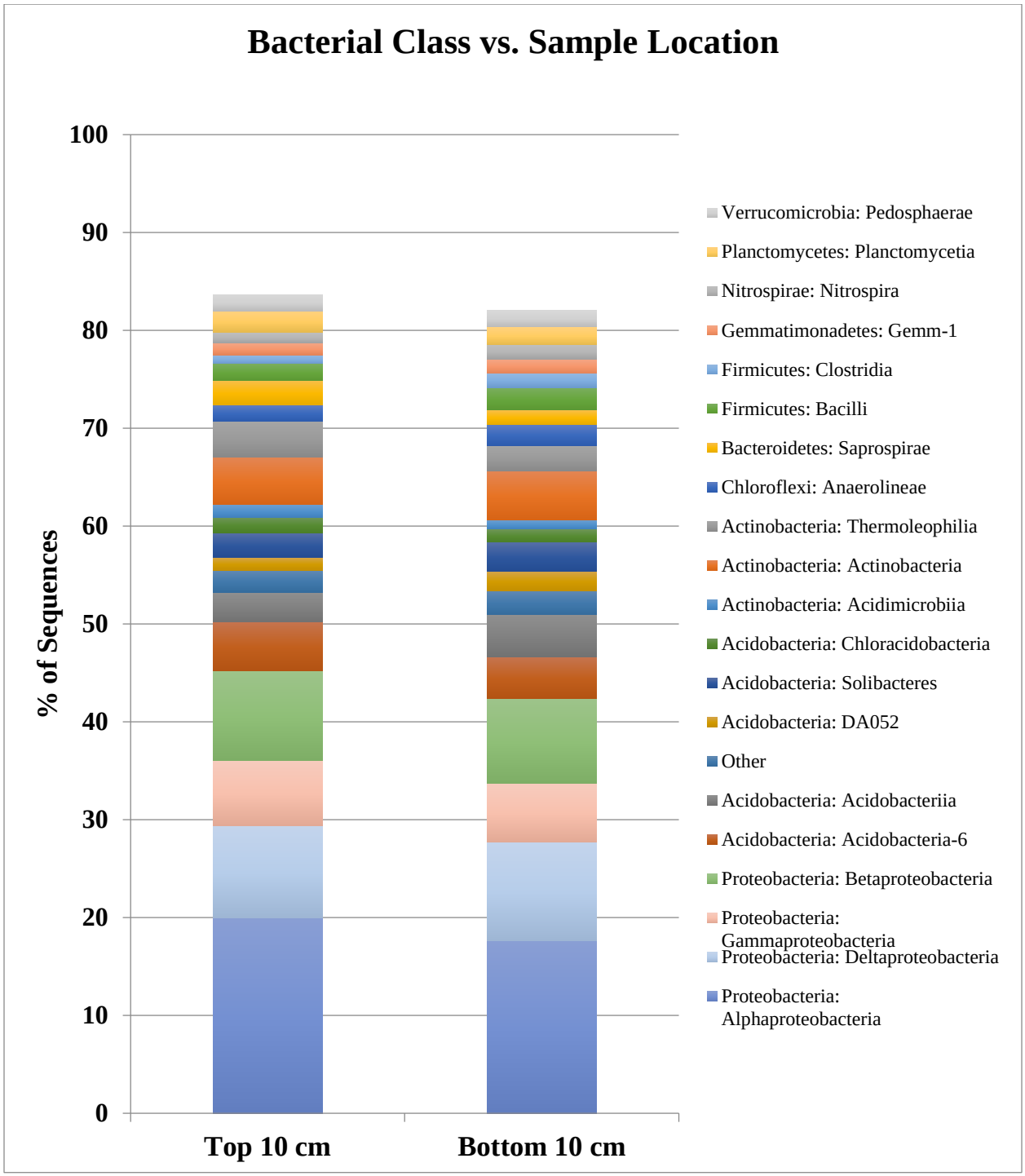

Figure 3.13. Averaged bacterial community composition by class within the top (n = 38) and bottom 10 cm (n = 37) samples.

3.4 Total Fungi

Fungal ITS gene abundances ranged from 6.82×10^5 copies/g field moist soil to 1.84×10^9 copies per g of soil. The mean and median gene copies were 3.13×10^8 copies/g field moist soil and 2.38×10^8 copies/g field moist soil, respectively. The model selection results shown for total fungi (Table 3.5) shows that inorganic N is the most important variable controlling fungal populations in both the top and bottom 10 cm of the sampled BRCs. The bottom 10 cm sample results report RVI values of 1 for inorganic N, cell depth, media mix composition, and TOC, while temperature and precipitation identified as the least important variable among the top models. RVI values of one indicate that these variables were contained in all of the top models and made up all of the Akaike's weight, or confidence in the models, for all response variable combinations. This indicates that all of these variables played an equal role in improving the ability of the model to predict ITS gene copy numbers. We are unable to distinguish which of these may contribute the most to fungal concentrations in the bottom 10 samples of BRCs. When all samples were included in the analysis, sample location and N concentrations were the top variables for the model and were equally important.

Our findings agree with a previous study which observed increasing fungal populations with increases in N amendments (Reeleder et al., 2006) but are opposite of Frey et al. (2004) who found decreasing fungal biomass with N additions. Increases in fungal populations with increasing N could be caused by a greater availability of N for biological assimilation, or increases in root exudation and C compounds released by vegetation. The interaction between fungi, plants, and resource availability may also explain differences in fungal community composition in relation to BRC vegetative cover. Similar to bacteria, when the bottom 10 samples were isolated, ITS gene copies significantly decreased with cell depth (Figure 3.14), agreeing with the findings of Fierer et al. (2003), who also observed decreasing fungi with depth. We hypothesize this was most likely due to decreases in oxygen availability, nutrients, and C in these layers.

The plotted centroids on the PCoA plot for the fungal sequencing data illustrate how the community structure is different in BRCs with various vegetative covers (Figure 3.15). Even though fungal communities from landscaped and overgrown BRCs are significantly different from each other (p-value of 0.0001), they are not very distinct in ordination space. Both, however, are clearly different from fungal communities in grassed BRCs. The PERMANOVA p-

values for dispersion, or a measure of variability among communities within a group, ranged from 0.13 to 0.55. The p-values for the centroid location were all < 0.0001, meaning that based on the number of sequences assigned to the individual operational taxonomic units (OTUs) across vegetation types, these communities were significantly different from each other.

Total Fungi: ITS

	Ammonium & Nitrite-Nitrate	Cell Depth	TOC	Media Mix Composition	pH	Vegetation	Temp & Precip	Location
Top 10 cm	1.00				0.80	0.18		
Bottom 10 cm	1.00	1.00	1.00	0.98			0.18	
Total Samples	1.00	0.89				0.63	0.79	1.00

Table 3.5. Model averaging results for total fungi in the soil media for the top 10 cm (n = 39), bottom 10 cm (n = 36), and total sample analysis (n = 83).

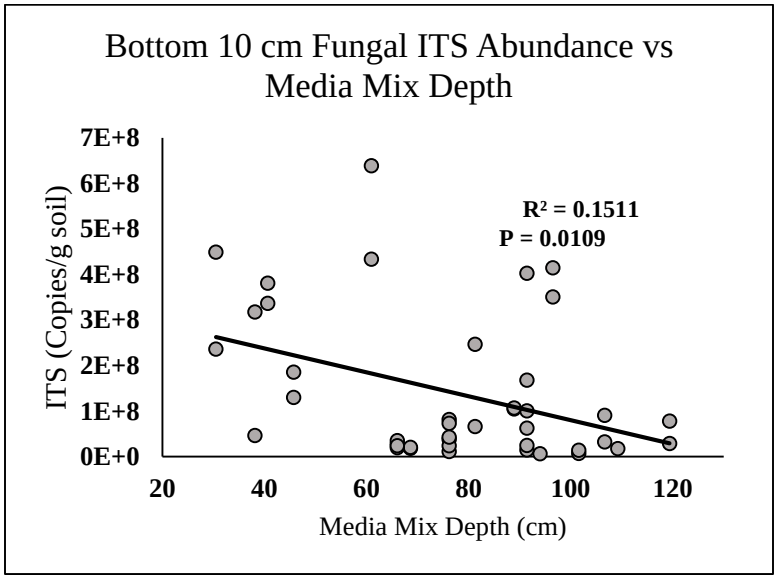

Figure 3.14. Total fungi vs. media mix depth in bottom 10 cm samples (n=43).

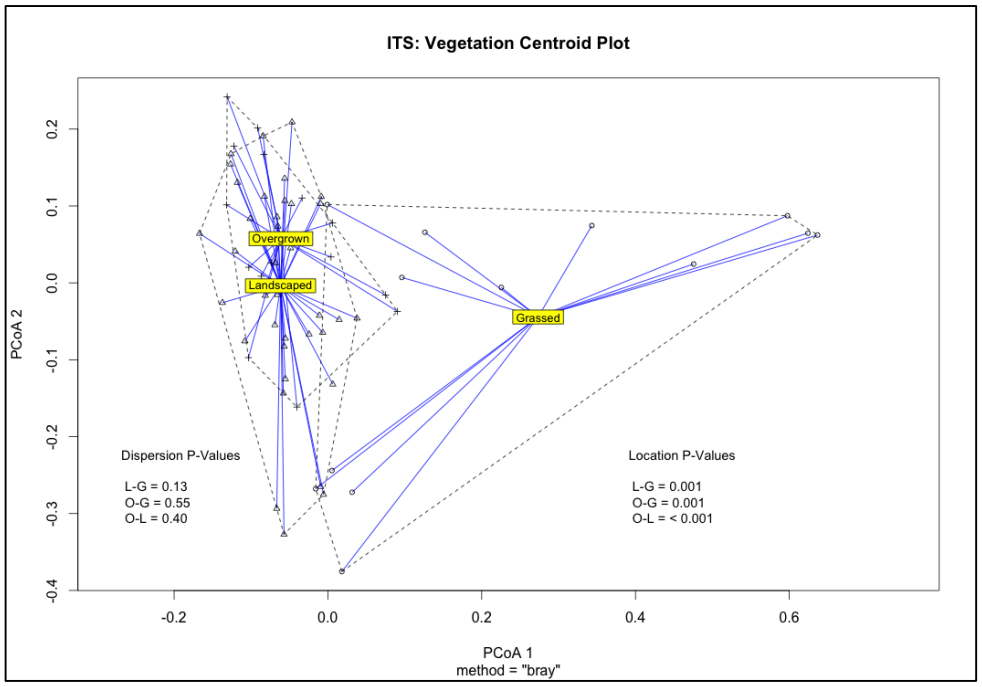

Figure 3.15. Fungal community centroids and PERMANOVA significance values by BRC vegetation type.

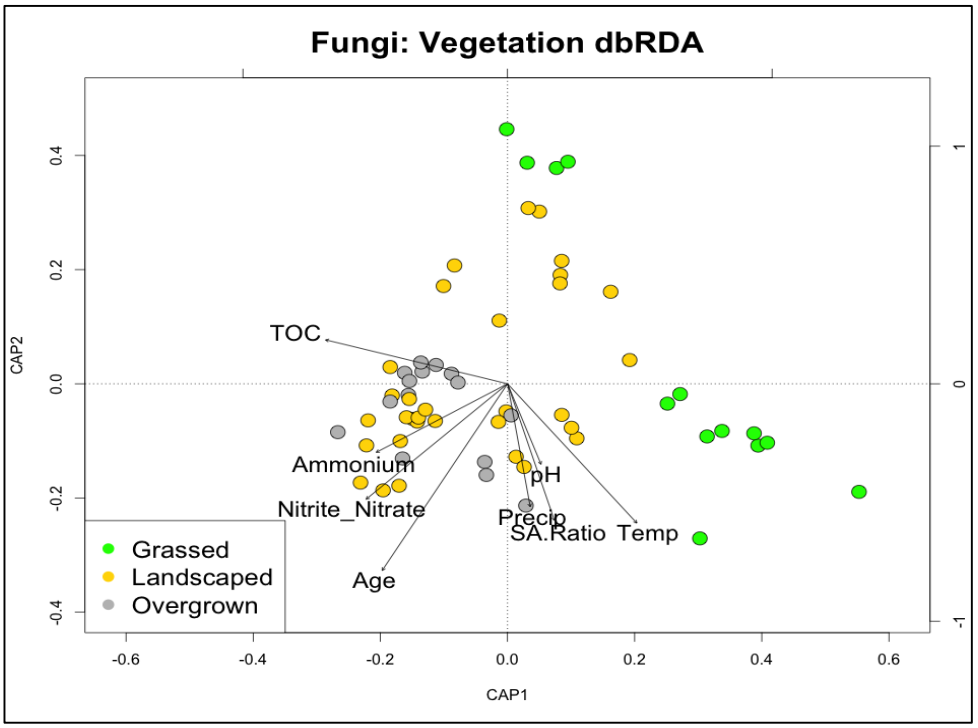

Figure 3.16. Fungi distance based redundancy analysis plot by BRC vegetation type.

The model selection results and linear regressions show that total fungal populations are largely driven by N concentrations (Table 3.5 and Figures 3.2B, 3.3B). Similarly, the dbRDA plot (Figure 3.16) suggests that fungal community composition in many of our samples is also influenced by both organic C and N concentrations. A large portion of the landscaped and overgrown BRC samples clustered around and in-between the ammonium, nitrite-nitrate, and TOC vectors, suggesting the fungal community composition in these samples were correlated with higher C and N concentrations. Samples that were collected from grassed BRCs cluster separately from the landscaped and overgrown BRCs and opposite of the TOC vector, an indication that these fungal communities were shaped by low C concentrations, which could have been a function of the vegetation. Several landscaped and overgrown samples clustered around the pH, precipitation, and surface area ratio vectors, making it difficult to infer which of these might have contributed most to fungal community composition. And unlike the bacterial dbRDA plot, there seems to be no samples clustered on or opposite of the age vector, an indication that time, at least on a relatively large scale, may not be as important in fungal communities.

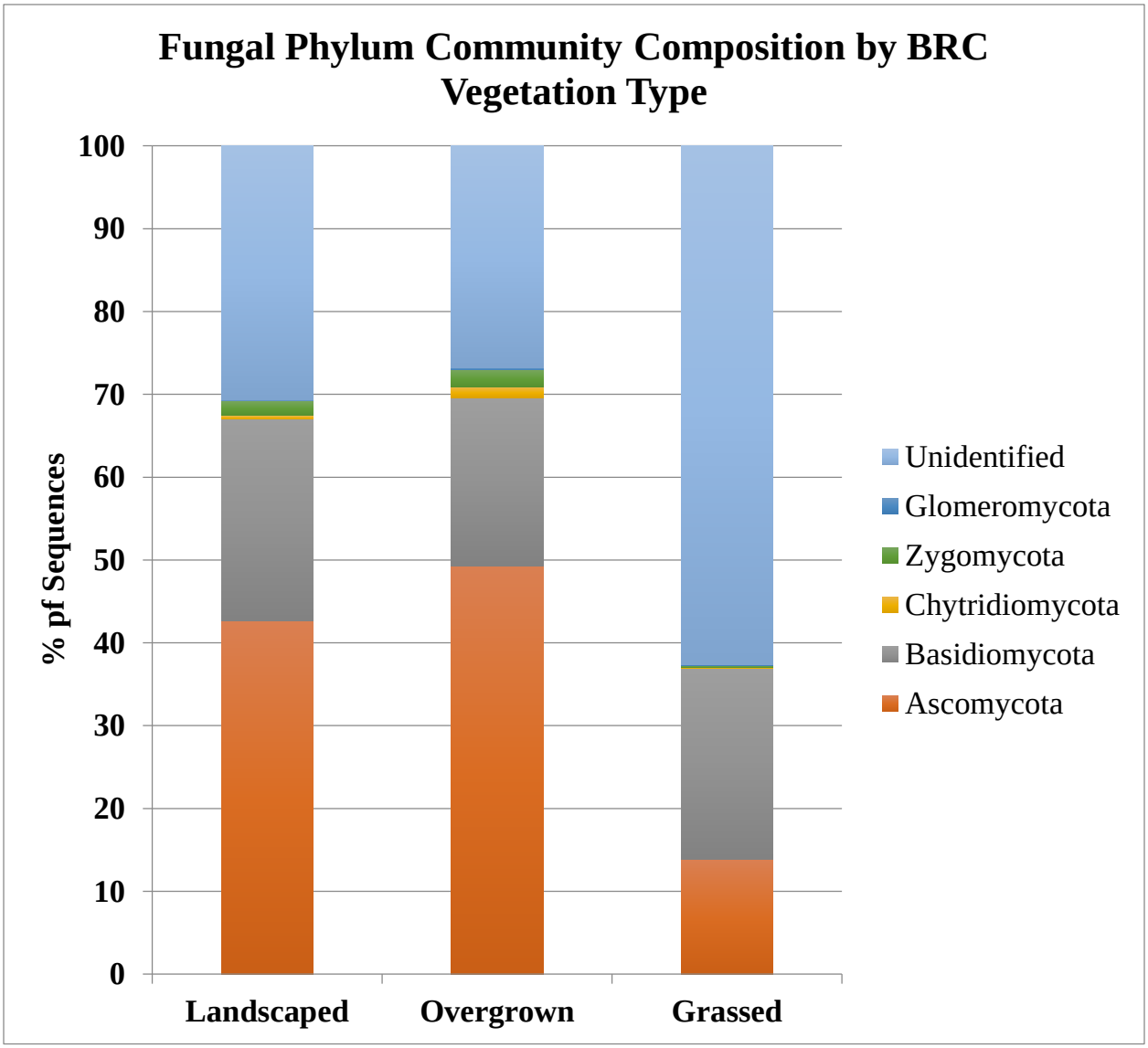

Figure 3.17. Average fungal community composition by phylum in landscaped (n=37), overgrown (n=17), and grassed (n=13) BRCs.

The phylum level classification results identified *Ascomycota* and *Basidiomycota* as the two dominant fungal phyla that collectively made up on average 67, 70, and 37% of the identified sequences on average in the landscaped, overgrown, and grassed BRCs, respectively. *Chytridiomycota*, *Glomeromycota*, and *Zygomycota* made up the greatest portion of the remaining identified sequences (Figures 3.16 & 3.17), representing an average of 2, 4, and < 1% of the sequences for landscaped, overgrown, and grassed cells, respectively. Unidentified phyla made up 31% of landscaped cells, 27% of overgrown cells, and a much higher 63% for grassed BRCs. Calculated Shannon diversity values for landscaped BRCs were 3.6, 3.3 for overgrown,

and 2.3 for grassed BRCs, suggesting that fungal communities were considerably less diverse in grassed BRCs.

Recent research has attempted to determine trends and averages of fungal community composition and composition across the globe and throughout biomes. Tedersoo et al. (2014) sequenced nearly 15,000 topsoil samples from 365 locations across the globe and found *Basidiomycota* to make up nearly half, *Ascomycota* a third, and *Mortierellomycotina* and *Mucoromycotina* together representing ten percent of the total sequences. Although *Ascomycota* and *Basidiomycota* made up the majority of our samples, neither *Mortierellomycotina* or *Mucoromycotina* were identified within our samples. Interestingly, they also identified the ratio of *Ascomycota* to *Basidiomycota* close to 1.7 in shrublands and tropical dry forests while lowest (0.88) in deciduous forest. Despite obvious differences between ecosystem climates and BRC conditions that make these systems difficult or impractical to compare, our results indicated the lowest *Ascomycota* to *Basidiomycota* ratio in grassed BRCs (0.6) and higher in the landscaped (1.8) and overgrown (2.4) BRCs.

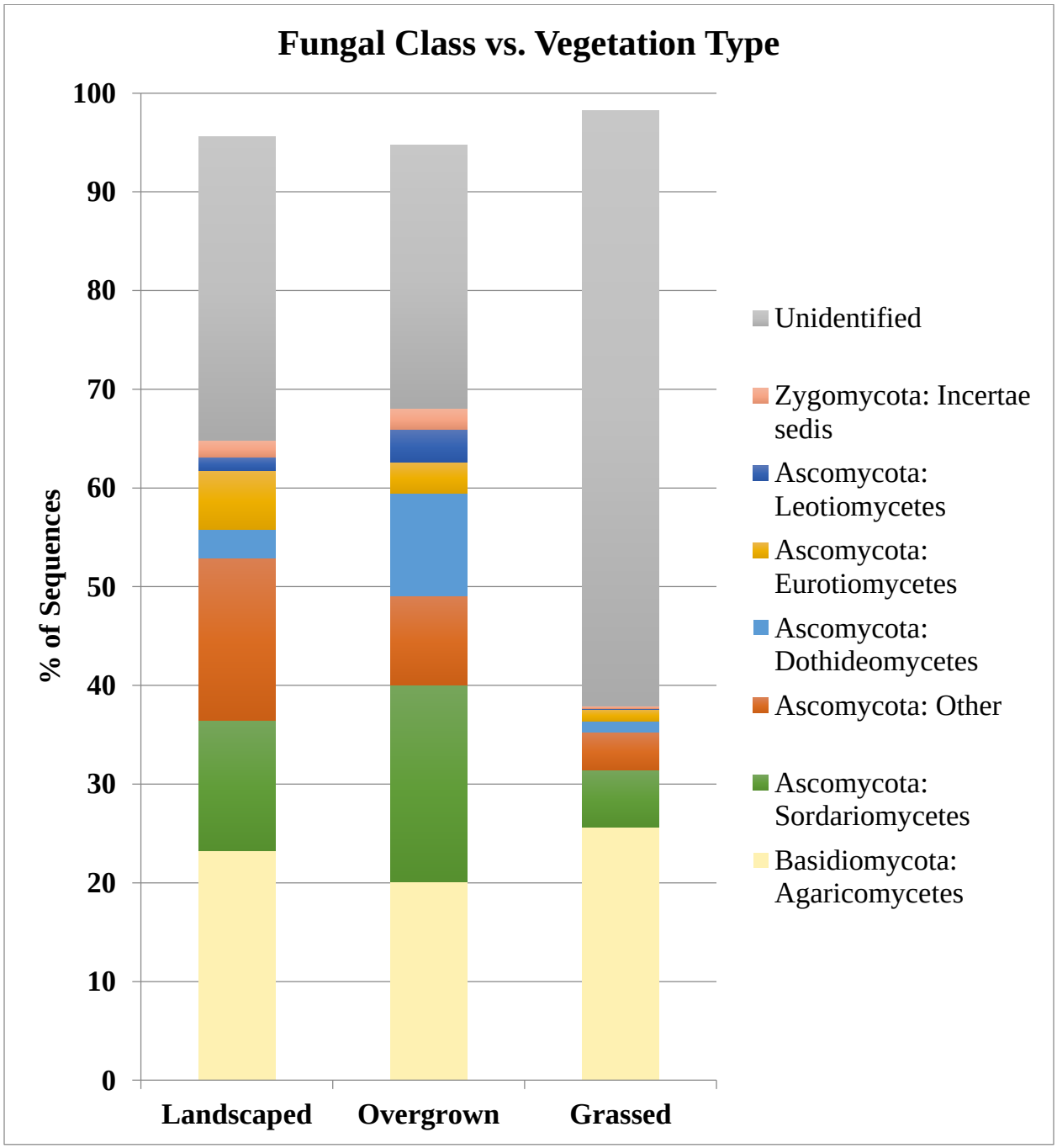

Figure 3.18. Average fungal community composition by phylum in landscaped (n=37), overgrown (n=17), and grassed (n=13) BRCs.

3.5 Fungal/Bacterial Ratio

Fungal/bacterial (F/B) ratios ranged from 0.02 to 0.50 with a mean of 0.17 and a median of 0.14. Our F/B ratios were in accordance to a previous study that quantified F/B ratios across ecosystem types, but our values were more similar to the desert and prairies studied, where F/B ratios are less than 0.5, rather than forest soils, where fungal genes were up to 4 times higher than those of bacteria (Fierer et al., 2005). The model selection results indicate vegetation type followed by the combined temperature and precipitation variable and the presence of a saturated zone to be the most important variables driving F/B ratios in the top 10 cm samples (Table 3.6). Bottom 10 cm results suggest media mix composition to be the primary variable effecting F/B ratios with region and the temperature and precipitation variable appearing in the top models but much less important than media mix. The total sample analysis indicates pH to be the most important variable followed by media mix composition, in controlling F/B ratios when all of the samples are taken into account.

Although the variables in model averaging results for the total sample analysis did not reach one, which is an indication of the highest possible confidence, pH was identified as the most important variable for F/B ratios. pH is often considered to be a "master variable" controlling microbial populations and many studies have reported positive relationships between F/B ratios and pH (Hogberg et al., 2007; Joergensen & Wichern, 2008; Rousk et al., 2009). However, upon further inspection, the linear regressions of bacterial/ fungal ratios with pH, (Figure 3.20) result in an inverse, weak and statistically insignificant correlation, contrary to the literature. The weak relationship between the bacterial and fungal ratios is likely caused by the limited range of pH values that were measured in the BRC media mix samples. All of the pH values were relatively neutral and narrow in range (5.6-8.3 with a mean and median of 6.9) and the lack of variability in the samples most likely played a minimal role in F/B ratios.

F/B ratios are thought to be controlled by many different environmental and soil conditions including nutrient availabilities and their respective ratios (Güsewell & Gessner, 2009; Suzuki et al., 2009), pH (Baath & Anderson, 2003; Rousk et al., 2010), and soil moisture/drying re-wetting events (Cosentino et al., 2006; Gordon et al., 2008). The RVI values for all of the variables in the model selection results were very low compared to those obtained for models of bacteria and fungi individually. When F/B ratios were individually examined, none

of the collected BRC design or environmental parameters, including the temperature and precipitation variable identified as important in the model selection results, indicated significant relationships or differences. However, when the F/B ratios were averaged across media mix compositions and the top and bottom 10 cm samples were analyzed separately, an interesting relationship appeared. In the top 10 cm samples F/B ratios were higher in media mixes with ≤ 50% sand, while in the bottom 10 cm samples, F/B ratios were higher in media mixes with ≥ 80% (Figure 3.19.A & B). Although only the bottom 10 cm sample differences were significant, the opposing relationships are interesting. It is difficult to infer what might have caused this relationship. Studies on fungi and F/B ratios have found varying resistances to moisture stress (Hamer et al., 2007; Williams et al., 1972) and C pools (Bailey et al., 2002; Busse et al., 2009), two factors which might be very different across these media mixes and locations. Ultimately there are likely many factors such as bacterial and fungal community composition, pH gradients, C pools, and nutrient availabilities that work together to shape the fungal and bacterial communities, making it difficult to determine which may play the largest role in controlling F/B ratios in BRCs.

Fungal/Bacterial Ratio

	Media Mix Composition	pH	Temp & Precip	Region	Vegetation	ISZ
Top 10 cm	0.26		0.45		0.31	0.27
Bottom 10 cm	0.92		0.28	0.44		
Total Samples	0.30	0.71				

Table 3.6. Model averaging results for the fungal/bacterial ratio in the soil media for the top 10 cm (n = 43), bottom 10 cm (n = 36), and total sample analysis (n = 67).

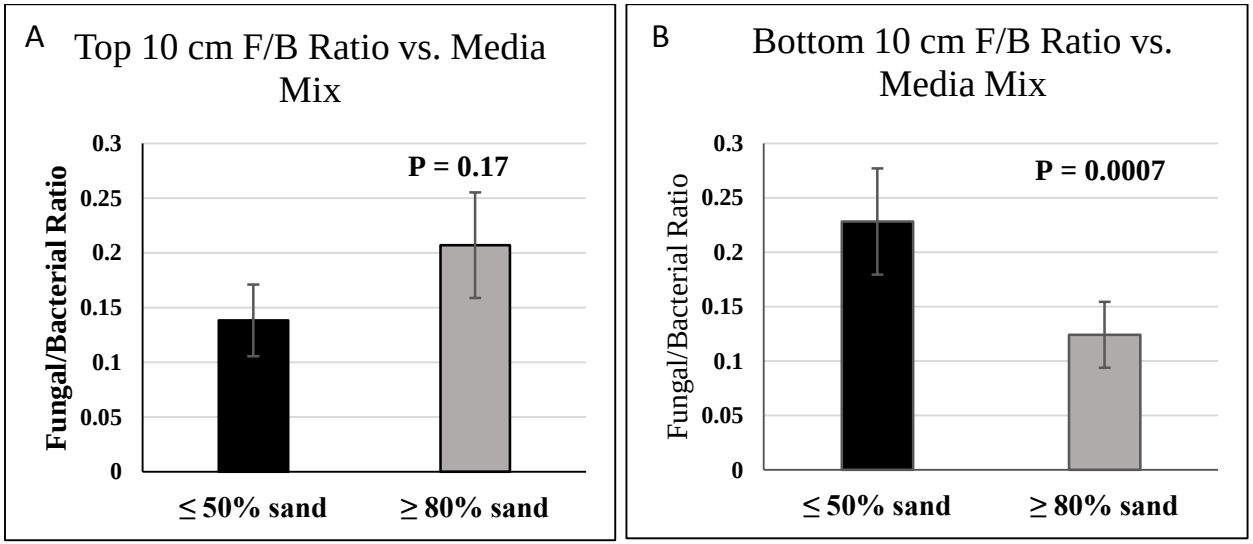

Figure 3.19. Average fungal/bacterial ratios in the top 10 cm (A) and bottom 10 cm (B) samples by media mix composition.

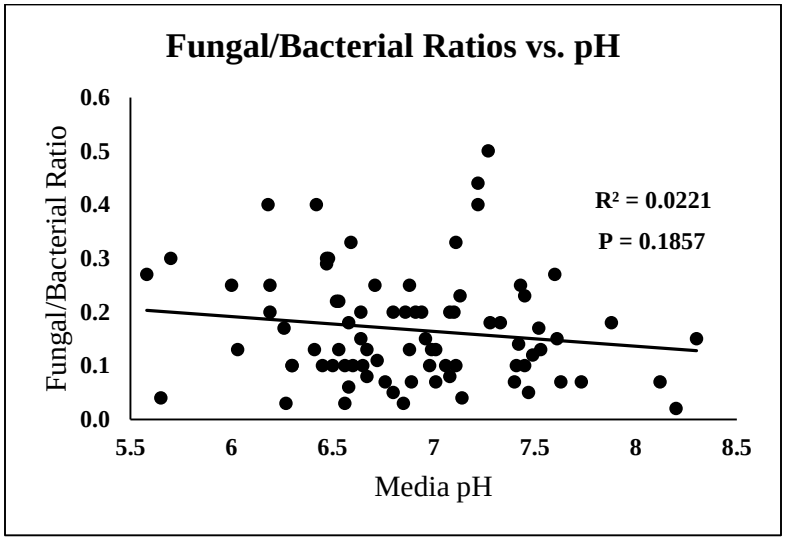

Figure 3.20. Linear regression of fungal/bacterial ratio vs. media mix pH (n = 79).

4. Conclusions and Future Research

Our results agree with previous research of bacterial populations in BRCs that observed positive relationships with N and negative relationships with soil depth. These findings highlight the need for future research into optimizing conditions in the lower layers of BRCs to be preferential to bacterial populations, particularly if this can be improved by simple alterations such as C amendments. Our analysis of the bacterial community structures indicated that the types of bacteria found in BRCs are largely controlled by N concentrations in the top 10 cm of the cell, while limited by C in the bottom layers. Larger proportions of *Proteobacteria* were observed in the top 10 cm samples, which are typically found in resource abundant environments and make up a large proportion of known denitrifiers. The bottom 10 cm samples were composed of larger populations of *Firmicutes*, a phylum that is commonly associated with nutrient and substrate limited environments. While it is difficult to speculate on how the composition and distribution of these species may contribute to nutrient cycling in BRCs, this work, to our knowledge, is the first instance in which bacterial and fungal communities have been characterized in a BRC. At a coarse level, it is clear that these communities share much in common with the microbiome structures observed in 'natural' soils, and this detailed view may pave the way for future studies that could potentially select for species that increase nutrient cycling or pollutant degradation.

Our investigation into the fungal populations in BRCs similarly identified N concentrations as an important variable driving both concentrations and community structure. *Ascomycota* and *Basidiomycota* made up the greatest contribution of fungal sequences, and the relative proportions of these species in most of the BRCs were similar to proportions of those in dry ecosystems. Fungal diversity considerably declined in the order of landscaped > overgrown > grassed BRCs, which may be attributed to the rooting systems or available C across these vegetation schemes. In contrast to the bacterial community, fungal composition did not change in response to the age of BRCs. This may be due to the hyphal systems of fungi, which allow for the translocation of nutrients and reduce their dependence on the accumulation of organic matter and nutrients. Insight into these relationships is currently a topic of interest in microbiology and ecology and will, hopefully, help contribute to the understanding and role of fungi in BRCs.

Strong, statistically significant relationships between microbial biomass, N, and C concentrations were observed that agree with previous findings in natural soil systems. These results could have important implications on nutrient cycling in BRCs. Engineering stormwater management structures that provide adequate C and nutrient sources may select for more and faster growing microbial biomass that can maximize nutrient transformations and pollutant degradation in stormwater. Research into a media mix that provides both C and nutrient amendments to supplement the microbial community, but does not cause excess leaching could improve N cycling and pollutant degradation in BRCs. Although the F/B ratio was only affected by media composition, future research on the interactions between fungi, bacteria, and pollutant removal will help in understanding the functioning of existing BRCs and informing design of future BMPs.

To our knowledge, this is the first instance in which the bacterial and fungal communities in any BRC have been characterized and by sampling multiple BRCs we were able to investigate relationships with varying design specifications across a spatial gradient. Across all of the collected design and environmental variables, C, N, and vegetation most strongly influence microbial populations and the bacterial and fungal community composition. Fortunately, all of these factors can be manipulated through BRC design. As the scientific community develops a better understanding of microbial communities and their relationship to ecosystem functioning, these results may be used to engineer best management practices to increase microbial biomass or select for a microbial community to increase pollutant removal efficiency from stormwater runoff.

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